INTEGRATIVNI PRISTUP U KONZERVACIJI VRSTA: GENETSKE, MORFOLOŠKE I EKOLOŠKE ZNAČAJKE SLATKOVODNIH RAKOVA PORODICE ASTACIDAE (DECAPODA)

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DOKTORSKI RAD

Mentor:

Prof. dr. sc. Ivana Maguire

Zagreb, 2022.

University of Zagreb

FACULTY OF SCIENCE

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Leona Lovrenčić

AN INTEGRATIVE APPROACH TO CONSERVATION: GENETICS, MORPHOLOGY AND ECOLOGY OF FRESHWATER CRAYFISH (DECAPODA: ASTACIDAE)

DOCTORAL DISSERTATION

Supervisor:

Dr. Ivana Maguire, Full Prof.

Zagreb, 2022

Ovaj je doktorski rad izrađen u Zoologijskom zavodu Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu, pod vodstvom prof. dr. sc. Ivane Maguire, u sklopu Sveučilišnog poslijediplomskog doktorskog studija Biologije pri Biološkom odsjeku Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu. Doktorski rad izrađen je uz novčanu potporu Hrvatske zaklade za znanost u sklopu znanstveno-istraživačkog projekta „Klimatske promjene i invazivne vrste - utvrđivanje utjecaja na bioraznolikost nativnih slatkovodnih rakova i pastrva i njihova konzervacija“ voditeljice prof. dr. sc. Ivane Maguire.

Informacije o mentoru

Ivana Maguire (MBZ: 217830) je redovita profesorica na Biološkom odsjeku Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu. Po završetku studija Biologije na Prirodoslovno-matematičkom fakultetu 1993. godine radi kao profesorica kemije u srednjoj školi, nakon čega upisuje magistarski studij na Université Catholique de Louvain-La-Neuve u Belgiji. Na Biološkom odsjeku počinje raditi 1997. godine u području istraživanja vezanom uz biologiju slatkovodnih rakova na čijoj tematici je doktorirala 2002. Njezin znanstveno-istraživački rad uključuje različite aspekte istraživanja slatkovodnih rakova porodice Astacidae. Sudjeluje u izvođenju nastave iz različitih kolegija na dodiplomskom, diplomskom i doktorskom studiju. Također, mentorica je diplomskih (30), magistarskih (4) i doktorskih radova (5) te studentskih radova za Rektorovu nagradu Sveučilišta u Zagrebu (4). Do sada je aktivno sudjelovala u provedbi 30 znanstvenih projekta od kojih je vodila 12 projekata, uključujući i projekt „Klimatske promjene i invazivne vrste - utvrđivanje utjecaja na bioraznolikost nativnih slatkovodnih rakova i pastrva i njihova konzervacija“ u sklopu kojeg je izrađen doktorski rad Leone Lovrenčić. Objavila je preko 60 znanstvenih radova u časopisima s međunarodnom recenzijom, aktivno sudjelovala na međunarodnim znanstvenim skupovima s više od 93 priopćenja te domaćim skupovima s 44 priopćenja. Do sada je recenzirala 68 znanstvenih radova za različite znanstvene časopise i 2 znanstvena projekta. Kao članica Uredničkog odbora sudjelovala je u radu znanstvenog časopisa Natura Croatica i časopisa Frontiers in Ecology and Evolution. Članica je nekoliko domaćih i međunarodnih strukovnih udruženja. Sudjelovala je u organizaciji jednog domaćeg i sedam međunarodnih znanstvenih skupova.

Zahvale

Pisanje ove doktorske disertacije može se usporediti s putovanjem dugim četiri godine, a ja sam imala sreću što sam putem sretala mnoge koji su to putovanje učinili poučnim i lijepim iskustvom. Nemoguće je zahvaliti svima koji su me hrabrili tijekom godina - trebao bi mi još jedna disertacija za to - ali nekima se mora odati počast.

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Sveučilište u Zagrebu Doktorski rad

Prirodoslovno-matematički fakultet

Biološki odsjek

INTEGRATIVNI PRISTUP U KONZERVACIJI VRSTA: GENETSKE, MORFOLOŠKE I EKOLOŠKE ZNAČAJKE SLATKOVODNIH RAKOVA PORODICE ASTACIDAE (DECAPODA)

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Ciljevi ove disertacije bili su utvrditi morfološku i genetsku raznolikost populacija potočnog i plemenitog raka u Hrvatskoj te razviti modele povoljnosti staništa koji će predvidjeti kako invazivne strane vrste rakova te klimatske promjene utječu na njihovu raznolikost i dugoročni opstanak. Geometrijska morfometrija ustanovila je postojanje morfološke raznolikosti i razlikovanje populacija ovisno o pripadnosti različitim linijama, genetskim grupama, riječnim slivovima i tipu staništa. Filogenetska rekonstrukcija potvrdila je veliku genetsku raznolikost, s filogeografskom strukturom i rasprostranjenošću evolucijskih linija oblikovanih geo-klimatskim procesima. Populacijsko-genetičke analize ustanovile su visoku razinu genetske raznolikosti i diferencijacije populacija, s ograničenim protokom gena. Modeli povoljnosti staništa pokazali su da će populacije plemenitog i potočnog raka biti ugroženije budućim klimatskim promjenama, nego širenjem invazivnih stranih vrsta rakova. Preklapanjem podataka o genetskoj raznolikosti s budućim povoljnim staništima identificirane su populacije i područja najveće vrijednosti kojima treba dati prioritet u budućim konzervacijskim planovima.

(239 stranica, 184 literaturnih navoda, jezik izvornika: hrvatski)

Ključne riječi: morfologija, filogenija, populacijska genetika, klimatske promjene, raznolikost

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Doctoral thesis

AN INTEGRATIVE APPROACH TO CONSERVATION: GENETICS, MORPHOLOGY AND ECOLOGY OF FRESHWATER CRAYFISH (DECAPODA: ASTACIDAE)

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Aims of this dissertation were to determine the morphological and genetic diversity of the stone crayfish and noble crayfish in Croatia, and to develop habitat suitability models that will predict how the invasive crayfish species and climate change affect their biodiversity and long-term survival. Geometric morphometrics revealed the existence of morphological diversity and differentiation of populations based on the crayfish affiliation to different genetic lineages, genetic clusters, river basins and habitat types. Phylogenetic reconstruction confirmed the existence of high genetic diversity, with geo-climatic processes shaping the contemporary geographic distribution of evolutionary lineages. Population genetics revealed high genetic diversity and differentiation of populations, with limited gene flow. Habitat suitability models suggested that climate change-driven habitat loss represents a greater threat than the potential future distribution of the invasive crayfish species. The obtained results enabled identification of populations and areas with the highest conservation value and which should be given the highest priority in conservation planning.

(239 pages, 184 references, original in Croatian)

Keywords: morphology, phylogeny, population genetics, climate change, diversity

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SADRŽAJ

1. UVOD

1. UVOD

1.1. Raznolikost i ugroženost slatkovodnih ekosustava

Voda predstavlja temeljni i neophodni čimbenik opstanka života na planeti Zemlji, a slatkovodni ekosustavi čine samo 0,01 % svih ekosustava na Zemlji (Dudgeon i sur., 2006.). Zauzimaju manje od 1 % Zemljine površine, a istovremeno su vruće točke bioraznolikosti (eng. biodiversity hotspots) koje podržavaju otprilike 10 % svih dosad opisanih vrsta (Dudgeon i sur., 2006.; Strayer i Dudgeon, 2010.). Rastući antropogeni pritisak izrazito negativno utječe na njihovu bioraznolikost, uzrokujući gubitak funkcija i usluga koje pružaju (Strayer i Dudgeon, 2010.; Darwall i sur., 2018.; Reid i sur., 2019.). Glavne prijetnje bioraznolikosti slatkovodnih ekosustava su prekomjerno iskorištavanje voda, izmjene vodotoka, onečišćenje, urbanizacija, gubitak i fragmentacija staništa, klimatske promjene i invazivne strane vrste (Dudgeon i sur., 2006.; Strayer i Dudgeon, 2010.). Međusobna interakcija i djelovanje ovih antropogenih pritisaka rezultirala je smanjenjem populacija i areala slatkovodnih vrsta diljem svijeta, stoga je ublažavanje brzog gubitka raznolikosti zbog negativnih antropogenih aktivnosti jedan od najvećih izazova čovječanstva (Darwall i sur., 2018.).

Slatkovodni ekosustavi Mediterana smatraju se jednom od vrućih točaka globalne bioraznolikosti. Današnja rasprostranjenost, genetska struktura i raznolikost slatkovodnih organizama ovog područja odraz je prošlih klimatskih uvjeta, geomorfoloških i hidrogeografskih procesa poput promjena razine mora, tektonskih aktivnosti i glacijacija (Hewitt, 2004.). Glacijacije tijekom pleistocena imale su značajan učinak na europske vrste čiji je dugoročni opstanak u prošlosti ovisio o glacijalnim refugijima smještenim na mediteranskim poluotocima (Iberski, Apeninski i Balkanski) iz kojih su se mnoge kopnene i vodene vrste u postglacijalnom periodu proširile u središnje i sjeverne dijelove Europe (Hewitt, 1999.; 2004.; 2011.). Jedna od posljedica ovih migracija jest postepeno smanjenje genetske raznolikosti prema sjevernim područjima i veća raznolikost u južnim refugijalnim populacijama. Ovakav uzorak genetske raznolikosti potvrđen je brojnim filogeografskim istraživanjima (Hewitt, 2000.). Dugotrajna izolacija populacija u različitim glacijalnim refugijima Balkanskog poluotoka dovela je do njihove povećane genetske divergencije i/ili specijacije te danas ovo područje karakterizira visoka bioraznolikost i endemičnost slatkovodne faune smještene u malim područjima i s malim veličinama populacija

(Bănărescu, 2004.). Ova slatkovodna staništa istovremeno su među najugroženijim i njihova se biološka raznolikost dramatično smanjuje, s visokim stopama izumiranja i velikim brojem ugroženih vrsta (Figueroa i sur., 2013.; Collen i sur., 2014.).

Hrvatska je smještena u jugoistočnoj Europi, dijelom na području Balkanskog poluotoka te zahvaljujući paleogeomorfološkim značajkama obiluje vodom. Obzirom da je smještena u četiri biogeografske regije (alpinska, kontinentalna, mediteranska i panonska) te obuhvaća različita geološka, pedološka, hidrološka i klimatska područja, u njoj postoje raznoliki slatkovodni i podzemni ekosustavi koji su kroz evolucijsku povijest omogućili razvoj brojnih, često endemskih vrsta (Verovnik i sur., 2004.; Bilandžija i sur., 2013.; Klobučar i sur., 2013.; Previšić i sur., 2014.; Jelić i sur., 2016.). Geološko-klimatski procesi u prošlosti i geomorfološka obilježja Balkanskog poluotoka snažno su utjecali na divergenciju i specijaciju lokalnih slatkovodnih organizama stvarajući mozaike divergentnih evolucijskih linija. Fragmentirana paleohidrografska mreža dinaridskog krša omogućila je stvaranje biogeografskih barijera, zaštitu tijekom glacijacija i stanište s konstantnim klimatskim uvjetima što je dovelo do velike raznolikosti i filogeografske strukturiranosti mnogih vrsta (Hewitt, 2011.).

U Hrvatskoj, kao i u svijetu, slatkovodni ekosustavi su pod sve izraženijim antropogenim pritiskom koji negativno utječe na njihovu bioraznolikost. Jedan od prioriteta Republike Hrvatske jest upravo zaštita i očuvanje bioraznolikosti na svim razinama organizacije, od ekosustava preko vrsta i populacija do jedinki i njihovog genetskog nasljeđa (NN 80/13), što je u skladu s Konvencijom o biološkoj raznolikosti (www.cbd.int).

1.2. Slatkovodni rakovi (Decapoda: Astacidea)

Predmet istraživanja u okviru ove doktorske disertacije su dvije nativne vrste slatkovodnih rakova osjetljive na promjene u staništu, *Austropotamobius torrentium* (Schrank, 1803) - potočni rak i *Astacus astacus* (Linnaeus, 1758) - plemeniti rak. Ugrožene su zbog negativnog antropogenog utjecaja na staništa, invazivnih vrsta i klimatskih promjena, sa zabilježenim smanjenjem i/ili nestankom populacija diljem areala (Maguire i sur., 2011.; 2018.). U današnje vrijeme, slatkovodni rakovi predstavljaju visoko ugroženu komponentu slatkovodnih ekosustava, s više od 30 % ugroženih vrsta (Richman i sur., 2015.). Istovremeno, slatkovodni rakovi su najveći beskralježnjaci i ključne vrste slatkovodnih ekosustava, s važnom ekonomskom i kulturnom ulogom u mnogim zajednicama. Održavaju ravnotežu staništa i značajna su komponenta hranidbenih mreža zbog veličine tijela, dugog

životnog vijeka, brojnosti i omnivornog načina prehrane (Usio i Townsend, 2002.). Osiguravaju normalno funkcioniranje ekosustava u kojima su prisutni, a gubitak njihovih populacija može ugroziti raznolikost cijelog staništa (Holdich, 2002.; Reynolds i sur., 2013.). Smatra ih se pokazateljima kvalitete vode (eng. water quality indicators), pokazateljima bioraznolikosti (eng. biodiversity indicators), trofičkim regulatorima (eng. keystone trophic regulators) i inženjerima ekosustava (eng. ecological engineers) (Reynolds i sur., 2013.). Nestanak rakova iz ekosustava dovodi do promjena u procesima i uslugama slatkovodnih ekosustava, strukturi i funkcioniranju staništa, promjena u dinamici transporta sedimenta te promjena u brojnosti i raznolikosti vrsta i sastavu zajednica (Reynolds i Souty-Grosset, 2012.; Reynolds i sur., 2013.).

1.2.1. Raznolikost i rasprostranjenost slatkovodnih rakova

Slatkovodni rakovi (Decapoda: Astacidea) su monofiletska grupa organizama s preko 640 opisanih vrsta podijeljenih u četiri porodice: Astacidae, Cambaridae, Cambaroididae i Parastacidae (Crandall i De Grave, 2017.). Rasprostranjeni su na gotovo svim kontinentima, s najvećom raznolikošću vrsta i podvrsta na području Sjeverne Amerike i Australije. Porodice Astacidae, Cambaridae i Cambaroididae rasprostranjene su na području sjeverne polutke (Azija, Europa i Sjeverna Amerika), a porodica Parastacidae na južnoj polutci (Južna Amerika, Australija, Madagaskar) (Crandall i Buhay, 2008.).

Na području Europe, zapadno od Urala, prirodno obitava samo porodica Astacidae Latreille, 1802 zastupljena s tri nativna roda: *Astacus* (Fabricius, 1775), *Austropotamobius* (Skorikov, 1907) i *Pontastacus* (Bott, 1950). U usporedbi s ostalim kontinentima, Europu karakterizira niska raznolikost vrsta i podvrsta, uz stalno prisutne taksonomske promjene i rasprave o točnom broju nativnih vrsta. Općenito je prihvaćeno mišljenje da na području Europe živi šest nativnih vrsta porodice Astacidae: *Austropotamobius pallipes* (Lereboullet, 1858) - bjelonogi ili primorski rak, *A. torrentium, A. astacus, Pontastacus leptodactylus* (Eschscholtz, 1823) - uskoškari, turski ili barski rak, *Astacus pachypus* (Rathke, 1837) (Holdich, 2002) i *Austropotamobius bihariensis* (Kouba i sur., 2014.; Pârvulescu, 2019.). Uz prisutnost nativnih vrsta rakova, u Europi danas žive i invazivne strane vrste koje se dijele se na stare, unesene su u Europu prije 1980. godine, i nove, unesene nakon 1980. godine (Kouba i sur., 2014.). Strane vrste rakova u Europu su unesene s namjerom uzgoja u konzumne svrhe i nadomještanja nativnih vrsta čija je brojnost bila u opadanju, a nedugo nakon introdukcije, pokazale su se invazivnima. Osim što su se u kompeticiji s nativnim vrstama pokazale

nadmoćnima (agresivnije, fertilnije, prilagodljivije na promjene u okolišu), ove su vrste u Europu donijele patogen *Aphanomyces astaci* Schikora, 1906 koji u nativnim vrsta uzrokuje letalnu bolest račju kugu. Najčešće stare invazivne vrste su sjevernoameričkog podrijetla - vrsta *Pacifastacus leniusculus* (Dana, 1852) - signalni rak iz porodice Astacidae te vrste *Faxonius limosus* (Rafinesque, 1817) - bodljobradi rak i *Procambarus clarkii* (Girard, 1852) iz porodice Cambaridae. U nove strane invazivne vrste ubrajaju se dvije australske vrste roda *Cherax* (Erichson, 1846): *Cherax destructor* (Clark, 1936) i *Cherax quadricarinatus* (Von Martens,1868), sjevernoameričke vrste roda *Faxonius*: vrsta *Faxonius immunis* (Hagen, 1870), *Faxonius juvenilis* (Hagen, 1870), *Faxonius cf. virilis* te tri vrste roda *Procambarus*: *Procambarus cf. acutus, Procamabarus virginalis* i *Procambarus alleni* (Kouba i sur., 2014.).

U Hrvatskoj su dosadašnjim istraživanjima utvrđene četiri nativne europske vrste; bjelonogi rak, potočni rak, plemeniti rak, uskoškari rak, i tri invazivne strane vrste podrijetlom većinom iz Sjeverne Amerike; signalni rak, bodljobradi rak i mramorni rak (Maguire i sur., 2018.). Bjelonogi rak rasprostranjen je na području Like, Dalmacije i nekih dalmatinskih otoka; potočni rak obitava u kontinentalnom dijelu Hrvatske u potocima na višim nadmorskim visinama; plemeniti rak, također na području kontinentalne Hrvatske i uskoškari rak, koji se proširio s područja Posavine i Slavonije prema zapadnom, južnom i sjevernom dijelu Hrvatske (Maguire i sur., 2018.). U Hrvatskoj je signalni rak prvi put zabilježen u rijeci Muri 2008. godine (Maguire i sur., 2008.) gdje se prirodno proširio nizvodnim putem iz Austrije i Slovenije. Iz rijeke Mure se proširio nizvodno u rijeku Dravu s procijenjenom brzinom nizvodnog širenja od 18-24,4 km godišnje, što je ujedno i najveća zabilježena brzina u Europi (Hudina i sur., 2009.). U 2011. godini zabilježen je u rijeci Korani u koju je unesen od strane ljudi (Hudina i sur., 2013.), a u kojoj se širi uzvodno i nizvodno te postupno istiskuje nativne vrste (Dragičević i sur., 2020.). Bodljobradi rak prvi put je zabilježen u močvarnom području Parka prirode Kopački rit 2003. godine gdje se prirodno proširio rijekom Dunav iz Mađarske (Maguire i Klobučar, 2003.). U usporedbi s ranijim podacima ova vrsta je značajno proširila svoj areal i nastavlja se uspješno širiti u hrvatskim slatkovodnim ekosustavima ugrožavajući nativnog plemenitog i uskoškarog raka (Maguire i sur., 2018.). Mramorni rak je zasad zabilježen samo u jezeru Šoderica (Samardžić i sur., 2014.; Maguire i sur., 2018.).

1.2.2. Ugroženost i zaštita slatkovodnih rakova

Klimatske promjene i invazivne strane vrste, zajedno s gubitkom i fragmentacijom staništa, prekomjernim iskorištavanjem prirodnih dobara i različitim onečišćivačima,

prepoznate su kao glavni uzroci globalnog smanjenja bioraznolikosti. Ove antropogeno uzrokovane okolišne promjene mijenjaju odnose među organizmima, uzrokuju pad broja vrsta i promjene u njihovoj rasprostranjenosti, a danas su značajno ubrzane uslijed sve intenzivnijeg antropogenog utjecaja i zbog njih se organizmi često nalaze u novim uvjetima koji zahtijevaju brz odgovor, brži nego ikad u njihovoj evolucijskoj povijesti (Palumbi, 2001.). Stoga je prepoznata sve veća potreba za praćenjem stanja i trendova biološke raznolikosti u svrhu određivanja i kvantifikacije ovih antropogeno uzrokovanih okolišnih promjena i poboljšanja zaštite. Usprkos znakovima brzih i razornih promjena u slatkovodnim ekosustavima, bioraznolikost slatkih voda ostaje niskog prioriteta u globalnim i lokalnim inicijativama (Gottstein i sur., 2011.). Zbog nedostatka konzervacijskih strategija s ciljem smanjenja i/ili uklanjanja štetnih promjena u slatkovodnim ekosustavima, u Hrvatskoj se povećava vjerojatnost gubitka slatkovodne bioraznolikosti.

Brojnost i veličina populacija nativnih vrsta rakova na području Europe značajno se smanjila tijekom posljednjeg stoljeća uslijed negativnih antropogenih pritisaka na njihova staništa, prekomjernog nekontroliranog izlova, klimatskih promjena s ekstremno sušnim razdobljima te širenja stranih invazivnih vrsta rakova (Kouba i sur., 2014.; Jussila i sur., 2021.). Antropogeni pritisak na staništa najviše se očituje u regulaciji vodenih tokova (izgradnja brana i hidro-akumulacija, kanaliziranje korita, melioracija) koja uzrokuje fragmentaciju i degradaciju prirodnih staništa. Dodatno, velike količine otpadnih tvari u slatkovodnim ekosustavima pogoršavaju kvalitetu vode i staništa, a time povećavaju ugroženost slatkovodnih organizama.

Negativni utjecaj klimatskih promjena na rakove očituje se u porastu temperature vode i promjenama u režimu vodotoka koji su rezultat promjena u oborinskim obrascima te promjena u intenzitetu i učestalosti ekstremnih vremenskih prilika (Moss i sur., 2009.; Capinha i sur., 2013., Markovic i sur., 2014.). Klima je jedan od glavnih ekoloških čimbenika oblikovanja biogeografije slatkovodnih organizama. Rasprostranjenost vrsta tijekom geološke povijesti stalno se prilagođavala klimatskim promjenama stoga možemo pretpostaviti da će sve snažnije i izraženije klimatske promjene (IPCC 2021) uzrokovati promjene i pomake u prirodnoj rasprostranjenosti vrsta. Klimatske promjene uzrokuju gubitak povoljnog staništa za brojne vrste, što rezultira pomicanjem areala, pogotovo prema višim geografskim širinama i nadmorskim visinama (Pecl i sur., 2017.). Također, klimatske promjene promiču širenje invazivnih stranih vrsta te širenje bolesti čiji su vektori invazivne strane vrste (Linders i sur.,

2019.). Slatkovodna bioraznolikost je posebno osjetljiva na klimatske promjene zbog ovisnosti o termalnim i hidrološkim režimima, ograničenoj mogućnosti rasprostranjivanja mnogih slatkovodnih organizama i sinergijskog učinka različitih antropogenih stresora (Woodward i sur., 2010.). Direktni utjecaj klimatskih promjena na slatkovodne ekosustave očituje se u povišenoj temperaturi vode i promjenama u protoku vode koji rezultiraju promjenama u fenologiji, rasprostranjenosti, gustoćama populacija i sastavu zajednica te promjenama u hranidbenim mrežama vodenih, ali i kopnenih ekosustava. Indirektni utjecaji klimatskih promjena očituju se u smanjenju količine kisika i promjene u kruženju nutrijenata.

Slatkovodne vrste osjetljivije su na klimatske promjene od kopnenih ili morskih vrsta zbog male veličine slatkovodnih staništa unutar kojih dolazi do intenzivnijeg/izraženijeg utjecaja antropogenih aktivnosti i invazivnih stranih vrsta (Strayer i Dudgeon, 2010.). Slatkovodni ekosustavi Mediterana istaknuti su kao jedno od najosjetljivijih područja na klimatske promjene zbog povećanja temperature i promjena u oborinskom režimu (smanjenje oborina, češći vremenski ekstremi, suša klima i izražen proces desertifikacije) (Markovic i sur., 2014.). Za mediteranske vrste predviđa se geografski pomak u njihovoj rasprostranjenosti prema višim geografskim širinama, a kao posljedicu možemo očekivati smanjenje populacija i povećani rizik od izumiranja vrsta. Populacije slatkovodnih vrsta koje su izložene naglim promjenama u okolišu moraju se prilagoditi novonastalim uvjetima ili migrirati u nova područja s povoljnim ekološkim uvjetima kako bi opstale. Sposobnost vrste i njenih populacija da se prilagode klimatskim promjena ovisi o postojećoj genetskoj varijabilnosti ili adaptivnom evolucijskom potencijalu (eng. standing adaptive variation) te mogućnosti migracije u klimatski povoljna područja.

Uz klimatske promjene, invazivne strane vrste su jedan od glavnih uzroka gubitka bioraznolikosti u slatkovodnim ekosustavima (Sala i sur., 2000.). Invazivne strane vrste rakova agresivnije su od nativnih europskih vrsta pa ih u kompeticiji za prostor i hranu istiskuju iz njihovih prirodnih staništa. Nadalje, odlikuju se obilježjima koja doprinose njihovom invazivnom uspjehu, poput brzog rasta, ranijeg postizanja spolne zrelosti, visokog fekunditeta i šire ekološke valencije (Souty-Grosset i sur., 2006.; Holdich i sur., 2009.; Hudina i sur., 2014.). Osim kompeticijom, invazivne strane vrste ugrožavaju nativne jer posjeduju veću toleranciju na promjene u uvjetima okoliša koji uključuju povišenje temperature, onečišćenje i veću količinu organskih tvari (Souty-Grosset i sur., 2006.). Širenje invazivnih stranih vrsta je često povezano s njihovom većom sposobnosti prilagodbe na

klimatske promjene (Linders i sur., 2019.). Invazivne strane vrste rakova kopanjem zaklona mehanički uništavaju stanište te time utječu na stabilnost obale, transport i karakteristike sedimenta, a udruženom interakcijom bioturbacije, ekskrecije i mehaničkog uništavanja mogu značajno promijeniti kvalitetu vode u nekom području (Harvey i sur., 2014.). Naposljetku, invazivne strane vrste dokazani su vektori širenja bolesti račje kuge koja je uzrokovana patogenom *A. astaci*, na koju su same većinom otporne, a koja je letalna za nativne vrste.

Na svjetskoj razini, većina europskih vrsta iz porodice Astacidae uvrštena je na Crveni popis ugroženih svojti (eng. Red List of Threatened Species) Međunarodne unije za očuvanje prirode (eng. International Union for Conservation of Nature, IUCN). Nalaze se i na Dodatku III Konvencije o zaštiti europskih divljih vrsta i prirodnih staništa (Bernska konvencija) te na Dodatku II i Dodatku V Direktive o zaštiti prirodnih staništa i divlje faune i flore. U Hrvatskoj su zaštićeni Zakonom o zaštiti prirode (NN 80/13) i Pravilnikom o proglašavanju divljih svojti zaštićenim i strogo zaštićenim (NN 144/13).

1.3. Integrativni pristup u konzervacijskoj biologiji

Konzervacijska biologija je mlada, ali brzorastuća biološka disciplina koja „proučava biološku raznolikost, utvrđuje prijetnje biološkoj raznolikosti te igra aktivnu ulogu u očuvanju biološke raznolikosti“ (Primack, 2006.). Razvijena je kao odgovor na postojeće prijetnje i ubrzani nestanak različitih sastavnica i razina biološke raznolikosti, a podržana velikim brojem znanstvenih dokaza o negativnom antropogenom utjecaju na vrste, zajednice i ekosustave (Groom i sur., 2006.; Primack, 2006.). Konzervacijska biologija podrazumijeva visoku razinu interdisciplinarnosti i spajanja niza bioloških disciplina (populacijska genetika, ekologija, biogeografija, fiziologija, morfologija) i ostalih znanosti (geologija, kemija, fizika, klimatologija, sociologija, antropologija, ekonomija, politika). Groom i sur. (2006.) definiraju konzervacijsku biologiju kao „integrativan pristup zaštiti i upravljanju biološkom raznolikošću“ što potvrđuje da se radi o interdisciplinarnoj disciplini koja povezuje prirodne i društvene znanosti s ciljem očuvanja biološke raznolikosti i sprječavanja izumiranja vrsta.

Prepoznavanje raznolikosti na svim razinama biološke organizacije osnovni je korak u konzervacijskoj biologiji. Biološka raznolikost obuhvaća tri organizacijske razine: genetsku raznolikost (raznolikost gena unutar populacija i vrsta), raznolikost unutar i između vrsta te raznolikost ekosustava (raznolikost ekosustava, zajednica, krajolika). Poznavanje i očuvanje genetske raznolikosti jedan je od neophodnih preduvjeta za razvoj učinkovitih mjera zaštite i

planova upravljanja, osobito kod ugroženih vrsta. Istraživanjem i očuvanjem genetske raznolikosti u svrhu opstanka vrsta bavi se konzervacijska genetika. Temelji se na principima molekularne biologije, populacijske genetike i evolucije, ali obuhvaća i elemente drugih bioloških disciplina.

Genetska raznolikost odražava adaptivni evolucijski potencijal vrste koji omogućava bolju prilagodbu na promjene u okolišu i njen dugoročni opstanak (Frankham i sur., 2002.; Toro i Caballero, 2005.). Definirana je kao raznolikost alela i genotipova prisutnih u populaciji koje rezultiraju morfološkim, fiziološkim i bihevioralnim razlikama između jedinki i populacija (Frankham i sur., 2002.). Osnovne mjere genetske raznolikosti su alelno bogatstvo, stupanj heterozigotnosti i postotak polimorfnih lokusa u populaciji. Veća genetska raznolikost čini vrstu otpornijom i bolje prilagođenom za opstanak u promjenjivim uvjetima okoliša. Niska genetska raznolikost može biti uzrokovana različitim čimbenicima, poput fragmentacije staništa, akumulacije štetnih alela te smanjenjem veličine populacije i nastankom malih izoliranih populacija koje su puno osjetljivije na stohastičke procese u okolišu (npr. genski pomak). Smanjenje populacije može dovesti do parenja u srodstvu (eng. inbreeding) čije međudjelovanje s fragmentacijom, gubitkom genetske raznolikosti, povećanom ekspresijom štetnih recesivnih alela i smanjenjem sposobnosti opstanka (eng. fitness), u konačnici može prouzročiti vrtložno izumiranje ili ekstinkcijski vorteks (eng. extinction vortex) (Frankham i sur., 2002.). Dugoročno očuvanje genetske raznolikosti osigurava održanje adaptivnog evolucijskog potencijala populacije ili vrste, dok kratkoročno očuvanje genetske raznolikosti osigurava održanje njene reprodukcijske sposobnosti (Frankham i sur., 2002.).

Kapacitet određene vrste da se prilagodi promjenama okoliša u velikoj mjeri ovisi o razini i prostornoj raspodjeli genetske raznolikosti. Glavna područja interesa konzervacijske genetike, kao što su gubitak genetske raznolikosti, parenje u srodstvu i fragmentacija populacija (Frankham i sur., 2002.), ujedno su i važni čimbenici koji određuju odgovor vrste na klimatske promjene (Etterson, 2008.). Kako bi opstale, vrste koje su izložene naglim promjenama u okolišu, moraju se prilagoditi novonastalim uvjetima ili migrirati u nova područja prateći povoljne ekološke uvjete (Aitken i sur., 2008.). Smanjena genetska raznolikost ograničava brzinu prilagodbe na nove klimatske uvjete, što može rezultirati geografskim promjenama u rasprostranjenosti vrste ili njenim izumiranjem. Osim prethodno navedenih, jedan od potencijalnih uzroka smanjenja genetske raznolikosti nativnih slatkovodnih vrsta su i invazivne strane vrste koje kroz direktnu i indirektnu kompeticiju

uzrokuju smanjenje veličine populacija nativnih vrsta (Jones i Closs, 2015.; Gallardo i sur., 2016.; Vera-Escalona i sur., 2019.).

Za identifikaciju potencijalnih klimatskih refugija (eng. climate change refugia) i refugija od invazivnih vrsta (eng. ark sites) te procjenu utjecaja istih na genetsku raznolikost i strukturu populacija, preporuča se integrirani pristup koji kombinira klasičnu populacijsku genetiku i modele rasprostranjenosti vrsta (eng. species distribution models). Ovi su modeli tijekom zadnjeg desetljeća našli vrlo široku primjenu u mnogim područjima biologije, uključujući istraživanja vezana uz rizike uzrokovane invazivnim stranim vrstama (Jimenez-Valverde i sur., 2011., Guisan i sur., 2014.) te evolucijsku i konzervacijsku biologiju (Pearson, 2007., Kozak i sur., 2008.). Kao jedan od važnih alata za razvoj učinkovitih konzervacijskih strategija i usmjeravanje odluka glede zaštite vrsta (Ferraz i sur., 2012.; Guisan i sur., 2013.; Sofaer i sur., 2019.), postali su ključni i gotovo neizbježni za predviđanje posljedica utjecaja invazivnih stranih vrsta i klimatskih promjena na vrste i staništa (Araújo i sur., 2012., Palaoro i sur., 2013.; Gallardo i Aldridge, 2013.).

Identifikacija klimatski stabilnih područja i područja bez invazivnih stranih vrsta u kojima populacije nativnih vrsta imaju veću vjerojatnost za preživljavanje mogu usmjeravati odluke glede *in situ* i *ex situ* mjera zaštite. Osnovni cilj konzervacijske biologije jest zaštita biološke raznolikosti u prirodi, odnosno *in situ*, a idealna strategija zaštite obuhvaća očuvanje prirodnih zajednica i populacija unutar njihova prirodnog areala i ekosustava. Međutim, u vrijeme sve intenzivnijih antropogenih pritisaka i klimatskih promjena, *in situ* konzervacija za mnoge ugrožene vrste nije dovoljna. Kao dopuna *in situ* konzervaciji, preporuča se *ex situ* konzervacija radi obnove populacija i povećanja vjerojatnosti njihova opstanka. Različite metode potpomognute migracije (eng. assisted migration), kao što su obnova populacija (eng. restocking; dodavanje jedinki u postojeću populaciju radi povećanja njene gustoće i/ili genetske raznolikosti), introdukcija (unošenje jedinki u područje unutar ili izvan prirodnog areala vrste kada je stanište u kojem se populacija nalazila uništeno i vrsta tamo ne može opstati ili nije moguće ukloniti čimbenik koji je doveo do njezino nestanka) i reintrodukcija (unošenje jedinki u područje unutar prirodnog areala vrste, s kojeg je ona prethodno nestala), označene su kao strategije upravljanja ugroženim vrstama i mjere prilagodbe na klimatske promjene i invazivne vrste (Weeks i sur., 2011.; Frankham i sur., 2017.). Iako su ove metode potpomognute migracije istaknute kao dobre konzervacijske strategije za očuvanje nativnih vrsta, one istovremeno mogu imati negativne posljedice na lokalne populacije, zajednice i ekosustave stoga je potrebno dobro planiranje i procjena rizika u svakom konkretnom slučaju

(Ricciardi i Simberloff, 2009.; Hewitt i sur., 2011.; Peterson i Bode, 2021.; Bucharova, 2017.). Navedeni rizici potpomognute migracije uključuju rizik da translocirane vrste postanu invazivne s negativnim biološkim, ekološkim i socioekonomskim učincima, potencijalno širenje bolesti i patogena te ugrožen opstanak izvorne populacije zbog uklanjanja jedinki iz postojećih populacija. Prevladavanje ovih rizika moguće je pažljivim planiranjem koje uključuje procjenu rizika (eng. risk assessments), analizu troškova i koristi (eng. cost-benefit analyses), praćenje potpomognute migracije, znanja o biologiji, ekologiji i genetskoj raznolikosti vrste, poznavanje kapaciteta rasprostranjivanja i mogućih barijera u migracijskim rutama te modeliranje rasprostranjenosti vrste pod utjecajem klimatskih promjena i invazivnih vrsta (Butt i sur., 2021.).

1.3.1. Integrativni pristup u konzervaciji slatkovodnih rakova

Smanjenje populacija nativnih slatkovodnih rakova diljem svijeta istaknulo je potrebu za razvojem učinkovitih konzervacijskih strategija i planova upravljanja (Richman i sur. 2015; Taylor i sur., 2019.; Jussila i sur., 2021.). Suvremeni multidisciplinarni pristup u konzervaciji ovih osjetljivih vrsta uključuje poznavanje genetske strukture i odabir klimatski stabilnih područja u kojima populacije imaju najveću vjerojatnost za preživljavanje (Souty-Grosset i Reynolds, 2009.; Kozák i sur., 2011.; Guisan i sur., 2013.; Capinha i sur., 2013.; Chucholl, 2017.).

Molekularno-genetičke analize u službi konzervacijske genetike rakova korištene su za precizno utvrđivanje stupnja ugroženosti vrsta i predlaganje djelotvornih metoda njihove zaštite jer omogućuju uvid u evolucijsku prošlost, srodstvene odnose, intraspecijsku genetsku raznolikost, populacijsku strukturu, efektivnu veličinu populacija, protok gena između populacija te razinu genetske diferenciranosti i izoliranosti populacija. Spoznaje o genetskoj strukturi i varijabilnosti omogućuju identifikaciju populacija koje imaju najveću konzervacijsku vrijednost i prioritet u zaštiti te koje se mogu u budućim planovima upravljanja koristiti kao izvorišne (donorske) za programe repopulacije/reintrodukcije kako bi se osigurao njihov opstanak. Također, na temelju istraživanja genetske raznolikosti i populacijske strukture upotrebom različitih biljega mitohondrijske ili jezgrine DNA moguće je identificiranje evolucijski značajnih jedinica (eng. evolutionarily significant units) kao i jedinica upravljanja (eng. management units). Definiranje konzervacijskih jedinica od iznimne je važnosti budući da većina vrsta nije genetski uniformna, već se sastoje od mozaika

povijesno izoliranih evolucijskih linija s jedinstvenom genetskom raznolikošću (Moritz, 1999.; Barbosa i sur., 2018.; Coates i sur., 2018.). Evolucijski značajne jedinice predstavljaju populacije koje su recipročno monofiletske za mitohondrijske i jezgrine alele (Moritz, 1999.). Predstavljaju važnu sastavnicu evolucijskog nasljeđa vrste jer se radi o linijama s ograničenim protokom gena prema drugim takvim linijama unutar vrste (Frazer i Bernatchez, 2001.). Jedinice upravljanja predstavljaju demografski nezavisne populacije, tj. funkcionalne komponente unutar evolucijski značajnih jedinica na kojima se mogu provoditi konkretni konzervacijski programi (Moritz, 1999.). Radi se o jedinicama koje su odijeljene geografskim barijerama, a ponekad se definiraju kao populacije među kojima ne postoji ili postoji mali protok gena, ali nisu odijeljene tijekom duljeg evolucijskog razdoblja. Ključni faktor u njihovom definiranju je demografska (ne)povezanost, a ne razina povijesnog protoka gena (Palsbøll i sur., 2007.). Identifikacija konzervacijskih jedinica kao važnih elemenata intraspecijske raznolikosti značajna je za genetsko spašavanje malih izoliranih populacija s velikim postotkom parenja u srodstvu, upravo kroz restauraciju protoka gena, obnovu vrsta, repopulaciju i/ili reintrodukciju (Coates i sur., 2018.). Moritz (1999.) u svrhu genetskog spašavanja ugroženih populacija preporuča miješanje jedinica upravljanja, ali ne i miješanje jedinki različitih evolucijski značajnih jedinica.

Najčešće korištene metode u istraživanjima genetske raznolikosti i konzervacijske genetike slatkovodnih rakova su genotipizacija mikrosatelitskih lokusa te sekvenciranje mitohondrijske i jezgrine DNA (Souty-Grosset i sur., 2003.; Fratini i sur., 2005.; Gouin i sur., 2006.; Vorburger i sur., 2014.; Bláha i sur., 2016.; Schrimpf i sur., 2017.; Berger i sur., 2018.). Mikrosateliti su često korišteni biljezi u populacijskoj i konzervacijskoj genetici. Također se nazivaju jednostavne ponavljajuće sekvence - SSR (eng. simple sequence repeats) ili kratke uzastopno ponavljajuće sekvence - STR (eng. short tandem repeats). Karakterizira ih kodominantnost, visoka stopa mutacija i polimorfnost koja proizlazi iz varijacije duljine alela koju uzrokuje različit broj ponavljanja mikrosatelitnog motiva. Zbog brze evolucije, služe u analizi nedavnih i trenutnih evolucijskih događaja. Mitohondrijska DNA zbog svog malog genoma jednostavne strukture i organizacije, haploidnosti, majčinskog nasljeđivanja, nedostatka rekombinacije i introna, također predstavlja vrlo koristan molekularni biljeg.

Pored istraživanja genetske raznolikosti, konzervacija nativnih vrsta rakova uključuje i identifikaciju povoljnih staništa te ispitivanje utjecaja klimatskih promjena i invazivnih vrsta (Richman i sur., 2015.). Modeli rasprostranjenosti vrsta, poznati i kao modeli povoljnosti staništa (eng. habitat suitability model) ili modeli ekološke niše (eng. ecological niche model)

koriste se za izradu karata potencijalne rasprostranjenosti vrsta, odnosno rasprostranjenosti za vrstu povoljnog staništa. Kroz modeliranje povoljnih staništa, korištenjem podataka o prisutnosti vrsta i niza okolišnih varijabli te različitih scenarija klimatskih promjena, procjenjuje se potencijalna trenutna ili buduća rasprostranjenost vrsta. Ovaj pristup omogućuje definiranje okolišno povoljnih i nepovoljnih područja za ugrožene vrste na temelju kojih se mogu izdvojiti prioritetna područja za konzervaciju. Ona obuhvaćaju područja koja će se naći pod najvećim pritiskom i na koja će trebati usmjeriti posebne mjere zaštite i upravljanja, kao i područja koja će moći poslužiti u budućim planovima upravljanja kao potencijalni refugiji bitni za opstanak vrsta, istovremeno uzimajući u obzir utjecaj invazivnih stranih vrsta i klimatskih promjena (Jiménez-Valverde i sur., 2011.; Guisan i sur., 2013., 2014.). Izrada modela povoljnosti staništa za proučavane vrste te njihova projekcija u budućnost prema različitim scenarijima klimatskih promjena omogućuje identifikaciju područja koja će biti izrazito nepovoljna za njihovo preživljavanje, kao i područja koja će osigurati dugotrajni opstanak vrste. Također, na temelju preklapanja podataka o genetskoj varijabilnosti vrsta s podacima o njihovoj potencijalnoj budućoj rasprostranjenosti, kao i s budućom potencijalnom rasprostranjenošću stranih invazivnih vrsta pod utjecajem klimatskih promjena, možemo identificirati populacije i područja koja imaju najveću konzervacijsku vrijednost i kojima treba dati najveći prioritet u zaštiti. Modeli rasprostranjenosti vrsta korišteni za istraživanje posljedica utjecaja klimatskih promjena i invazivnih vrsta, predviđaju velike gubitke povoljnih staništa za europske vrste slatkovodnih rakova do kraja ovog stoljeća (Capinha i sur., 2013.; Ghia i sur., 2013.; Markovic i sur., 2014.; Chucholl i sur., 2016., 2017.; Piyapong i sur., 2020.; Préau i sur., 2020.).

1.4. Plemeniti rak (*Astacus astacus*)

1.4.1. Geografska rasprostranjenost, ekologija, morfologija, filogenija, ugroženost i zaštita

Plemeniti rak je najrasprostranjenija europska vrsta porodice Astacidae. Prirodno je rasprostranjen širom Europe, od Skandinavije na sjeveru, do Francuske na jugozapadu, Rusije na istoku te Grčke na jugu (Kouba i sur., 2014.). Zbog visokocijenjene vrijednosti kao konzumne vrste često je prenošen i izvan prirodnog areala. U Hrvatskoj prirodno obitava u rijekama, potocima i jezerima dunavskog sliva, ali je unesen i u slatkovodna staništa jadranskog sliva, stoga se može naći u kontinentalnoj, alpinskoj i mediteranskoj biogeografskoj regiji (Maguire i sur., 2018.). Nastanjuje rijeke, potoke i jezera s ilovastim,

pjeskovitim i šljunkovitim dnom, s puno zaklona i razvijenom obalnom vodenom vegetacijom te visokom koncentracijom kisika u vodi.

Plemeniti rakovi narastu do ukupne dužine tijela do 15 cm. Karakteristično obojenje tijela je varijabilno, obično s leđne strane tamnosmeđe (maslinasto zelena do crna, ponekad plavičasta ili crvenkasta), a s trbušne strane zeleno-smeđe boje. Od vrsta roda *Austropotamobius* razlikuju se po dva para postorbitalnih grebena na gornjoj strani karapaksa, što je ujedno i karakteristika cijelog roda *Astacus*. Karapaks je gladak bez trnova s malim granuliranim izbočenjima iza cervikalne brazde (red sitnih izbočenja obično sa samo jednim jače izraženim tupim trnom). Rostrum im je dobro razvijen i najčešće ravan s glatkim rubovima (strane su paralelene ili trapezoidne), a apeks rostruma je istaknut i dosta dugačak.

Istraživanjima mitohondrijske DNA i mikrosatelitnih lokusa plemenitog raka ustanovljeno je postojanje najveće genetske raznolikosti u jugoistočnoj Europi gdje su populacije ove vrste preživjele pleistocenske oledbe u glacijalnim refugijima te bile izvorište rekolonizacije europskih voda nakon oledbi (Schrimpf i sur. 2014; Laggis i sur. 2017). Utvrđeno je postojanje šest mitohondrijskih linija koje su se diverzificirale zahvaljujući preživljavanju populacija u izoliranim glacijalnim refugijima na području zapadnog i južnog dijela Balkana (Schrimpf i sur., 2017.; Laggis i sur., 2017.) te zapadnog dijela crnomorskog bazena (Schrimpf i sur., 2017.). U dosadašnjim je istraživanjima bio uključen mali broj uzoraka iz Hrvatske koji su se izdvojili u zasebnu liniju s visokom genetskom raznolikošću. Današnja rasprostranjenost i filogeografska struktura ove vrste oblikovana je geološko-klimatskim događajima i utjecajima čovjeka (translokacije, onečišćenje, utjecaj stranih invazivnih vrsta rakova). Naime, plemeniti rak predmet je razmjene i trgovine već više od 2000 godina (Skurdal i Taugbøl, 2002.). Farme slatkovodnih rakova postoje i danas u mnogim europskim zemljama, naročito u središnjoj i sjevernoj Europi (Jussila i sur., 2021.). Spomenuta razmjena i trgovina te pokušaji obnavljanja prirodnih populacija rezultirali su antropogenim translokacijama koje su doprinijele uništavanju i/ili promjeni prirodne genetske strukture i rasprostranjenosti ove vrste (Schrimpf i sur., 2014.).

Smanjenje populacija plemenitog raka zabilježeno je diljem Europe (Jussila i sur., 2021.). Usporedbom povijesnih i recentnih podataka ustanovljeno je da je 55 % hrvatskih populacija plemenitog raka izgubljeno (Maguire i sur.. 2018.). Ova je vrsta ugrožena regulacijom vodenih tokova, velikim količinama otpadnih tvari u vodenim ekosustavima te

širenjem invazivnih stranih vrsta rakova koje su vektori širenja račje kuge, od koje plemeniti rakovi ugibaju, stoga je proglašen ugroženom vrstom te zaštićen nacionalnim i međunarodnim zakonima. Na nacionalnoj razini vrsta je zaštićena Zakonom o zaštiti prirode (NN 80/13) i Pravilnikom o strogo zaštićenim vrstama (NN 144/13), dok je u sklopu zakonske zaštite na međunarodnoj razini uvrštena na Dodatak III Konvencije o zaštiti europskih divljih vrsta i prirodnih staništa (Bernska konvencija) te na Dodatak V Direktive o zaštiti prirodnih staništa i divlje faune i flore. Plemeniti rak ima status osjetljive vrste (eng. vulnerable, VU) na Crvenom popisu rakova slatkih i bočatih voda Hrvatske (Gottstein i sur., 2011.) i na Crvenoj listi ugroženih svojti Međunarodne unije za očuvanje prirode (IUCN). Također, prema Članku 17 Direktive o staništima, status plemenitog raka u tri biogeografske regije u Hrvatskoj označen je kao nepovoljan-neodgovarajuć (https://nature-art17.eionet.europa.eu/article17/).

1.4.2. Pregled istraživanja morfološke i genetske raznolikosti plemenitog raka

1.4.2.1. Pregled dosadašnjih istraživanja morfološke raznolikosti plemenitog raka

Prva istraživanja o taksonomiji i biogeografiji plemenitog raka temeljena su na morfološkim karakteristika vrste (Karaman, 1929.; Bott, 1950., 1972.; Karaman, 1962., 1963.; Albrecht, 1983.).

Sint i sur. (2005.) istraživali su morfološku varijabilnost plemenitog i bjelonogog raka u austrijskoj pokrajini Tirol korištenjem velikog broja morfometrijskih značajki u kombinaciji s multivarijatnim statističkim metodama. U istraživanju su koristili 21 morfometrijsku značajku kako bi utvrdili postoje li morfološke razlike između različitih populacija te određivanja koje morfometrijske značajke najbolje doprinose njihovom razlikovanju. Rezultati su pokazali da postoje razlike u morfometrijskim značajkama na razini spola, različitih populacija i geografskih regija te su utvrđene značajke koje najviše doprinose razlikovanju različitih populacija. Ovo istraživanje poslužilo je kao temelj budućim istraživanjima morfološke varijabilnosti slatkovodnih rakova (Sint i sur., 2006., 2007.; Maguire i Dakić, 2011.; Vlach i Valdmanová, 2015.; Rudolph i sur., 2016.; Maguire i sur., 2017.; Mijošek i sur., 2017.; Đuretanović i sur., 2017.; Berger i sur., 2018.).

Sint i sur. (2007) su u svom istraživanju fenotipske karakterizacije bjelonogog, plemenitog i potočnog raka koristili 21 morfometrijsku značajku u kombinaciji s hijerarhijskom klaster analizom i diskriminatnom analizom. Rezultati su pokazali jasno razdvajanje vrsta i populacija u morfometrijskim značajkama te ukazali da geografski bliže

populacije posjeduju sličnije morfometrijske značajke za razliku od geografski udaljenih populacija. Potvrdili su uspješno korištenje morfometrijskih metoda u karakterizaciji različitih populacija slatkovodnih rakova, čiji se rezultati mogu koristiti za identifikaciju potencijalnih donorskih populacija za programe reintroducije i/ili jedinica upravljanja. Proporcije glavopršnjaka i kliješta pokazale su se kao najvažniji čimbenik razdvajanja intraspecijskih grupa.

Đuretanović i sur. (2017.) su analiziranjem morfometrijske varijabilnosti plemenitog raka iz različitih regija Balkanskog poluotoka ustanovili postojanje razlika između populacija iz različitih slatkovodnih ekosustava, koje odražava njihovu geografsku udaljenost. Morfometrijske značajke koje najviše doprinose razdvajanju ženki iz različitih populacija su bile dužina abdomena, širina rostruma, ukupna dužina te dužina i širina kliješta, dok razdvajanju mužjaka iz različitih populacija najviše doprinose težina, širina glave te širina karapaksa i dužina kliješta.

1.4.2.2. Pregled dosadašnjih istraživanja genetske raznolikosti plemenitog raka

Dosadašnja istraživanja genetske raznolikosti i populacijske strukture plemenitog raka koristila su proteinske (alozimi) i molekularne biljege (mitohondrijski geni za *COI* i *16S* rRNA) te manji ili veći broj mikrosatelitnih lokusa. Rana istraživanja genetske raznolikosti plemenitog raka temeljila su se na elektroforezi proteina (alozima), međutim pokazala su nisku razinu varijabilnosti i mali broj polimorfnih lokusa (Fevolden i Hessen, 1989.; Agerberg, 1990.; Fevolden i sur., 1994.). Prve molekularne biljege za procjenu genetske varijabilnosti populacija plemenitog raka upotrijebili su Schulz (2000.) i Edsman i sur. (2002.).

Schulz (2000) je istraživao genetsku raznolikost njemačkih populacija plemenitog raka primjenom analize nasumično umnožene polimorfne DNA – RAPD (eng. random amplified polymorphic DNA) koja je omogućila dobro razdvajanje populacija s obzirom na pripadnost različitim „stockovima“.

Edsman i sur. (2002.) su analiziranjem varijacija u veličini mikrosatelita unutarnje transkribirajuće razmaknice 1 – *ITS1* (eng. internal transcribed spacer 1) ribosomalne DNA detektirali genetsku raznolikost i diferencijaciju između švedskih populacija plemenitog raka. Nadalje, Alaranta i sur. (2006.) analizirali su genetsku diferencijaciju populacija plemenitog raka iz Finske, Švedske i Estonije korištenjem navedene *ITS1* regije. Međutim, kasnijim

istraživanjem ustanovljeno je da je *ITS1* regija dio gena s više kopija i ne može biti tretirana kao kodominantni Mendelov biljeg; intragenomska varijabilnost regije *ITS1* u slatkovodnih rakova ograničava njenu upotrebu za detaljnije filogenetske rekontrukcije (Harris i Crandall, 2000.).

Kõiv i sur. (2008., 2009.) su razvili prve vrsno specifične mikrosatelitne lokuse za plemenitog raka koji su kasnije korišteni u istraživanjima genetske raznolikosti i populacijske strukture (Gross i sur., 2013.; Schrimpf i sur., 2014.; Blaha i sur., 2016.; Laggis i sur., 2017.). Međutim, broj upotrebljivih lokusa u ovim istraživanjima bio je prilično nizak (šest do 10 lokusa) zbog nedosljednog umnažanja te mnogo dodatnih fragmenata (eng. sttuter bands) i fragmenata nastalim nespecifičnim umnažanjem što je otežavalo pouzdanu genotipizaciju (Gross i sur., 2017.).

Schrimpf i sur. (2011.) su proveli prvo opsežno filogeografsko istraživanje plemenitog raka na temelju kratkog fragmenta mitohondrijskog gena za *COI*. Istraživanje su proveli s ciljem utvrđivanja genetske strukture i raznolikosti plemenitog raka na području središnje i jugoistočne Europe koje obuhvaća crnomorski, sjevernomorski i baltički sliv. Rezultati su pokazali veliku raznolikosti haplotipova u jugoistočnoj Europi (crnomorski sliv) u usporedbi s niskom raznolikosti haplotipova u središnjoj Europi (sjevernomorski i baltički sliv). Velika haplotipska raznolikost i broj privatnih haplotipova u populacijama crnomorskog sliva upućivali su na postojanje glacijalnih refugija na području Balkana. Dominantna prisutnost haplotipa H01 u istraživanom području i niska haplotipska raznolikost indiciraju rekolonizaciju iz područja Balkanskog poluotoka prema središnjoj Europi.

Gross i sur. (2013.) su proveli prvu populacijsko-genetičku analizu plemenitog raka na području baltičkog i crnomorskog sliva koristeći mikrosatelitne biljege. Rezultati su ukazali na postojanje dvije visoko diferencirane grupe populacija koje odgovaraju navedenim slivovima. Populacije baltičkog sliva bile su manje genetski varijabilne i s manjim brojem privatnih alela u usporedbi s populacijama crnomorskog sliva.

Schrimpf i sur. (2014.) su napravili veliko istraživanje genetske raznolikosti i filogeografske povijesti plemenitog raka na području sjevernomorskog, baltičkog, crnomorskog, jadranskog i egejskog sliva. Molekularnim analizama mitohondrijske (*COI, 16S*) i nuklearne (mikrosateliti) DNA je utvrđeno da je najveća genetska raznolikost ove vrste u jugoistočnoj Europi, gdje su populacije preživjele pleistocenske oledbe u glacijalnim

refugijima te poslužile za rekolonizaciju europskih voda nakon oledbi. Rekonstrukcijom filogenetskih odnosa ustanovili su postojanje četiri evolucijske linije koje su se diverzificirale tijekom pleistocena. Uz pomoć migracijskih modela otkrili su da su kolonizacije sjevernomorskog i baltičkog sliva tekle neovisno jedna o drugoj putem različitih migracijskih putova iz istočnog crnomorskog sliva. Područje zapadnog Balkana nije doprinijelo spomenutim kolonizacijama, ali je vjerojatno doprinijelo kolonizaciji donjeg dijela dunavskog sliva. U kasnijem istraživanju Schrimpf i sur. (2017.) su predložili četiri jedinice upravljanja za očuvanje genetske raznolikosti plemenitog raka na području zapadne Europe korištenjem mitohondrijske DNA i mikrosatelita.

Makkonen i sur. (2015.) su istraživali genetsku raznolikost populacija plemenitog raka na području Finske i Estonije korištenjem mitohondrijskog gena za *COI*. Ustanovili su postojanje samo jednog haplotipa na cijelom području istraživanja što ukazuje na izuzetno nisku genetsku raznolikost u sjevernim dijelovima njegovog areala. Uočena niska raznolikost može biti rezultat male veličina populacije utemeljitelja, antropogenih translokacija, širenja invazivnih stranih vrsta rakova koji nose račju kugu i uništenja originalne populacije zbog epidemije račje kuge.

Bláha i sur. (2016.) su istraživali efekt utemeljitelja i njegov utjecaj na genetsku raznolikost translociranih populacija plemenitog raka, 10 godina nakon unosa. Korištenjem mikrosatelitnih lokusa otkrili su nisku genetsku raznolikost izvorišne populacije te visok stupanj parenja u srodstvu što ukazuje na njenu dugotrajnu izoliranost i malu veličinu. Unatoč tome, translocirana populacija nije pokazivala značajno smanjenje genetske raznolikosti u odnosu na izvorišnu populaciju. Iako je izvorišna populacija imala nisku raznolikost, dovoljno je raznolika da se može koristiti u konzervacijske svrhe i programe reintrodukcije i/ili repopulacije.

Skuza i sur. (2016.) su analizirali genetsku raznolikost plemenitog raka na području Poljske korištenjem mitohondrijskih gena (*COI* i *16S*). U istraživanom području ustanovili su postojanje dva *COI* haplotipa i jednog *16S* haplotipa.

Laggis i sur. (2017.) su koristili mitohondrijsku DNA (*16S* i *COI*) za istraživanje filogenetskih odnosa populacija plemenitog raka te mikrosatelitne lokuse za analiziranje genetske strukture i demografske povijesti plemenitih rakova u Grčkoj. Ustanovili su veliku genetsku raznolikost grčkih populacija plemenitog raka i njihovu jasnu odvojenost od ostalih

europskih populacija. Filogenetske analize otkrile su postojanje šest evolucijskih linija plemenitog raka u Europi, od kojih su dvije evolucijske linije sadržavale isključivo grčke haplotipove i identificirane su prvi put, dok su haplotipovi u ostalim linijama identični s prethodno opisanima u Schrimpf i sur. (2014.). Procjena vremena odvajanja ukazuje na divergenciju grčkih mitohondrijskih linija tijekom pleistocena što potvrđuje postojanje južnog glacijalnog refugija (Grčka, južni Balkan). Analiza mikrosatelitnih lokusa ustanovila je prostorno strukturiranje genetske raznolikosti na istraživanom području i postojanje šest do devet genetskih grupa te osam potencijalnih genetskih barijera.

Gross i sur. (2017.) su razvili nove tetranukleotidne mikrosatelitne biljege pogodne za populacijsko-genetičke analize plemenitog raka te otkrivanje hibrida plemenitog i uskoškarog raka. Multipleks reakcija sastavljena od dobroumnažajućih i lako genotipizirajućih 19 mikrosatelitnih lokusa omogućila je istraživanje genetske strukture, analizu srodnosti, identifikaciju i genetsko upravljanje „stockovima“ (izbor donorskih populacija, planiranje parenja, izbjegavanje razmnožavanja u srodstvu).

Mrugała i sur. (2017.) istražuju rasprostranjenost slatkovodnih rakova u dotad neistraženom području Balkanskog poluotoka (Albanija) te analiziraju genetsku raznolikost plemenitog raka upotrebom gena za *COI*.

Panicz i sur. (2019.) su istraživali genetsku raznolikost i strukturu prirodnih populacija plemenitog raka na sjeverozapadu Poljske na temelju analize mikrosatelitnih lokusa i polimorfizma dužine umnoženih fragmenata - AFLP (eng. amplified fragment length polymorphism).

Dannewitz i sur. (2020.) su istraživali genetsku strukturu plemenitog raka na području Švedske, Norveške i Finske, te su rekonstruirali postglacijalnu kolonizaciju s juga prema baltičkom slivu i proučavali utjecaj antropogenih translokacija na genetsku strukturu populacija. Analiziranjem većeg broja mikrosatelitnih lokusa otkrili su tri genetske grupe populacija koje odgovaraju sjeveru, sredini i jugu Fenoskandije. Među trima grupama, najveća genetska raznolikost zabilježena je u južnoj, koja je ujedno genetski slična populacijama središnje Europe. Uočena genetska struktura odražava dvije postglacijalne kolonizacijske rute baltičkog bazena iz glacijalnih refugija na jugoistoku Europe i prošle antropogene translokacije plemenitog raka.

1.5. Potočni rak (*Austropotamobius torrentium*)

1.5.1. Geografska rasprostranjenost, ekologija, morfologija, filogenija, ugroženost i zaštita

Potočni rak je najmanja vrsta porodice Astacidae. Prirodno je rasprostranjen u središnjoj i jugoistočnoj Europi. Areal mu se proteže od Njemačke i Češke na sjeveru, Luksemburga na zapadu, Grčke na jugu te Turske i Bugarske na istoku (Kouba i sur., 2014.). Nastanjuje vodotoke na višim nadmorskim visinama te je u Hrvatskoj rasprostranjen u rijekama crnomorskog i jadranskog sliva (Maguire i sur., 2018.). Prilagođen je na uvjete u hladnim vodotocima te je njegova rasprostranjenost u Hrvatskoj usko povezana s krškim reljefom (Klobučar i sur., 2013.). Prirodna staništa vrste nalaze se u izvorišnim te gornjim dijelovima manjih lotičkih sustava prosječne godišnje temperature vode do 10 °C, s koncentracijom kisika iznad 4 mg/L, brzog strujanja i kamenog supstrata (Maguire i sur., 2002.; Maguire i Gottstein – Matočec, 2004.). Jedinkama odgovaraju staništa s puno zaklona i razvijenom obalnom vodenom vegetacijom. Ova vrsta je izrazito osjetljiva na onečišćenje i modifikacije vodotoka i preferira staništa visoke kvalitete vode (Machino i Füreder, 2005.).

Potočni rakovi narastu do ukupne dužine tijela do 11 cm, maksimalno do 15 cm (mužjaci dosegnu duljinu tijela od 8 do 10,5 cm, a ženke 6 do 9 cm). Od vrsta roda *Astacus* razlikuju se po samo jednom paru postorbitalnih grebena na gornjoj strani karapaksa, što je ujedno karakteristika cijelog roda *Austropotamobius*. Od druge vrste roda *Austropotamobius*, bjelonogog raka, razlikuje se po nedostatku trnova iza cervikalne brazde. Karakteristično obojenje tijela je varijabilno, tamnosmeđe do maslinastozeleno, uz rjeđu pojavnost svjetlije ili plave nijanse. Rostrum je najčešće oblika jednokostraničnog trokuta, uz postojeće intraspecijske varijacije u dužini i obliku vršnog dijela (apeks rostruma).

Rezultati molekularno-filogenetičkih istraživanja roda *Austropotamobius* pokazali su da je vjerojatno mjesto nastanka ovog roda bio sjeverozapad Balkanskog poluotoka, odakle su se dvije vrste ovog roda proširile u područje današnjeg areala (Trontelj i sur., 2005.; Klobučar i sur., 2013.; Jelić i sur., 2016.). Alpska i dinaridska orogeneza tijekom srednjeg i kasnog miocena smatraju se uzrokom filogeografskog razdvajanja ancestralne populacije koja je, naposljetku, dovela do nastanka dviju različitih vrsta; potočnog raka sa sjeverne strane Dinarida i bjelonogog raka s južne strane (Trontelj i sur., 2005., Klobučar i sur., 2013.). Istraživanjima mitohondrijskih gena potočnog raka utvrđeno je postojanje sedam geografski i

genetski izoliranih filogrupa, od kojih je šest rasprostranjeno u slatkovodnim ekosustavima Hrvatske, s najvećim brojem linija i raznolikošću u vodotocima sjeverno-središnjih Dinarida (Klobučar i sur., 2013.). Ustanovljena velika genetska raznolikost i filogeografski uzorak ove vrste smatraju se rezultatom procesa okršavanja Dinarida koje je uzrokovalo fragmentaciju paleohidrografske mreže i geografsku izolaciju te omogućilo uvjete za alopatrijsku specijaciju (Trontelj i sur., 2005., Klobučar i sur., 2013.). Također, današnja rasprostranjenost i genetska struktura potočnog raka, rezultat je pleistocenskih klimatskih promjena te brze postglacijalne rekolonizacije središnje Europe iz glacijalnih refugija kroz dunavski sliv.

Nedavnim istraživanjima u Europi i Hrvatskoj, kao i kod ostalih nativnih vrsta, zabilježen je značajni pad broja jedinki potočnog raka (Maguire i sur., 2018.). Smatra se da je 29 % populacija potočnog raka u Hrvatskoj izgubljeno tijekom posljednjeg stoljeća. Njegova su prirodna staništa često izolirana te nakon nestanka lokalne populacije, ne dolazi do prirodne rekolonizacije (Maguire i sur., 2018.). Uzroci smanjenja broja populacija su mnogobrojni, a najčešći je negativni antropogeni utjecaj na staništa ove vrste, klimatske promjene i invazivne strane vrste rakova koje prenose bolest račju kugu. Potočni rak je vrsta osjetljiva na promjene u kvaliteti i temperaturi vode, stoga onečišćenja i klimatske promjene koje se manifestiraju kao periodi ekstremne i dugotrajne suše, predstavljaju izazov za preživljavanje populacija ove vrste (Kouba i sur., 2016.).

Potočni rak je ugrožena vrsta, zaštićena nacionalnim i međunarodnim zakonima. Na nacionalnoj razini zaštićena je Zakonom o zaštiti prirode (NN 80/13) i Pravilnikom o strogo zaštićenim vrstama (NN 144/13) te je uvrštena u Crveni popis rakova slatkih i bočatih voda Hrvatske kao osjetljiva vrsta (eng. vulnerable, VU) (Gottstein i sur., 2011.). Na međunarodnoj razini, potočni rak nalazi se na Crvenom popisu ugroženih svojti Međunarodne unije za očuvanje prirode (IUCN) u kategoriji nedovoljno istražene vrste (eng. data deficient, DD) (Füreder i sur., 2010.). Također, uvršten je u Dodatak III Konvencije o zaštiti europskih divljih vrsta i prirodnih staništa i u Dodatak II i Dodatak V Direktive o zaštiti prirodnih staništa i divlje faune i flore (Council directive 92/43/EEC, 2000.). Prema Članku 17 Direktive o staništima, status potočnog raka u tri biogeografske regije u Hrvatskoj označen je kao nepovoljan-neodgovarajuć (https://nature-art17.eionet.europa.eu/article17/).

Dosadašnjim su istraživanjima prikupljeni podaci o biologiji, ekologiji i morfološkoj varijabilnosti potočnog raka u Hrvatskoj. Također, utvrđena je njegova detaljna rasprostranjenost te činjenica da se broj populacija značajno smanjio u posljednjem stoljeću

(Maguire i Gottstein Matočec, 2004., Maguire i sur., 2011., Maguire i sur., 2018.). Nadalje, dosadašnja su istraživanja uključila i analizu molekularno-filogenetskih i filogeografskih odnosa roda *Austropotamobius* (Trontelj i sur., 2005.; Klobučar i sur., 2013.). Do danas nisu provedena istraživanja genetske strukture populacija potočnog raka, a čiji bi rezultati pomogli u identifikaciji vrućih točaka genetske raznolikosti na hrvatskoj, odnosno europskoj razini.

1.5.2. Pregled istraživanja morfološke i genetske raznolikosti potočnog raka

1.5.2.1. Pregled dosadašnjih istraživanja morfološke raznolikosti potočnog raka

Morfometrijska mjerenja često se koriste za istraživanje morfološke varijabilnosti slatkovodnih rakova (Grandjean i sur., 1997.; Grandjean i Souty-Grosset, 2000.; Sint i sur., 2005.; Sint i sur., 2006.; Sint i sur., 2007.; Maguire i Dakić, 2011.; Vlach i Valdmanová, 2015.; Đuretanović i sur., 2017.; Mijošek i sur., 2017.; Maguire i sur., 2017.; Berger i sur., 2018.; Pârvulescu, 2019). Danas se morfometrijska analiza koristi i u integriranom pristupu konzervaciji kako bi se ustanovila morfološka kompatibilnost donorskih populacija. Uz tradicionalnu i geometrijsku morfometriju, u istraživanjima morfologije slatkovodnih rakova koriste se i merističke značajke, kao što je broj trnova na merusu trećeg maksilipeda ili prisutnost medijane rostralne kriste (Füreder i Machino, 2002.; Harlioğlu, 2002.; Karaman, 1961., 1962.; Trontelj i sur., 2005.).

Morfologija vrsta iz roda *Austropotamobius* često je proučavana, međutim većina morfoloških istraživanja odnosi se na bjelonogog raka, dok je na potočnom raku proveden manji broj istraživanja. Istraživanja morfološke raznolikosti potočnog raka do sada su bila temeljena većinom na tradicionalnim morfometrijskim i merističkim značajkama. Prva istraživanja morfološke raznolikosti potočnog raka napravljena su u svrhu razlikovanja različitih populacija i podvrsta na temelju morfometrijskih i merističkih značajki (Karaman, 1929.; Bott, 1950.; Karaman, 1961., Albrecht, 1982.; Starobogatov, 1996.). Međutim, napravljena su korištenjem malog broja jedinki i morfoloških značajki koje nisu bile dovoljno pouzdane za razlikovanje različitih populacija (Holdich i sur., 2002.). Trenutno su prihvaćene tri podvrste roda *Austropotamobius* na temelju morfologije, ali nisu potvrđene molekularnim podacima: *A. t. torrentium*, *A. t. danubicus* i *A. t. macedonicus* (Crandall i De Grave, 2017.).

Trontelj i sur. (2005.) su prilikom istraživanja molekularno-filogenetskih odnosa roda *Austropotamobius* uočili izraženu medijanu rostranu kristu kao dijagnostičku značajku za razlikovanje divergentne mitohondrijske filogrupe rasprostranjene u dijelu sjevernih Dinarida.

Sint i sur. (2005.) su istraživali morfološku raznolikost europskih vrsta rakova primjenom multivarijantne statističke analize. U svom istraživanju koristili su 21 tradicionalnu morfometrijsku značajku za razlikovanje populacija bjelonogog i plemenitog raka iz austrijske pokrajine Tirol. Kasnije su iste morfometrijske značajke koristili za istraživanje morfološke varijabilnosti miješane populacije bjelonogog i potočnog raka (Sint i sur., 2006.). Rezultati su pokazali dobro odvajanje ovih dviju vrsta u morfološkim karakteristikama te su pronađene jedinke koje bi mogle predstavljati interspecijske hibride. Zatim su napravili genotipsku karakterizaciju triju vrsta slatkovodnih rakova, bjelonogog, potočnog i plemenitog raka (Sint i sur., 2007.). Primjena hijerarhijske klaster analize i diskriminantne analize omogućila je razlikovanje vrsta te populacija iste vrste, koje je odraz geografske udaljenosti. Nadalje, na temelju ustanovljene morfologije, identificirali su jedinice upravljanja.

Vlach i Valdmanová (2015.) istraživali su alometriju i spolni dimorfizam potočnog raka u Češkoj. Multivarijantnom analizom tradicionalnih morfometrijskih značajki ustanovili su postojanje značajnih razlika između spolova čime je potvrđeno postojanje spolnog dimorfizma u potočnih rakova. Također, ustanovili su da razlikovanju mužjaka i ženki najviše doprinose morfometrijske značajke abdomena, karapaksa i kliješta. Uočili su da, nakon postizanja spolne zrelosti, većina tijela raste alometrijski; pozitivna alometrija ustanovljena je u dužini karapaksa kod mužjaka, širini karapaksa i abdomena te omjeru dužine i širine kliješta ženki. Negativni alometrijski rast zabilježen je u duljini rostro-cervikalnog dijela karapaksa, širini i visina kliješta.

Dakić i Maguire (2016.) su istraživale životni ciklus i morfometrijske karakteristike potočnog raka u dvije krške rijeke u Hrvatskoj. Rezultati su pokazali da se mužjaci i ženke značajno razlikuju u svim mjerenim morfometrijskim obilježjima i težini tijela, dok se jedinke iz dviju populacija međusobno značajno razlikuju u širini karapaksa, dužini, širini i debljini kliješta kod mužjaka te ukupnoj dužini tijela, širini karapaksa, debljini kliješta i širini prve abdominalne pleure kod ženki. Pretpostavljaju da su zabilježene morfometrijske razlike između dviju populacija posljedica adaptacije na specifične stanišne i ekološke uvjete.

Maguire i sur. (2017.) istraživali su morfološku varijabilnost potočnog raka multivarijantnom analizom morfometrijskih i merističkih značajki populacija u Parku prirode Žumberak-Samoborsko gorje u Hrvatskoj. Istraživanjem su utvrdili postojanje morfoloških razlika između jedinki koje pripadaju trima prethodno otkrivenim mitohondrijskim

filogrupama. Morfometrijske karakteristike koje su doprinosile razdvajanju mužjaka iz različitih filogrupa su kliješta, karapaks i rostrum, a kod ženki karapaks, rostrum i ukupna dužina. Iako su se populacije razlikovale morfološki i genetski, nije utvrđena značajna korelacija između genetskih i morfometrijskih udaljenosti.

Berger i sur. (2018.) primijenili su integrativni pristup u konzervaciji potočnog raka na temelju istraživanja genetske i morfološke raznolikosti kako bi identificirali potencijalne donorske populacije za buduće repopulacije i reintrodukcije.

Pârvulescu (2019.) je opisao novu vrstu roda *Austropotamobius* na temelju molekularno-filogenetičkih analiza i morfologije. Vrsta *Austropotamobius bihariensis* označena je kao endem planinskog masiva Apuzeni u Rumunjskoj, a uočene morfološke karakteristike koje razlikuju ovu vrstu od ostalih su nedostatak dentikulacije na donjem rubu antenalnog egzopoda, kratki rostrum zvonolikog oblika i manji broj tuberkula na kliještima.

1.5.2.2. Pregled dosadašnjih istraživanja genetske raznolikosti potočnog raka

Dosadašnja istraživanja genetske raznolikosti potočnog raka temeljena su na analizi mitohondrijskih gena za citokrom c oksidazu podjedinica I (*COI*) i *16S* rRNA (Trontelj i sur., 2005.; Schubart i Huber, 2006.; Klobučar i sur., 2013.; Berger i sur., 2018.; Pârvulescu i sur., 2019.) te mikrosatelitnih lokusa iz blisko srodnih vrsta (Iorgu i sur., 2011.; Berger i sur., 2018.; Pârvulescu i sur., 2020.) ili korištenjem malog broja vrsno specifičnih mikrostatelita (Vorburger i sur., 2014.).

Trontelj i sur. (2005.) su proveli prvo istraživanje molekularno-filogenetskih odnosa vrsta roda *Austropotamobius* na većem dijelu njihovog areala korištenjem mitohondrijskog gena *COI*. Analize su otkrile veliku genetsku raznolikost na području sjeverozapada Balkanskog poluotoka, koji se smatra mjestom nastanka i centrom radijacije ovog roda, odakle su se dvije vrste diverzificirale i proširile u područje današnjeg areala. Alpska i dinaridska orogeneza tijekom srednjeg i kasnog miocena smatraju se uzrokom filogeografskog odvajanja ancestralne populacije roda *Austropotamobius* koja je, naposljetku, dovela do nastanka dviju različitih vrsta, bjelonogog raka i potočnog raka. Naredna tektonska zbivanja stvorila su niz novih staništa i uvjetovala daljnja razdvajanja genetskih linija i migracije populacija. Također, ustanovljeno je postojanje triju odvojenih genetskih linija unutar potočnog raka, linija na području gornjeg dijela rijeke Kupe (dinaridski krš na granici

Hrvatske i Slovenije), linija na području južnog Balkana, i linija koja obuhvaća populacije s ostatka areala.

Schubart i Huber (2006.) istraživali su genetsku raznolikost potočnog raka u Njemačkoj te proširili istraživanje od Trontelj i sur. (2005.) sekvenciranjem dodatnog molekularnog biljega, mitohondrijskog gena za *16S* rRNA. Na temelju analize mitohondrijskih gena *COI* i *16S* rRNA potvrdili su da je genetska raznolikost potočnog raka u središnjoj Europi, sjeverno od Alpa uvelike smanjena i homogena u odnosu na veliku genetsku raznolikost južno od Alpa i na području Balkana. Uočeni obrazac povećane genetske raznolikosti južnih populacija te postepeno opadanje genetske raznolikosti prema sjevernijim populacijama objašnjavaju postglacijalnim širenjem i genetskim uskim grlom (eng. genetic bottleneck) koji se dogodio tijekom brze rekolonizacije središnje Europe iz južnih glacijalnih refugija.

Iorgu i sur. (2011.) su testirali upotrebu mikrosatelitnih lokusa razvijenih za druge vrste deseteronožnih rakova za potrebe istraživanja genetske raznolikosti i populacijske strukture potočnog raka. Od velikog broja testiranih lokusa (55), uspješno su umnožili pet polimorfnih mikrosatelitnih lokusa, uz zabilježena odstupanja od Hardy-Weinbergove ravnoteže i prisutnost nul alela.

Klobučar i sur. (2013.) su istraživali filogeografiju i filogeniju potočnog raka u svrhu objašnjavanja utjecaja paleogeologije dinaridskog krša i okršavanja u oblikovanju evolucijske povijesti i genetske raznolikosti slatkovodnih vrsta na području zapadnog Balkana. Na temelju analize mitohondrijskih gena za *COI* i *16S* rRNA utvrdili su postojanje velike genetske raznolikosti, prisutne u obliku sedam geografski i genetski izoliranih monofiletskih filogrupa, od kojih je šest rasprostranjeno u slatkovodnim ekosustavima Hrvatske, s najvećim brojem linija i raznolikošću u sjeverno-središnjoj dinaridskoj regiji (eng. northern - central Dinaric region, NCD). Filogrupe su nazvane prema geografskim područjima na kojima su populacije rasprostranjene: Banovina (BAN), Središnja i jugoistočna Europa (CSE), Gorski kotar (GK), Lika i Dalmacija (LD), južni Balkan (SB), Zeleni vir (ZV) te Žumberak, Plitvice i Bjelolasica (ŽPB). Velike vrijednosti genetskih udaljenosti i nepostajanje jasnih razlika u morfologiji između jedinki različitih filogrupa ukazali su na moguće postojanje kriptičnih vrsta. Također, autori su istaknuli da bi ove visoko divergentne linije trebale imati prioritet u konzervaciji vrste jer nastanjuju vrlo malo geografsko područje.

Vorburger i sur. (2014.) su analizirali genetsku raznolikost i povezanost populacija potočnog raka u Švicarskoj korištenjem osam novorazvijenih mikrosatelitnih biljega, uz četiri dodatna mikrosatelitna biljega razvijenih za srodne vrste deseteronožnih rakova. Rezultati su pokazali nisku genetsku varijabilnost istraživanih populacija, ali istovremeno visoku genetsku diferenciju. Autori su zaključili da se navedeni mikrosateliti mogu koristiti u konzervaciji potočnog raka i identifikaciji potencijalnih donorskih populacija za buduće pokušaje reintrodukcije.

Petrušek i sur. (2017.) su istraživali genetsku varijabilnost i podrijetlo populacija potočnog raka u Češkoj korištenjem mitohondrijskog gena za *COI*. Molekularno-filogenetička analiza otkrila je da proučavane populacije imaju dva različita podrijetla. Jedinke iz geografski izolirane populacije na istoku Češke nose haplotip koji je prethodno utvrđen u Sloveniji i Hrvatskoj, na temelju čega zaključuju da je ova populacija vjerojatno introducirana od strane čovjeka. Suprotno tome, u većini čeških populacija prisutan je dominatni haplotip, inače široko rasprostranjen u Njemačkoj te nekoliko haplotipova koji se međusobno razlikuju u točkastoj mutaciji. Navedeni uzorak govori u prilog scenarija postglacijalna rekolonizacije te dvostrukog podrijetla potočnog raka u Češkoj, s prirodnim i antropogenim čimbenicima koji su oblikovali sadašnju raznolikost i areal ove vrste.

Berger i sur. (2018.) koristili su integrativni pristup u konzervaciji potočnog raka u Austriji, Njemačkoj i Švicarskoj temeljem molekularno-filogenetičkih, morfometrijskih i populacijsko genetičkih analiza. Rezultati su pokazali prethodno utvrđeno (Klobučar i sur., 2013.), populacije središnje Europe pokazuju nizak stupanj varijabilnosti u odnosu na jugoistočne.

Pârvulescu i sur. (2019.) otkrili su novu mitohondrijsku liniju potočnog raka na području gorja Apuzeni u Rumunjskoj koja je naknadno opisana kao nova endemska vrsta roda *Austropotamobius (Austropotamobius bihariensis)* na temelju merističkih značajki i molekularno-filogenetičkih analiza (Pârvulescu, 2019.).

1.6. Utjecaj klimatskih promjena i invazivnih vrsta na slatkovodne rakove

Slatkovodni rakovi izdvojeni su kao posebno osjetljiva skupina organizama na klimatske promjene zbog ograničene mogućnosti rasprostranjivanja. Predviđene promjene u termalnim i hidrološkim režimima reducirat će količinu klimatski povoljnih područja za rakove, uzrokujući suženja njihovog prirodnog areala i veću simpatriju s invazivnim stranim

vrstama rakova koji su nosioci bolesti račje kuge te na taj način doprinijeti daljnjem smanjenju populacija nativnih vrsta rakova diljem Europe (Capinha i sur., 2013.). Imajući na umu da je za mnoge vrste rakova, migracija u klimatski povoljna staništa ugrožena zbog ograničenih mogućnosti rasprostranjivanja i izoliranosti staništa, stručnjaci predlažu asistiranu migraciju, reintrodukciju i/ili repopulaciju kao glavne konzervacijske strategije (Souty-Grosset i Reynolds, 2009.; Olden i sur., 2011.; Capinha i sur., 2013.). Rezultati istraživanja procjene osjetljivosti slatkovodnih rakova na klimatske promjene pokazali su da je 87 % istraživanih vrsta visoko osjetljivo na klimatske promjene, 35 % ima nisku adaptivnu sposobnost, dok je 57 % visoko izloženo klimatskim promjenama (Hossain i sur., 2018.). Moguće posljedice utjecaja klimatskih promjena na rasprostranjenost slatkovodnih vrsta rakova istražene su u brojnim publikacijama, koristeći modele ekološke niše ili modele povoljnosti staništa (Liu i sur., 2011.; Capinha i sur., 2013.; Ghia i sur., 2013.; Gallardo i Aldridge, 2013.; Markovic i sur., 2014.; Chucholl, 2016., 2017.; Hossain i sur., 2018.; Yarra i sur., 2018.; Préau i sur., 2020.; Zhang i sur., 2019.; Piyapong i sur., 2020.). Tako je na primjer pomoću takvih modela potvrđeno da će populacije nativnih europskih vrsta rakova biti posebno ugrožene zbog klimatskih promjena, ali i invazivnih stranih vrsta. Capinha i sur. (2013.) istraživali su učinak klimatskih promjena i invazivnih vrsta rakova na rasprostranjenost nativnih europskih vrsta. Modeliranjem povoljnih staništa na temelju različitih klimatskih scenarija predvidjeli su veliko smanjenje areala; gubitak povoljnih staništa za plemenitog raka iznosio je 71 %, potočnog raka 52 %, uskoškarog raka 20 %, bjelonogog raka 19 % i za vrstu *A. pachypus* 31 %. Istovremeno, većina klimatski povoljnih područja za slatkovodne rakove predviđena je u vodotocima koji su nedostupni zbog prirodnih barijera i izoliranosti slatkovodnih staništa. Predviđeno je da će povoljni uvjeti za plemenitog raka ostati uglavnom unutar staništa koje trenutno nastanjuju i pristupačnih vodotokova (85 %), dok će budući povoljni uvjeti za bjelonogog, uskoškarog i potočnog raka biti unutar trenutno nenaseljenih i nedostupnih vodotokova (57 %, 58 % i 67 %, redom). Temeljem preklopa potencijalne buduće rasprostranjenosti nativnih i invazivnih stranih vrsta rakova, predvidjeli su preklapanje u njihovoj rasprostranjenosti i povoljnim staništima. Stoga, autori predlažu premještanje vrsta u klimatski povoljna područja izvan njihovog povijesnog areala u svrhu konzervacije nativnih vrsta. Ghia i sur. (2013.) su modeliranjem ekološke niše bjelonogog raka u sjevernoj i središnjoj Italiji otkrili da je povećanje temperature u najtoplijem razdoblju godine kritično za rizik od lokalnog izumiranja. Učinak klimatskih promjena i invazivnih vrsta na ugrožene slatkovodne vrste analizirali su Gallardo i Aldridge

(2013.). U svom istraživanju procjenjivali su utjecaj klimatskih promjena i rasprostranjenosti invazivnog signalnog raka na rasprostranjenost nativnog bjelonogog raka. Iako su modeliranjem predvidjeli da će obje vrste biti podložne negativnom utjecaju klimatskih promjena, procijenjeno smanjenje areala i gubitak povoljnih staništa biti će veći za signalnog raka. Štoviše, preklapanje u rasprostranjenosti obje vrste smanjit će se za 13-17 %, što može značiti smanjenje utjecaja signalnog raka na bjelonogog. Markovic i sur. (2014.) su u svom istraživanju utjecaja klime na slatkovodne vrste Europe predvidjeli da će 6 % čestih vrsta i 77 % rijetkih vrsta izgubiti 90 % svog trenutnog areala. Također, potvrdili su osjetljivost slatkovodnih rakova na buduće klimatske promjene predviđanjem gubitka areala od 22%.

Modeliranje potencijalne rasprostranjenosti i/ili ekološke niše izrađeno je u brojnim publikacijama s ciljem istraživanja utjecaja invazivnih stranih vrsta rakova na nativne europske vrste. Chucholl (2016.) je koristio multivarijatne statističke analize i modele ekološke niše za procjenu sadašnje i buduće rasprostranjenosti devet vrsta rakova (šest invazivnih stranih vrsta rakova i tri nativne vrste) u jugozapadnoj Njemačkoj. Zaključio je da su ekološke niše nativnih vrsta povezane s hladnijim temperaturama (izuzev bjelonogog raka), nižim indeksom ljudskog utjecaja i većim nagibom terena, u usporedbi s ekološkom nišom invazivnih stranih vrsta. Analize su pokazale najveće preklapanje u povoljnim staništima između signalnog raka i nativnih vrsta. Također, ustanovljeno je da signalni rak predstavlja najveću prijetnju konzervaciji nativnih vrsta rakova u Njemačkoj zbog najviše brzine širenja. Naredne godine, Chucholl (2017.) je koristio modele ekološke niše za planiranje konzervacije potočnog i plemenitog raka u jugozapadnoj Njemačkoj. Modeliranjem su otkrivene dotad nepoznate populacije bjelonogog raka u Njemačkoj te su identificirani klimatski povoljni vodotoci za buduće programe reintrodukcije. Préau i sur. (2020.) su modeliranjem ekoloških niša ugroženog bjelonogog raka i invazivnog signalnog raka u Francuskoj predvidjeli povoljna staništa ovih vrsta pod utjecajem različitih klimatskih scenarija. Identificirali su povoljna područja za konzervaciju postojećih populacija i buduće programe zaštite i/ili reintrodukcije. Modeli su pokazali preklapanje ekoloških niša bjelonogog i signalnog raka te veliko smanjenje povoljnih staništa za obje vrste. Piyapong i sur. (2020.) su u svom istraživanju procjenjivali potencijalnu rasprostranjenost nativnog bjelonogog raka te dviju invazivnih stranih vrsta, bodljobradog i signalnog raka. Istraživanje je pokazalo da je bjelonogi rak više ugrožen povišenjem temperature uslijed globalnog zatopljenja nego potencijalnom budućom rasprostranjenošću invazivnih vrsta. Autori su istaknuli važnost pronalaska okolišnih varijabli koje objašnjavaju trenutnu rasprostranjenost vrsta u predviđanju

interakcija nativnih i invazivnih stranih vrsta te predlaganje učinkovitih strategija upravljanja ovim vrstama kako bi se smanjio negativni utjecaj invazivnih vrsta.

1.7. Ciljevi i hipoteze istraživanja

Općeniti cilj ove doktorske disertacije je istražiti morfološku i genetsku raznolikost potočnog i plemenitog raka te razviti modele povoljnosti staništa koji će predvidjeti kako će klimatske promjene te prisutnost i širenje invazivnih stranih vrsta rakova utjecati na njihovu buduću rasprostranjenost i dugoročni opstanak.

Specifični ciljevi istraživanja:

1. Utvrditi morfološke značajke koje omogućuju razlikovanje jedinki različitih populacija potočnog i plemenitog raka korištenjem morfometrijskih podataka.

2. Istražiti genetsku raznolikost unutar i među populacijama potočnog i plemenitog raka upotrebom mitohondrijskih (*COI*, *16S* rRNA) i nuklearnih (*ITS2*, mikrosateliti) DNA biljega te doprinijeti novim spoznajama o njihovim srodstvenim odnosima i evolucijskoj povijesti te identificirati evolucijski bitne jedinice i jedinice upravljanja kao osnovu za razvoj strategija zaštite i upravljanja ovim ugroženim vrstama.

3. Izraditi modele povoljnosti staništa za potočnog i plemenitog raka te njihovu projekciju u budućnosti korištenjem različitih scenarija klimatskih promjena u svrhu identifikacije područja koja će biti izrazito nepovoljna za njihovo preživljavanje, kao i područja koja će osigurati njihov dugotrajni opstanak.

4. Izraditi modele potencijalno povoljnih staništa za invazivne vrste rakova u svrhu predviđanja staništa i nativnih populacija rakova koja će biti pod njihovim najvećim pritiskom u budućnosti.

5. Postaviti temelje za pravilnu zaštitu i planove upravljanja populacijama potočnog i plemenitog raka preklapanjem podataka o genetskoj raznolikosti proučavanih nativnih vrsta s podacima o njihovoj potencijalnoj budućoj rasprostranjenosti, kao i s budućom potencijalnom rasprostranjenošću stranih invazivnih vrsta pod utjecajem klimatskih

promjena te identificirati populacije i područja koja imaju najveću konzervacijsku vrijednost i kojima treba dati najveći prioritet u zaštiti.

6. Identificirati potencijalne buduće refugije potočnog i plemenitog raka tijekom klimatskih promjena.

Hipoteze istraživanja:

1. Populacije potočnog i plemenitog raka razlikuju se u morfološkim značajkama ovisno o pripadnosti različitim evolucijskim linijama, slivovima i vrsti staništa.

2. Populacije potočnog i plemenitog raka u Hrvatskoj karakterizira velika genetska raznolikost i složena evolucijska povijest.

3. Populacije potočnog i plemenitog u Hrvatskoj su genetski i geografski izolirane te su obje vrste posljedično genetski strukturirane.

4. Klimatske promjene uzrokovati će pomicanje areala potočnog i plemenitog raka na više nadmorske visine, u dijelove vodotoka s konstantnim protokom i hladnijom vodom.

5. Klimatske promjene i invazivne strane vrste negativno će utjecati na razinu genetske raznolikosti potočnog i plemenitog raka.

2. ZNANSTVENI RADOVI

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2.1. Popis znanstvenih radova

1. Lovrenčić L, Ferrón HG, Grbin D, Maguire I (2022) Insight into the noble crayfish morphological diversity: a geometric morphometric approach. Knowledge and management of aquatic ecosystems 423: 9.

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RESEARCH PAPER OPEN ACCESS

Insight into the noble crayfish morphological diversity: a geometric morphometric approach

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Abstract – The noble crayfish (*Astacus astacus*), a keystone species of high ecological, economic, and cultural importance in Europe, is threatened due to a long-term population decline caused by anthropogenic pressure on its habitats, the presence of non-indigenous invasive crayfish species and climate change. Since the effective protection of the remaining populations requires conservation measures based on the comprehensive knowledge of the species, including good understanding of its genetic and morphological variability, our aim was to study morphological features of the noble crayfish in Croatia using geometric morphometrics for the first time. We applied two-dimensional geometric morphometrics to find morphological differences among 15 populations of the noble crayfish from Croatian freshwater habitats, grouped according to previously established (a) mitochondrial (genetic) lineages, (b) genetic clusters inferred from nuclear microsatellites, as well as (c) river basins and (d) habitat types (lotic, lentic). Overall, the results indicated the existence of morphological diversity among the studied populations of the noble crayfish in Croatia. Shape analysis showed differences in cephalon based on crayfish affiliation to different genetic lineages, genetic clusters, river basins and habitat types. Our study provided novel insights into morphological diversity of the endangered noble crayfish in the area of its high genetic diversity.

Keywords: *Astacus astacus* / freshwater crayfish / biodiversity / landmarks / shape analysis

1 Introduction

Freshwater ecosystems in the Mediterranean Basin are considered one of the global biodiversity hotspots (Myers *et al.*, 2000). Concurrently, they are among the most endangered habitats and their biodiversity is declining dramatically, with high extinction rates and copious amount of threatened species (Dudgeon *et al.*, 2006; Strayer and Dudgeon, 2010; Collen *et al.*, 2014). One of Mediterranean countries is Croatia, located in the south-eastern Europe, on the dividing line between four biogeographical regions. Due to its outstandingly diverse ecological, climatic, and geomorphologic features, alongside complex paleo-hydrogeology, Croatia hosts unique and various freshwater biodiversity (Previšić *et al.*, 2009; Maguire *et al.*, 2018; Lovrenčić *et al.*, 2020b, Buj *et al.*, 2020; Gross *et al.*, 2021). It is recognized as an important centre of native and endemic animal and plant species diversity as well as an important wildlife refuge area.

Croatian freshwaters also represent hotspot of native European astacofauna by harbouring four native crayfish species with high genetic and morphological diversity comparing to other parts of Europe (Maguire *et al.*, 2014; Jelić *et al.*, 2016; Maguire *et al.*, 2017; Lovrenčić *et al.*, 2020a, b; Gross *et al.*, 2021). However, crayfish as keystone species and ecosystem engineers are negatively affected and highly threatened by anthropogenic pressure on their habitat (Jussila *et al.*, 2021). The noble crayfish *Astacus astacus* (L.) is the most highly appreciated indigenous crayfish species in Europe, known for its economic and cultural value. Previous studies have shown that the noble crayfish populations harbour the greatest genetic diversity in the south-eastern Europe where populations survived Pleistocene glaciations and subsequently re-colonised European freshwaters (Schrimpf *et al.*, 2014; Gross *et al.*, 2021). Natural distribution and genetic structure of *A. astacus* have been greatly altered and diminished across Europe due to habitat degradation, pollution, climate change, the introduction of non-indigenous invasive crayfish species, and a high amount of manmade translocations and stockings (Schrimpf *et al.*, 2017). As a result, *A. astacus* has been classified as vulnerable by the IUCN Red List of Threatened Species (Edsman *et al.*, 2010) and

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protected by international legislation (Annex III of the Bern Convention, Annex V of Habitat Directive (92/43/EEC). Moreover, *A. astacus* is listed as vulnerable in Croatia with a decreasing population trend (Gottstein *et al.*, 2011), and is protected by national legislation (NN 80/13). Effective protection of its existing populations requires conservation measures based on the comprehensive knowledge of the species, including information on its genetics and morphology (Sint *et al.*, 2005; Souty-Grosset and Reynolds, 2009). Moreover, the outcomes of (re)introduction programmes, as one of the conservation approaches, highlighted the importance of using stocking material that will fit into its new habitat (Sint *et al.*, 2005; Souty-Grosset and Reynolds, 2009).

Large-scale genetic analyses indicated the existence of seven divergent mitochondrial lineages within *A. astacus* in Europe, with highly differentiated populations characterised by limited gene flow in the south-eastern Europe (Schrimpf *et al.*, 2014, Laggis *et al.*, 2017, Gross *et al.*, 2021, Lovrenčić *et al.*, 2022). Phylogenetic reconstruction based on the mitochondrial genes revealed that Croatian samples nested within two mitochondrial lineages, Lineage 2 and 4 *sensu* Schrimpf *et al.* (2014), with some populations harbouring crayfish from both lineages. Results of microsatellite analyses revealed low genetic diversity of *A. astacus* in central and north Europe due to frequent human translocations and/or founder effects due to postglacial re-colonization, and the highest genetic diversity in the south-eastern Europe (Laggis *et al.*, 2017; Gross *et al.*, 2021). Moreover, population genetic analysis showed the existence of two genetic groups (clusters) of *A. astacus* in Croatia (Lovrenčić *et al.*, 2022).

Research of the freshwater crayfish morphology showed that they display great morphological diversity and phenotypic plasticity due to the influence of environment and/or genetic background (Haddaway *et al.*, 2012; Perry *et al.*, 2013). Furthermore, studies have been focused on evaluating morphological traits based on meristic and traditional morphometric in order to study different European crayfish species (Sint *et al.*, 2005; Maguire and Dakić, 2011; Maguire *et al.*, 2017; Đuretanović *et al.*, 2017), while geometric morphometric characters were used in studies of *Austropotamobius torrentium* (Lovrenčić *et al.*, 2020a), *A. pallipes* (Scalici and Bravi, 2012), *Cambarus* species (Helms *et al.*, 2015) and *Procambarus clarkii* (Malavé *et al.*, 2018). The first studies on *A. astacus* biogeography and taxonomy were based on the analyses of morphological and meristic characteristics (Albrecht, 1983; Karaman, 1929; Karaman, 1962; Karaman, 1963). Later, morphometric variation among *A. astacus* populations was studied based on the statistical analyses of a large set of morphometric parameters per crayfish in order to define characteristics that will distinguish different populations (Sint *et al.*, 2005; Đuretanović *et al.*, 2017). However, traditional morphometric multivariate analyses possess a comparatively low power in describing and visualising shape variation given that variables are usually strongly correlated with size and do not encode information about the relative location of the measurements. On the other hand, geometric morphometrics provides a solution to the problems inherent to traditional morphometric procedures by analysing shape using a Cartesian landmark coordinate system (Zelditch *et al.*, 2004). This further allows for various methods of visualisation that can communicate complex morphological changes and detect subtle morphological differences within and among species (Klingenberg, 2013). Thus, in the present study we analysed the morphological features of *A. astacus* using geometric morphometrics for the first time.

The aim of this study was to verify whether or not there are significant morphological differences among *A. astacus* populations belonging to different previously established groups (Lovrenčić *et al.*, 2022): (a) mitochondrial (genetic) lineages, (b) genetic clusters inferred from nuclear microsatellites, as well as (c) river basins and (d) habitat types (lotic, lentic). We hypothesized that morphology of *A. astacus* cephalon differs among different groups, and that our study will provide novel insights into biodiversity of this endangered species in the area of its high genetic diversity.

2 Material and methods

2.1 Sampling

This study was conducted by collecting adult crayfish from 15 populations across *A. astacus* distribution range in Croatia (Tab. 1, Fig. 1). Crayfish were collected with permission obtained from Ministry of Environmental Protection and Energy of the Republic of Croatia and in accordance with ethical standards. Catching was conducted either by hand during the night or with baited LiNi traps (Westman *et al.*, 1978) that were left in the water body overnight. All sampled specimens were euthanised by freezing and identified to the species level according to the key of crayfish families in Europe (Holdich *et al.*, 2006). Only adult (crayfish longer than 60 mm total length (Holdich *et al.*, 2006)), uninjured, intermolt crayfish were used in the further analyses in order to minimise the influence of ontogenetic allometry. Also, the sexes were merged in the further analyses since previous studies showed that morphological differences in cephalon shape were not affected by sexual dimorphism in crayfish (Scalici *et al.*, 2010, Helms *et al.*, 2015; Lovrenčić *et al.*, 2020a).

Crayfish specimens were grouped into *a priori* defined genetic lineages and genetic clusters, as well as their affiliation to river basins and habitat types (Tab. 1). Previous molecular phylogenetic analyses based on the two mitochondrial genes (*COI* and *16S* RNA) were conducted on the same samples by Lovrenčić *et al.* (2022), and the results revealed the presence of two mitochondrial lineages of *A. astacus* in Croatia. These results enabled *a priori* classification of our specimens into three groups: Lineage 2 and Lineage 4 *sensu* Schrimpf *et al.* (2014) and the group containing specimens from both Lineages 2 and 4 (in further text abbreviated as Lineage 2/4). Likewise, previous population genetic analysis using nuclear microsatellites was conducted on the same specimens, and the results of Bayesian assignment test in the software STRUCTURE enabled *a priori* classification of each specimen into one of two genetic clusters: Genetic Cluster I and Genetic Cluster II *sensu* Lovrenčić *et al.* (2022). Moreover, specimens were also assigned to certain river basin (Drava, Sava or Danube) and habitat type (lentic or lotic).

Table 1. List of studied *Astacus astacus* populations including information on the sample size (N), genetic lineage defined according to Lovrenčić *et al.* (2022), genetic cluster (I and II) defined according to Lovrenčić *et al.* (2022), river basin and habitat type. *L2–Lineage 2 sensu* Schrimpf *et al.* (2014), *L4-Lineage 4 sensu* Schrimpf *et al.* (2014), L2/L4–both lineages present in the same population.

Population	Abbr.	N	Genetic lineage	Genetic cluster	Basin	Habitat
Bednja	BED	25	L2	I, II	Drava	lotic
Bijela	BIJ	7	L4	II	Sava	lotic
Breznica	BRE	11	L4	II	Sava	lotic
Glogovica	GLO	26	L4	II	Sava	lotic
Ilova	ILO	17	L4	II	Sava	lotic
Kikovac	KIK	18	L4	II	Sava	lotic
Kutjevačka	KUT	12	L4	II	Sava	lotic
Motičnjak	MOT	20	L2/L4	II	Drava	lentic
Otuča	OTU	2	L2/L4	II	Zrmanja	lotic
Peratovica	PER	4	L4	II	Sava	lotic
Vuka	VUK	19	L4	II	Danube	lotic
Sloboština	SLO	18	L4	II	Sava	lotic
Totovec	TOT	19	L4	I	Drava	lentic
Veličanka	VEL	29	L4	II	Sava	lotic
Jaruga	JAR	2	L2	II	Sava	lotic

2.2 Geometric morphometrics and multivariate analyses

Geometric morphometric methods (GM) focus on the geometry of shape that is estimated using the relative locations of landmarks and/or outlines rather than on linear measurements. In comparison with traditional morphometrics, it provides better quantification and visual presentation of the morphological structures, and allows high statistical sensitivity that detects small changes in the shape of morphological units (Adams *et al.*, 2013). Due to numerous advantages, geometric morphometrics represent an innovative advancement in the analysis of morphology, and is now routinely used across the tree of life in all sorts of biological studies (Adams *et al.*, 2013). Moreover, morphometrics is usually applied in combination with multivariate statistics to complement studies on crayfish genetics (Sint *et al.*, 2007; Bertocchi *et al.*, 2008) and ecology (Inoue *et al.*, 2013). In the present study, following the procedure detailed in Lovrenčić *et al.* (2020a), GM analysis was focused on the cephalon shape using dorsal photographs of specimens. Images for digitisation were obtained by scanning dorsal view of crayfish cephalon using Epson Perfection V600 Photo scanner. Namely, dorsally positioned specimens were placed in a water basin on the flatbed scanner in order to obtain two-dimensional digital images. The GM analyses were performed using TPS 1.49 series software (Rohlf, 2015) and MorphoJ 1.06d (Klingenberg, 2011). Total of 14 landmarks were digitized using TpsDig v.2.17 (Rohlf, 2015) (Appendix 1). The number and selection of landmarks were based on the combination of points from Scalici *et al.* (2010), Scalici and Bravi (2012), and Lovrenčić *et al.* (2020a). Non-shape variation in translation, rotation and size from the original landmark configurations was removed by Generalized Procrustes Analysis (GPA) in MorphoJ 1.06d. The Canonical Variate Analysis (CVA) was performed using the same software in order to discriminate groups based on cephalon shape variation in different groups referred to in Table 1. In order to find the shape characteristics that the best distinguish groups of specimens, CVA was chosen over Principal Component Analysis (PCA) since it maximizes the differences between groups, constructing variables in the way that it describes relative positions of groups in the sample, while PCA not, and it represents general variation in the sample (Zelditch *et al.*, 2004). Further, CVA is one of the most used approaches to discriminate among groups since it maximizes distinction among groups in relation to the variation within groups (Campbell and Atchley, 1981). The results were reported as Procrustes and Mahalanobis distances with the respective *p*-values for these distances after permutation tests (10,000 iterations). Scatterplots of canonical scores were built to visualize the relationships among the *a priori* defined groups, while shape variation described by CVA was graphically depicted in the form of wireframes. In addition, CVA results were represented in scatterplots (CV1 vs CV2) with marginal density plots obtained using the packages *ggplot2* v.3.3.5 (Wickham, 2016), *cowplot* v.1.1.1 (Wilke *et al.*, 2019) and *ggpubr* v.0.4.0 (Kassambara, 2020) in R 4.0.0 (R Core Team, 2021).

We checked the existence of allometry (*i.e.*, shape change related to size) by performing correlation analysis between Procrustes coordinates and centroid size. To assess whether or not differences between *a priori* groups are explained by size differences and allometry, we performed alternative CVAs removing the shape component due to allometry (Klingenberg, 2016). Both analyses were implemented in MorphoJ 1.06d (Klingenberg, 2011).

In order to account for the impact of uneven sample size in the *a priori* groups we created a routine that allows generating subsamples where each group has the same sample size by randomly selecting specimens. The number of specimens of each group was constrained by the group with the smallest sample size (*i.e.*, genetic lineages = 22, genetic clusters = 40, river basins = 18, and habitat types = 18). This routine was repeated 250 times for each of the *a priori* classifications and CVA was carried out in all subsamples. Separation between

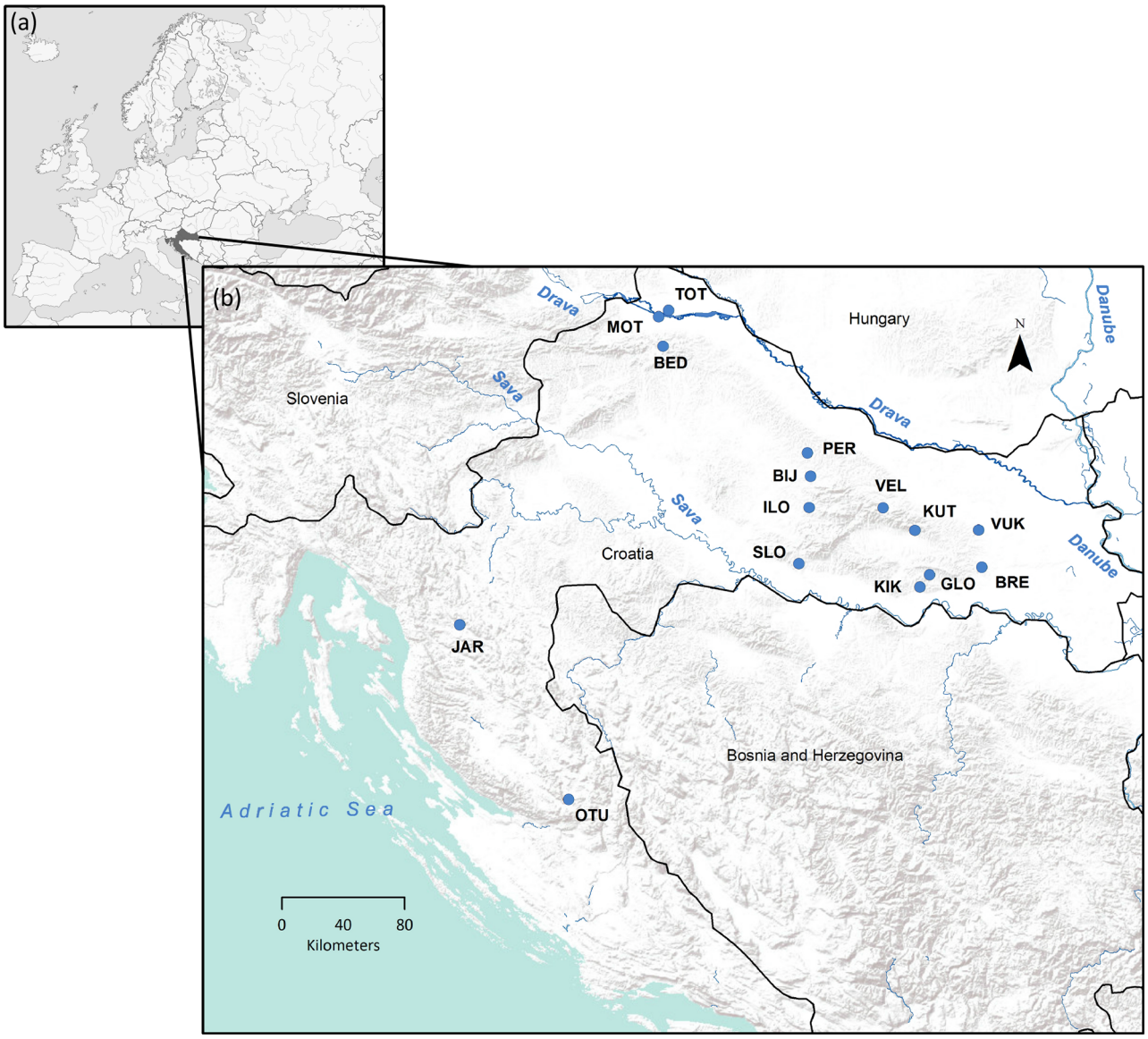

Fig. 1. Geographical location of the studied *Astacus astacus* populations (details about sampling sites are provided in Tab. 1). Map of Europe was adapted from https://commons.wikimedia.org/wiki/File:Croatia_in_Europe.svg. Map was prepared in QGIS 3.10 software and edited in Inkscape v. 0.91.

groups was assessed visually with scatterplots (CV1 or CV1 vs CV2) and by plotting Mahalanobis distance dendrograms (only in classifications with more than two groups, *i.e.* genetic lineages and river basins). Analyses were performed in R 4.0.0 (R Core Team, 2021) using the packages *geomorph* v.4.0.1 (Adams and Otárola-Castillo, 2013), *Morpho* v.2.9 (Schlager *et al.*, 2021), and *shapes* v.1.2.6 (Dryden, 2021). Complementarily, we tested for potential confounding effects between *a priori* classifications with similar partitioning of specimens within their groups (*i.e.*, genetic lineage *vs* river basin, genetic cluster *vs* habitat type, and river basin *vs* habitat type). For this, we performed Principal Component Analysis (PCA) on the Procrustes coordinates and fitted linear models considering the first three PC scores (cumulative proportion of variance explained ~ 56 %) as dependent variables and *a priori* classifications as factors. We compared models including each *a priori* classification separately (~ genetic lineage; ~ river basin; ~ genetic cluster; ~ habitat type) with models including combinations of these by pairs (~ genetic lineage + river basin; ~ genetic cluster + habitat type; ~ river basin + habitat type). Akaike information criterion (AIC) was then employed to assess which models fit the data better (*i.e.*, those with lower AIC values). A better support for models containing a single factor over those containing pairs of factors would be in agreement with the presence of important confounding effects; while the contrary would support that both factors contribute to explain shape variability within our sample. Linear models were fitted with the *aov()* function in the R package *Stats* v. 4.2.0 (R Core Team, 2021) and AIC values were obtained in the R package *AICcmodavg* v. 2.3.1 (Mazerolle, 2021). We considered a significance threshold of $p < 0.05$ for all the analyses.

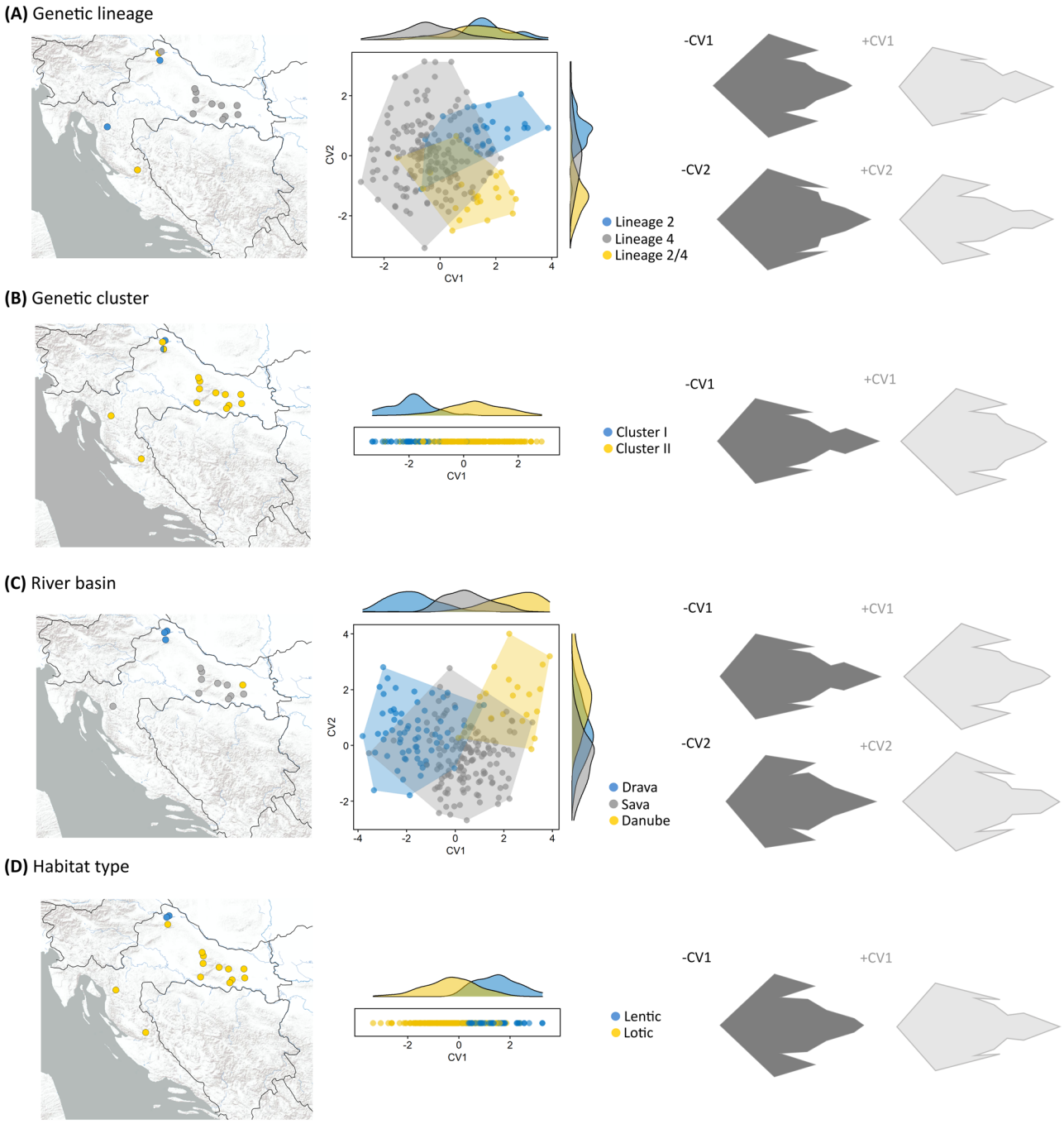

Fig. 2. Cephalon shape variation of *Astacus astacus* revealed by Canonical Variate Analysis. Scatter plots of the first two canonical variate axes (CV1 and CV2) with associated wireframe representations depicting shape changes along positive and negative extremes of the axes. Dark grey landmark wireframe configurations represent cephalon morphologies along negative extremes, while light grey landmark wireframe configurations represent cephalon morphologies along positive extreme. Different colour dots represent different groups: (A) genetic lineage, (B) genetic cluster, (C) river basin, (D) habitat type, with maps showing their distribution in Croatia.

3 Results

3.1 Cephalon shape variation according to *A. astacus* genetic lineages

Cephalon shape variation analyses included 229 crayfish (Tab. 1). Results of CVA showed the differentiation among *A. astacus* genetic lineages based on the cephalon shape (Fig. 2). The first two canonical variates (CVs) explained entire shape variation: CV1 accounted for 71.65%, while the CV2 accounted for 28.35% of the variability. The CVA separated to some extent the specimens into three groups, corresponding to the previously defined genetic lineages. CV1 separated, with somewhat overlap, the Lineage 4 from Lineage 2 and

Table 2. Procrustes distances (below diagonal) and Mahalanobis distances (above diagonal) between different groups of *Astacus astacus* with *p*-values from permutation tests (10,000 permutation rounds).

Procrustes and Mahalanobis distances			
A) Genetic lineage	Lineage 2	Lineage 2/4	Lineage 4
Lineage 2		2.103**	2.175**
Lineage 2/4	0.031**		2.038**
Lineage 4	0.034**	0.024*	
B) Genetic cluster	I	II	
I		2.324**	
II	0.029**		
C) River basin	Drava	Danube	Sava
Drava		4.393**	2.397**
Danube	0.057**		2.899**
Sava	0.026**	0.036**	
D) Habitat type	Lentic	Lotic	
Lentic		1.811**	
Lotic	0.025**		

*Indicates statistical significance at $p < 0.05$.
**Indicates statistical significance at $p < 0.0001$.

Lineage 2/4, while CV2 separated, with certain overlap, the Lineage 2 and mixed Lineage 2/4 (Fig. 2A). Overall cephalon shape, as quantified by Procrustes and Mahalanobis distances, differed among the genetic lineages, with all distances being statistically significant (Tab. 2). Randomised subsampling revealed much better separation among genetic lineages with slight overlap (Appendix 2).

Shape changes that contributed to the distinction among crayfish from different lineages were mostly visible in the apical part of the cephalon (rostrum), the lateral edge of the cervical groove, the placement of the postorbital ridges, and the overall cephalon length and width (Fig. 2A).

Shape change along the CV1 axis was mostly determined by the length and width of the cephalon, the width of lateral edge of the cervical groove, and the placement of the postorbital ridges (Fig. 2A). Shape changes along +CV1 were characterised by enlargement of the rostrum, elongating and narrowing of the cephalon, narrowing of the lateral edge of the cervical groove, and the two postorbital ridges placed closer to the lateral edge of the body. These characteristics were more pronounced in some specimens belonging to the Lineage 2 and Lineage 2/4. In contrast, −CV1 was related to the reduction of rostrum size, both length and width, shortening and widening of the cephalon, widening of the lateral edge of the cervical groove, with two postorbital ridges placed distant from the lateral edge of the body. These characteristics prevailed in some specimens from Lineage 4.

Shape changes along +CV2 were generally characterised by larger rostrum, narrower cephalon, shortening of the lateral edge of the carapace, and the two postorbital ridges placed distant from the lateral edge of the body and rostrum. These morphological characteristics were generally pronounced in the crayfish from the Lineage 2 and partially Lineage 4. Shape changes along −CV2 were pronounced in the crayfish from the Lineage 2/4 and partially Lineage 4, and were characterised by smaller rostrum, wider cephalon, widening of the lateral edge of the carapace, and with the two postorbital ridges placed closer to the lateral edge of the body (Fig. 2A).

3.2 Cephalon shape variation according to *A. astacus* genetic cluster

Results of CVA showed the differentiation between crayfish from different genetic clusters (Fig. 2B). Shape changes along the positive extreme (+CV1) included crayfish from the Genetic Cluster II *sensu* Lovrenčić *et al.* (2022, Tab. 1) that were characterised by shorter and narrower rostrum, wider cephalon, and wider lateral edge of the cervical groove. Contrary, shape changes along the negative extreme (-CV1) indicated crayfish from the Genetic Cluster I *sensu* Lovrenčić *et al.* (2022, Tab. 1), and were characterised by longer and larger rostrum, elongated and narrower cephalon, and narrower lateral edge of the cervical groove (Fig. 2B). Procrustes and Mahalanobis distances separated significantly crayfish from different clusters (Tab. 2B). Randomised sampling revealed similar results (Appendix 3).

3.3 Cephalon shape variation according to river basin

The CVA showed that shape variation among crayfish from three river basins was explained in the first two dimensions of the shape space (Fig. 2C). The first two canonical variates (CVs) explained the shape variation: CV1 accounted for 78.70%, while the CV2 accounted for 21.30% of the variability. Procrustes and Mahalanobis distances separated crayfish from different basins, all being statistically significant (Tab. 2C). Randomised sampling showed much better separation among crayfish belonging to different river basins with no overlap (Appendix 4).

The CV1 differentiated crayfish from the Drava and Danube River basins: the crayfish from the Danube basin (shape associated with the positive extreme, along +CV1) had smaller rostrum, smaller and wider cephalon, wider lateral edge of the cervical groove, and the two postorbital ridges placed distant from the lateral edge of the body and closer to the rostrum, than the crayfish from the Drava basin

(shape associated with the negative extreme, along −CV1) that were characterised by narrower and elongated cephalon with larger rostrum, narrower lateral edge of the cervical groove, and the two postorbital ridges placed closer to the lateral edge of the body (Fig. 2C). Crayfish from Sava basin occupied an intermediate position in the morphospace with a high degree of overlapping with two other basins.

The CV2 partly separated crayfish from the Danube and Sava River basins with the shape changes mostly pronounced in the width of the lateral edge of the carapace and cervical groove, the rostrum, and the placement of the postorbital ridges (Fig. 2C). Shape changes along the positive extreme (+CV2) were characterised by wider lateral edge of the carapace and cervical groove, wider rostrum apex, and the two postorbital ridges and spines placed closer to the lateral edge of the body. These characteristics were mostly present in the specimens from the Danube basin. In contrast, some of the crayfish from the Sava basin (shape associated with the negative extreme, along −CV2) were characterised by narrower lateral edge of the carapace and cervical groove, and the two postorbital ridges placed distant from the lateral edge of the body.

3.4 Cephalon shape variation according to habitat type

Results of CVA showed the differentiation between crayfish from different habitat types (Fig. 2D). Shape changes along the positive extreme (+CV1) designated the crayfish from the lentic habitats (lakes), and were characterised by longer and larger rostrum, elongated and narrower cephalon, narrower lateral edge of the cervical groove, but wider lateral edge of the carapace. Contrary, shape changes along the negative extreme (−CV1) indicated crayfish from the lotic habitats (rivers and streams) that were characterised by shorter and narrower rostrum, shorter and wider cephalon, wider lateral edge of the cervical groove, but narrower lateral edge of the carapace (Fig. 2D). Randomised sampling revealed similar results (Appendix 5). Procrustes and Mahalanobis distances separated significantly crayfish from different habitats (Tab. 2D).

3.5 Allometry analyses

We detect allometric change affecting the cephalon of *A. astacus* (p-value < 0.0001), where bigger specimens show an expansion of posterior region of the head, narrower rostrum, and longer lateral edge of the carapace, in comparison with smaller specimens (Appendix 6). However, proportion of variation for which the allometric regression accounts is comparatively low (7.66 %). Groups within each *a priori* classification are still separated by CVA when removing allometric component of shape and the morphological changes associated to each CV are equivalent to those of the original analysis (Appendix 7). Therefore, size of individuals did not affect our results.

3.6 Linear model fit

The results of the linear modelling showed that crayfish cephalon shape significantly differed among genetic lineages (p-value $= 3.11*10^{-6}$), genetic clusters (p-value $= 1.64*10^{-4}$), and habitat types (p-value $= 3.05*10^{-2}$), while we did not observe significant effect based on river basins (p-value $= 9.1*10^{-1}$) (see Appendix 8 for detailed results). The models including genetic clusters + habitat types and river basins + habitat types as factors fit the data better than the models including those separately ($AIC_{\text{genetic clusters + habitat types}} = 1236.70$; $AIC_{\text{river basins + habitat types}} = 1256.68$; $AIC_{\text{genetic clusters}} = 1250.80$; $AIC_{\text{habitat types}} = 1260.42$; $AIC_{\text{river basins}} = 1267.03$); while the model including genetic lineages + river basins as factors fits the data better than the model including only river basins and slightly worse than the model including only genetic lineages ($AIC_{\text{lineages + river basins}} = 1244.28$; $AIC_{\text{lineages}} = 1241.53$; $AIC_{\text{river basins}} = 1267.03$).

4 Discussion

The present study provides important insight into the morphological diversity of the studied *A. astacus* populations through the application of geometric morphometrics. This approach was used for depicting cephalon shape differences/differentiation among *A. astacus* specimens. Our study revealed existence of variation in the cephalon shape among *A. astacus* inhabiting Croatian freshwater habitats. Results indicated that cephalon morphology differs among mitochondrial lineages, but also between microsatellite clusters, among river basins and between habitat types. Additionally, these results were consistent after applying randomization routines to correct uneven sample size of *a priori* defined groups, supporting that this aspect has no major impact on our analyses. Even though the presence of allometry is well supported, this seems to play a minor role in the morphological differentiation of the different *a priori* defined groups, as results remain the same after removing allometric component of shape. Moreover, differences in the crayfish cephalon shape among *a priori* defined groups are also supported by PCA and derived linear models, except for the case of river basins. However, the later might be due to the fact that results from PCA, contrary to those from CVA, not necessarily reflect differences between predefined groups, even if they exist (Krzanowski, 2000).

Majority of studies on the morphological variability of freshwater crayfish have been based on traditional morphometrics that includes multivariate analyses of crayfish body linear measures to quantify shape (Ghia *et al.*, 2006; Sint *et al.*, 2006; Bertocchi *et al.*, 2008; Bök *et al.*, 2010; Maguire and Dakić, 2011; Benzer *et al.*, 2017; Maguire *et al.*, 2017). In our study, we applied geometric morphometrics, in combination with CVA, on *A. astacus* for the first time. This approach has allowed us to establish a comparative framework, based on quantitative and statistically tractable data, where new specimens can be included and classified into predefined groups, with associated probabilities. This is in fact one of the direct applications of CVA (McKeown and Schmidt, 2013) and, in our case, it would allow to infer, for example, genetic or ecological aspects from morphology.

Freshwater crayfish exhibit high intraspecific morphological variation that reflects both environmental influence and/or genetic background (Sint *et al.*, 2005; Sint *et al.*, 2007; Ghia *et al.*, 2006; Bertocchi *et al.*, 2008; Mathews *et al.*, 2008; Cataudella *et al.*, 2010; Haddaway *et al.*, 2012; Perry *et al.*, 2013;

Helms *et al.*, 2015; Rudolph *et al.*, 2016). Moreover, they display phenotypic plasticity, meaning that individual phenotype is not only determined by its genotype, but can also be shaped through habitat and its local environmental conditions (Haddaway *et al.*, 2012). In general, species can exhibit a wide phenotypic variation due to their widespread distribution within heterogeneous habitats, genetic and behavioural differences among individuals or populations, and ontogenetic and evolutionary forces that can also affect their phenotype (Rudolf *et al.*, 2016). Phenotypic plasticity can affect not only the morphological, but also physiological and behavioural aspects of an organism, including its life history traits (Sommer, 2020). Phenotypic plasticity enables adaptations to the local ecological conditions that may lead to pronounced morphological differences between populations, but also may portray populations living under similar ecological conditions that in the end will exhibit similar morphology (Sint *et al.*, 2005). Even though crayfishes exhibit morphological plasticity as a consequence of genetic variation or environmental factors or their interaction, our study corroborates geometric morphometrics as a valuable tool in studying variability and slight morphological differences. In our case, application of GM on the *a priori* defined groups (*e.g.,* genetic groups) helped us to perceive morphological features (*e.g.,* shape of rostrum) that characterise those groups and to separate them to some extent. Moreover, this study confirmed the anterior part of the crayfish body, *e.g.* cephalon and rostrum shape, as a source of variation within and among populations as demonstrated for *Austropotamobius torrentium* (Lovrenčić *et al.*, 2020a). Further, it confirmed the advantage of using carapace and particularly cephalon in studying variation, since measurements are not affected by loss, regeneration, or abdominal muscle contractions (Sint *et al.*, 2005).

Mitochondrial DNA sequences revealed the existence of several distinct evolutionary lineages within *A. astacus* in Europe (Schrimpf *et al.*, 2014, Laggis *et al.*, 2017, Gross *et al.*, 2021, Lovrenčić *et al.*, 2022), so one of our aims was to determine whether two genetic lineages identified in Croatian populations differ in cephalon shape, and if it can be used as a feature that can be used to distinguish lineages. Our study showed slight differences in the cephalon shape between crayfish from different lineages revealed by mitochondrial DNA analyses (Lovrenčić *et al.*, 2022). Observed differences could be the consequence of species evolutionary history. According to Schrimpf *et al.* (2014), *A. astacus* probably survived last glaciations in the western and southern parts of Balkans, as well as in the lower part of the Danube basin, and then spread northward and westward along the Danube drainage system. The observed differences among genetic lineages in morphology probably mirror the long-term separation of *A. astacus* populations, experienced during the cold periods of the Pleistocene when nearly all of the central and northern Europe was unsuitable for crayfish, and therefore, they were mainly confined to refugia in the Iberian, Italian, and Balkan Peninsulas (Hewitt, 2011). The results of phylogenetic analysis are congruent with shape variation to some extent; genetic lineages described within *A. astacus* are still evolutionary very young and mostly unsupported in the phylogenetic reconstruction (Gross *et al.*, 2021), which is also reflected in morphology. In conclusion, observed changes/differences in cephalon shape reflect phylogenetic reality with numerous individuals overlapping /without clear separation among/between genetic lineages. Since model including genetic lineages + river basin fits the data better than the model including only river basins and slightly worse than the model including only genetic lineages we can conclude that river basin does not contribute to obtained separation.

Considering groups based on the previous microsatellite genotyping (Lovrenčić *et al.*, 2022), studied crayfish belong to two distinct genetic clusters. Most of the individuals were assigned to Genetic Cluster II, whereas the Genetic Cluster I grouped individuals from populations TOT and partly BED, with the sign of possible admixture in BED. Our results, both nonrandomised and randomised, revealed that morphology of cephalon differs between the two genetic clusters. Since models including genetic cluster and habitat slightly better fit the data than single models, we may say that both factors contribute to explain shape variability.

Astacus astacus showed variation in the cephalon shape among three different river basins: Sava, Drava and Danube. The geometric morphometrics suggested separation among populations belonging to separate river basin which was especially pronounced for the individuals from the Drava and Danube River basins. This is contrary to findings by Malato *et al.* (2017) who studied differences between fish from genus *Rhoadsia* and concluded that fish body shape from different drainages overlaps. However, our results are concordant with findings of Jerry and Cairns (1998) who studied Australian bass and revealed morphological differences among populations belonging to different riverine drainages.

Finally, our results, both nonrandomised and randomised, showed morphological variation between different habitat types, *i.e.* lakes and rivers. Crayfish from lentic sites had a narrower, more elongated cephalon and rostrum than those from lotic sites with shorter and wider cephalon (generally smaller head, rostrum and apex). The morphological characteristics that differentiate specimens from different habitats can be correlated with lifestyle habits resulting in an improved performance of a species (Rudolph *et al.*, 2016). For example, streamlined body shape is associated with a reduced resistance to the water flow, which might mitigate drag and increase opportunities of finding shelters. This pattern of morphological difference is similar to that observed in other decapods crustaceans, such as the case reported by Perry *et al.* (2013) for *Orconectes rusticus*. Moreover, our results showed that crayfish from lotic habitats possess less pronounced rostrum that can, according to Perry *et al.* (2013), facilitate their life among the rocks on the river bed, the search for food and shelter from predators. This finding is consistent with the results of Rudolph *et al.* (2016) that studied the only South American parastacid that inhabits both rivers and lakes, *Samastacus spinifrons*. Their study revealed that lake specimens exhibit more robust body, more pronounced rostrum and more elongated, less thick claws and longer phallic papillae compared to fluvial populations. This phenomenon explains variations in morphological forms that occur as a result of the ability of organisms/genotype to produce distinct phenotypes in response to different environmental conditions (Sommer, 2020). Furthermore, Perry *et al.* (2013) studied the effect of water velocity on the size and shape of invasive crayfish *Orconectes rusticus*, and reported that specimens that inhabit streams with high-velocity water flow are slender than those that inhabit lakes and streams with low-velocity flow. They

confirmed that elevated water velocity can affect crayfish morphology with crayfish size as an important factor mediating the response to water velocity what is in accordance with results obtained in our study. Therefore, both genetic variation and phenotypic plasticity play important roles in predictable phenotypic differentiation across flow regimes (Langerhans, 2008; Perry *et al.*, 2013).

In conclusion, we applied geometric morphometrics to address cephalon shape variation of the noble crayfish, and it proved as appropriate for studying its morphology. Moreover, analyses of cephalon showed as suitable for separation of different groups. Overall, our results indicated the existence of morphological diversity of the noble crayfish in Croatia. Our study showed differences of the cephalon shape based on crayfish affiliation to different genetic lineages, genetic clusters, river basins and habitat types (lotic, lentic). Despite some equivalence in the partitioning of specimens within the groups of the different *a priori* classifications exists, our analyses based on PCA and linear modelling show that these factors contribute in different ways to the shape variation of our sample which denotes that, if present, confounding effects are not important. This is further supported by the fact that shape changes related to the separation of groups are different for each of the considered *a priori* classifications. Therefore, observed morphological characteristics are attributed not only to genetics but also to environmental factors that speaks in favour of their phenotypical plasticity.

Supplementary Material

Appendix 1. Position of the 14 landmarks on the dorsal side of *Astacus astacus* cephalon. Landmarks 1, 2 and 14 describe apex; Landmarks 1, 2, 3, 4, 12, 13 and 14 describe rostrum; Landmarks 7 and 8 describe lateral edge of cervical groove; Landmarks 4, 5, 6, 10, 11 and 12 describe postorbital ridges.

Appendix 2. Cephalon shape variation of *Astacus astacus* revealed by Canonical Variate Analysis on *a priori* defined genetic lineages accounting for uneven sample size. Scatter plots of the first two canonical variate axes (CV1 and CV2) are shown. Different colour dots represent individuals assigned to certain genetic lineages (blue, Lineage 2; grey, Lineage 4; yellow, Lineages 2/4). Separation of the genetic lineages is also represented by Mahalanobis distance dendrograms.

Appendix 3. Cephalon shape variation of *Astacus astacus* revealed by Canonical Variate Analysis on *a priori* defined genetic clusters accounting for uneven sample size. Scatter plots of the first canonical variate axis (CV1) are shown. Different colour dots represent individuals assigned to certain genetic cluster (blue, Cluster I; yellow, Cluster II).

Appendix 4. Cephalon shape variation of *Astacus astacus* revealed by Canonical Variate Analysis on individuals affiliated to different river basin accounting for uneven sample size. Scatter plots of the first two canonical variate axes (CV1 and CV2). Different colour dots represent individuals assigned to certain river basin (blue, Drava; grey, Sava; yellow, Danube). Separation of the river basins is also represented by Mahalanobis distance dendrograms.

Appendix 5. Cephalon shape variation of *Astacus astacus* revealed by Canonical Variate Analysis on individuals affiliated to different habitat type accounting for uneven sample size. Scatter plots of the first canonical variate axis (CV1). Different colour dots represent individuals assigned to certain habitat type (blue, lentic; yellow, lotic).

Appendix 6. Regression analysis between shape and centroid size showing associated shape changes corresponding to positive (upper) and negative (lower) extremes (shape changes are displayed with scale factors magnified x100 to ease interpretation).

Appendix 7. Cephalon shape variation of *Astacus astacus* revealed by Canonical Variate Analysis after removing allometric component of shape. Scatter plots of the first two canonical variate axes (CV1 and CV2) with associated wireframe representations depicting shape changes along positive and negative extremes of the axes. Dark blue landmark wireframe configurations represent cephalon morphologies along extremes, while light blue average landmark wireframe configurations. Different colour dots represent different groups: (A) genetic lineage, (B) genetic cluster, (C) river basin, (D) habitat type.

Appendix 8. Detailed results of linear models.

The Supplementary Material is available at https://www.kmae.org/10.1051/kmae/2022006/olm.

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Znanstveni rad 2

Genetic diversity and structure of the noble crayfish populations in the Balkan Peninsula revealed by mitochondrial and microsatellite DNA markers

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ABSTRACT

Background. The noble crayfish (*Astacus astacus*) is a native European species in decline, with a contracting range and diminishing populations and abundance. Previous studies revealed this species significant genetic diversity in the south-eastern Europe, with populations from the western and the southern part of the Balkan Peninsula being the most divergent. However, sampling of populations from the western part of the Balkans was limited and insufficient for investigating genetic diversity and population divergence for the purpose of conservation planning and management. Thus, the major aim of this study was to fill in this knowledge gap by studying mitochondrial and microsatellite DNA diversity, using 413 noble crayfish from 18 populations from waterbodies in the western part of the Balkan Peninsula.

Methods. Phylogenetic analysis of studied populations and their mitochondrial diversity were studied using *COI* and *16S* sequences and population genetic structure was described using 15 microsatellite loci.

Results. Phylogeographic analysis revealed new divergent mitochondrial haplotypes for the populations in the westernmost part of the Balkan Peninsula in the tributaries of the Sava and Drava rivers. Microsatellite data indicated that these populations harbour an important component of genetic diversity within *A. astacus*. The results suggest that the western part of the Balkans played an important role as microrefugia during the Pleistocene climate fluctuations, allowing the long term persistence of *A. astacus* populations in this region. These results will also be important to supporting conservation decision making and planning.

Subjects Aquaculture, Fisheries and Fish Science, Conservation Biology, Genetics, Zoology, Freshwater Biology
Keywords *Astacus astacus*, South-east Europe, Cytochrome oxidase subunit I, Conservation, Glacial refugia

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INTRODUCTION

Freshwater crayfishes are important organisms for normal functioning of freshwater food webs in many parts of the world (*Usio & Townsend, 2004*) and they are considered flagship species for the conservation of aquatic systems (*Füreder & Reynolds, 2003*), hence an understanding of their biodiversity and conservation status is a priority (*Souty-Grosset & Reynolds, 2009*). One of the most widely distributed native European freshwater crayfish species is *Astacus astacus* (noble crayfish) whose range and abundance have declined rapidly due to a negative anthropogenic influence upon their habitats (*e.g.*, fragmentation, destruction and pollution), overfishing and adverse impacts of invasive alien crayfish species (*Kouba, Petrusek & Kozák, 2014*). Besides being able to outcompete native crayfish (*Hudina et al., 2016*), invasive crayfish are also vectors of the pathogen *Aphanomyces astaci*, the causative agent of the crayfish plague, which is mostly lethal to native European crayfish species (*Alderman, Holdich & Reeve, 1990*; *Kozubíková-Balzarová & Horká, 2015*). As a result, *A. astacus* is listed by the IUCN as a Vulnerable species (*Edsman et al., 2010*). Also, it is listed in the Appendix III of the Bern Convention, and in Appendix V of Habitat Directive (92/43/EEC). In order to ensure that noble crayfish conservation is effective, it is necessary to develop management plans based on sound knowledge of the species ecology, biology and genetics (*Schulz & Grandjean, 2005*; *Souty-Grosset & Reynolds, 2009*).

Further complicating the understanding of the diversity within the noble crayfish is that it has been frequently translocated, especially in the central and northern Europe (*Policar & Kozák, 2015*; *Jussila et al., 2016*; *Gross et al., 2017*), as it is a valued human food source, potentially over millennia, and is economically important, attracting a premium price in the market place. As a consequence, the natural distribution of genetic variation in the noble crayfish is thought to have been impacted by historical introductions in many European regions (*Schrimpf et al., 2011*; *Schrimpf et al., 2014*; *Gross et al., 2013*; *Gross et al., 2017*), necessitating comprehensive geographic sampling, to distinguish original and remnant populations from recent translocations, and identify potentially missed or hybrid populations.

Nevertheless, it is presumed that noble crayfish populations in south-eastern Europe have maintained their original genetic structure, since this species has been of little commercial interest in the region and although diminishing, may reflect historical evolutionary and phylogenetic patterns (*Simić et al., 2008*; *Maguire, Jelić & Klobučar, 2011*; *Pârvulescu & Zaharia, 2014*; *Slavevska-Stamenković et al., 2016*; *Đuretanović et al., 2017*). Conversely, crayfish from south-eastern Europe (Croatia, Slovenia and Bosnia and Herzegovina) have been used for restocking of freshwaters in the central Europe that were devastated by crayfish plague in the late 19th century (cf. *Jussila et al., 2016*; *Schrimpf et al., 2014*). Thus genetic signatures from crayfish native to this region may be present in central Europe, further highlighting the need for a comprehensive understanding of the genetic structure throughout the species range.

Large-scale studies of mitochondrial cytochrome oxidase subunit I (*COI*), and the *16S* rRNA sequences by *Schrimpf et al. (2014)* indicated the existence of four mitochondrial DNA (mtDNA) lineages within the noble crayfish in Europe, with populations from the

south-eastern Europe (Black Sea basin) having the highest genetic diversity in Europe. The populations from the western part of the Balkan Peninsula were the most divergent; however, the authors further more detailed analysis of the south-eastern European populations as their sampling was not exhaustive. Recently, *Laggis et al. (2017)* studied populations from the southernmost part of the south-eastern Europe (Greece) using the same mtDNA markers. Their research included extensive sampling of noble crayfish and the results revealed the existence of two new noble crayfish mtDNA lineages (they called groups) endemic to Greece, and showed that newly discovered lineages possessed the highest haplotype richness and genetic diversity found so far.

The major aim of this study was to fill in the gap in our understanding of the genetic diversity of the noble crayfish in south-eastern Europe by studying mitochondrial and microsatellite DNA variation in 18 previously unstudied populations from this region. The first aim was to examine mitochondrial variation using the *COI* barcoding gene and *16S* rRNA in *A. astacus* populations from less sampled parts of the south-eastern Europe and compare diversity and genealogical relationships with previously studied European populations using these markers (*Schrimpf et al., 2014*; *Laggis et al., 2017*; *Mrugała et al., 2017*). The second aim of this study was to describe genetic structure and characteristics of 18 *A. astacus* populations from waterbodies in the western part of the Balkan Peninsula using a suite of 15 microsatellite loci developed by *Gross et al. (2017)* to assist conservation planning and the protection of priority populations of this species in this region. Results of the present study will help guide efficient and effective conservation plans and management strategies for protecting the genetic diversity and maintaining the adaptive potential of *A. astacus* populations on the regional level in south-eastern Europe.

MATERIALS & METHODS

Sample collection and DNA isolation

This study used more than 400 noble crayfish samples from 18 populations in the western part of the Balkans (Fig. 1, Table 1). The specimen collection was conducted in Croatia, Romania, Serbia and Slovenia with the approval of local authorities (Croatia UP/I-612-07/18-48/148; Romania 408/CJ/27.11.2018; Serbia 324-04-10/2021-04 and 03 No. 026-419/2; Slovenia 35601-1262 150/2006-6 and 35601-135/2010-9). Crayfish were collected by hand or with trapped baited traps (*Westman, Pursiainen & Vilkman, 1978*; *Kozák et al., 2015*). Crayfish samples from Albania were purchased from a fisherman on the Prespa Lake. The Prespa Lake and Ohrid Lake are approximately 10 km apart, and waters from the Prespa Lake feed Ohrid Lake through underground karst channels. Out of the Ohrid Lake springs the only outlet, the Black Drim River, which flows in a north direction into Albania and thus into the Adriatic Sea. A pereiopod from each specimen was removed and preserved in 96% ethanol. Wild caught crayfish were then released back into the water. This sampling method does not harm crayfish as these appendages regenerate after the next moulting. Total genomic DNA was extracted from the pereiopod muscle tissue with the Sigma GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, USA) following the manufacturer's protocol and stored at −20 °C.

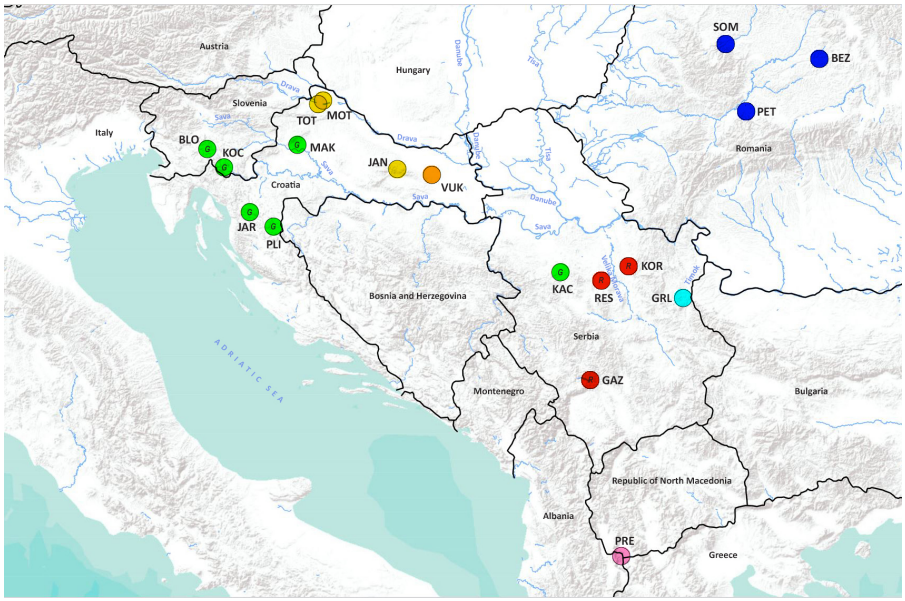

Figure 1 Geographical location of the studied *Astacus astacus* populations. Details about sampling sites are provided in Table 1. Map was prepared in QGIS 3.10 software (available at: https://qgis.org/en/site/) and finished in GIMP 2.10 (available at: https://www.gimp.org/). In order to distinguish red and green circles on the map, we included letters *R* for red, and *G* for green.

Full-size DOI: 10.7717/peerj.11838/fig-1

Mitochondrial DNA analyses

Mitochondrial *16S* and *COI* gene fragments were amplified and sequenced with primers 16Sar/16Sbr (*Simon et al., 1994*) and LCO-1490/HCO-2198 (*Folmer et al., 1994*) allowing comparisons to be made with *16S* and *COI* sequences from previous studies on this species (*Schrimpf et al., 2014*; *Laggis et al., 2017*; *Mrugała et al., 2017*) available from GenBank. Polymerase chain reactions (PCR) for *COI* were prepared in a total volume of 25 µl containing 10–50 ng/µl DNA template, 1.5 mM Promega Buffer, 0.04 U HotStart Polymerase, 0.15 mM of each dNTP, 0.7 mM $MgCl_2$ and 0.4 µM of each primer. PCR conditions for *COI* were as follows: initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 48 °C for 60 s and extension at 72 °C for 60 s, and the final extension of 10 min at 72 °C. The final reaction mix in a total volume of 10 µL for *16S* gene contained 0.05 U GoTaq G2 HotStart Polymerase, 1.5 mM GoTaq FlexiBuffer, 0.2 mM of each dNTP, 0.275 µM of each primer, and 10–50 ng/µl of DNA template. PCR conditions for *16S* were as follows: initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 1 min, annealing at 52 °C for 1 min and extension at 72 °C for 1 min, and the final extension of 5 min at 72 °C. The purification of PCR products was performed with EXOAnP Mix (20 U/µl of Exonuclease I (New England Biolabs), 5 U/µl of Antarctic phosphatase (New England Biolabs). The sequencing of purified PCR products was prepared by Macrogen, Inc. (Seoul, South Korea), with the same forward primers used for the gene amplifications.

Sequences were edited in SEQUENCHER v. 5.3 (Gene Codes Corporation, Ann Arbor, USA) and aligned using MAFFT v.7.187 (*Katoh & Standley, 2013*). The final alignment for

Table 1 Information on sampling sites. Information on sampling sites with abbreviation (Abbr.), habitat type, country, river basin and major tributary, sea basin (BS, Black Sea; AS, Adriatic Sea), coordinates, putative refugial area (WBA, western Balkans; WBS, western Black Sea; SBA, southern Balkans), year of sampling, sample size and origin (status) of studied *Astacus astacus* populations.

Site	Abbr.	Habitat	Country	Basin/Tributary	Sea basin	Coordinates	Refugial area	Year	Sample size	Status
Kočevska	KOC	river	Slovenia	Danube/Sava	BS	45.573N, 14.797E	W BA	2015	10	native
Bloke	BLO	lake	Slovenia	Danube/Sava	BS	45.786N, 14.516E	W BA	2015	28	native
Jaruga	JAR	river	Croatia	Danube/Sava	BS	45.048N, 15.225E	W BA	2008	23	native
Plitvice	PLI	lake	Croatia	Danube/Sava	BS	44.879N, 15.615E	W BA	2008	9	probably introduced
Maksimir	MAK	lake	Croatia	Danube/Sava	BS	45.831N, 16.026E	W BA	2016	30	introduced
Kačer	KAC	river	Serbia	Danube/Sava	BS	44.222N, 20.280E	W BA	2014	29	native
Motičnjak	MOT	lake	Croatia	Danube/Drava	BS	46.305N, 16.386E	W BA	2016	32	probably introduced
Totovec	TOT	lake	Croatia	Danube/Drava	BS	46.345N, 16.469E	W BA	2016	30	probably introduced
Jankovac	JAN	stream	Croatia	Danube/Drava	BS	45.522N, 17.684E	W BA	2016	30	native
Vuka	VUK	river	Croatia	Danube	BS	45.438N, 18.258E	W BA	2016	31	native
Resnički	RES	stream	Serbia	Danube/Velika Morava	BS	44.090N, 20.937E	W BA	2014	30	native
Korenica	KOR	lake[a]	Serbia	Danube/Velika Morava	BS	44.228N, 21.413E	W BA	2014	17	native
Gazivode	GAZ	lake[a]	Serbia	Danube/Velika Morava	BS	42.942N, 20.648E	W BA	2014	26	native
Grliško	GRL	lake[a]	Serbia	Danube/Timok	BS	43.812N, 22.232E	W BA	2014	13	native
Somesul	SOM	river	Romania	Danube/Tisa	BS	46.712N, 23.338E	W BS	2016	22	native
Petresti	PET	river	Romania	Danube/Tisa	BS	45.909N, 23.559E	W BS	2016	11	native
Bezid	BEZ	lake	Romania	Danube/Tisa	BS	46.413N, 24.878E	W BS	2016	9	native
Prespa	PRE	lake	Albania	L. Ohrid/Black Drim	AS	40.865N, 20.944E	S BA	2014	33	native

Notes.
[a]reservoirs

the *COI* gene fragment was 655 bp long, while for *16S* was 475 bp long. New *16S* and *COI* sequences were submitted to GenBank and BOLD data bases, and their GenBank accession numbers are MW726211–MW726336 and MW726338–MW726635 for *16S* and *COI* sequences, respectively (Table S1 in Supplements). Additionally, all available sequences of *16S* and *COI* genes of *A. astacus* were downloaded from GenBank (*COI* sequences from *Schrimpf et al. (2014)* and *Laggis et al. (2017)* were 350 bp-long, while sequences from *Mrugała et al. (2017)* were 635 bp long, and *16S* sequences were 475 bp long). GenBank accession numbers of the haplotypes obtained in the present study, as well as the ones from *Schrimpf et al. (2014)*, *Laggis et al. (2017)* and *Mrugała et al. (2017)* are reported in Tables S1 and S2.

In order to reconstruct phylogenetic tree that will be comparable with trees obtained in previous studies (*Schrimpf et al., 2014*; *Laggis et al., 2017*) we concatenated *COI* and *16S* sequences, and used only those samples for which sequences of both genes were available. This made concatenated data set (used in phylogenetic reconstruction) smaller compared to the *COI* data set that was used for other analyses. The 350 bp long *COI* sequences and 475 bp *16S* sequences from the same individual were concatenated and collapsed to unique haplotypes using FaBox (*Villesen, 2007*). The full concatenated *COI*/*16S* data set included 83 haplotype combinations (Table S2) and the final alignment was 825 bp long including a single gap-containing position observed in *16S* fragment. The optimal models of nucleotide evolution for each partition of the concatenated data set were selected under the Bayesian information criterion (BIC) using the jModelTest (*Darriba et al., 2012*). The selected model for *16S* was HKY+I, while for *COI* was HKY+G. Phylogenetic tree was reconstructed using the concatenated haplotypes in BEAST v.2.5.2 (*Bouckaert et al., 2019*). Since the null hypothesis of equal evolutionary rate throughout the tree was rejected at a 5% significance level, we used a relaxed uncorrelated lognormal clock model and the arthropod substitution rate of 2.3% pairwise sequence divergence for *COI* (0.0115 substitutions/s/Ma/l) (*Brower, 1994*) along with an estimated molecular clock for the *16S*. The tree prior was set as the birth-death and independent substitution models were assigned to each mtDNA gene. The Markov Chain Monte Carlo (MCMC) analysis run comprised 300,000,000 generations, sampled every 10,000 generations. In order to determine convergence, the Effective Sample Size (ESS) values were checked in Tracer (*Rambaut et al., 2018*). The best fit tree was produced using the Maximum clade credibility tree option in TreeAnnotator 2.5.2 after the 20% of the sampled trees was discarded as burn-in.

Additionally, the phylogenetic relationships were estimated using Bayesian analysis (BA) in MrBayes ver.3.2. (*Huelsenbeck & Ronquist, 2001*; *Ronquist & Huelsenbeck, 2003*) with priors set according to the suggested model for each partition (*16S*: HKY+I, *COI*: HKY+G). Two separate runs with four Metropolis-coupled Monte Carlo Markov chains (MMCM) were performed for 10,000,000 generations, and trees were sampled every 1,000 generations. After effective sample size (ESS values $>$ 200) for each parameter was confirmed with Tracer, the first 25% of sampled trees were eliminated as burn-in, and a 50% majority-rule consensus tree was constructed, with nodal values representing the

posterior probabilities. Sequences of *Pontastacus leptodactylus* (Acc. No. KX279350) were used as an outgroup.

The median-joining network (MJ) approach (*Bandelt, Forster & Röhl, 1999*) was used in order to establish non-hierarchical phylogeographic and phylogenetic relationships among samples. To that end, three data sets were prepared; two including only *COI* sequences, and one including concatenated (*16S+COI*) sequences. The *COI* sequence data set I comprised 655 bp sequences obtained in this study, while data set II comprised 350 bp long sequences published in *Schrimpf et al. (2011)*; *Schrimpf et al. (2014)*) and *Laggis et al. (2017)*, combined with sequences obtained in this study and the study by *Mrugała et al. (2017)*. The sequences obtained in this study and the study by *Mrugała et al. (2017)* were trimmed to 350 bp in order to match size of data sets from *Schrimpf et al. (2011)*; *Schrimpf et al. (2014)*) and *Laggis et al. (2017)*. This approach enabled us to associate *COI* haplotypes obtained in the present study to the *COI* haplotypes obtained in their research and indirectly to the lineages *sensu Schrimpf et al. (2014)* and groups *sensu Laggis et al. (2017)*. Since one of our aims was to position our new samples into previously constructed phylogenies (*Schrimpf et al., 2014*; *Laggis et al., 2017*), we adopted their naming of lineages or/and groups. The MJ networks were generated using the program PopART v.1.7 (*Leigh & Bryant, 2015*) with all parameters set to default values.

Genetic diversity indices (number of segregating sites, number of haplotypes, haplotype diversity, nucleotide diversity, average number of nucleotide differences) were calculated in program DNASP v.6 (*Rozas et al., 2017*).

Analysis of molecular variance (AMOVA) (*Excoffier, Smouse & Quattro, 1992*) was performed in Arlequin v.3.5 (*Excoffier & Lischer, 2010*) in order to estimate hierarchical distribution of genetic diversity of *A. astacus*. Populations were grouped on the basis of major tributaries of the Danube River (Drava, Sava, Vuka, Velika Morava, Timok, Tisa) and Prespa Lake. Standard AMOVA computations were performed with three hierarchical levels: among groups (river basins/tributaries), among populations within groups, and within populations. The variance components were tested statistically by non-parametric randomisation tests using 10,000 permutations. Genetic differentiation among populations and river catchments was analysed through estimation of pairwise values of Φ_{ST}.

Microsatellite DNA analyses

A total of 19 species-specific tetranucleotide repeat microsatellite loci were amplified following the protocols and procedures described by *Gross et al. (2017)* with the following modifications; initial screening of a few Balkan *A. astacus* populations revealed that they possess much higher variability and wider allele size range at many loci than the eastern European (Czech Republic and Estonia) populations used in the study of *Gross et al. (2017)*. Therefore, we split the single 19-plex microsatellite panel into two multiplex (10-plex and 9-plex) panels (Table S3). Only 15 loci were used for data analysis, as it later became apparent that at four loci, the allele size ranges still overlapped in some populations (Aast4_26, Aast4_47, Aast4_10 and Aast4_30) (Table S3). The PCR reaction (10 µl) contained 1x Type-it Multiplex PCR Master Mix (QIAGEN, Germany), 200 to 400 nM of each primer (Table S3), and ca 5 ng/ µl of DNA template. Touchdown program was

used for PCR amplification: initial activation of 5 min at 95 °C, followed by 20 cycles of 30 s at 95 °C, 90 s at 60 °C, 30 s at 72 °C, with the annealing temperature decreasing 0.5 °C per cycle, followed by 10 cycles of 30 s at 95 °C, 90 s at 50 °C, 30 s at 72 °C and a final extension for 30 min at 60 °C. The multiplex panels of microsatellite loci were genotyped on Applied Biosystems 3500 Genetic Analyser (Life Technologies, USA) using internal GeneScan 600 LIZ Size Standard v2.0 (Life Technologies, USA) and microsatellite genotypes were scored using GeneMapper v.5 software (Life Technologies, USA). Microsatellite genotypes were initially scored automatically and were double-checked manually by two experts. Subsample of 50 individuals (12% of total 413 analysed individuals) was genotyped twice to check the genotyping consistency.

MICRO-CHECKER 2.2.3 (*Van Oosterhout et al., 2004*) was used to assess the potential presence of genotyping errors due to scoring of stutter peaks, large allele dropouts and null alleles. FSTAT v. 2.9.3.2 programme package (*Goudet, 2001*) was used for calculating allele frequencies, F_{IS} and pair-wise F_{ST} values (as estimated by Weir and Cockerham's θ), for estimating expected (unbiased genetic diversity) and observed heterozygosities (H_E, H_O) and rarefied allelic richness (A_R), and for testing the significance of differences in average values of A_R, H_E and H_O among groups of populations (1,000 permutations, two-sided tests). The private allelic richness (A_{PrRar}) was estimated by rarefaction method using HP Rare v.Feb-2-2009 (*Kalinowski, 2005*) and multiplied by number of loci in order to make it comparable to the number of observed private alleles (A_{Pr}). GENEPOP v. 3.3 (*Raymond & Rousset, 1995a*) was used to test genotypic distributions for conformance to Hardy-Weinberg (HW) expectations and to test the loci for genotypic disequilibria. All probability tests were based on the Markov chain method (*Guo & Thompson, 1992*; *Raymond & Rousset, 1995b*) using 1,000 de-memorization steps 100 batches and 1,000 iterations per batch. Sequential Bonferroni adjustments (*Rice, 1989*) were applied to correct for the effect of multiple tests. Analysis of molecular variance (AMOVA) incorporated in ARLEQUIN v. 3.5.1.2 (*Excoffier & Lischer, 2010*) was used to partition genetic variance hierarchically between population groups, between populations within groups and among individuals within the populations. Populations were grouped based on major tributaries of the Danube River (Drava, Sava, Tisa, Velika Morava, Timok and Vuka) and the Prespa Lake.

Principal Coordinates Analysis (PCoA), implemented in PAST v. 4.05 (*Hammer, Harper & Ryan, 2001*), was used to explore and to visualize D_A genetic distance (*Nei, Tajima & Tateno, 1983*) matrix between populations in multidimensional space. The D_A distances were calculated using POPULATIONS v1.2.3.1 software (*Langella, 1999*).

Long-term effective population size (N_e) was assessed from the microsatellite data using the coalescent-based approach implemented in Lamarc v.2.1 (*Kuhner, 2006*), considering a mutation rate of 5×10^{-4} and a mixed mutational model with 30% KAM as in *Gouin et al. (2011)*. We ran three replicates of two initial chains retaining 10,000 genealogies sampled every 200 and discarding the first 1,000 as burn-in, followed by one final chain retaining 30,000 genealogies sampled every 200 and discarding the first 5,000 as burn-in.

Combined mtDNA and microsatellite analyses

In order to visualise the distribution of *A. astacus* genetic diversity across geographic space and connect genetic diversity patterns to geographic features we applied Alleles in Space (AIS) package (*Miller, 2005*), with software implementation of Mantel test and interpolation of genetic landscape shape (GLS). The Mantel test was used to test for correlations between genetic and geographic distances using three datasets: (a) *COI* data set including all sequences (350 bp long), (b) *COI* data set including only sequences obtained in this study (655 bp long), (c) genotypes of 15 microsatellite loci. Mantel tests were performed at the individual level using an analogue of Nei's genetic distances (*Nei, Tajima & Tateno, 1983*) between pair of individuals. For the Mantel test and other analyses run in AIS package, significance was tested using 10,000 permutations.

GLS interpolation was performed using two datasets: (a) full *COI* data set including all sequences (350 bp long) aiming to get insight into diversity on the European level, and (b) genotypes from 15 microsatellite loci in order to get insight into local diversity on the Balkan Peninsula. First, sampling sites were connected through a network based on the Delaunay triangulation, in which the simple mismatch molecular distances between connected sampling sites were calculated based on the molecular data obtained from all individuals. Surface calculation was based on midpoints of edges derived from Delaunay triangulation. Surface heights were calculated based on residual genetic distances.

The values of molecular distance were set in the mid-points of each connection in the network using the Alleles in Space (AIS) software (*Miller, 2005*). The raw molecular distances were interpolated. The matrix of the 'elevation' values was imported into QGIS 3.10 software (available at: https://qgis.org/en/site/) to generate molecular divergence surface image using the inverse distance weighted (IDW) algorithm, plotted over a map of Europe.

RESULTS

Phylogenetic relationships among populations using mitochondrial *COI* and *16S*

The phylogenetic tree inferred by BEAST using concatenated data set indicated six mostly unsupported previously described genetic lineages *sensu Schrimpf et al. (2014)* and groups *sensu Laggis et al. (2017)* (Fig. 2A), with all newly obtained concatenated haplotypes (Hap49, Hap50, Hap51, Hap52, Hap53, Hap54) nested within them. Most of the lineages diversified during Pleistocene, within the period between 1.7 and 0.5 mya (Fig. 2A). Phylogenetic reconstruction using BA in MrBayes revealed unresolved relationships among lineages/groups and weak nodal support with numerous polytomies (Fig. S1).

New haplotypes from Serbian populations (Hap49, Hap52 and Hap53) grouped with haplotypes from Kosovo, Montenegro and Germany (Fig. 2A). Haplotype 51 from Slovenia and haplotype 54 from Montenegro recovered within the same unsupported clade together with haplotypes from Croatia, Austria and Czechia (Fig. 2A). The haplotype representing specimens from the Lake Prespa in Albania (Hap50) was positioned within the clade encompassing haplotypes from Greece (Fig. 2A).

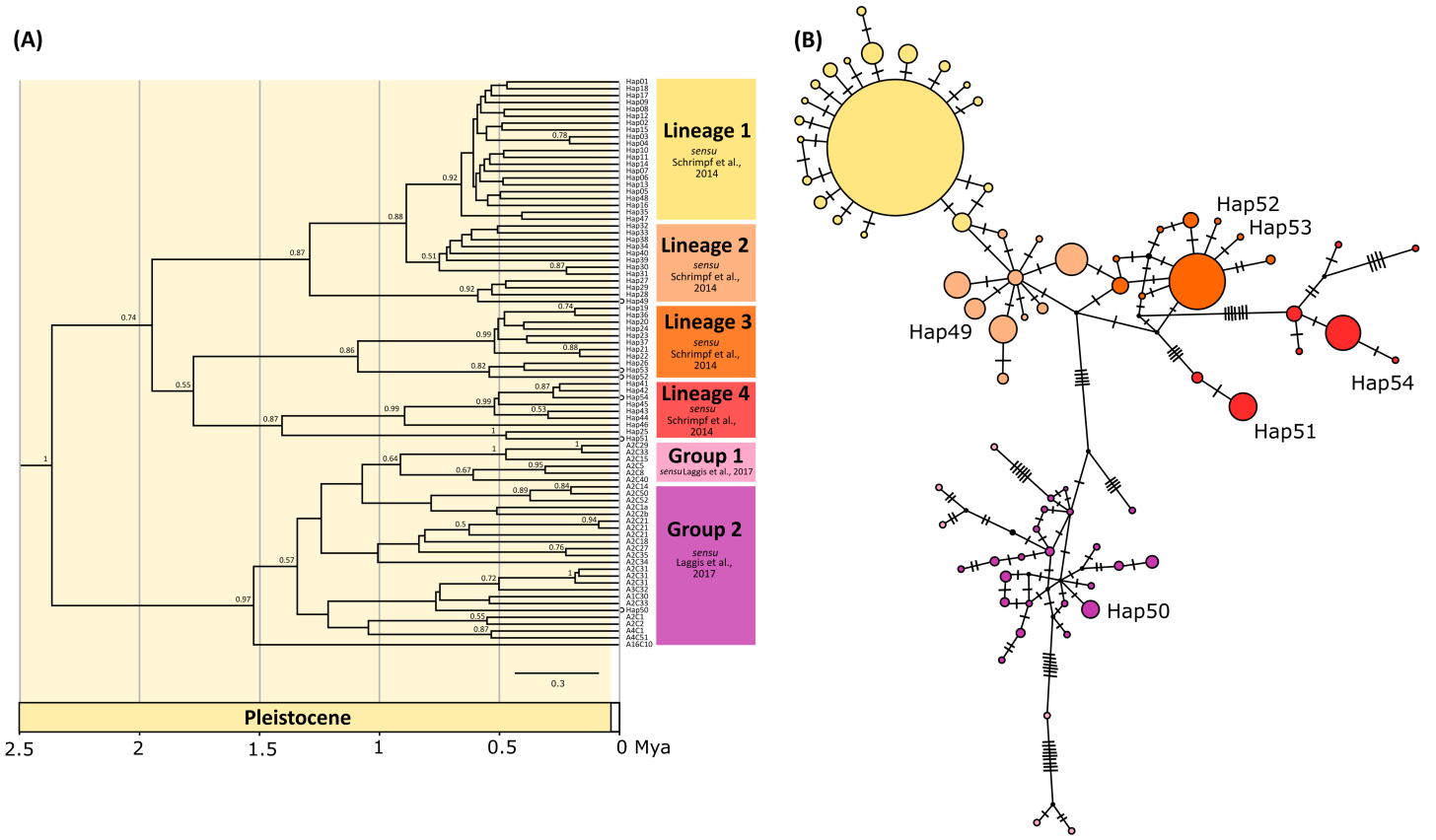

Figure 2 Bayesian phylogram and Median joining network. (A) Bayesian phylogram based on the concatenated *COI* and *16S* haplotypes of the noble crayfish using BEAST. Values at nodes represent posterior probabilities >0.5. Phylogenetic clades are represented as in *Schrimpf et al. (2014)* (Lineages 1–4) and *Laggis et al. (2017)* (Group 1 and 2), and the position of new haplotypes is indicated by circle at the end of the branch. (B) Median joining (MJ) network of concatenated sequences (*COI* and *16S*) of the noble crayfish. Numbers of mutational steps are given as hatch marks. The size of the circle is proportional to the frequencies of the haplotype, with black dots indicating extinct ancestral or unsampled haplotypes. Haplogroups are represented by different colour as in (A).

Full-size DOI: 10.7717/peerj.11838/fig-2

The median-joining (MJ) networks for concatenated data set (Fig. 2B), as well as for two *COI* data sets (Figs. 3 and 4), were mostly congruent and they depicted haplotype relatedness and distribution within *A. astacus*. The MJ network based on long *COI* sequences (655 bp) revealed the existence of 11 unique haplotypes, separated by different number of mutational steps (Fig. 3). The analysis of short-sequences data set (350 bp) produced MJ network containing 60 haplotypes, with 12 established in the present study, six of them (ssh1, ssh4, ssh5, ssh6, ssh10, ssh11) obtained for the first time (Fig. 4). The MJ network based on the concatenated data set encompassed 83 haplotypes, six of them (Hap49-Hap54) obtained for the first time (Fig. 2B).

In the long-sequences MJ network, haplotype Lshm9 (detected in the Slovenian populations BLO and KOC (Sava River, tributary of the Danube River)) and haplotype Lshm10 (discovered in Croatia, in the TOT and MAK populations (Drava and Sava, tributaries of the Danube River, respectively)) were separated by five mutational steps, whereas 10 bp changes were observed between Lshm9 and Lshm3, and 11 between Lshm9

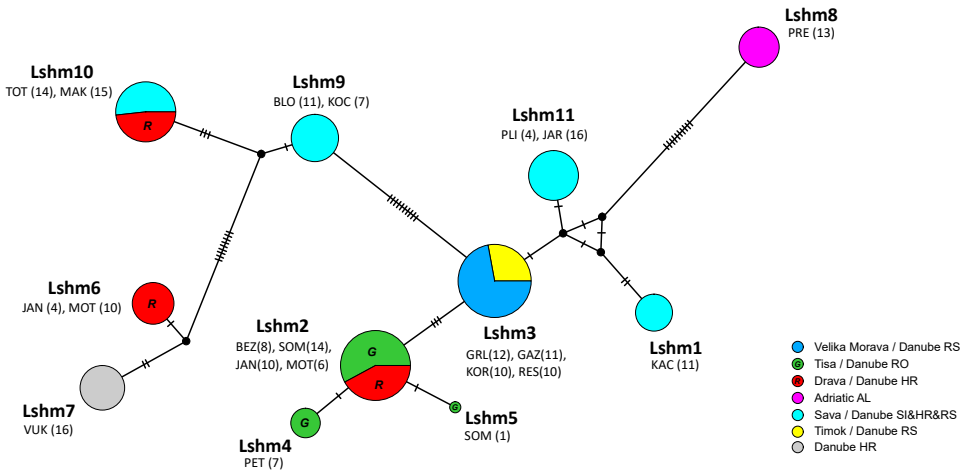

Figure 3 Median-joining network of *COI* sequences (655 bp long) obtained from *Astacus astacus* populations. Frequencies of haplotypes are proportional to the size of circles. The black dots indicate the median vectors, and numbers of base pair changes are indicated by hatch marks. Circles are coloured according to samples affiliation to the river tributary/ river basin/ country (RS-Serbia, RO-Romania, HR-Croatia, AL-Albania, SI-Slovenia). Haplotypes are labelled from Lshm1 to Lshm11 (Lshm –**l**ong **s**equence **h**aplotypes (samples) used also for **m**icrosatellites analyses). The three letter codes indicate sampling locality (for details see Table 1) with numbers of analysed specimens in brackets. In order to distinguish red and green circles in the network, we included letters *R* for red, and *G* for green.

Full-size DOI: 10.7717/peerj.11838/fig-3

and Lshm6 (Fig. 3). In the short-sequences MJ those two haplotypes (ssh10 and ssh11, respectively) were separated by one and three bp changes, respectively, from the closest haplotype that was recorded in Austria and Czech Republic (Fig. 4). The same was observed from the concatenated MJ; Hap51 (*COI* haplotype ssh10) and Hap25 (*COI* haplotype Aas18) were separated by one base pair change (Fig. 2B). Moreover, when BEAST tree was reconstructed with concatenated sequences the same grouping was observed; Hap51 and Hap25 form well supported subclade within unsupported lineage 4 *sensu Schrimpf et al. (2014)* (Fig. 2A), while this subclade was less supported in the Bayesian phylogram (Fig. S1). The Lsmh2/ssh2 *COI* haplotype was recorded in Croatian populations from the Drava River system (MOT and JAN) as well as in the Romanian populations (BEZ and SOM) from the Tisa River system where also Lshm4/ssh5 was recorded (Figs. 3 and 4). The former (ssh2 haplotype) is widely distributed in Europe (Fig. 4). Interestingly, in the populations MOT and JAN another *COI* haplotype was recorded (Lshm6/ssh7, Figs. 3 and 4) indicating presence of crayfish belonging to two distinct lineages/groups in the Drava River system. In concatenated data set, in both MJ and BEAST tree, Hap42 (*COI* haplotype ssh7) was closely related to Hap41 (*COI* haplotype Aas26) and Hap54 (*COI* haplotype ssh6) distributed also in Germany and Montenegro, respectively (Fig. 2). Some of the *A. astacus* specimens from Serbian waterbodies had ssh4 (in concatenated data set Hap53) haplotype, while most of them possessed the Lshm3/ssh3 haplotype (Figs. 3 and 4). Both haplotypes were geographically restricted to the Danube River basin tributaries Velika Morava and Timok. In addition, in concatenated data set the later formed Hap26 that is positioned close to haplotypes distributed also in Romania and Kosovo (Fig. 2).

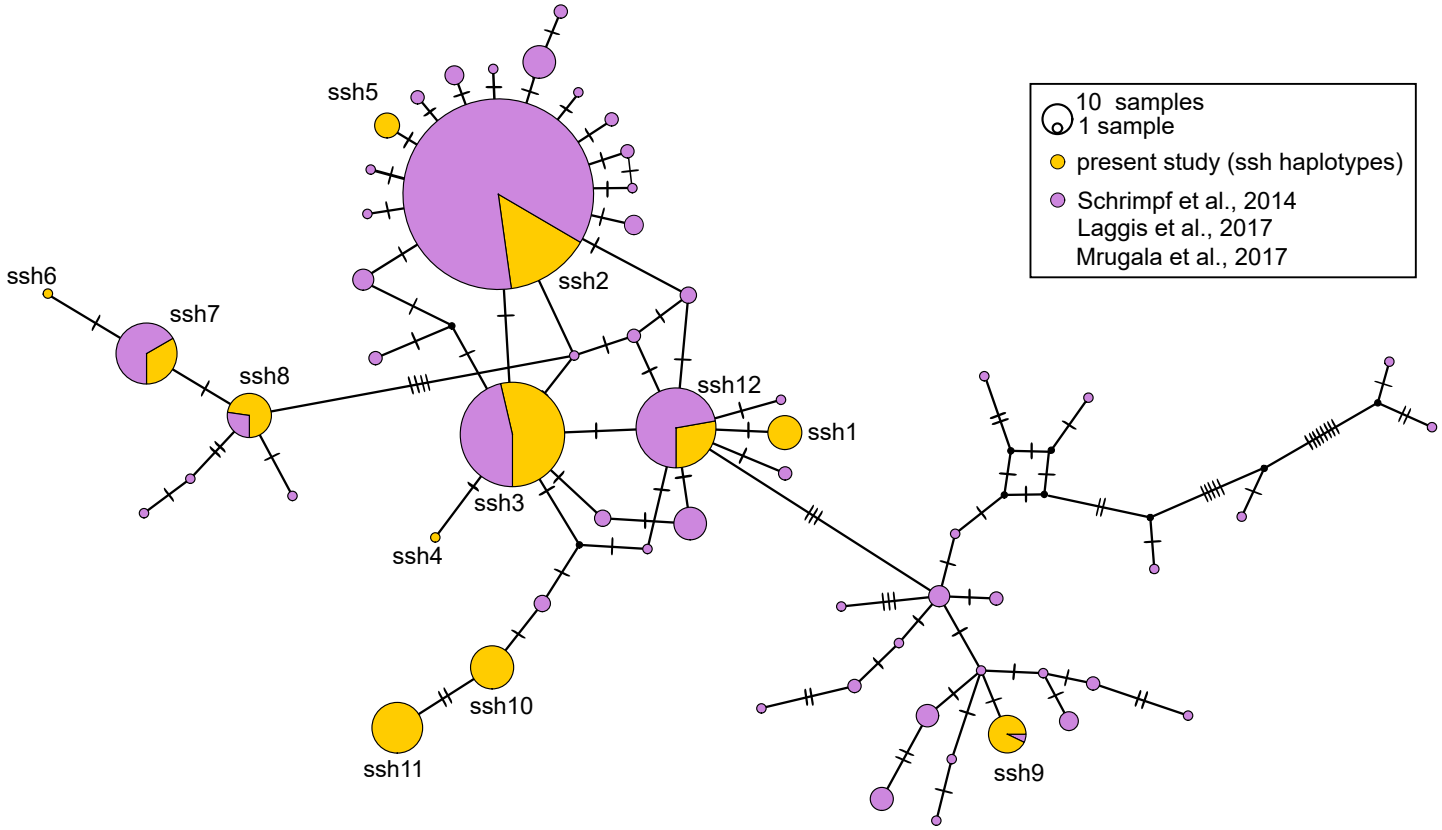

Figure 4 **Median-joining network of 350 bp long *Astacus astacus COI* sequences.** The black dots indicate the median vectors, and numbers of base pair changes are indicated by hatch marks. Frequencies of haplotypes are proportional to the size of circles. Purple colour presents share of previously haplotyped sequences (*Schrimpf et al., 2014*; *Laggis et al., 2017*; *Mrugała et al., 2017*) and yellow colour presents share of sequences obtained in the present study (marked as ssh–short sequences haplotypes: ssh1 to ssh11) (for details see Table S1).

Full-size DOI: 10.7717/peerj.11838/fig-4

The remaining specimens from Serbia were collected from the Sava River system (KAC) and they carried haplotype Lshm1/ssh1 (Figs. 3 and 4) that in concatenated data set forms Hap49, positioned closely to haplotype Hap28 from Croatia (Fig. 2). The Lshm7/ssh8 haplotype was established in the Vuka population, and in concatenated data set (Hap43) analyses is positioned closely to haplotypes recovered from the Save River in Croatia, but also haplotypes found in Germany (Figs. 2, 3 and 4). Finally, haplotype Lshm11/ssh12 was found in Croatian populations, and in concatenated data set it formed Hap28 that was positioned central to haplotypes from Danube's tributaries in Croatia, Serbia and Romania, but also from the Rhine River system (Figs. 2, 3 and 4).

Summarised results of genetic diversity indices of 655 bp long *COI* sequences revealed that populations from the Danube's tributaries Sava, Drava and Tisa rivers possessed higher number of haplotypes and nucleotide diversity compared to other river systems that were characterised by only one haplotype (Table 2).

Genetic differentiation of populations based on pairwise comparison of *COI* sequences revealed very high Φ_{ST}-values indicating genetically isolated populations with limited gene flow (Table S4). However, some of the population pairs' Φ_{ST}-values were extremely low

Table 2 DNA polymorphism indices. DNA polymorphism indices calculated using 655 bp-long *COI* sequences obtained from 18 *Astacus astacus* populations grouped according the river system: n, number of specimens; *S*, number of segregating sites; *h*, number of haplotypes; H_d, haplotype diversity; standard deviation in brackets, *Pi*, nucleotide diversity; standard deviation in brackets, *k*, average number of nucleotide differences, BS, Black Sea basin, AS, Adriatic Sea basin.

River system	Basin	n	*S*	*h*	H_d	*Pi*	*k*
Sava	BS	64	19	4	0.750 (0.0003)	0.01274 (0.0004)	8.344
Drava	BS	44	20	3	0.681 (0.0003)	0.01423 (0.0004)	9.323
Vuka	BS	16	0	1	0	0	0
Velika Morava	BS	31	0	1	0	0	0
Timok	BS	12	0	1	0	0	0
Tisa	BS	30	2	3	0.421 (0.087)	0.0007 (0.0002)	0.437
Prespa Lake	AS	13	0	1	0	0	0
Total		210	34	11	0.876 (0.008)	0.0145 (0.0005)	9.473

Table 3 Analysis of molecular variance. Analysis of molecular variance using mitochondrial *COI* sequences (655 bp long) and 15 microsatellite loci of *Astacus astacus*. For mtDNA data, populations were grouped based on their affiliation to major tributaries of the Danube River (Drava, Sava, Vuka, Velika Morava, Timok, Tisa) and Prespa Lake. For microsatellite DNA data, populations were grouped based on their affiliation to major tributaries of the Danube River (Drava, Sava, Tisa, Velika Morava, Timok and Vuka) and the Prespa Lake.

DNA marker	d.f.	Sum of squares	Variance components	Percentage of variation
Mitochondrial data				
Among groups	6	530.89	1.66	31.10
Among populations within groups	11	391.22	3.21	61.05
Within populations	192	88.46	0.46	8.56
Microsatellite data				
Among groups	6	1285.12	0.92	14.62
Among populations within groups	11	1096.26	2.34	37.18
Within populations	778	2360.32	3.03	48.21

Notes.
df, Degrees of freedom

and not statistically significant. If populations are grouped according to the Danube River tributaries (Sava, Drava, Velika Morava, Vuka, Timok, Tisa) and the Prespa Lake, values of genetic differentiation varied from 0.193 (Drava-Sava) to 1.00 (Prespa Lake vs Vuka, Velika Morava and Timok), again demonstrating genetically different groups (data not shown).

Results of AMOVA conducted on 655 bp long *COI* sequences grouped according to specimen's affiliation to major tributaries of the Danube River (Drava, Sava, Vuka, Velika Morava, Timok, Tisa) and Prespa Lake, revealed that most of the variance is contained among population within groups (61.05%, $P < 0.001$; Table 3), followed by variance among groups (31.1%), and a small amount of the variance was found within populations (8.56%).

Table 4 Genetic diversity parameters inferred from 15 microsatellite loci for 18 sites of the noble crayfish (see Table 1 for full names of populations).

Population	Basin/tributary	Sea basin	*n*	*P*	*A*	A_r	A_{pr}	A_{prRar}	H_e	H_o	F_{IS}	P_{HWE}
KOC	Danube/Sava	BS	10	0.73	2.07	2.05	0	0.30	0.344	0.333	0.032	NS
BLO	Danube/Sava	BS	28	1.00	3.40	2.90	2	1.65	0.465	0.464	0.003	NS
JAR	Danube/Sava	BS	23	0.93	3.47	3.05	4	3.00	0.562	0.577	−0.027	NS
PLI	Danube/Sava	BS	9	1.00	2.87	2.79	2	2.25	0.422	0.380	0.106	NS
MAK	Danube/Sava	BS	30	0.93	2.93	2.39	8	6.15	0.355	0.350	0.013	NS
KAC	Danube/Sava	BS	29	0.93	4.07	3.37	10	9.30	0.522	0.547	−0.049*	NS
Aver. Sava				0.92	3.14	2.76	4.33	3.75	0.45	0.44	0.03	
MOT	Danube/Drava	BS	32	1.00	3.87	3.34	2	2.85	0.573	0.562	0.020	NS
TOT	Danube/Drava	BS	30	1.00	3.27	3.09	1	1.65	0.577	0.557	0.036	NS
JAN	Danube/Drava	BS	30	1.00	4.13	3.26	3	1.80	0.557	0.529	0.051	<0.05
Aver. Drava				1.00	3.76	3.23	2.00	2.10	0.57	0.55	0.04	
VUK	Danube	BS	31	0.87	2.67	2.33	0	0.75	0.404	0.411	−0.017	NS
RES	Danube/V. Morava	BS	30	0.93	3.67	2.96	8	4.95	0.510	0.529	−0.037	NS
KOR	Danube/V. Morava	BS	17	0.73	2.00	1.91	0	0.60	0.306	0.267	0.133	NS
GAZ	Danube/V. Morava	BS	26	0.47	1.53	1.48	0	0.15	0.126	0.133	−0.063	NS
Aver. V. M.				0.71	2.40	2.12	2.67	1.95	0.31	0.31	0.01	
GRL	Danube/Timok	BS	13	0.33	1.33	1.31	0	0.65	0.106	0.113	−0.069	NS
SOM	Danube/Tisa	BS	22	0.93	2.53	2.22	1	0.30	0.334	0.342	−0.024	NS
PET	Danube/Tisa	BS	11	0.73	1.93	1.89	1	1.05	0.313	0.309	0.013	NS
BEZ	Danube/Tisa	BS	9	0.47	1.73	1.69	0	0.00	0.144	0.163	−0.143*	NS
Aver. Tisa				0.71	2.06	1.93	0.67	0.45	0.26	0.27	−0.01	
PRE	L. Ohrid/Black Drim	AS	33	0.73	2.27	1.83	11	11.25	0.237	0.230	0.027	NS

Notes.

BS, Black Sea; AS, Adriatic Sea; *n*, sample size; *P*, proportion of polymorphic loci; *A*, average number of alleles/locus; A_R, mean allelic richness; A_{pr}, number of private alleles; A_{prRar}, rarefied values of number of private alleles; H_e, expected and H_o, observed heterozygosity; F_{IS}, inbreeding coefficient; P_{HWE}, probability of deviations from expected Hardy–Weinberg proportions after sequential Bonferroni adjustments (15 simultaneous tests per population).

*$P < 0.05$.

Microsatellites analyses

Genetic diversity

A total of 180 alleles were observed across the 15 microsatellite loci with an average of 12.0 alleles per locus, ranging from 6 alleles at *Aast4_7* to 26 alleles at *Aast4_17* (Table S3). The average observed heterozygosity of the studied loci was 0.407 and varied from 0.254 (*Aast4_2*) to 0.518 (*Aast4_17*) (Table S3).

All microsatellite loci in studied crayfish populations were in linkage equilibrium (data not shown). Only a single population (JAN from Croatia) displayed significant deviation from expected HW proportions after applying sequential Bonferroni correction (Table 4). MICROCHECKER software provided evidence for putative null alleles at 4 out of 15 microsatellite loci in four populations (one to two loci per population, data not shown). However, as only 4 out of 270 tests were significant (1.5%, less than the expected Type-I error level), we decided not to exclude any loci from further analysis.

Genetic variation, expressed as the proportion of polymorphic loci (P), mean allelic richness (A_R) and observed heterozygosity (H_O), was on an average higher in the

populations from the Black Sea basin than in the Adriatic Sea basin ($P = 0.82$ and 0.73, $A_R = 2.5$ and 1.8, $H_O = 0.386$ and 0.230, respectively). Among the major tributaries of the Danube River, the highest average variation was observed in the Drava River populations ($P = 1.00$, $A_R = 3.2$, $H_O = 0.549$), while the lowest average variation was recorded in the Tisa River populations ($P = 0.71$, $A_R = 1.9$, $H_O = 0.271$) (Table 4). Overall, the most variable populations were JAR and KAC from the Sava River, MOT, TOT and JAN from the Drava River and RES from the Velika Morava River, while the least variable populations were GAZ from the Velika Morava River, GRL from the Timok River and BEZ from the Tisa River (Table 4).

The results of the hierarchical gene diversity analysis by AMOVA revealed that for the total data set, the highest percentage of variation was present within populations (48.21%), followed by variation among populations within river systems (37.18%) and among river systems (14.62%) (Table 3).

Of the total 180 alleles, 127 were shared by the Black Sea and Adriatic Sea basin populations, 11 alleles were confined to the Adriatic Sea basin and 42 alleles were found only in the Danube River basin of the Black Sea basin. Among major tributaries of the Danube, 26, 6, 8 and 2 alleles were confined to the Sava, Drava, Velika Morava and Tisa rivers, respectively (averaged values are shown in Table 4). When the number of private alleles was rarefied, the highest private allelic richness (average number of rarefied private alleles) was again confined to the Prespa population from the Adriatic Sea basin, followed by Sava, Drava and Velika Morava populations of the Danube basin, while the lowest private allelic richness was indicated for the Vuka, Timok and Tisa populations of the Danube basin (Table 4).

Genetic differentiation and population structure

The overall level of genetic differentiation between all studied samples was high (global $F_{ST} = 0.501$) with pairwise estimates of F_{ST} ranging from 0.211 (between JAR and MOT populations in Croatia) to 0.838 (between Serbian GRL and Romanian BEZ populations) (Table S4). The average level of differentiation among populations from different major tributaries of the Danube (Sava, Drava, Velika Morava, Tisa) was relatively high (average pair-wise F_{ST} ranging from 0.360 between Sava and Drava to 0.623 between Velika Morava and Tisa) but lower than their differentiation from the PRE population of the Adriatic Sea basin (average pair-wise F_{ST} from 0.578 between Drava and PRE to 0.736 between Tisa and PRE). Within the major tributaries of the Danube, populations were more differentiated in Sava, Velika Morava and Tisa (average pair-wise F_{ST} 0.425, 0.473 and 0.502, respectively) than in Drava (average pair-wise $F_{ST} = 0.281$) (data not shown).

PCoA analysis provided good resolution of spatial population relationships reflecting their affiliation to the river systems (Fig. 5). The Adriatic Sea basin PRE population was clearly separated from the Black Sea basin populations on both PCo1 and PCo2. Further, PCo1 clearly separated populations belonging to the Tisa, Timok and Velika Morava tributaries from the populations belonging to the Sava and Vuka rivers (Fig. 5). PCo2 separated populations from the Tisa River tributaries and populations from Serbia (both Timok and Velika Morava tributaries) (Fig. 5).

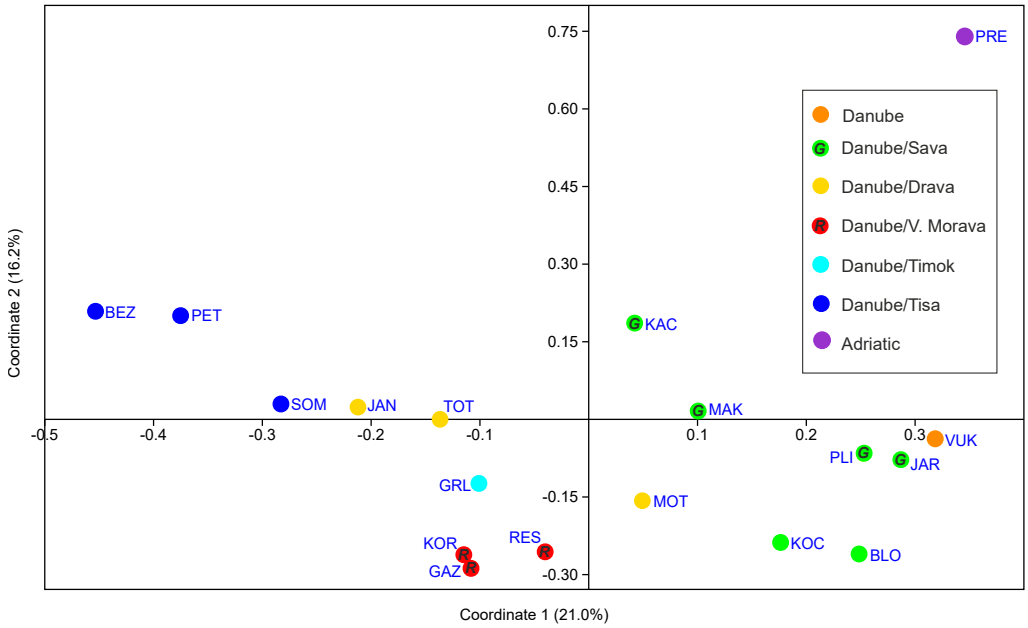

Figure 5 **PCoA scatterplot of the first two coordinates.** See Table 1 for full names of populations. In order to distinguish red and green circles on the map scatterplot, we included letters *R* for red, and *G* for green.

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Effective population size

Effective population size estimates varied strongly between populations and within river basins, ranging from 9 to 4920 (Table 5). The highest values were found in the Croatian populations of MOT, TOT and JAN located in the north-western part of the study area in the Drava River basin, with N_e values of 4920, 4864 and 4742, respectively. Globally, the Croatian populations displayed the highest effective population sizes (mean $N_e = 2951$), while lower estimates appeared mainly in the eastern populations of Serbia (mean $N_e = 1113$; the value drops to 539 if the north westernmost population of KAC from the Sava River basin is not considered), Romania (mean $N_e = 272$) and Albania ($N_e = 491$). The lowest N_e values were found in the Romanian population of BEZ ($N_e = 11$), and the Serbian populations of GAZ ($N_e = 18$) and GRL ($N_e = 9$).

Spatial analysis

Mantel's tests showed significant correlations between genetic and geographical distances ($r = 0.317$ ($P_{Mantel} < 0.001$) for 655 bp long *COI* sequences dataset; $r = 0.096$ ($P_{Mantel} < 0.001$) for 350 bp long *COI* sequences dataset; $r = 0.493$ ($P_{Mantel} < 0.001$) for microsatellite dataset). Alleles in Space (AIS) analysis was used to visualise the general genetic divergence on the European level for the *COI* data set (Fig. 6). Also, AIS was used to delineate genetic divergence in the area of interest (Balkan Peninsula) for the microsatellite data set (Fig. 7). The GLS interpolation on the *COI* data set showed that the area of the highest genetic divergences for *A. astacus* was located in the southern part of the Balkan Peninsula (Greece, Albania and North Macedonia) (Fig. 6). Moderate level of genetic divergence was observed

Table 5 Effective population size estimates - Theta (Θ), effective population size (N_e) and its 90% confidence interval (CI) estimated from the microsatellite data with Lamarc for the 18 *Astacus astacus* populations from the Balkans. $\Theta = 4N_e\mu$. $\mu = 5 \times 10^{-4}$.

Pop.	Θ	N_e	90% CI
KOC	0.668	334	200–572
BLO	2.043	1021	962–1704
JAR	4.485	2243	1987–3384
PLI	1.117	558	336–790
MAK	3.152	1576	1044–2106
KAC	6.812	3406	2356–4800
MOT	9.839	4920	4050–5041
TOT	9.727	4864	3777–5005
JAN	9.483	4742	3435–5006
VUK	3.512	1756	1171–2224
RES	3.628	1814	1288–2260
KOR	0.634	317	288–560
GAZ	0.037	18	14–38
GRL	0.019	9	1–11
SOM	1.193	597	319–643
PET	0.419	209	166–345
BEZ	0.023	11	8–17
PRE	0.981	491	352–985

among populations in Croatia and Slovenia, whereas the lowest divergences were indicated among populations from southern Serbia, Romania, Germany and Poland. The GLS interpolation on the microsatellite dataset indicated the highest genetic divergences was across a large area in the western part of Balkans (covering Sava and Drava River basins in Croatia and Slovenia) and the area in the southern Balkans (approximately corresponding to North Macedonia) (Fig. 7). Areas with the lowest genetic divergence were from parts of Romania, Serbia and Hungary.

DISCUSSION

In the present study we have updated the current knowledge on the mtDNA diversity of *A. astacus* in Europe by analysing numerous unstudied populations in the western part of the Balkan Peninsula. Study revealed new haplotypes (both *COI* and *16S*) that nested among haplotypes belonging to different lineages described in previous studies (*Schrimpf et al., 2014*; *Laggis et al., 2017*). Moreover, genetic structure of studied *A. astacus* population revealed by microsatellites indicated that populations in the western part of the Balkans harbour important components of genetic diversity for the species as anticipated in the previous studies (*Schrimpf et al., 2011*; *Schrimpf et al., 2014*; *Schrimpf et al., 2017*; *Gross et al., 2013*; *Laggis et al., 2017*). The finding of population structuring at both local, and larger geographic scales in this study is consistent with other studies of *A. astacus* across its distribution range (*Schrimpf et al., 2011*; *Schrimpf et al., 2014*; *Schrimpf et al., 2017*; *Gross et*

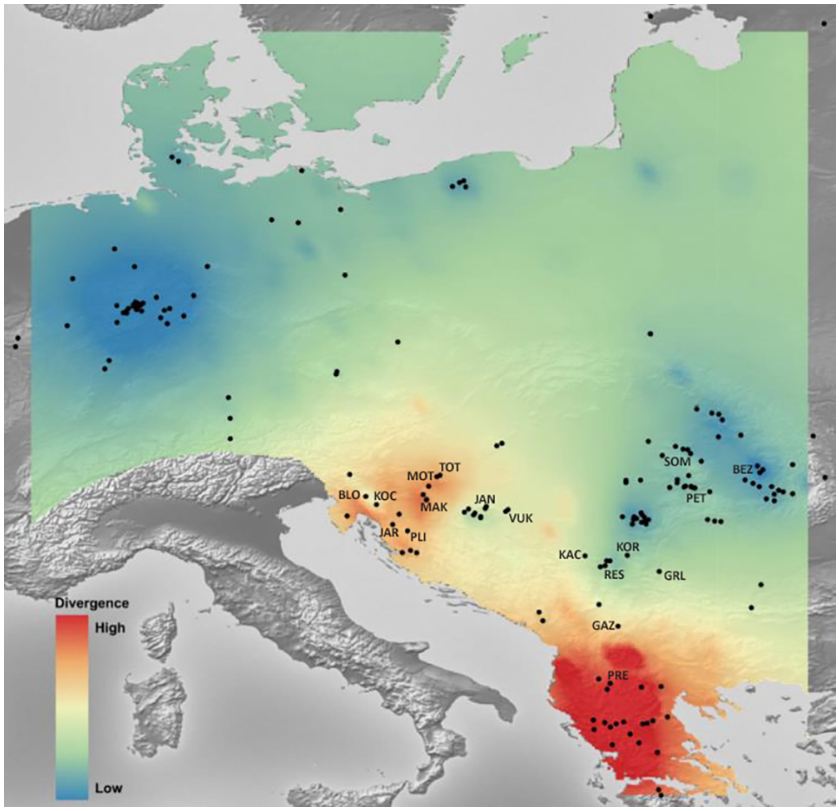

Figure 6 The genetic landscape map inferred by 350 bp long *Astacus astacus COI* sequences. A genetic landscape map based on *COI* was overlaid onto a relief map of Europe. Black dots refer to the sampling sites, red colour presents high molecular divergence between neighbouring populations, while blue colour correspond to areas of lower molecular divergence among populations.

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al., 2013; *Makkonen, Kokko & Jussila, 2015*; *Bláha et al., 2016*; *Laggis et al., 2017*; *Mrugała et al., 2017*; *Panicz et al., 2019*).

Astacus astacus is characterised by the complex evolutionary history as it has a large distributional range across a number of large catchments, and phylogeographic patterns and genetic diversity shaped through past geo-climatic processes and recent anthropogenic activities (translocations, reintroductions) (*Kouba, Petrusek & Kozák, 2014*; *Policar & Kozák, 2015*). Climate oscillations during the Pleistocene followed by postglacial (re)colonization processes from southern refugia, shaped the biodiversity of current European fauna (*Hewitt, 1999*), including *A. astacus*. Populations isolated in the southern refugia and micro-refugia accumulated genetic variation throughout the glacial period, however some of that diversity was lost due to bottlenecks and the founder effects experienced by populations during subsequent range expansions to the north (*Hewitt, 1999*). The observed pattern of reduced genetic diversity indicated by low haplotype diversity, allelic richness and fewer private alleles, in the north and central Europe (areas with pronounced glaciations) point to more recent range expansion into these regions

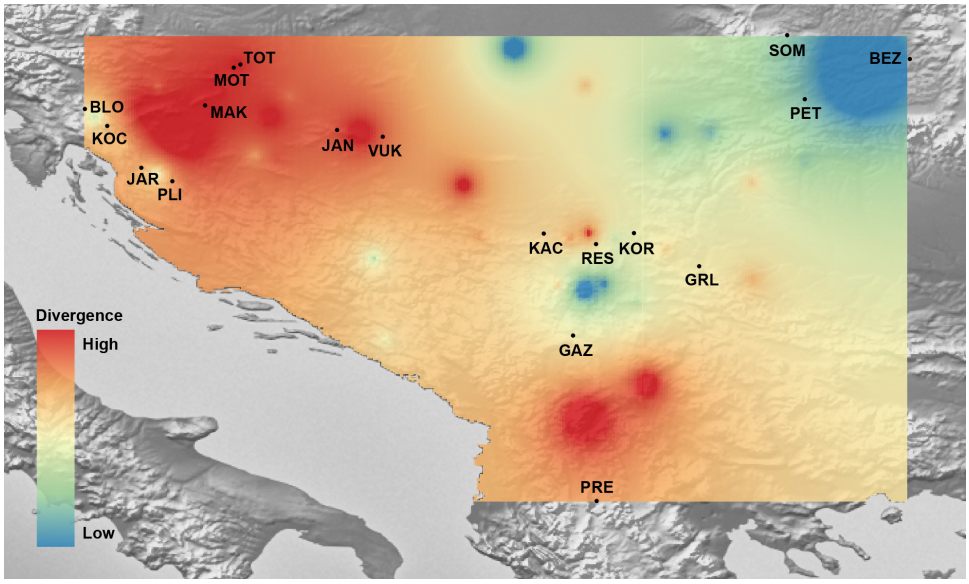

Figure 7 The genetic landscape map inferred by multilocus microsatellite genotypes of 18 *Astacus astacus* populations. To avoid extrapolating beyond the spatial extent of collection points, the genetic landscape is clipped to the extent of the original network (sampling extent) and to the boundaries of the region of analysis. Details about sampling sites (abbreviations) are provided in Table 1.

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(*Gross et al., 2013*; *Klobučar et al., 2013*; *Schrimpf et al., 2014*; *Laggis et al., 2017*; *Berger et al., 2018*, this study).

Previous studies suggested that *A. astacus* populations persisted through glaciations on the Balkan Peninsula, in three refugia: (I) western part of the Balkan Peninsula (waterbodies of the Adriatic and the eastern Black Sea basins in Croatia and Montenegro (*Schrimpf et al., 2014*); (II) the eastern Black Sea basin (waterbodies of the lower Danube in Romania, Bulgaria, Hungary (*Schrimpf et al., 2014*); (III) southern Balkans (Greece) (*Laggis et al., 2017*). These suggestions are supported by our finding of high genetic diversity between and within populations on the Balkan Peninsula compared to those in central and northern Europe (*Schrimpf et al., 2014*; *Laggis et al., 2017*; *Mrugała et al., 2017*; this study). The most likely postglacial colonization route of *A. astacus* towards north and central Europe was through the Danube River system (*Schrimpf et al., 2014*; *Laggis et al., 2017*). Also, human activities, such as translocations and re/introductions of *A. astacus*, also probably strongly influenced the natural genetic structure and diversity (*e.g.*, population mixing and introgression between introduced and indigenous populations) across Europe (*Souty-Grosset & Reynolds, 2009*; *Schrimpf et al., 2011*; *Schrimpf et al., 2014*; *Gross et al., 2013*; *Gross et al., 2017*; *Makkonen, Kokko & Jussila, 2015*).

Phylogenetic analyses based on the concatenated mtDNA data positioned newly obtained haplotypes within previously described haplogroups (*Schrimpf et al., 2014*; *Laggis et al., 2017*) which are mostly weakly supported. The median-joining network for concatenated data set as well as for 350 bp *COI* data set reflected those relationships, while the analysis of longer *COI* sequences (655 bp) refined relations and indicated existence of undescribed

diversity. These results uncovered how length of *COI* sequences could influence on discrimination and relationships between haplotypes. Even though short barcode sequences are suitable for species identification, frequently they are not accurate for resolving phylogenetic relationships (*Min & Hickey, 2007*; *Vecchioni et al., 2017*; *Meiklejohn, Damaso & Robertson, 2019*) and therefore in the future studies that will use longer sequences data sets possibly clearer insight into phylogenetic relationships among different haplotype groups within *A. astacus* will be gained.

It is worth mentioning that the number of mutational steps between *A. astacus* mtDNA lineages/groups is much lower when compared to the number of mutational steps between mtDNA lineages within other European freshwater crayfish species, such as *Austropotamobius pallipes* (varying from 1 to 26) and *Austropotamobius torrentium* (varying from 1 to 36) (*Fratini et al., 2005*; *Klobučar et al., 2013*; *Jelić et al., 2016*). Compared to other European freshwater crayfish species, *A. astacus* exhibits lower genetic diversity (*Klobučar et al., 2013*; *Maguire et al., 2014*; *Akhan et al., 2014*; *Jelić et al., 2016*). Based on the Barcode of Life Data Systems (BOLD system; http://www.boldsystems.org) records, all published *A. astacus COI* sequences form a single BIN (barcode index number) (cluster), while other European crayfish species form 17, 5 and 5 BINs (*A. torrentium*, *A. pallipes* and *Pontastacus leptodactylus,* respectively). Therefore, it can be inferred that all *A. astacus* mtDNA lineages/groups that have been described up to now (*Schrimpf et al., 2014*; *Laggis et al., 2017*) belong to a single species with indication that southernmost populations (haplotypes from well supported Group 1 and 2 *sensu Laggis et al. (2017)* could present subspecies *A. astacus balcanicus* (*Laggis et al., 2017*)/ *A. balcanicus balcanicus* (*Crandall & De Grave, 2017*). Future studies will probably clarify/resolve this taxon status as suggested in the study of *Astacus colhicus* by *Bláha et al. (2021)*. Nevertheless *A. astacus* displays relatively low level of genetic variation across its large geographic range compared to other members of the Astacidae family.

It is important to highlight the distribution range of haplotypes Lshm9 and Lshm10, that is limited to the westernmost part of the Drava and Sava tributaries in Croatia, and in the Sava River tributaries in Slovenia, likely indicating that this area of species distribution might have had an important role as microrefugia allowing *A. astacus* populations to survive Pleistocene climate fluctuations.

The present study established presence of crayfish with *COI* haplotype Lshm2 (ssh2, identical to *COI* haplotype Aas01 from *Schrimpf et al. (2014)*) in Croatia. This haplotype was recorded exclusively in the Drava River drainage, and is the most widely distributed haplotype in Europe. Moreover, haplotype Lshm2, as well as closely related haplotypes (Lshm4 and Lshm5) were recorded in the *A. astacus* populations from Romanian waterbodies. According to our results and *Schrimpf et al. (2014)*, it can be presumed that this haplotype did not originate in the Croatian freshwaters, but rather in Romania. Presence of closely related haplotypes in tributaries of the Tisa and Drava Rivers provide evidence of historical hydrological connection between those rivers (both tributaries of the Danube River). Moreover, the microsatellites based genetic distances (observed in PCoA plot) also indicated closer relationships of Drava's populations JAN and TOT to Romanian Tisza populations than to Sava or V. Morava populations.

Our results regarding populations TOT and MOT showed discrepancy between observations and expected results. Since the geographical distance between these two lakes is only 8 km, it was expected that specimens from both populations would share similar/identical haplotypes. However, this was not the case; the crayfish from TOT possessed distinct *COI* haplotype that grouped together with samples from MAK, BLO and KOC, while in the MOT population we discovered the presence of two distant haplotypes (Lshm2 and Lshm6). The observed genetic structure indicated complex evolutionary history of *A. astacus* in the Drava drainage that possibly played an important corridor for crayfish post-glacial range expansion. Moreover, it should be pointed out that both TOT and MOT are gravel pits that were part of the Drava River, so it is possible that these *A. astacus* populations represent remnant astacofauna formerly present in local river systems but now lost because of invasive species (*Hudina et al., 2009*) and therefore represent important ark sites (*Peay, 2009*). On the other hand, as the gravel pits are regularly used by fisherman the possibility of introduction of translocated crayfish from unknown locations cannot be excluded (*Maguire, Jelić & Klobučar, 2011*). Similar scenarios of crayfish translocations were found in different crayfish species across the globe: *e.g.*, Australia (*Nguyen et al., 2002*) and Europe (*Petrusek et al., 2017*).

The Serbian noble crayfish populations possess widely distributed haplotypes that have pan-European distribution, without an apparent geographical pattern.

The Prespa Lake samples analysed in the present study, and the Ohrid Lake samples studied in *Mrugała et al. (2017)* share a single *COI* haplotype what possibly indicates a historical bottleneck that reduced their diversity. Possible bottleneck for PRE population was also indicated by relatively small effective population size, what is similar to findings in *Laggis et al. (2017)* for *A. astacus* in Greece, and *Gouin, Grandjean & Souty-Grosset (2006)*; *Gouin et al. (2011)*) for *A. pallipes* in France.

The AMOVA results are not congruent between *COI* dataset and microsatellites what might be a consequence of different effective population sizes or/and mutation rates of the bi-parentally and maternally inherited markers (*Chesser & Baker, 1996*). While *COI* suggested that majority of variance exist among populations within major tributaries of the Danube River (Drava, Sava, Vuka, Velika Morava, Timok, Tisa) and Prespa Lake, AMOVA for microsatellite revealed that most of genetic variation is within populations, which is similar to findings in *Schrimpf et al. (2017)* and *Panicz et al. (2019)*. Observed genetic structuring might be an indication that *A. astacus* used to be widely distributed, and nowadays populations are isolated within drainages (tributaries/basins) but still retain a part of their original diversity, which is also consistent with high Φ_{ST} pairwise values.

Genetic structuring of *A. astacus* populations in the studied area was influenced by isolation by distance at a moderate level as observed using both *COI* and microsatellites datasets. However, isolation by distance probably was not the only factor that contributed to the observed genetic differences between populations; significant differences could be a consequence of landscape characteristics that produced geographically isolated population which have no surface water connection. Hydrogeography and complex landscape character of studied area played an import role onto the genetic differentiation of different freshwater taxa (*e.g.*, *Previšić et al., 2009*; *Klobučar et al., 2013*; *Jelić et al., 2016*). Furthermore, genetic

structure of *A. astacus* populations in Europe was shaped through anthropogenic influence (*Schrimpf et al., 2014*; *Laggis et al., 2017*), and possibly unexpected similarity between KAC (Serbia) and JAR and PLI (Croatia) populations can be explained by artificial stockings between the two countries.

The *COI* data set GLS interpolation indicated that, on the European level, the entire Balkan area was an important refugium for *A. astacus* in the past. Within the Balkan area, as shown by microsatellite GLS interpolation, there is a spatial subdivision of genetic diversity pattern within this large refugium suggesting several microrefugia (*e.g.*, western and southern parts of the Balkan Peninsula) also known for the occurrence of distinct freshwater taxa, such as amphipods, fish or insects (*Economidis & Banarescu, 1991*; *Previšić et al., 2009*; *Grabowski et al., 2017*; *Vucić et al., 2018*).

Using microsatellite markers, we revealed high genetic diversity (12.0 alleles per locus) and high differentiation ($F_{ST} = 0.512$) among populations, however relatively low diversity within populations (on an average 2.8 alleles per locus), indicating a long-term isolation of small refugial populations. This strong population subdivision may limit local adaptation and facilitate random genetic drift which might result in diminished evolutionary potential of *A. astacus* (*Nguyen et al., 2004*; *Steeves, Johnson & Hale, 2017*; *Hoffmann, Miller & Weeks, 2020*). The pairwise F_{ST} values obtained in our analyses indicated clear signs of high differentiation among populations within different tributaries of the Danube River and the Prespa Lake, as well as between population-pairs. This study revealed the highest value of global F_{ST} compared to other studies ($F_{ST} = 0.264$ in *Gross et al. (2013)*; $F_{ST} = 0.232$ in *Schrimpf et al. (2014)*; $F_{ST} = 0.400$ in *Laggis et al. (2017)*).

Even though the number of samples per population was not even, application of microsatellite data rarefaction showed that results of rarefied and not rarefied data are congruent, and different sample number did not influence much the results. The highest genetic diversity, revealed by microsatellites, was found in JAR and KAC (Sava), MOT, TOT and JAN (Drava), and in RES (Velika Morava) populations. These crayfish populations exhibit the highest values of average number of alleles per locus, allelic richness, expected and observed heterozygosity of all studied populations. Furthermore, those genetic diversity estimates were higher than the ones reported in previous studies where the same set of tetranucleotide microsatellite loci were used (*Gross et al., 2017*). When comparing other microsatellite studies on *A. astacus*, higher values of genetic diversity estimates were detected in populations from the southern Balkans (*Laggis et al., 2017*), while lower values of genetic diversity estimates were observed in the populations from central, northern and eastern Europe (*Gross et al., 2013*; *Schrimpf et al., 2014*; *Panicz et al., 2019*). The values of indices obtained in the present study suggested the postglacial re-colonisation towards north followed by decreasing genetic diversity, as it is observed for numerous taxa (*Taberlet et al., 1998*; *Hewitt, 1999*). Since JAR, KAC, MOT, TOT, JAN and RES populations possess the highest reservoir of genetic diversity, and high effective population sizes, they could play an important role in future conservation programs. Crayfish from those populations could be a source for repopulation/restocking but bearing in minds their genetic background as well as composition of recipient population need to be taken into account in order to avoid inbreeding/outbreeding depression (*Souty-Grosset & Reynolds, 2009*; *Hoffmann,*

Miller & Weeks, 2020). Furthermore, special effort should be taken in conservation of these populations not only because they harbour the highest genetic diversity, but also have the greatest number of private alleles, along with populations from PRE and MAK. Rare alleles are often considered a minor element in genetic conservation programmes, but they can be very important for the long-term response to selection and the survival of populations and species (*Allendorf & Luikart, 2007*). Moreover, PCoA analysis also revealed that the most distinct population was PRE (Black Drim/Adriatic Sea basin) and the rest of populations were well distinguished and grouped mainly according to their affiliation to river system. On the other hand, the lowest genetic diversity was recorded in GAZ (Velika Morava), GRL (Timok) and BEZ (Tisa) populations, which are also characterised with low effective populations sizes. These values of genetic diversity estimates are among the lowest compared to all previous population genetic studies (*Gross et al., 2017*).

In several populations the expected heterozygosity was higher than the observed heterozygosity indicating homozygote excess. In order to estimate the deviation from the Hardy-Weinberg equilibrium caused by inbreeding, which reduces the amount of genetic diversity in a population, we calculated the inbreeding coefficient (F_{IS}; proportional to the loss of genetic diversity and consequently loss of adaptive evolutionary potential of the species (*Frankham, 2005*)). Since the F_{IS} values in populations were slightly higher/lower than zero, we could conclude that inbreeding/outbreeding occurs, but is still not significant, and the intra-population variability is still evident.

Compared to previous population genetic studies of *A. astacus* (*Gross et al., 2013*; *Schrimpf et al., 2014*; *Mrugała et al., 2017*; *Panicz et al., 2019*), the overall genetic diversity in the study area was notably high. Our study confirms higher haplotype diversity and number of private haplotypes/alleles in the Black and Adriatic Sea basins compared to the North and Baltic Sea basins further corroborate a glacial refugium in the Balkan area.

It is necessary to know the genetic structure of the species in order to be able to preserve its integrity and within species diversity (*Schrimpf et al., 2014*). The combination of phylogenetic information and the degree of threat to species are both important for establishing conservation priorities (*Owen et al., 2015*). The results we obtained could be used as a starting point for developing future management plans. We would suggest that crayfish from populations with distant mtDNA haplotypes and, to some extent, between different tributaries of the Danube River (Sava, Drava, Tisa, Velika Morava, Timok) and the Prespa Lake should be treated separately in the future conservation projects (*e.g.*, restocking/repopulations) that will require careful and balanced approach in order to avoid outbreeding and inbreeding depression (*Schrimpf et al., 2017*). Preserving genetic variability between and within *A. astacus* populations will ensure their evolutionary potential and long-time survival.

CONCLUSIONS

In the present study we updated the current knowledge on the mtDNA diversity of *A. astacus* in Europe by including previously understudied populations and geographic regions. New haplotypes were discovered restricted to the western part of the Balkan

Peninsula. Analyses of microsatellites revealed population structuring at both local, and larger geographic scales observed across the *A. astacus* distributional range and indicates a complex genetic structure and confirmed that populations in the western part of the Balkans harbour important component of genetic diversity of the species. This information will help inform future conservation and management programs.

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ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Riho Gross, Mišel Jelić and Ivana Maguire conceived and designed the experiments, performed the experiments, analysed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Leona Lovrenčić and Frederic Grandjean performed the experiments, analysed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.

- Simona Đuretanović, Vladica Simić, Oksana Burimski and Marius-Ioan Groza performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Lena Bonassin analysed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.

Field Study Permissions

The following information was supplied relating to field study approvals (i.e., approving body and any reference numbers):

Samples were collected with permissions from local authorities: Croatia –the permission from Ministry of Environmental Protection and Energy of the Republic of Croatia (UP/I-612-07/18-48/148); Serbia - the permissions from Ministry of Environmental Protection, Republic of Serbia Number: 324-04-10/2021-04 and Institute for nature conservation of Serbia, Republic of Serbia 03 No. 026-419/2; and Slovenia –permission from the Environmental Agency of the Republic of Slovenia (35601-1262 150/2006-6 and 35601-135/2010-9), and in Romania samples were collected under permission of Comisia pentru Ocrotirea Monumentelor Naturii Nr. 408/CJ/27.11.2018.

Data Availability

The following information was supplied regarding data availability:

The *16S* (MW726211–MW726336) and *COI* sequences (MW726338–MW726635) are available in GenBank.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.11838#supplemental-information.

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Integrating population genetics and species distribution modelling to guide conservation of the noble crayfish, *Astacus astacus*, in Croatia

Leona Lovrenčić[1,4], Martina Temunović[2,4], Riho Gross[3], Marin Grgurev[1] & Ivana Maguire[1]✉

The noble crayfish, *Astacus astacus*, is an indigenous European freshwater species. Its populations show significant declines caused by anthropogenic pressure on its habitats, climate change and the spread of invasive species. Diminishing populations' trends and loss of genetic diversity highlight the need for effective conservation that will ensure their long-term survival. We combined population genetics and species distribution modelling (SDM) to reveal the impact of climate change and invasive species on the noble crayfish, and to guide future conservation programs of current populations. Our study showed that Croatian populations of *A. astacus* harbour an important part of species genetic diversity and represent significant genetic reservoir at the European level. The SDM results predicted substantial reductions of suitable habitats for *A. astacus* by the 2070; only 13% of its current potential distribution is projected to remain stable under pessimistic Representative Concentration Pathway (RCP 8.5) emission scenario. Moreover, most of the populations with high genetic diversity are located in the areas predicted to become unsuitable, and consequently have a high probability of being lost in the future. Further, SDM results also indicated considerable decrease of future habitat suitability for invasive crayfish species in Croatia, suggesting that climate change poses a major threat to already endangered *A. astacus*. The obtained results help in the identification of populations and areas with the highest conservation value which should be given the highest priority for protection. In order to preserve present diversity in areas that are predicted as suitable, we propose assisted migration and repopulation approaches, for enhancing populations' size and saving maximum genetic variability. The result of our research emphasizes once again the benefits of multidisciplinary approach in the modern biodiversity conservation.

One of the greatest challenges faced by humanity is the mitigation of rapid biodiversity loss, associated with negative anthropogenic activities[1]. Indigenous crayfish species are among the most threatened animal taxa in European freshwaters where they are experiencing substantial population declines across their entire distribution ranges[2,3]. Thus, the requirement for appropriate conservation actions and policies are urgently needed[4].

The noble crayfish, *Astacus astacus,* is a keystone species of high ecological, economic, and cultural importance in Europe[5]. It is an indigenous European freshwater species whose gene pool and wide current distribution have been shaped by geo-climatic events (i.e. Pleistocene glaciations) and anthropogenic impacts (i.e. translocations, pollution, habitat degradation). In Croatia, *A. astacus* is recorded in all three biogeographical regions: Continental, Alpine and Mediterranean. It is naturally distributed in the waterbodies of the Black Sea drainage, with a few recorded populations in the Adriatic Sea drainage that are of anthropogenic origin[6]. Large-scale genetic analyses revealed that *A. astacus* encompasses several mitochondrial lineages that have separated and diversified during the Pleistocene glaciations in the western and southern Balkans[7–9], as well as in the lower Danube basin[7]. Results of microsatellite analysis revealed a differentiation of northern European populations from central European populations, with the former exhibiting a lower genetic diversity[10]. Furthermore, Schrimpf et al.[7,11] and Laggis et al.[8] revealed that this species harbours the highest genetic diversity in south-eastern Europe, while, low genetic diversity was detected in central and northern Europe, resulting from founder effects due to postglacial re-colonization and frequent human translocations for economic reasons[7]. Climate change is also

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among the major pressures for a decline in *A. astacus* populations, and its recognised vulnerability[12] prompted the protection afforded by international legislation (Annex III of the Bern Convention, Annex V of Habitat Directive (92/43/EEC). In Croatia, its conservation status in all three biogeographical regions is unfavourable-inadequate following EU Habitats Directive (details are provided at https://nature-art17.eionet.europa.eu/article17/); during the last decade, 55% of *A. astacus* populations extirpated[6], demonstrating the inadequacy of the species conservation. Consequently, as decreasing population trends continued[13], *A. astacus* is listed as vulnerable even though protected by national legislation (NN 80/13).

Apart from being threatened by climate change, habitat loss and deterioration, the introduction of non-indigenous crayfish species (NICS), and their pathogens, are of additional major concern for indigenous crayfish species decline in freshwater ecosystems across Europe[5]. The NICS displace indigenous crayfish species through transmission of diseases such as crayfish plague caused by the oomycete *Aphanomyces astaci*, which is listed among the World's 100 Worst Invasive Alien Species[14]. Furthermore, NICS' success is attributed to competitive exclusion, tolerance to poor water quality and altered habitat[5]. In addition, apart from direct competition with indigenous crayfish populations, NICS possess the ability to change food webs and entire ecosystems[5]. During the last two decades, three NICS have been recorded in Croatian freshwaters: the marbled crayfish (*Procambarus virginalis*), the spiny-cheek crayfish (*Faxonius limosus*) and the signal crayfish (*Pacifastacus leniusculus*)[6,15]. Since the presence of *P. virginalis* remains limited to one local population[16], and *P. leniusculus* and *F. limosus* have more extensive ranges, they represent the most problematic NICS for *A. astacus* populations in Croatian freshwaters. *Faxonius limosus* was first recorded in the Nature Park Kopački rit (eastern part of Continental Croatia) in 2003 where it has spread naturally along the Danube River from Hungary[6]. Compared to previous data this species has significantly expanded its range and continues to successfully spread in Croatian freshwater ecosystems displacing native *A. astacus* and *Pontastacus leptodactylus*[6,15]. *Pacifastacus leniusculus* is one of the most successful crayfish invaders in Europe[2]. Indeed, Chucholl[3] found *P. leniusculus* to be the greatest threat to indigenous crayfish species among six NICS, in south-western Germany. This species is the most widespread NICS in Croatia due to its high dispersal rate which is among the highest recorded rates in Europe[15]. It is distributed in the continental part of the country, particularly in the Mura and Drava Rivers[6], as well as in the Korana River, where it was illegally introduced[15]. The Korana River and its tributaries are the hotspots of indigenous Croatian astacofauna diversity, namely, *A. astacus*, *Austropotamobius torrentium* and *P. leptodactylus*, which encompass various divergent lineages, are distributed in those freshwater ecosystems[6,17]. To guide best-practice conservation and management actions for *A. astacus* populations in Croatia, we aimed to identify areas of potential current and future habitat suitability overlap between the indigenous *A. astacus* and the two problematic NICS using Species Distribution Modelling (SDM) (details are provided below). Identifying such areas will enable perceiving locations where endangered *A. astacus* may overlap with its invasive competitors, currently or in the future, under different climate change scenarios. This information, combined with genetic data, is a crucial piece of information for selecting *A. astacus* populations with the highest priority for protection.

Recent substantial declines and local extinctions of indigenous European crayfish populations have highlighted the need for developing appropriate conservation programmes and policies[4]. Contemporary, conservation planning includes genetic screening and selection of potentially suitable habitats for long-term preservation[4]. Genetic variability within the species is essential for species survival by securing its evolutionary and adaptive potential which result in populations' capability of responding to new environmental conditions[18]. Reintroductions and/or restocking, as an approach for conservation of endangered species, are frequently disputed[19,20], but they are still emphasized as management strategies for the conservation of indigenous crayfish species[20,21]. For successful conservation, greater insight into genetic diversity and structure of endangered crayfish populations is needed[4]. Microsatellites are among the most popular and versatile genetic markers with wide applications in population genetics, conservation biology, and evolutionary biology[22]. Population genetics analyses using microsatellites have been used successfully in several studies of *A. astacus* by providing insights into patterns of contemporary genetic diversity and structure, gene flow, effective population sizes, evolutionary history, and fates of introductions[7–11,23,24]. Conservation of indigenous crayfish species does not only include the assessment of a population's genetic diversity in order to obtain restocking material for restoring their populations, but also includes the selection of potentially suitable habitats/sites in the future. The translocation of crayfish for reintroduction or restocking has been attempted many times across Europe, but the low rate of this measure's success showed the need to improve site selection[4]. Species distribution modelling, also known as ecological niche modelling, is increasingly suggested as part of conservation decision making by forecasting environmental suitability for an endangered species[25,26]. Species distribution models require georeferenced biodiversity observations (e.g., species occurrences) and geographic layers of environmental information (e.g., climate, land cover, soil attributes). This approach represents a useful tool for selecting suitable habitats for conservation actions, such as translocation, and selection of sites for protection, and at the same time taking into consideration impacts of invasive species and climate change on species and habitats[3,27–30]. Global climate changes impact the size and extent of areas that may potentially be inhabited by many species[31,32]. Moreover, climate change is causing distributional shifts of many species worldwide due to altering environmental conditions to which they are adapted[32]. Factors like increasing water temperatures and long-term droughts could, undoubtedly impact dispersal-limited freshwater crayfish impairing their survival[33], while favouring the future spread of warm-water adapted NICS[34].

Maintaining genetic diversity in an indigenous species is a pivotal goal at the global (European) and local (Croatian) levels (EU-Biodiversity strategy for 2030). Therefore, the present study has combined population genetic analyses and SDM as a guide for the future conservation actions of *A. astacus* genetic diversity. To provide such a baseline for conservation programs, the aims of our study were:

Population	Abbr	N	P	N_A	A_R	A_{PR}	H_E	H_O	F_{IS}	P_{HWE}	Null alleles
Motičnjak	MOT	21	1.00	3.73	3.33	2.70	0.580	0.562	0.032	ns	4_42
Breznica	BRE	14	1.00	3.67	3.20	2.10	0.541	0.495	0.087	*	4_35
Burgeti	BUR	19	1.00	3.33	2.94	0.15	0.450	0.453	-0.005	ns	4_42
Ilova	ILO	24	1.00	5.20	4.19	1.95	0.684	0.630	0.081	ns	4_17, 4_20
Otuča	OUT	9	1.00	3.47	3.41	1.50	0.573	0.511	0.114	ns	4_3
Bijela	BIJ	21	1.00	4.47	3.72	3.30	0.590	0.568	0.037	ns	4_42, 4_48
Glogovica	GLO	28	1.00	5.20	3.95	0.90	0.638	0.569	0.109	ns	4_2, 4_3
Kikovac	KIK	30	1.00	4.00	3.42	0.60	0.554	0.566	-0.021	ns	
Sloboština	SLO	27	0.93	4.07	3.32	2.70	0.565	0.455	0.198*	*	4_17, 4_37, 4_42, 4_32, 4_3, 4_35
Bednja	BED	30	1.00	6.00	4.18	2.25	0.624	0.582	0.069	ns	4_35, 4_3
Kutjevačka	KUT	16	1.00	4.93	4.23	3.30	0.674	0.586	0.133	*	4_37
Veličanka	VEL	30	1.00	5.27	4.07	3.90	0.600	0.515	0.145*	ns	4_38, 4_37, 4_3
Jaruga	JAR	23	0.93	3.47	3.05	2.10	0.562	0.577	-0.03	ns	
Maksimir	MAK	30	0.93	2.93	2.39	3.30	0.355	0.350	0.013	ns	4_3
Totovec	TOT	30	1.00	3.27	3.09	1.35	0.577	0.557	0.036	ns	4_42
Jankovac	JAN	30	1.00	4.13	3.26	0.30	0.557	0.529	0.051	*	4_32, 4_44
Vuka	VUK	31	0.87	2.67	2.33	0.15	0.404	0.411	-0.02	ns	

Table 1. Summarizing results across 15 microsatellite loci of population genetic diversity of studied *A. astacus* populations (N number of specimens, P proportion of polymorphic loci, N_A average number of alleles/locus, A_R allelic richness, A_{PR} rarefied number of private alleles, H_E expected heterozygosity, H_O observed heterozygosity, F_{IS} inbreeding coefficient and P_{HWE} probability of deviation from Hardy–Weinberg equilibrium after Bonferroni adjustments (not significant (ns) or significant (*)), null alleles—loci showing null alleles. Reference populations from Gross et al. (2021): JAR, MAK, TOT, JAN, VUK.

(1) To reveal genetic diversity and population structure of *A. astacus* from 17 localities in Croatia (Table 1), using mitochondrial DNA (mtDNA) and nuclear DNA (microsatellite) markers;
(2) To assess potential suitable habitats for the current and future period under different climate change scenarios for endangered *A. astacus* as well as for two NICS (*P. leniusculus* and *F. limosus*), and to identify areas of their potential current and future distribution overlap in Croatia using SDM;
(3) To combine genetic data from *A. astacus* with its potential future distribution areas, as well as with future potential distribution of both NICS in order to identify populations and areas of the highest conservation value and priority for protection.

We expect that a combination of SDM and genetic data will provide the required information needed to develop conservation programs for endangered *A. astacus*. Genetic characterisation will help identifying populations that should be given the highest priority in conservation, and which can also serve as suitable donor populations for possible repopulation and reintroduction programs not only in Croatia, but also in other European countries. Furthermore, we will be able to define areas and habitats that will be under the greatest pressure from NICS and climate change, as well as potential ark sites for this species long-time survival.

Results

Phylogenetic assignment of studied populations using mtDNA sequencing. Intraspecific phylogenetic relationships and haplotype relatedness within *A. astacus* were described by the Median-joining (MJ) networks (Fig. 1). Reconstruction based on concatenated mtDNA data indicated the existence of six previously reported genetic lineages *undefined sensu* Schrimpf et al.[7] and Laggis et al.[8] within *A. astacus* in Europe (Fig. 1). Both *COI* and *16S* + *COI* MJ networks exhibited comparable results and based on the number of mutational steps could possibly indicate the presence of a new distinct lineage containing haplotypes from the two Croatian populations and several Slovenian populations (Lsh18/Hap51 and Lsh19/Hap61 in Fig. 1**,** Supplementary Table S1). Remaining novel concatenated haplotypes obtained from studied Croatian populations (Hap55-Hap60) were nested within formerly recognised mtDNA lineages. Precisely, haplotypes were recovered within two lineages, Lineages 2 and 4 *sensu* Schrimpf et al.[7], with some populations harbouring crayfish with haplotypes from both lineages (populations JAN, MOT, OTU) (Fig. 1, Supplementary Table S1). The most widespread were populations belonging to Lineage 4 *sensu* Schrimpf et al.[7] encompassing the whole *A. astacus* distribution range in Croatia, while Lineage 2 *sensu* Schrimpf et al.[7] was found only in a few populations (Supplementary Fig. S1 and Supplementary Table S1).

Population genetics. *Genetic diversity.* The final data set for the microsatellite analyses comprised 413 samples and 15 microsatellite loci; 269 successfully genotyped noble crayfish samples from 12 populations in this study, and reference data from five populations obtained in Gross et al.[9]. No evidence of linkage disequilibrium between pairs of loci tested over all populations was detected after Bonferroni correction ($p = 0.0004$), hence all

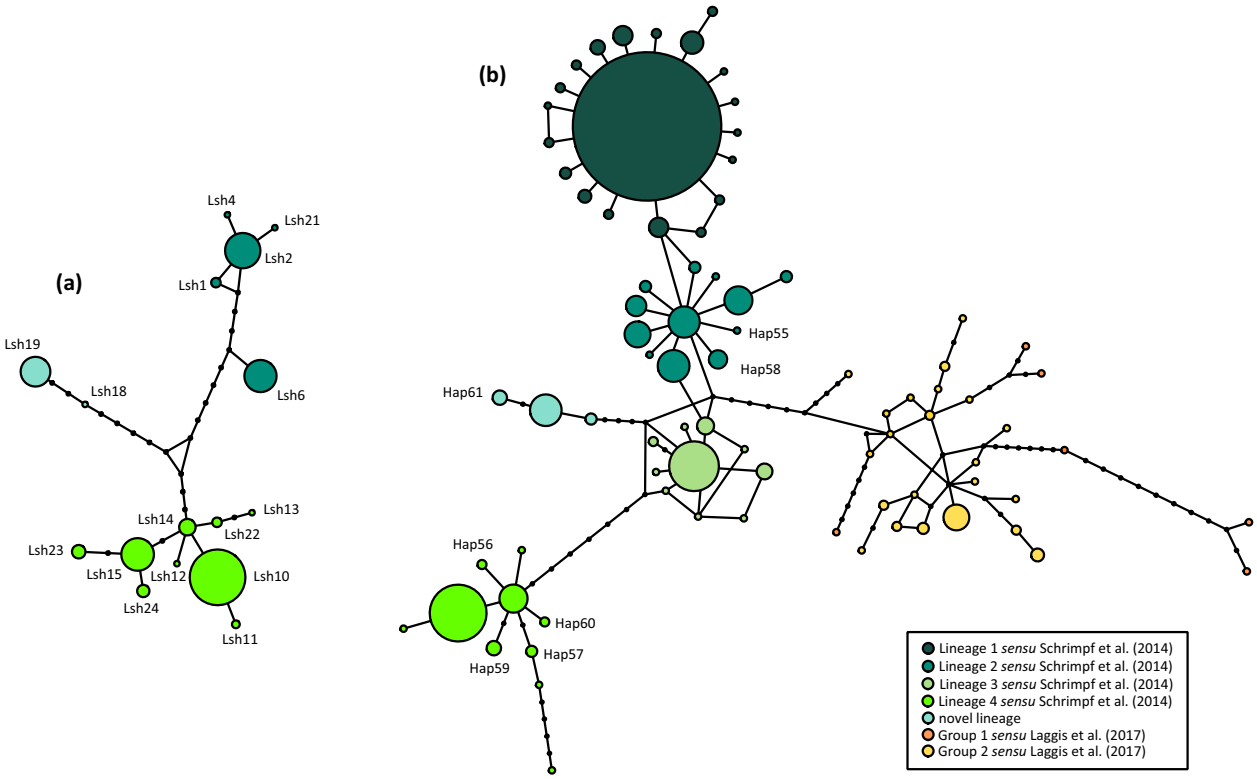

Figure 1. Median joining networks showing intraspecific phylogenetic relationships among **(a)** *COI* haplotypes from studied Croatian populations, and **(b)** concatenated *COI* + *16S* haplotypes from a European-wide dataset of *Astacus astacus,* with novel haplotypes labelled. Colours depict samples affiliation to mitochondrial lineages *sensu* Schrimpf et al.[7] and groups *sensu* Laggis et al.[8].

microsatellites were considered as independent markers. Likewise, no signs for genotyping error due to stuttering or large allele dropout were observed as assessed by MICRO-CHECKER software. Null alleles were detected within several loci and populations (Table 1). Estimated null allele frequencies were mostly low, ranged from 0.00001 (several combinations) to 0.284 (4_37 in population VEL). Only two loci (4_37 and 4_42) exhibited a high null allele frequency, > 0.2, according to Chapuis and Estoup[35]. Even though the F_{ST} values increased slightly when recalculated using adjusted allele frequencies (Table 2), no significant differences were observed between uncorrected F_{ST} values and F_{ST} values corrected for null alleles (t-test = 0.44, p = 0.32). Furthermore, no locus showed null alleles across all populations and a bias on our evaluation of the population structure due to null alleles was very unlikely. Therefore, all subsequent analyses were conducted using the original data set.

The summary statistics of the genetic diversity indices, for each population across 15 microsatellite loci, is shown in Table 1. All 15 microsatellite loci were polymorphic across all studied populations (Table 1). A total of 175 alleles were observed across the 15 microsatellite loci with an average of 12 alleles per locus, ranging from 5 alleles at locus 4_19 to 22 at locus 4_17. The mean number of alleles across loci ranged from 2.67 (VUK) to 6.00 (BED), mean allelic richness from 2.33 (VUK) to 4.23 (KUT), and H_E from 0.355 (MAK) to 0.684 (ILO) (Table 1). Total number of private alleles was 32. Rarefied number of private alleles ranged from 0.15 to 3.90, with the highest number observed in populations VEL, BIJ, KUT and MAK. The lowest number of private alleles was indicated for populations BUR, VUK, JAN and KIK (Table 1).

The H_O among populations ranged from 0.350 (MAK) to 0.630 (ILO), and the H_E from 0.355 (MAK) to 0.684 (ILO). H_E and H_O averaged to 0.560 and 0.524, respectively. The inbreeding coefficient per population was low to moderate and statistically not significant in the majority of populations (F_{IS} = -0.027 up to 0.198), so the intra-population variability was still evident. Only populations SLO and VEL exhibited statistically significant higher values of F_{IS} (0.198 and 0.145, respectively) indicating homozygote excess/heterozygote deficit. Significant deviations from Hardy–Weinberg equilibrium (HWE) were observed in four populations (BRE, SLO, KUT, JAN). Deviations from HWE were accompanied by positive F_{IS} values, indicating the heterozygote deficit and homozygote excess. Also in those populations, null alleles were detected (Table 1). Bottleneck analysis revealed consistent signs for recent contraction of population size in the populations JAR and TOT, which showed significant ($p < 0.05$) heterozygote excess according to the three mutational models tested (Supplementary Table S2).

Genetic differentiation and structure. The pairwise F_{ST} values ranged from 0.116 (between population KUT and OTU) to 0.556 (between MAK and BUR), with the global F_{ST} = 0.319 (Table 2). Populations MAK and VUK exhibited the highest F_{ST} values in comparison with other populations.

	MOT	BRE	BUR	ILO	OTU	BIJ	GLO	KIK	SLO	BED	KUT	VEL	JAR	MAK	TOT	JAN	VUK
MOT		0.292	0.305	0.207	0.228	0.271	0.224	0.294	0.229	0.147	0.228	0.209	0.240	0.462	0.267	0.272	0.422
BRE	0.296		0.371	0.227	0.254	0.278	0.228	0.339	0.278	0.285	0.202	0.227	0.287	0.500	0.307	0.346	0.381
BUR	0.311	0.377		0.274	0.309	0.313	0.235	0.288	0.334	0.255	0.251	0.286	0.204	0.550	0.382	0.336	0.478
ILO	0.212	0.241	0.281		0.191	0.146	0.160	0.207	0.171	0.188	0.157	0.169	0.222	0.392	0.245	0.247	0.331
OTU	0.230	0.262	0.316	0.196		0.245	0.222	0.288	0.182	0.202	0.121	0.171	0.239	0.447	0.267	0.290	0.426
BIJ	0.270	0.284	0.317	0.150	0.246		0.180	0.270	0.226	0.243	0.213	0.207	0.241	0.491	0.328	0.323	0.435
GLO	0.228	0.236	0.242	0.164	0.228	0.183		0.134	0.207	0.178	0.182	0.243	0.187	0.448	0.266	0.273	0.361
KIK	0.297	0.345	0.298	0.206	0.289	0.273	0.129		0.268	0.249	0.224	0.293	0.275	0.498	0.346	0.309	0.448
SLO	0.237	0.293	0.344	0.179	0.190	0.235	0.209	0.273		0.235	0.172	0.212	0.237	0.461	0.279	0.321	0.386
BED	0.149	0.286	0.261	0.195	0.206	0.243	0.184	0.251	0.241		0.221	0.234	0.203	0.408	0.217	0.154	0.409
KUT	0.233	0.216	0.255	0.167	0.116	0.218	0.192	0.226	0.188	0.225		0.130	0.239	0.440	0.266	0.260	0.358
VEL	0.213	0.242	0.291	0.177	0.180	0.213	0.250	0.296	0.222	0.236	0.147		0.256	0.438	0.268	0.291	0.377
JAR	0.239	0.293	0.205	0.228	0.246	0.241	0.188	0.277	0.240	0.204	0.245	0.259		0.506	0.312	0.310	0.439
MAK	0.464	0.506	0.556	0.393	0.446	0.492	0.450	0.496	0.457	0.406	0.441	0.440	0.509		0.361	0.462	0.538
TOT	0.278	0.321	0.394	0.259	0.278	0.338	0.276	0.356	0.293	0.227	0.281	0.284	0.321	0.378		0.276	0.383
JAN	0.284	0.360	0.349	0.258	0.300	0.331	0.287	0.318	0.336	0.160	0.274	0.303	0.320	0.469	0.289		0.438
VUK	0.424	0.389	0.486	0.332	0.428	0.435	0.365	0.449	0.386	0.411	0.364	0.382	0.442	0.539	0.392	0.448	

Table 2. Pairwise uncorrected F_{ST} values (below) and F_{ST} values corrected for null alleles (above) from 15 microsatellite loci between all populations pairs (all values are statistically significant, $p < 0.05$; see Table 1 for populations' abbreviation).

Population genetic structure was detected by the Bayesian clustering analysis implemented in the software STRUCTURE. The Bayesian Assignment Test was applied in order to assign individuals into clusters. The Evanno method, as implemented in STRUCTURE HARVESTER, revealed that the optimal number of clusters was two ($\Delta K = 2$). Individuals were assigned to a certain cluster if their assignment probability was ≥ 0.8, where individuals with membership to a cluster below this threshold were considered to be admixed. Most individuals showed a high assignment to one genetic cluster. The cluster I included individuals from populations MAK, TOT, JAN and BED, whereas the cluster II comprised crayfish from populations MOT, BRE, BUR, ILO, BIJ, GLO, KIK, SLO, KUT, VEL, JAR, VUK and OTU (Fig. 2). In the populations OTU and BED evidence of admixture was observed in some individuals (Fig. 2). In addition, with the purpose of getting finer insight into genetic structure of *A. astacus*, we report the second most probable number of distinct genetic clusters, $\Delta K = 5$: I) MOT, BED, JAN; II) BRE, VUK; III) BUR, GLO, KIK, JAR; IV) ILO, OTU, BIJ, SLO, KUT, VEL; and V) MAK, TOT (Supplementary Fig. S2).

Structure in the distribution of genetic variation was also depicted by the principal coordinates analysis (PCoA) (Fig. 2), where the PCo1 axis accounted for 25.94%, while the PCo2 axis accounted for 16.84% of the variation in the data. The PCoA revealed the existence of two well separated distinct clusters, with indication of another cluster between them. These results were congruent with the results of STRUCTURE (Fig. 2).

In order to reveal partitioning of genetic variance by AMOVA, populations were grouped according to their affiliation to the genetic clusters inferred by the Bayesian clustering analysis (Fig. 2). The results of the hierarchical genetic diversity analysis by AMOVA revealed that most of the genetic variation was represented among crayfish within populations (66.67% of variance) followed by variation among populations within clusters (27.01% of variance), while there was less variation between genetic clusters (6.31% of variance) (Supplementary Table S3).

Species distribution models (SDMs). *Model performances.* We evaluated model performance using area under the receiver operating characteristic curve (AUC)[36]. All SDMs for all species had excellent performance following interpretations for AUC values given in the literature[37,38], with AUC > 0.9, regardless of the method used (Supplementary Table S4). The current ensemble model for *A. astacus* had an AUC value of 0.998, while for the NICS (*P. leniusculus* and *F. limosus*) AUC values were 0.999 for both species.

Current and future Habitat suitability. Based on model projections under current environmental conditions, *A. astacus* habitat suitability values (ranging from 0, indicating areas of no or low suitability, to 1 indicating areas of the highest suitability) largely corresponded to current known distribution of this species in Croatia (Fig. 3a). Largest continuous suitable habitat for this species was projected into Continental Croatia, along and between the Drava and Sava Rivers, and along the Kupa River towards the south into Alpine Croatia, while smaller and more isolated areas of suitable habitat were predicted in the area of Mediterranean Croatia, where this species is not indigenous.

Current projections for NICS revealed highly suitable habitats for *F. limosus* in the easternmost part of Croatia corresponding to the regions along the Danube River and lower parts of the Sava River, and the small areas of suitable habitat were predicted along the middle part of the Sava River that could enable this species spreading towards the west of Croatia (Fig. 3b). For *P. leniusculus,* suitable habitats under current conditions were predicted

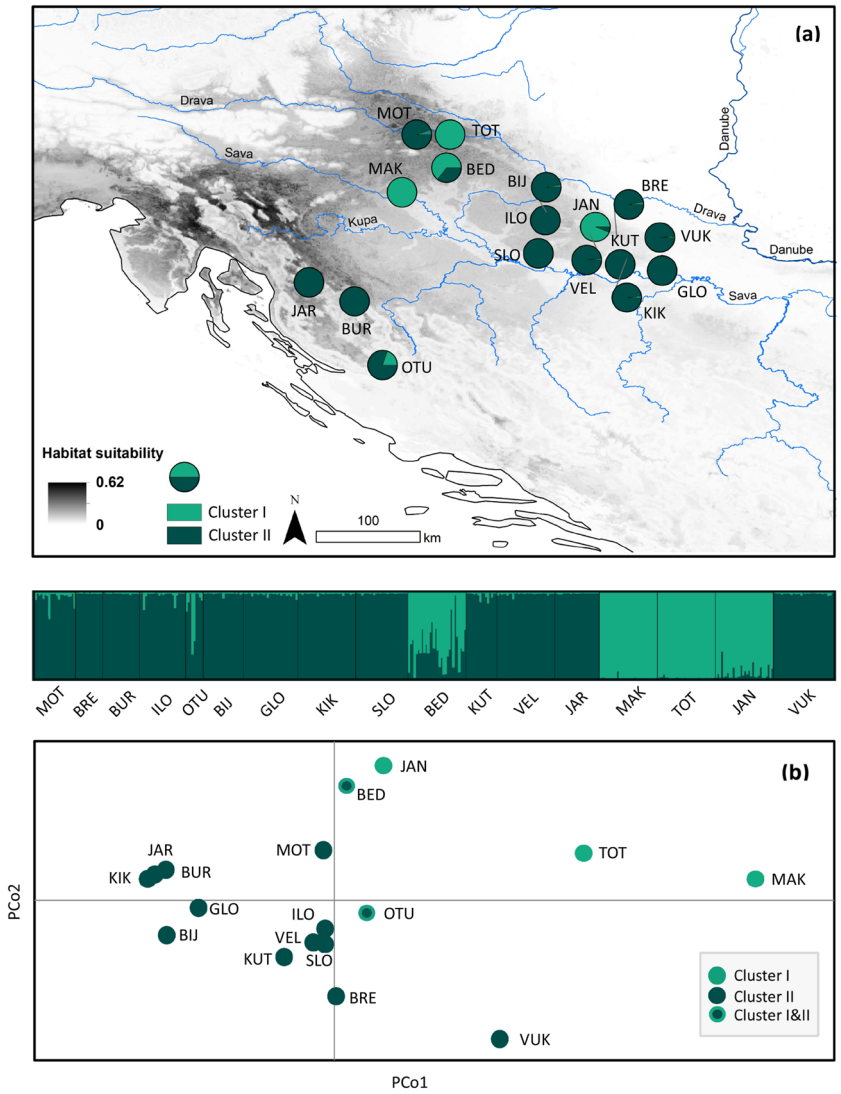

Figure 2. Genetic structure of the 17 studied *Astacus astacus* populations (see Table 1 for abbreviation) based on 15 microsatellites. **(a)** Genetic clustering inferred by STRUCTURE with the suggested K = 2 clusters. **(b)** Plots of the first two axes of a principal coordinates analysis (PCoA) based on Nei' D_A genetic distances. Each dot represents one population with colours depicting genetic cluster identified in STRUCTURE. Grey shading in the map indicates projected future habitat suitability for *A. astacus* under RCP 8.5 scenario in 2070. Map was produced in ArcGIS 10.3 program package by authors of this study.

in the Continental Croatia. The suitable habitats were anticipated along and between the Sava and Drava Rivers, as well as along the Kupa River, overlapping with habitats suitable for *A. astacus* (Fig. 3c).

Main trends in projected future habitat suitability under two considered RCP scenarios were similar for all species; therefore, we only report and show results for the more extreme RCP 8.5 pessimistic scenario (Fig. 3), while results for mid-range RCP 4.5 scenario are in the Supplement (Supplementary Fig. S3).

Future projections for *A. astacus* suggest considerable negative impact of climate change on habitat suitability of this endangered species in Croatia (Fig. 3d). In particular, future climate change projections forecasted severe reduction in suitable habitat by 2070 in the easternmost parts of the distribution in Croatia (along and between the Sava and Drava Rivers) and to some (lesser) extent in the western part along the Kupa River towards the Alpine Croatia. In addition, future maximum habitat suitability values did not exceed 0.62, compared to current maximum of 0.98. Overall, potential future distribution of *A. astacus* was predicted to shift towards north-west with some gain of suitable habitat predicted in the area of Slovenia (Figs. 3 and 4). Ensemble model projections suggested that 87% of the current suitable habitat will be lost by 2070 under pessimistic RCP 8.5 scenario and only 13% will remain suitable (Fig. 4). Under mid-range RCP 4.5 scenario 65% of the current suitable habitat is projected to be lost and 35% remains stable.

Although the projected future suitable areas for NICS were wider compared to current ones, we found a severe decrease in habitat suitability values for both NICS under future climate predictions which were the

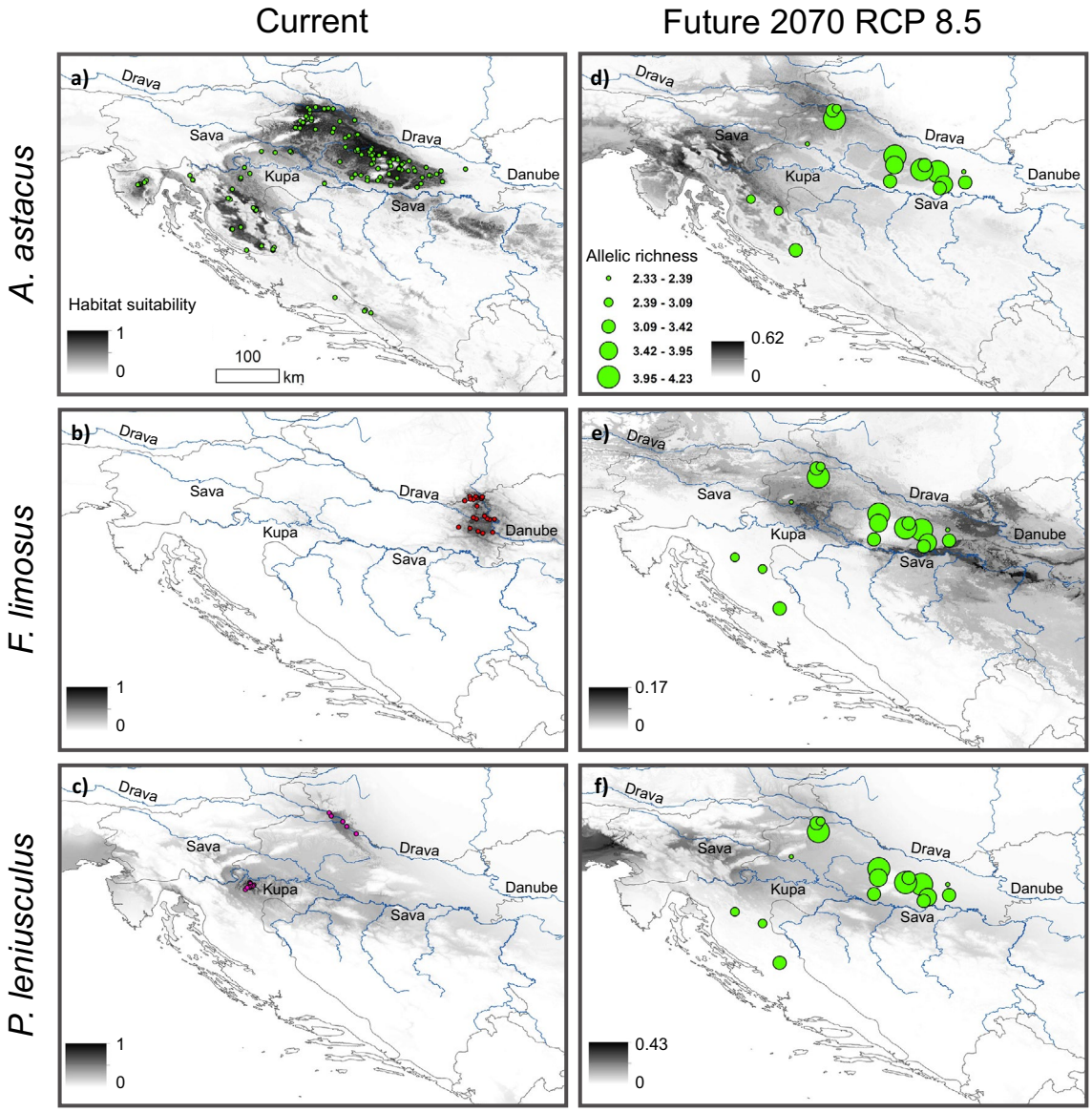

Figure 3. Ensemble potential habitat suitability for indigenous *Astacus astacus* and the two NICS, *Pacifastacus leniusculus* and *Faxonius limosus* in Croatia under current conditions **(a–c)** and future RCP 8.5 scenario in 2070 **(d–f)** based on SDMs. Occurrences of each species used for building SDMs are shown with coloured points **(a–c)**. Note that habitat suitability values in current projections are on the scale from 0 (unsuitable) to 1 (high suitability), while in the future projections habitat suitability values are on the scale from 0 (unsuitable) to maximum projected habitat suitability value. Future projections **(d–f)** of all species are shown in relation with the distribution of *A. astacus* allelic richness. Maps were produced in ArcGIS 10.3 program package by authors of this study.

most pronounced in *F. limosus* (Fig. 3e,f). Future maximum habitat suitability values did not exceed 0.43 for *P. leniusculus* and 0.17 for *F. limosus* (Fig. 3e,f). In most global circulation model (GCM) projections, maximum habitat suitability values were below the threshold maximizing the sum of sensitivity and specificity. Consequently, binary maps did not provide any suitable areas for NICS in the future, regardless of the RCP scenario. We therefore show and interpret only continuous future habitat suitability projections for NICS. Under future environmental conditions *F. limosus* is predicted to gain suitable habitats towards the west from its current distribution, along the Sava and Drava Rivers, although with very low probability, while suitable habitats for *P. leniusculus* are predicted to remain relatively similar to current ones, however with lower probability (Fig. 3e,f). Under current conditions we found an overlap between suitable habitats for *A. astacus* and *P. leniusculus* in the north-west Croatia along the Drava River and southern tributaries of the Sava River, which seem to be suitable for both species (Fig. 4a). Contrary, no overlap was detected between current suitable areas for *A. astacus* and *F. limosus* (Fig. 4a).

Overlapping genetic variation of *A. astacus* with projected changes between its current and future habitat suitability indicated that majority of the areas harbouring highly diverse *A. astacus* populations are expected to

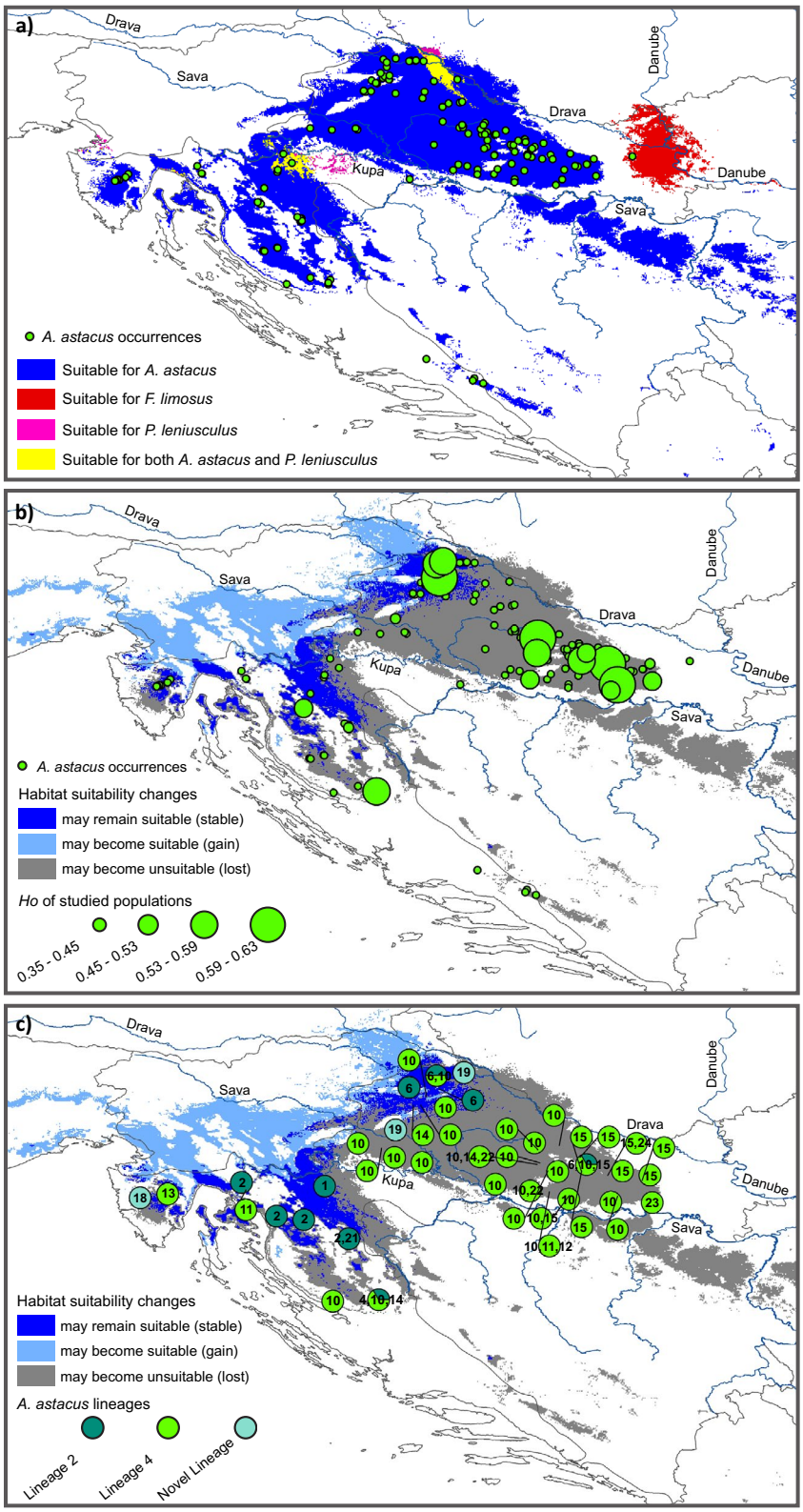

Figure 4. Potential overlap between suitable habitats for *Astacus astacus* and the two NICS. (**a**) Potential overlap between suitable habitats for *Astacus astacus* and the two NICS, *Pacifastacus leniusculus* and *Faxonius limosus* shown under current conditions**.** Projected changes between current and future habitat suitability for *A. astacus* under RCP 8.5 scenario in 2070 in relation to (**b**) observed heterozygosity (H_o) and (**c**) *COI* haplotypes depicted by numbers and coloured according to mitochondrial lineages (both corresponding to Fig. 1). Known species occurrences are also shown in (**a,b**). Maps were produced in ArcGIS 10.3 program package by authors of this study.

become unsuitable in the future (Figs. 3d and 4b). The only populations (sampled for microsatellites) remaining in areas predicted to remain suitable in 2070 under RCP 8.5 scenario are MOT, BUR, BED, JAR and TOT (Fig. 4b). Considering mtDNA, overlap of *COI* haplotypes and future habitat suitability indicated that 50% (8/16) of *COI* haplotypes recorded in Croatia may be lost (namely, Lsh1, Lsh4, Lsh12, Lsh15, Lsh18, Lsh22, Lsh23 and Lsh24) (Fig. 4c). Majority of those haplotypes are distributed only in the eastern part of the Continental Croatia (Fig. 4c).

Discussion

In our research, the fine scale phylogenetics, population genetics and species distribution modelling were used to explore genetic diversity and structure of *A. astacus*, as well as the impact of climate changes and invasive species on its populations. Analyses of genetic data coupled with species distribution models revealed the vulnerability of this keystone species to climate change.

The phylogenetic network based on mtDNA displayed intraspecific relationships within *A. astacus* consistent with the findings of previous studies[7–9]. Our results confirmed the existence of several genetic lineages, with the indication of novel divergent lineage containing haplotypes from Croatia and Slovenia (Hap51/Lsh18 and Hap61/Lsh19). Phylogenetic analysis indicates that all Croatian haplotypes belong to two mtDNA lineages (Lineages 2 and 4 *sensu* Schrimpf et al.[7]) that were also recorded in different countries across Europe. *Astacus astacus* exhibits lower mtDNA diversity and lower genetic structuring, without an obvious geographical pattern[7], compared to other native European crayfish species[17,39–42]. Precisely, the MJ network showed weak phylogeographic structure and high haplotype-sharing even between geographically distant populations. This finding is consistent with the results of previous studies[9,11] showing that the contemporary distribution and genetic structure of *A. astacus* were shaped through past geo-climatic events, strong anthropogenic influence on its habitat and frequent human mediated translocations that partly eroded their genetic structure. Similarities between distant *A. astacus* populations in several cases were explained by artificial stockings from different countries or populations[10,11]. Such a case was also observed in our study; crayfish from population JAR were used for aquaculture in the geographically distant hatchery Otočac, and consequently samples from both populations belonged to the same mtDNA lineage and shared the same haplotype (Lsh2). The genetic lineages of *A. astacus* diversified during the late Pliocene and throughout the Pleistocene, within the period between 1.7 and 0.5 mya[9]. Current *A. astacus* lineage distribution shows a divergence pattern congruent with the phenomena of insularity and isolation of multiple southern glacial refugia during repeated climatic pulses in the Pleistocene that produced a mosaic of lineages[43].

Population genetic analyses on *A. astacus* across the sampled localities revealed high within-population genetic diversity and moderate differentiation among populations, that differed from the results of previous studies using the same[9] or different microsatellite loci[7,8,10,11,24]. Overall, we detected a high number of alleles, proportion of polymorphic loci (P), allelic richness (A_R) and observed heterozygosity (H_O) in the study area. Genetic diversity, expressed as the P, A_R and H_O was higher in populations ILO, BED, KUT, VEL, while the level of genetic diversity was lower in populations MAK, VUK, BUR. Reduced genetic diversity in the populations MAK and BUR could be explained by the fact that they represent introduced populations[44,45]. Overall genetic diversity across the sampled localities of *A. astacus* was high when compared to the results of Gross et al.[10], Schrimpf et al.[7,11], Laggis et al.[8] and Panicz et al.[24] that used different set of microsatellite loci. A considerable number of private alleles was found in the majority of populations suggesting the presence of the unique genetic variation. Besides, private alleles are considered important in the long-term response to selection and the survival of populations and species[46]. We found that two populations (SLO and VEL), with significant homozygote excess, are vulnerable to inbreeding which may reduce the populations' genetic diversity, and consequently lead to the loss of adaptive evolutionary potential of the species[47]. Furthermore, we analysed whether the recent bottleneck events influenced the observed genetic structure of the studied populations, and found that two populations did experience a recent bottleneck event (JAR and TOT). Bottlenecks in small remnant populations with limited gene flow could lead to low effective population sizes and cause fitness reductions across at least part of the species distribution. In Croatia, as elsewhere in Europe, *A. astacus* populations are mostly isolated by natural (i.e., watershed boundaries) or artificial (i.e., anthropologically influenced) barriers, and their distribution being frequently limited to small fragmented areas (geographical regions). Therefore, there is a reasonable concern that they may undergo significant declines in effective population size and that much of their genetic diversity might be lost.

The results of STRUCTURE and PCoA indicated the presence of genetic structuring among *A. astacus* populations in Croatia by identifying two main genetic clusters. Moreover, they revealed the presence of admixed individuals/populations assigned to different genetic cluster reflecting contributions of different ancestral groups or artificial translocation. Furthermore, results indicated to some extant populations' structuring according to different river basins what is similar to that found by Gross et al.[9]. The pairwise F_{ST} values and AMOVA indicated moderate to high levels of genetic differentiation among studied populations demonstrating isolated populations with limited gene flow. The most genetically differentiated populations were MAK, TOT, JAN and VUK when compared to other studied populations. Contrary to other populations, the Vuka River (population VUK) flows directly into the Danube River which may explain the high F_{ST}. Lower F_{ST} values obtained in this study reflected well geographical proximity, with the exception of populations OTU and KUT. The native range of *A. astacus* is restricted to the rivers of the Black Sea basin, whereas population OTU belongs to the Adriatic Sea basin. Thus, low F_{ST} value obtained for this population pair could indicate anthropogenic translocation between those two populations. Likewise, high values of F_{ST} for MAK populations could also be explained by artificial stockings from an unknown source. Moreover, it should be pointed out that MAK is recorded in an urban lake in Zagreb City, and that it may have been introduced from the Sava River where Karaman[48] recorded *A. astacus*. Therefore, it is possible that this population represents a remnant astacofauna formerly present in the Sava River, with unique genetics that no longer exists elsewhere. This study discovered a higher value of global F_{ST} (= 0.319) compared

to the study of *A. astacus* in central and northern Europe by Gross et al. ([10]; $F_{ST} = 0.264$) and Schrimpf et al. ([7]; $F_{ST} = 0.232$), but a lower value than that was found by Laggis et al. ([8]; $F_{ST} = 0.400$) and Gross et al. ([9]; $F_{ST} = 0.512$), in *A. astacus* populations in Greece and across the Balkan Peninsula, respectively. A pattern of isolated populations of freshwater species that contain high genetic diversity is characteristic for the Balkan Peninsula, that is recognised as one of the freshwater biodiversity hotspots[43,49,50]. Currently this region is characterised by fragmented and complex habitats with frequently no suitable surface water connections. Therefore, restricted dispersal and gene flow among populations probably led to genetic isolation of numerous freshwater species in this area, including crayfish. However, limited gene flow may lead to reduced effective population sizes, lower genetic diversity and increase the risk of local extinction, resulting in cascading effects through freshwater ecosystems[51]. Moreover, geographically isolated populations with low dispersal capabilities such as crayfish could experience problems in accommodating to ongoing climate changes due to limited possibilities for migration and a shift in their distribution toward more climate-suitable habitats.

Sensitivity to climate change in freshwater taxa was proved to be higher than in terrestrial taxa[52], and vulnerability of freshwater crayfish to climate change, as well as to NICS has been demonstrated in many studies[3,30,33]. To evaluate the impact of climate change and NICS on the endangered *A. astacus*, we performed SDM. The models were able to capture the known ranges of *A. astacus* and two NICS in Croatia. Our predictions are concordant with previous studies of *A. astacus* distribution in Croatia[6]; majority of areas currently suitable for *A. astacus* are located in the area of Continental Croatia, including parts of Alpine Croatia and small isolated areas in Mediterranean Croatia where species was introduced[6]. Likewise, current projections for NICS, *F. limosus* and *P. leniusculus,* revealed highly suitable habitats corresponding to their present distribution in Croatia, but also encompassing areas for their potential spread. Our current projections suggested overlap between suitable habitats for *A. astacus* and *P. leniusculus*, a competitor which negatively affects *A. astacus* populations in the rivers of the continental part of Croatia through competitive exclusion and *A. astaci* transmission[5,15]. On the contrary, modelling the current potential distribution of *F. limosus* in Croatia did not detect any overlap between current suitable areas with *A. astacus,* as expected, since waterbodies of eastern Croatia are inhabited by *P. leptodactylus*[6].

Overall, our future projections demonstrated that climate change may have major negative effects on the distribution of *A. astacus* by reducing the surface of climate-suitable areas available for this native European species. This result is in line with findings for other endangered aquatic species in Europe[54]. Change in thermal and precipitation regimes caused by global warming will probably lead to drastic range contractions of *A. astacus*. Consequently, this could drive population declines across the species distribution range in Croatia. This conclusion supports the alarming studies of Capinha et al.[34] and Hossain et al.[52] that predicted extreme loss of habitat suitability for freshwater crayfish due to climate change. Thus, our results indicate that climate change-driven habitat loss represents a greater threat to *A. astacus* than the potential future distribution of the two studied NICS. A similar scenario was found for *Austropotamobius pallipes* in relation to the invasive *P. leniusculus* ([30,53,54], see below).

Future SDM projections suggested that the suitable habitat for *A. astacus* will likely shift towards the northwest and practically disappear from the easternmost parts of Croatia due to the severe reduction (87% of currently suitable habitats) in habitat suitability by 2070. Furthermore, the most suitable areas for *A. astacus* in the future were forecasted to be in the western Croatian waterbodies, some of which are at high altitudes where *Austropotamobius torrentium* is currently recorded[6]. Even though the Alpine region and its freshwater ecosystems represent suitable habitats for most crayfish species[53], these two indigenous species might compete for habitats and resources[29]. To overcome this, potential ark sites for *A. astacus* should be placed in the rivers and artificial lakes at lower altitudes in the Alpine region, as well as within gravel pits and oxbows alongside the Drava and Sava Rivers in the north-western part of Continental Croatia that were predicted as suitable in the future. These lower altitude waterbodies of the Alpine region would provide suitable ark sites for the crayfish from mtDNA Lineage 2 and/or Genetic cluster II, while crayfish from mtDNA Lineage 4 and/or Genetic cluster I could find refugia within gravel pits along the Drava and Sava Rivers in the north-western part of Continental Croatia. Keeping in mind that those suitable habitats are inaccessible to *A. astacus* due to natural dispersal barriers, human interventions would be needed. Assisted migration (AM) as an adaptation strategy for mitigating the projected effects of climate change on species is widely proposed[20,21], especially for those with a life history features that prevents them from migrating to suitable habitats. However, it is a controversial topic among conservation biologists, with numerous identified risks. Arguments against AM include: risk of translocated species becoming invasive with associated negative biological, ecosystem and socioeconomic effects; spread of diseases and pathogens that can be transferred into new host species; removing individuals from existing populations increases the extinction risks facing those source populations[19,55]. In order to overcome those arguments, careful planning encompassing risk assessments, cost–benefit analyses, conducting AMs on a small scale, with robust monitoring that would enable prompt corrective actions to be taken if needed, along with political and public promotion, could insure successful AM implementations[21].

Regardless of the RCP scenarios, our binary projections did not forecast any suitable areas for NICS in the future which should be interpreted with caution due to (a) the small number of available occurrences for NICS in the Croatian waterbodies used for SDMs; (b) models that do not account for human-mediated dispersal of NICS[56]; (c) the naturalised climatic niches of NICS that can differ from their natives' climatic niches[28]; and (d) underestimated potential range expansion in the future due to the known issue of non-equilibrium of NICS with the environment within the invaded range[57]. Nevertheless, our continuous future habitat suitability projections showed that, even though the projected future suitable areas for NICS were more extensive than the current ones, drastic decrease in habitat suitability values for both NICS were displayed under future climate predictions. Explicitly, potential areas where *A. astacus* would overlap and compete with NICS virtually disappeared by 2070 under both climate change scenarios of high-warming (RCP 8.5) and low-warming conditions (RCP 4.5). This result is consistent with the results of Préau et al.[30] showing no overlap between future suitable areas for *A. pallipes*

and *P. leniusculus* in France based on SDMs, despite substantial ecological niche overlap between the two species. Likewise, Gallardo & Aldridge[54] found that both endangered *A. pallipes* and invasive *P. leniusculus* were predicted to be negatively affected by climate changes in Europe. However, the range contraction was predicted to be more dramatic for the invasive *P. leniusculus*, leading to decreased overlap and consequently competition between the two species in the future, particularly in our study area. A more recent study by Zhang et al.[58] confirmed that invasive *P. leniusculus* may lose a substantial portion of suitable habitat in Europe by 2070 in response to climate change. Moreover, Capinha et al.[34] studied the potential distribution of indigenous crayfish species and NICS in Europe and found that climate-suitable areas were predicted to decrease by nearly 70% for *A. astacus*, 42% for *P. leniusculus*, and 49% for *F. limosus* by 2080. However, their models predicted that overlap of suitable ranges for native European crayfishes and invasive crayfishes would increase in the future which is contrary to our results. This may be because south-eastern Europe seems to be less suitable for *P. leniusculus* under changing climatic conditions[54,58]. It is therefore crucial to continue the monitoring of NICS invasions in the future.

Estimated reduction in habitat suitability by the end of this century indicates potential loss of a significant portion of the *A. astacus* genetic variability, especially in the eastern part of Continental Croatia that may potentially lose populations with high and unique genetic diversity. Minimising such possible losses in the future requires viable *A. astacus* populations to be established and maintained in ark sites/climate change refugia. Our results exposed an alarming need to prioritise conservation planning and management that will support existing populations and potentially establish new ones in the areas of stable habitat suitability that are expected to sustain *A. astacus* into the future. Species responses to climate change will depend on their distribution shifts to accommodate climate changes, and/or rely on the adaptation based on the standing genetic variation. Keeping in mind low dispersal abilities and isolated populations, we argue that assisted migration and population mixing approaches will be probably needed in the future to enhance the size and genetic diversity of remnant populations in order to maintain the long-term survival of the species[30,34,59]. Based on our results, we propose several donor populations for future restocking and reintroduction strategies. Namely, populations ILO, KUT, VEL, BAČ and BIJ, contain high and unique genetic diversity both at the mitochondrial and nuclear level, but they are predicted to be lost due to unsuitable habitats in the future. Dispersal as a fundamental behavioural mechanism is of great importance for adaptation and species' responses to rapidly changing climate[26]. Strong dispersal limitations, habitat discontinuities and limited gene flow have a major effect on the ability of crayfish populations to withstand climate changes. Thus, assisted migration in climate change refugia seems a logical solution for slowing down genetic diversity erosion, reducing genetic load and the detrimental consequences of inbreeding, but also allows variations in allele frequencies[60–62].

The adoption of such approaches for conservation purposes has gained significant momentum over the last few decades; reintroduction of the *A. astacus* into restored waterbodies has become common practice, even though the genetic origin of stocking material has rarely been considered[10]. Thus, potential ark sites should represent areas that maintain the highest contemporary genetic diversity in the species and predicted climate-suitable habitats for the future. *Astacus astacus* relocation should be preceded with a careful assessment regarding potential negative consequences of assisted gene flow that can impact the success of relocated populations, particularly when populations exhibit local adaptation to factors other than climate[63,64]. Also, introgression between local and translocated populations could result in outbreeding depression[60,61]. Still, Bláha et al.[23] found no significant decline in genetic diversity between the source and translocated *A. astacus* populations after introduction. Furthermore, their study showed that even though the source populations did not possess high genetic diversity, their distinctiveness still made them suitable for conservation purposes. In addition, it is critical that climatically suitable sites outside *A. astacus* historical range for conservation purposes should be free from diseases, such as crayfish plague caused by oomycete *Aphanomyces astaci*.

In conclusion, our results suggest that securing the future of *A. astacus* will require significant interventions. This paper provides a baseline to guide these actions. Specifically, SDM combined with population genetics provided essential guidance for conservation actions aimed at safeguarding endangered *A. astacus* in Croatia by revealing genetic structure and identifying sites most suitable for protection and sites where climate change constitutes a threat. In addition, our study corroborates SDM as a valuable tool for conservation planning of threatened crayfish species by identifying areas within a species' distribution that may be vulnerable and suitable areas for assisted migration as shown in studies on European crayfish[34,52], *A. pallipes* complex[3,29,30,53,65], and *A. torrentium*[3].

Material and methods

Genetic diversity and population structure. *Sampling and DNA extraction.* We collected *A. astacus* samples across its entire distribution range in Croatia (Supplementary Table S1, Fig. 5). Specimens were collected by hand or baited traps in accordance with ethical standards and with permissions of local authorities. One pereiopod from each individual was sampled and stored in 96% ethanol at 4 °C. Genomic DNA was extracted from the pereiopod's muscle tissue using GenElute Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich, St. Louis, MO) following the manufacturer's protocol and stored at −20 °C.

Phylogenetic assignment of studied populations using mtDNA. Samples used for phylogenetic reconstruction are reported in Supplementary Table S1. Mitochondrial *16S* and *COI* genes were amplified and sequenced with universal primers 16Sar/16Sbr[66] and LCO-1490/HCO-2198[67] allowing comparison with previously published *A. astacus* sequences[7–9,68]. Polymerase chain reactions (PCR), purification and sequencing were performed according to Gross et al.[9]. Sequences were edited and aligned in Bioedit v. 7.2.5[69]. The final *COI* alignment did not contain any length variants or ambiguous sites and its final length was 623 bp, while *16S* alignment contained 1 length variation and its final length was 475 bp. In order to perform phylogenetic analysis comparable with

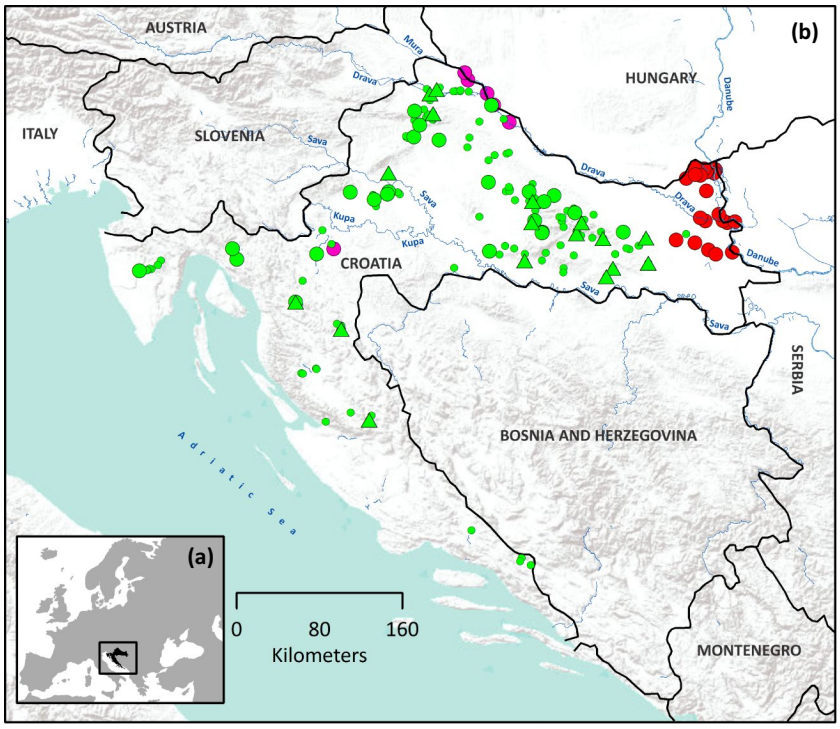

Figure 5. **(a)** Position of Croatia in Europe and **(b)** Geographical distribution of indigenous *Astacus astacus* and the two NICS, *Pacifastacus leniusculus* and *Faxonius limosus* in Croatia. Pink dots—*P. leniusculus*; red dots—*F. limosus*; small green dots—*A. astacus* occurrences; bigger green dots—*A. astacus* populations included into mtDNA analyses; green triangles—*A. astacus* populations included in both mtDNA and microsatellites analyses. Map was produced in ArcGIS 10.3 program package by authors of this study.

previous studies of *A. astacus*, *COI* sequences (trimmed to 350 bp length) and *16S* sequences (475 bp length) from the same individual were concatenated (final alignment was 825 bp long). Sequences were collapsed to unique haplotypes using FaBox[70], and all newly obtained haplotypes were submitted to GenBank (Supplementary Table S1).

Median-joining (MJ) network approach[71] was used to visualise intraspecific relationships among haplotypes within *A. astacus* using PopArt[72]. Since *A. astacus* is characterised by low diversification in mtDNA[9] and within-species data sets have fewer characters for phylogenetic analysis which diminish the statistical power of traditional phylogenetic methods[73], we used phylogenetic networks that are better suited for description of intraspecific evolutionary relationships. Two MJ networks were reconstructed in order to determine non-hierarchical phylogenetic relationships between *A. astacus* haplotypes. Median-joining network I comprised *COI* sequences (623 bp long) from Croatian populations obtained in this study and in the study by Gross et al.[9]. Median joining network II included concatenated *16S* + *COI* sequences (825 bp long) obtained in this study and assembled with all available sequences at European level[7–9,11,68]. This approach enabled us to associate haplotypes obtained in the present study to the haplotypes obtained in previous research and indirectly to the lineages *sensu* Schrimpf et al.[7] and groups *sensu* Laggis et al.[8].

Population genetics of studied populations using microsatellites. For microsatellite analyses we amplified 19 species-specific tetranucleotide repeat microsatellite loci developed by Gross et al.[74], and following modified protocols and procedures as in Gross et al.[9]. Microsatellite loci were genotyped on Applied Biosystems 3500 XL Genetic Analyser (Life Technologies, USA) using internal GeneScan 600 LIZ Size Standard v2.0 (Life Technologies, USA). Genotypes were scored using GeneMapper v.5 software (Life Technologies, USA), and were double-checked manually by two experts. Since four loci had overlapping allele size ranges (Aast4_26, Aast4_47, Aast4_10 and Aast4_30) they were omitted from further data analyses which were performed using 15 microsatellite loci. Also, several samples from different populations with more than two non-amplified loci were omitted from further analysis. Microsatellite loci were tested for potential presence of genotyping errors due to null alleles, stutter peaks and large allele dropout using MICRO-CHECKER v.2.2.3[75]. Pairwise linkage disequilibrium between all pairs of loci was tested using Fisher's exact test in GENEPOP v. 4.7.2[76]. Null allele frequencies based on the expectation–maximization (EM) algorithm[77] and corrected F_{ST} values using the ENA method were estimated using FreeNA[35] with a number of bootstrap replicates fixed to 10,000. The estimations of F_{ST}, with and without null allele correction, were compared for each population using t-test in STATISTICA 13 (StatSoft. Inc).

Population genetics analyses were conducted with the microsatellite genotype data of 12 *A. astacus* populations obtained in this study that were supplemented with the microsatellite genotype data of five Croatian *A.*

Variable ID	Variable description (unit)	*Astacus astacus*	NICS	Reference
bio2	Mean Diurnal Range (°C)	x	x	Hijmans et al., 2005[87]
bio4	Temperature Seasonality (SD × 100)	x	x	Hijmans et al., 2005[87]
bio5	Max Temperature of Warmest Month (°C)		x	Hijmans et al., 2005[87]
bio9	Mean Temperature of Driest Quarter (°C)	x		Hijmans et al., 2005[87]
bio14	Precipitation of Driest Month (mm)	x	x	Hijmans et al., 2005[87]
bio15	Precipitation Seasonality (CV)	x	x	Hijmans et al., 2005[87]
bio18	Precipitation of Warmest Quarter (mm)	x	x	Hijmans et al., 2005[87]
bio19	Precipitation of Coldest Quarter (mm)	x	x	Hijmans et al., 2005[87]
alt	Altitude (m)	x	x	https://www2.jpl.nasa.gov/srtm
slope	Slope derived from altitude (%)	x	x	https://www2.jpl.nasa.gov/srtm
forest_clc	Percentage of forest cover in 1km^2 (%)	x		https://land.copernicus.eu/pan-european/corine-land-cover

Table 3 . Environmental predictor variables used for building SDMs of indigenous *Astacus astacus* and the two NICS, *Pacifastacus leniusculus* and *Faxonius limosus*. x—used in model building.

astacus populations (JAR, MAK, TOT, JAN and VUK) from the study by Gross et al.[9] in order to enlarge data set and make analyses more robust.

Population genetic diversity. Population genetic diversity was assessed with standard descriptive statistics using GenAlEx v. 6.51[78]. Statistics included the percentage of polymorphic loci (P), mean number of alleles (N_A), observed heterozygosity (H_O) and unbiased expected heterozygosity (H_E). Further, FSTAT v.2.9.4[79] was used for estimation of allelic richness (A_R) that was calculated as the number of alleles per locus independent of sample size, and the inbreeding coefficient (F_{IS}).

The number of private alleles (A_{PR}) was estimated by rarefaction method using HP Rare v.Feb-2-2009[80] and multiplied by the number of used loci. Deviations from the Hardy–Weinberg equilibrium (HWE) for each population across all loci were tested using GENEPOP v. 4.7.2[76]. All probability tests were based on the Markov chain algorithm using 10,000 dememorization steps, 100 batches and 5000 iterations per batch. Significance levels were adjusted applying the Bonferroni correction to correct for the effect of multiple tests. Recent reductions in the effective population size using allele frequency data and potential signatures of recent bottlenecks were tested using the heterozygosity excess method implemented in BOTTLENECK v.1.2.02[81] under three different mutational models: infinite allele model (IAM), stepwise mutation model (SMM) and two-phase model (TPM). Significant deviations from mutational-drift equilibrium were tested using the Wilcoxon sign rank test with 10,000 simulations.

Population genetic differentiation and structure. Genetic differentiation between all population pairs was estimated through pairwise F_{ST} values using FSTAT v.2.9.4[79]. Genetic structure among studied populations and assembling of individuals into groups (genetic clusters) was assessed using the Bayesian model-based clustering approach implemented in STRUCTURE v.2.3.4.[82]. The conditions performed were 10 runs for each genetic cluster (K) between 1 and 17 using a 100,000 burn-in period followed by 100,000 MCMC iterations, under the admixture model, with correlated allelic frequencies. The number of optimal K was inferred using the protocol defined by Evanno et al.[83] as implemented in STRUCTURE HARVESTER v. 0.6.93[84]. STRUCTURE graphical results were plotted with CLUMPAK[85]. In addition, structure in the distribution of genetic variation was visualized by principal coordinates analysis (PCoA) using Nei's genetic distance in GenAlEx v. 6.51. Hierarchical analysis of molecular variance (AMOVA) was carried out using ARLEQUIN v. 3.5.1.2[86] in order to estimate partitioning of genetic variance among groups, among populations within groups and within population. Populations were grouped according to their affiliation to the genetic clusters inferred from STRUCTURE; The cluster I included individuals from populations MAK, TOT, JAN and BED, and the cluster II comprised crayfish from populations MOT, BRE, BUR, ILO, BIJ, GLO, KIK, SLO, KUT, VEL, JAR, VUK and OTU (Fig. 2).

Species distribution models (SDMs). *Species occurrence data.* We compiled all known presence-only occurrences of *A. astacus* and the two NICS (*P. leniusculus* and *F. limosus*) from across Croatia from our own published and unpublished field sampling[6]. This resulted in a total of 174 occurrences for *A. astacus*, 22 for *F. limosus* and 17 for *P. leniusculus* (Fig. 3).

Environmental data. We initially considered 22 environmental variables from various sources and databases describing climate, topography and forest cover of the study area (Table 3). The 19 bioclimatic variables were obtained from the WorldClim 1.4 database[87], altitude and slope were derived from a digital elevation model from the NASA Shuttle Radar Topography Mission (SRTM) elevation data (https://www2.jpl.nasa.gov/srtm), while the variable percentage of forest cover in 1 km^2 was calculated from the Corine Land Cover 2018 dataset (https://land.copernicus.eu/pan-european/corine-land-cover). All environmental variables were used at a spatial resolution of ~ 1 km^2. Predictor variables for SDMs of *A. astacus* and the two NICS were then selected based

on our expert knowledge about their ecological relevance for the target species (potentially influencing species' physiology and life history), excluding highly correlated ones based on variance inflation factor, VIF < 10 (usdm R package;[88]). Thus, the final predictor set for *A. astacus* included ten, and for NICS nine environmental variables (see Table 3).

Modelling procedure. To assess the potential current and future habitat suitability of *A. astacus* and two NICS (*P. leniusculus and F. limosus*), we developed SDMs using an ensemble approach implemented in R package BIOMOD2 ver. 3.3-7[89,90]. For each species we applied three different modelling methods (Random Forest—RF, Generalized Boosted Model—GBM and Maximum Entropy—Maxent) with ten replicates for each method (a total of 30 models for each species). Occurrences were combined with 10,000 random pseudo-absences drawn across the study area for methods that require absences[91]. In each run, 70% of the occurrences were used for model calibration, and the remaining 30% were used for model evaluation using AUC[36].

To build the current ensemble model we used only highly reliable models with AUC > 0.9[37] and obtained this ensemble as an AUC weighted average. The obtained current ensemble model was then projected under both current and future environmental conditions to obtain potential habitat suitability maps for each species. For future projections we used two RCP scenarios (mid-range emission scenario RCP 4.5 and pessimistic scenario RCP 8.5) and four global circulation models (GCMs) suitable for Europe[92] (CCSM4, MIROC5, MPI-ESM-LR and HadGEM2-CC) for the 2070-time period (average for 2061–2080). Since future projections for variables slope, altitude and forest cover were not available, we kept them as constant in our future projections, assuming that they will not change for our study area during the considered time period. The available data on forest cover change in Croatia during the last decades and current forest management structure and practices provide confidence that at least for our study area, forest cover may remain stable in the future[93] [https://forest.eea.europa.eu]. An ensemble future projection for each RCP scenario was obtained by taking an average of the four GCM projections. Multiple RCPs and GCMs were used to address the associated uncertainties arising from different climate change predictions[94].

To obtain binary presence/absence maps helpful in model interpretation and for calculating changes in habitat suitability for *A. astacus*, we applied a threshold maximizing the sum of sensitivity and specificity[95] to ensemble current and future continuous habitat suitability maps.

Finally, to estimate the effects of climate change on genetic diversity and structure of the focal species, we overlapped genetic data of *A. astacus* with its potential current and future suitable areas, as well as with future potential distribution of both NICS.

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Author contributions

I.M., L.L. and M.T. conceived the study and designed the experiments. L.L. and I.M. collected the samples. L.L., I.M. and R.G. conducted laboratory work. L.L., M.T., I.M. and M.G. analysed and interpreted the data. L.L., M.T. and I.M., prepared the figures and tables. L.L. wrote the original version of the manuscript, and all authors read, edited, enhanced, and approved the final version of the manuscript.

Competing interests

The authors declare no competing interests.

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GAP ANALYSIS REVEALED A MODERATE EFFICIENCY OF PROTECTED AREAS FOR THE CONSERVATION OF THE ENDANGERED NOBLE CRAYFISH IN CROATIA

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Lovrenčić, L. & Maguire, I.: Gap analysis revealed a moderate efficiency of protected areas for the conservation of the endangered noble crayfish in Croatia. Nat. Croat., Vol. 30, No. 2, 493–500, 2021, Zagreb.

The noble crayfish is a native European freshwater species, endangered due to the strong anthropogenic influence on its habitats, climate change, and invasive crayfish species. In the present study, we aimed to assess the effectiveness of nationally designated protected areas and the pan-European Natura 2000 network in representing and maintaining over time the noble crayfish diversity using a comprehensive species occurrence dataset. Overall, our gap analysis indicated moderate efficiency of the existing protected areas in covering the noble crayfish diversity. Overlapping the distribution map of the noble crayfish with the map of protected areas revealed that protected areas encompass 50% of recorded populations. This study can serve as an evaluation of the protected areas in conservation of this key freshwater crayfish species.

Key words: *Astacus astacus*, Astacidae, Natura 2000 network, conservation planning, biodiversity conservation

Lovrenčić, L. & Maguire, I.: Gap analiza pokazuje umjerenu učinkovitost zaštićenih područja u očuvanju plemenitog ili riječnog raka u Hrvatskoj. Nat. Croat., Vol. 30, No. 2, 493–500, 2021, Zagreb.

Plemeniti ili riječni rak je autohtona europska vrsta slatkovodnih rakova, ugrožena mnogobrojnim antropogenim pritiscima na njegova staništa, klimatskim promjenama i stranim invazivnim vrstama rakova. Cilj ovog istraživanja je bio procijeniti učinkovitost zaštićenih područja i pan-europske mreže Natura 2000 u očuvanju raznolikosti plemenitog raka u Hrvatskoj. U tu svrhu korišteni su podaci o rasprostranjenosti vrste u slatkovodnim ekosustavima Hrvatske koji su preklopljeni s kartom zaštićenih područja što je poslužilo u gap analizi. Rezultati analize su pokazali da su populacije plemenitog raka umjereno dobro pokrivene zaštićenim područjima. Preklapanjem mape zaštićenih područja s mapom nalaza rakova zaključeno je da je 50% populacija plemenitog raka unutar zaštićenih područja. Rezultati ovog istraživanja mogu poslužiti u izradi budućih planova upravljanja ovom ugroženom vrstom.

Ključne riječi: *Astacus astacus*, Astacidae, Natura 2000, konzervacijski planovi, zaštita bioraznolikosti

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INTRODUCTION

Freshwater ecosystems around the world have been critically imperilled, mostly due to growing anthropogenic pressure that negatively influence their biodiversity, and consequently led to major population declines in freshwater species (Strayer & Dudgeon, 2010). Currently, one in three freshwater species is threatened with extinction worldwide, and crayfish are among the most endangered groups (Collen *et al*., 2014). Concurrently, crayfish are recognised as ecosystem engineers and keystone species due to their high impact on freshwater ecosystems and their biodiversity (Reynolds *et al*., 2013).

The noble crayfish, *Astacus astacus*, is a widely distributed native European freshwater species (Kouba *et al*., 2014). In Croatia, it is recorded in all three biogeographical regions (Continental, Alpine and Mediterranean), and naturally distributed in the waterbodies of the Black Sea drainage, with a few recorded populations in the Adriatic Sea drainage that are of anthropogenic origin (Maguire *et al*., 2018). Currently, the noble crayfish is endangered due to the strong anthropogenic influence on its habitats, climate change, and spreading of non-indigenous invasive crayfish species and their pathogens (Jussila *et al*., 2021). European-wide population declines resulted in a protection of the species under IUCN Red List of Threatened Species (Edsman *et al*., 2010) and EU Habitat Directive (Annex III of the Bern Convention, Annex V of Habitat Directive (92/43/EEC)). In Croatia, comparison of its previous and current distributional data, in the overlapping localities, revealed that 55% of the populations disappeared during the last decades (Maguire *et al*., 2011, 2018). According to the Croatian National Red List of freshwater crayfish, it is recognised as vulnerable with decreasing population trends (Gottstein *et al*., 2011), and is protected by the Croatian Law of Nature Protection (NN 80/13). The conservation status of the noble crayfish, as assessed within the framework of the EC Habitats Directive (Council of the European Communities, 1992), was noted as 'unfavourable-inadequate' in all three biogeographical regions in Croatia (Ministry of Environmental Protection and Energy, 2019). This is the second worst possible conservation status under Article 17 of the Directive, based on four parameters: range, population, habitat of species and future prospects. According to this status, 'a change in management or policy is required to return the habitat type or species to favourable status, but there is no danger of extinction in the foreseeable future'.

Previous genetic studies revealed that Croatian populations of the noble crayfish constitute an important part of within-species genetic diversity (Gross *et al*., 2021; Lovrenčić *et al*., under review). At the same time, species distribution models forecasted substantial reductions in its habitat suitability by the end of this century. Namely, 87% of the currently suitable habitat is predicted to be lost by the 2070 time-period under the high-emissions 'RCP8.5' global warming scenario (Lovrenčić *et al*., under review). Bearing in mind that most of the populations with high genetic diversity are located in the areas predicted to become unsuitable, estimated reduction in habitat suitability entails potential loss of a significant portion of the noble crayfish genetic variability. This raises a question of how much of its diversity is actually covered by some level of protection. Since the prime task for the conservation is to secure the long-term survival of species, it is important to evaluate the extent to which existing protected areas in Croatia conserve the noble crayfish diversity.

In Europe, the cornerstone of conservation has been the Natura 2000 network. Natura 2000 is designated to support the long-term survival of the important habitats and species throughout Europe, maintaining listed habitat and species at ''favourable conservation status''(European Commission, 2000). In Croatia, the implementation of Natura 2000 led to the rise in the quantity of the conservation areas, which previously covered 9.3% of the Croatian territory. Currently, Croatia has one of the most extensive Natura 2000 networks in Europe covering 36.7% of the country and 15.4% of the seashore.

Here we assess the effectiveness of both national protected areas and the pan-European Natura 2000 network in representing and maintaining long-term survival of the noble crayfish diversity using a comprehensive species occurrence dataset. The aims of our study were to (1) overlap the distribution map of the noble crayfish with the national protected areas and Natura 2000 network in order to reveal the effectiveness of the protected areas in safeguarding the noble crayfish, and (2) evaluate if the effectiveness of the protected areas varies between biogeographical regions. In order to achieve our goals, we performed gap analysis, a GIS-based approach that overlays species distribution data onto a map of protected areas aiming to assess the effectiveness of protected areas in preserving species diversity (Jennings, 2000; Rodrigues *et al*., 2004).

MATERIAL AND METHODS

Study area

Study area is located in Croatia, a country recognised as one of the hot spots of the noble crayfish diversity. It encompasses the distribution of the noble crayfish in three biogeographical regions in Croatia: Continental, Alpine and Mediterranean. Those regions cover freshwater habitats belonging to the Black Sea drainage, and also the Adriatic Sea drainage, where the noble crayfish was introduced (Maguire *et al*., 2018). Characteristic habitat of the noble crayfish in the study area includes rivers, streams and lakes with loam, sand or gravel bottom, where shelter availability is high. The noble crayfish prefers water with high oxygen level, and soft banks where they construct simple burrows for the shelters.

Species distribution data

Research was performed on the data from previously published work on the distribution of the noble crayfish in Croatia (Maguire & Gottstein-Matočec, 2004; Maguire *et al*., 2011, 2018; Gross *et al*., 2021; Lovrenčić *et al*., under review). Geographic Information System (ArcGIS) was used for preparation of distribution data (i.e. the point occurrences) of each noble crayfish population.

Gap Analysis

We performed the gap analysis, a GIS-based approach for 'assessing the effectiveness of protected areas in representing species diversity' (Rodrigues *et al*., 2004), by comparing the distribution of the noble crayfish with the extent of the protected area in Croatia. Gap analysis represents well-established conservation tool for the identifi-

cation of the areas in which selected elements of biodiversity (e.g., species, habitats, ecosystems) are represented, and through comparison with existing protected areas recognises areas/regions that require additional protection. Precisely, we performed gap analysis to assess how many of the noble crayfish populations are covered by protected areas (Special Protection Areas (SPAs) designated under the Birds Directive 2009/147/EC, Special Areas of Conservation (SACs) designated under the Habitats Directive 1992/43/EEC), and national and regional parks). We computed the number of populations that were included or excluded from such areas in order to detect populations and areas that need greater attention. In the gap analysis, the species/populations is considered as 'covered' if any protected area overlapped with its recorded distribution, and otherwise to be a 'gap species/population' (Rodrigues *et al*., 2004). ArcGIS program package was used to overlap occurrence data with the map of protected areas obtained from the online platform Bioportal (http://www.bioportal.hr). Upon processing and overlapping layers, a single layer was produced, and it was used to calculate the number and the percentage of populations covered by protected areas.

RESULTS AND DISCUSSION

Overlap of the noble crayfish distribution and protected areas

Results presented in this study introduce, according to our best knowledge, the first gap analysis of the noble crayfish. Thus, they can be used as an evaluation of the protected areas in conservation of this key freshwater crayfish species. Gap analysis including a total of 164 populations indicated moderate efficiency of protected areas in covering the noble crayfish diversity (Fig. 1, Tab. 1). Overlapping the distribution map of the noble crayfish with the map of protected areas revealed that 83 populations (51%) were covered, while 81 (49%) were located outside the existing protected areas (Fig. 1, Tab. 1). The coverage gaps varied across different biogeographical regions. The biogeographical region with the highest percentage of coverage (34 out of 36 populations; 94%) was Alpine since in this region numerous areas are under protection. In contrast, the Continental region that represents an important area with high noble crayfish presence exhibited the lowest coverage (42 populations are within (36%), and 74 outside (64%) protected areas) (Tab. 1). This region embodies the natural distribution area of the noble crayfish in Croatia, and thus it harbours populations with the greatest genetic diversity on mitochondrial and nuclear level (Gross *et al*., 2021; Lovrenčić *et al*., under review). Since various human activities, pollution, habitat degradation and fragmentation are progressing, this region needs special attention in future conservation management and planning. In the Mediterranean region, into which noble crayfish was introduced, 7 out of 12 populations (58%) are distributed within protected areas.

How well protected areas safeguard the endangered noble crayfish in Croatia?

Representation of species and ecosystems in protected areas, and conservation strategies is a core principle of global conservation priority setting approaches (Rodrigues *et al*., 2004). Our study revealed that the current protected areas in Croatia

(Natura 2000 network, national and regional parks) partially encompass the areas with high diversity of the noble crayfish, and in that sense, provide moderate level of protection (Fig. 1, Tab. 1). As revealed in the gap analysis, there are coverage gaps in the conservation of the noble crayfish, particularly in the habitats of the Continental region in Croatia. Contrary, study by Lovrenčić *et al.* (2020) that evaluated representation of the stone crayfish (*Austropotamobius torrentium*) by protected areas in Croatia, revealed its much greater coverage compared to the results of this study. The effectiveness of the protected areas, especially Natura 2000 network, in fulfilling their

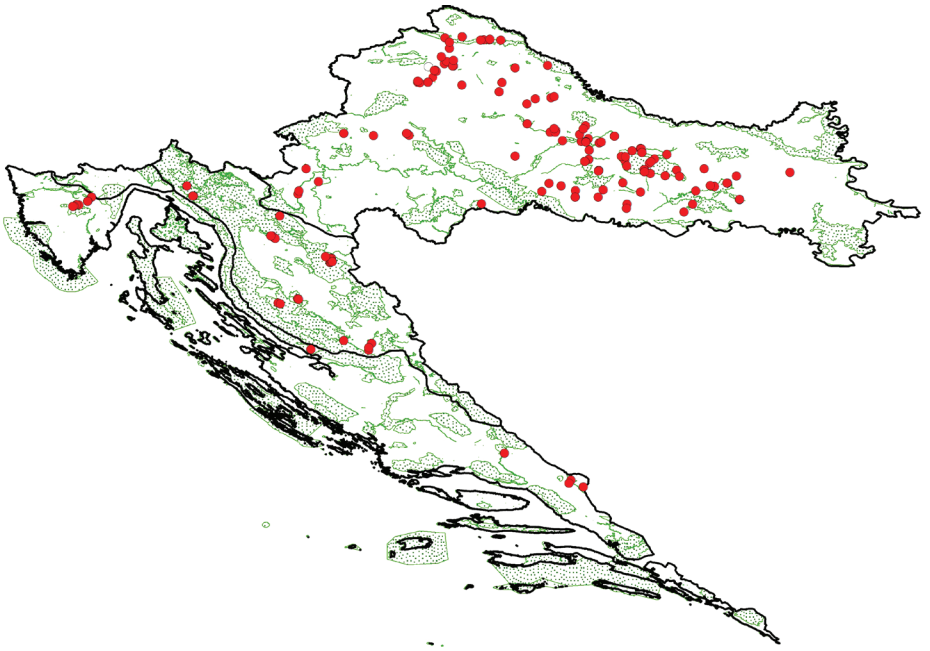

Fig. 1. Distribution map of recorded noble crayfish populations in Croatia overlapped with the protected areas. Protected areas include Special Protection Areas (SPAs) designated under the Birds Directive 2009/147/EC, Special Areas of Conservation (SACs) designated under the Habitats Directive 1992/43/EEC), and national and regional parks. Red circles represent distribution data, while protected areas are shown in green doted surfaces. Also black lines represent borders of/between biogeographical regions.

Tab. 1. Number of the noble crayfish populations per each biogeographical region when overlapped with the map of protected areas in Croatia. Protected areas include Special Protection Areas (SPAs) designated under the Birds Directive 2009/147/EC, Special Areas of Conservation (SACs) designated under the Habitats Directive 1992/43/EEC), and national and regional parks.

Biogeographical region	Within protected areas	Outside protected areas	Total
Mediterranean	7 (58.3%)	5 (41.7%)	12
Alpine	34 (94.5%)	2 (5.5%)	36
Continental	42 (36.2%)	74 (63.8%)	116
Total	83 (50.6%)	81 (49.4%)	164

role of protecting biodiversity has been evaluated through gap analysis in numerous studies at global or regional scales with the varying outcomes (Rodrigues *et al*., 2004; Verovnik *et al*., 2011; Gruber *et al*., 2012; Bagella *et al*., 2013; Abellan & Sanchez-Fernandez, 2015; Maiorano *et al*., 2015; Orlikowska *et al*., 2016; Yang *et al*., 2020; Ahmadi *et al*., 2020; Spiliopoulou *et al*., 2021). Some studies reported great effectiveness of protected areas and/or Natura 2000 in safeguarding various groups on the European level, such as butterflies (Verovnik *et al*., 2011), birds of prey (Mazaris *et al*., 2013), plants (Fois *et al*., 2017), and freshwater crayfish (Lovrenčić *et al*., 2020). In contrast, others revealed numerous gaps in the existing networks of protected areas making them inadequate for the long-term preservation of biodiversity, as found for terrestrial vertebrates in Italy (Maiorano *et al*., 2006, 2015), birds in tropical Andes (O'dea *et al*., 2006), endangered flora of Almería (Mendoza-Fernandez *et al*., 2009), European wetland species (Jantke *et al*., 2011), and endemic species in Mediterranean temporary freshwater habitats (Bagella *et al*., 2013).

Freshwaters in Croatia, which belong to the Mediterranean biodiversity hotspot, are characterised by high levels of diversity and endemism, but at the same time exposed to higher levels of threat than the rest of Europe (Médail & Quézel 1999; Myers *et al*., 2000). Moreover, a study by Carrizo *et al.* (2017) showed that many Natura 2000 sites in freshwater ecosystems of southern and eastern Europe are managed poorly, with the current level of protection not being sufficient. Therefore, the effectiveness of the national protected areas and the Natura 2000 network should be enhanced by better local management of both native and invasive species, habitat restoration, public acceptance and engagement, collaboration among local and state agencies, researchers, as well as landholders and funding bodies (Blicharska *et al*., 2016; Carrizo *et al*., 2017).

CONCLUSION

Our results exhibited moderate efficiency of protected areas in Croatia in covering the noble crayfish distribution, and thus its diversity. About 50% of the noble crayfish populations were covered by some of the national protected areas. The percentage of covered populations varied among biogeographical regions; the best covered was Alpine region (94%), then the Mediterranean region (58%), and the least covered was Continental region (36%). We propose that, in order to achieve better and effective conservation of the noble crayfish, other available approaches, such as habitat restoration, enhancing local management, raising public awareness through local campaigns, should be included in future management plans and actions for this vulnerable species.

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RESEARCH PAPER OPEN ACCESS

Morphological diversity of the stone crayfish – traditional and geometric morphometric approach

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Abstract – *Austropotamobius torrentium* is one of four native European crayfish species inhabiting Croatian freshwaters. Existence of eight divergent monophyletic mtDNA phylogroups was described within *A. torrentium*; six of them are distributed in Croatia, with the highest genetic diversity established in its northern-central Dinaric region. Recent small-scale study of the stone crayfish morphological variability indicated significant differences among different phylogroups. In the present study larger sample size, covering populations from five phylogroups, was analysed with the aim of determining whether there are morphological characteristics that reliably separate stone crayfish from different phylogroups. Aiming this, 245 stone crayfish were analysed through traditional (TM) and, for the first time, geometric morphometric (GM) analyses. Multivariate discriminant analyses included 24 TM characteristics per crayfish, while GM comprised analyses of 22 landmarks on the dorsal side of cephalon. Both methods revealed congruent results, and significant differences among phylogroups in analysed features were obtained, with the cephalon shape contributing the most to crayfish discrimination. Research confirmed that both approaches, combined with statistical methods, are useful in distinguishing and separating crayfish phylogroups. Findings of present study are compatible with the previous molecular findings; stone crayfish present several distinct evolutionary lineages whose species status are currently undefined and require urgent clarification.

Keywords: *Austropotamobius torrentium* / Generalized Procrustes Analysis / landmark / multivariate discriminant analysis / semilandmark

Résumé – Diversité morphologique de l'écrevisse des torrents – approche morphométrique traditionnelle et géométrique. *Austropotamobius torrentium* est l'une des quatre espèces indigènes d'écrevisses européennes qui peuplent les eaux douces croates. L'existence de huit phylogroupes divergents d'ADNmt monophylétiques a été décrite chez *A. torrentium* ; six d'entre eux sont distribués en Croatie, avec la plus grande diversité génétique établie dans sa région dinarique nord-centre. Une étude récente à petite échelle de la variabilité morphologique de l'écrevisse des torrents a révélé des différences significatives entre les différents phylogroupes. Dans la présente étude, on a analysé des échantillons de plus grande taille, couvrant des populations de cinq phylogroupes, dans le but de déterminer s'il existe des caractéristiques morphologiques qui séparent de façon fiable les écrevisses des torrents des différents phylogroupes. Dans ce but, 245 écrevisses des torrents ont été analysées par des analyses traditionnelles (TM) et, pour la première fois, morphométriques géométriques (GM). Les analyses discriminantes à plusieurs variables comprenaient 24 caractéristiques TM par écrevisse, tandis que les analyses GM comprenaient des analyses de 22 repères sur la face dorsale du céphalon. Les deux méthodes ont révélé des résultats congruents, et des différences significatives entre les phylogroupes dans les traits analysés ont été obtenues, la forme du céphalon contribuant le plus à la discrimination des écrevisses. La recherche a confirmé que les deux approches, combinées à des méthodes statistiques, sont utiles pour distinguer et séparer les phylogroupes d'écrevisses. Les résultats de la présente étude sont compatibles avec ceux des études moléculaires précédentes ; les écrevisses des torrents présentent

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plusieurs lignées évolutives distinctes dont le statut d'espèce n'est pas encore défini et nécessite une clarification urgente.

Mots-clés : *Austropotamobius torrentium* / Analyse procrustéenne généralisée / points de repère / analyse discriminante multivariée / marques de semi-marques

1 Introduction

The stone crayfish *Austropotamobius torrentium* (Schrank, 1803) is one of four native European crayfish species inhabiting streams and smaller rivers at higher altitudes of both the Black Sea and the Adriatic Sea basins in Croatian freshwater ecosystems (Maguire *et al.*, 2011). In the last few decades, noticeable declines of stone crayfish populations have been recorded across its distribution range in Europe (Weinländer and Füreder, 2009; Kouba *et al.*, 2014). In Croatia, losses are mainly caused by anthropogenic pressure onto the stone crayfish habitats (fragmentation, waterbody engineering), presence and spread of non-native invasive crayfish species and diseases they transmit (*e.g.* crayfish plague), as well as climate changes (Maguire *et al.*, 2018). Therefore, the stone crayfish is considered a threatened species, protected by national (NN 80/13, 144/13) and international legislation (Appendix III of the Bern Convention, Appendices II and V of the Habitat Directive (92/43/EEC and 97/62/EU)). Efficient protection of existing populations requires development of conservation and management plans based on the sound knowledge of species, including information on genetics and morphology (Peay, 2009; Souty-Grosset and Reynolds, 2009).

Previous molecular-phylogenetic research based on mtDNA by Trontelj *et al.* (2005) indicated that the stone crayfish should be considered a species complex. Later, Klobučar *et al.* (2013) discovered the existence of at least seven divergent monophyletic phylogroups within *A. torrentium*, with the highest genetic diversity recorded in the Dinaric region of Croatia, while recently Pârvulescu *et al.* (2019) described another phylogroup endemic to the Romanian Apuseni region. Moreover, latest analyses of meristic and morphometric features of several stone crayfish populations, belonging to different phylogroups, indicated their significant differences and point to necessity of additional analyses that would cover bigger sample size and wider area (Maguire *et al.*, 2017). Following that idea, a new *Austropotamobius* crayfish species was just described (Pârvulescu *et al.*, 2019).

In this study we included larger sample size, complementing data set from Maguire *et al.* (2017) covering populations from five out of seven previously described mtDNA phylogroups (Klobučar *et al.*, 2013). Also, apart from traditional morphometry, we used, for the first time geometric morphometrics on *A. torrentium*. Geometric morphometrics (GM) is a method with a growing application in different organisms' studies (Caumul and Polly, 2005; Cardini *et al.*, 2007; Becking *et al.*, 2016). It proved itself as a powerful tool in detecting differences in morphology within and among species (Lawing and Polly, 2010). Up till now, in the freshwater crayfish research, GM has been applied in the studies on the white-clawed crayfish (*Austropotamobius pallipes* (Lereboullet, 1858)) (Scalici *et al.*, 2010; Scalici and Bravi, 2012), red swamp crayfish (*Procambarus clarkii* (Girard, 1852)) (Malavé *et al.*, 2018), rusty crayfish (Perry *et al.*, 2013) and two *Cambarus* species (*Cambarus halli* Hobbs, 1968 and *Cambarus englishi* Hobbs & Hall, 1972) (Helms *et al.*, 2015).

The aim of this study was to verify if there are significant differences among stone crayfish belonging to different phylogroups, as previous preliminary results indicated (Maguire *et al.*, 2017). Further, the intention was to find which traditional morphometric features discriminate the best different phylogroups. Another objective was to validate if landmark-based GM analyses are suitable for detecting differences in shape among different *A. torrentium* phylogroups. Finally, the goal was to verify if results of the two methods (traditional and geometric morphometrics) yield congruent results. We expected that morphology of stone crayfish differs among different phylogroups and that results obtained by the two methods are congruent and compatible with the previous molecular findings.

2 Materials and methods

2.1 Sampling

Prior to field work, all of the required permits (working in protected areas, studying strictly protected species) were obtained from the Ministry of Environmental Protection and Energy of the Republic of Croatia. Crayfish were collected from 15 localities covering populations from five previously described mtDNA phylogroups: Gorski Kotar (GK); Žumberak, Plitvice and Bjelolasica (ŽPB); Lika and Dalmatia (LD), Banovina (BAN) and central and south-eastern Europe (CSE) (Trontelj *et al.*, 2005; Klobučar *et al.*, 2013) (Tab. 1, Fig. 1). Sampling was performed by hand during the night or with hand-made traps (Maguire *et al.*, 2002) placed along banks of the stream and left in the water overnight. All captured crayfish specimens were identified to the species level and their sex was determined. Only adult (total length $>$ 5 cm (Huber and Schubart, 2005; Maguire and Klobučar, 2011)), uninjured, intermolt crayfish were examined and measured; 245 individuals were included into traditional morphometric (TM) analyses, and out of them 209 into geometric morphometric (GM) analyses. Discrepancy in the number is due to the fact that some of the landmark positions on some of the crayfish were indistinct, so those crayfish were omitted from the GM analyses.

2.2 Traditional morphometric analyses

In order to perform traditional morphometric analyses, we measured 24 morphometric characteristics per each crayfish, using a digital calliper with precision of 0.01 mm; 21 were taken from Sint *et al.* (2005); claw width (CLW), claw length (CLL), claw height (CLH), length of the claw finger (CFL), length of the claw palm (CPL), rostrum width (ROW), rostrum length (ROL), head width (HEW), head length (HEL), areolar width (ARW), areolar length (ARL), abdomen width (ABW), abdomen height (ABH), abdomen length (ABL), telson width

Table 1. List of samples; mtDNA phylogroups *sensu* Klobučar *et al.* (2013); (Banovina (BAN), Lika and Dalmatia (LD), Gorski Kotar (GK), Žumberak, Plitvice and Bjelolasica (ŽPB), and central and south-eastern Europe (CSE)) with sampled localities (streams) and number of stone crayfish per group (total, and number of males and females).

mtDNA phylogroup (locations)	Crayfish number (Male/Female)
BAN (Bručina, Maja)	42 (16/26)
LD (Krasulja, Orašnica)	61 (45/16)
GK (Bresni, Delnički, Vele vode)	30 (20/10)
ŽPB (Slapnica, Jarak, Sopotski slap, Sartuk)	33 (31/2)
CSE (Bliznec, Dolje, Okičnica, Jarak-Stojdraga)	79 (40/39)

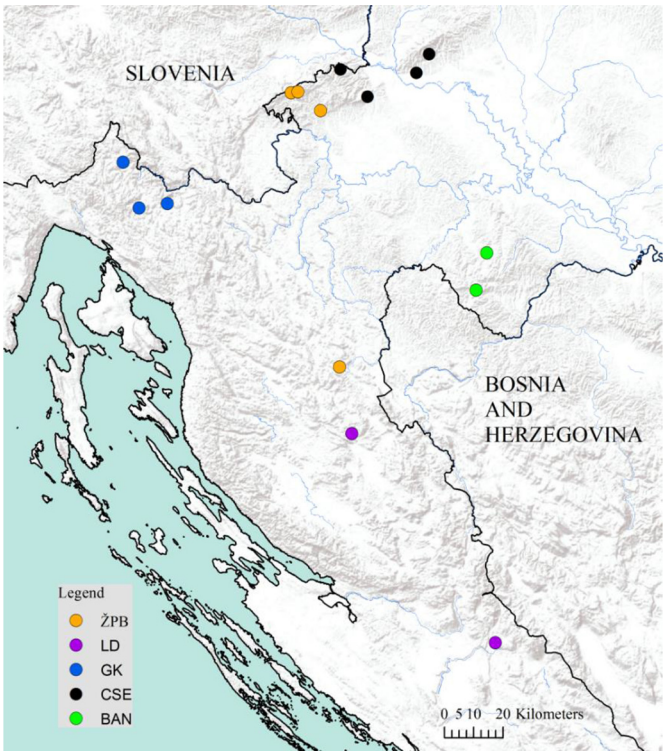

Fig. 1. Map of the study area with sampling sites produced in ArcGIS 10.3 program package. Different colours represent different phylogroups *sensu* Klobučar *et al.* (2013); ŽPB – Žumberak, Plitvice and Bjelolasica; LD – Lika and Dalmacija; GK – Gorski Kotar; CSE – Central and south-eastern Europe; BAN – Banovina

(TEW), telson length (TEL), width of the carapace at the hind edges (CEW), carapace height (CPH), carapace width (CPW), width at the cervical groove (CGW), and total length (TL); and extra three included cephalothorax length (CEF), apex length and width (APL and APW, respectively). Bilateral characteristics were recorded on the right body side because it was proven that bilateral characteristics are symmetrical and show no differences between the two body sides (Maguire *et al.*, 2017). Further, all of the measured morphometric characteristics were normalised by dividing their value with the corresponding postorbital length (POL = HEL + ARL) to avoid comparison of different sized crayfish that could lead to false results (Chambers *et al.*, 1979; Palma and Andrade, 2002; Sint *et al.*, 2005). Since *t*-test showed that males and females differed significantly in characteristics describing claws and abdomen, the two sexes were analysed separately (Sint *et al.*, 2007; Berger *et al.*, 2017; Maguire *et al.*, 2017; Vlach and Valdmanová, 2015). Observed differences are consequence of sexual dimorphism that is characteristic of crayfish and appears upon crayfish attein sexual maturity (Grandjean *et al.*, 1997; Streissl and Hödl, 2002; Vlach and Valdmanová, 2015). Analyses were performed in Microsoft Excel (version 2010) and Statistica 13 (StatSoft. Inc). ANOVA with Bonferroni post-hoc test was applied to verify if there were differences among phylogroups in the measured morphometric characteristics. The multivariate discriminant analysis (MDA) was conducted in order to analyse differentiation of phylogroups based on their morphometric characteristics, and to select morphometric characteristics that made the most significant contribution to separation of crayfish belonging to different phylogroups. The results of the canonical discriminant analysis were visualised by scatterplots for the two discriminant functions.

2.3 Geometric morphometric analyses

Geometric morphometric (GM) method was applied for a detailed analysis of shape variations of crayfish cephalon, especially focussing on the shape of the rostrum and apex. Data for both sexes were merged because no significant differences were found between the two sexes in log-transformed centroid sizes ($t = 0.416$; $p = 0.677$) what is in accordance with previous GM studies by Scalici *et al.* (2010) on *A. pallipes* (*A. torrentium* sister species) which revealed lack of sexual dimorphism in GM variables. In order to perform landmark-based morphometrics analyses, a digital picture of the dorsal side of each crayfish cephalon was obtained by scanning cephalon portion of each animal on the Epson Perfection V600 Photo scanner. Each specimen was positioned with dorsal side downwards in a water basin placed on flatbed scanner, while the area of interest (cephalon) was kept parallel to scanner by hand. Images were scaled to cm scale. GM analyses were performed using TPS series (Rohlf, 2015) and MorphoJ (Klingenberg, 2011). The location of 22 equally distributed two-dimensional specific measuring points (12 landmarks – LM and 10 semilandmarks – SM) on the dorsal side of the crayfish cephalon were digitised using software TpsDig2 (Rohlf, 2015) (Fig. 2). Position of LMs and SMs was modified according to Scalici *et al.* (2010) and Scalici and Bravi (2012). Semilandmarks were used to describe variation in the shape of rostrum apex and were treated as equivalent to landmarks when computing superimposition. They were defined as equally distant points between corresponding landmarks as described in Zelditch *et al.* (2004). Digitalisation of the LM and SM was made in the same order on each picture, after setting a scale factor. Each specimen was subjected to Generalized Procrustes Analysis (GPA), a procedure that separates the form of an organism into two components, centroid size and shape, by eliminating the non-shape variation resulting from positioning, orientation and

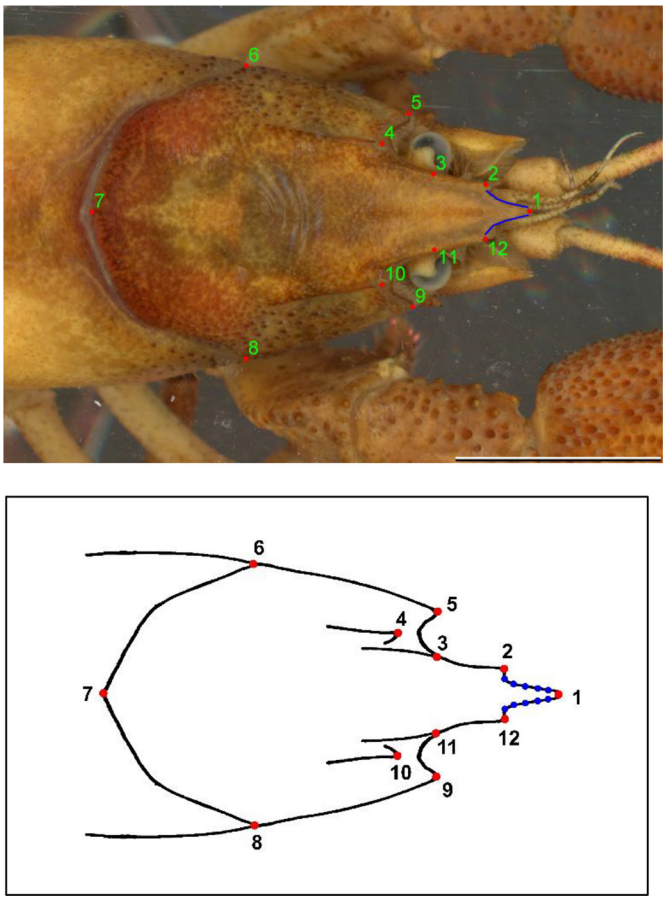

Fig. 2. Position of the landmarks and semilandmarks on the dorsal side of stone crayfish. Above – original scan with 12 landmarks (red dots) and 10 semilandmarks (blue lines); Under – drawing 12 landmarks (red dots) and 10 semilandmarks (blue dots)

scaling (Rohlf and Slice, 1990; Bookstein, 1991). Procrustes coordinates represent shape variables, while centroid size represents size variable independent of shape; it equals the square root of the sum of squared distances of each landmark from their centroid (Zelditch *et al.*, 2004). The measurement error is important when analysing shape in GM, due to possible errors in the landmark and semilandmark digitisation (Klingenberg and McIntyre, 1998; Klingenberg *et al.*, 2002). In order to evaluate the significance of measurement error, a sample of 50 randomly picked individuals was digitised twice by the same operator. Procrustes ANOVA was conducted to access the measurement error by comparing the mean square values of the individual variation with the mean square values of the digitised Error 1.

The patterns of shape variation among five *A. torrentium* phylogroups were examined by carrying out a canonical variate analysis (CVA). Since our aim was to analyse if stone crayfish from different phylogroups differ significantly in the shape, we chose performing CVA over PCA (principle component analysis). It is recommended to use PCA when variation among individuals is analysed, whereas CVA is suggested to be used for describing differences between groups (Zelditch *et al.*, 2004). Variation and changes in shape described by CVA were graphically depicted in the form of wireframe to visualise the differentiation of the crayfish belonging to different phylogroups.

Table 2. Standardized canonical discriminant function coefficients for morphometric characteristics of *A. torrentium* males classified in five phylogroups (LD, GK, ŽPB, BAN and CSE) *sensu* Klobučar *et al.* (2013); for each discriminant function (root1-root4).

Characteristic	Root 1	Root 2	Root 3	Root 4
CLH-d	–0.414	2.313	0.067	–1.273
CPL-d	1.616	–0.212	–0.617	–1.133
TEL	–0.477	–0.183	–0.207	–0.849
ABL	–0.013	0.255	0.759	–0.359
CEF-d	–0.018	–0.215	0.546	–0.304
CGW	–0.743	0.276	0.270	–0.106
ABH	0.185	0.025	0.296	0.143
CFL-d	–0.278	0.201	0.161	0.148
CPH	0.227	0.106	0.179	0.156
HEW	0.475	–0.181	0.061	0.189
TEW	0.265	0.036	–0.785	0.247
ABW	–0.071	–0.193	0.158	0.250
HEL	0.083	0.049	0.283	0.266
ARW	0.271	–0.237	–0.490	0.280
CPW	0.3291	–0.128	–0.846	0.283
ROW	–0.6921	0.073	0.019	0.342
CLW-d	–0.3221	0.169	–0.628	0.427
APW	0.489	0.362	0.017	0.550
CLL-d	–0.801	–2.655	0.672	1.838
Eigenvalue	10.587	1.208	0.718	0.183
Cum. Prop.	0.834	0.929	0.985	1.000
% Expl. Var.	83.381	92.902	98.557	100.000
Canonical R	0.955	0.739	0.646	0.393

In order to establish correlation between TM and GM distances, Mantel test was conducted (Mantel, 1967) in XLSTAT ver. 2019, implemented in Excel, using 9999 permutations. For TM distance matrix, Euclidian distances for measured morphometric characteristics among phylogroups were calculated, while for GM distance matrix we used Procrustes distances among phylogroups.

A level of significance of 1% ($P < 0.01$) was used in all statistical analyses.

3 Results

3.1 Traditional morphometry

Traditional morphometry (TM) analyses included 245 crayfish from 15 populations assigned to five phylogroups, of which 89 were females and 156 males. Results of ANOVA displayed significant differences between phylogroups in the measured morphometric characteristics, for both males ($F = 4.01$; $P < 0.01$) and females ($F = 3.35$; $P < 0.01$) (details are not shown), therefore we proceeded with multivariate discriminant analysis. MDA revealed that morphometric characteristics with the highest discriminatory impact (highest loadings in discriminant functions) for males were those describing claws (CLL, CPL, CLH) and head region (ROW, CGW) (Tab. 2), while for females they included claws (CLL, CLH), head (ROL, HEL and APW) and abdomen (TEW, ABW

Table 3. Standardized canonical discriminant function coefficients for morphometric characteristics of *A. torrentium* females classified in five phylogroups (LD, GK, ŽPB, BAN and CSE) *sensu* Klobučar *et al.* (2013); for each discriminant function (root1-root4).

Characteristic	Root 1	Root 2	Root 3	Root 4
CLL-d	–0.698	–0.398	1.506	–1.117
TEW	–0.527	0.348	0.567	0.038
ARW	–0.486	0.129	0.328	–0.398
APW	–0.465	–0.408	–0.281	0.791
ABH	–0.433	–0.015	0.037	–0.042
HEW	–0.323	0.069	–0.351	–0.190
ABW	–0.246	–0.515	0.756	0.341
CPW	–0.196	–0.328	0.333	0.155
HEL	–0.127	–0.644	0.059	0.062
CFL-d	–0.018	–0.154	–0.576	0.492
TL	0.059	–0.138	–1.746	0.586
ABL	0.187	0.465	0.101	–0.420
CLH-d	0.199	0.564	–0.866	0.237
CLW-d	0.218	–0.126	–0.176	0.488
CEW	0.242	0.317	–0.610	–0.538
CGW	0.251	–0.406	0.129	0.282
TEL	0.518	0.526	0.287	0.546
CPH	0.576	0.156	–0.255	0.265
ROL	1.132	–0.156	0.344	0.026
Eigenvalue	5.316	1.399	0.978	0.635
Cum.Prop	0.638	0.806	0.923	1.000
% expl. Var.	63.820	80.625	92.370	99.993
Canonical R	0.917	0.763	0.703	0.623

Table 4. Percentages of correctly classified individuals of *A. torrentium* based on the function of the corresponding discriminant analyses for males (% males) and females (% females); Lika and Dalmatia (LD), Gorski Kotar (GK), Žumberak, Plitvice and Bjelolasica (ŽPB), Banovina (BAN) and central and south-eastern Europe (CSE).

Phylogroup	% males	% females
LD	95.55	100.00
GK	95.00	90.00
ŽPB	93.55	50.00
BAN	58.82	80.00
CSE	90.90	85.71
Total	89.80	86.36

and CEW) (Tab. 3). Stepwise analysis revealed high number of correctly classified males and females per phylogroups: 89.81% of males and 86.36% of females were classified correctly (Tab. 4).

Canonical variate analyses were performed on populations classified in five *a priori* defined phylogroups: LD, GK, ŽPB, BAN and CSE. Scatter plots for the discriminant functions (roots) are shown in Figure 3a (males) and b (females). The first discriminant function discriminated well males belonging to the phylogroups ŽPB and LD from males belonging to the

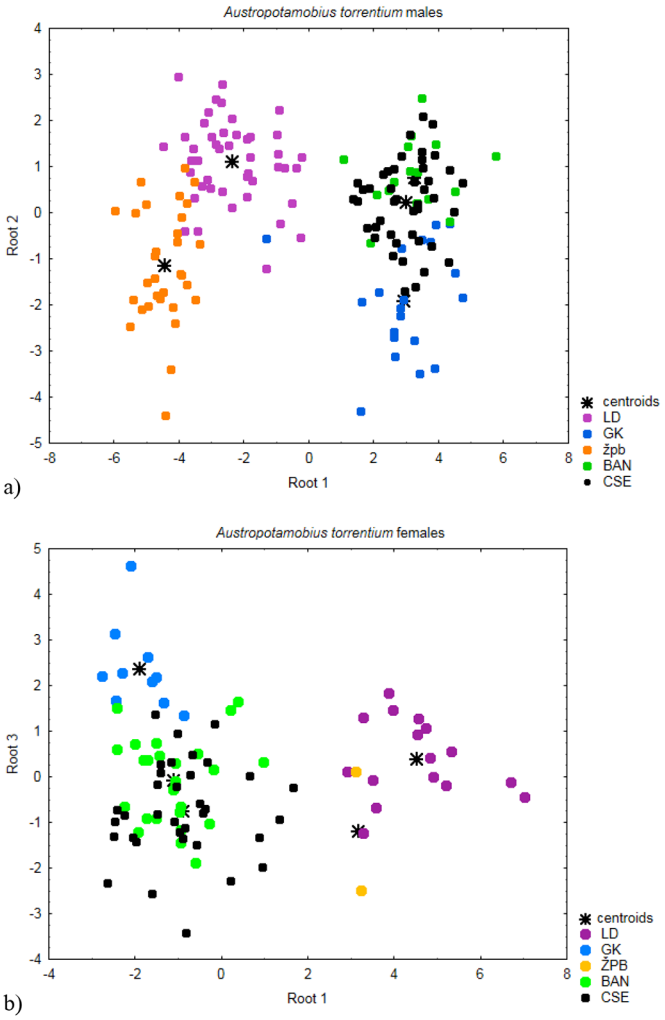

Fig. 3. Discrimination of the different phylogroups of a) males of *A. torrentium* by the two discriminant function (Root 1/Root 2) and b) females of *A. torrentium* by the first and third discriminant functions (Root 1/Root 3). Different colour dots represent different phylogroups: green dots – Banovina (BAN), black dots – central and south-eastern Europe (CSE), blue dots – Gorski Kotar (GK), purple dots – Lika and Dalmatia (LD), orange dots – Žumberak, Plitvice and Bjelolasica (ŽPB)

phylogroups GK, CSE and BAN (Fig. 3a). The first discriminant function was marked with high negative loadings for CLL, CGW and ROW and with high positive loadings for CPL and APW, therefore the smaller the values of CLL and ROW, the more likely males belong to the phylogroups ŽPB or LD, and the higher the values of CPL and APW, the more likely males belong to the phylogroups GK, CSE and/or BAN. The second discriminant functions discriminated to some extant males from the phylogroups ŽPB and GK from the rest. Since second discriminant function was marked with high negative loadings for CLL and high positive loadings for CLH and APW, we may assume the smaller the values of CLL, the more likely males belong to the phylogroups ŽPB or GK, and the higher the values of CLH and APW, the more likely males belong to the phylogroups LD, CSE and/or BAN.

Table 5. Measurement error assessed by performing Procrustes ANOVA on centroid size and shape of stone crayfish. Sums of squares (SS) and mean squares (MS) are in units of Procrustes distances.

Effect	SS	MS	df	F	*P* (param.)	Pillai tr.	*P* (param.)
Centroid size							
Individual	0.000038	0.000001	49	2.03	0.007		
Error 1	0.000019	0.000000	50				
Shape							
Individual	0.18727328	0.0001910952	980	3.07	<.0001	14.02	<.0001
Error 1	0.02474525	0.0000123726	2000				

For females, the first discriminant function (root1) discriminated individuals belonging to the phylogroup LD and ŽPB from those belonging to GK, BAN and CSE (Fig. 3b). The first discriminant function was marked with high negative loadings for CLL and TEW or ARW and with high positive loadings for ROL and CPH. Accordingly, it can be presumed the smaller the values of CLL and TEW or ARW the more likely females belong to the phylogroups CSE, BAN and/or GK, and the higher the values of ROL and CPH, the more likely females belong to the phylogroups LD and/or ŽPB. The third discriminant function (root3) discriminated females belonging to phylogroups GK from the rest. Since the third discriminant function was marked with high negative loadings for TL and CLH and with high positive loadings for CLL and ABW, we may say the smaller the values of TL and CLH the less likely females belong to the phylogroup GK, while the higher the values of CLL and ABW the more likely females belong to the phylogroup GK.

3.2 Geometric morphometrics

Geometric morphometric analyses included 209 crayfish from 15 populations belonging to five previously described mtDNA phylogroups. Generalized Procrustes Analysis revealed the centroid size as size variables and Procrustes coordinates as shape variables. The Procrustes ANOVA was applied to assess the measurement error, and results showed that the mean square for individual variation exceeded the measurement error; therefore it was negligible (Tab. 5).

Results of CVA showed the differentiation among five *A. torrentium* phylogroups caused by the cephalon shape variation (Fig. 4). The first two canonical variates (CV1 and CV2) explained 71.41% of the total variation of the cephalon shape; CV1 accounted for 45.95% of the variability while the CV2 accounted for 25.46% of the variability. The CV1 and CV2 mostly separated phylogroups ŽPB, GK, LD, while there was an overlapping between the phylogroups BAN and CSE. The cephalon shape, as quantified by Procrustes distances, differed between the phylogroups (Tab. 6). The highest values of the Procrustes distances were obtained between the phylogroups ŽPB and BAN (0.0599), followed by ŽPB and CSE (0.0548), while the lowest values of Procrustes distances were observed between CSE and BAN (0.0210), and CSE and LD (0.0269). All Procrustes distances between the phylogroups were statistically significant with p-values lower than 0.01.

Morphological variability of cephalon was mainly visible in the anterior part, while the posterior area was less variable in the shape (Fig. 4). Shape changes that contribute to the distinction among crayfish from different phylogroups were visible in the apical part of the cephalon, particularly in the rostrum apex, rostrum base and width of the lateral edge of the head. Shape changes along +CV1 are characterised by general widening of the rostrum, shortening (LM 7) and narrowing (LM 6 and 8) of the head, shifting of the lateral edge of the head towards its apical part (LM 5 and 9), and elongation of the apex (SM 1-2 and 1-12). These characteristics were present in the majority of the specimens belonging to the phylogroup GK, CSE, BAN and partly ŽPB. In contrast, −CV1 was related to reduction of rostrum size and length, elongation (LM 7) and widening (LM 6 and 8) of the head, shifting of the lateral edge of the head towards its distal part (LM 5 and 9), and shortening of the apex (SM 1-2 and 1-12). These morphological characteristics were generally present in the phylogroup LD and partly in the phylogroup ŽPB. Shape changes along +CV2 were characterised by larger head, narrower elongated rostrum (LM 2-3 and 11-12 and SM 13-17 and 18-22), and longer apex (SM 1-2 and 1-12). Shape changes along −CV2 were characterised by wider and shorter rostrum (LM 2- 3 and 11-12; SM 13-17 and 18-22), less robust head and shorter apex (SM 1-2 and 1-12). These morphological characteristics were generally pronounced in the crayfish from the phylogroup ŽPB, GK and partly LD.

The Mantel test showed significant correlation between TM and GM distance matrices ($r = 0.756$, $p = 0.007$).

4 Discussion

This study aimed to determine morphological characteristics which separate stone crayfish belonging to different mtDNA phylogroups through analyses of traditional morphometric features, and for this species for the first time through geometric morphometric approach. Also, the goal was to verify if the two methods yield congruent results, and if results of morphometry are consistent with the results obtained in previous molecular research (Klobučar *et al.*, 2013). Overall, results of morphometry were in agreement with results obtained in previous molecular research (Klobučar *et al.*, 2013) and established significant differences in morphology among stone crayfish from different phylogroups, with characteristics of cephalon (both TM and GM) and claws (TM) contributed the most to their divergence.

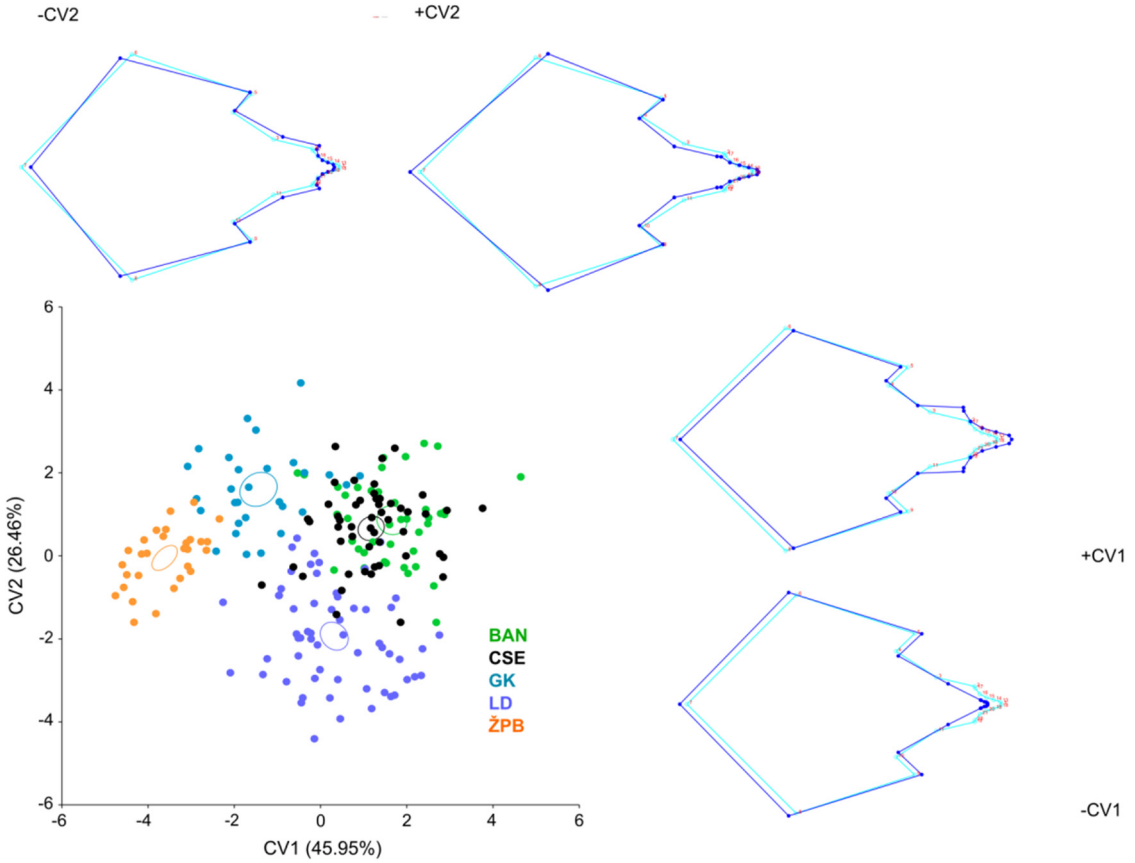

Fig. 4. Cephalon shape variation among the phylogroups of *A. torrentium* revealed by canonical variate analysis. Scatter plott of the two first canonical variate axes (CV1 and CV2) with associated wireframe representation depicting shape changes among phylogroups along positive and negative extremes of the axes. The light blue wireframes represent average cephalon shape, while the dark blue wireframes represent most extreme shape changes. Different colour dots represent different phylogroups: green dots – Banovina (BAN), black dots – central and south-eastern Europe (CSE), blue dots – Gorski Kotar (GK), purple dots – Lika and Dalmatia (LD), orange dots – Žumberak, Plitvice and Bjelolasica (ŽPB).

Table 6. Procrustes distances between the phylogroups of *A. torrentium* (above diagonal, bold) with *p*-values from permutation tests (10,000 permutation rounds) (below diagonal); Banovina (BAN), central and south-eastern Europe (CSE), Gorski Kotar (GK), Lika and Dalmatia (LD), Žumberak, Plitvice and Bjelolasica (ŽPB).

Phylogroup	BAN	CSE	GK	LD	ŽPB
BAN		**0.0210**	**0.0455**	**0.0307**	**0.0599**
CSE	<0.0001		**0.0452**	**0.0269**	**0.0548**
GK	<0.0001	<0.0001		**0.0337**	**0.0316**
LD	<0.0001	<0.0001	<0.0001		**0.0316**
ŽPB	<0.0001	<0.0001	<0.0001	<0.0001	

Majority of recent studies on the freshwater crayfish morphological variability were based on the statistical analyses of morphometric characteristics using traditional morphometrics. Analyses of large number of morphological characteristics per specimen, in combination with multivariate statistical analyses, enabled the identification of statistically significant differences between populations of the same species, *e.g. A. pallipes* (Ghia *et al.*, 2006; Bertocchi *et al.*, 2008), *A. leptodactylus* (Deniz *et al.*, 2010; Maguire and Dakić, 2011; Benzer et al., 2017), *A. torrentium* (Sint *et al.*, 2006; Maguire *et al.*, 2017), *A. astacus* (Đuretanović *et al.*, 2017) as well as between different crayfish species (Sint *et al.*, 2006, 2007; Larson *et al.*, 2012).

Application of geometric morphometric approach in freshwater crayfish studies was not frequently used (Scalici *et al.*, 2010; Scalici and Bravi, 2012; Helms *et al.*, 2015; Malavé *et al.*, 2018).

Various studies on the crustaceans showed that this group of animals displays great morphological diversity and plasticity (Wills, 1998; García-Dávila *et al.*, 2006; Oda *et al.*, 2007; Stillman *et al.*, 2008; Tanaka, 2009; Zimmermann *et al.*, 2011; Yampolsky *et al.*, 2014). The variations in their phenotype could be a consequence of adaptation to the environment (Zimmermann *et al.*, 2011; Yampolsky *et al.*, 2014) or genetic factors (Atashbar *et al.*, 2016; Hidayani *et al.*, 2018). Likewise, freshwater crayfish exhibit intraspecific morphometric variation that reflects environmental influence (Sint *et al.*, 2005, 2006; Ghia *et al.*, 2006; Haddaway *et al.*,

2012; Perry *et al.*, 2013; Rudolph *et al.*, 2016) or genetic background (Sint *et al.*, 2007; Cataudella *et al.*, 2010; Maguire *et al.*, 2017; Pârvulescu, 2019) or probably combination of both (Baric *et al.*, 2005a, 2005b; Bertocchi *et al. et al.*, 2008; Mathews *et al.*, 2008; Helms *et al.*, 2015; Berger *et al.*, 2017).

Former molecular phylogenetic and phylogeographic research of the stone crayfish based on the mtDNA revealed existence of at least seven (Klobučar *et al.*, 2013) or eight (Pârvulescu *et al.*, 2019) highly divergent monophyletic phylogroups within *A. torrentium*, with the highest genetic diversity recorded in the north-central Dinarids in Croatia. Especially high distances were obtained for phylogroups ZV, GK, ŽPB and LD. Since at that time there was no sufficiently stable diagnostic feature that would reliable distinguish crayfish from different phylogroups, it was suggested that they represent cryptic species (Klobučar *et al.*, 2013). Resolving status of cryptic species is a challenge that requires, beside molecular techniques, morphological approach (Mound *et al.*, 2010; Larson *et al.*, 2012; Singhal *et al.*, 2018). In a small scale preliminary morphometric study of *A. torrentium* populations originating from three phylogroups (ZV, GK and ŽPB) Maguire *et al.* (2017) revealed the characteristics that clearly separate crayfish in a similar way as molecular methods. Current study included larger number of populations per phylogroup, and it covered five out of seven (eight) phylogroups.

Analyses of variance for both sexes displayed significant differences in morphometric characteristics among crayfish belonging to different phylogroups what is in accordance with previous research by Maguire *et al.* (2017). Similarly to results of previous studies (Sint *et al.*, 2005, 2007; Maguire *et al.*, 2017; Mijošek *et al.*, 2017), males differ in more TM characteristics than females. Multivariate discriminant analyses, for both sexes, distinguished characteristics describing claws, head and rostrum (Tabs. 3 and 4) as characteristic that separate populations/phylogroups the best what confirmed results of previous study (Maguire *et al.*, 2017). Percentage of correctly classified female and male stone crayfish per phylogroup was relatively high (from 50 to 100%; Tab. 4) and similar to values obtained in other studies (Sint *et al.*, 2007; Maguire *et al.*, 2017). Misclassification of some of individuals could have been a consequence of paucity of samples caused by limited sampling in certain areas (*e.g.* males in BAN and females in ŽPB) (Larson *et al.*, 2012). Scatterplots of canonical analyses, for both sexes, displayed relatively well separation of individuals from different phylogroups (Fig. 3a and b), what is concordant with previous findings (Maguire *et al.*, 2017). Differentiation between individuals from different phylogroups by CVA and high values of correctly classified males and females per phylogroup indicated clear morphological differentiation of phylogroups and supported the use of traditional morphometrics in phylogroups delimitation.

Geometric morphometrics analyses were applied previously on *A. torrentium*'s sister species *A. pallipes* in order to study allometry during ontogenesis (Scalici and Gibertini, 2009; Scalici *et al.*, 2010) and systematic relations of *A. pallipes* species complex (Scalici and Bravi, 2012). In the present study, significant variation in cephalon shape of *A. torrentium* was observed by GM, and successful intraspecific delimitation, based on morphological variation, was obtained (Fig. 4). Each of the five phylogroups showed a significantly different cephalon shape. The variation was noticeable on the anterior part of the crayfish body, especially rostrum, while the posterior part of the head was less variable in shape, what is consistent with the findings by Bertocchi *et al.* (2008); Larson *et al.* (2012) or Rudolph *et al.* (2016). Results of CVA showed clear separation of phylogroups ŽPB, GK and LD, while there was an overlapping between the phylogroups BAN and CSE. Geometric morphometrics, as a new approach in the research of the stone crayfish morphological features, demonstrate itself as an improvement and complement to TM studies as well as useful in the future population genetics and ecological research of the stone crayfish phylogroups.

Results of both TM and GM canonical discriminant analyses showed congruent topologies: separation among phylogroups, with exception of an overlap of the crayfish from the phylogroups BAN and CSE, which coincides with the results of molecular phylogenetic research of stone crayfish (Klobučar *et al.*, 2013). Further, the results of Mantel test established significant correlation between distances obtained by the two approaches. Correlations between TM and GM were already recorded for different organisms (*e.g.* crustaceans (Malavé *et al.*, 2018; de Melo and Masunari, 2017), honeybees (Tofilski, 2008), cichlids (Parsons *et al.*, 2003), oaks (Viscosi *et al.*, 2009)).

Overall, our results confirmed traditional and geometric morphometrics as useful tools for identification and delimitation of stone crayfish phylogroups and highlighted the urgent need for taxonomic revision of stone crayfish phylogroups status. At the same time, obtained results draw attention to the importance of thorough knowledge about species, including genetics, morphology and ecology, that are necessary for development of proper conservation plans. Application of scientifically based results in conservation measures could help preservation of evolutionary potential and heritage, as well as enable insight into historical biogeography of the species.

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New insights into the genetic diversity of the stone crayfish: taxonomic and conservation implications

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Abstract

Background: *Austropotamobius torrentium* is a freshwater crayfish species native to central and south-eastern Europe, with an intricate evolutionary history and the highest genetic diversity recorded in the northern-central Dinarides (NCD). Its populations are facing declines, both in number and size across its entire range. By extanding current knowledge on the genetic diversity of this species, we aim to assist conservation programmes. Multigene phylogenetic analyses were performed using different divergence time estimates based on mitochondrial and, for the first time, nuclear DNA markers on the largest data set analysed so far. In order to reassess taxonomic relationships within this species we applied several species delimitation methods and studied the meristic characters with the intention of finding features that would clearly separate stone crayfish belonging to different phylogroups.

Results: Our results confirmed the existence of high genetic diversity within *A. torrentium*, maintained in divergent phylogroups which have their own evolutionary dynamics. A new phylogroup in the Kordun region belonging to NCD has also been discovered. Due to the incongruence between implemented species delimitation approaches and the lack of any morphological characters conserved within lineages, we are of the opinion that phylogroups recovered on mitochondrial and nuclear DNA are cryptic subspecies and distinct evolutionary significant units.

Conclusions: Geographically and genetically isolated phylogroups represent the evolutionary legacy of *A. torrentium* and are highly relevant for conservation due to their evolutionary distinctiveness and restricted distribution.

Keywords: *Austropotamobius torrentium*, Species delimitation, Species validation, MOTU, ESU, Phylogeographic patterns, nuDNA, mtDNA, Evolutionary history

Background

The stone crayfish (*Austropotamobius torrentium* (Schrank, 1803)) is an indigenous European crayfish species (ICS) [1]. It is the smallest of all European ICS and is considered a keystone species in freshwater ecosystems [2]. The stone crayfish is a cold-adapted species active at water temperatures > 5 °C with a mean annual water temperature that does not exceed 10 °C [2]. It inhabits smaller pristine waterbodies at high altitude in central and south-eastern Europe (Fig. 1) that are related to karstic formations [1, 2]. The species exhibits high genetic diversity represented by the eight distinct mtDNA lineages/phylogroups discovered so far [3–5].

Lately, studies have shown that populations of the stone crayfish, as well as those of other ICS, are declining [1, 6]. They are threatened by habitat deterioration [2], water

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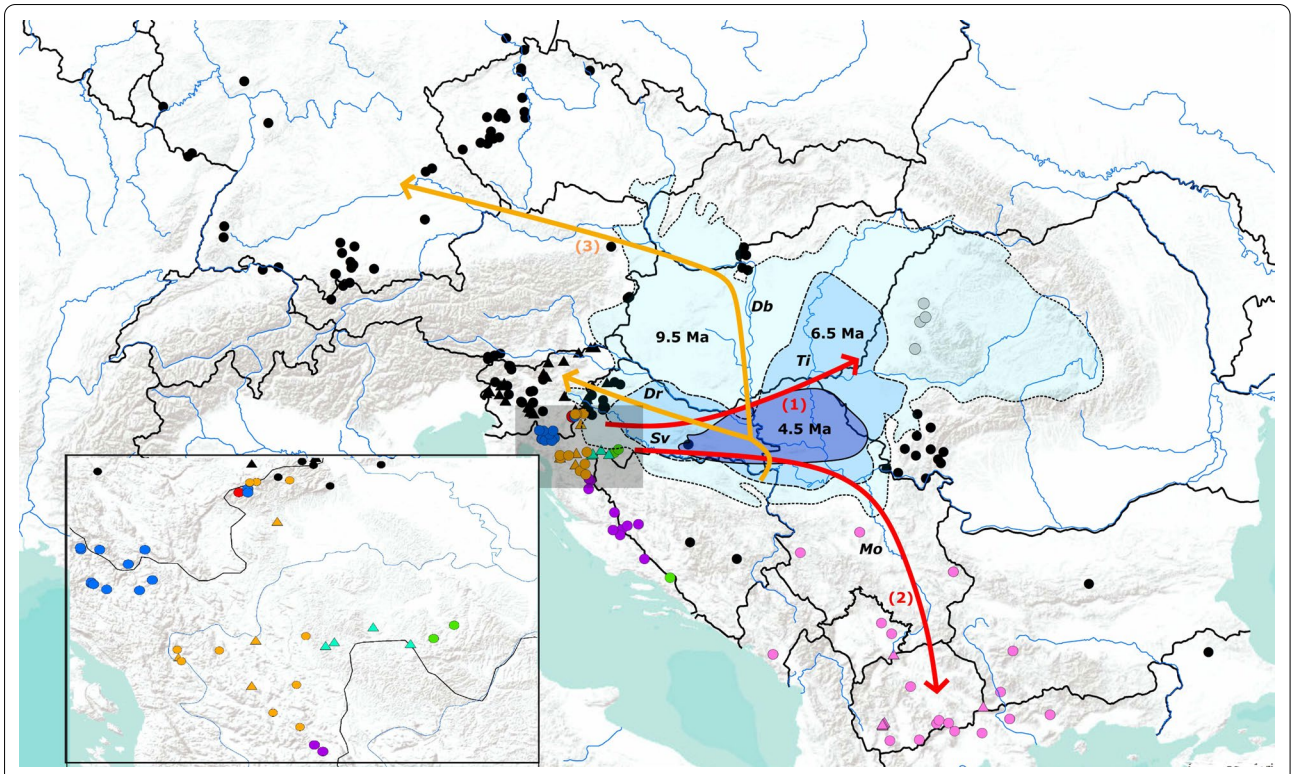


Fig. 1 Geographical distribution of different *A. torrentium* mtDNA phylogroups in Europe produced in ArcGIS 10.3 program package and finished in the program Inscape 1.0 by authors of this study. Symbols used on the map: dots represent samples from previous research [3–5], and triangles samples from this study. Colours depict mitochondrial phylogroups: black—central and south-eastern Europe (CSE), blue—Gorski Kotar (GK), purple—Lika and Dalmatia (LD), orange—Žumberak, Plitvice and Bjelolasica (ŽPB), pink—southern Balkans (SB), green—Banovina (BAN), red—Zeleni Vir (ZV), gray—Apuseni Mountain (APU) and turquoise blue—Kordun (KOR), new phylogroup discovered in the present study. River systems abbreviations: *Db* Danube, *Dr* Drava, *Sv* Sava, *Ti* Tisza, *Mo* Morava. Also shown: the extent of the Lake Pannon at 9.5 Ma, 6.5 Ma and 4.5 Ma (adapted according to Magyar et al. [76]) and shaded in blue. Possible pre-glacial and post-glacial dispersal routes are indicated; red arrows indicate: (1) possible colonisation of the Apuseni Mountains through delta systems of paleo-Danube and paleo-Tisza on the northern shelf margin of the Lake Pannon and (2) colonization of southern Balkan after formation of the freshwater Danube drainage system. Orange arrows (3) indicate post-glacial recolonisation of northern part of *A. torrentium* areal through leading edge expansion of CSE phylogroup (adapted according to Klobučar et al. [4]). Shaded gray area in the main map is enlarged in the bottom left corner

quality decline [7], climate change [6, 8], the presence/spreading of non-indigenous invasive American crayfish species and their pathogens, e.g., *Aphanomyces astaci* causative agent of the disease crayfish plague [9, 10]. ICS are offered differing levels of protection under international and national laws, with the stone crayfish listed in the Annexes II and V of the EU Habitats Directive [11]. The conservation status of *A. torrentium* remains unresolved as it noted as being "data deficient" on the global IUCN Red List [12], whilst in Croatia it is classed as vulnerable [13].

The maintenance of genetic diversity is considered fundamental to modern conservation efforts, as it is essential for securing the evolutionary potential and long-term survival of a species [14]. In order to protect vulnerable species adequate conservation plans are urgently needed on a global scale which requires sound knowledge of both the morphologic and genetic diversity of this species in addition to the identification of evolutionary independent lineages within the species [2, 15, 16].

The first morphological studies aimed to distinguish between different populations of the stone crayfish [17] which resulted in the identification of four subspecies: *Austropotamobius torrentium torrentium* [17], *A. t. macedonicus* [18], *A. t. dalmatinus* [18] and *A. t. danubicus* [19, 20]. Later, studies based mainly on meristic characteristics confirmed previously described subspecies [20, 21]. Recently, Maguire et al. [22] discovered differences among distinct populations (representing different mtDNA phylogroups defined by preciding genetic analyses [4]) of the stone crayfish in a small geographical region in Croatia. This was achieved by analysing a number of individual morphometric and meristic characteristics with these findings corroborated by a large scale

geometric morphometric analyses applied to stone crayfish populations for the first time [23]. Even though these studies confirmed morphological delineation between phylogroups (the cephalon shape being pertinent), it was shown that morphological variation within phylogroups is also present [22, 23]. Freshwater crayfish are known to exhibit high intraspecific morphological variation and plasticity reflecting environmental influence and/or genetic background [22, 24, 25]. Hence, it is hard to find unique and unambiguous morphological character specific only for one phylogroup that would be suitable to clearly distinguish between phylogroups thus resolving the taxonomic status of *A. torrentium* phylogroups in addition to describing a new species.

Until now, molecular phylogenetic studies of *A. torrentium* were based on the analyses of mitochondrial genes for cytochrome *c* oxidase subunit I (*COI*) and *16S* ribosomal RNA (*16S* rRNA) [3–5, 26, 27]. Trontelj et al. [3] discovered three highly divergent mtDNA phylogroups: one distributed in the southern part of the Balkan Peninsula, another in a small area on the border between Slovenia and Croatia, and the third that encompasses the rest of Europe. This finding indicated that the stone crayfish should be considered a species complex. Later, Klobučar et al. [4] confirmed Trontelj et al. [3] findings, and discovered the existence of four additional phylogroups, with the highest genetic diversity found in the Dinaric region of Croatia. The phylogroups were named after geographical areas of their distribution: central and south-eastern Europe (CSE), southern Balkans (SB), Banovina (BAN), Gorski Kotar (GK), Lika and Dalmatia (LD), Zeleni Vir (ZV), Žumberak, Plitvice and Bjelolasica (ŽPB), with the five latter situated in the north and central Dinarides (NCD). Recently, Pârvulescu et al. [5] discovered the existence of a new phylogroup, endemic to the Romanian Apuseni Mountain region (APU). Combining the molecular mtDNA analyses with morphological data, the APU phylogroup was proposed as a new species *Austropotamobius bihariensis* [28].

Species delimitation requires integrative taxonomic approach that combines molecular, morphological, ecological, and geographical data to build species hypotheses [29, 30]. This approach enables taxonomy to go beyond naming the species and assists in understanding the processes that shape the species [31, 32].

Even though mitochondrial genes (*COI* and *16S* rRNA) are appropriate for resolving taxonomic relationships between genera and species [33–35], they show some drawbacks in species delimitations (e.g., higher failure rate at proposing species delimitation hypothesis compared to nuclear markers) [31, 36, 37]. Therefore, the need for a nuclear marker that can be used in the reconstruction of genetic relationships as well as in the species delimitation has been recognised [38, 39]. Yao et al. [40] proposed the second internal transcribed spacer (*ITS2*) as a nuclear marker that is complementary to mitochondrial *COI* and *16S* rRNA, and is suitable in studying relationships of lower taxonomic categories (e.g. genera, species) [41] as well as for species delimitation [42].

In order to extand current knowledge about the stone crayfish diversity and provide baseline for conservation programs, the aims of our study were:

(a) to update phylogenetic findings based on the largest dataset used so far that includes new samples from previously unstudied stone crayfish populations from Croatia, Slovenia and Republic of North Macedonia
(b) to test if *ITS2* is a good nuclear marker for phylogenetic inference on *A. torrentium* and verify phylogenetic congruence between mitochondrial and nuclear DNA markers
(c) to evaluate alternative scenarios in the background of the currently observed distribution, genetic variability and phylogeographic patterns via varied molecular clock calibrations
(d) to apply species delimitation methods aiming to identify Molecular Operational Taxonomic Units (MOTUs) within *A. torrentium,* and to reassess their taxonomic status
(e) to study meristic characteristics on a large data set in order to find reliable character/characters that will clearly and undoubtedly distinguish MOTUs
(f) to give new perspectives in *A. torrentium* conservation programs through the identification of Evolutionary Significant Units (ESUs).

Results

Sequence data

We obtained a total of 153 (58 new) *COI* and 72 (24 new) *16S* rRNA unique haplotypes. The concatenated *COI*/*16S* rRNA data set included 151 (78 new) haplotype combinations (Additional file 1). Analyses of *COI* gene revealed 166 (27.95%) variable sites, of which 141 (23.74%) were parsimony informative, while 65 (13.59%) sites were variable in *16S* rRNA sequences, with 51 (10.27%) of them being parsimony informative. Obtained *ITS2* sequences showed only 27 (2.45%) variable sites, and 20 (1.81%) were parsimony informative. Analysis of the *ITS2* sequences using FastGap revealed gapmatrix with 26 (2.35%) gap sites, 13 being informative.

Phylogenetic reconstruction

All implemented criteria of phylogenetic reconstruction (BA, MP and ML) yielded mostly congruent topologies

for *COI*/*16S* rRNA concatenated data set (Fig. 2a). The new phylogroup, belonging to the Kordun region (part of NCD) was discovered, while majority of the newly obtained sequences nested within the eight previously reported phylogroups [4, 5]. Moreover, phylogroups belonging to the NCD region (ZV, GK, ŽPB, LD, BAN, KOR) and APU appeared as monophyletic clades, well supported by bootstrap values and Bayesian posterior probabilities (Fig. 2a). The 'Southern Balkans' (SB) phylogroup was not supported as monophyletic; it comprised four sub-clades and two individual haplotypes represented in a basal polytomy with the monophyletic CSE clade. Numerous sub-clades also existed within well-supported monophyletic CSE phylogroup.

The Bayesian inference of phylogeny based on the nuclear gene *ITS2* yielded a tree topology with seven well supported phylogroups and the KOR lineage (Fig. 2b). Unlike in mtDNA phylogeny, CSE haplotypes have not formed separate monophyletic clade, but rather combined with SB haplotypes in a well supported clade. Phylogenetic relations among groups were not resolved with the majority of them form polytomy within *A. torrentium*, with the exception of well-supported separation of APU phylogroup (Fig. 2b).

In general, the common feature of the phylogenetic recontructions of both datasets was that phylogroups were well supported in the phylogenetic trees, but the relationship among them was unresolved, showing weak support for deeper nodes.

Phylogeographic analysis and genetic diversity

A median-joining (MJ) network for concatenated *COI*/*16S* rRNA data set was used to visualise haplotype relatedness and haplotype distribution within *A. torrentium* (Fig. 2c). All nine phylogroups were highly divergent and separated by large numbers of mutational steps. The newly discovered KOR phylogroup was 42 mutational steps distant from closely related ŽPB phylogroup. The CSE phylogroup showed a complex structure consisting of large number of closely related haplotypes with a broad geographical distribution, separated by a small number of mutational steps. The SB phylogroup comprised six subclades separated by a large number of mutational steps. The SB and CSE phylogroups showed the smallest between-group number of mutational steps, whilst the ZV phylogroup showed the largest number of mutational steps when related to its closest neighbouring phylogroup BAN. Further, contrary to the relations in the phylogenetic tree, the APU phylogroup was closest to the BAN and not to the ZV phylogroup.

The results of TCS network analysis, based on the *COI* data set (used also as species delimitation approach; Additional file 2), were concordant with MJ results. The TCS network revealed the existence of 18 MOTUs with CSE, GK, ZV, LD, KOR, APU and ŽPB phylogroup each representing one MOTU. The SB phylogroup was split into nine separated MOTUs. The BAN phylogroup was split into two MOTUs; haplotype 41 formed the first one, and the second contained all other BAN haplotypes.

The obtained values of uncorrected sequence divergences (p-distances) and patristic distances within and between phylogroups for *COI, 16S* rRNA and *ITS2* are shown in Additional file 3. The obtained values of genetic distances for all genes were calculated using p-distances and K2P distances were congruent. The p-distances between phylogroups ranged from 4.98 to 9.62% for *COI*, and from 0.00 to 5.05% for *16S* rRNA gene. The highest values of genetic distances were observed when ZV, GK and APU clades were compared with other phylogroups, for both *16S* rRNA and *COI* markers. The range of p-distances within phylogroups for the *COI* gene was between 0.17 and 5.33%, and between 0.00 and 2.75% for *16S* rRNA gene. The values of p-distances for the *ITS2* gene, which showed less genetic variation than mitochondrial genes, were mostly congruent to the results obtained for mitochondrial genes, ranging from 0.00 to 0.79% between groups, and from 0.00 and 0.29% within groups (Additional file 3). Patristic distances between the phylogroups indicated various molecular divergence between several phylogroup pairs with values ranging from 0.08 to 0.22.

Time of divergence

Divergence time estimates based on a mitochondrial data set using three molecular clock and four geological calibrations are presented in Fig. 3 (for details see Additional file 4). The results of divergence time approximations overlapped, with the mean values of three molecular calibration approaches as follows: (a) ~ 17.90 Ma for the split between *A. pallipes* and *A. torrentium*, (b) ~ 8.80 Ma for the split between populations belonging to the NCD + APU from BAN + SB + CSE phylogroups, (c) ~ 5.01 Ma for the split of SB + CSE phylogroups from BAN phylogroup, and (d) ~ 3.12 Ma for the split between SB and CSE phylogroups.

Geological calibration points showed a wider range of different divergence times estimates. The Tisza–Dacia microplate tectonic displacement that, according to Pârvulescu et al. [5], occurred ~ 16 Ma, gave the largest intervals of possible divergence times, and was not consistent with other geological and molecular calibrations. The results of new geological calibration point used in this research (Fig. 3), based on the contact between the paleo-Tisza and paleo-Danube river systems [43], accompanied by the process of desalination of the Lake Pannon, indicated that this dispersion route could have enabled colonisation of the species north-east distribution range

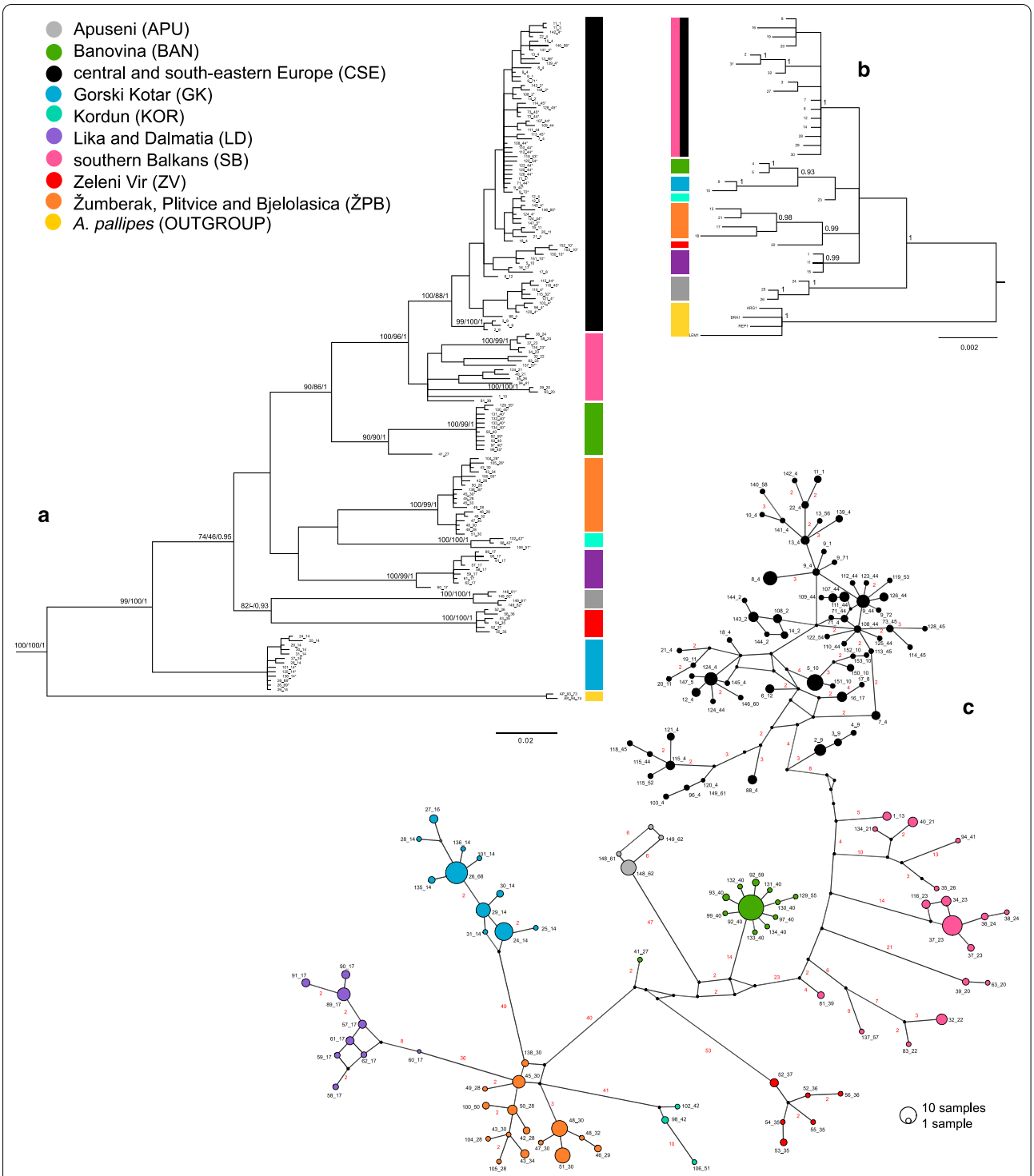


Fig. 2 Phylogenetic recontruction for *A. torrentium*. **a** Phylogram inferred from concatenated *COI/16S* rRNA (new haplotypes obtained in this study are marked with an asterisk) and **b** phylogram inferred from *ITS2* haplotypes depicting the phylogenetic relationships within *A. torrentium*. Numbers at the nodes indicate maximum likelihood and maximum parsimony nonparametric bootstrap support values and Bayesian posterior probabilities, respectively. **c** Median joining (MJ) network for concatenated *COI/16S* rRNA. Numbers of mutational steps are given in red above branches except when it equals one. The size of the circle is proportional to the frequencies of the haplotype. The black dots indicate extinct ancestral or unsampled haplotypes. Phylogroups are represented by different colour: black—central and south-eastern Europe (CSE), blue—Gorski Kotar (GK), purple—Lika and Dalmatia (LD), orange—Žumberak, Plitvice and Bjelolasica (ŽPB), pink—southern Balkans (SB), green—Banovina (BAN), red—Zeleni Vir (ZV), gray—Apuseni Mountain (APU) and turquoise blue—Kordun (KOR)

(See figure on next page.)

Fig. 3 Chronogram of 95% highest posterior density intervals (HPD) of divergence time estimates (in Ma) obtained with the mean values in brackets **a** using arthropod evolutionary rate [134, 135], **b** using decapod evolutionary rate [136], **c** using mid-points of a uniform distribution [137], **d** using geological calibration based on the connection of paleo-Tisza–paleo-Danube river systems **e** and **g** using geological calibration based on the uplift of the Dinaric Mountains [4, 61], **f** using geological event based on the separation of the Tisza–Dacia microplate from Dinarides [5]. Different colours denote the HPD of distinct lineages: dark blues—split of *A. pallipes* and *A. torrentium*; light blue—split of NCD (north and central Dinaric phylogroups = ZV, GK, LD, KOR, ŽPB) + APU from the BAN, SB and CSE phylogroups; purple—split of BAN from CSE + SB phylogroups; grey—split of CSE and SB phylogroups. In the upper right corner BEAST estimates of divergence times for *A. torrentium* based on the paleo-Danube–paleo-Tisza geological calibration is given; maximum clade credibility tree based on concatenated sequence. Horizontal node bars depict the 95% HPD intervals and are coloured according to posterior probability support (blue bars—posterior probabilities > 0.95; orange bars—posterior probabilities 0.50–0.95, green bars—posterior probabilities < 0.50. *APU* Apuseni, *ZV* Zeleni Vir, *GK* Gorski Kotar, *LD* Lika and Dalmatia, *KOR* Kordun, *ŽPB* Žumberak, Plitvice and Bjelolasica, *BAN* Banovina, SB southern Balkans, *CSE* central and southeastern Europe. *Austropotamobius pallipes*, *Astacus astacus* and *Pontastacus leptodactylus* were used as outgroups

including the Apuseni region. Estimates of this calibration approach yielded results consistent with molecular and geological calibrations based on the intense Dinarides uplift. The median values for the key points in *A. torrentium* evolution based on this geological calibration were: (a) 13.24 Ma (HPD 18.70–8.55 Ma) for the split of *A. pallipes* and *A. torrentium*, (b) 6.13 Ma (HPD 8.21–4.46 Ma) for the split of NCD + APU from BAN + SB + CSE phylogroups, (c) 3.67 Ma (HPD 5.22–2.46) for the split between BAN and CSE + SB phylogroups and (d) 2.25 Ma (HPD 3.21–1.51 Ma) for the split between CSE and SB phylogroups.

Species delimitation and validation

Species delimitation analyses (ABGD, GMYC, bPTP, mPTP, TCS) for mtDNA (*COI*) confirmed the existence of different *A. torrentium* MOTUs (Fig. 4, Additional file 5). The number of supported groups varied depending on the applied method. In the ABGD analysis for the majority of prior intraspecific divergence values (P), initial partitioning identified nine MOTUs (ABGD lumper approach), while the results from recursive partitioning singled out the existence of 18 MOTUs (ABGD splitter approach). The ABGD lumper, as the most conservative approach, recognised six phylogroups as a single MOTU: APU, GK, KOR, LD, ZV, ŽPB, while BAN phylogroup was split into two MOTUs. CSE and SB phylogroups were lumped into one MOTU. The ABGD splitter revealed nine MOTUs in the SB phylogroup, while haplotypes belonging to CSE phylogroup were recovered as a single MOTU. Delimitation results from ABGD splitter were consistent with the results obtained by TCS method. The mPTP method delimited 21 putative MOTUs that were mostly congruent with the results from ABGD splitter and TCS. The bPTP recognised between 26 and 45 MOTUs, 9 with Bayesian support values over 0.95. The GMYC *single threshold* approach identified 22 ML clusters (confidence interval: 19–36) and 29 entities (confidence interval: 25–53), but most of them lacked statistical support. Overall, PTP and GMYC yielded unrealistically high number of MOTUs, and relying only on the supported groups, the number of recognised groups was lower. Nested sampling analysis yielded marginal likelihood estimations ranging from − 4195 to − 4539 (Additional file 6). The model receiving the highest marginal likelihood score was GMYC, and calculated Bayes factor values showed decisive support for species tree topology associated with this species delimitation.

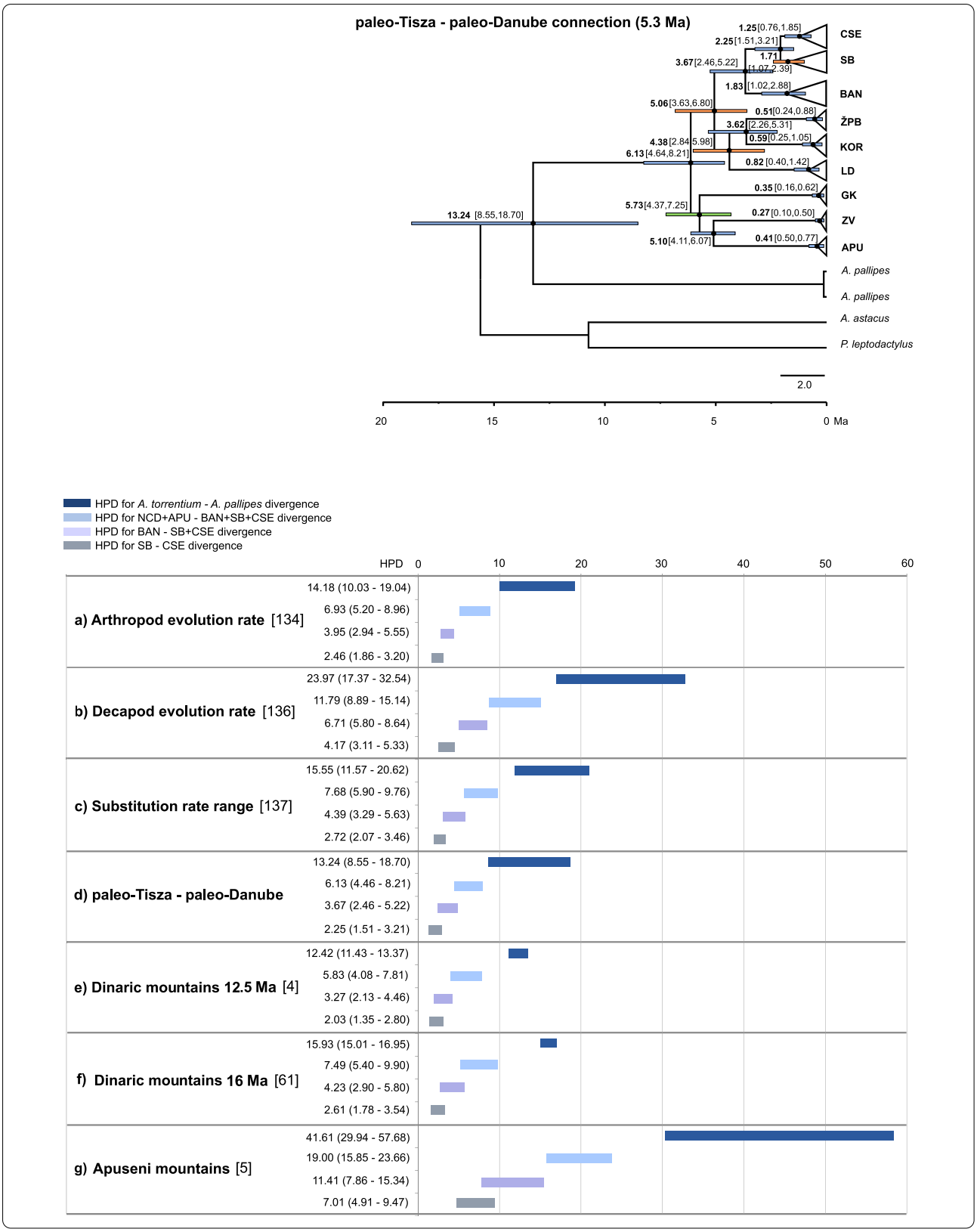
Single-locus species tree (*COI*) based on GMYC and multi-locus species tree (*COI* + *16S* rRNA + *ITS2*) based on the phylogeny showed a pattern of divergence between phylogroups; all phylogroups formed own monophyletic clades (Fig. 5). Species trees were congruent, showing the pattern of high genetic diversity, with no clear separation of genetic clusters (phylogroups).

Meristics

Within-phylogroup variation in the number of spines on the ventral side of the merus of the third maxilliped was apparent, while significant difference between studied phylogroups was obtained ($H\ (7,\ N = 732) = 112.94$, $P < 0.001$), with crayfish from ZV possessing more spines compared to crayfish from other phylogroups, except KOR (Additional file 7).

Presence and pronunciation of rostral crista was inconsistent, and differed among phylogroups ($\chi^2_{21,\ N = 735} = 491.58$, $P < 0.01$); some of the crayfish from KOR, CSE and LD did not have rostral crista; in the rest of phylogroups rostral crista were present, and variation in the level of pronunciation exists. Crayfish from GK did not have weak crista, and in the SB phylogroup we did not record any crayfish with strong crista (Additional file 7).

In the studied phylogroups all three types of denticulation on the lower surface of antennal exopodite (smooth = no denticulation, tubercles, spines) were recorded, with phylogroups differed in the percentage of different type of denticulation ($\chi^2_{14,\ N = 735} = 176.22$, $P < 0.01$) (Additional file 7).


paleo-Tisza - paleo-Danube connection (5.3 Ma)
1.25[0.76,1.85]
2.25[1.51,3.21]
3.67[2.46,5.22]
1.71[1.07,2.39]
1.83[1.02,2.88]
5.06[3.63,6.80]
0.51[0.24,0.88]
3.62[2.26,5.31]
0.59[0.25,1.05]
4.38[2.84,5.98]
6.13[4.64,8.21]
0.82[0.40,1.42]
0.35[0.16,0.62]
5.73[4.37,7.25]
13.24[8.55,18.70]
0.27[0.10,0.50]
5.10[4.11,6.07]
0.41[0.50,0.77]
CSE
SB
BAN
ŽPB
KOR
LD
GK
ZV
APU
A. pallipes
A. pallipes
A. astacus
P. leptodactylus
2.0
20
15
10
5
0 Ma
HPD for A. torrentium - A. pallipes divergence
HPD for NCD+APU - BAN+SB+CSE divergence
HPD for BAN - SB+CSE divergence
HPD for SB - CSE divergence
HPD 0 10 20 30 40 50 60
a) Arthropod evolution rate [134]
14.18 (10.03 - 19.04)
6.93 (5.20 - 8.96)
3.95 (2.94 - 5.55)
2.46 (1.86 - 3.20)
b) Decapod evolution rate [136]
23.97 (17.37 - 32.54)
11.79 (8.89 - 15.14)
6.71 (5.80 - 8.64)
4.17 (3.11 - 5.33)
c) Substitution rate range [137]
15.55 (11.57 - 20.62)
7.68 (5.90 - 9.76)
4.39 (3.29 - 5.63)
2.72 (2.07 - 3.46)
d) paleo-Tisza - paleo-Danube
13.24 (8.55 - 18.70)
6.13 (4.46 - 8.21)
3.67 (2.46 - 5.22)
2.25 (1.51 - 3.21)
e) Dinaric mountains 12.5 Ma [4]
12.42 (11.43 - 13.37)
5.83 (4.08 - 7.81)
3.27 (2.13 - 4.46)
2.03 (1.35 - 2.80)
f) Dinaric mountains 16 Ma [61]
15.93 (15.01 - 16.95)
7.49 (5.40 - 9.90)
4.23 (2.90 - 5.80)
2.61 (1.78 - 3.54)
g) Apuseni mountains [5]
41.61 (29.94 - 57.68)
19.00 (15.85 - 23.66)
11.41 (7.86 - 15.34)
7.01 (4.91 - 9.47)

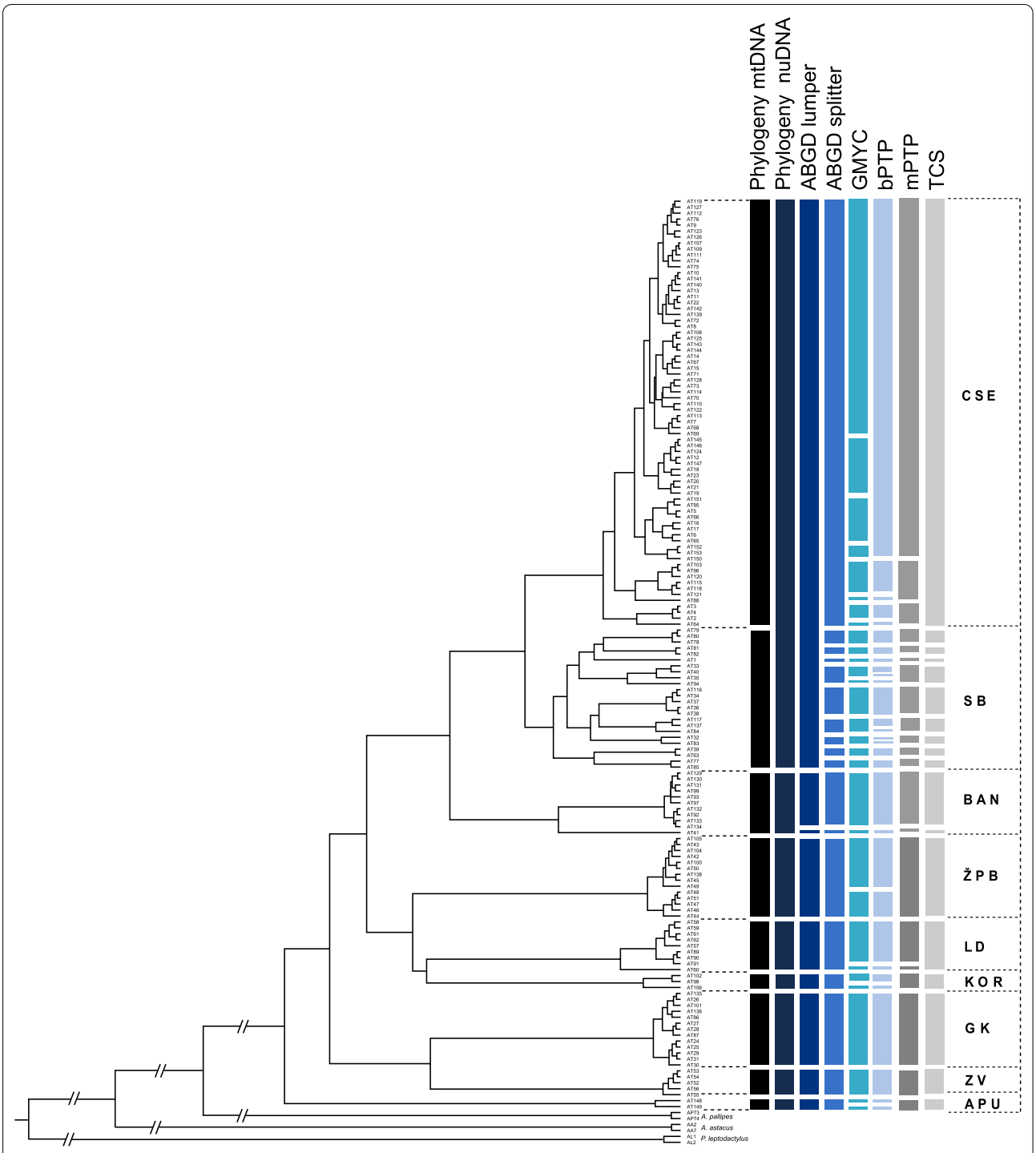


Fig. 4 Species delimitation results visualised as bars on an ultrametric Bayesian maximum clade credibility tree of *A. torrentium COI* gene. Next to the tree phylogroups obtained according to reconstruction of mtDNA and nuDNA are presented. Then follows two partitions of Automatic Barcode Gap Discovery (lumper and splitter) (ABGD [101]), General Mixed Yule Coalescent (GMYC [102]); Bayesian implementation of the Poisson Tree Processes (bPTP [140]); Multi-rate Poisson Tree Processes (mPTP [141]), and Templeton, Crandall and Sing method (TCS [129]). Also, in the last column phylogroups' abbreviations are given: *ZV* Zeleni Vir, *GK* Gorski Kotar, *ŽPB* Žumberak, Plitvice and Bjelolasica, *LD* Lika and Dalmatia, *BAN* Banovina, *SB* southern Balkans, *CSE* central and southeastern Europe, *APU* Apuseni. As outgroups *Austropotamobius pallipes*, *Astacus astacus* and *Pontastacus leptodactylus* were used

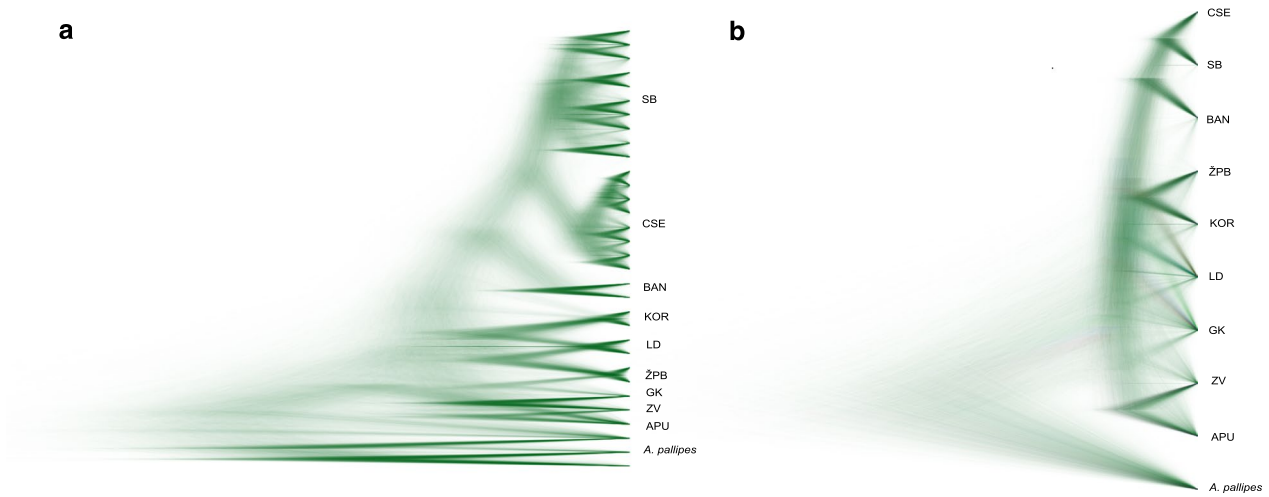


Fig. 5 Species tree of *A. torrentium* inferred with *BEAST and visualised with DENSITREE, based on GMYC species delimitation model (**a**) and multilocus species delimitation (**b**). Next to the trees phylogroups' abbreviations are given: *ZV* Zeleni Vir, *GK* Gorski Kotar, *ŽPB* Žumberak, Plitvice and Bjelolasica, *LD* Lika and Dalmatia, *BAN* Banovina, *SB* southern Balkans, *CSE* central and southeastern Europe, *APU* Apuseni. *Austropotamobius pallipes* was used as an outgroup

The "shape" of the tip of the endopodit of the first gonopod differed among males from different phylogroups ($\chi^2_{7,\ N\ =\ 414} = 151.67$, $P < 0.01$), with a small percentage of males from CSE, ŽPB and KOR possessing a 1st gonopod tip measuring half the length of the gonopod (Additional file 7). The length of the second gonopod tip did not differ among males from different phylogroups ($\chi^2_{7,\ N\ =\ 414} = 7$, $P = 0.42$), with all males, but a small percentage from ŽPB, posses the tip of the 2nd gonopod that was a third of the length of the gonopod (Additional file 7). The length of the exopodite of the second gonopod differed among males from different phylogroups ($\chi^2_{7,\ N\ =\ 414} = 209.82$, $P < 0.01$). Variation within phylogroups was evident, with the exception of males from ZV who all possessed an exopodite that was half the length of the second gonopod (Additional file 7).

Discussion

Phylogenetic structure, genetic diversity and phylogeographic analysis

This study confirmed the complexity of *A. torrentium*'s phylogenetic structure which consists of nine highly divergent and genetically diverse phylogroups [3–5]. An important discovery of this study was the establishment of novel haplotypes distributed in the Kordun region (part of NCD) forming a new Kordun phylogroup (KOR) (Fig. 2a, c). This is the result of the comprehensive sampling of a previously poorly studied region and indicates that future studies could potentially reveal more diversity within stone crayfish. All phylogroups were well supported as deeply divergent monophyletic clades, with the exception of the SB phylogroup that shows a paraphyletic relationship towards CSE phylogroup on both a mitochondrial and nuclear phylogenetic reconstructions. Even though the phylogroups were highly supported, their phylogenetic relationship is best described as unresolved polytomy (Fig. 2a, b). This lack of resolution could have emerged from a rapid and simultaneous divergence of the phylogroups [4, 44, 45].

For both mitochondrial genes, ranges of genetic distances between and within the phylogroups (Additional file 3) were in accordance with previously reported in Trontelj et al. [3], Klobučar et al. [4], Petrusek et al. [27] and Berger et al. [46]. Some of the observed ranges of the *COI* genetic distances between phylogroups were within the range of genetic distances found between *Astacus* species [3, 47], *Austropotamobius* species [3, 48] or Australian Parastacidae crayfish [49]. The lowest value of sequence divergence calculated between SB and CSE demonstrate their genetic similarity. Namely, ancestors of SB phylogroup went through a southern expansion [4, this study], presumably through paleo-Morava, right tributary of the paleo-Danube. This idea is supported by the fact that the oldest SB clades are distributed nowadays in Serbia's Morava tributaries (Fig. 1). Populations of SB sub-clades were probably isolated during glaciations in the numerous micro-refugia in the southern part of the Balkan Peninsula and did not come into the secondary contact post-glacially which resulted in high genetic distances among them, which is similar to the findings of Laggis et al. [50] for the noble crayfish (*Astacus astacus*) and Economidis and Banarescu [51] for freshwater fishes.

Further, the CSE phylogroup experienced a fast and far-reaching range expansion during the post-glacial recolonization and is currently spread over the largest area of *A. torrentium* distribution in Europe (Fig. 1), so consequently this phylogroup shows numerous haplotypes separated by small number of mutational steps (Fig. 2c).

Ranges of pairwise patristic distances found between several phylogroups were equal to, or exceeded the typical crustacean species level distinction value of 0.16 substitutions per site, which point to the existence of cryptic species (Additional file 3). However, our *COI* patristic distances between phylogroups are much lower compared to the ranges of patristic distances found for other cryptic crustacean species that represent deep and old divergent lineages [52–54]. We may conclude that the phylogroups within *A. torrentium* are highly divergent but "young" in evolutionary terms, and in a shifting phase from genetic lineages to species, where additional studies of meristic characteristics and their high intra-phylogroup variation (Additional file 7) failed to provide distinct morphological characters that would unambiguously distinguish genetic lineages into species, or that would, at least, further advocate specific status of those highly divergent genetic lineages. To avoid taxonomic inflation, results that indicate an incongruence between morphologic and genetic data should be considered carefully during delimitation of species, whilst leaving the possibility of cryptic species and/or subspecies being in existence [55]. Contrary to this reasoning, Pârvulescu [28] recently described a new species *Austrobotamobius bihariensis*. If accepted as a new species and considering its position in the phylogenetic tree that is not basal, *A. torrentium* would become paraphyletic.

It is difficult to find a suitable nuclear marker with enough resolution to delimit closely related species amongst others because of slower evolution rate of non-coding nuDNA as previously observed for many different species [56, 57]. In the present study the nuclear *ITS2* marker was found suitable for inferring *A. torrentium* phylogenetic tree. Phylogenies inferred from single nuclear genes often have low resolution and low statistical support of the clades [58], but we achieved better resolution by including gaps through simple indel coding method [59] to render the indels phylogenetic information for these tree search methods. We identified lineages recognised also by mtDNA (GK, ZV, APU, LD, ŽPB, KOR, BAN), except for CSE and SB phylogroups that clustered together (Fig. 2b). This clustering was expected since CSE and SB share close evolutionary history [3, 4]. The relationship among phylogroups was unresolved probably due to the lower genetic variability and slower evolutionary rate of *ITS2*, also demonstrated by low intraspecific genetic distances (Additional file 3). The findings agree with other studies that evaluated the diversity of this nuclear gene in crustaceans [47, 60–62]. Obtained values of genetic distances within and between phylogroups were of intraspecific level compared to the interspecific distances found for other European Astacidae (e.g., 1–5% between sister species *Pontastacus* (*Astacus) pachypus* and *Pontastacus (Astacus) leptodactylus* vs. 0.00–0.79% between phylogroup pairs in this study) [47].

The accumulation of characters that contribute to high genetic diversity and intricate phylogeographic patterns are a consequence of numerous events such as vicariant processes and isolation. This is especially pronounced in organisms of limited dispersal potential such as crayfish [63]. Furthermore, such setups are frequently found in organisms distributed in the karst habitats known for their complex and fragmented (paleo)hydrography [54, 64, 65]. One such region is the Dinaric Karst that possesses a high level of biodiversity, with many endemic species of freshwater surface and subterranean fauna [61, 64, 66–68]. A similar effect is observed in the karstic Apuseni Mountains, which represented a refugium that preserved some endemic and relic species of Gastropoda, Isopoda and Diplopoda species [69].

Evolutionary history

It has been shown that southern Europe and the Balkan Peninsula are regions possessing high plant and animal genetic diversity and are recognised as European biodiversity hotspots [70]. Previous studies of *Austropotamobius torrentium* [3, 4, 27] revealed that its complex evolution was formed from Miocene, to Pleistocene. Distinct evolutionary phylogroups emerged through the intensification of Neotectonic movements and the development of karstification that has a heavily fragmented palaeohydrography, along with periodic climatic shifts during the Pleistocene [3, 4]. Recently, a different perspective on the evolutionary history of *A. torrentium* was proposed [5]. Namely, a new calibration point for species divergence time estimates was used: the separation of the Tisza–Dacia Mega-Unit from the Dinarides that was dated to ~ 16 Ma [5]. According to the authors, this process included "the Tisza–Dacia Mega-Unit (which includes the Apuseni Mountains), which broke away from a larger plate that included the Dinarides and traveled toward the northeast during the Miocene". Apparently, the process caused the split of the APU phylogroup ancestor, trapped on the "floating island", from the rest of *A. torrentium*. Reconnection of the Apuseni Mountains freshwater system with other freshwater systems in the area occured ~ 5 Ma [5]. This approach yielded much earlier separation dates for *A. torrentium* and its sister species *A. pallipes*, ~ 42 Ma (HPD 32–54 Ma), as well as the

split between *Astacus* and *Austropotamobius* ~ 48.8 Ma (HPD 62.4–37.5 Ma), and among all mtDNA phylogroups of *A. torrentium*, compared to previous estimations. Although Pârvulescu et al. [5] brought a new perspective to the geological history of the *A. torrentium* species complex, it lacked congruence with previous research and molecular clock calibrations [3, 4, 61]. Furthermore, contemporary geological literature indicates an ongoing debate about the geodynamic evolution of the Apuseni Mountains during the Neogene [71 and references within]. Recent integrative studies [72 and references within] point to Paleozoic origin of the Apuseni Mountains that were shaped during Mesozoic and strongly influenced by the contact between Tisza and Dacia Mega-Units during Triasic and Early Jurassic, what indicate the dissconection of the Apuseni and the Dinarids since the Triassic period. Shifts in the region that occurred during the Miocene were primarily related to the deformation and bending of the Eastern Carpathians, and not to the tectonic separation of the Apuseni Mountains from the Dinarides [73]. Until the beginning of the Pliocene, there was a continuous sea or brackish lake between the two areas, while continental conditions with the freshwater lake system began at about 4.5 Ma [74]. Keeping in mind this data, the present study attempted to reconcile both geological calibration approaches and bring a new plausible perspective on *A. torrentium* evolutionary history.

The uplift of Dinarides caused the genus *Austropotamobius* to split into *A. pallipes* to the west of Dinarides, and *A. torrentium* on the east [3, 4, 61]. The uplift of the Dinaric and Carpathian Mountains [75] triggered the isolation of the Pannonian basin from the rest of the Paratethys and the formation of the large brackish/ freshwater Lake Pannon [76]. The complete isolation of the Lake Pannon from the inflow of saline water was estimated to ~ 11.7 Ma [77–79] which coincides with the emergence of the paleo-Danube, discharging directly into the Lake Pannon through its large delta [76, 78]. This caused a change in the depth and water salinity of Lake Pannon, turning it into a shallow brackish/freshwater environment [80]. Together with its northern tributaries, such as the paleo-Tisza, the paleo-Danube formed a shelf margin that prograde from the northwest to the southeast [78]. Klobučar et al. [4] assumed that during this period (probably until ~ 6.5 Ma) the populations of *A. torrentium* in the NCD region were isolated from the east by the large, mostly brackish Lake Pannon and, from the north and west, by mountain ridges of uplifting Dinarides and Alps. Crayfish could survive only in the shallow parts of the lake due to the strong freshwater influx from surrounding rivers. Freshwater conditions are corroborated by findings of freshwater molluscs that were widespread in the shallow parts of the lake ~ 4.5 Ma [74, 76]. Magyar et al. [76] also observed that the paleo-Danube delta lobes in the central part of the Pannonian Basin approached the lower flow of the paleo-Tisza River. This was later confirmed as the shelf margins of the paleo-Danube and the paleo-Tisza were observed as coalesced, and their original, almost perpendicular strike, can be detected until 5.3 Ma [78]. We argue that the connection between the paleo-Danube and Paleo-Tisza Rivers could have allowed the ancestor of the current APU phylogroup to colonise the Apuseni Mountains around 5.3 Ma (Fig. 3).

The paleo-Tisza–paleo-Danube connection coincides with the end of the Messinian Salinity Crisis (MSC) that lasted from ~ 5.96 Ma until ~ 5.33 Ma [81, 82]. The MSC, besides having a strong influence on hydrology, caused increased temperature, aridity and evaporation in the Northern Hemisphere [83]. It is also speculated that the MSC caused a lowering of the water level of the Lake Pannon, at least in its northern part [84]. Thus, *A. torrentium* colonisation of the Apuseni Mountains would be possible at the end of the MSC (~ 5.3 Ma), throughout the northern margin of Lake Pannon (Fig. 1), which is indicated by the lowest genetic distances between ZV/GK phylogroups and APU phylogroup, previously also observed by Pârvulescu et al. [5], and confirmed in this study. Also, during MSC, the sea-level dropped for 50–200 m in the Dacian Basin connected to the Black Sea, situated to the east from Lake Pannon [85]. It is assumed that during the MSC, paleo-Danube ran across the south Carpathians and overflowed from the freshwater Pannonian into the saline Dacian Basin [86]. Therefore, we consider that the northern dispersal route of *A. torrentium* towards the Apuseni region is equally, if not more likely than the previously proposed scenario [5]. It is possible that numerous populations existed in the northern areas, and on the northern dispersal route, but did not survive the adverse climatic conditions during glaciations unlike populations in Apuseni that survived in refugia in karst, similar to the NCD populations. The remnant populations exhibited limited or non post-glacial range expansion and contact indicating the existence of multiple 'refugia within refugia' [87], as previously suggested by Klobučar et al. [4].

Formation of the Danube River basin and its drainage network, as we know it today, with its right-sided tributaries (e.g., Velika Morava and Sava), is estimated to Pliocene [78, 88–90]. This, along with the cold climatic conditions [91–93] which are favourable for *A. torrentium* [2] could have allowed its south-eastward spreading. Estimated divergence times between BAN (the most eastern NCD phylogroup) and SB + CSE coincide with this period (Fig. 3 and Additional file 4), which also indicates their closer genetic relatedness compared to other phylogroups (Figs. 1, 2). Further, our results

indicate that the divergence between CSE and SB coincide with the beginning of glaciations that started in the Northern Hemisphere during the late Pliocene-early Pleistocene [89, 94] and continued with CSE spreading northward through the Danube River drainage showing a post-glacial leading edge effect as previously suggested in Klobučar et al. [4]. Similar scenarios of post-glacial (re) colonisation of Europe from the southern refugia were recorded for numerous aquatic and terrestrial taxa [51, 95, 96].

Species delimitation

Molecular species delimitation proved to be a valuable tool for the species identification as a stand-alone method or as part of an integrative taxonomic approach [97]. Contrary to this, a large number of papers reported taxa oversplitting, overlumping or the incongruence among implemented methods [36, 37, 55, 65, 98]. Furthermore, the MOTUs delimited by the analyses of mtDNA represent a hypothesis that should be considered with caution even if well-supported [31]. Species delimitation conducted on our dataset showed a high degree of discordance among methods, with a majority suggesting an unrealistically high number of MOTUs/potential species (9–30) within *A. torrentium* (Fig. 4, Additional file 5). While some of these MOTUs might be the result of revealing previously undescribed diversity, others may be the result of discovering isolated populations currently undergoing speciation [37, 99]. However, in many cases, it is obvious that the analyses oversplit taxa, because the intra-specific genetic divergence for majority of these identified MOTUs is too low to currently consider them as distinct species (Additional file 3). Relatively high genetic divergence (Figs. 2, 5, Additional file 3) indicates that identified MOTUs are in the process of splitting and may evolve into different species in the future [100]. The single locus-based species delimitation approaches, as ABGD, GMYC, bPTP, are known to oversplit taxa and their performance is sensitive to many factors such as higher substitution rates, the number of species included, uneven sampling, varying population sizes, level of gene flow, the number of singletons in the input trees and unresolved nodes [36, 37, 42, 97, 101–105]. Further, species delimitation results inferred on single locus data are known to reflect locus variability, as more variable loci led to a higher number of proposed MOTUs [65]. However, the majority of these delimitations are taxonomically uninformative. Furthermore, most of these methods have been designed for species-rich data sets [32, 106]. Performance of species delimitation approaches can also be affected by the ratio of population sizes to species divergence times [97]. Failure to sample intermediate haplotypes could also be the reason causing the oversplit in the phylogroup CSE, BAN and especially SB due to incomplete geographical coverage, so further sampling could help resolving this oversplitting scenario. The higher number of MOTUs obtained by the tree-based analyses could be a consequence of the fact that those methods tend to overestimate the number of species and they actually reflect genetic structure of the data showing the population structure within the species [107]. This could be the reason why BFD species delimitation recovered GMYC as the most appropriate model for our data set (Additional file 6), reflecting prominent substructure within *A. torrentium*.

Obtained results again suggested the presence of deep divergence within *A. torrentium*, harbouring monophyletic and geographically isolated phylogroups with their own evolutionary trajectories. It is important to point out that strong divergence is not necessarily dependent on the intrinsic characteristics of a species, but could also represent the landscape dynamics of a species habitat [108]. Dinaric karst with fragmented palaeohydrography created important biogeographical barriers that led to diversification events and strong phylogeographic structure in many taxa on the Balkan Peninsula. Currently observed distribution patterns and diversity of freshwater biota are often connected with the geomorphological features of this region and its geo-climatic history [70, 109, 110].

Meristics

The meristics ([22], this study Additional file 7) and geometric morphometrics [23] could separate crayfish belonging to different phylogroups to some extent, but variation in studied characters, within groups, was evident. Obtained results demonstrated freshwater crayfish plasticity and high intraspecific morphological variation which reflects both the environmental influence and genetic background. Our research on the morphology of *A. torrentium* has not indicated sufficiently stable diagnostic characters that would be helpful in distinguishing crayfish from different phylogroups. Hence we may conclude that morphological traits are not conserved among phylogenetic lineages. The lack of denticulation on the lower edge of antennal scale (antennal exopodite) was pointed out by Pârvulescu [28] as among the most important distinguishing morphological feature to separate newly described *A. bihariensis* from *A. torrentium* belonging to CSE phylogroup and analysed in his study. Contrary to this, in our study of the largest data set analysed so far and including crayfish from all phylogroups but APU, we found this character variable; as the absence of denticulation was observed in all phylogroups (Additional file 7). Accordingly, neither can we use this character, nor any other tested characters reliably in the

description of a new species. At the moment, based on the obtained results, we may conclude that observed mtDNA/nuDNA phylogroups present cryptic subspecies [111–114] that should be treated as separate ESUs and, especially ones belonging to the NCD region, should have conservation priority.

Conservation

Our multigene phylogenetic analyses as well as species delimitation methods revealed that the genetic diversity and evolutionary history of *A. torrentium* is complex and intricate with an everlasting need for further studying (Figs. 1, 2, 3). The geoclimatic processes have left distinguishing signatures in the current distribution and genetics of *A. torrentium* giving rise to highly divergent phylogroups with their own independent evolution. Discovered phylogroups play a fundamental role in the long-term survival and evolution dynamics of *A. torrentium*. Considering that *A. torrentium* shows a decreasing population trend and is listed as vulnerable species in Croatia [13], one of the most important aims of our study was to provide a baseline for the conservation and management of unique genetic variability found within this species through the identification of evolutionary significant units (ESUs). Recognition of ESUs facilitates conservation planning and management without the necessity of formally naming new species or elevating taxa to species level [63]. Taxonomic revision with the description of new species must be a thoughtful process, which considers the whole genus *Austropotamobius* and not only the taxons/groups within *A. torrentium* species-complex, so the number of species would not be over- or underestimated. Due to the incongruence between implemented approaches, including lack of morphological characters associated with phylogroups that would be conserved among them (Additional file 7), we were conservative in the inferences drawn from the analyses, and declared phylogroups recovered both on mitochondrial and nuclear DNA as cryptic subspecies and distinct ESUs (ESU1 = BAN, ESU2 = CSE, ESU3 = GK, ESU4 = KOR, ESU5 = LD, ESU6 = SB, ESU7 = ZV, ESU8 = ŽPB, ESU9 = APU) (Additional file 8).

Geographically and genetically isolated phylogroups represent the evolutionary legacy of *A. torrentium* which is highly relevant for conservation due to their mostly small distribution ranges and evolutionary distinctness. Since human mediated translocation and restocking of crayfish for repopulation are encouraged with the aim of increasing the genetic diversity of endangered populations [115], future conservation programs should consider conducting translocations and repopulations only within the same ESU [46, 116–118].

Furthermore, one of the fundamental issues in the conservation of freshwater species is in maintaining genetic diversity by defining the degree of connectivity between populations [119] and finding a balance between outbreeding and inbreeding depression that represent potential threat while restocking/repopulating, so future research should be focused on the study of the genetic structure of phylogroups. Population genetic analyses based on microsatellites can contribute to the understanding of the degree of genetic variation within and among populations, potentially identify management units (MUs) and source populations for future introductions, as well as to reveal recent evolutionary changes and possible population-level hybridisation events through secondary contacts [46, 50, 117, 118, 120]. In addition, cytogenetic research, next generation sequencing and genomic approaches may advance understanding of phylogenetic relationships and taxonomic status of mt and nuDNA phylogroups which, without doubt, play a pivotal role in long term future evolution of *A. torrentium*.

Conclusions

Results corroborate high genetic diversity within *A. torrentium* preserved in divergent phylogenetic groups.

Because there was no congruence between implemented species delimitation approaches, and we lack establishing morphological characters conserved within lineages, we conclude that established phylogroups, recovered both on mitochondrial and nuclear DNA, are cryptic subspecies and distinct evolutionary significant units that present evolutionary legacy of *A. torrentium* and are highly relevant for conservation due to their mostly small distribution ranges and evolutionary distinctness.

Methods

To accomplish our aims, we applied a multi-gene molecular approach in the phylogenetic reconstructions and several methods of species delimitation analyses, as well as divergence time estimates using both molecular evolutionary rates and geological/hydrological calibration.

Sampling, DNA extraction, gene amplification and sequencing

Total of 279 crayfish from 63 locations from Croatia, Slovenia and Republic of North Macedonia were sampled and analysed (Fig. 1, Additional file 1). One pereiopod from each individual was sampled and stored in 96% ethanol at 4 °C until DNA isolation. Sampling was conducted in accordance with ethical standards and all required permissions were obtained from Ministry of Environmental Protection and Energy of the Republic of Croatia. The specimen collections in Slovenia and

Republic of North Macedonia were conducted with permissions of local authorities.

Genomic DNA was extracted from muscle tissue using GenElute Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich, St. Louis, MO) following the manufacturer's protocol, and stored in a freezer until PCR. Mitochondrial *COI* and *16S* rRNA, and nuclear *ITS2* genes were amplified and sequenced, with details provided in the Additional file 9.

Sequence data and phylogenetic analyses

Sequences were edited using SEQUENCHER 5.4.6 (Gene Codes Corporation, Ann Arbor, MI USA) and aligned using MAFFT [121]. The chromatograms were checked manually for base pair ambiguities and indications for nuclear–mitochondrial pseudogenes (numts) as recommended by Buhay [122]. The *COI* alignment did not contain any length variants or ambiguous sites, while the sequences of the *16S* rRNA cointained length variation. The *ITS2* region contained length variations and nine ambiguous sites. The final alignments were 582 and 476 bp long for *COI* and *16S* rRNA, respectively, while *ITS2* region was 1102 bp long. Number of haplotypes, number of polymorphic sites, and number of parsimony informative sites for each gene alignment was calculated using MEGA X [123] and DnaSP 6.12.03 [124].

The phylogenetic analyses encompassed a total of 1114 *16S* rRNA and *COI* genes sequences of which 642 mtDNA sequences (431 *COI* and 211 *16S* rRNA) were downloaded from GenBank, and 472 sequences (198 *COI* and 274 *16S*) were obtained in this study (Additional file 1). The sequences were collapsed to unique haplotypes with DnaSP 6.12.03 [124]. New haplotypes from this study were deposited in the GenBank and will be publicly available after manuscript acceptance. Phylogenetic analyses were performed on two data sets: the first data set consisted of concatenated *COI* and *16S* rRNA sequences, and the second data set included only *ITS2* sequences. Prior to concatenation, the incongruence length difference test [125] as implemented in PAUP* 4.0a164 [126] was applied to assess congruence between two mitochondrial genes. There was no significant heterogeneity amongst the partitions ($P = 0.78$), and the final alignment for concatenated mitochondrial sequences was 1058 bp long. *Austropotamobius pallipes* was chosen as an outgroup (GenBank accession numbers for *COI*: KX369673, KX369674; and *16S* rRNA: KX370093, KX370094). Phylogenetic relationships were reconstructed using three different optimality criteria: maximum parsimony (MP), maximum likelihood (ML) and Bayesian analysis (BA), with settings provided in the Additional file 9. Nodes in the phylogenetic trees with bootstrap values $P \geq 75$ in ML and MP, and posterior probabilities (pp) values ≥ 0.95 in BA were considered supported.

Haplotype networks and genetic diversity

Median-joining (MJ) network approach [127] was used to visualise intraspecific evolutionary relationships and haplotype relatedness within *A. torrentium* on concatenated mitochondrial data set using the PopArt [128]. Phylogenetic network using statistical parsimony was constructed for the *COI* gene using the TCS 1.21 software [129] and visualised using tcsBU [130].

Pairwise comparison of uncorrected sequence divergences (p-distances) and corrected Kimura's two-parameter distances (K2P) between and within phylogroups for *COI*, *16S* rRNA and *ITS2* was performed in MEGA X [123]. The pairwise patristic distances were computed from the ML tree using the program PATRISTIC v1.0 [131] with the aim of comparing obtained values with the proposed crustacean species delimitation threshold of 0.16 substitutions per site in the mitochondrial *COI* gene [39].

Time of divergence

In order to estimate divergence times among mtDNA phylogroups, concatenated data set (*COI* and *16S* rRNA) was used in the Bayesian statistical framework implemented in BEAST 2.5.2 [132]. The analyses were run on the Cipres Science Gateway [133]. For this purpose, seven different calibration approaches were employed (three molecular and four geological). Molecular clock calibrations were based on the arthropod substitution rate of 2.3% pairwise sequence divergence (0.0115 subs/s/Ma/l) [134, 135], and the decapod substitution rate of 1.4% pairwise sequence divergence (0.007 subs/s/Ma/l) [136] for *COI* partition along with an estimated molecular clock for the *16S* rRNA partition of mtDNA data set. In the third approch, we implemented substitution rates according to Schubart et al. [137] with setting the meanRate prior as a uniform distribution between 0.0083–0.01165 subs/s/Ma/l for *COI* and 0.00325–0.0044 subs/s/Ma/l for *16S* rRNA. Following Klobučar et al. [4] we used mid-points of these intervals (0.0099 for *COI* and 0.0038 for *16S* rRNA) as an ucld.mean prior. For the geological calibrations of the molecular clock, we used three previously described approaches. Firstly, we used the episode of intense uplifting of the Dinarids [138] that caused the split between *A. pallipes* and *A. torrentium* estimated to ~ 12.5 Ma and ~ 16 Ma [for details see [4] and [61]]. TreeModel prior distribution was set to normal, with a mean of 12.5 Ma or 16 Ma and a standard deviation of 0.5. The second approach was based on the tectonic separation of the Apuseni Mountains (Tisza–Dacia microplate) from Dinarides that, according to Pârvulescu et al.

[5], took place 16 Ma and it was used as a calibration point for splitting between APU and other NCD phylogroups. TreeModel prior distribution was set to normal, with a mean of 16 Ma and standard deviation of 0.5. For the fourth geological calibration point, we used the occurence of the fluvial connection between the paleo-Danube River and paleo-Tisza River systems that took place around 5.3 Ma [78]. That event could have enabled the colonisation of nowadays north-eastern areal of *A. torrentium* distribution. TreeModel prior distribution was set to normal, with a mean of 5.3 Ma and standard deviation of 0.5. Divergence time estimates were calculated using relaxed molecular clock with log normal distribution, birth–death model of speciation, independent substitution models assigned to mtDNA genes, and run for 150,000,000 generations with details provided in Additional file 9.

Species delimitation and validation

Application of multiple species delimitation approaches is generally preferable comparing to reliance on a single method [55]. Several methods of single-locus species delimitation were conducted using: the Automatic Barcode Gap Discovery (ABGD) method of Puillandre et al. [101], the General Mixed Yule Coalescent (GMYC, single threshold algorithm) method of Pons et al. [139], the Bayesian implementation of the Poisson Tree Processes (bPTP) method of Zhang et al. [140] and multi-rate Poisson Tree Process (mPTP) method of Kapli et al. [141]. Molecular species delimitation methods generate a certain number of MOTUs and were applied only to the *COI* dataset due to the largest number of available sequences and higher variation levels compering to other markers (e.g. *16S* rRNA and *ITS2*).

The ABGD, genetic pairwise distances based method, was performed using the online version of the program [101] with default parameters and Kimura 2 parameter (K2P) model. Tree-based methods, such as GMYC, bPTP and mPTP, employ a phylogenetic tree as input for the analysis. The GMYC method was performed using the time-calibrated ultrametric tree based on *COI* gene obtained using BEAST 2.5.2, and was run using the SPLITS package [142] in R. The same input tree was used for both bPTP and mPTP methods [140, 141]. The details regarding reconstruction of input tree for species delimitation analyses are reported in the supplementary data (Additional file 9). Boundaries of potential species were also inferred by using the statistical parsimony network reconstruction software TCS [129].

We estimated *A. torrentium* single-locus species trees using *BEAST v.2.5.2 [143] with the same parameters as for species delimitation. The *COI* haplotypes were assigned into different species trees topologies according to the results of phylogeny and species delimitation analyses (ABGD lumper and splitter partitions strategy—in the further text ABGD lumper and splitter [144], TCS, GMYC, bPTP, mPTP), as well as the assumption that all crayfish belong to the same species. Bayes factor delimitation (BFD) approach was applied to compare candidate *BEAST species tree models based on Bayes factors (BF) [145]. Nested sampling analysis [146] was used for the marginal likelihood estimation (MLE) of each species tree [147] in order to calculate the BFs between two models, with details in Additional file 9. The multi-locus species tree was estimated using *BEAST on data set comprising three loci (*COI*, *16S* rRNA, *ITS2*) sampled from 38 individuals representing nine phylogroups of the stone crayfish. We imported three aligments along with additional file with recoded gaps as matrix of binary characters. *BEAST co-estimated three gene trees embedded in a shared species tree and the analysis was run for 150,000,000 generations using the birth–death tree prior and a relaxed molecular clock with an uncorrelated log-normal distribution. Previously established substitution models were assigned to each datasets, with *A. pallipes* as outgroup. The substitution rate for *COI* and *16S* rRNA were set according to Schubart et al. [138] and estimated rate for *ITS2*. Gene trees for mitochondrial genes were linked, while nuclear unlinked. Species tree was visualised in DensiTree v.2.2.6 [199].

Meristics

Meristic characteristics were examined under a magnifying glass by the same researcher. In total, 749 crayfish collected during the period of the last 20 years, were examinded and 735 were included into analyses, covering all phylogroups except APU that was previously analysed by Pârvulescu [28] (Additional file 1, Additional file 9).

We recorded: number of spines on the ventral side of the merus of the third maxilliped, presence and pronunciation of rostral crista, and absence/presence and type of denticulation (spines or tubercles) on the lower surface of the antennal exopod. Additionaly, in males, shape of the tip of the endopodit of the first and the second gonopod, and the length of the exopodit of the second gonopod were noted. All bilateral characters were recorded for the right side of the body, because previous studies showed that there are no significant differences in their distribution on the two body sides [22]. All details on studied meristic characteristics are given in, Additional file 7, Additional file 9.

Differences in the recorded meristic characters (ordinal variables) between phylogroups were tested by nonparametric Kruskal–Wallis ANOVA and chi-square test in STATISTICA 13.5.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12862-020-01709-1.

Additional file 1: List of *Austropotamobius torrentium*, as well as *Austropotamobius pallipes*, *Astacus astacus* and *Pontastacus leptodactylus* sequences used in the analyses. The information comprises the name of the country and sampling site, mtDNA phylogroup, *COI* haplotype ID and their GenBank Accession numbers, *16S* haplotype ID and their GenBank Accession numbers, concatenated sequences ID, *ITS2* ID and their GenBank Accession numbers and bibliographic references.

Additional file 2: TCS phylogenetic network based on *Austropotamobius torrentium COI* gene.

Additional file 3: Estimates of evolutionary divergence over sequence pairs of *Austropotamobius torrentium*. Range values of within and between genetic distances of nine *A. torrentium* mtDNA phylogroups (ZV—Zeleni Vir; GK—Gorski Kotar; ŽPB—Žumberak, Plitvice and Bjelolasica; LD—Lika and Dalmatia; BAN—Banovina; SB—southern Balkans; CSE—central and southeastern Europe; APU—Apuseni) for *COI*, *16S* rRNA and *ITS2*. Observed ranges of pairwise patristic distances for *COI* within and between the phylogroups measured on ML tree are also provided.

Additional file 4: Estimation of divergence times based on *Austropotamobius torrentium* mitochondrial data set using three molecular clock and four geological calibrations.

Additional file 5: Results of species delimitation analyses performed on *Austropotamobius torrentium COI* dataset applying different methods (ABGD (lumper, splitter), TCS, bPTP, mPTP and GMYC).

Additional file 6: Results of Bayes factor species delimitation (BFD) based *Austropotamobius torrentium COI* dataset.

Additional file 7: Results of *Austropotamobius torrentium* meristic characteristics analyses.

Additional file 8: Map of proposed evolutionary significant units (ESUs)/cryptic subspecies for *Austropotamobius torrentium*. The map depicted in figure was produced in ArcGIS 10.3 program package and finished in the program package FreeHand MXa by authors of this study.

Additional file 9: Material and methods extended. The detailed information about gene amplification and sequencing, phylogenetic reconstruction, time of divergence estimates, species delimitation and validation, and analyses of meristic characteristics. Additional file also includes list of used references.

Abbreviations

ABGD: Automatic barcode gap discovery; ANOVA: Analysis of variance; BA: Bayesian analysis; BEAST: Bayesian evolutionary analysis sampling trees; BF: Bayes factors; BFD: Bayes factor delimitation; BIC: Bayesian information criterion; bPTP: Bayesian implementation of the Poisson tree processes; BP: Base pair; BS: Bootstrap; *COI*: Cytochrome *c* oxidase subunit I; CSE: Central and South-eastern Europe; SB: Southern Balkans; BAN: Banovina; GK: Gorski Kotar; LD: Lika and Dalmatia; ZV: Zeleni Vir; ŽPB: ŽUmberak, Plitvice and Bjelolasica; APU: Apuseni Mountain; KOR: Kordun; ESS: Effective sample size; ESU: Evolutionary significant unit; GMYC: General mixed yule coalescent; HKY + G: Hasegawa–Kishino–Yano model with gamma distribution; HKY + I + G: Hasegawa–Kishino–Yano model with invariabe sites and gamma distribution; HKY + F + I + G: Hasegawa–Kishino–Yano model with empirical base frequencies, invariabe sites and gamma distribution; HPD: Highest posterior density; ICS: Indigenous crayfish species; *ITS2*: Second internal transcribed spacer; IUCN: International Union for Conservation of Nature; JC: Jukes–Cantor model; K2P: Kimura 2 parameter model; K3Pu + F + I + G: Kimura 3-parameter with unequal, empirical base frequencies, invariabe sites and gamma distribution; Ma: Million years ago; MCMC: Markov Chain Monte Carlo; MJ: Median-joining; ML: Maximum likelihood; MLE: Marginal likelihood estimation; MMCM: Metropolis-coupled Monte Carlo Markov chains; MOTU: Molecular operational taxonomic unit; MP: Maximum parsimony; mPTP: Multi-rate Poisson tree process; MSC: Messinian salinity crisis; mtDNA: Mitochondrial deoxyribonucleic acid; MU: Management unit; NCD: Northern-central dinarides; nuDNA: Nuclear deoxyribonucleic acid; PCR: Polymerase chain reaction; PP: Posterior probabilities; TCS: Templeton, Crandall and Sing method.

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Authors' contributions

IM, LL, LB, LJLB and MP conceived and designed the study. LL, LB, LJLB, MJ, GK, MJ, VS, JH and IM collected crayfish in the field. LL, LB, LJLB, MJ and IM conducted laboratory work; LL and IM conducted meristic study; LL, LB, LJLB and MP processed and analysed molecular data. LL, LB, LJLB and IM drafted the initial version of the manuscript. All authors read, edited, enhanced, and approved the final version of the manuscript.

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Availability of data and materials

Materials (samples) used in this study are stored in the astacological collection at the Department of Biology, University of Zagreb, while the DNA sequence data supporting the results of this article are available in the GenBank® repository (https://www.ncbi.nlm.nih.gov) under accession numbers (Additional file 1).

Ethics approval and consent to participate

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in this study were in accordance with the ethical standards of the institution and all required permissions were obtained from Ministry of Environmental Protection and Energy of the Republic of Croatia (UP/I-612–07/18–48/148) and the Enviromental Agency of the Republic of Slovenia (35601–150/2006–6 and 35601–135/2010–9).

Consent to publish

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Publisher's Note

Znanstveni rad 7

Climate change threatens unique genetic diversity within the Balkan biodiversity hotspot - the case of the endangered crayfish *Austropotamobius torrentium*

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Abstract

Climate change imperils the persistence of many species essential for ecosystem functioning and services, including European freshwater crayfish. The stone crayfish, *Austropotamobius torrentium*, is globally endangered due to anthropogenic pressure on its habitats, climate change and the invasive crayfish species, particularly the signal crayfish (*Pacifastacus leniusculus*). Aiming to guide *A. torrentium* conservation within the hotspot of its genetic diversity in the western Balkan Peninsula, we combined population genetics and species distribution models (SDMs) to reveal the impact of climate change and the invasive *P. leniusculus*. Population genetic analyses based on newly developed microsatellites revealed moderate within-population genetic diversity and high differentiation among populations, reflecting isolated populations with limited gene flow. Alongside strong genetic structuring, we discovered high level of inbreeding coefficient indicating homozygote excess within the majority of populations. The SDMs results predicted substantial reductions of suitable habitats for *A. torrentium* by 2070; 80% of its currently suitable habitat is predicted to be lost under high-warming climate change scenario. Obtained results indicated that 44% of populations with high and/or unique genetic diversity, including three highly divergent and geographically restricted evolutionary lineages, are located in the areas predicted to become unsuitable in the future, highlighting their vulnerability to extinction. Further, SDMs revealed considerable decrease of future habitat suitability for *P. leniusculus*, suggesting that climate change represents greater threat to *A. torrentium*. Our study highlights the importance of conserving remnant *A. torrentium* populations through assisted migration and population mixing that can help populations overcome the risks of inbreeding and maladaptation, and thus enabling *A. torrentium* to withstand the ongoing climate change.

Keywords

stone crayfish, population genetics, species distribution modelling, ecological niche modelling, habitat suitability, conservation

Founding

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Introduction

Climate change is recognised as one of the leading threats to freshwater biodiversity (Markovic et al., 2014; Dudgeon, 2019). For freshwater organisms, such as crayfish, ongoing climate change imperil populations through rising water temperatures and altered river flow regimes due to changes in precipitation patterns and the frequency of extreme weather events (Moss et al., 2009). In addition, climate change accelerates the potential spread of non-indigenous crayfish species (NICS) and their associated pathogens (Rahel and Olden, 2008; Hulme, 2017). Narrow distribution ranges limited by watershed boundaries and preferences for higher altitudes or habitats where their dispersal is limited enhance the indigenous crayfish species (ICS) vulnerability to both to climate change and NICS. Given the insular nature of freshwater habitats, ICS will have limited ability to shift their distribution towards climate suitable habitats in the future due to their low dispersal capacity and geographic barriers (Strayer and Dudgeon, 2010). In addition, life history characteristics that include low reproductive output and long-life span coupled with small population sizes, limit the ability of ICS populations to respond to environmental change via local adaptation (Foden et al., 2013). In particular, recent global assessment of freshwater crayfish vulnerability to climate change has shown that 36% of species have low adaptive capacity and 87% can be considered as highly sensitive to climate change (Hossain et al., 2018). The former study also highlighted that four out of five indigenous European freshwater crayfish are vulnerable to climate change. Consequently, effective conservation plans are challenging tasks and are urgently needed for ensuring the persistence and long-term survival of highly imperilled crayfish species under multiple threats.

The stone crayfish, *Austropotamobius torrentium* (Schrank, 1803), is often emphasized as one of the most threatened freshwater crayfish species in Europe (Chucholl and Schrimpf, 2016; Maguire et al., 2018; Pârvulescu et al., 2020). It is indigenous to central and south-eastern Europe (Kouba et al., 2014), with the centre of its genetic diversity located in the western Balkan Peninsula (Trontelj et al., 2005, Klobučar et al., 2013; Lovrenčić et al., 2020). This cold-adapted species inhabits small pristine waterbodies at higher altitudes with great hydro-morphological heterogeneity (Streissl and Hödl, 2002; Pöckl and Streissl, 2005). *Austropotamobius torrentium* is a keystone species and an ecosystem engineer that influences both species composition and trophic pathways in freshwater habitats (Weinländer and Füreder, 2016). Due to its small size, it was never of economic importance and there are no records of its translocations around the county, therefore we believe that its distribution and genetic structure in Croatia is mostly natural.

Over the last decades, dramatic population declines and local extinctions have been reported throughout its geographic range, including its diversity hotspot in Croatia (Maguire et al., 2011, 2018; Chucholl and Schrimpf 2016; Berger et al., 2018; Pârvulescu et al., 2020). Observed declines are likely driven by several co-occurring threating processes, including anthropogenic impacts on its freshwater habitats, climate change, and the introduction of NICS (Richman et al., 2015; Jussila et al., 2021). Even though declines are observed, the conservation status of *A. torrentium* remains unresolved as it is listed as “data deficient” on the global IUCN Red List (Füreder et al., 2010). It is protected by international (Annex III of the Bern Convention, Annex II and V of the EU Habitats Directive (92/43/EEC)) and European national legislations. Following EU Habitats Directive, A. *torrentium* is considered to be a priority species for which each member state needs to designate specific Natura 2000 sites (Special Areas of Conservation, SACs). In Croatia, it is classified as vulnerable due to the decreasing populations trends (Gottstein et al., 2011), and is protected by national legislation (NN 80/13, NN 144/2013).

Large-scale genetic studies revealed that *A. torrentium* harbours high genetic diversity characterised by nine geographically and genetically isolated mitochondrial lineages (Lovrenčić et al., 2020). Seven of these divergent lineages are distributed in the western Balkan Peninsula, and six of them have highly restricted distributions

within the northern-central Dinaric region in Croatia (Trontelj et al., 2005; Klobučar et al., 2013; Lovrenčić et al., 2020). Each of these mitochondrial lineages is recognised as distinct evolutionary significant unit (ESU) requiring conservation prioritisation due to their genetic distinctiveness and limited distributions (Coates et al., 2018; Lovrenčić et al., 2020). In contrast, more widespread central European populations of *A. torrentium* belong to a single lineage with low haplotype diversity (Schubart and Huber, 2006; Petrusek et al., 2017; Berger et al., 2018; Pârvulescu et al., 2020).

In the context of declining populations, small population size can result in the loss of genetic variation and the adaptive potential of the species overall, and is one of the core issues in conservation biology. Both inbreeding and drift reduce genetic diversity in small populations, which limits the capacities of populations to adapt to rapidly changing environments, lowers fertility, decreases disease resistance and increases the risk of local population extinction (Frankham et al., 1996 and 2005; Lande et al., 1996; Reed et al., 2003; Smith et al., 2006). Since loss of intraspecific genetic diversity associated with declining population trends directly impacts species' long-term survival, protection of genetically unique lineages of *A. torrentium* is a priority for maintaining the overall adaptive capacity of this species (Frankham et al., 2002; Lovrenčić et al., 2020). Furthermore, conservation planning requires a detailed understanding of intraspecific genetic diversity both within and among populations. In this regard, microsatellite markers are recognised as a valuable tool in studying population genetics and conservation biology of imperilled species (Abdul-Muneer, 2014). However, due to relatively low number of usable loci available from previous studies of *A. torrentium* genetic diversity (Iorgu et al., 2011; Vorburger et al., 2014; Berger et al., 2018; Pârvulescu et al., 2020), the need for the development of new microsatellite loci for conservation genetic purposes of *A. torrentium* was identified as a priority.

NICS and their pathogens are one of the major threats to ICS (Holdich et al., 2009). NICS represent major global change drivers that can alter biodiversity and ecosystem functioning (Linders et al., 2019) due to transforming the structure and species composition of ecosystems by dominating and supressing or excluding native species (Souty-Grosset et al., 2016). Furthermore, NICS displace ICS through transmission of deadly diseases such as crayfish plague caused by the pathogenic oomycete *Aphanomyces astaci* to which they are relatively resistant (Jussila et al., 2021). This alien pathogen is known to cause devastating mass mortalities and numerous population collapses in all native European crayfish species (Grandjean et al., 2017; Jussila et al., 2021). Three NICS are currently present in Croatia: the marbled crayfish *Procambarus virginalis*, the spiny-cheek crayfish *Faxonius limosus* and the signal crayfish *Pacifastacus leniusculus* (Maguire et al., 2018). With the exception of *P. virginalis,* with a distribution limited to one local population, *P. leniusculus* and *F. limosus* have expanded their ranges over the last two decades, and continue to spread successfully in Croatian freshwater ecosystems (Maguire et al., 2018; Dragičević et al., 2020). Moreover, apart from different competitive advantages, NICS success may be enhanced by a superior ability to adapt to climate change (Linders et al., 2019).

The most problematic invasive competitor for the *A. torrentium* is *P. leniusculus*, one of the most successful crayfish invaders in Europe (Ercoli et al., 2021), and most widespread NICS in Croatia (Dragičević et al., 2020). It is distributed in the continental part of the country, including the Mura, Drava, and Korana Rivers, which together with their tributaries host three ICS (*Astacus astacus*, *A. torrentium* and *Pontastacus leptodactylus*) (Maguire et al., 2018; Dragičević et al., 2020). In other countries, *P. leniusculus* also poses a high threat to other ICS (Chucholl, 2016; Préau et al., 2019; Jussila et al., 2021). For example, in Germany, among the six invasive NICS, *P. leniusculus* was found to have the highest habitat overlap with the local ICS, including *A. torrentium* (Chucholl, 2016). Likewise, the signal crayfish has been shown to have significant impact on the populations of the endangered *Austropotamobius pallipes* in France due to equivalent niches of these two species (Préau et al., 2019), however, up to our knowledge, no similar information on niche quantification exists for *P. leniusculus* in relation to *A. torrentium.*

Species distribution models (SDMs) have become increasingly useful tools to assess the impact of climate change on species of conservation concern (Guisan and Thuiller, 2005; Guisan et al., 2013; Araújo et al., 2019; Casazza et al., 2021; Zhang et al., 2020). They have become an important component of conservation planning as they link science to policy and decision making processes (Araújo et al., 2019; Randin et al., 2020; Taheri et al., 2021; Blair et al., 2022). For example, SDMs can be useful to identify areas of habitat suitability where populations can be maintained in the future and can thus serve as climate refugia ("ark sites"), as well as to identify sites or drainages that are at risk from invasive species (Jiménez-Valverde et al., 2011; Briscoe et al., 2016) or potential alien invasive species-free refugia for endangered species (Johovic et al. 2020). Also, SDMs represents a valuable tool for selection of suitable habitats for the purpose of assisted migration or translocation of species of conservation significance (Guisan et al., 2013). Several studies, using SDMs for crayfish conservation, have already emphasised the vulnerability of European crayfish by predicting the extreme loss of suitable habitats due to climate change and increased interactions with NICS (Capinha et al., 2013; Ghia et al., 2013; Chucholl et al., 2016 and 2017; Piyapong et al., 2020; Préau et al., 2019; Lovrenčić et al., 2022).

Since maintenance of genetic diversity of an indigenous crayfish is considered a cornerstone of species conservation, we here combined population genetics and SDMs to provide a baseline for design of the future conservation strategies for *A. torrentium* under climate change. The specific aims of our study were: (1) to understand the genetic diversity and population structure of *A. torrentium* within its diversity hotspot in Croatia using a set of newly developed microsatellite markers; (2) to assess potential current and future habitat suitability of indigenous *A. torrentium* in relation to its standing genetic variation and currently designated Natura 2000 sites in Croatia, as well as in relation to potential current and future distribution of invasive *P. leniusculus* under different climate change scenarios; (3) to quantify niche overlap between *A. torrentium* and *P. leniusculus.* We anticipate that our results will enable the identification of populations and areas of the highest conservation value and priority for protection of *A. torrentium* under ongoing climate change and NICS threats. As such the results will provide valuable information for the development of appropriate conservation and management plans for *A. torrentium* including the hotspot of its unique genetic diversity.

Materials and methods

Sample collection and DNA isolation

Austropotamobius torrentium samples were collected throughout its distributional range in Croatia (Fig. 1, Table 1) for population genetic analysis from natural habitats. A total of 422 specimens were collected from 16 localities that were carefully chosen to represent all seven previously identified ESUs in Croatia: Banovina (BAN), central and south-eastern Europe (CSE), Gorski Kotar (GK), Kordun (KOR), Lika and Dalmatia (LD), Zeleni Vir (ZV), Žumberak, Plitvice and Bjelolasica (ŽPB), (Lovrenčić et al., 2020) (Fig. 1). Crayfish were collected by hand or baited traps, along approximately 100m of shoreline, in accordance with ethical standards and the approval of local authorities. Tissue samples were obtained non-destructively by taking a pereopod from each individual (stored in 96% ethanol), enabling the crayfish to be returned to the water. Genomic DNA was extracted from the pereopod muscle tissue with GenElute Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich, St. Louis, MO) following the manufacturer's protocol, and stored at -20 °C.

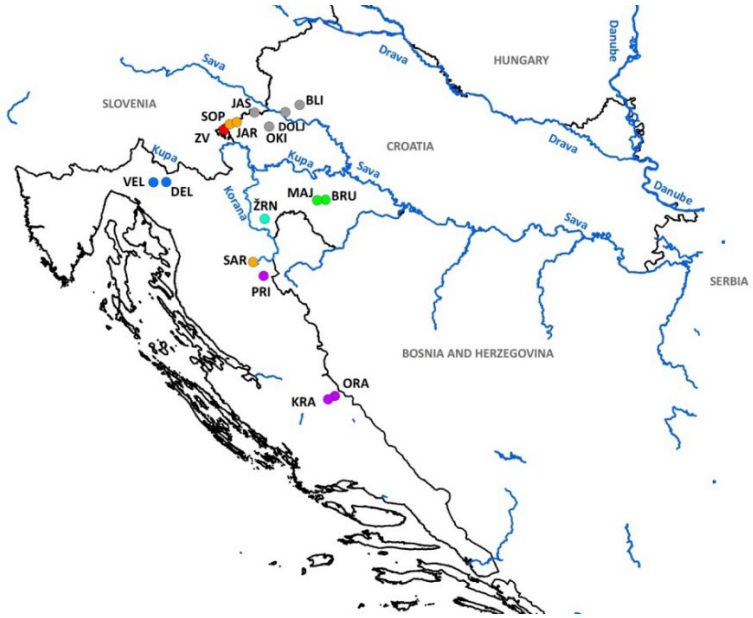


Figure 1 Geographical locations of the studied *Austropotamobius torrentium* populations. Colours depict populations affiliation to specific Evolutionarily Significant Unit (ESU) of *A. torrentium sensu* Lovrenčić et al. (2020): green - Banovina (BAN), gray - central and south-eastern Europe (CSE), blue - Gorski Kotar (GK), turquoise blue - Kordun (KOR), purple - Lika and Dalmatia (LD), red - Zeleni Vir (ZV), orange - Žumberak, Plitvice and Bjelolasica (ŽPB).

Table 1. Genetic diversity within populations of *Austropotamobius torrentium*. Pop. – population, Pop. Abb. – population abbreviation, ESU - Evolutionarily Significant Unit *sensu* Lovrenčić et al. (2020), ESU Abb. – Evolutionarily Significant Unit abbreviation**,** N – sample size, P - proportion of polymorphic loci, N_A – average number of alleles/locus, A_R - allelic richness, A_{PR} - private alleles with rarefied private allele frequency in parentheses, uH_E - unbiased expected heterozygosity, H_O - observed heterozygosity, F_{IS} - inbreeding coefficient and P_{HWE} - probability of deviation from Hardy-Weinberg equilibrium after Bonferroni adjustments (not significant (ns) or significant (*)).

Pop.	Pop. Abb.	ESU	ESU Abb.	N	P	N_A	A_R	A_{PR}	uH_E	H_O	F_{IS}	P_{HWE}
Brućina	BRU	Banovina	BAN	31	1.00	4.75	3.62	1 (0.40)	0.551	0.473	0.144	ns
Maja	MAJ	Banovina	BAN	30	1.00	4.13	3.19	1 (1.12)	0.456	0.313	0.317*	*
			BAN average		1.00	4.44	3.41	1 (0.76)	0.504	0.393	0.231	
Bliznec	BLI	Central and South-eastern Europe	CSE	29	1.00	4.13	3.48	5 (2.72)	0.524	0.431	0.180*	ns
Jarak (Stojdraga)	JAS	Central and South-eastern Europe	CSE	21	1.00	3.88	2.90	3 (1.12)	0.362	0.351	0.032	ns
Okićnica	OKI	Central and South-eastern Europe	CSE	32	1.00	5.00	3.84	5 (2.88)	0.543	0.440	0.193*	*
Dolje	DOLJ	Central and South-eastern Europe	CSE	31	1.00	4.50	3.86	2 (2.08)	0.595	0.465	0.221*	*
			CSE average		1.00	4.38	3.52	4 (2.24)	0.506	0.422	0.157	
Delnički Potok	DEL	Gorski Kotar	GK	29	0.75	2.75	2.50	0 (0.64)	0.393	0.349	0.113	*
Vele Vode	VEL	Gorski Kotar	GK	31	0.88	3.13	2.49	0 (0.24)	0.344	0.363	-0.055	ns
			GK average		0.81	2.94	2.49	0 (0.44)	0.369	0.356	0.029	
Žrvnica	ŽRN	Kordun	KOR *average*	11	0.88	3.88	3.79	3 (2.80)	0.585	0.455	0.232*	ns
Krasulja	KRA	Lika and Dalmatia	LD	30	0.88	3.13	2.72	0 (0.64)	0.454	0.434	0.045	ns
Orašnica	ORA	Lika and Dalmatia	LD	33	0.88	3.25	2.53	0 (0.80)	0.307	0.227	0.262*	*
Prijeboj	PRI	Lika and Dalmatia	LD	24	1.00	3.50	3.11	0 (0.08)	0.532	0.485	0.090	*
			LD average		0.92	3.29	2.79	0 (0.51)	0.431	0.382	0.132	
Zeleni Vir	ZV	Zeleni Vir	ZV *average*	29	1.00	4.50	3.89	10 (8.00)	0.584	0.402	0.316*	*
Jarak (Sošice)	JAR	Žumberak, Plitvice and Bjelolasica	ŽPB	10	0.75	2.38	2.32	0 (0.08)	0.307	0.325	-0.061	ns
Sartuk	SAR	Žumberak, Plitvice and Bjelolasica	ŽPB	27	0.88	2.88	2.60	1 (1.52)	0.457	0.315	0.315*	*
Sopotski slap	SOP	Žumberak, Plitvice and Bjelolasica	ŽPB	24	0.75	2.63	2.18	2 (0.88)	0.262	0.058	0.782*	*
			ŽPB average		0.79	2.63	2.37	1 (0.83)	0.342	0.233	0.345	

Development of novel microsatellite markers in *A. torrentium* suitable for population genetics

Partial genome sequencing

For the purpose of development of novel microsatellite markers approximately 1 µg of genomic DNA was extracted from a crayfish muscle sample using DNAeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). The purified genomic DNA was quantified with Qubit HS (Invitrogen, USA) and normalized to 2 ng/µL and subsequently processed using Nextera-based library preparation (Illumina, San Diego, CA) following the manufacturer's instructions. Quantification and size estimation of the library was performed on a Bioanalyzer 2100 High Sensitivity DNA chip (Agilent, Santa Clara, CA). Next, the library was normalized to 2 nM and sequenced on a MiSeq Benchtop Sequencer (2 x 250 bp paired-end reads) (Illumina, USA at the Monash University Malaysia Genomics Facility). The reads were assembled de novo into contigs using IDBA-UD (–mink 31 –maxk 251 setting) (Peng et al. 2012).

Detection of microsatellite motifs and initial testing of loci for amplification success and polymorphism

The open-source QDD version 3 (Meglécz et al., 2014) was used to identify contigs possessing microsatellite motifs as well as to design primer pairs suitable for the amplification of these loci. Primers were subsequently filtered based on suggestions by the authors of the software (Meglécz et al., 2014). A selection of contigs including di-, tri-, and tetra-nucleotide repeats were used for subsequent analysis. As a result, a total of 35 primer pairs were selected and initially tested for amplification success and presence of polymorphism in seven individuals of *A. torrentium* (each specimen representing one ESU present in Croatia). Forward primers were designed with a 19 bp M13-tail that was labelled during the PCR reaction using a universal fluorescently (either 6-FAM, NED, PET or VIC) labelled M13 primer (CACGACGTTGTAAAACGAC). Additionally, previously published species-specific microsatellite loci from Vorburger et al. (2014) (AT1, AT9, AT22, AT37) and Berger et al. (2018) (ATOR37) were included. Furthermore, six microsatellite loci (Aas5, Aas6, Aas3040, AP1, AP6 and Aitali3), designed for closely related crayfish species (*A. astacus, Austropotamobius pallipes, Austropotamobius italicus*), were suitable for cross-amplification (Iorgu et al., 2011; Vorburger et al., 2014; Berger et al., 2018) and were tested for polymorphism in *A. torrentium*. The PCR reaction (10 µl) contained 1x GoTaq® Colorless Master Mix (Promega), 75 nM of forward primer, 300 nM of reverse primer, 300 nM of M13, and ~10-50 ng of DNA template. Touchdown program was used for PCR amplification: initial activation of 3 min at 95 °C, followed by 20 cycles of 30 s at 95 °C, 90 s at 60 °C, 30 s at 72 °C, with the annealing temperature decreasing 0.5 °C per cycle, followed by 12 cycles of 30 s at 95 °C, 90 s at 50 °C, 30 s at 72 °C and a final extension for 30 min at 60 °C. Electrophoresis was performed in Macrogen, Inc. (Seoul, South Korea), while the length of fragments was determined using internal GeneScan 600 LIZ Size Standard and software GeneMapper v.4 (Life Technologies, USA).

Selection of the suitable microsatellite markers for population genetics of A. torrentium

From the initially tested microsatellite loci, eight polymorphic microsatellite markers were selected for population genetic studies of *A. torrentium*: ATM57, ATM78, ATM79, ATM64, AT1, AT37, ATOR37 and Aas3040. Details on these primers are provided in Supplement Table S1. The PCR reactions and touchdown program were used for PCR amplification as described above. Selected primers were split into two sets for avoiding the overlap of the allele size ranges: (set I) ATM57, ATM64, ATM78 and ATM79, and (set II) AT1, AT37, ATOR37 and Aas3040. Single PCR reactions for the same individual were performed for each primer pair, and the PCR products of the same set consisting four primer pairs (labelled with four different fluorescent dyes) were then pooled together. Genotyping was performed by capillary electrophoresis using Macrogen, Inc. (Seoul, South Korea). Raw alleles were scored manually in two independent scorings using GeneMapper v.4.0 (Life Technologies, USA) and allele sizes were binned with TANDEM v.1.08 (Matschiner and Salzburger, 2009).

Pairwise linkage disequilibrium (LD) was investigated for all pairs of loci using GENEPOP v.4.7.2 (Rousset, 2008), and significance levels were adjusted for multiple comparisons by applying Bonferroni correction. Microsatellite loci were assessed for potential presence of genotyping errors due to scoring of null alleles, stuttering and large allele dropout using MICRO-CHECKER v.2.2 (Van Oosterhout et al., 2004). Null allele frequencies based on the expectation-maximization (EM) algorithm (Dempster et al., 1997) were estimated using FreeNA (Chapuis and Estoup, 2007) with the ENA method and a number of bootstrap replicates fixed to 10,000. The estimations of F_{ST}, with and without null allele correction, were compared for each population using t-test in STATISTICA v.13 (StatSoft. Inc).

Population genetic diversity

The within-population genetic diversity was described by calculating the percentage of polymorphic loci (P), mean number of alleles (N_A), observed heterozygosity (H_O), unbiased expected heterozygosity (H_E) inferred using GenAlEx v.6.51 (Peakall and Smouse, 2012), and allelic richness (A_R) which was calculated and corrected for sample size by rarefaction using FSTAT v.2.9 (Goudet, 2003). Private alleles (A_{PR}) were identified using GenAlEx, while the private allele frequency was corrected for sample size in HPRARE (Kalinowski, 2005), and multiplied by number of used loci. The inbreeding coefficient, F_{IS}, was calculated for each population in FSTAT, with significance assessed using 1,000 permutations. Deviations from the Hardy-Weinberg equilibrium (HWE) for each population across all loci were estimated using GENEPOP v.4.7 (Rousset, 2008). Probability tests were based on the Markov chain algorithm using 10,000 dememorization steps, 100 batches and 5,000 iterations per batch, while significance levels were adjusted for multiple comparisons by applying the Bonferroni correction. Genetic signatures of demographic contraction and recent bottlenecks were assessed using the heterozygote excess and the mode-shift tests, both implemented in BOTTLENECK v.1.2 (Piry et al., 1999) under three different mutational models (infinite allele model, stepwise mutation model and two-phase model). Significant deviations from mutational-drift equilibrium were tested using the Wilcoxon sign rank test with 10,000 simulations, and the distribution of allele frequency classes was examined for a deviation from the normal L-shaped distribution (Luikart et al., 1998).

Genetic differentiation and population structure

Levels of genetic differentiation between population pairs were estimated by pairwise comparisons of F_{ST} values using FSTAT, and evaluated using 1,000 permutations. The analysis of molecular variance (AMOVA) was used to partition the total genetic variance among ESUs, among populations within ESU and within population in ARLEQUIN v.3.5 (Excoffier and Lischer, 2010). Population genetic structure and assignment of individuals into genetic clusters was inferred using Bayesian model-based clustering method as implemented in STRUCTURE v.2.3 (Pritchard et al., 2000) and model-free Discriminant Analysis of Principle Components (DAPC) (Jombart et al., 2010). For STRUCTURE, the conditions performed were 10 runs for each genetic cluster (K) between 1 and 16 with a 100,000 burn-in period followed by 100,000 Markov Chain Monte Carlo iterations, using correlated allele frequencies under a straight admixture model. The best value of K that fits our data was determined using the Evanno method (ΔK method; Evanno et al., 2005) as implemented in STRUCTURE HARVESTER (Earl and von Holdt, 2012). STRUCTURE graphical results were plotted with CLUMPAK (Kopelman et al., 2015). The DAPC, which is a model-free approach that does not use a priori geographical assumptions on sample origins, was carried out using the *dapc* function of the 'adegenet' package (Jombart, 2008) in the R statistical package (http://www.R-project.org/). The number of clusters within the data set (K) was selected using the *find.clusters* function and Bayesian information criterion (BIC). The chosen number of K was based on the minimum number of clusters after which the BIC decreased by a negligible amount. Genetic relationships among populations were estimated via pairwise D_A genetic distances and visualised using Principal Coordinate Analysis (PCoA) implemented in GenAlEx and as an unrooted neighbour-joining dendrogram in POPULATIONS v.1.2 (Langella, 1999).

Species distribution models (SDMs)

Species occurrence and environmental data

We obtained the known occurrence data for *A. torrentium* and for NICS *P. leniusculus* from literature and our field surveys. In total, we compiled 124 occurrences for *A. torrentium* and 17 for *P. leniusculus* used as modelling inputs. We considered a combination of seven bioclimatic and two topographic variables as environmental predictors for our SDMs (Table 2). The bioclimatic variables were obtained from the WorldClim 1.4 database (Hijmans et al., 2005), while altitude and slope were derived from a digital elevation model from the NASA Shuttle Radar Topography Mission (SRTM) data (Farr et al., 2007). All variables were used at a resolution of 30 arc-s (~1 km). Environmental predictors were selected based on our expert knowledge about their potential relevance for the ecology of the focal species, influencing their eco-physiology and distribution, as well as based on previously published literature using SDMs for crayfish (Chucholl, 2017; Lovrenčić et al., 2022). In addition, our selection was guided to avoid multicollinearity between the predictors, thus we removed variables with variance inflation factor VIF >10 using the R package *usdm* (Naimi, 2014; 2015).

Table 2 Environmental predictor variables used for building SDMs of *A. torrentium* and *P. leniusculus.*

Variable	Variable description (unit)	Reference
bio2	Mean Diurnal Range (°C)	Hijmans et al., 2005
bio4	Temperature Seasonality (SD ×100)	Hijmans et al., 2005
bio5	Max Temperature of Warmest Month (°C)	Hijmans et al., 2005
bio14	Precipitation of Driest Month (mm)	Hijmans et al., 2005
bio15	Precipitation Seasonality (CV)	Hijmans et al., 2005
bio18	Precipitation of Warmest Quarter (mm)	Hijmans et al., 2005
bio19	Precipitation of Coldest Quarter (mm)	Hijmans et al., 2005
alt	Altitude (m)	https://www2.jpl.nasa.gov/srtm
slope	Slope derived from altitude (%)	https://www2.jpl.nasa.gov/srtm

Modelling approach and habitat suitability changes

Species distribution models for each species were built using three commonly-used modelling techniques (Random Forest—RF, Generalized Boosted Model—GBM and Maximum Entropy—Maxent) implemented in the R package BIOMOD2 v 3.3.7 (Thuiller et al., 2009; 2016). We sampled 10,000 random pseudo-absences across the study area as background data for modelling techniques that require absences (Barbet-Massin et al., 2012). For each method, we generated 10 replicates and, in each run, 70% of the initial occurrences were used for model calibration, and the remaining 30% for model evaluation. Model performance was evaluated using the area under the receiver operating characteristic curve (AUC) (Hanley and McNeil, 1982) following the values proposed by Araújo et al. (2005). Resulting SDMs from different model techniques and runs were assembled into the current ensemble model based on AUC weighted average and including only highly reliable models with AUC > 0.9 (Thuiller et al., 2009).

The obtained current ensemble model was projected under both current and future environmental conditions to obtain potential habitat suitability maps for each species. For the future projections, we used the same set of environmental predictors and two representative concentration pathways (RCPs) describing moderate and pessimistic potential future emission scenarios for the 2070-time horizon (2061–2080 average): RCP 4.5 and RCP 8.5, respectively. Future projections of habitat suitability were based on four different general circulation

models (GCMs) shown to be suitable for Europe (CCSM4, MIROC5, MPI-ESM-LR and HadGEM2-CC; McSweeney et al. 2015). Different modelling techniques, RCPs and GCMs projections were chosen to account for known uncertainties in the modelling process (Araújo and New, 2007; Thuiller et al., 2019). To obtain a consensus future prediction of habitat suitability for each RCP, we averaged the SDM projections arising from the four different GCMs.

In order to have comparable predictions between the two species and across time periods, habitat suitability values were shown on the scale between 0 (unsuitable) to 1 (high suitability). Aiming to estimate changes in habitat suitability between current and future conditions posed by climate change, we first converted continuous habitat suitability outputs into binary presence-absence predictions using the conservative minimum training presence (MTP) threshold. This threshold presents the lowest predicted habitat suitability value associated with any of the occurrences used in the model calibration, thus predicting all known species observations as present (Pearson et al., 2007). To gain a more specific view of the climate change threat to endangered *A. torrentium*, we calculated overall habitat suitability changes between current and future, as well as habitat suitability changes within the currently designated Natura 2000 sites for this species in Croatia. Finally, to estimate the impacts of climate change on genetic variation of the focal species, we overlaid genetic variation data of *A. torrentium* with its potential current and future suitable areas. In addition, current and future habitat suitability of *A. torrentium* were estimated in relation to potential current and future distribution of invasive *P. leniusculus.*

Niche comparison between the indigenous *A. torrentium* and invasive *P. leniusculus*

We compared and quantified the niche overlap between the two crayfish species within the shared available environmental space in Croatia using the PCA-env approach and procedures implemented in the R package *ecospat* (Broennimann et al., 2012; Di Cola et al., 2017). For this purpose, we used the same species' occurrences and environmental variables as for our SDMs. Briefly, species occurrences were transformed into densities by kernel smoother and the niche overlap was then calculated in a gridded available environmental space (here 1,000x1,000 grid) summarized by the first two PCA axes (Broennimann et al., 2012). We used the Schoener's D as niche overlap metrics which ranges from 0 (no overlap) to 1 (complete overlap) (Warren et al., 2008). Finally, we carried out tests of niche equivalency and similarity to test whether two niches of the native and invasive crayfish species in Croatia are equivalent (identical) and/or more similar than expected by chance (we used the alternative "greater" in the similarity test to specifically test for niche conservatism) (for details see Warren et al., 2008 and Di Cola et al., 2017). In both tests, the observed D value was compared to the simulated null distribution of D values obtained by 1,000 replications to assess the significance.

Results

Amplification success and polymorphism of microsatellite loci in *A. torrentium*

From the total of 35 newly developed markers that were selected for initial testing, 11 amplified successfully, but only four (ATM57, ATM78, ATM79, ATM64) were polymorphic among the seven tested *A. torrentium* individuals. Three previously published markers for this species (AT1, AT37, ATOR37) were also found suitable based on these individuals. Markers from closely related species applied to *A. torrentium* were unsuccessful, with the exception of one locus (Aas3040) from *A. astacus* that cross-amplified and was polymorphic. Therefore, we used a set of eight polymorphic microsatellites: ATM57, ATM78, ATM79, ATM64, AT1, AT37, ATOR37, and Aas3040.

No signs of genotyping error due to stuttering or large allele dropout were observed across loci. A total of 114 alleles were observed across all loci with the number of alleles per locus ranged from 7 (ATM78, ATM79) to 22 (ATM57, AT37), and the H_O varied from 0.194 (ATOR37) to 0.571 (AT37) (Table S1). Linkage disequilibrium was found in three out of 28 pairwise comparisons of loci after Bonferroni corrections. Estimated null allele

frequencies were mostly low, ranging from 0.00001 (12 combinations) to 0.39 (ATOR37 in population SAR) (Table S1). Two loci (AT1 and ATOR37) exhibited a high null allele frequency, > 0.2, according to Chapuis and Estoup (2007). Omitting those two loci from analyses did not have any substantial effect on the results (data not shown). The F_{ST} values increased slightly when recalculated using adjusted allele frequencies, but no significant differences were observed between uncorrected F_{ST} values and F_{ST} values corrected for null alleles (t-test, p=0.2). Also, the FreeNA analysis, after applying the ENA correction, indicated that potential bias on average F_{ST} calculations caused by null alleles was minor (global F_{ST} with correction for null alleles = 0.442). Therefore, all loci were included in further population structure analyses.

Population genetic diversity

The summary statistics of the genetic diversity indices for studied *A. torrentium* populations is shown in Table 1. Over all, the microsatellite markers showed a moderate level of polymorphism (Table 1). The N_A ranged from 2.38 (JAR) to 5.00 (OKI), and A_R from 2.18 (SOP) to 3.89 (ZV). Total number of A_{PR} was 33, whereas rarefied number of A_{PR} ranged from 0.08 (JAR, PRI) to 7.68 (ZV). The greater number of A_{PR} was observed in populations ZV, ŽRN, BLI, OKI, while the lower number was found in populations DEL, JAR, KRA, ORA, PRI, SOP and VEL (Table 1). The H_O ranged from 0.058 (SOP) to 0.485 (PRI), and the H_E from 0.262 (SOP) to 0.595 (DOLJ). The multilocus F_{IS} per population was high in the majority of populations (F_{IS} ranging between -0.061 in JAR to 0.782 in SOP), indicating homozygote excess/heterozygote deficit. Significant deviation from Hardy-Weinberg equilibrium was observed in nine populations, and was accompanied by null alleles and positive F_{IS} values, indicating the existence of a heterozygote deficit/homozygote excess. Bottleneck analysis did not reveal consistent signs for recent contraction of population size according to the three mutational models tested (Table S2). Moreover, no recent bottlenecks were detected for any population as suggested by the L-shape graph that shows a characteristic L-shape distribution (alleles with low frequency are the most numerous) (Figure S1).

Genetic diversity, expressed as the P, N_A, A_R and H_O was, on the average, higher in the populations from the ESUs BAN, CSE, KOR and ZV, than in the ESUs GK, LD, and ŽPB (Table 1). Overall, the most variable populations were BRU from BAN; BLI, OKI, DOLJ from CSE; ŽRN from KOR; PRI from LD; and ZV from ZV, while the least variable populations were DEL from GK, ORA from LD, JAR, SAR, SOP from ŽPB. The highest private allelic richness was found in the ESU ZV, followed by KOR and CSE, while the lowest private allelic richness was indicated for the BAN, GK, LD and ŽPB.

Genetic differentiation and population structure

Genetic differentiation among 16 studied populations showed high level of genetic differentiation (global F_{ST} = 0.463, $p < 0.001$ for all pairs), with pairwise F_{ST} values ranging from 0.078 (between populations MAJ and BRU) to 0.689 (between populations ORA and SOP) (Table S3). As expected, lower F_{ST} values were recorded between populations within the same ESU, while higher F_{ST} were found between populations belonging to different ESUs. The results of the analysis of molecular variance (AMOVA) indicated that the majority of genetic variation occurs among crayfish within populations (49.10%), followed by the variance among the ESUs (36.08%), and variance among populations within the same ESU (14.82%) (Table S4).

The Bayesian clustering analysis performed in STRUCTURE revealed that the individuals from 16 populations grouped into eight distinct genetic clusters encompassing following populations (ΔK = 8): 1) BRU, MAJ; 2) BLI, JAS, OKI, DOLJ; 3) VEL, DEL; 4) ŽRN; 5) KRA, ORA, PRI; 6) ZV; 7) JAR, SAR, SOP; 8) several individuals from DOLJ and OKI (Figure 2, Figure S2). All individuals were well partitioned into the eight clusters according to their assignment probabilities, showing good separation among individuals from distinct ESUs (Figure 2). Precisely, populations from the same ESU constitute the same genetic cluster, with a minimal amount of admixture

present. The population ORA exhibited a minor proportion of ancestry from the genetic cluster 1 (ESU BAN). Moreover, both populations, ORA and PRI, included several individuals presenting a genetic profile characteristic of population from cluster 4 (ESU KOR). The DAPC analysis resulted in the relatively congruent assignment of individuals according to their affiliation to the same ESU; the proposed number of clusters for the DAPC was eight (Figure 2, Figure S3). Furthermore, individuals from the ESUs GK, ZV, CSE and ŽPB were more separated than others that clustered closely together (ESUs LD, BAN and KOR). LD was split into two genetic subclusters, placed closely together. The majority of the genetic structure in DAPC was captured in the first two principal components (scree plot of the eigenvalues, Figure 2). Structure in the distribution of genetic variation was also depicted by the Principal Coordinates Analysis (PCoA) (Figure 2), where the PCo1 axis accounted for 45.17%, the PCo2 axis accounted for 20.79%, and the PCo3 axis accounted for 10.93% of the variation in the data. PCo1 separated population belonging to ESUs LD, KOR and BAN from the populations belonging to CSE, GK, ZV and ŽPB. PCo2 separated populations from the ŽPB and ZV from the rest of the populations. PCo3 additionally separated GK and CSE. As observed for Bayesian analysis, PCoA and NJ dendrogram provided good resolution of spatial population relationships reflecting their affiliation to the same ESU and relatively high level of differentiation.

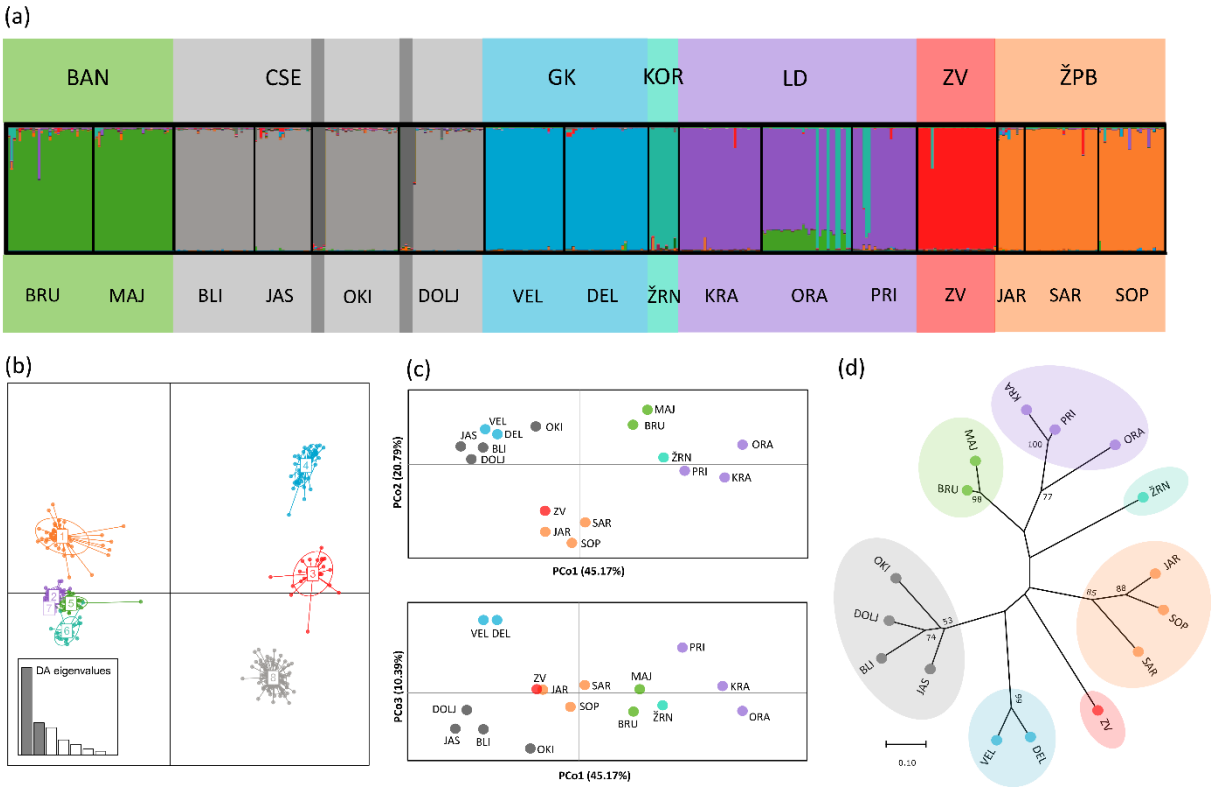


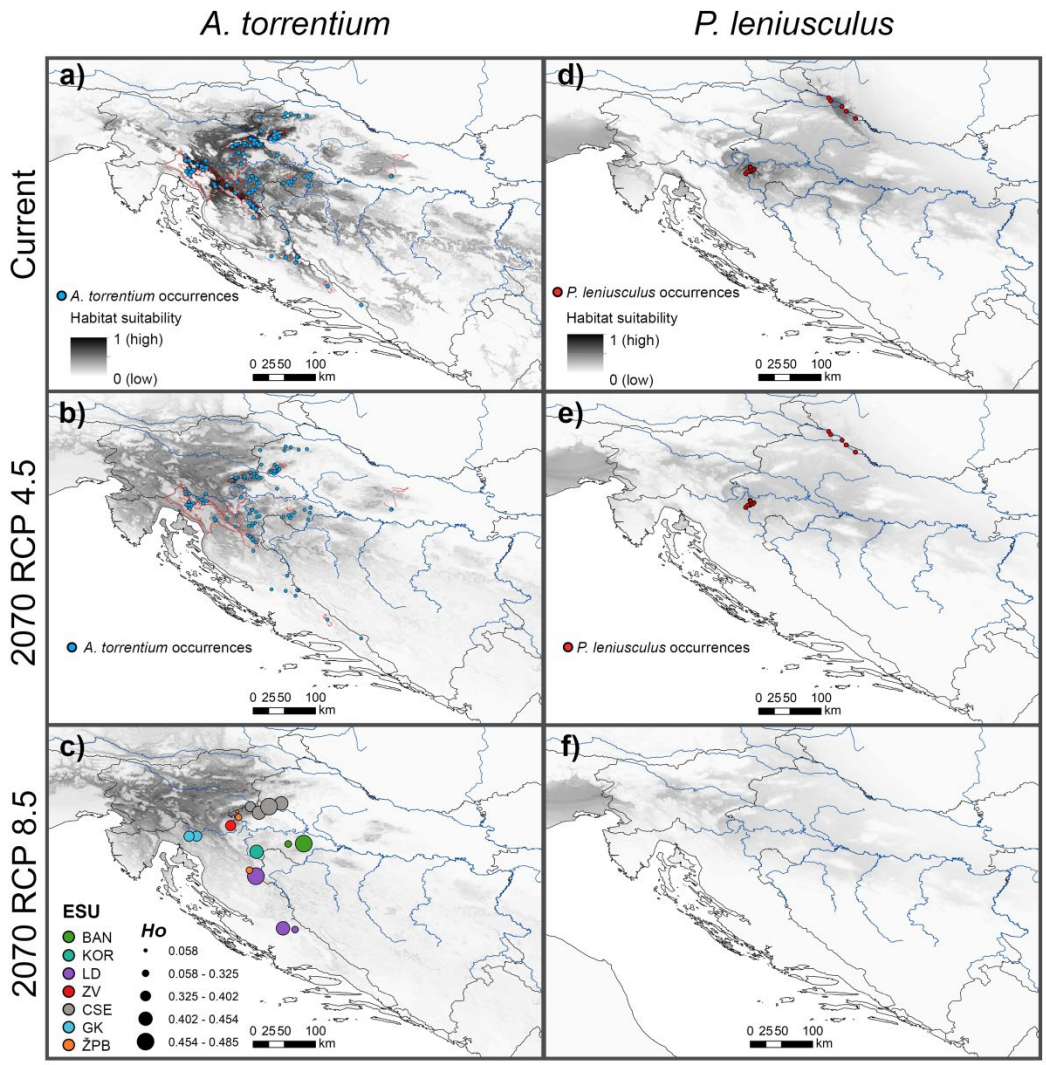
Figure 2 (a) Genetic structure of the 16 studied *Austropotamobius torrentium* populations based on 8 microsatellites inferred by STRUCTURE with the suggested K = 8 clusters. **(b)** DAPC scatterplot of the first two principal components for genetic clusters of *Austropotamobius torrentium* (K = 8). Clusters are represented by distinguishable colors and numbers (1-ŽPB; 2,7-LD; 3-ZV; 4-GK; 5-BAN; 6-KOR; 8-CSE). The inset shows the discriminant analysis (DA) eigenvalue. **(c)** PCoA scatterplot of the first three coordinates. **(d)** Neighbour-joining dendrogram based on D_A distances. For details on populations (geographical location, sample size, ESU) see Figure 1 and Table 1. Colours depict populations' affiliation to evolutionarily significant unit (ESU) of *A. torrentium sensu* Lovrenčić et al. (2020).

Predicted changes between current and future suitable habitat

Model performance metrics indicated excellent performance across methods and runs for both species, with AUC ranging from 0.931 to 0.991 (mean 0.986 ± 0.013) for *A. torrentium* and from 0.886 to 0.99 (mean 0.983 ± 0.032) for *P. leniusculus*. The current ensemble model for *A. torrentium* and *P. leniusculus* had an AUC of 0.998 and 0.999, respectively.

According to our ensemble model predictions under current conditions, highly suitable areas for *A. torrentium* agreed well with the known distribution of this species in the study area and mainly occurred in the north-west of the country, roughly corresponding to the Alpine biogeographical region in Croatia, while in the Continental biogeographical region they corresponded to areas of higher altitudes (Figure 3a). Under current projection, 3,700 km^2 are covered with Natura 2000 sites designated for *A. torrentium* (Figure S4a). Future projections under both climate change scenarios (RCP4.5 and RCP8.5) suggested that this endangered crayfish species may experience a drastic decrease of its potential suitable habitat in the study area, as well as displacement of the suitable habitat towards north-west, outside of its currently designated Natura 2000 sites (Figure 3b,c). In particular, when we quantified the changes in suitable habitat for *A. torrentium* between current and future binary ensemble predictions, 57% of its total currently suitable area is predicted to be lost by the 2070 under the moderate RCP4.5, and 80% under pessimistic RCP8.5 scenarios, while only 43% and 20% is predicted to remain stable, respectively (Figure 4a, Figure S5). Predicted decline of suitable habitat was, however, less pronounced within the designated Natura 2000 sites. When we compared the relative proportional change in size of the suitable habitat residing within these Natura 2000 sites, no change is predicted under RCP4.5; however, 40% of the currently suitable habitat is predicted to remain stable and 60% is predicted to be lost in the future under RCP8.5 scenario (Figure S4b,c). In addition, following our predictions, four out of 16 (25%) investigated populations and 23% of all currently known occurrences fall within the areas predicted to become unsuitable in the future under the RCP4.5. Under pessimistic RCP8.5 scenario, seven out of 16 (44%) studied populations, namely BRU, MAJ, ŽRN, KRA, ORA, PRI and SAR, as well as 65% of known occurrences are predicted to be lost (Figure 3c, Figure 4a). This suggests that some of the populations with the highest genetic diversity and three unique ESUs (BAN, KOR and LD) may be lost in the future (Figure 3c, Figure 4a).

For invasive *P. leniusculus* areas of suitable habitat were predicted mainly in the Continental biogeographical region of the country along the Drava and Sava Rivers, as well as along the Kupa River (Figure 3d). Currently, the two crayfish species overlap their potential geographic distributions in the area of the Kupa and Korana Rivers (Figure 4b). Although the extent of the potentially suitable habitat for *P. leniusculus* remains relatively similar under both future projections (Figure 3e,f), habitat suitability values were predicted to decrease, indicating lower probability of NICS occurrence. Thus, our future predictions did not identify any areas of overlap between indigenous *A. torrentium* and invasive *P. leniusculus* when continuous habitat suitability projections were converted into binary using the MTP thresholds.



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451 **Figure 3** Ensemble model predictions of habitat suitability under current and future conditions for the
452 indigenous *Austropotamobius torrentium* (**a-c**) and invasive *Pacifastacus leniusculus* (**d-f**) within the study area.
453 In **a)** and **b)** *A. torrentium* habitat suitability is shown in relation to its currently known distribution and
454 designated Natura 2000 sites for its protection in Croatia, while in **c)** future habitat suitability is shown in
455 relation to observed heterozygosity (Ho) of the studied populations and their affiliation to different ESUs
456 depicted by corresponding colours (as in Figure 1).

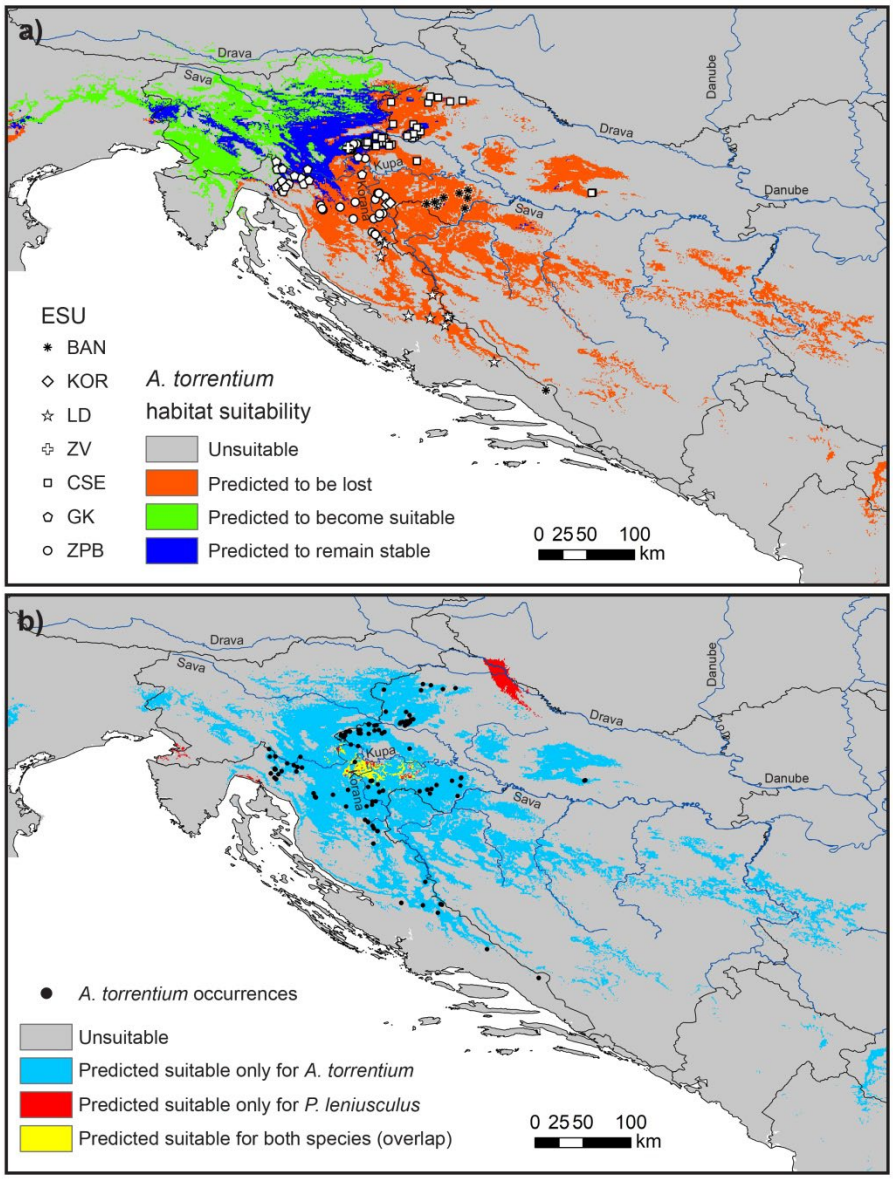

Figure 4 a) Predicted changes between current and future (2070) suitable habitat for the endangered crayfish *Austropotamobius torrentium* under RCP 8.5 scenario in relation to its currently known distribution and affiliation of known occurrences to different ESUs. **b)** Predicted overlap between potential current distribution of *A. torrentium* and invasive *Pacifastacus leniusculus* within the study area. Shown predictions are based on ensemble SDMs.

Niche overlap analysis

The first two PCA-env axis explained 48.9% and 30.5% of the environmental variability in the study area (cumulatively 79.45%), with altitude (alt) and mean diurnal range (bio2) contributing the most to the PC1 and precipitation variables - precipitation seasonality (bio15) and precipitation of warmest quarter (bio18) - to the PC2 (Figure 5d). Niche quantification analysis showed very limited niche overlap between the indigenous *A. torrentium* and invasive *P. leniusculus* in Croatia with D = 0.02 (see the classification by Rödder and Engler (2011)) with *P. leniusculus* currently exploiting a smaller part of the available environment in Croatia compared to indigenous *A. torrentium* (Figure 5a-c). The equivalency test showed that the two niches were not identical because the observed D overlap value was significantly lower than 95% of the simulated values ($p<0.05$; Figure

5e). In addition, the niche similarity test indicated that the two niches were not more similar than the random expectations because the observed D value was within the 95% confidence interval (p>0.05), thus rejecting the niche conservatism between the two crayfish species within the study area (Figure 5f).

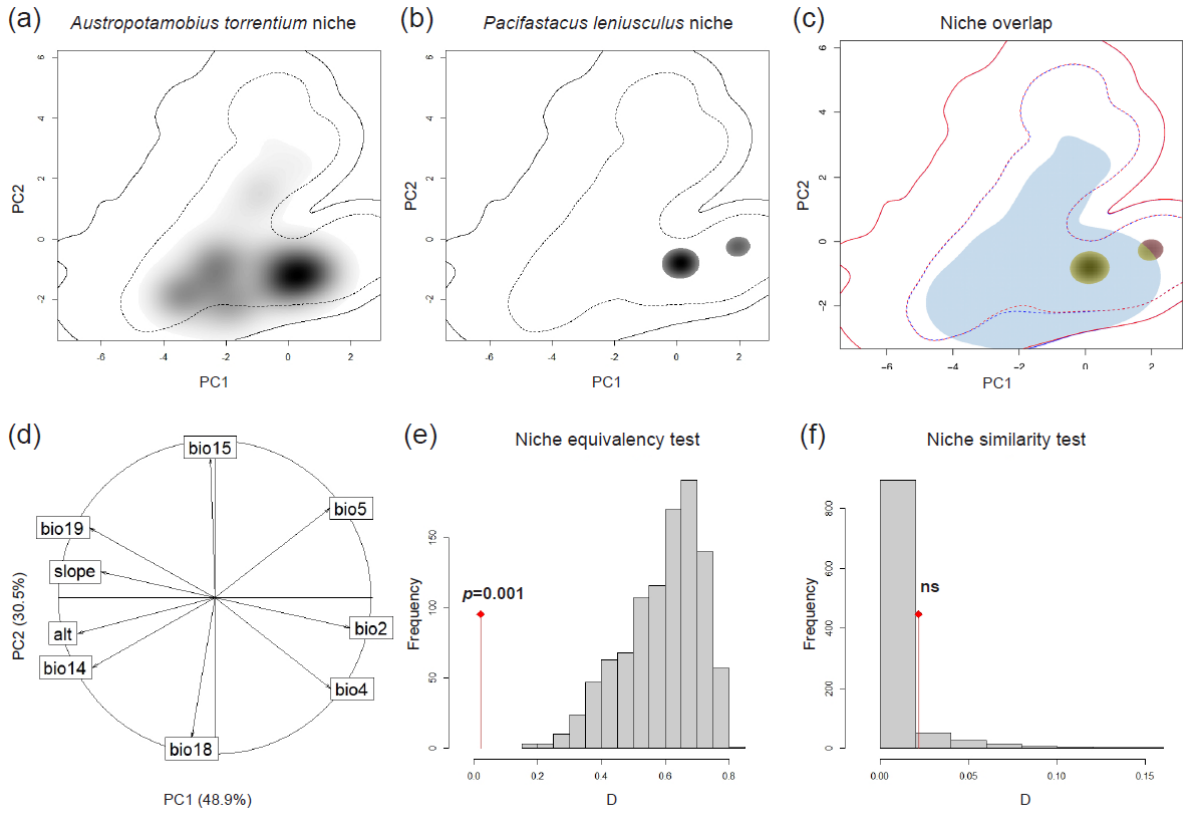

Figure 5 Environmental niches of *A. torrentium* and *P. leniusculus* along the first two PCA-axes (**a-b**). Gray shading indicates the species occurrence density by grid cell, while solid and dashed lines in **a-c** depict 100% and 50% of the available environmental space in Croatia. **c)** Pairwise niche overlap between the two crayfish species in Croatia (blue = *A. torrentium*, red = *P. leniusculus*, yellow = overlap). **d)** The PCA biplot showing the contribution of the nine environmental variables on the first two PCA-axes (for variable abbreviations see Table 2.). Niche equivalency (**e**) and niche similarity test (**f**). Observed D value (D=0.02, red diamond) is shown in relation to simulated null distribution of D values obtained by 1000 replications (gray bars).

Discussion

Our study provides an integrated approach combining population genetics, assessment of potential climate change impacts based on SDMs and niche overlap quantification between target and invasive crayfish to guide the conservation prioritisation of endangered crayfish *A. torrentium* and its populations residing within the hotspot of its genetic diversity located in the western Balkan Peninsula. Based on newly developed set of microsatellite loci we revealed moderate to high within-population genetic diversity and high differentiation among populations concordant with ESUs previously established using mtDNA data (Lovrenčić et al., 2020).

SDM projections suggest that climate change may considerably reduce the total area of suitable habitat for *A. torrentium* under future scenarios by the 2070, thus directly threatening its unique genetic diversity and restricted divergent lineages occurring within the study area. Moreover, a majority of areas currently suitable for *A. torrentium* are predicted to shift outside of Croatia in a north-west direction, as well as beyond currently

designated Natura 2000 sites for the focal species. This points to potentially reduced efficiency of the current Special Areas of Conservation in the future and highlights the possible need for putative assisted migration or translocations of target populations. We discuss our results in the light of possible future conservation strategies.

Development and optimization of microsatellite loci

Microsatellite loci are commonly used markers in population genetic studies with frequent application in conservation genetics (Guichoux et al., 2011; Vieira et al., 2016), including crayfish (Gouin et al., 2006; Vorburger et al., 2014; Bláha et al., 2016; Schrimpf et al., 2017; Berger et al., 2018; Gross et al., 2021; Lovrenčić et al., 2022). However, we experienced a number of experimental challenges during their optimisation, amplification and genotyping (e.g., stuttering, monomorphic loci, null alleles, artefactual bands, triallelic patterns, and extensive manual corrections). Consequently, out of all tested microsatellite loci, only eight were polymorphic and scorable for this study similar to previous studies on crayfish that used only a small number of microsatellite loci (Gouin et al., 2002 and 2006; Kõiv et al., 2008; Iorgu et al., 2011; Berger et al., 2018; Pârvulescu et al., 2020). Those studies also observed a significant departure from Hardy-Weinberg equilibrium across numerous loci, mostly due to null alleles. Nevertheless, selected loci provided useful insights into genetic diversity and population structure of *A. torrentium*. The total number of recorded alleles in our study (98 across eight loci) was sufficient for estimation of genetic diversity parameters given the number of loci used (Kalinowski, 2002). Moreover, the number of alleles per selected locus was almost three times higher than in the previous studies of *A. torrentium* (Kõiv et al., 2008; Iorgu et al., 2011; Vorburger et al., 2014; Berger et al., 2018). The selected loci enabled the identification of populations with the highest conservation value and priority for protection, as well as the selection of suitable donor populations for potential future restocking and reintroduction programs (see below).

Population genetic diversity

Understanding the genetic diversity and structure of an endangered species is the foundation of all conservation efforts as the genetic diversity is essential for evolutionary adaptation that is a key to the long-term survival of species (Yarra and Magoulick, 2019; Clay et al., 2020; Victoriano and Elía, 2021). Here, we used microsatellite loci to assess genetic diversity, population structure, and connectivity of the *A. torrentium* populations in Croatia. The study area constitutes an important part of its distribution range because it harbours the highest genetic diversity on mtDNA and nuDNA level (Lovrenčić et al., 2020). However, our study using microsatellites revealed lower genetic diversity, additionally corroborating its vulnerable status (Maguire et al., 2018).

Overall genetic diversity was moderate to high when compared to populations from other parts of the range. In particular, genetic diversity indices, A_R and H_O were higher in our populations than in the populations occurring at species' range edges in Austria, Switzerland and Germany (Vorburger et al., 2014; Berger et al., 2018), but lower than in the populations from Romania (Iorgu et al., 2011; Pârvulescu et al., 2020), even though not directly comparable due to difference in microsatellite loci used. Also, a high frequency of private alleles was found in several populations (e.g., BLI, OKI, ŽRN, ZV) suggesting local genetic divergence and isolation, and enhancing the long-term response to selection pressures. In contrast, populations without private alleles, with lower heterozygosity and allelic richness (e.g., DEL, VEL, KRA, ORA, JAR, SOP) might be less able to adapt to future changes.

We may presume that many studied *A. torrentium* populations are vulnerable due to their small and isolated nature. Specifically, we found that several populations (e.g., ORA, SAR, SOP) with significant homozygote excess and low genetic diversity indices are vulnerable to inbreeding which may further reduce the level of genetic diversity, and consequently lead to the loss of adaptive evolutionary potential of the species overall (Frankham,

2002). Based on microsatellite data, which may be informative for demographic events within the past 10-50 generations (Peery et al., 2012), we have not found evidence for population bottlenecks, which appears to be in conflict with the observed heterozygote deficit. Also, numerous populations (MAJ, OKI, DOLJ, DEL, ORA, PRI, ZV, SAR and SOP) significantly deviated from HWE, mainly caused by heterozygote deficiency, reflected in positive F_{IS} values. Deviations in HWE and significant local homozygote excess are frequently attributed to the presence of null alleles, inbreeding or the Wahlund effect (De Meeûs, 2018; Manangwa et al., 2019). The presence of null alleles might be the explanation for the observed departures from HWE and increased F_{IS} values in some populations (e.g., SAR, SOP). The instability of the flanking regions of microsatellites leads to weak or null amplification of one or both alleles resulting in artificial homozygote excess (Selkoe and Toonen, 2006). In populations with homozygote excess, but with low frequency of null alleles, homozygote excess may be caused by mating of close relatives (Van Oosterhout et al., 2004; Selkoe and Toonen, 2006), or caused by recent translocations as shown in studies of Australian crayfish species (Miller et al., 2014; Whiterod et al., 2016). Also, given the recent decrease in population size (Maguire et al., 2018), inbreeding is a plausible explanation for the observed heterozygote deficit. A Wahlund effect due to unrecognized population substructure could be ruled out as the cause of the observed heterozygote deficit, as concluded in the study by Yue et al. (2010) and Bláha et al. (2016). Here, no population substructure was found based on results from both Bayesian clustering analyses implemented in STRUCTURE and DAPC, with the exception of populations OKI and DOLJ where a small proportion of individuals belonged to different unique cluster. Despite the potential influence of one or more of the above mentioned processes, additional studies using more populations per ESU, higher number of loci and/or genome-level scans are required to further investigate the complex demographic history and intriguing patterns of genetic diversity within *A. torrentium*.

Population differentiation and structure

Population genetic analyses revealed strong genetic structuring among *A. torrentium* across Croatia, demonstrating isolated populations with limited gene flow, typical for species with low vagility (Clay et al., 2020). The fixation index (F_{ST}) indicated high overall genetic differentiation and low genetic connectivity among populations. Obtained pairwise F_{ST} values reflected populations' affiliation to certain ESU and geographical proximity. As expected, the F_{ST} values were the lowest between populations within the same ESU, and higher among geographically distant populations. Namely, the most genetically differentiated population was ORA when compared to other studied populations, which is geographically the most distant population. The F_{ST} values among our populations are higher compared to values estimated in other studies of *A. torrentium* (Vorburger et al., 2014; Berger et al., 2018; Pârvulescu et al., 2020), which indicates long periods of isolation and limited gene flow, and is in line with divergence times among ESUs within *A. torrentium* that was estimated to Miocene and Pliocene (Klobučar et al., 2013; Lovrenčić et al., 2020). The results of the AMOVA show that the greatest diversity is contained within populations, and the least between populations within the same ESU, which is congruent with F_{ST} values. This is similar to findings for European crayfish species, the noble crayfish, *A. astacus* (Panicz et al., 2019; Gross et al., 2021), narrowed-clawed crayfish, *Pontastacus leptodactylus* (Khoshkholgh and Nazari, 2019), and white-clawed crayfish, *A. pallipes* (Gouin et al., 2006), as well as for Australian *Euastacus armatus* (Whiterod et al., 2016). Genetic structuring with minimal indications of admixture was also identified by clustering analysis in STRUCTURE and DAPC, with both clustering methods showing similar grouping with the earlier ESUs identification (Lovrenčić et al., 2020).

Predicted impacts of climate change on distribution and genetic variation of *A. torrentium* - implications for conservation prioritization

Projections of our SDMs are consistent with previous studies pointing to severe contractions of suitable habitat in response to future climate change scenarios for many freshwater organisms that require colder temperatures, including crayfish (Capinha et al., 2013; Markovic et al., 2014; Bush and Hoskins, 2017; Préau et

al., 2019). According to our projections, *A. torrentium* may lose up to 80% of currently suitable habitat within the study area and 7 out of the 16 (44%) studied populations, including three highly divergent and geographically restricted evolutionary lineages (KOR, LD and BAN) may be at risk of extinction by 2070 under RCP 8.5 scenario. This includes some of the most genetically diverse and/or unique *A. torrentium* populations (e.g. BRU, MAJ, ŽRN, KRA, PRI, SAR and ORA). Such loss of genetic diversity and distinct ESUs may in turn substantially decrease overall species adaptive capacity to rapidly changing environments and jeopardize the long-term survival in the uncertain future (Wright et al., 2008). Decreasing genetic diversity increases the extinction risk of populations due to a decline in fitness, which can lead to further reduction in population size and a higher risk of stochastic demographic extinction (Markert et al., 2010).

Potential climate refugia (ark sites), defined as areas of stable habitat under future climate conditions that may support long term species survival (Barrows et al., 2020), are predicted to occur along the north-west border of Croatia and further north-west into Slovenia, to higher-altitude regions. Hydrology in the study area of the western Balkan Peninsula is highly fragmented and frequently lacks suitable surface water connections due to complex geo-climatic history and environmental heterogeneity, leaving remnant crayfish populations isolated (Klobučar et al., 2013). Pattern of isolated populations of freshwater species that contain high genetic diversity is characteristic for area of Dinaric karst in the western Balkan Peninsula, recognised as one of the freshwater biodiversity hotspots (Hewitt, 2004; Myers et al., 2000). Moreover, evidence of local genetic structuring and highly divergent evolutionary lineages within *A. torrentium* reflects their limited dispersal ability, which is characteristic for many aquatic species (Monaghan et al., 2002; Yamamoto et al., 2004), including freshwater crayfish (Barnett et al., 2020; Gross et al., 2021). Further, limited dispersal ability and low gene flow/connectivity may lead to reduced population sizes, reproductive success, and genetic diversity, consequently decreasing the likelihood of population persistence (Lowe and Allendorf, 2010). Thus, compensatory movements needed to reach ark sites/climate refugia will be particularly difficult for isolated populations and lineages that cannot disperse fast enough due to topographic and hydrological barriers, as well as low dispersal capacity in general (Barnett et al., 2019; Dudgeon, 2019; Clay et al., 2020). Consequently, conservation efforts should focus on improving the genetic connectivity of populations through waterbodies connectivity restoration programs (Erős et al., 2018) that could be beneficial for native species, but at the same time could enable non-native invasive species to enter/spread into previously unattainable habitats/non-invaded areas (Manenti et al., 2019). Alternative conservation approach includes assisted migrations/translocations of populations that are at risk of extinction/disappearance (Miller et al., 2014; Whiterod et al., 2016; Lovrenčić et al., 2022). Even tough species translocations carry the potential risk of further eroding biodiversity and disrupting ecosystems, if carefully planned, assisted migration and population mixing approaches could be a valuable conservation strategy for connecting crayfish populations and overcoming risks of genetic erosion in highly fragmented landscapes under ongoing climate change (Butt et al., 2020).

Based on our findings, prioritised populations for the *in situ* conservation should include BLI, OKI, DOLJ, and ZV because they hold high levels of genetic diversity which promotes population resilience and overall species adaptive potential in the face of rapidly changing environmental pressures (Frankham, 2002). Their *in situ* conservation will be plausible because, under both RCP scenarios, their habitats are predicted to remain stable. High level of genetic diversity was also recorded for ŽRN, but since its habitat is predicted to be lost under RCP 8.5 scenario or reduced under RCP 4.5 scenario, alternative adequate stable habitats for possible translocation should be considered (e.g. western part of Croatia, towards Slovenian border or higher altitudes in the same region, respectively). In addition, some populations (e.g., ESU LD-ORA, PRI, KRA; ESU BAN- BRU/MAJ; ESU ŽPB-SOP, SAR, PRI) should be given particular attention because they are highly divergent, and despite their low/lower genetic diversity they may be disproportionately important for the species/ESUs survival due to their relict character (Hampe and Petit, 2005). Additionally, our SDM predictions for those populations indicated loss of suitable habitat (RCP 8.5 scenario), or significant habitat reduction (RCP 4.5. scenario) in the future.

Consequently, their survival will depend on human-mediated translocations into suitable habitats/climate refugia (either to western part of Croatia, towards Slovenian border (RCP 8.5 scenario) or regionally, into streams on higher altitudes (RCP 4.5 scenario)). For the populations in ESU GK (DEL and VEL) that were predicted to persist within the stable habitats (both RCP scenarios), but show low level of genetic diversity, we would suggest mixing approaches (reinforcement) that could reduce further genetic erosion (Lavrik, 2022). Moreover, all of the mentioned ESUs have restricted distribution ranges and their unique genetic architecture should be given prioritization in conservation because they significantly contribute to overall diversity of the species and local biodiversity (Crandall, 1998; Yarra and Magoulick, 2019).

Finally, although decline of the suitable habitat was predicted to be less severe within the currently designated Natura 2000 sites for the focal species, we still detected a decrease of 60% under RCP 8.5 compared to current suitable area. Since suitable habitats are predicted to shift towards north-west into Slovenia, establishment of transboundary (Croatia-Slovenia) sites to maintain genetic and phylogeographical diversity in the future should also be considered. This approach should include, amongst other, enhanced transboundary collaboration of local and state agencies and researchers that could provide effective long-term crayfish protection (Carrizo et al., 2017; Lovrenčić et al., 2020).

Predicted impacts of climate change on NICS

We found relatively low overlap between potential current geographic distribution of *A. torrentium* and invasive *P. leniusculus* currently located along the Kupa and Korana Rivers, while no areas of distributional overlap were predicted in the future within the study area. Even more limited niche overlap was identified within the available environmental space in Croatia and the two crayfish niches were neither identical, nor more similar than expected by chance, thus not supporting the niche conservatism pattern (Wiens and Graham, 2005; Warren et al., 2008). In contrast, Préau et al. (2019) found relatively high levels of niche overlap between *P. leniusculus* and *A. pallipes* in France where the two species seem to exploit equivalent niches in the available environment. Likewise, Chucholl (2016) reported relatively high niche overlap between *P. leniusculus* and *A. torrentium* for Germany, however their study assessed environmental niche overlap based on SDMs only. On the other hand, pattern of decreased distribution overlap between native and invasive crayfish in response to climate change has been previously reported for Europe and specific countries (Gallardo and Aldridge, 2013; Préau et al., 2019, Lovrenčić et al., 2022).

We acknowledge that our SDM predictions for *P. leniusculus* may have underestimated the potential future distribution of this invasive competitor within the study area due to known caveats when modelling invasive species (Jarnevich et al., 2015). In particular, *P. leniusculus* is in the expansion phase of invasion in our study area with the recorded rate of downstream dispersion among the highest in Europe (Hudina et al., 2009; Dragičević et al., 2020). Thus we may assume that it did not yet reach its distributional equilibrium in this part of the invaded range, which could also explain the narrow environmental space currently occupied as well as limited niche overlap with *A. torrentium*. Moreover, we cannot account for human-mediated dispersal of NICS in the future which is a common practice (Hudina et al., 2013; Strand et al., 2019; Jussila et al., 2021).

In conclusion, although *A. torrentium* needs to undoubtedly confront challenges from both climate change-related impacts and invasive crayfish, at least for the moment, climate change seems to pose the greatest threat to its future in this disproportionally important part of its distribution range. In the light of these uncertainties, continuous monitoring of the *P. leniusculus* invasion is needed, including transboundary monitoring schemes to validate our results (Black and Bartlett, 2020; Madzivanzira et al., 2021). Finally, the findings of this study have relevance to other crayfish species of conservation importance, and also emphasize the importance of multidisciplinary approach in the modern biodiversity conservation.

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Supplementary Tables

Table S1 Characterization of eight selected microsatellite loci for population genetics of *Austropotamobius torrentium*. ATM57, ATM78, ATM79, ATM64 represent microsatelite loci developed in this study; AT1 and AT37 from Vorburger et al. (2014); ATOR37 from Berger et al. (2018); Aas3040 from Iorgu et al., (2011).

Locus	Repeat motif	Dye	Set	Primer sequences 5′ → 3′*	No. of alleles	Observed allele size range (bp)	H_O
ATM57	AAGG	6-FAM	I	F: M13-TCTGGGTCTAGAGCAGCGG R:TGGCAAATGGTGAGGAGGAT	22	303-459	0.503
ATM64	AGG	PET	I	F: M13-TACTTGAGGGATCGACCAGC R: TGACAAAGGTGGCTCGTGAT	10	281-308	0.316
ATM78	AGGC	VIC	I	F: M13-GCGTCCGGGATACTCTTGAA R: CATCTTCTGTGGTGCCACCT	7	236-264	0.342
ATM79	ACG	NED	I	F: M13-TACGACTCTCCTGGACCTCC R: TTGATTCTCAAGGAGCGGCC	7	184-205	0.282
AT1	(AGG)…(AGC)	NED	II	F: M13-GAGGTCTAAGGCGACGAGG R: CAAGTAAGGGCCGGGTGAG	12	209-257	0.357
AT37	TAACC	6-FAM	II	F: M13-ACTATCCGACCGAACGAACC R: ACAGAACCGATTCTTGGCAT	22	252-362	0.571
ATOR37	AC	PET	II	F: M13-GTGCTTCTGTTGTTCGCTCTT R: TGTTGTGCTAACTGTTGTGAGTCC	15	156-184	0.194
AAS3040	TA	VIC	II	F: M13-GTTGTGTGGTAACTCCTGACGA R: CAATCGTATCCCACATGCAG	19	224-260	0.380

* Forward primers with 19 bp M13-tail (TGTAAAACGACGGCCAGT).

Table S2. Probability (bold indicates significant p-values; $p < 0.05$) of bottleneck for *Austropotamobius torrentium* population using Wilcoxon sign rank test under three different mutational models: infinite allele model (IAM), stepwise mutation model (SMM) and two-phase model (TPM).

Pop.	ESU	Wilcoxon sign-rank test 1-tail			Wilcoxon sign-rank test 2t		
		IAM	TPM	SMM	IAM	TPM	SMM
BRU	BAN	0.16	0.63	0.97	0.31	0.84	0.07
MAJ	BAN	0.32	0.73	0.99	0.64	0.64	0.04
BLI	CSE	**0.01**	0.53	0.98	**0.02**	1.00	0.05
JAS	CSE	0.96	0.99	0.99	0.20	0.04	0.02
OKI	CSE	**0.04**	0.19	0.73	0.07	0.38	0.64
DOLJ	CSE	**0.01**	**0.04**	0.47	**0.01**	0.07	0.95
DEL	GK	**0.01**	**0.02**	0.34	**0.02**	0.05	0.69
VEL	GK	0.41	0.71	0.96	0.81	0.69	0.11
ŽRN	KOR	**0.00**	**0.02**	0.15	**0.01**	**0.04**	0.30
KRA	LD	**0.01**	**0.04**	0.41	**0.02**	0.08	0.81
ORA	LD	0.77	0.96	0.96	0.58	0.11	0.11
PRI	LD	**0.00**	0.16	0.68	**0.00**	0.31	0.74
ZV	SB	**0.01**	0.10	0.37	**0.01**	0.20	0.74
JAR	ZV	0.66	0.78	0.92	0.84	0.56	0.44
SAR	ŽPB	**0.00**	**0.00**	0.19	**0.01**	**0.01**	0.38
SOP	ŽPB	0.78	0.96	0.98	0.56	0.11	0.05

Table S3 Pairwise F_{ST} values from 8 microsatellite loci between 16 *Austropotamobius torrentium* population pairs (all values are statistically significant, $p < 0.05$).

	BRU	MAJ	BLI	JAS	OKI	DOLJ	DEL	VEL	ŽRN	KRA	ORA	PRI	ZV	JAR	SAR	SOP
BRU																
MAJ	0.078															
BLI	0.385	0.462														
JAS	0.452	0.521	0.188													
OKI	0.339	0.421	0.182	0.249												
DOLJ	0.372	0.437	0.119	0.133	0.172											
DEL	0.479	0.520	0.444	0.489	0.446	0.374										
VEL	0.509	0.554	0.476	0.539	0.485	0.409	0.242									
ŽRN	0.343	0.393	0.426	0.528	0.377	0.380	0.512	0.551								
KRA	0.381	0.441	0.496	0.581	0.480	0.463	0.564	0.597	0.404							
ORA	0.359	0.381	0.582	0.666	0.522	0.545	0.645	0.667	0.484	0.452						
PRI	0.316	0.359	0.444	0.539	0.426	0.409	0.501	0.536	0.338	0.107	0.390					
ZV	0.394	0.451	0.395	0.457	0.366	0.340	0.445	0.495	0.374	0.462	0.534	0.411				
JAR	0.478	0.558	0.473	0.573	0.487	0.407	0.591	0.625	0.513	0.565	0.680	0.492	0.388			
SAR	0.441	0.497	0.445	0.523	0.439	0.379	0.525	0.560	0.427	0.487	0.575	0.417	0.352	0.271		
SOP	0.526	0.601	0.531	0.635	0.540	0.473	0.639	0.661	0.564	0.588	0.689	0.518	0.426	0.089	0.326	

0	0.1	0.2	0.3	0.4	0.5	0.6	0.7

Table S4 Analysis of Molecular Variance (AMOVA) within and among 16 populations of *Austropotamobius torrentium* (level of significance is based on 10,000 iterations). Populations were grouped based on their affiliation to Evolutionary Significant Unit (ESU).

Sourse of variation	d.f.	Sum of squares	Percentage of variation
Among ESU	6	905.19	36.08
Among populations within ESU	9	229.71	14.82
Within populations	828	1236.01	49.10

Supplementary Figures

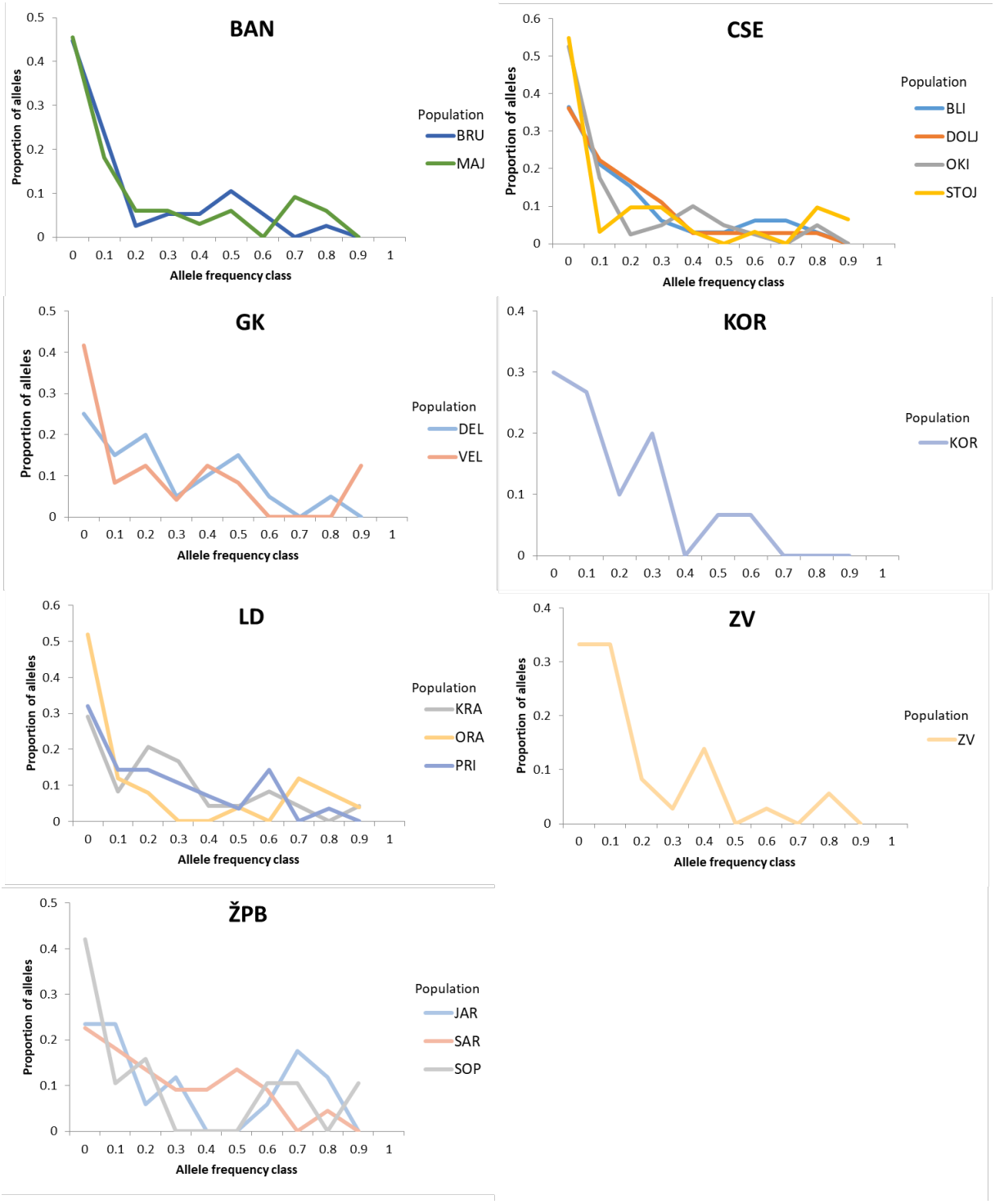


Figure S1 Distribution of allele frequencies in different allele frequency class for each population based on mode-shift test using eight microsatellite loci in *Austropotamobius torrentium*. Populations are grouped according to ESUs (Evolutionary Significant Units).

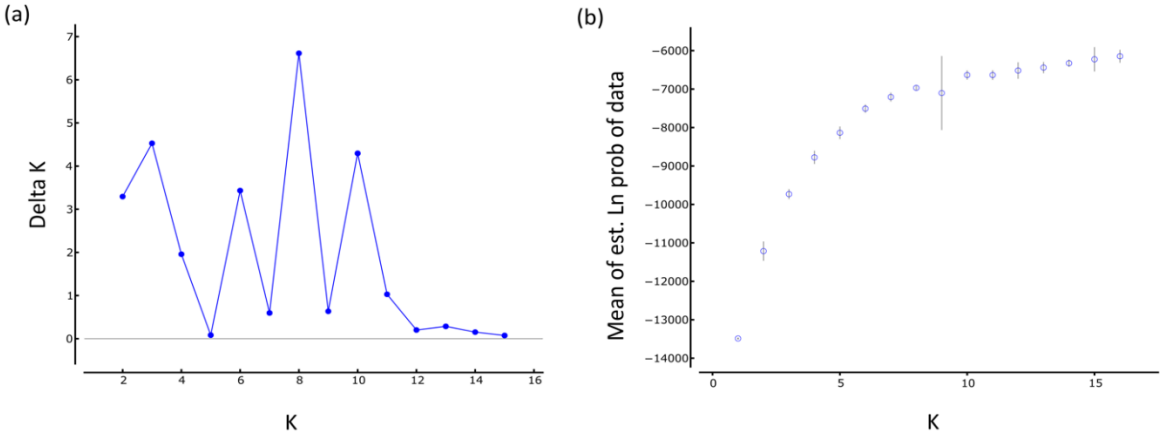


Figure S2 (a) Delta K and (b) posterior probability plots representing the most probable number (K = 8) of genetic clusters of *Austropotamobius torrentium* in Croatia (obtained with Structure Harvester using Evanno method). The optimum K value is 8 – the point where the plateau in the posterior probability starts with maximum delta K value.

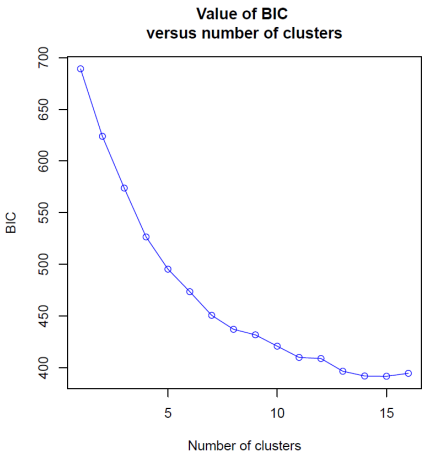
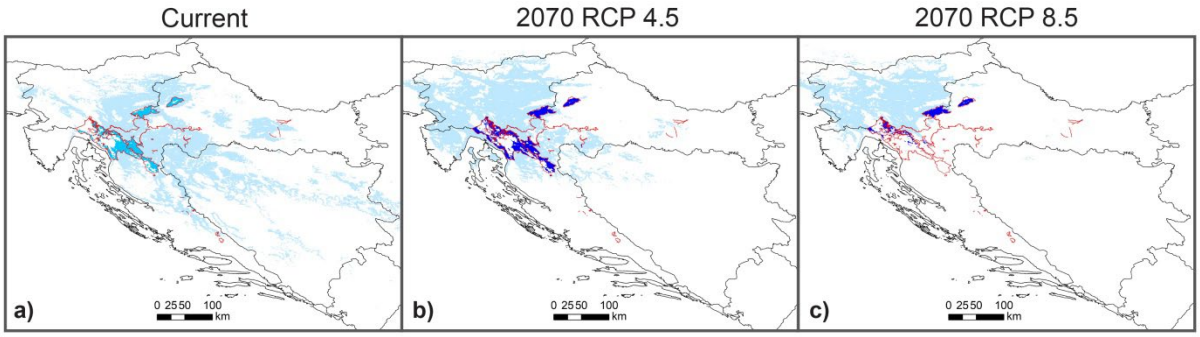
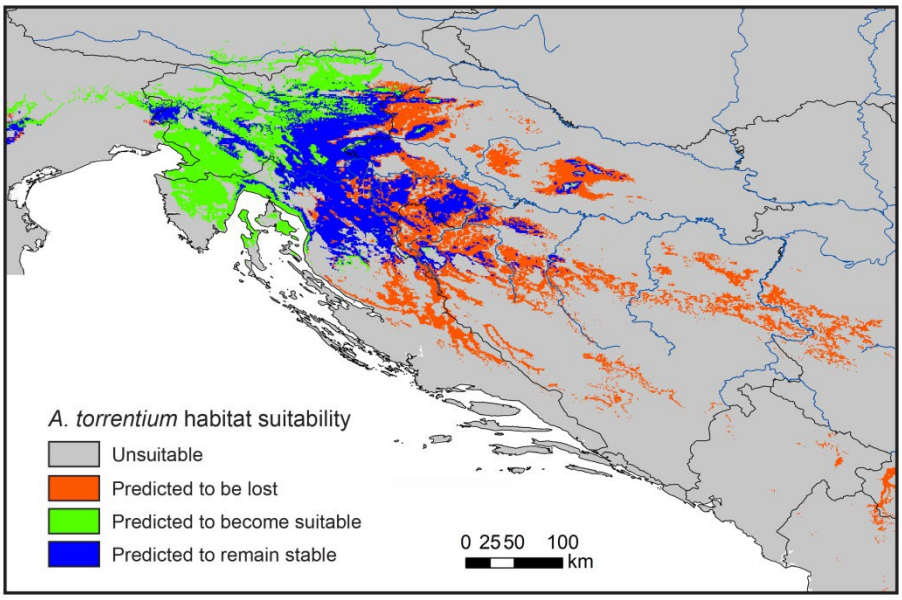


Figure S3 Inference of number of clusters. Graphical representations of Bayesian information criterion (BIC) for every number of clusters, using DAPC.



1080

1081 **Figure S4** Habitat suitability changes within Natura 2000 sites designated for *A. torrentium* under a) current b)
1082 RCP 4.5 c) RCP 8.5.



1083

1084 **Figure S5** Predicted changes between current and future (2070) suitable habitat for the endangered crayfish
1085 *Austropotamobius torrentium* under RCP 4.5 scenario.

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HOW WELL DOES NATURA 2000 PROTECT THREATENED STONE CRAYFISH IN CROATIA?

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Lovrenčić, L., Temunović, M. & Maguire, I.: How well does NATURA 2000 protect threatened stone crayfish in Croatia? Nat. Croat., Vol. 29, No. 2, 241-253, 2020, Zagreb.

The stone crayfish *Austropotamobius torrentium* (Schrank, 1803) is a threatened native European freshwater crayfish species for which Natura 2000 network represents the most important conservation effort at the European level. In Croatia, there are altogether 25 Natura 2000 sites defined specifically for this species. In the present study, we aimed to assess the effectiveness of Natura 2000 sites in preserving stone crayfish diversity through gap analysis, a GIS-based approach that overlays species distribution data on a map of designated Natura 2000 sites. Our results showed that the existing Natura 2000 network in Croatia encompasses most of the areas with a high diversity of *A. torrentium*; currently designated sites harbour 73.3% of recorded *A. torrentium* populations. Future conservation planning efforts, and possible expansion of Natura 2000, should be focused on newly discovered *A. torrentium* populations that present divergent evolutionary lineages.

Key words: *Austropotamobius torrentium*, Astacidae, gap analysis, conservation planning, biodiversity conservation

Lovrenčić, L., Temunović, M. & Maguire, I.: Koliko dobro postojeća Natura 2000 mreža štiti ugroženog potočnog raka u Hrvatskoj? Nat. Croat., Vol. 29, No. 2, 241-253, 2020, Zagreb.

Potočni rak *Austropotamobius torrentium* (Schrank, 1803) je ugrožena autohtona europska vrsta slatkovodnog raka porodice Astacidae za kojeg mreža Natura 2000 predstavlja najvažniju inicijativu u konzervaciji na europskoj razini. U Hrvatskoj uključuje 25 područja određenih posebno za ovu vrstu. Cilj ovog istraživanja je bio procijeniti učinkovitost Natura 2000 područja u očuvanju raznolikosti potočnog raka kroz *gap* analizu koja se temelji na preklapanju karata rasprostranjenosti ove vrste i Natura 2000 određenih područja korištenjem GIS programskog paketa. Rezultati su pokazali da postojeća mreža Natura 2000 u Hrvatskoj obuhvaća 73,3% populacija potočnog raka te uključuje i područja njegove najveće raznolikosti. Pri izradi budućih planova konzervacije ove vrste i mogućih proširenja Natura 2000 područja posebnu pažnju treba obratiti na novootkrivene populacije koje ujedno predstavljaju evolucijski divergentne linije.

Ključne riječi: *Austropotamobius torrentium*, Astacidae, *gap* analiza, konzervacijski planovi, zaštita bioraznolikosti

INTRODUCTION

Austropotamobius torrentium (Schrank, 1803), the stone crayfish, is a cold-adapted crayfish species native to smaller pristine water bodies related to karstic formations at higher altitudes in central and south-eastern Europe (Kouba *et al*., 2014). In Croatia it

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is distributed mainly in water bodies of the Black Sea drainage, with a few populations in the Adriatic Sea basin (Maguire *et al*., 2018). It is characterised by complex evolutionary history and high genetic diversity represented by nine distinct mitochondrial phylogroups, seven of them inhabiting freshwaters of Croatia (Klobučar *et al*., 2013; Pârvulescu *et al*., 2019; Lovrenčić *et al*., 2020). Nowadays numerous populations belonging to different phylogroups are threatened by human activities and protection of them is indispensable (Maguire *et al*., 2018).

Stone crayfish conservation activities ought to secure the long-term survival of species by taking into account the complexity of natural ecosystems involving all levels of biological diversity and organisation (Rodrigues *et al*., 2004; Jantke *et al*., 2013). Therefore, the development of broad-scale conservation networks of protected areas is considered to be a fundamental step for preventing future biodiversity loss through enabling the continuation of eco-evolutionary processes (Poiani *et al*., 2000; Rodrigues *et al*., 2004; Maoirono *et al*., 2006) and adaptation of species to changes in the distribution of suitable habitats under the ongoing climate change (Araújo *et al*., 2011). Effective preservation includes: the evaluation of the existing conservation areas in the representation of biodiversity, identification of underrepresented elements, and recommendation how the conservation could be enhanced (Abellan & Sanchez-Fernandez 2015; Bosso *et al*., 2016). For this reason, systematic approaches to conservation planning for protecting *A. torrentium* should be focused on the preservation of its unique genetic diversity so as it ensures adaptive potential and evolutionary response to the fast changes in the environment and pressures on its habitats.

One of the most important conservation efforts at European level is the Natura 2000 network. Natura 2000 is the largest network of protected areas in the world, established by the European Union (EU) with the aim of ensuring the long-term survival of its most valuable and threatened species and habitats. The network includes Special Protection Areas (SPAs) designated under the Birds Directive 2009/147/EC and Special Areas of Conservation (SACs) designated under the Habitats Directive 1992/43/EEC. The effectiveness of this network in representing biodiversity has been assessed in numerous studies at global or regional scales through gap analysis (Rodrigues *et al*., 2004; Jantke *et al*., 2011; Verovnik *et al*., 2011; Gruber *et al*., 2012; Bagella *et al*., 2013; Abellan & Sanchez-Fernandez, 2015; Maiorano *et al*., 2015; Orlikowska *et al*., 2016), and the outcomes of these studies vary.

Gap analysis is a GIS-based approach for assessing the effectiveness of protected areas in representing species diversity by comparing the distribution of species with the extent of the conservation network (Rodrigues *et al*., 2004; Jennings, 2000; Bosso *et al*., 2016). It is a widely implemented and useful tool for the identification of different elements (e.g., species, habitats, ecosystems) that require greater and/or stronger protection (Jennings, 2000; Scott *et al*., 2001; Oldfield *et al*., 2004; Dietz & Czech, 2005; O'Dea *et al*., 2006).

Croatia has one of the most extensive Natura 2000 networks in Europe covering 36.73% of the land territory and 15.42% of the seashore. Moreover, Natura 2000 in Croatia includes 25 designated sites (Sites of Community Importance – SCI) for *A. torrentium* that were defined according to historical and recent data on the distribution, size of populations, and abundance of this vulnerable species (Maguire *et al*., 2011). The state members propose their SCI areas to the European Union and once approved,

they can be appointed as SACs (Special Areas of Conservation) that are targeted to important measures in order to conserve the habitats and species in question.

From a practical conservation perspective, it is critical to evaluate the extent to which present and future protected areas cover *A. torrentium* diversity in freshwater ecosystems. Therefore, the aim of this research was to assess the effectiveness of the Natura 2000 network in preserving stone crayfish diversity in Croatia, through performing gap analysis that overlays *A. torrentium* distribution data on a map of Natura 2000 sites. This study provides the first comprehensive analysis of the effectiveness of conservation areas in protecting the endangered stone crayfish in Croatia.

MATERIAL AND METHODS

Study area

The karstic freshwaters of the north-central Dinarides in the western part of the Balkans are considered the primary centre of radiation of the stone crayfish, harbouring the highest number of lineages and the greatest genetic diversity, while diversity outside this area is greatly reduced (Trontelj *et al*., 2005; Klobučar *et al*., 2013; Berger *et al*., 2018; Pârvulescu *et al*., 2019; Lovrenčić *et al*., 2020) (Fig. 1). Nowadays the phylogeographic pattern of the stone crayfish is connected to the palaeo-hydro-geomorphological and climatic history of the Balkan Peninsula. The karstification processes fragmented the palaeohydrography of the area, which facilitated geographical isolation and enabled allopatric speciation shaping this species' intricate evolution (Trontelj *et al*., 2005; Klobučar *et al*., 2013). Because habitat deterioration, decline in water quality, climate change and the spreading of invasive alien crayfish species and their pathogens caused populations decline throughout its distribution range (Kouba *et al*., 2014; Maguire *et al*., 2018),

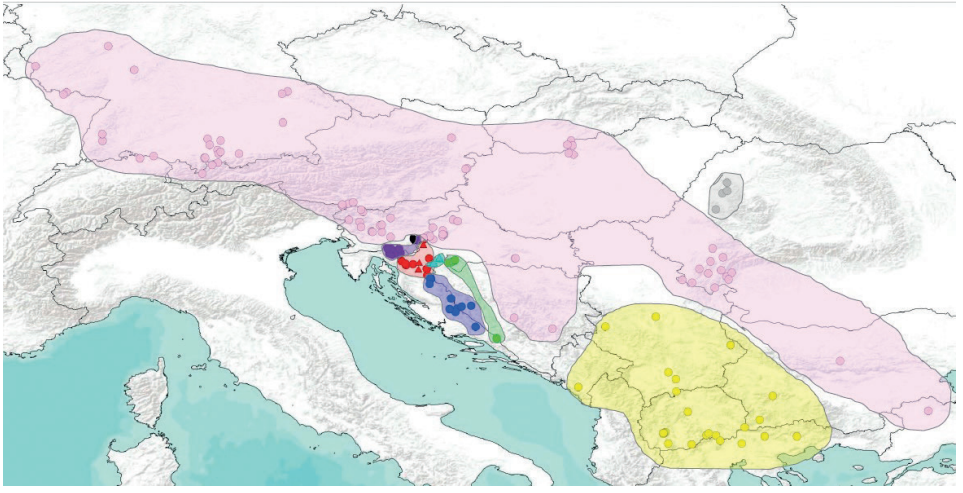

Fig. 1. Distribution of *Austropotamobius torrentium* phylogroups in Europe. Colours depict distribution of different mitochondrial phylogroups: pink – central and south-eastern Europe (CSE), purple – Gorski Kotar (GK), blue – Lika and Dalmatia (LD), red – Žumberak, Plitvice and Bjelolasica (ŽPB), green – Banovina (BAN) and black – Zeleni Vir (ZV), yellow – southern Balkans (SB), grey – Apuseni Mountain (APU) and turquoise – Kordun (KOR, new phylogroup, Lovrenčić *et al*., 2020).

the stone crayfish is listed as threatened and protected in Appendix III of the Bern Convention and Annexes II and V of the EU Habitats Directive (92/43/EEC and 97/62/EU). Furthermore, it was declared a Natura 2000 species in 2013.

Species distribution data

This study was performed on a dataset that included previously published work on the distribution of *A. torrentium* in Croatian freshwater bodies (Maguire & Gottstein-Matočec, 2004; Maguire *et al*., 2011; Klobučar *et al*., 2013; Maguire *et al*., 2018). Distribution data (i.e. the point occurrences) of each *A. torrentium* population were prepared using a geographic information system (ArcGIS). The dataset was split into three subsets according to the chronology of population sampling: (I) distribution dataset based on the historical data collected from the literature (Grube, 1861; Šoštarić, 1888; Car, 1901; Brusina, 1907; Entz, 1914; S. Karaman, 1929; M. Karaman, 1961, 1962, 1963; Albrecht, 1982, Sket, 1988; Sekulić *et al*., 1989; Delić, 1993; Gottstein, 1998; Gottstein & Kerovec, 1998; Gottstein *et al*., 1999; Maguire *et al*., 2002; Maguire & Gottstein-Matočec, 2004), and the fieldwork conducted in order to establish Natura 2000 sites (before 2014) (Maguire *et al*., 2011); (II) a distribution dataset that included additional populations discovered during the NIP project (EU Natura 2000 Integration Project with the main aim of gathering new distributional data about concerned taxonomic groups in Croatia in order to assess threat status and plan conservation activities; 2014-2016) (Maguire *et al*., 2018); (III) combined distribution dataset (all literature and documented occurrences available before 2016; I+II).

Gap Analysis

Gap analysis was applied to assess how much of *A. torrentium* diversity is covered by Natura 2000 network with a computation of the amount of populations included or excluded in order to detect areas that need better protection. It is a methodology that "identifies the gaps in representation of biological diversity (biodiversity) in areas managed exclusively or primarily for the long-term maintenance of populations of native species and natural ecosystems" (Scott *et al*., 1993). In gap analysis, a species is considered as a 'covered' by the conservation network if at least one occurrence was recorded inside the reserve network, while species is considered as a 'gap' if it is not represented in any of the protected areas (Rodrigues *et al*., 2004).

First, in order to determine how well Natura 2000 represents *A. torrentium* diversity in Croatia, we overlapped its occurrence data with the map of the Natura 2000 sites using ArcGIS. We used the distribution datasets mentioned above (I, II, III) together with GIS data layers (the entire Natura 2000 network for Croatia and SCI for *A. torrentium*; available at http://www.bioportal.hr/gis/) supplied by Institute for Environment and Nature Conservation. Layers were processed, and then combined to produce a single layer of Natura 2000 in Croatia as currently defined. Second, we evaluated the percentage of populations outside and inside Natura 2000 by analysing several settings: (a) distribution map of dataset I overlapped with a map of *A. torrentium*-designated Natura 2000 sites (SCI); (b) distribution map of dataset II overlapped with a map of *A. torrentium*-designated Natura 2000 sites (SCI); (c) distribution map of dataset III overlapped with a map of *A. torrentium*-designated Natura 2000 sites (SCI); (d) distribution map of dataset III overlapped with a map of the entire Natura 2000 network in Croatia.

RESULTS AND DISCUSSION

Natura 2000 and Stone Crayfish distribution overlapped

To the best of our knowledge, there have been no previously published studies on the effectiveness of the Natura 2000 network on preventing further stone crayfish losses. Thus, this study represents the first evaluation of the conservation value of Natura 2000 for a species sensitive to environmental and human activities, one that is ecologically important and presents a key component of the biodiversity in the freshwater habitats (Reynolds *et al*., 2013).

One of the first stages of systematic conservation planning for *A. torrentium* was the review of conservation areas in th e Natura 2000 network. Our gap analysis, including 61 populations of *A. torrentium* in Croatia belonging to seven major mtDNA phylogroups, showed that the Natura 2000 network performs well in representing its diversity, which is confirmed by high percentage of covered populations (Figs. 2-5, Tab. 1).

Overlapping the distribution map of *A. torrentium* populations (dataset I; occurrences recorded before 2014) with the *A. torrentium*-designated Natura 2000 sites (SCI) revealed that 39 populations (79.6%) were covered, while 10 (20.4%) were located outside the designated sites (Fig. 2, Tab. 1). It should be noticed that *A. torrentium* populations recorded in dataset I served as the basis for the designation of Natura 2000 sites.

Tab. 1. Number of populations per mitochondrial phylogroup in four different settings (a-d): (a) distribution map of dataset I overlapped with a map of *Austropotamobius torrentium*-designated Natura 2000 sites (SCI); (b) distribution map of dataset II overlapped with a map of *A. torrentium*-designated Natura 2000 sites (SCI); (c) distribution map of dataset III overlapped with a map of Natura 2000 sites designated for *A. torrentium* (SCI); (d) distribution map of dataset III overlapped with a map of the entire Natura 2000 network in Croatia. Central and south-eastern Europe (CSE), Gorski Kotar (GK), Lika and Dalmatia (LD), Žumberak, Plitvice and Bjelolasica (ŽPB), Banovina (BAN), Zeleni Vir (ZV), Kordun (KOR).

	Phylogroup	a	b	c	d
Number of populations covered by Natura 2000	GK	13	2	15	15
	ZV	1	/	1	1
	ŽPB	8	2	10	11
	LD	5	/	5	7
	CSE	11	1	12	13
	BAN	1	/	1	1
	KOR	/	/	0	0
Total		**39 (79.6%)**	**5**	**44 (73.3%)**	**48 (80.0%)**
Number of populations not covered by Natura 2000	GK	0	/	0	0
	ZV	0	/	0	0
	ŽPB	2	/	2	1
	LD	3	/	3	1
	CSE	3	2	5	4
	BAN	2	/	2	2
	KOR	/	4	4	4
Total		**10 (20.4%)**	**6**	**16 (26.7%)**	**12 (20.0%)**
Total number of populations		**49**	**11**	**60**	**60**

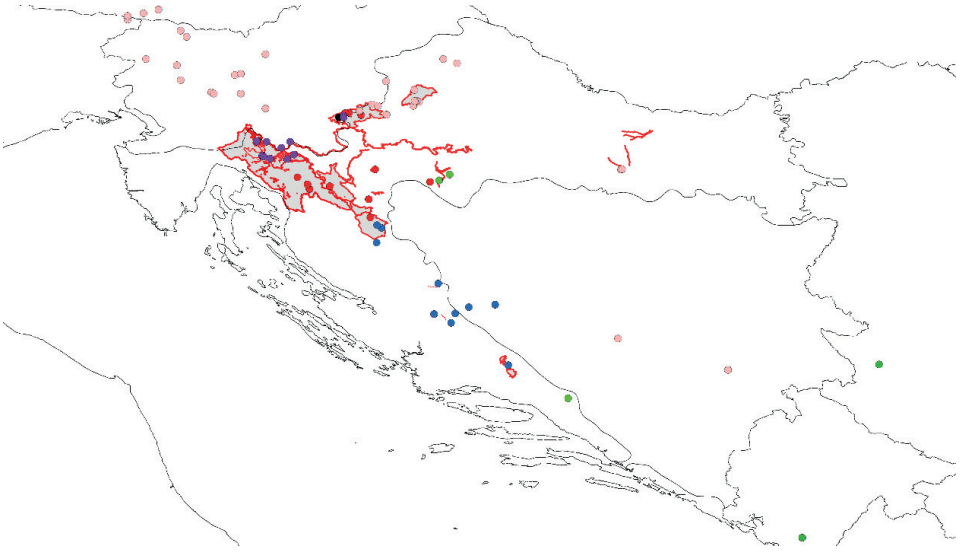

Fig. 2. Distribution map of recorded *A. torrentium* populations (dataset I) overlapped with the designated Natura 2000 sites for *A. torrentium* (SCI). Dots represent distribution data based on the literature and fieldwork conducted in order to establish Natura 2000 sites for *A. torrentium* (recorded occurrences before 2014). Colours depict different mitochondrial phylogroups present in Croatia: pink – central and south-eastern Europe (CSE), purple – Gorski Kotar (GK), blue – Lika and Dalmatia (LD), red – Žumberak, Plitvice and Bjelolasica (ŽPB), green – Banovina (BAN) and black – Zeleni Vir (ZV). Natura 2000-designated sites are represented by red lines and grey areas.

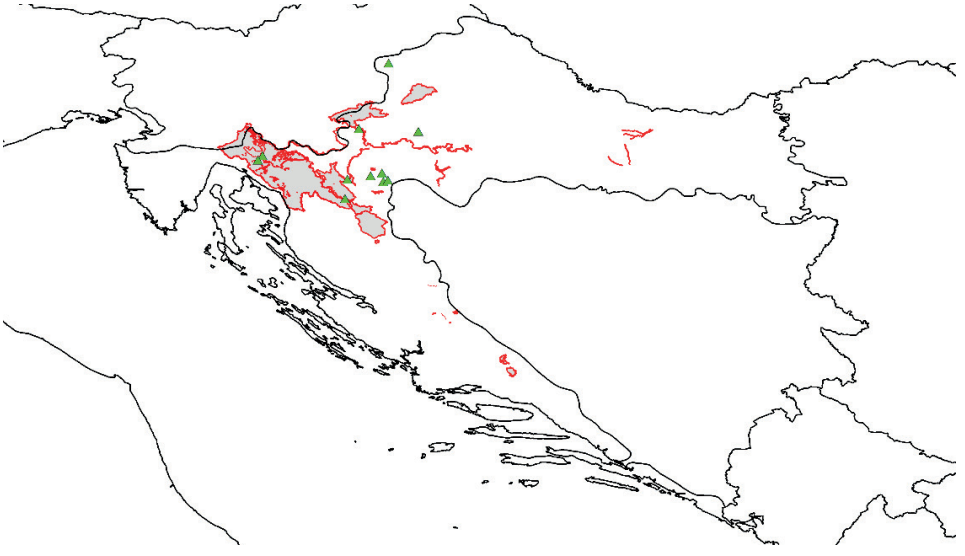

Fig. 3. Distribution map of additional *A. torrentium* populations discovered during the NIP project (dataset II; occurrences represented by green triangles recorded from 2014 to 2016) overlapped with the Natura 2000 sites designated for *A. torrentium* (SCI) that are represented by red lines and grey areas.

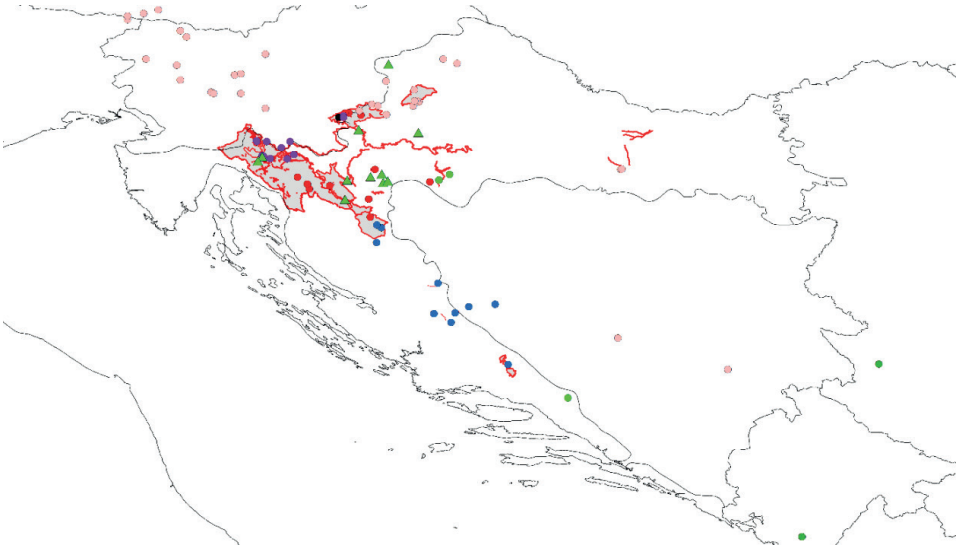

Fig. 4. Distribution map of all recorded *A. torrentium* populations in Croatia (dataset III; occurrences based on the literature and fieldwork conducted in order to establish Natura 2000 sites (different coloured dots represent different mitochondrial phylogroups) with additional populations discovered during the NIP project (green triangles) (all documented occurrences before 2016) overlapped with the Natura 2000 sites designated for *A. torrentium* (SCI; represented by red lines and grey areas).

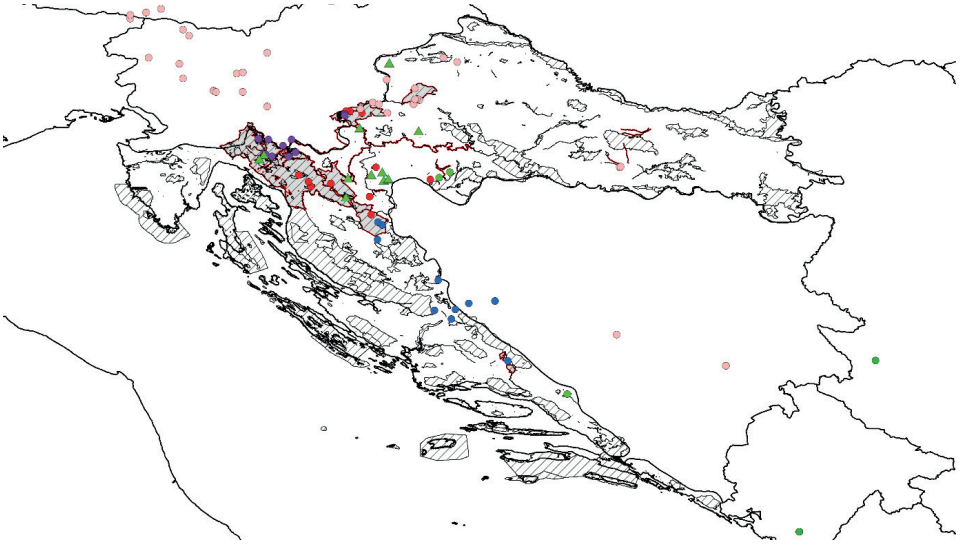

Fig. 5. Distribution map of all recorded *A. torrentium* populations in Croatia (dataset III; occurrences based on the literature and fieldwork conducted in order to establish Natura 2000 sites (different coloured dots represent different mitochondrial phylogroups) and additional populations discovered during the NIP project (green triangles) (all documented occurrences before 2016) overlapped with the entire Natura 2000 network in Croatia (SPAs and SACs for Croatia with SCI for *A. torrentium*). Natura 2000 designated sites (SCI for *A. torrentium*) are represented by red lines and grey areas, while SPAs and SACs for Croatia are represented by areas with black diagonal lines.

During the NIP project 11 new populations of *A. torrentium* were recorded (dataset II; recorded occurrences 2014-2016) (Maguire *et al*., 2018). Gap analysis revealed an additional five populations located inside the Natura 2000 sites designated previously for *A. torrentium*, while six populations were outside the protected area (Fig. 3, Tab. 1). Detailed molecular analyses of collected crayfish samples during NIP project revealed the existence of a new mtDNA phylogroup (phylogroup KOR in Fig. 1; Lovrenčić *et al*., 2020) that was not covered by the currently designated Natura 2000 sites for *A. torrentium*.

Our results using a combined distribution dataset (dataset III; all literature and documented occurrences available before 2016) showed that *A. torrentium*-designated Natura 2000 sites (SCI) covered the species range relatively well, with currently designated sites harbouring 73.3% of recorded populations in Croatia (Fig. 4, Tab. 1).

Overlapping the map of the entire Natura 2000 network in Croatia with a complete distribution map of *A. torrentium* (dataset III) revealed four additional populations included in the Natura 2000 network (altogether 48 populations out of 60). Hence, altogether 80.0% of recorded *A. torrentium* populations were covered by the Natura 2000 network (Fig. 5, Tab. 1).

The distribution of mitochondrial phylogroups and the number of populations per phylogroup are displayed in Fig. 1 and Tab. 1. Overlapping all the known distribution data with the entire Natura 2000 network in Croatia showed that the mtDNA phylogrups with the highest percentage of coverage were GK and ZV, which are among the oldest phylogroups in the species phylogenetic tree (all point occurrences covered by some level of protection), while the phylogroup with the least coverage was KOR (all occurrences outside Natura 2000). Phylogroup KOR represents a newly discovered genetically divergent lineage distributed in the Kordun region (Lovrenčić *et al*., 2020).

How Natura 2000 works for Crayfish in Croatia?

Freshwater ecosystems are among the most diverse habitats in the world and, at the same time, the most threatened by human activities (Strayer & Dudgeon, 2010). High intra- and inter-specific diversity are particularly widespread in freshwater environments, where the isolation of species with limited capacity for dispersal, such as freshwater crayfish, often leads to high genetic divergence. Erosion of the freshwater biodiversity at European level associated with habitat degradation, overexploitation, invasive alien species, pollution and climate change indicate the need to find an effective way of managing species. Therefore, the aim of maintaining existing biodiversity underlies most of the conservation efforts (e.g., Natura 2000) nowadays.

Our study results revelaed that the current Natura 2000 network in Croatia encompasses most of the areas with high diversity of *A. torrentium*, and in that sense, provides good protection (Figs. 2-5, Tab. 1). Even though this finding is in agreement with other studies evaluating the effectiveness of Natura 2000 (Verovnik *et al*., 2011; Mazaris *et al*., 2013; Kallimanis *et al*., 2015; Fois *et al*., 2017), there are numerous gap analyses revealing that coverage of species and ecosystems by existing networks of protected areas is insufficient for the long-term maintenance of biodiversity (Dietz & Czech 2005; Maiorano *et al*., 2006; O'Dea *et al*., 2006; Araújo *et al*., 2011; Jantke *et al*., 2011). Since some of the studies showing poor Natura 2000 network effectiveness were based on species that are not listed in EU directives (Mendoza-Fernandez *et al*., 2009; Jantke *et al*., 2011; Bagella *et al*., 2013; Maiorano *et al*. 2015), it was expected that our species of

interest would have a high level of coverage. *Austropotamobius torrentium* is, indeed, a Natura 2000 species, protected on both national and international level, with sites designated for ensuring its survival and persistence.

The Natura 2000 network in Croatia is conceived in such a way as to be able to prevent further *A. torrentium* diversity loss; nonetheless gap analysis based on protected area coverage alone does not necessarily reflect this efficacy (Carrizo *et al*., 2017; Hermoso *et al*., 2019). Although the Natura 2000 coverage may be satisfactory in terms of encompassing the recorded occurrences of this species, there are several drawbacks. For example, from a practical point of view, single occurrences within reserve networks and site protection alone are considered insufficient to ensure long-term survival and to safeguard freshwater biodiversity, especially of species with demanding habitat requirements, and do not take into account climate change (Rodrigues *et al*., 2004a; Carrizo *et al*., 2017). Global climate changes impact the size and extent of areas that may potentially be inhabited by species (Parmesan, 2006). So, in the case of *A. torrentium* , which is a cold-adapted species, we may assume distribution shifts to higher and colder habitats in the future under further climate change.

Moreover, an additional limitation of gap analysis is that it does not predict species viability and does not reveal previous habitat losses, which can lead to a misleading result in the context of present distribution (Jennings *et al*., 2000). Furthermore, despite the overlap with protected areas, many Natura 2000 sites in freshwater ecosystems of southern and eastern Europe are managed poorly (Carrizo *et al*., 2017). Since habitat destruction, climate change and invasive alien species present major threats to freshwater biodiversity (Cardinale *et al*., 2012; Castro *et al*., 2015), the efficacy of the Natura 2000 network must be enhanced by better local management of freshwater resources. Essential practical actions would require a range of activities/measures, from habitat restoration works to managing invasive species. An important and unavoidable activity that should be incorporated is building collaboration among various participants involved, including such stakeholders as local and state agencies, researchers, landholders and funding bodies (Blicharska *et al*., 2016; Carrizo *et al*., 2017).

Even though the gaps in the diversity coverage could be overcome by extension of Natura 2000 network, there is a strong emphasis on directing resources to the most threatened populations with high conservation value instead of constantly increasing the size of protected areas. Improving existing conservation sites rather than designating new ones should be the primary focus of the future efforts in increasing conservation outcomes (Hermoso *et al*., 2019). The Natura 2000 network is considered a rigid network due to the limited potential of adding new sites or adjusting the locations of existing sites (Orlikowska *et al*., 2016). However, there is a potential to improve its effectiveness through better management of sites and by implementing local legislation and regulations (Fois *et al*., 2017). Currently, management practices which are not receiving adequate attention in policy or implementation, low level and quality of public participation with lack of flexibility on the part of the authorities are the greatest challenges to the functioning of the Natura 2000 network (Blicharska *et al*., 2016). One of the focal points in the future conservation of threatened species such as the stone crayfish should be public acceptance and engagement (Davis *et al*., 2014; Blicharska *et al*., 2016; Fois *et al*., 2017). Developing and implementing guidelines for public participation are needed for successful conservation and improvement of the existing Natura 2000 network across Europe.

CONCLUSIONS

This study provides the first gap analysis that estimates the effectiveness of the Natura 2000 network in supporting and maintaining *A. torrentium* diversity in Croatia using a comprehensive and recently available species occurrence dataset. This analysis can serve as a model for other Natura 2000 species and as a base for possible extension of the Natura 2000 network.

Our results showed that the Natura 2000 network in Croatia covers species distribution relatively well, and, with good management, we could consider the Natura 2000 network well suited for the long-term conservation of *A. torrentium* diversity.

Since conservation planning implies securing the evolutionary potential of the species, it is important to point out that a recently discovered phylogroup from the Kordun region is not covered by the current Natura 2000 network; thus this region should be proposed as an additional area of protection in the future extension of the Natura 2000 network.

In order to achieve effective conservation plans, one of our future goals is to develop species distribution models under different climate change scenarios to effectively address future distribution under climate changes. Furthermore, since population genetics plays an important role in conservation planning, greater insight into genetic structure of the populations is needed for the identification of the populations that have the highest conservation value.

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3. RASPRAVA

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Integracijom rezultata niza bioloških disciplina (molekularna filogenija, populacijska genetika, geometrijska morfometrija, modeliranje povoljnosti staništa) u ovom istraživanju nastojala se utvrditi morfološka i genetska raznolikost potočnog i plemenitog raka te istražiti utjecaj klimatskih promjena i invazivnih stranih vrsta rakova na njihovu raznolikost. Navedeni podaci poslužit će kao kvalitetna osnova za izradu ciljanih konzervacijskih strategija i planova upravljanja s ove dvije osjetljive i ključne vrste slatkovodnih ekosustava. Rezultati su potvrdili postojanje velike intraspecijske genetske i morfološke raznolikosti potočnog i plemenitog raka u Hrvatskoj što upućuje na važnost njihovog očuvanja. Također, Bayesovskom analizom populacijske strukture utvrđena je specifična raspodjela genetske raznolikosti i postojanje više odvojenih genetskih grupa, dvije za plemenitog raka i osam za potočnog raka. Nadalje, modeli povoljnosti staništa pokazali su da bi klimatske promjene mogle imati veliki negativni utjecaj na buduću količinu potencijalno povoljnih staništa te posljedično smanjenje areala potočnog i plemenitog raka, ali i invazivnih vrsta. Ovo istraživanje predstavlja temelj za upravljanje i očuvanje genetskih resursa potočnog i plemenitog raka uslijed klimatskih promjena te identificiranje potencijalnih budućih klimatskih refugija.

3.1. Integrativni pristup u konzervaciji plemenitog raka

3.1.1. Morfološka raznolikost populacija plemenitog raka u Hrvatskoj

Morfološka raznolikost populacija plemenitog raka u Hrvatskoj istraživana je metodom geometrijske morfometrije (Znanstveni rad 1). Ustanovljeno je postojanje razlika u morfološkim značajkama prednjeg dijela glavopršnjaka između populacija ovisno o njihovoj pripadnosti različitim mitohondrijskim linijama (Linija 2, Linija 4 ili Linija 2/4 *sensu* Schrimpf i sur., 2014), genetskim grupama na temelju nuklearnih mikrosatelita (Genetska grupa I, Genetska grupa II), slivovima (Sava, Drava, Dunav) i tipovima staništa (lentičko, lotičko) (Znanstveni rad 1). Rezultati rekonstrukcije filogenetskih odnosa plemenitog raka (Znanstveni radovi 2 i 3) u velikoj su mjeri podudarni s rezultatima geometrijske morfometrije; populacije koje pripadaju dvjema različitim evolucijskim linijama imaju različit oblik glavopršnjaka (Znanstveni rad 1). Naime, genetske linije plemenitog raka evolucijski su mlade i većinom nepodržane u filogenetskoj rekonstrukciji (Schrimpf i sur., 2014.; Laggis i

sur., 2017.; Znanstveni rad 2), što je vidljivo i u njihovoj morfologiji. Nadalje, utvrđeno je da se dvije genetske grupe plemenitog raka razlikuju u obliku glavopršnjaka i pogotovo rostruma, što dijelom odražava pripadnost različitim stanišnim tipovima. Prilikom proučavanja genetske raznolikosti plemenitog raka na području zapadnog Balkana utvrđeno je grupiranje populacija prema riječnim slivovima (Znanstveni rad 2). U ovom istraživanju, utvrđene su morfološke razlike između tri sliva u Hrvatskoj, s osobito izraženim razlikama između jedinki iz dravskog i dunavskog sliva (Znanstveni rad 1). Nadalje, rezultati geometrijske morfometrije su pokazali da jedinke iz lentičkog staništa (stajačice) imaju užu te izduženiju glavu i rostrum, dok jedinke iz lotičkih staništa (tekućice) posjeduju širu i manju glavu i rostrum. Ovaj rezultat je usporediv s rezultatima istraživanja na vrsti *Samastacus spinifrons*, jedinoj južnoameričkoj vrsti iz porodice Parastacidae koja nastanjuje i rijeke i jezera (Rudolph i sur., 2016.). Jezerske populacije ove vrste imaju robusnije tijelo, izraženiji rostrum te izdužena i uža kliješta u usporedbi s riječnim populacijama. Slično je pronađeno i u istraživanju Perry i sur. (2013.) vrste *Orconectes rusticus*, u kojem jezerske populacije imaju veće i robusnije tijelo, izraženiji rostrum te izdužena i uža kliješta u usporedbi s riječnim populacijama. Uočene morfološke razlike između jedinki različitih staništa vjerojatno su povezane sa životnim uvjetima u kojima žive (Rudolph i sur., 2016.). Hidrodinamičan (vretenasti) oblik predstavlja morfološku prilagodbu za svladavanje otpora vode i veću pokretljivost. Rakovi iz lotičkih staništa imaju manje izražen rostrum što, prema Perryju i sur. (2013.), može olakšati kretanje među kamenjem i korijenjem korita tekućica te olakšati potragu za hranom i skloništem od predatora. Također, Perry i sur. (2013.) su prilikom proučavanja utjecaja brzine strujanja vode na veličinu i oblik vrste *O. rusticus*, uočili da jedinke koje žive u potocima s visokom brzinom strujanja vode su manjih tjelesnih proporcija od onih koje žive u jezerima ili potocima s manjom brzinom strujanja vode. Time je potvrđeno da povišenje brzine strujanja vode može utjecati na morfologiju slatkovodnih vrsta rakova, što je u skladu s rezultatima ovog istraživanja. Sposobnost organizma i/ili genotipa da stvara različit fenotip u različitim ekološkim uvjetima može objasniti postojanje razlika u morfologiji između rakova iz različitih staništa (Sommer, 2020.). Naime, genetska varijabilnost i fenotipska plastičnost igraju važnu ulogu u oblikovanju morfologije slatkovodnih rakova (Langerhans, 2008.; Haddaway i sur., 2012.; Perry i sur., 2013.; Znanstveni rad 1).

Većina prethodnih istraživanja morfološke raznolikosti populacija slatkovodnih vrsta rakova iz porodice Astacidae, uključujući plemenitog raka, temelji se na tradicionalnoj

morfometriji velikog broja značajki u kombinaciji s multivarijantnim statističkim analizama (Ghia i sur., 2006.; Sint i sur., 2005.; 2006.; Bertocchi i sur., 2008.; Bök i sur., 2010.; Maguire i Dakić, 2011.; Maguire i sur., 2017.; Berger i sur., 2018.). Geometrijska morfometrija prvi put je korištena za istraživanje morfološke varijabilnosti plemenitog raka (Znanstveni rad 1) i pokazala se kao dobar alat za detekciju morfoloških razlika unutar vrste te analizu varijabilnosti veličine i oblika prednjeg dijela glavopršnjaka. Značajna prednost geometrijske morfometrije jest proučavanje oblika neovisno o veličini i mogućnost grafičkog predstavljanja varijabilnosti oblika.

3.1.2. Genetska raznolikost i struktura populacija plemenitog raka u Hrvatskoj

Genetska raznolikost populacija plemenitog raka proučavana je analizom mitohondrijskih gena (*COI, 16S*) i nuklearnih mikrosatelitnih markera na području Hrvatske i Balkana (Znanstveni radovi 2 i 3).

Filogenetski odnosi populacija plemenitog raka rekonstruirani na temelju mitohondrijskih gena u skladu su s rezultatima dosadašnjih istraživanja (Schrimpf i sur., 2014.; Laggis i sur. 2017.). Ustanovljeno je postojanje šest prethodno utvrđenih evolucijskih linija s naznakom postojanja nove divergentne linije koja obuhvaća nove haplotipove iz Hrvatske i Slovenije (Znanstveni radovi 2 i 3). Hrvatske populacije plemenitog raka pripadaju dvjema evolucijskim linijama, L2 i L4 *sensu* Schrimpf i sur. (2014.), koje su također zabilježene i u drugim europskim zemljama. Velik broj zabilježenih kao i novootkrivenih haplotipova u ovom istraživanju idu u prilog činjenici o području zapadnog dijela Balkana, odnosno Hrvatske, kao vrućoj točki genetske raznolikosti plemenitog raka, ali i drugih slatkovodnih organizama (Hewitt, 2011.).

Međutim, u usporedbi s drugim vrstama rakova iz porodice Astacidae, plemenitog raka karakterizira niža raznolikost na razini mitohondrijske DNA i slabije genetsko strukturiranje, bez očiglednog geografskog obrasca. Rezultati filogenetičkih analiza održavaju slabo filogeografsko strukturiranje i prisutnost istih haplotipova čak i u geografski udaljenim populacijama što je u skladu s rezultatima prijašnjih istraživanja koja su pokazala da je današnja rasprostranjenost i genetska struktura plemenitog raka oblikovana geološko-klimatskim događajima u prošlosti i čestim antropogenim translokacijama koje su djelomično narušile njegovu prirodnu genetsku strukturu (Schrimpf i sur., 2014.; Laggis i sur., 2017.; Znanstveni radovi 2 i 3). Procijenjena vremena odvajanja linija pokazala su da se plemeniti

rak kao vrsta diverzificirao relativno nedavno, što se odražava i na slabim podržanostima grana na filogenetskom stablu (Znanstveni rad 2).

Korištenjem molekularnog sata na temelju mitohondrijskih gena procijenjeno je vrijeme divergencije genetskih linija plemenitog raka (Znanstveni rad 2). Sva odvajanja dogodila su se u geološkom razdoblju pleistocena kojeg karakteriziraju snažne oscilacije klimatskih i ekoloških uvjeta za vrijeme izmjene glacijala i interglacijala. Promjene klime i reljefa utjecale su na gubitak kontakta između rezidualnih slatkovodnih površina i na taj način pridonijele genetskoj divergenciji vrste te stvorile mozaike evolucijskih linija (Hewitt, 2004.). Današnja rasprostranjenost genetskih linija plemenitog raka pokazuje navedeni uzorak divergencije u skladu s fenomenom postojanja izoliranih južnih glacijalnih refugija tijekom pleistocena iz kojih je tekla postglacijalna rekolonizacija ostatka Europe (Hewitt, 2004.).

Populacijsko-genetičke analize na temelju mikrosatelita ustanovile su postojanje visoke genetske raznolikosti populacija plemenitog raka u Hrvatskoj (Znanstveni radovi 2 i 3). Ustanovljen je velik broj alela, alelnog bogatstva, privatnih alela, i visoke razine uočene heterozigotnosti te su identificirane populacije s različitim vrijednostima indeksa genetske raznolikosti. Ovi se podaci mogu koristiti za razvoj programa reintrodukcije ili repopulacije. Genetska raznolikost populacija na području Balkana, i pogotovo Hrvatske (Znanstveni radovi 2 i 3), viša je u usporedbi s raznolikosti populacija iz središnje i sjeverne Europe (Gross i sur., 2013.; Schrimpf i sur., 2014.; 2017.; Laggis i sur., 2017.; Panicz i sur., 2019.). Značajne pozitivne vrijednosti koeficijenta parenja u bliskom srodstvu i velik udio homozigotnih jedinki koji ukazuju na potencijalni gubitak genetske raznolikosti pronađeni su u samo dvije populacije na području Hrvatske. Također, rezultati ukazuju da su neke populacije nedavno prošle kroz usko grlo, što u malim izoliranim populacijama s ograničenim protokom gena može dovesti do smanjenja efektivne veličine populacije, njenog fitnesa, adaptivne vrijednosti i u konačnici nestanka (Frankham 2005).

Određivanje genetske strukture hrvatskih populacija plemenitog raka provedeno je na temelju Bayesovske analize pomoću programa STUCTURE. Utvrđeno je da se populacije u Hrvatskoj grupiraju u dvije genetske grupe te da postoje populacije koje sadrže jedinke iz obje grupe što odražava pripadnost zajedničkoj ancestralnoj grupi ili antropogene translokacije. Ovakva genetska struktura djelomično se poklapa s rezultatima filogenije na temelju mitohondrijskih gena (Znanstveni rad 3). Vrijednosti koeficijenta diferencijacije i rezultati analize molekularne varijance ukazuju na postojanje srednje do visoke razine genetske diferenciranosti između istraživanih populacija koja je odraz njihove prirodne izoliranosti i

smanjenog protoka gena. U usporedbi s globalnim vrijednostima koeficijenta diferencijacije iz prijašnjih istraživanja, vrijednost dobivena u istraživanju genetske diferenciranosti hrvatskih populacija (Znanstveni rad 3) veća je od one između populacija središnje i sjeverne Europe (Schrimpf i sur., 2014.; Gross i sur., 2013.), ali manja od one između grčkih populacija na jugu Balkanskog poluotoka (Laggis i sur., 2017.) i populacija koje su uključivale cijeli Balkanski poluotok (Znanstveni rad 2). Visoka razina genetske raznolikosti i diferenciranosti populacija iz ovog istraživanja u skladu je s očekivanjem s obzirom da se Hrvatska nalazi na području zapadnog Balkana koji je vruća točka bioraznolikosti i jedan od potvrđenih glacijalnih refugija plemenitog raka (Schrimpf i sur., 2014.).

3.1.3. Potencijalni utjecaj klimatskih promjena i invazivnih vrsta rakova na genetsku raznolikost plemenitog raka

Modeli povoljnosti staništa pokazali su da će populacije plemenitog raka biti ugroženije budućim klimatskim promjenama, nego širenjem invazivnih vrsta rakova. Rezultati sugeriraju gubitak oko 87 % trenutno povoljnih staništa do kraja stoljeća te pomicanje potencijalno povoljnih staništa plemenitog raka prema sjeverozapadu, što može negativno utjecati na ukupnu razinu genetske raznolikosti ove vrste. Naime, velik dio genetske raznolikosti plemenitog raka bit će izgubljen ukoliko populacije zaista nestanu zbog gubitka staništa uslijed klimatskih promjena. Preklapanjem podataka o genetskoj raznolikosti i budućoj rasprostranjenosti ove vrste ustanovljeno je da su najugroženije populacije ujedno one koje imaju najveću i jedinstvenu genetsku raznolikost. Gubitak takvih populacija u kontinentalnoj Hrvatskoj mogao bi negativno utjecati na razinu ukupne genetske raznolikosti plemenitog raka na europskoj razini.

Odgovor vrste na klimatske promjene velikim dijelom ovisi o postojećoj genetskoj raznolikosti koja omogućuje prilagodbu na nove uvjete okoliša te sposobnosti rasprostranjivanja pojedine vrste. Slatkovodni rakovi su osobito ugrožena skupina jer ih karakteriziraju često geografski i genetski izolirane populacije te niska mogućnost rasprostranjivanja. U istraživanju provedenom u okviru Znanstvenog rada 3 po prvi put su predložena takva područja koje možemo smatrati klimatskim refugijima za plemenitog raka. Potencijalni budući refugiji bitni za opstanak plemenitog raka tijekom predstojećih klimatskih promjena identificirani su u slatkovodnim ekosustavima na nižim nadmorskim visinama alpinske regije (Linija 2, Genetska grupa II) te u šljunčarama i mrtvajama uz rijeke Dravu i Savu u sjeverozapadnom dijelu kontinentalne Hrvatske (Linija 4, Genetska grupa I). S

obzirom da su ova područja izvan mogućnosti prirodnog rasprostranjivanja plemenitog raka i nedostupna zbog prirodnih barijera širenja, bit će potrebno premještanje potpomognuto od strane čovjeka. Asistirana migracija je često predložena kao konzeravacijska strategija za ublažavanje učinaka klimatskih promjena i invazivnih vrsta (Butt i sur., 2019.). Naime, repopulacija i reintrodukcija nativnih vrsta rakova smatra se temeljem njihove konzervacije (Souty-Grosset i Reynolds, 2009.; Kozák i sur., 2011.; Olden i sur., 2011.; Capinha i sur., 2013.), stoga su rezultati ovog istraživanja od posebne važnosti za upravljanje i očuvanje genetskih resursa plemenitog raka uslijed klimatskih promjena i invazivnih vrsta (Znanstveni rad 3). Potencijalna područja reintrodukcije trebala bi biti smještena unutar prirodnog areala vrste, zadovoljavati njihove ekološke potrebe i biti sigurna od invazivnih stranih vrsta rakova i patogena koje oni nose (Armstrong i Seddon, 2008.; Kozák i sur., 2011.; Souty-Grosset i Reynolds, 2009.; Chucholl, 2017.).

Modeli potencijalne rasprostranjenosti invazivnih vrsta uslijed budućih klimatskih promjena, predvidjeli su drastično smanjenje povoljnih staništa za signalnog i bodljobradog raka u Hrvatskoj (Znanstveni rad 3). Shodno tome, potencijalna podrucja gdje bi se areali ovih invazivnih vrsta rakova mogli u budućnosti preklopiti s arealom plemenitog raka će gotovo nestati. Sličan obrazac smanjenja buduće rasprostranjenosti invazivnih vrsta rakova ustanovljen je u istraživanju Préau i sur. (2020.) gdje u slučaju bjelonogog raka i signalnog raka ne dolazi do preklapanja buduće rasprostranjenosti unatoč preklapanju njihovih ekoloških niša. Također, Zhang i sur. (2021.) su potvrdili da će signalni rak izgubiti veliki dio povoljnih staništa u Europi do kraja stoljeća uslijed klimatskih promjena.

3.1.4. Smjernice za konzervaciju plemenitog raka u Hrvatskoj

Kombinacijom podataka različitih bioloških disciplina postavljeni su temelji konzervacije plemenitog raka u Hrvatskoj. Integracijom morfoloških, genetičkih i ekoloških podataka identificirane su populacije plemenitog raka i područja koja imaju najveću konzervacijsku vrijednost i kojima treba dati najveći prioritet u zaštiti. U istraživanju provedenom unutar Znanstvenog rada 3 su prvi put identificirani potencijalni budući klimatski refugiji za plemenitog raka te populacije koje su potencijalni izvor jedinki za reintrodukcije i repopulacije. Nadalje, predložene su jedinice upravljanja plemenitog raka vezane uz genetske linije i grupe unutar pojedinih rijeka i pritoka. S obzirom da su današnja rasprostranjenost i genetska struktura plemenitog raka pod velikim utjecajem antropogenih translokacija, njegove mitohondrijske linije nisu striktno monofiletske i ne predstavljaju evolucijski značajne

jedinice (Scrhimpf i sur., 2017.); identifikacija jedinica upravljanja u ovom slučaju smatra se prikladnijom.

Osim prepoznavanja genetske i morfološke raznolikosti te procjene utjecaja klimatskih promjena i invazivnih vrsta, prilikom izrade konzervacijskih planova za ugrožene vrste potrebno je procijeniti učinkovitost postojećih zaštićenih područja u očuvanju njihove raznolikosti, usporedbom rasprostranjenosti vrste s opsegom zaštićenih područja (Rodrigues i sur., 2004.; Jennings, 2000.; Bosso i sur., 2016.). U svrhu procjene učinkovitosti nacionalnih zaštićenih područja i pan-europske mreže Natura 2000 u očuvanju raznolikosti plemenitog raka u Hrvatskoj putem gap analize (eng. gap analysis), korišteni su podaci o rasprostranjenosti ove vrste koji su preklopljeni s kartom zaštićenih područja (Znanstveni rad 4). Rezultati analize su pokazali da su populacije plemenitog raka umjereno dobro pokrivene zaštićenim područjima budući da se 50 % populacija nalazi unutar zaštićenih područja. Velik broj populacija s jedinstvenom genetskom raznolikošću, a koje su istovremeno ugrožene budućim klimatskim promjenama nalaze se izvan zaštićenih područja; kontinentalna regija u kojoj se nalazi većina prirodnih populacija plemenitog raka najmanje je pokrivena zaštićenim područjima (Znanstveni radovi 3 i 4). Istraživanje Carrizo i sur. (2017.) pokazalo je da se mnogim slatkovodnim Natura 2000 područjima u južnoj i istočnoj Europi upravlja loše i da trenutna razina zaštite nije dovoljna. Naime, samo proglašenje zaštite područja ne može postići očuvanje vrijednosti zbog kojih je proglašeno, već je potrebno trajno pratiti promjene i provesti aktivnosti za poboljšanje stanja područja, kao što su upravljanje stranim invazivnim vrstama, restauracija slatkovodnih staništa, povećanje sudjelovanja javnosti te suradnje lokalnih i državnih agencija, znanstvenika i tijela za financiranje (Blicharska i sur., 2016.; Carrizo i sur., 2017.).

3.2. Integrativni pristup u konzervaciji potočnog raka

3.2.1. Morfološka raznolikost populacija potočnog raka u Hrvatskoj

Morfološka raznolikost potočnog raka u Hrvatskoj i morfološke značajke koje razlikuju jedinke iz različitih populacija proučavane su na temelju geometrijske i tradicionalne morfometrije te analize merističkih značajki (Znanstveni radovi 5 i 6). Dobiveni podaci korišteni su za rješavanje taksonomskog statusa mitohondrijskih filogrupa i identifikaciju potencijalnih donorskih populacija za buduće reintrodukcije i/ili repopulacije. Također, ovo istraživanje potvrdilo je prednji dio glavopršnjaka, rostrum i apeks (vrh rostruma) kao dobre

morfološke značajke za razlikovanje različitih populacija, što je u skladu s prethodnim istraživanjima slatkovodnih rakova (Taylor, 2000.; Bertocchi i sur., 2008.; Scalici i sur., 2010.; Rudolph i sur., 2016.). Geometrijska morfometrija, kao noviji pristup u istraživanju morfološke varijabilnosti po prvi je puta upotrebljena u istraživanju morfologije potočnog raka i pokazala se korisnom za opisivanje oblika glavopršnjaka (Znanstveni rad 5). Rezultati ukazuju na postojanje fenotipske plastičnosti i visoke intraspecijske morfološke varijabilnosti koja odražava dvojaki utjecaj okoliša i genetske pozadine.

Geometrijsko-morfometrijska analiza pokazala je da su populacije potočnog raka u Hrvatskoj morfološki raznolike (Znanstveni rad 5). Naime, utvrđeno je da se populacije dijelom razlikuju u morfološkim značajkama prednjeg dijela glavopršnjaka (od vrha rostruma do cervikalne brazde) ovisno o pripadnosti različitim mitohondrijskim linijama te da se rezultati morfoloških istraživanja djelomično poklapaju s rezultatima molekularno-filogenetičkih analiza (Klobučar i sur., 2013.). Istraživanje merističkih značajki na velikom broju jedinki potočnog raka utvrdilo je veliku intraspecijske varijabilnost te postojanje raznolikosti unutar i između različitih filogrupa (Znanstveni rad 6). Ustanovljeno je da su morfološke značajke varijabilne i da ne postoji determinacijska značajka koja bi pomogla razlikovanju filogrupa te omogućila mogući opis vrsta. Također, nedostatak dentikulacije na donjem rubu antenalnog egzopoda, koje Pârvulescu (2019.) ističe kao jednu od glavnih morfoloških karakteristika za razlikovanje novo opisane vrste *A. bihariensis*, pokazala se kao iznimno varijabilna značajka u istraživanju provedenom unutar Znanstvenog rada 6 (nedostatak dentikulacije zabilježen je u svim filogrupama). Stoga, velika intraspecijska varijabilnost morfometrijskih i merističkih značajki dovodi u pitanje validnost vrste *A. bihariensis*. Nadalje, zbog nepodudarnosti rezultata implementiranih metodologija (morfometrija, meristika, molekularne metode razgraničavanja potencijalnih vrsta), uključujući nedostatak morfoloških značajki koje bi bile specifične za svaku filogrupu, genetski i geografski izolirane filogrupe potočnog raka proglašene su kriptičnim podvrstama i evolucijski značajnim jedinicama (Znanstveni radovi 5 i 6).

3.2.2. Genetska raznolikost i struktura populacija potočnog raka u Hrvatskoj

Istraživanje genetske raznolikosti i rekonstrukcija filogenetskih odnosa upotrebom mitohondrijskih i nuklearnih DNA markera potvrdili su veliku genetsku raznolikost i složenu filogeografsku strukturu potočnog raka u Hrvatskoj (Znanstveni rad 6). Utvrđeno je postojanje devet genetski i geografski izoliranih monofiletskih filogrupa koje karakterizira

bazalna politomija kao odraz brze diverzifikacije ancestralnih linija (Trontelj i sur., 2005.; Klobučar i sur., 2013.; Jelić i sur., 2016.; Pârvulescu i sur., 2019.). Tijekom ovog istraživanja i opsežnog uzorkovanja prethodno neistraženog područja ustanovljeno je postojanje nove divergentne filogrupe na području Korduna (filogrupa KOR), što ukazuje na potencijalno postojanje još neotkrivene raznolikosti.

Utvrđena velika genetska raznolikost na razini mitohondrijske DNA, ovim istraživanjem potvrđena je i na razini nuklearne DNA. Naime, pronalazak informativnog nuklearnog markera pokazao se kao problem u istraživanju filogenije slatkovodnih rakova zbog sporije stope evolucije i niske genetske varijabilnosti nuklearne DNA (Chu i sur., 2001.; Blaha i sur., 2016.; Jelić i sur., 2016.). Tako i u istraživanju provedenom u okviru Znanstvenog rada 6, upotrebom nuklearne regije *ITS2* potvrđene su gotovo sve filogrupe, ali filogenetski odnosi između njih su ostali neriješeni. Mitohondrijski geni pokazali su se kao informativniji filogenetski biljezi jer sadrže veći broj varijabilnih mjesta koja osiguravaju dovoljan broj informativnih značajki za filogenetičke analize pa time i bolje razlučivanje filogenetskih odnosa.

Kompleksna filogeografska struktura i današnja rasprostranjenost filogrupa potočnog raka odraz su paleoklimatskih i paleogeoloških procesa koji uključuju alpsku i dinaridsku orogenezu, proces okršavanja, pleistocenske izmjene glacijala i interglacija te postpleistocensku rekolonizaciju kroz dunavski sliv (Trontelj i sur., 2005.; Klobučar i sur., 2013., Znanstveni rad 6). Šest upotrijebljenih kalibracija molekularnog sata (tri geološke i tri molekularne) pokazale su da su geografski izolirane i duboko divergentne filogrupe nastale uslijed intenzifikacije tektonskih gibanja tijekom pliocena i početkom pleistocena te procesa okršavanja koje je fragmentiralo paleohidrografiju ovog područja (Znanstveni rad 6). Navedene promjene klime i reljefa utjecale su na gubitak kontakta između rezidualnih slatkovodnih površina i na taj način pridonijele genetskoj diverzifikaciji vrste (Trontelj i sur., 2005.; Klobučar i sur., 2013.). Otkrićem nove filogrupe potočnog raka na području planinskog masiva Apuzeni u Rumunjskoj, Parvulescu i sur. (2019.) predložili su novi pristup u objašnjenju današnje rasprostranjenosti filogrupa potočnog raka. Kao mogući uzrok odvajanja apuzenske filogrupe od blisko srodnih filogrupa sjeverno-središnje dinaridske regije (GK, LD, BAN, ZV, ŽPB) navode tektonsko pomicanje i odvajanje Tisa-Dacia mikroploče, koje je započelo u miocenu (prije 16 milijuna godina) i prema autorima prenijelo dio ancestralne populacije potočnog raka do današnjeg položaja u gorju Apuzeni. Navedeni geološki događaj

koristili su za geološko kalibriranje molekularnog sata čiji su rezultati pokazali puno starije odvajanje filogrupa unutar potočnog raka, ali i unutar cijelog roda *Austropotamobius*. Obzirom da navedeni proces nije bio u skladu s prethodnim istraživanjima i tri molekularne kalibracije, u istraživanju u sklopu Znanstvenog rada 6 ponuđeno je drugačije objašnjenje podrijetla apuzenske filogrupe. Korištena je nova geološka kalibracija za procjenu vremena divergencije filogrupa prema kojoj je uspostava hidrološke veze između dviju rijeka, paleo-Dunava i paleo-Tise, prije 5.3 milijuna godina mogla omogućiti pretku apuzenske filogrupe prirodno širenje i kolonizaciju prema gorju Apuzeni. Upotrijebljena geološka kalibracija rezultirala je procijenjenim vremenima odvajanja koje su u skladu s prethodnim istraživanjima i molekularnim kalibracijama. Također, jedno od potencijalnih objašnjenja podrijetla apuzenske filogrupe jest da su brojne populacije potočnog raka postojale u sjevernim područjima areala, ali nisu preživjele klimatske uvjete tijekom pleistocenskih glacijala, za razliku od populacije u Apuzenskom gorju. Naime, planinski masiv Apuzeni identificiran je kao jedan od pleistocenskih refugija i mogao je omogućiti preživljavanje u krškim staništima, slično populacijama sjeverno-središnje dinaridske regije (Pullaiah, 2019.).

Taksonomski status filogrupa potočnog raka i pitanje postojanja kriptičnih vrsta nastojao se riješiti opsežnijim istraživanjem morfometrijskih i merističkih značajki te molekularnim metodama razgraničavanja potencijalnih vrsta (eng. species delimitation) (Znanstveni rad 6). Međutim, primijenjene morfološke analize i metode razgraničavanja vrsta rezultirale su nepodudarnošću morfoloških i genetskih podataka. Morfološke analize (geometrijska i tradicionalna morfometrija te meristika) pokazale su da je intraspecijska i unutarpopulacijka raznolikost prevelika i da ne postoji jedinstvena morfološka značajka za razlikovanje filogrupa. Metode razgraničavanja potencijalnih vrsta (ABGD, GMYC, bPTP, mPTP) predložile su nerealno visok broj potencijalnih vrsta (9-30) koje nužno ne predstavljaju samo neotkrivenu raznolikost, već i izolirane populacije koje prolaze proces specijacije (Hofmann i sur., 2019.; Loretán i sur., 2020.). Naime, učinkovitost molekularnih metoda razgraničavanja vrsta osjetljiva je na brojne čimbenike kao što su visoka supstitucijska stopa korištenog gena, broj uključenih vrsta, nejednoliko uzorkovanje, varijabilna veličina populacija, razina protoka gena te politomije i neriješena filogenetska grananja (Dellicour i Flot, 2015.; Ahrens i sur., 2016.; Luo i sur., 2018.; Hofmann i sur., 2019.). Nadalje, veći broj predloženih potencijalnih vrsta može biti posljedica tendencije metoda razgraničavanja da precjenjuju broj vrsta i odražavaju genetsku strukturu podataka pritom prikazujući populacijsku strukturu unutar vrste (Sukumaran i Lacey, 2017.). Stoga smo

u ovom istraživanju zauzeli konzervativni pristup u svrhu izbjegavanja „taksonomske inflacije“ (Isaac i sur., 2004.) (i istovremenog ostavljanja mogućnosti postojanja kriptičnih vrsta i/ili podvrsta) te filogrupe proglasili kriptičnim podvrstama i evolucijski značajnim jedinicama potočnog raka. Filogrupe su genetski visoko divergentne, ali evolucijski mlade, u prijelaznoj fazi između genetskih linija i vrsta. Vrijednosti genetskih udaljenosti unutar i između grupa ukazuju na relativno stare evolucijske linije ili vrste u nastanku.

Postojanje sedam genetski i geografski izoliranih evolucijski značajnih jedinica na području Hrvatske potvrđeno je i korištenjem mikrosatelitnih markera (Znanstveni rad 7). Rezultati populacijsko-genetičkih analiza pokazali su srednju razinu genetske raznolikosti populacija, što zapravo predstavlja nižu genetsku raznolikost od očekivane, čime je dodatno potvrđena ugroženost ove vrste. Prosječno najveću genetsku raznolikost pokazuju populacije filogrupa/ESU BAN, CSE, KOR i ZV s visokim brojem alela, vrijednostima alelnog bogatstva i heterozigotnosti, dok populacije iz filogrupa GK, LD i ŽPB pokazuju niže vrijednosti. Genetska raznolikost populacija potočnog raka u Hrvatskoj je umjerena do visoka u usporedbi s rezultatima Iorgu i sur. (2011.), Vorburger i sur. (2014.), Berger i sur. (2018.), te Pârvulescu i sur. (2020.). Indeksi genetske raznolikosti, izraženi kao broj alela, alelno bogatstvo i uočena heterozigotnost, bili su viši u populacijama iz Hrvatske nego u populacijama iz Austrije, Švicarske i Njemačke (Vorburger i sur., 2014.; Berger i sur., 2018.), ali niži od populacija iz Rumunjske (Iorgu i sur., 2011.; Pârvulescu i sur., 2020.). Populacije analizirane u istraživanju provedenom unutar Znastvenog rada 7 pokazuju veći broj privatnih alela u odnosu na populacije iz drugih istraživanja (Berger i sur., 2018., Pârvulescu i sur., 2020.). Prisutnost velikog broja privatnih alela i alelnog bogatstva ukazuje na područja velike genetske raznolikosti koja su kroz duga razdoblja bila stabilna i u kojima su postojali uvjeti za kontinuirane evolucijske procese kao što su npr. glacijalni refugiji (Hewitt, 2011.). Dosadašnja istraživanja rakova porodice Astacidae pokazala su da je područje zapadnog Balkana poznato kao vruća točka raznolikosti ovih vrsta (Trontelj i sur., 2005.; Klobučar i sur., 2013.; Schrimpf i sur., 2014.; Jelić i sur., 2016.; Znanstveni radovi 2 i 6). Ovo područje služilo je kao glacijalni refugij iz kojeg je krenula rekolonizacija središnjih i sjevernih dijelova Europe nakon posljednjeg ledenog doba, što je vidljivo i iz podataka o smanjenoj genetskoj raznolikosti rakova u tim područjima (Gross i sur., 2013., Bláha i sur., 2016., Berger i sur., 2018.). Nadalje, postojanje velikog broja privatnih alela i jedinstvene genetske varijabilnosti može biti ključno u dugoročnom odgovoru vrste na selekcijske pritiske. Populacije bez privatnih alela, s nižom heterozigotnošću i alelnim bogatstvom mogle bi imati

problema u prilagodbi na promjene u okolišu. Smanjenje genetske raznolikosti povećava rizik od izumiranja populacija zbog smanjenja fitnesa, što može dovesti do smanjenja efektivne veličine populacije i većeg rizika od stohastičkog demografskog izumiranja (Markert i sur., 2010.). Suprotno tome, populacije s većim šansama za dugoročni opstanak su one s visokom razinom genetske raznolikosti koja promiče opstanak populacije i jamči adaptivni potencijal populacija suočenih s brzim promjenama okolišnih pritisaka (Frankham, 2003.). U većini populacija zabilježene su više vrijednosti koeficijenta parenja u bliskom srodstvu što ukazuje na parenje u bliskom srodstvu, a predstavlja udio uočenih heterozigota u odnosu na očekivani broj heterozigota u populaciji i označava gubitak raznolikosti (Frankham, 2005.). Mnoge populacije potočnog raka su osjetljive na promjene u okolišu i stresore zbog male veličine i prirodne izoliranosti. Naime, populacija s većim udjelom homozigota i niskim vrijednostima indeksa genetske raznolikosti su osjetljive na parenje u bliskom srodstvu što može dodatno smanjiti količinu genetske raznolikosti populacija i posljedično dovesti do gubitka adaptivnog evolucijskog potencijala vrste (Frankham 2005). Nadalje, brojne istraživane populacije pokazale su odstupanje od Hardy-Weinbergove ravnoteže što može biti uzrokovano prisustvom nul alela, parenjem u bliskom srodstvu ili Wahlundovim efektom. Odstupanje od Hardy-Weinbergove u istraživanim populacijama je najčešće združeno sa znatnim deficitom heterozigota i pozitivnim vrijednostima F_{IS} koeficijenta ili zabilježenim nul alelima. Smanjenje heterozigotnosti i općenito genetske raznolikosti može se objasniti i učinkom uskog grla (eng. bottleneck). Međutim, korištenjem mikrosatelita koji se smatraju informativnim markerima za demografske događaje unutar zadnjih 10-50 generacija (Peery i sur., 2012.), nije pronađeno da je ijedna od istraživanih populacija nedavno prošla kroz „usko grlo“ (Znanstveni rad 7).

Populacije potočnog raka u Hrvatskoj geografski su strukturirane, s ograničenim protokom gena i slabom povezanosti. Vrijednosti fiksacijskog indeksa upućuju na visoku diferencijaciju i genetsku izoliranost populacija koje odražavaju njihovu prirodnu izoliranost i slabu pokretljivost. Vrijednosti fiksacijskog indeksa su manje između populacija unutar istih evolucijski značajnih jedinica, a veće što su populacije geografski udaljenije. Također, vrijednosti fiksacijskog indeksa za hrvatske populacije veće su u odnosu na one procijenjene u drugim istraživanjima potočnog raka (Vorburger i sur., 2014.; Berger i sur., 2018.; Pârvulescu i sur., 2020.) što ukazuje na dulju izoliranost populacija i ograničeni protok gena, što je u skladu s procijenjenim vremenima odvajanja filogrupa potočnog raka u miocenu i pliocenu (Znanstvenim rad 6).

Utvrđivanje genetske strukture populacija potočnog raka provedena je na temelju Bayesovske analize pomoću programa STRUCTURE. Utvrđeno je da se populacije u Hrvatskoj grupiraju u osam genetskih grupa što je podudarno s evolucijski značajnim jedinicama (Znanstveni rad 7). Ovakva genetska struktura populacija se u velikoj mjeri podudara s filogeografskom strukturom potočnog raka na temelju mtDNA (Znanstveni rad 6). Utvrđeni obrazac genetske strukture posljedica je navedenih geološko-klimatskih procesa u prošlosti i krškog reljefa koji predstavlja fragmentirano stanište s fizičkim barijerama za rasprostranjivanje i protok gena. Poznato je da divergencija staništa može uzrokovati ekološku izolaciju i genetsku diferencijaciju čak i geografski bliskih populacija kao što je utvrđeno i u našim istraživanjima (Znanstveni rad 6).

3.2.3. Potencijalni utjecaj klimatskih promjena i invazivnih vrsta rakova na genetsku raznolikost potočnog raka

Modeli povoljnosti staništa pokazali su da bi klimatske promjene mogle značajno smanjiti ukupnu površinu povoljnih staništa potočnog raka na području vruće točke njegove genetske raznolikosti do kraja 21. stoljeća, čime bi izravno ugrozile njegovu genetsku raznolikost i jedinstvene evolucijske linije (Znanstveni rad 7). Naime, rezultati predviđaju gubitak oko 80 % trenutno povoljnih staništa do 2070. godine te pomicanje potencijalno povoljnih područja potočnog raka prema sjeverozapadu i višim nadmorskim visinama, kao i izvan trenutno određenih Natura 2000 područja za ovu vrstu. Slično, veliko smanjenje povoljnih staništa za potočnog raka predviđa se na području cijelog areala vrste; Capinha i sur. (2013.) predviđaju gubitak oko 52 % trenutno povoljnih staništa za potočnog raka na europskoj razini. Preklapanjem podataka o genetskoj raznolikosti i budućoj rasprostranjenosti ove vrste ustanovljeno je da će 44 % istraživanih populacija i 65 % zabilježenih točaka potočnog raka potencijalno biti izgubljeno, uključujući tri visoko-divergentne, geografski izolirane evolucijske linije malih areala (Znanstveni rad 7). Takav gubitak genetske raznolikosti i evolucijskih linija mogao bi značajno smanjiti sposobnost prilagodbe ove vrste na brze promjene u okolišu i ugroziti dugoročni opstanak (Wright i sur., 2008.). Smanjenje genetske raznolikosti povećava potencijalni rizik od izumiranja populacija zbog smanjenja fitnesa, što može dovesti do daljnjeg smanjenja veličine populacije i većeg rizika od stohastičkog demografskog izumiranja (Markert i sur., 2010.). Evolucijski značajne linije potočnog raka karakterizira mali areal i jedinstvena genetska arhitektura, stoga im treba dati

prioritet u konzervaciji jer značajno doprinose ukupnoj raznolikosti vrste, ali i lokalnoj biološkoj raznolikosti (Crandall, 1998.; Yarra i Magoulick, 2019.).

U istraživanju provedenom u okviru Znanstvenog rada 7 po prvi put su predložena područja koje možemo smatrati klimatskim refugijima za potočnog raka tijekom predstojećih klimatskih promjena. Potencijalni budući refugiji bitni za opstanak potočnog raka uključuju područja uz sjeverozapadnu granicu Hrvatske i prema staništima na višim nadmorskim visinama u Sloveniji. S obzirom da su ova područja nedostupna zbog prirodnih granica širenja, predložene strategije očuvanja ove vrste uključuju programe repopulacije i reintrodukcije. Potreba za ovim strategijama naglašena je i zbog potencijalno smanjene učinkovitosti trenutnih Natura 2000 područja određenih za potočnog raka u budućnosti (Znanstveni rad 7).

Modeli potencijalne rasprostranjenosti signalnog raka u Hrvatskoj uslijed budućih klimatskih promjena predvidjeli su veliko smanjenje povoljnih staništa, stoga su područja gdje bi se eventualno njegova rasprostranjenost mogla preklopiti s rasprostranjenošću potočnog raka u budućnosti virtualno nestala (Znanstveni rad 7). Smanjeno preklapanje rasprostranjenosti između nativnih i invazivnih vrsta rakova kao odgovor na klimatske promjene zabilježeno je ranijim istraživanjima na području određenih europskih zemalja (Gallardo i Aldridge, 2013.; Preau i sur., 2020., Lovrenčić i sur., 2022.). Također, istraživanjem u sklopu Znanstvenog rada 7 nije utvrđeno preklapanje ekoloških niša potočnog i signalnog raka u Hrvatskoj. Suprotno tome, Chucholl (2016.) je utvrdio preklapanje ekoloških niša potočnog i signalnog raka u Njemačkoj. Slično je pronađeno u istraživanju Préau i sur. (2020.) gdje u slučaju bjelonogog raka i signalnog raka ne dolazi do preklapanja buduće rasprostranjenosti u Francuskoj, ali se njihove ekološke niše preklapaju.

3.2.4. Smjernice za konzervaciju potočnog raka u Hrvatskoj

Integracijom morfoloških, genetičkih i ekoloških podataka postavljeni su temelji konzervacije potočnog raka u Hrvatskoj. Identificirane su populacije i područja koja imaju najveću konzervacijsku vrijednost i kojima treba dati najveći prioritet u zaštiti te su predložene evolucijski značajne jedinice i jedinice upravljanja. Također, prvi put su identificirani potencijalni budući klimatski refugiji za potočnog raka te populacije koje su potencijalni izvor jedinki za reintrodukcije i repopulacije. Nadalje, procijenjena je

učinkovitost Natura 2000 područja u očuvanju raznolikosti potočnog raka kroz gap analizu koja se temelji na preklapanju karata rasprostranjenosti potočnog raka i Natura 2000 određenih područja korištenjem GIS programskog paketa (Znanstveni rad 8). Rezultati su pokazali da postojeća mreža Natura 2000 u Hrvatskoj obuhvaća 73,3 % populacija potočnog raka te uključuje i područja njegove najveće raznolikosti (Znanstveni rad 8), s izuzetkom novootkrivene filogrupe na području Korduna (Znanstveni rad 6). Visok postotak pokrivenosti populacija je očekivan s obzirom da je potočni rad jedna od Natura 2000 vrsta, s 25 područja određenih posebno za ovu vrstu. Pri izradi budućih planova konzervacije ove vrste posebnu pažnju treba obratiti na novootkrivene populacije koje ujedno predstavljaju evolucijski divergentne linije.

4. ZAKLJUČAK

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- Molekularno-filogenetičke analize na temelju mitohondrijske i nuklearne DNA potvrdile su veliku genetsku raznolikost potočnog i plemenitog raka u Hrvatskoj; paleogeološki i paleoklimatski procesi u prošlosti oblikovali su današnju rasprostranjenost evolucijskih linija.

- Integracija rezultata različitih analiza (molekularna filogenija, populacijska genetika, geometrijska morfometrija) omogućila je identifikaciju populacija s visokom i niskom raznolikosti za potrebe upravljanja i konzervacije ovim dvjema ugroženim vrstama.

- Unutar plemenitog raka potvrđeno je postojanje šest evolucijskih mitohondrijskih linija koje predstavljaju jedinice upravljanja, uz naznaku postojanja nove linije koja sadrži haplotipove iz slovenskih i hrvatskih populacija.

- Populacije plemenitog raka u Hrvatskoj posjeduju veliku genetsku raznolikost i visoku razinu diferencijacije te predstavljaju dvije genetske grupe na temelju analize mikrosatelitnih lokusa.

- Istraživanjem mitohondrijskih gena utvrđeno je sedam genetski i geografski izoliranih filogrupa potočnog raka u Hrvatskoj koje su na temelju svoje jedinstvenosti i malog areala proglašene kriptičnim podvrstama ili evolucijski značajnim jedinicama. U ovom istraživanju otkrivena je nova filogrupa na području Korduna što ukazuje na postojanje još neotkrivene raznolikosti u neistraženim područjima.

- Populacije potočnog raka u Hrvatskoj su genetski i geografski strukturirane te predstavljaju osam genetskih grupa na temelju analize mikrosatelitnih lokusa. Karakterizira ih umjerena genetska raznolikost i visoka diferencijacija populacija s ograničenim protokom gena. U brojnim populacijama zabilježene su značajne vrijednosti koeficijenta parenja u bliskom srodstvu koje potencijalno objašnjavaju niže vrijednosti parametara genetske raznolikosti.

- Klimatske promjene potencijalno će uzrokovati negativne promjene u rasprostranjenosti potočnog i plemenitog raka smanjenjem areala i klimatski povoljnih staništa.

- Modeli povoljnosti staništa pokazali su da će populacije plemenitog i potočnog raka biti ugroženije budućim klimatskim promjenama, nego širenjem invazivnih vrsta rakova. Rezultati sugeriraju gubitak oko 87 % trenutno povoljnih staništa do kraja stoljeća za plemenitog raka te 80 % za potočnog raka, što može negativno utjecati na ukupnu razinu njihove genetske raznolikosti.

- Preklapanjem podataka o genetskoj raznolikosti i budućoj rasprostranjenosti potočnog i plemenitog raka ustanovljeno je da su najugroženije populacije ujedno one koje imaju najveću i jedinstvenu genetsku raznolikost. Njihov nestanak može negativno utjecati na ukupnu razinu genetske raznolikosti, a time i na adaptivni potencijal vrsta.

- Potencijalni budući klimatski refugiji nalaze se u područjima za koje su modeli predvidjeli da predstavljaju dugoročno klimatski povoljna područja i u koje će rakovi biti uneseni ili već postoje na području.

- Rezultati ovog istraživanja mogu se iskoristi za izradu konzervacijskih strategija i planove upravljanja ovim ključnim vrstama slatkovodnih ekosustava.

5. POPIS LITERATURE

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6. ŽIVOTOPIS

Leona Lovrenčić upisuje preddiplomski smjer Biologije na Prirodoslovno-matematičkom fakultetu (PMF) Sveučilišta u Zagrebu 2009. godine. Titulu sveučilišne prvostupnice biologije stječe 2012. godine te se iste godine upisuje na diplomski studij smjera Eksperimentalna biologija na PMF-u u Zagrebu. Diplomski rad „Molekularno-filogenetička i filogeografska analiza populacija vrste *Holandriana holandrii* (C. Pfeiffer, 1828) (Mollusca: Gastropoda) u Hrvatskoj“ izrađuje 2015. godine te ga uspješno obranjuje pod mentorstvom dr.sc. Martine Podnar i izv. prof. dr. sc. Jasne Lajtner. Po završetku studija radi kao pripravnica u DNA laboratoriju Hrvatskog prirodoslovnog muzeja u Zagrebu te kao profesorica biologije u srednjoj školi. Godine 2018. upisuje doktorski studij Biologije i počinje raditi na Biološkom odsjeku PMF-a u Zagrebu kao znanstveni novak - asistent na znanstvenom projektu „Klimatske promjene i invazivne vrste - utvrđivanje utjecaja na bioraznolikost nativnih slatkovodnih rakova i pastrva i njihova konzervacija“ pod vodstvom prof. dr. sc. Ivane Maguire. Tijekom doktorskog studija znanstveno se usavršava izvan Hrvatske (Slovenija, Sveučilište u Kopru; Estonija, Sveučilište u Tartu; Španjolska, Sveučilište u Valenciji) i sudjeluje na osam tečajeva i radionica. Objavila je 11 znanstvenih radova u časopisima s međunarodnom recenzijom (na njih 8 je prvi autor), aktivno sudjelovala na međunarodnim znanstvenim skupovima s osam priopćenja te domaćim skupovima sa šest priopćenja.

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Gross R*, **Lovrenčić L***, Jelić M, Grandjean F, Đuretanović S, Simić V, Burimski O, Bonassin L, Groza M, Maguire I (2021) Genetic diversity and structure of the noble crayfish populations in the Balkan Peninsula revealed by mitochondrial and microsatellite DNA markers. PeerJ 9: e11838.

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Lovrenčić L, Temunović M, Gross R, Grgurev M, Maguire I (2021) Conservation of the Croatian noble crayfish populations confronted with invasive crayfish and climate change – when population genetics meet species distribution modelling. In: Books of abstracts, p. 42. 4th Croatian Symposium on Invasive Species - with international participation, Zagreb, Croatia. (usmeno priopćenje)

Lovrenčić L, Gross R, Kovačević M, Maguire I (2021). Genetic diversity and population structure of the noble crayfish in Croatia. Book of Abstracts - 5. PhD Student Symposium, Faculty of Science, University of Zagreb, p. 215-216. (postersko priopćenje)

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Lovrenčić L, Podnar M, Besendorfer V, Šašić M, Toth V, Koren T, Tvrtković N (2017) The Balkan Green-veined White (*Pieris napi balcana*): did the barcoding approach resolve its taxonomic status? Book of Abstracts, p. 7. 20th European Congress of Lepidopterology, Podgora, Croatia. (usmeno priopćenje)

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Radionice i tečajevi

Tečaj "Conservation Genomics" (Physalia-courses, Berlin, Njemačka, 24h)
Tečaj "Introduction to statistics in R" (Physalia-courses, Berlin, Njemačka, 24h)
Tečaj "Ecological niche modelling in R" (Physalia-courses, Berlin, Njemačka, 30h)
Tečaj "Introduction to Qgis" (Exaatoo, Hrvatska, Zagreb, 24h)
Tečaj "Geometric morphometrics and phylogeny" (Transmitting Science, Španjolska, 40h)
Tečaj "Introduction to R" (Sveučilište u Zagrebu, SRCE, 20h)
Tečaj "Modern plant and animal applied genomics driven by genotype and sequence data" (Sveučilište u Zagrebu, Agronomski fakultet, 18h)
Radionica "Remote sensing for Conservation & Biodiversity" (NASA's ARSTE, 6h)

Znanstveno i stručno usavršavanje

Znanstveno usavršavanje izvan Hrvatske, mobilnost u svrhu stručne prakse u okviru Erasmus+ programa ključne aktivnosti 1 unutar programskih zemalja, Sveučilište u Valenciji, Španjolska (2 mjeseca).

Znanstveno usavršavanje izvan Hrvatske, prekogranična akademska mobilnost prema visokoškolskim i znanstvenim ustanovama, Sveučilište u Tartu, Estonija (2 tjedna).

Znanstveno usavršavanje izvan Hrvatske, prekogranična akademska mobilnost prema visokoškolskim i znanstvenim ustanovama, Sveučilište na Primorskom, Slovenija (1 tjedan).