

# Utjecaj ekoloških čimbenika na genetičku varijabilnost poljskog jasena (*Fraxinus angustifolia* Vahl, Oleaceae)

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Sveučilište u Zagrebu

PRIRODOSLOVNO-MATEMATIČKI FAKULTET  
BIOLOŠKI ODSJEK

Martina Temunović

**UTJECAJ EKOLOŠKIH ČIMBENIKA NA  
GENETIČKU VARIJABILNOST  
POLJSKOG JASENA  
(*Fraxinus angustifolia* Vahl, OLEACEAE)**

DOKTORSKI RAD

Zagreb, 2013.



University of Zagreb

FACULTY OF SCIENCE  
DIVISION OF BIOLOGY

Martina Temunović

**INFLUENCE OF ECOLOGICAL  
FACTORS ON GENETIC VARIATION OF  
*Fraxinus angustifolia* Vahl (OLEACEAE)**

DOCTORAL THESIS

Zagreb, 2013.

Ovaj je doktorski rad izrađen na Zavodu za šumarsku genetiku, dendrologiju i botaniku Šumarskog fakulteta Sveučilišta u Zagrebu, pod vodstvom prof. dr. sc. Joze Franjića te na Botaničkom zavodu Biološkog odsjeka PMF-a Sveučilišta u Zagrebu pod vodstvom prof. dr. sc. Zlatka Libera, u sklopu Sveučilišnog poslijediplomskog doktorskog studija Biologije pri Biološkom odsjeku Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu. Dio istraživanja proveden je u laboratoriju za ekologiju, sistematiku i evoluciju (*Laboratoire Ecologie, Systématique et Evolution, CNRS UMR 8079*) Sveučilišta Paris-Sud 11 u Francuskoj, a sufinanciran je od strane bilateralnog projekta „Potencijalni utjecaj klimatskih promjena na vrste jasena na Mediteranu“ u okviru programa COGITO 2011./12. (šifra projekta: 25031UM), od strane europskog projekta „EVOLTREE“ u okviru FP7 programa, od strane projekta Hrvatskih šuma „Istraživanja morfološke varijabilnosti hrvatskih hrastova“ (šifra projekta: 1.1.27.), te stipendijama Hrvatske zaklade za znanost (šifra projekta: 03.01/69) i Vlade Francuske Republike.

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## **TEMELJNA DOKUMENTACIJSKA KARTICA**

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Sveučilište u Zagrebu  
Prirodoslovno-matematički fakultet  
Biološki odsjek

Doktorski rad

### **Utjecaj ekoloških čimbenika na genetičku varijabilnost poljskog jasena (*Fraxinus angustifolia* Vahl, Oleaceae)**

Martina Temunović

Zavod za šumarsku genetiku, dendrologiju i botaniku, Šumarski fakultet Sveučilišta u  
Zagrebu, Svetošimunska 25, 10000 Zagreb, Hrvatska

Ciljevi ove disertacije bili su utvrditi genetičku varijabilnost populacija poljskog jasena u različitim staništima na području Hrvatske i Europe, istražiti utjecaj ekoloških čimbenika i klimatskih promjena na unutarvrstu genetičku varijabilnost, te istražiti razinu i mehanizme hibridizacije s običnim jasenom. U mediteranskoj regiji Hrvatske utvrđena je značajno niža unutarpopulacijska genetička raznolikost i veća međupopulacijska divergencija u odnosu na kontinentalnu regiju. Nadalje, varijabilnost okoliša značajno je korelirana s genetičkom varijabilnošću. Rezultati ukazuju da heterogeni okoliš potiče ekološko i genetičko odvajanje populacija, da istraživane populacije u Hrvatskoj potencijalno predstavljaju dva ekotipa (kontinentalni i mediteranski), te potvrđuju važnu ulogu ekoloških čimbenika u oblikovanju genetičke varijabilnosti. U Europi je utvrđen gradijent genetičke varijabilnosti koja značajno opada u dva smjera: od zapada prema istoku, te od sjevera prema jugu. Predviđene klimatske promjene ukazuju na mogućnost pomicanja rasprostranjenosti poljskog jasena i njegovih hibrida prema višim geografskim širinama, kao i na negativan utjecaj na ukupnu razinu genetičke varijabilnosti i adaptivni potencijal vrste. Buduća utočišta tijekom klimatskih promjena za poljski jasen predviđena su u sjevernom dijelu današnjeg areala. Naime, u ovim područjima nalaze se populacije s najvišom genetičkom varijabilnošću, a stanište će i u budućnosti ostati povoljno. Stvaranje hibridnih populacija između poljskog i običnog jasena omogućeno je na područjima gdje im se ekološke niše preklapaju, dok mraz te ljetne temperature i oborine ograničavaju stvaranje hibridnih zona.

(110 stranica, 6 slika, 113 literaturnih navoda, jezik izvornika hrvatski)

Rad je pohranjen u središnjoj biološkoj knjižnici Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu, Marulićev trg 20/II, 10000 Zagreb

**Ključne riječi:** *Fraxinus angustifolia*, genetička varijabilnost, heterogenost okoliša, modeli ekološke niše, klimatske promjene, hibridne zone

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Prof. dr. sc. Sven Jelaska  
Prof. dr. sc. Zlatko Šatović

Rad prihvaćen: 11. rujna 2013.

## **BASIC DOCUMENTATION CARD**

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University of Zagreb  
Faculty of Science  
Division of Biology

Doctoral thesis

### **Influence of ecological factors on genetic variation of *Fraxinus angustifolia* Vahl (Oleaceae)**

Martina Temunović

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Aims of this dissertation were to determine genetic variation of narrow-leaved ash populations across divergent habitats in Croatia and Europe, to explore the influence of ecological factors and climate changes on genetic variation, and to examine the degree and mechanisms of hybridization with common ash. In Croatia, significantly lower genetic diversity and higher differentiation was revealed in the Mediterranean region when compared to the Continental region. In addition, genetic variation was significantly correlated with the environmental variation. Results suggest that environmental heterogeneity may promote genetic and ecological divergence of populations, that known populations in Croatia may represent two divergent ecotypes and confirm an important role of ecological factors in shaping genetic variation. A bidirectional cline of genetic diversity was revealed across Europe: it was declining significantly from West to East and from North to South. Climate warming may enable northwards expansion of *F. angustifolia* and its hybrids. Consequently, ongoing climate changes may negatively affect the overall genetic diversity and possibly adaptive potential of this species. Results indicate that refugia from climate change are potentially located in the northerly parts of the current distribution, where core high-diversity populations occur and suitable habitat is predicted to remain stable under future climate. Hybrid populations between *F. angustifolia* and *F. excelsior* are mostly found in areas where the niches of the two species are predicted to overlap. Number of days of frost in January, summer precipitation and summer temperature can potentially limit the extent of hybrids.

(110 pages, 6 figures, 113 references, original in Croatian)

Thesis deposited in the Central Biological Library, Division of Biology, Faculty of Science, University of Zagreb, Marulićev trg 20/II, 10000 Zagreb

**Keywords:** *Fraxinus angustifolia*, genetic variation, environmental heterogeneity, ecological niche modelling, climate change, hybrid zones

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Reviewers: Dr. Toni Nikolić, Associate Prof.  
Dr. Sven Jelaska, Associate Prof.  
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## **1. UVOD**

## 1.1. Genetička varijabilnost

Genetička varijabilnost predstavlja ukupnu raznolikost molekula nasljeđa (gena) između jedinki, populacija i različitih taksonomskih kategorija, ali najčešće se odnosi na varijabilnost populacija unutar iste vrste. Bez genetičke varijabilnosti neki od osnovnih mehanizama evolucije poput prirodne selekcije ne bi mogli djelovati. Recentna genetička varijabilnost i genetička struktura populacija neke vrste rezultat su djelovanja osnovnih mikroevolucijskih procesa na nekom prostoru: prirodne selekcije, mutacije, genetičkog pomaka (eng. drifta) i protoka gena. Prostorna i vremenska raspodjela jedinki, populacija, okolišnih čimbenika i povoljnog staništa može znatno utjecati na razinu i distribuciju genetičke varijabilnosti. Genetička varijabilnost neke vrste odražava njen adaptivni evolucijski potencijal i omogućava joj bolju prilagodbu na različite uvjete okoliša i preživljavanje u nepovoljnim uvjetima. Veća genetička raznolikost čini vrstu otpornijom i bolje prilagođenom za opstanak u promjenjivim uvjetima okoliša, kao i u uvjetima stresa (primjerice prošle i sadašnje klimatske promjene, ili onečišćenje okoliša). Stoga, razumijevanje postanka i poznavanje razine i strukture genetičke varijabilnosti ostaje temeljno pitanje evolucijske biologije i jedan je od neophodnih preduvjeta za razvoj učinkovitih mjera zaštite i planova upravljanja, osobito kod gospodarski važnih vrsta.

## 1.2. Prostorna i vremenska raspodjela genetičke varijabilnosti

Ako je protok gena između populacija prostorno ograničen, zbog primjerice ograničenog rasprostiranja plodova i sjemenki ili pak zbog ograničenja oprašivanja, dolazi do formiranja karakterističnog uzorka prostorne raspodjele genetičke varijabilnosti uslijed procesa koji nazivamo izolacija uslijed udaljenosti („*Isolation by distance*“; Wright 1943). Najjednostavnije rečeno, to znači da očekujemo kako će populacije koje su geografski bliže biti genetički sličnije i da genetička udaljenost između populacija raste linearno s geografskom udaljenošću. Ova pretpostavka se može lako testirati uspoređujući genetičku i geografsku udaljenosti između populacija. Važno je pritom naglasiti kako je izolacija uslijed udaljenosti isključivo neutralan proces na koji okoliš nema utjecaja i koji dovodi do formiranja neutralne genetičke strukture populacija. Međutim, ukoliko genetička varijabilnost populacija prati varijabilnost u okolišu, a populacije koje žive u sličnom staništu su genetički sličnije, bez obzira na njihovu geografsku udaljenost, to ukazuje da ekološki čimbenici (temperatura, padaline, nadmorska visina, ali i primjerice povoljnost staništa) utječu na

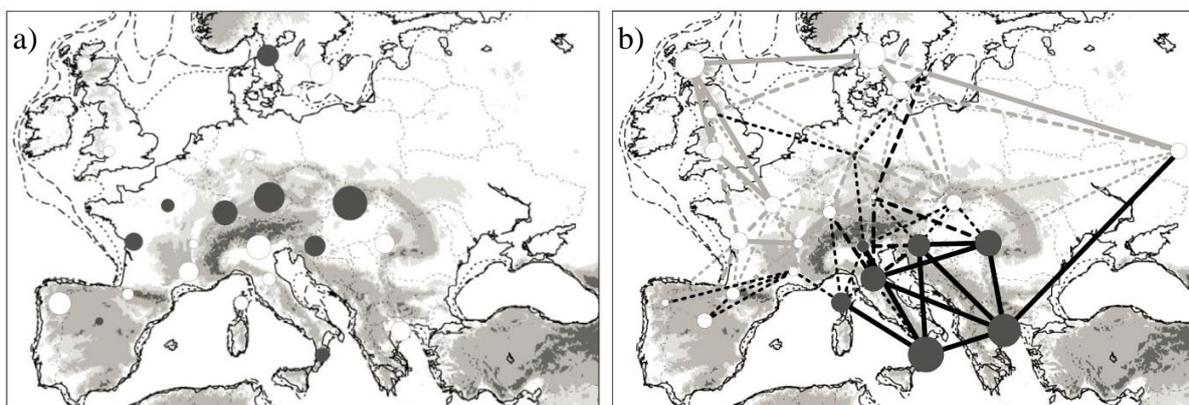
genetičku diferencijaciju populacija (Bockelmann i sur. 2003; Pilot i sur. 2006). Ukoliko je varijabilnost ekoloških čimbenika značajno korelirana s genetičkom varijabilnošću, takav uzorak je nedavno opisan kao izolacija uslijed djelovanja okoliša („*isolation by environmental distance*“; Mendez i sur. 2010) i sugerira interakciju prirodne selekcije (moguća lokalna adaptacija) i neutralnih mikroevolucijskih procesa (npr. protok gena, genetički drift) (Gram i Sork 2001; Parisod i Christin 2008; Sork i sur. 2010). Kako je varijabilnost ekoloških čimbenika često i sama geografski strukturirana, to treba prilikom analize uzeti u obzir.

Postoji još nekoliko važnih hipoteza koje predviđaju kako bi se teoretski trebao mijenjati uzorak prostorne raspodjele genetičke varijabilnosti duž geografskog areala određene vrste. Prema centralno-marginalnoj hipotezi očekuje se da jedinke i populacije određene vrste budu najbrojnije u središtu svog areala gdje su uvjeti staništa najpovoljniji, te da postepeno postaju sve rjeđe prema granicama areala gdje uvjeti postaju suboptimalni (Vucetich i Waite 2003; Eckert i sur. 2008). Kao posljedica, očekuje se da populacije na periferiji areala budu manje, međusobno udaljenije i prostorno izoliranije, što dovodi do smanjenog protoka gena između marginalnih populacija, te naposljetku do njihove genetičke divergencije i smanjene razine genetičke raznolikosti (Vucetich i Waite 2003; Eckert i sur. 2008). Opadanje genetičke raznolikosti od središta prema marginama areala sugerira da upravo središnje populacije imaju najveću genetičku raznolikost, a time i najveći adaptivni potencijal, odnosno sposobnost prilagodbe na promjenjive uvjete okoliša. Stoga bi upravo populacije u središtu areala trebale imati najveću konzervacijsku vrijednost.

Suprotno centralno-marginalnoj hipotezi, za mnoge vrste umjerenog pojasa Sjeverne hemisfere prostorni uzorak genetičke varijabilnosti je drugačiji (Hewitt 2000) jer su u prošlosti bile izložene ekstremnim promjenama klime za vrijeme izmjena glacijala i interglacijala posljednjeg ledenog doba koje je nastupilo u kvartaru (prije otprilike 2 milijuna godina) i čiji vrhunac je bio u pleistocenu (prije cca. 20.000 godina). Naime, ledeno doba je uzrokovalo masovne migracije živih bića i drastično utjecalo na njihovu rasprostranjenost, a time i genetičku varijabilnost. Primjerice, široko rasprostranjene vrste diljem Europe su za vrijeme glacijala bile potisnute daleko na jug, sklanjajući se pred debelim ledenim pokrivačem koji je pokrivao veći dio europskog kontinenta. Glavna pribježišta za europsku floru i faunu, tzv. glacijalni refugiji, nalazili su se na tri mediteranska poluotoka (Balkanski, Apeninski i Iberijski) gdje je klima tijekom ledenog doba ostala povoljna za preživljavanje većine vrsta (Hewitt 2000). Po završetku posljednje oledbe, vrste su se počele ponovo širiti iz utočišta u sjevernije dijelove areala pomoću učinka osnivača (eng. „*founder effect*“). Kao

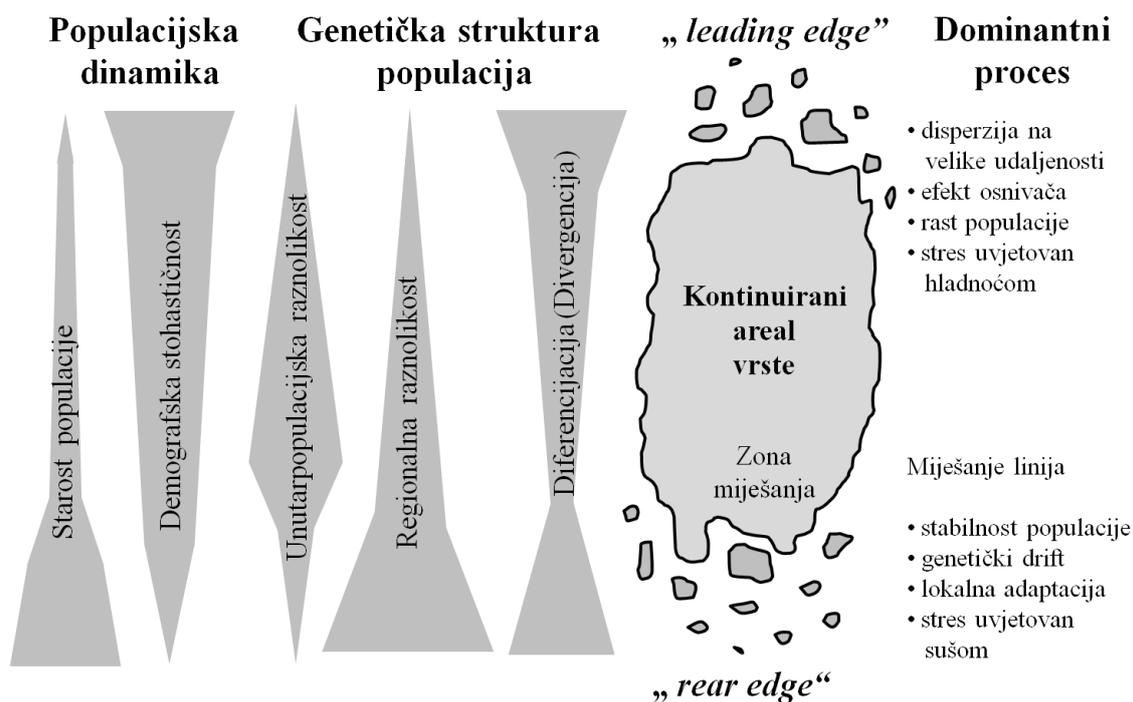
genetička posljedica ovih migracija javlja se povećana genetička raznolikost južnih refugijalnih populacija, te postepeno opadanje genetičke raznolikosti prema sjevernijim populacijama, tzv. “*leading edge expansion*” teorija ili “*southern richness versus northern purity*” uzorak genetičke raznolikosti (Hewitt 2000). Ovaj uzorak potvrđen je brojnim filogeografskim istraživanjima na mnogim vrstama, uključujući drvenaste vrste umjerenog pojasa (Magri i sur. 2006). Dugotrajna izolacija populacija u različitim refugijima dovela je također do njihove povećane genetičke divergencije ili specijacije.

Međutim, mnoge vrste umjerenog pojasa se ne uklapaju u predloženi sjever-jug gradijent genetičke raznolikosti. Za mnoge drvenaste vrste utvrđeno je primjerice da je njihova unutarvrsta genetička raznolikost najveća u središnjoj Europi, što je vjerojatno posljedica spajanja i miješanja divergentnih rekolonizacijskih linija iz različitih refugija na srednjim geografskim širinama (Slika 1a; Petit i sur. 2003). Petit i sur. (2003) su također istaknuli kako se genetički najdivergentnije šume u Europi nalaze u mediteranskom području u Italiji, na Korzici, te na Balkanu, uključujući Hrvatsku (Slika 1b). Dodatno, za tipične mediteranske drvenaste vrste nedavno je utvrđen i specifični gradijent opadanja genetičke raznolikosti od istoka prema zapadu (Fady i Conord 2010).



**Slika 1.** Genetička raznolikost (a) i divergencija (b) 25 istraživanih šuma u Europi. U svakoj šumi su uzorkovane 22 drvenaste vrste, a analiza je provedena temeljem kloroplastne DNA (kloroplastnih haplotipova); crni krugovi – vrijednosti više od prosječnih; bijeli krugovi: vrijednosti niže od prosječnih; veličina kruga je proporcionalna odstupanju od srednje vrijednosti; isprekidanim linijama su označene razine mora prije 21.000, 15.000 i 12.000 godina prije sadašnjosti (slika preuzeta iz Petit i sur. 2003).

Na kraju, „*rear edge versus leading edge*” koncept naglašava evolucijski značaj i konzervacijsku vrijednost perifernih populacija koje se nalaze na marginama areala vrste na nižim geografskim širinama („*rear edge*“) u odnosu na vodeću frontu populacija („*leading edge*“) koja se obično širi prema višim geografskim širinama (Slika 2; Hampe i Petit 2005). Naime, smatra se da periferne mediteranske populacije nisu bile izvorišne populacije za rekolonizaciju sjevernijih dijelova Europe nakon oledbe, već da su perzistirale *in situ* od posljednjeg ledenog doba pa sve do danas u relativno stabilnom okolišu mediteranske regije (Hampe i Petit 2005; Petit i sur. 2005). Iako su periferne populacije obično male i imaju nižu genetičku raznolikost zbog dugotrajne izolacije, one su obično vrlo stare, genetički divergentnije i bolje adaptirane na lokalne, često suboptimalne okolišne uvjete (Slika 2; Petit i sur. 2003; Hampe i Petit 2005). Stoga se često nazivaju i reliktnim populacijama koje posjeduju jedinstvenu genetičku raznolikost koja značajno doprinosi evolucijskom potencijalu vrste (Hampe i Petit 2005; Petit i sur. 2005).



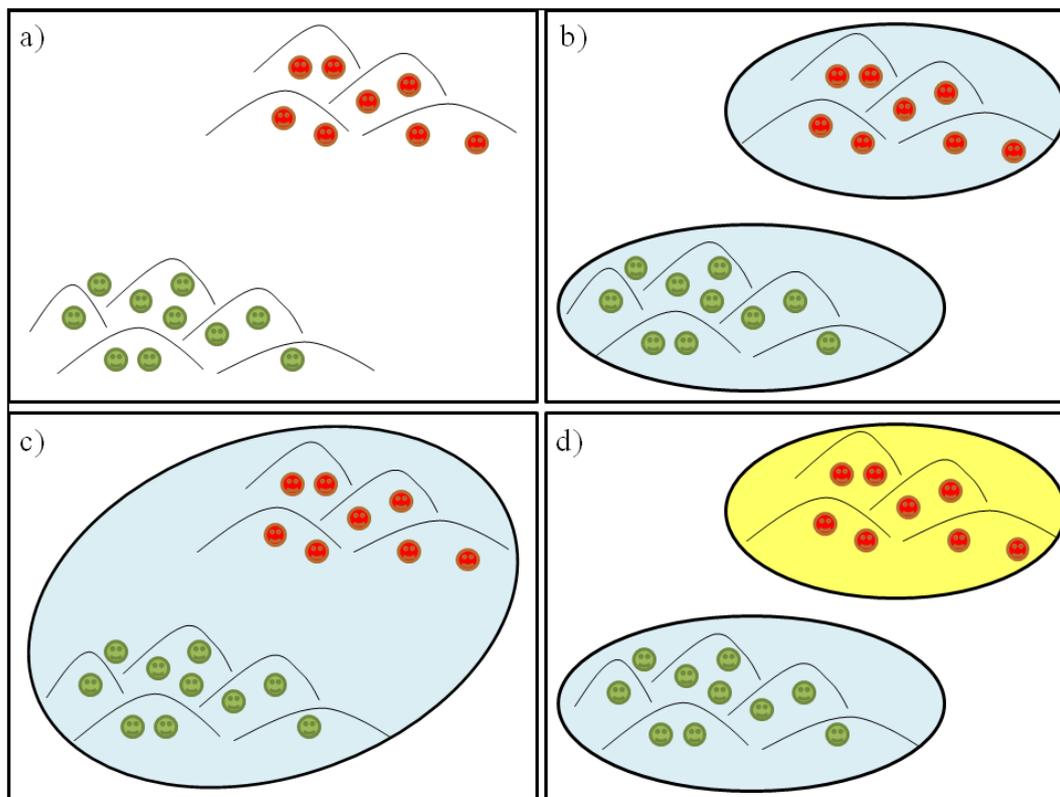
**Slika 2.** Različiti procesi na vodećem („*leading edge*“) i stražnjem rubu („*rear edge*“) areala (slika preuzeta i prilagođena iz Hampe i Petit 2005).

### 1.3. GIS i Modeli ekološke niše u evolucijskoj biologiji

Ekološka niša je centralni koncept u ekologiji i evoluciji. Iako je pojam ekološke niše prvi uveo Grinnell (1917), najraširenija definicija je ona koju navodi Hutchinson (1957): ekološka niša je skup ekoloških čimbenika (biotičkih i abiotičkih) u kojima neka vrsta može opstati i održavati dugoročno stabilnu populaciju. Modeli ekološke niše („*Ecological niche model*“) poznati i kao modeli povoljnosti staništa („*Habitat suitability model*“) ili modeli rasprostranjenosti vrsta („*Species distribution model*“) se primarno koriste za izradu karata potencijalne rasprostranjenosti vrsta, odnosno rasprostranjenosti za vrstu povoljnog staništa. Modeli ekološke niše su često neophodni u mnogim ekološkim i biogeografskim istraživanjima. Temelje se na principu interakcije vrsta-okoliš gdje pomoću poznatih podataka o prisutnosti/ili odsutnosti vrste i niza ekoloških za dotičnu vrstu važnih okolišnih varijabli (za koje smatramo da utječu na povoljnost staništa) pomoću neke od raspoloživih statističkih metoda pokušavamo predvidjeti distribuciju vrste u ekološkom prostoru, te je zatim projicirati u geografski prostor (Guisan i Zimmermann 2000). Također, pomoću modela ekološke niše možemo predvidjeti potencijalnu distribuciju vrste u nekom drugom prostoru (primjerice za invazivne vrste) ili u nekom drugom vremenskom razdoblju (primjerice u prošlosti ili budućnosti) (Ficetola i sur. 2007; Fløjgaard i sur. 2009; Carroll 2010). Trenutno postoji cijeli niz statističkih metoda i algoritama za izradu modela ekološke niše, a njihov odabir često ovisi o tipu i kvaliteti ulaznih podataka, te o cilju istraživanja (Elith i sur. 2006).

Ubrzanim razvojem GIS tehnologije i uslijed velike količine dostupnih podataka putem interneta, modeli ekološke niše našli su tijekom zadnjeg desetljeća vrlo široku primjenu u mnogim područjima biologije, uključujući evolucijsku i konzervacijsku biologiju (Pearson 2007; Kozak i sur. 2008). Tako su danas modeli ekološke niše jedan od važnih alata za testiranje evolucijskih i biogeografskih hipoteza, i ako ih kombiniramo s podacima o genetičkoj varijabilnosti, mogu nam pružiti bolji uvid u evolucijsku povijest i proces specijacije ili hibridizacije srodnih vrsta (Kozak i Wiens 2006; Raxworthy i sur. 2007; Jakob i sur. 2007; Swenson 2008). Primjerice, ako model ekološke niše ukazuje da dvije skupine populacija nerazjašnjenog taksonomskog statusa (dvije potencijalne kriptične vrste, podvrste ili evolucijske linije; Slika 3a) imaju različite ekološke niše, te da se između njih nalazi nepovoljno stanište (koje je van granica ekološke niše), tada se radi o potencijalno dvije odvojene evolucijske linije ili vrste, pogotovo ako ovaj uzorak prati i genetička struktura populacija (Slika 3d; Wiens i Graham 2005; Raxworthy i sur. 2007). Međutim, ako su ekološke niše dvije skupine populacija slične, a okolišni uvjeti između njih su povoljni

(nalaze se unutar granice ekološke niše), tada je omogućen protok gena između ove dvije skupine populacija i one vjerojatno ne predstavljaju različite evolucijske linije ili vrste (Slika 3c) (Wiens i Graham 2005; Rissler i Apodaca 2007).



**Slika 3.** Teorijski primjer koji ilustrira „*niche conservatism*“ i primjenu modela ekološke niše u razgraničavanju vrsta. a) Dvije alopatrijske skupine populacija (crveni i zeleni kružići) nerazjašnjenog taksonomskog statusa (evolucijske linije, podvrste, vrste). Svaka skupina populacija živi na drugoj planini, a dvije planine odvojene su dolinom. b) Model ekološke niše ukazuje da su ekološke niše dva seta populacija slične, ali ekološki uvjeti između planina su van granica niše pa je protok gena kroz dolinu smanjen ili prekinut, što upućuje na mogućnost da se radi o dvije potencijalno odvojene vrste. c) Ekološke niše dva seta populacija su slične i ekološki uvjeti u dolini su unutar granica niše, pa je protok gena između populacija omogućen (ukoliko nema fizičkih barijera). Ovakav rezultat ukazuje da se ne radi o dvije odvojene vrste. d) Ekološke niše dva seta populacija su različite i okolišni uvjeti u dolini su van granica niše. Divergencija ekološke niše je odgovorna za izolaciju populacija, a ovakav uzorak podržava hipotezu da se radi o potencijalno dvije odvojene vrste (slika preuzeta i prilagođena prema Wiens i Graham 2005).

Nadalje, s razvojem GIS-a pojavila se nova znanstvena disciplina: krajobrazna genetika („*landscape genetics*“; Manel i sur. 2003; Storfer i sur. 2007), koja istražuje kako i na koji način ekološki čimbenici i struktura krajobraza utječu na prostornu raspodjelu genetičke varijabilnosti unutar i između populacija. Krajobrazna genetika je interdisciplinarna znanstvena disciplina i predstavlja spoj populacijske genetike, krajobrazne ekologije i metoda prostornih analiza. Za razliku od filogeografije koja se bavi evolucijskim procesima na

velikim prostornim razlučenjima kroz duga vremenska razdoblja, krajobrazna genetika proučava nedavne mikroevolucijske procese na malim prostornim razlučenjima (Manel i sur. 2003). Unatoč naglom procvatu ove discipline zadnjih nekoliko godina, većina istraživanja odnosi se na životinjske vrste (Storfer i sur. 2010).

Naposljetku, modeli ekološke niše postali su gotovo neizbježan alat za predviđanje posljedica utjecaja klimatskih promjena na vrste i staništa (Thuiller et al. 2005).

#### **1.4. Klimatske promjene i njihov potencijalni utjecaj na rasprostranjenost i genetičku varijabilnost**

Kako bi opstale, populacije raznih drvenastih vrsta 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). Kao što je već ranije spomenuto, kapacitet određene vrste i njenih populacija da se prilagode promjenama okoliša u velikoj mjeri ovisi o razini i prostornoj raspodjeli genetičke varijabilnosti.

Kako se prikupljaju znanstveni rezultati postalo je nedvojbeno da trenutne klimatske promjene uzrokuju gubitak povoljnog staništa za biljne i životinjske vrste, što može dovesti do promjena i pomaka u njihovoj rasprostranjenosti (Parmesan i Yohe 2003; Parmesan 2006). Međutim, ne znači nužno da će sve regije svijeta biti jednako pogođene gubitkom staništa, a osjetljivost pojedine regije na klimatske promjene primarno ovisi o njejoj regionalnoj klimi i topografiji. Primjerice, Mediteranska regija istaknuta je kao jedno od najranjivijih područja. Predviđa se da će ekosustavi Mediterana biti iznimno pogođeni klimatskim promjenama zbog povećanja temperature i poremećaja u oborinskom režimu (smanjenje oborina, češći oborinski ekstremi, tendencija prema sušoj klimi i izražen proces desertifikacije) (IPCC 2007; Giorgi i Lionello 2008; Lindner i sur. 2010). Stoga možemo očekivati da će odgovor široko rasprostranjenih drvenastih vrsta na promjene u okolišu biti različit u različitim dijelovima njihovih areala (Lindner i sur. 2010).

Za mediteranske vrste predviđa se da će uslijed zatopljenja doći do geografskog pomaka u njihovoj rasprostranjenosti prema sjeveru, tj. prema višim geografskim širinama. Kao posljedicu možemo očekivati povećani rizik od izumiranja kod perifernih populacija u odnosu na one koje se nalaze u središtu areala ili na njegovom vodećem rubu (Slika 2). Pritom su dugoživuće drvenaste vrste izdvojene kao posebno osjetljiva skupina organizama na

klimatske promjene zbog spore evolucije, dugog generacijskog vremena i ograničene mogućnosti disperzije (Petit i sur. 2005). Međutim, drveće može prevladati ovaj nedostatak i oduprijeti se promjenama u okolišu ukoliko ima dovoljno veliku postojeću genetičku varijabilnost, odn. adaptivni evolucijski potencijal („*standing adaptive variation*“) (Savolainen i sur. 2011). Ako primjerice zbog smanjene povoljnosti staništa u mediteranskoj regiji periferne populacije izumru, određena vrsta izgubiti će dio svoje ukupne genetičke varijabilnosti, što može ugroziti njen adaptivni potencijal i dugoročni opstanak u promjenjivim uvjetima okoliša (Hampe i Petit 2005; Eckert i sur. 2008).

Hipoteze i moguće posljedice utjecaja klimatskih promjena na rasprostranjenost vrsta istražene su u brojnim publikacijama, koristeći modele ekološke niše (Thuiller i sur. 2005). Tako je na primjer pomoću takvih modela potvrđeno da će populacije drvenastih vrsta umjerenog pojasa biti najugroženije na Mediteranu (Benito Garzón i sur. 2008). Pod pretpostavkom da je ekološka niša vrste konzervirana („*niche conservatism*“, Wiens i Graham 2005), možemo pomoću modela ekološke niše utvrditi koji dijelovi areala su potencijalno najosjetljiviji na klimatske promjene. Područja gdje je prema modelu stanište trenutno povoljno za vrstu, te će ostati povoljno u bliskoj budućnosti unatoč promjeni klime, posebno su važna jer predstavljaju tzv. potencijalne *in situ* refugije od klimatskih promjena („*refugia from climate change*“), Ashcroft (2010). Iz aspekta konzervacijskih programa i planova upravljanja vrstama nužno je identificirati takva klimatski stabilna područja u kojima populacije imaju najveću vjerojatnost za preživljavanje, jer mogu usmjeravati odluke glede *in situ* i *ex situ* mjera zaštite.

Međutim, klasične metode modeliranja ekološke niše ne uzimaju u obzir unutarvrstu genetičku varijabilnost, niti njezinu prostornu raspodjelu. Stoga se za identifikaciju potencijalnih refugija tijekom antropogenih klimatskih promjena i procijene utjecaja istih na genetičku raznolikost i strukturu populacija, preporuča multidisciplinarni pristup koji kombinira modele ekološke niše i klasičnu populacijsku genetiku. Samo na ovaj način moguće je dobiti bolji uvid u potencijalni odgovor vrste na promjene u okolišu i razraditi efikasnije planove zaštite i upravljanja (Alsos et al. 2009; D’Amen i sur. 2012; Keppel i sur. 2012). Tako su Collevatti i sur. (2011) na primjeru endemične drvenaste vrste iz Brazila pokazali kako se genetička varijabilnost naglo smanjuje kada povoljnost staništa padne ispod određenog praga. Pod pretpostavkom da dugoživuće drvenaste vrste imaju vrlo ograničenu sposobnost migracije (Thuiller i sur. 2005) one mogu dugoročno opstati samo na područjima stabilnih staništa gdje se sadašnji i budući povoljni okolišni uvjeti prostorno preklapaju (tzv.

makrorefugiji tijekom klimatskih promjena). Ako mikrorefugiji (Rull 2009) ne pruže utočište ugroženim lokalnim populacijama u područjima smanjene povoljnosti staništa, klimatske promjene mogu dovesti do njihovog izumiranja, a time i do smanjenja ukupne genetičke varijabilnost vrste.

U novije vrijeme, predložena je nova metoda modeliranja od strane Jay i sur. (2012) pomoću koje su autori predvidjeli promjene u genetičkoj strukturi nekoliko alpskih biljnih vrsta kao potencijalni odgovor na zatopljenje klime. Ove modele nazvali su „*ancestry distribution models*“, a temelje se na kombinaciji prostorne Bayesovske analize genetičke strukture populacija i skrivene regresijske analize u kojoj koristimo okolišne varijable kao zavisne, prediktorske varijable (Durand et al. 2009; Jay i sur. 2011; Jay i sur. 2012). Osnovna razlika u odnosu na modele ekološke niše je u tome što se ovdje umjesto podataka o prisutnosti/odsutnosti vrste koriste genotipovi jedinki. Temeljem regresijskih koeficijenata između udjela pripadnosti pojedine jedinke pojedinom genskom skupu i odabranih klimatskih varijabli možemo predvidjeti prostornu genetičku strukturu populacija u budućnosti temeljem nekog od ponuđenih scenarija klimatskih promjena (Jay i sur. 2012). Kao rezultat umjesto potencijalne distribucije vrste, dobivamo potencijalnu distribuciju genetičke varijabilnosti uslijed klimatskih promjena. Ovaj metodološki okvir uklopljen je u program POPS (<http://membres-timc.imag.fr/Olivier.Francois/pops.html>) i komplementaran je modelima ekološke niše.

Međutim, kao i kod klasičnih modela ekološke niše, korištenje ove metode podrazumijeva nekoliko unaprijed zadanih pretpostavki i pojednostavljenja (Jay i sur. 2012):

- Ekološka niša vrste je konzervirana („*niche conservatism*“), tj. vrsta će pratiti promjene povoljnosti staništa u geografskom prostoru, što podrazumijeva određeni migracijski kapacitet vrste
- Utvrđena korelacija, tj. odnos između genetičke strukture i okolišnih varijabli bit će nepromijenjen u bliskoj budućnosti
- Adaptacija na promijenjene uvjete okoliša nije rezultat novih mutacija, već postojeće razine genetičke varijabilnosti („*adaptive standing variation*“)
- U analizu se uzimaju u obzir samo genotipovi jedinki, a ne i njihovi fenotipovi
- Predviđene promjene genetičke strukture i potencijalne migracije gena bit će iste za neutralne i adaptivne dijelove genoma, što podrazumijeva da genetička struktura

populacija odražava izolaciju uslijed adaptacije („*Isolation by adaptation*“; Nosil i sur. 2009)

Pod ovim pretpostavkama „*ancestry distribution models*“ mogu predvidjeti promjene u genetičkoj strukturi populacija uslijed klimatskih promjena, jer se smatra da će genotipovi adaptirani na lokalne okolišne uvjete migrirati prateći promjene u okolišu (Jay i sur. 2012). Međutim, potencijalne modele buduće rasprostranjenosti genotipova treba vrlo pažljivo interpretirati uzimajući u obzir migracijsku sposobnost vrste koju istražujemo, geografske barijere koje mogu ograničiti protok gena i dužinu vremenskog perioda za koji simuliramo promjene.

Kakve će biti posljedice klimatskih promjena na genetičku varijabilnost vrsta danas je otvoreno i goruće pitanje u svjetskoj znanosti. Unatoč tome, istraživanja na tu temu još su oskudna i u samim začetcima (Gienapp i sur. 2008; Rubidge i sur. 2012). Rezultati ovakvih istraživanja imaju posebnu vrijednost i praktičnu primjenu kod gospodarski važnih i široko rasprostranjenih vrsta, kao što su mnoge šumske vrste drveća (npr. kvalitetnije planiranje zaštite i gospodarenja šumama u svijetlu trenutnih klimatskih promjena).

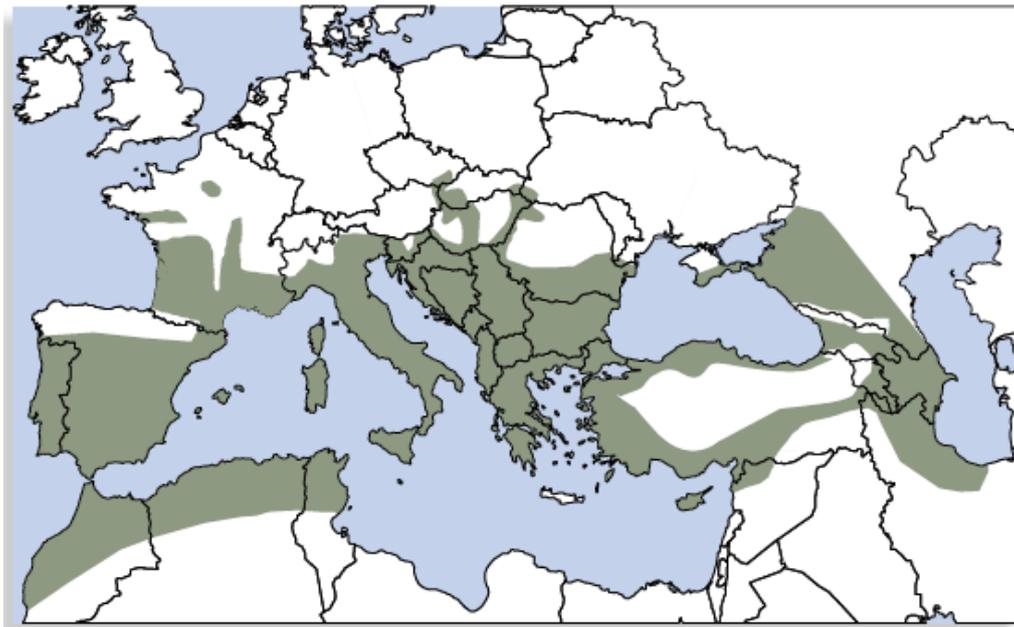
### **1.5. Poljski jasen (*Fraxinus angustifolia* Vahl) – ekologija, rasprostranjenost, taksonomija i kratki pregled dosadašnjih istraživanja**

Poljski jasen (*Fraxinus angustifolia* Vahl) je listopadna i anemofilna drvenasta biljna vrsta iz porodice Oleaceae (Slika 4). Prirodno je rasprostranjen u južnoj, središnjoj i jugoistočnoj Europi (Slika 5). Areal mu se na zapad proteže do Portugala i Španjolske, na istok do Turske i obala Crnog mora, dok na sjeveru ne seže dalje od Slovačke i Češke. Stoga se često u literaturi naziva mediteranskom vrstom, iako se najveći kompleksi šuma poljskog jasena nalaze u kontinentalnim dijelovima jugoistočne Europe.



**Slika 4.** Sastojina i detalj poljskog jasena (*Fraxinus angustifolia* Vahl)

Poljski jasen je higrofilna i uglavnom termofilna vrsta koja voli duboka, ilovasta i vlažna tla s povremenim plavljenjem (Fukarek 1983). Iako na svom širokom arealu obitava u vrlo raznolikim ekološkim uvjetima i smatra se eurivalentnom vrstom, poljski jasen u Srednjoj Europi, na Balkanu i u Panonskoj nizini najčešće nalazimo uz obale velikih nizinskih rijeka i njihovih pritoka, te na poplavnim i močvarnim područjima gdje tvori velike i kontinuirane sastojine (Fraxigen 2005; Bogdan i sur. 2007). Na Mediteranu su mu populacije male, fragmentirane i raštrkane uz rijeke i rijetke mediteranske močvare, a dolazi i na sušim tlima te na višim nadmorskim visinama (500-2000 m) (Fraxigen 2005; Bogdan i sur. 2007). U nizinskim poplavnim šumama poljski jasen ima ključnu ulogu jer je pionirska vrsta koja vrlo dobro raste u močvarnim uvjetima koji su često nepovoljni za ostale drvenaste vrste, stoga gotovo nema kompeticije na ovakvim staništima i tvori granicu šume prema močvari (Anić 1999). Nizinske poplavne šume poljskog jasena i hrasta lužnjaka u Hrvatskoj jedni su od najbolje očuvanih kompleksa nizinskih poplavnih šuma u Europi.



**Slika 5.** Rasprostranjenost poljskog jasena u Europi (Slika preuzeta iz Fraxigen 2005)

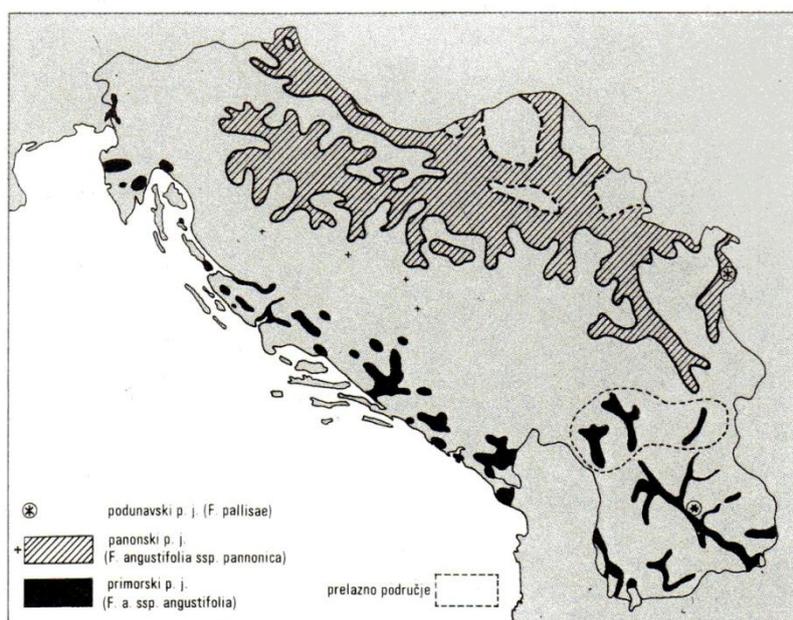
Od približno 43 vrste iz roda *Fraxinus* (Wallander i sur. 2008), u Europi, pa tako i u Hrvatskoj, dolaze tri autohtone vrste jasena – poljski jasen (*Fraxinus angustifolia* Vahl), obični jasen (*Fraxinus excelsior* L.) i crni jasen (*Fraxinus ornus* L.). O njima postoji prilično opsežna literatura, posebno u radovima koji se bave vegetacijom nekog područja. Poljski jasen kao zasebna vrsta jasena u Europi opisan je posljednji, 1804. godine od strane švedskog botaničara Vahl-a na primjercima iz Španjolske. U Hrvatskoj ga prvi navodi i istražuje Fukarek 1954. godine. Na našim prostorima istraživanja poljskog jasena bila su uglavnom usmjerena na njegovu distribuciju, morfološku varijabilnost i taksonomsku problematiku (detaljno razrađena u radovima Fukarek-a (1954, 1960, 1983), kao i na ekologiju i uzgojnu problematiku na području Posavine i Pokuplja (Dekanić 1970; Prpić 1974; Matić 1989; Matić i sur. 1996; Anić 1997, 1999, 2001).

O taksonomiji poljskog jasena postoji mnogo različitih mišljenja i zbog iznimne morfološke varijabilnosti opisan je cijeli niz podvrsta i varijeteta od kojih su mnogi sinonimi (*Fraxinus oxycarpa*, *F. syriaca*, *F. pallisae*, *F. potamophila*, *F. sogdiana*, *Fraxinus angustifolia* var. *oxyphylla*, *Fraxinus angustifolia* var. *obliqua*...; Fukarek 1954). Međutim, u literaturi prevladava podjela vrste *Fraxinus angustifolia* na tri geografski diferencirane podvrste (Tutin i sur. 1972; Fraxigen 2005):

- *Fraxinus angustifolia* ssp. *angustifolia* (zapadni Mediteran),
- *Fraxinus angustifolia* ssp. *oxycarpa* (M. Bieb. ex Willd.) Franco & Rocha Afonso (središnja i jugoistočna Europa),
- *Fraxinus angustifolia* ssp. *syriaca* (Boiss.) Yalt. (Turska i istočnije do Irana).

Temeljem novije molekularne filogenije roda *Fraxinus* Wallander (2008) potvrđuje kako ova tri navedena taksona mogu zadržati status podvrste, ali također zaključuje da se svi dosad opisani taksoni (podvrste i varijeteti) poljskog jasena mogu svesti pod *F. angustifolia*.

Fukarek (1983) je temeljem svojih istraživanja morfologije poljskog jasena opisao dvije podvrste na području Hrvatske i bivše Jugoslavije: pretpostavio je da populacije poljskog jasena uz jadransku obalu (uz rijeke Mirnu, Krku, Cetinu, Jadro, Zrmanju, Neretvu i Bojanu, otoci Rab, Pag, niža krška polja, Skadarsko jezero) pripadaju tipičnoj podvrsti ssp. *angustifolia* (Fuk.) Soó & Simon (sredozemni poljski jasen), dok u kopnenom i u panonskom nizinskom dijelu (uz rijeke Dravu, Muru, Savu, Dunav, Tisu, Moravu) opisuje podvrstu ssp. *pannonica* (panonski poljski jasen) za koju je smatrao da je morfološki sličnija podvrsti ssp. *oxycarpa* (Slika 6).



AREALI PODVRSTA POLJSKOG JASENA U JUGOSLAVIJI (original)

**Slika 6.** Rasprostranjenost podvrsta poljskog jasena (ssp. *angustifolia* i ssp. *pannonica*) u Hrvatskoj i na području bivše Jugoslavije prema Fukareku (1983), slika preuzeta iz Šumarske enciklopedije.

U novije vrijeme morfološka varijabilnost poljskog jasena bila je ponovo predmet istraživanja na području Slovenije (Jarni i sur. 2011) gdje nisu utvrđene značajne razlike između panonskih i mediteranskih populacija, međutim autori navode kako mogu potvrditi isključivo prisutnost podvrste ssp. *oxycarpa* na području sjeverozapadnog Balkana.

Iako je poljski jasen ekološki i gospodarski vrlo važna vrsta, njegova genetička varijabilnost u Hrvatskoj, ali i šire, dosad nije bila predmet sustavnog istraživanja upotrebom molekularnih biljega. Detaljno je analizirana jedino varijabilnost kvantitativnih svojstava hrvatskih populacija poljskog jasena u testovima polusrodnika kojom nisu utvrđene značajne razlike između posavskih populacija, niti značajna diferencijacija između kontinentalnih i mediteranskih populacija (Bogdan 2006; Bogdan i sur. 2007). Dosadašnja molekularna istraživanja u europskim zemljama bila su uglavnom posvećena običnom jasenu (*Fraxinus excelsior* L.) (Lefort i sur. 1999; Heuertz i sur. 2001; Heuertz i sur. 2004a, 2004b; Fraxigen 2005; Hebel i sur. 2006; Ferrazzini i sur. 2007; Ballian i sur. 2008; Sutherland i sur. 2010; 5th FP project RAP: [www.teagasc.ie/advisory/forestry/rap](http://www.teagasc.ie/advisory/forestry/rap)), a istraživana je i razina hibridizacije između poljskog i običnog jasena na morfološkoj i genetičkoj razini (Morand i sur. 2002; Gerard i sur. 2006a, b; Fernandez-Manjarrés i sur. 2006). Naime, zbog velike morfološke sličnosti ove dvije vrste jasena nekada je teško razlučiti na terenu, pa su često zamjenjivane ili krivo određene u literaturi (Fukarek 1954; Fraxigen 2005).

Do danas jedini poznati objavljeni podaci o genetičkoj varijabilnosti poljskog jasena pomoću molekularnih biljega ostaju rezultati europskog projekta Fraxigen (2005) i rezultati filogeografske analize roda *Fraxinus* na europskoj razini (Heuertz i sur. 2006) koji se temelje na istraživanju kloroplastne DNA. Rezultati ovih istraživanja pokazali su da je raznolikost kloroplastnih haplotipova poljskog jasena relativno niska, da su populacije jasno geografski strukturirane, te da su se glacialni refugiji jasena vjerojatno nalazili na području Iberijskog poluotoka, sjevernog dijela Apeninskog poluotoka, na Balkanskom poluotoku, te potencijalno na području Dinarida (Heuertz i sur. 2006). Također, utvrđeno je da poljski jasen dijeli većinu haplotipova s običnim jasenom, što upućuje na historijski protok gena između ove dvije srodne vrste. Varijabilnost jezgrine DNA analizirana je pomoću mikrosatelitnih biljega samo u grčkim, talijanskim i španjolskim populacijama. Utvrđena je vrlo visoka razina genetičke varijabilnosti, međutim genetička diferencijacija između istraživanih populacija bila je jako mala (Fraxigen 2005; Papi i sur. 2012). Naposljetku, unatoč upotrebi modernih molekularnih metoda (npr. sekvenciranja gena izabranih za univerzalni DNA barkod; Arca i sur. 2012) do danas nije u potpunosti razjašnjen taksonomski status poljskog i običnog jasena.

## 1.6. Mehanizmi hibridizacije na primjeru poljskog i običnog jasena

Drvenaste vrste mogu formirati hibridne zone duž nekoliko stotina kilometara, što često ovisi o ekološkim čimbenicima (Dodd i Afzal-Rafii 2004; Kamiya i sur. 2011; Wang i sur. 2012). Hibride obično stvaraju srodne vrste koje nisu reproduktivno izolirane ili je došlo do sekundarnog kontakta dvije divergentne evolucijske linije koje su se razvile zasebno u izolaciji (Givnish 2010). Općenito, postoji nekoliko tipova zona hibridizacije, a njihov postanak ovisi o tipu selekcijskog pritiska i o kapacitetu disperzije jedinki (kako roditeljskih vrsta, tako i njihovih hibrida). Klasična teorija hibridizacije (Endler 1977; Barton i Hewitt 1985, 1989) predviđa da raspon i oblik hibridne zone ovise o ravnoteži između selekcije i migracije. Pritom selekcija uključuje egzogene (uvjetovane vanjskim okolišnim čimbenicima) i endogene pritiske (uvjetovane unutarnjim genetičkim čimbenicima) (Barton 2001). Na lokalnoj razini, razina hibridizacije drvenastih vrsta ovisi prije svega o načinu razmnožavanja (oprašivanja) i relativnoj brojnosti pojedine roditeljske vrste (Field i sur. 2011), što može dovesti do asimetrije u protoku gena. Međutim, ukoliko nemamo jasan dokaz da je riječ o selekciji, teško je razlučiti koji od ova dva procesa (migracija ili selekcija) ima dominantnu ulogu u formiranju hibridne zone.

Koristeći modele ekološke niše možemo utvrditi barem dva tipa hibridnih zona (Swenson 2008). Ako se distribucija roditeljskih vrsta i hibrida predviđena modelom ne poklapa sa stvarnom opaženom distribucijom hibridne zone i granicama distribucije roditeljskih vrsta, tada je vjerojatno riječ o tzv. „*tension zone*“ modelu (Barton i Hewitt 1985). U ovom modelu, kombinirano djelovanje selekcije i migracije određuje položaj i veličinu hibridne zone koja u pravilu nije pod utjecajem okolišnih čimbenika, te se stoga može pomicati u prostoru. Međutim, ako su križanci superiorni u određenom okolišu u odnosu na svoje roditeljske vrste (imaju veći fitnes unutar, a manji fitnes van hibridne zone), tada očekujemo da distribucija hibridne zone predviđena modelom odgovara njenoj stvarnoj opaženoj distribuciji. Ovaj slučaj opisan je kao tzv. „*bounded hybrid superiority*“ model (Moore 1977) i dopušta da predviđena distribucija roditeljskih vrsta zalazi u hibridnu zonu jer križanci kontroliraju širenje roditeljskih vrsta unutar hibridne zone, dok predviđena distribucija križanaca ne bi smjela prelaziti granice opažene hibridne zone (Swenson 2008). Ovaj model ovisan je o vanjskim okolišnim čimbenicima i podrazumijeva da različit okoliš favorizira različite taksone. Na primjer, ako je distribucija pojedine roditeljske vrste jasno povezana s različitim okolišnim uvjetima, a njihovi križanci se nalaze u intermedijarnom okolišu između okoliša dvije roditeljske vrste, možemo zaključiti kako je egzogena selekcija glavna evolucijska sila

koja oblikuje i stabilizira dinamiku hibridnih zona (Moore 1977). Tako pomoću modela ekološke niše možemo relativno lako utvrditi simpatrijske zone dvije roditeljske vrste (tamo gdje im se ekološke niše preklapaju) i u tim zonama analizirati razinu hibridizacije pomoću molekularnih ili morfoloških biljega.

Poljski i obični jasen predstavljaju izvrstan model za istraživanje mehanizma hibridizacije jer se radi o dvije široko rasprostranjene i visoko srodne vrste za koje je rezultatima dosadašnjih istraživanja potvrđeno da hibridiziraju kako u laboratorijskim uvjetima, tako i u prirodi kada dolaze u simpatriji (Raquin i sur. 2002; Morand-Prieur i sur. 2002; Fernandez-Manjarrés i sur. 2006; Gerard i sur. 2006a, b). Također, ove dvije vrste preferiraju različite ekološke uvjete i imaju različitu fenologiju cvjetanja. Obični jasen je najraširenija vrsta jasena u Europi i raste uglavnom u brdskim i gorskim područjima s obilnijom zračnom vlagom i dubokim, vlažnim i dobro prozračenim tlima, dok je poljski jasen termofilnija vrsta nizinskih, močvarnih i priobalnih šuma koja voli duboka, ilovasta i vlažna tla s povremenim plavljenjem, te puno svjetla (Fukarek 1983). Poljski jasen cvjeta uvijek prije običnog jasena u istom području (nekad već u prosincu pa sve do ožujka) i u tom razdoblju osjetljiv je na hladnoću, a posebno na kasni mraz. Međutim, u određenim godinama i klimatskim uvjetima cvjetanje poljskog jasena može biti pomaknuto (zbog primjerice blage zime), pa dolazi do vremenskog preklapanja s cvjetanjem običnog jasena, što omogućava njihovu hibridizaciju (Gerard i sur. 2006a). Hibridne zone poljskog i običnog jasena poznate su i najbolje istražene u Francuskoj u dolinama rijeka Loire i Saône, gdje je potvrđeno da vremenska izolacija u periodu cvjetanja igra važnu ulogu u reproduktivnoj izolaciji ove dvije vrste, te da blaga klima omogućava molekularnu i morfološku introgresiju poljskog jasena u obični jasen (Gerard i sur. 2006a; Fernandez-Manjarrés i sur. 2006). Hibridne populacije spominju se još uz rijeku Rajnu i Dunav, zatim u Španjolskoj, Češkoj, Mađarskoj, te na Balkanu (FRAXIGEN 2005; usmeno priopćenje: J. Dufour, B. Heinze, F. Starlinger, H. Sainz, J. Franjić).

## 1.7. Hipoteze i ciljevi istraživanja

Sukladno dosadašnjim spoznajama i pregledom literature kako one vezane za populacijsku genetiku, modeliranje ekoloških niša, krajobraznu genetiku tako i za taksonomiju, genetičku raznolikost, ekologiju i hibridizaciju poljskog jasena definirane su slijedeće hipoteze:

1. Populacije poljskog jasena u Hrvatskoj strukturirane su tako da postoji značajna razlika u genetičkoj varijabilnosti populacija iz mediteranske i kontinentalne biogeografske regije.
2. Ekološki čimbenici utječu na genetičku varijabilnost vrste.
3. Najveću genetičku raznolikost u Europi imaju južne populacije iz područja glacijalnih refugija na Iberijskom, Apeninskom i Balkanskom poluotoku.
4. Klimatske promjene omogućit će pomicanje areala poljskog jasena prema većim geografskim širinama, a negativno će utjecati na razinu genetičke raznolikosti.
5. Ekološki čimbenici uvjetuju stupanj i smjer hibridizacije tako da je stvaranje hibridnih populacija s običnim jasenom omogućeno na područjima gdje se vrste nalaze u simpatriji te im se ekološke niše preklapaju.

i ciljevi ove doktorske disertacije:

1. Utvrditi genetičku varijabilnost populacija poljskog jasena rasprostranjenih u različitim staništima duž Hrvatske i Europe.
2. Testirati zavisnost genetičke varijabilnosti u odnosu na ekološku varijabilnost.
3. Analizirati ekološku nišu poljskog jasena i istražiti odražava li genetičku strukturu populacija.
4. Testirati postoji li specifičan geografski gradijent tj. prostorna raspodjela genetičke raznolikosti unutar europskog areala.
5. Izraditi potencijalni model povoljnosti staništa uslijed klimatskih promjena.

6. Identificirati potencijalne buduće refugije poljskog jasena tijekom klimatskih promjena.
7. Procijeniti kako će klimatske promjene utjecati na genetičku varijabilnost vrste u budućnosti.
8. Utvrditi razinu molekularne introgresije između poljskog i običnog jasena, identificirati područja simpatrije te analizirati distribuciju hibridnih populacija u odnosu na modelirana područja simpatrije.
9. Utvrditi koji lokusi su pod potencijalnim utjecajem selekcije i pomoću njih identificirati glavne ekološke čimbenike koji su odgovorni za održavanje granica rasprostranjenosti između roditeljskih vrsta te za formiranje hibridnih zona.

**2. ZNANSTVENI RADOVI**

### 2.1. Popis znanstvenih radova

1. Temunović M, Franjić J, Satovic Z, Grgurev M, Frascaria-Lacoste N, Fernández-Manjarrés JF (2012) Environmental heterogeneity explains the genetic structure of Continental and Mediterranean populations of *Fraxinus angustifolia* Vahl. PLoS ONE, 7 (8), e42764.
2. Temunović M, Frascaria-Lacoste N, Franjić J, Satovic Z, Fernández-Manjarrés JF (2013) Identifying refugia from climate change using coupled ecological and genetic data in a transitional Mediterranean-temperate tree species. Molecular Ecology, 22 (8), 2128-2142.
3. Gérard PR, Temunović M, Sannier J, Bertolino P, Dufour J, Frascaria-Lacoste N, Fernández-Manjarrés JF (2013) Chilled but not frosty: understanding the role of climate in the hybridization between the Mediterranean *Fraxinus angustifolia* Vahl and the temperate *Fraxinus excelsior* L. (Oleaceae) ash trees. Journal of Biogeography, 40 (5), 835-846.

**ZNANSTVENI RAD BR. 1**

# Environmental Heterogeneity Explains the Genetic Structure of Continental and Mediterranean Populations of *Fraxinus angustifolia* Vahl

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## Abstract

Tree species with wide distributions often exhibit different levels of genetic structuring correlated to their environment. However, understanding how environmental heterogeneity influences genetic variation is difficult because the effects of gene flow, drift and selection are confounded. We investigated the genetic variation and its ecological correlates in a wind-pollinated Mediterranean tree species, *Fraxinus angustifolia* Vahl, within a recognised glacial refugium in Croatia. We sampled 11 populations from environmentally divergent habitats within the Continental and Mediterranean biogeographical regions. We combined genetic data analyses based on nuclear microsatellite loci, multivariate statistics on environmental data and ecological niche modelling (ENM). We identified a geographic structure with a high genetic diversity and low differentiation in the Continental region, which contrasted with the significantly lower genetic diversity and higher population divergence in the Mediterranean region. The positive and significant correlation between environmental and genetic distances after controlling for geographic distance suggests an important influence of ecological divergence of the sites in shaping genetic variation. The ENM provided support for niche differentiation between the populations from the Continental and Mediterranean regions, suggesting that contemporary populations may represent two divergent ecotypes. Ecotype differentiation was also supported by multivariate environmental and genetic distance analyses. Our results suggest that despite extensive gene flow in continental areas, long-term stability of heterogeneous environments have likely promoted genetic divergence of ashes in this region and can explain the present-day genetic variation patterns of these ancient populations.

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## Introduction

Understanding how environmental heterogeneity influences the distribution of genetic variation among natural populations along different spatial scales remains a central question in evolutionary biology and population genetics [1,2]. Genetic divergence of natural plant populations can be influenced by several evolutionary processes including gene flow, genetic drift, and natural selection [3]. If gene flow is locally restricted because of limited pollen and seed dispersal of the species, then the genetic differentiation of populations will show a pattern of isolation-by-distance (IBD) [4], which is considered to be the main force in the establishment of neutral genetic structure in plant populations.

Yet, greater genetic divergence than expected among populations inhabiting different environments has been used to suggest that contrasting ecological conditions may have a strong influence on the genetic differentiation of local populations [5,6]. Several studies have shown statistical associations between putatively neutral genetic variation and environmental variation in plant

species, and such correlations may be interpreted as evidence of diversifying selection acting over the whole genome [3,7–9]. In cases in which the genetic distance between populations correlates with their environmental distance, the pattern has been described as “isolation by environmental distance” (IBED) [10]. Nevertheless, the removal of geographic effects is necessary to detect the unique contribution of environmental gradients to genetic divergence because climatic differences are typically correlated with geographic distance [11]. A significant positive partial correlation after removing the effect of geographic distance suggests that genetic divergence is associated with environmental gradients and that natural selection may interact with neutral processes of gene flow and genetic drift [9–11].

Ecological niche modelling (ENM) allows the generation of biogeographical hypotheses and, when coupled with genetic data, provides new insights into the evolutionary history of animal and plant species [11–15]. For instance, ENM indicates that two population sets of unresolved taxonomic status with non-overlap-

ping ecological niches and separated by a portion of unsuitable habitat may represent distinct evolutionary lineages ([16,17], see example for geckos in Madagascar in [12]). As such, the application of ENM to delimit cryptic species has become an emergent field of ecological genetics [11,17,18]. In particular, the ability of ENM to test for a potential lack of spatial overlap in subpopulations of the same species allows the generation of different gene flow hypotheses that otherwise would be difficult to formulate. For example, if population differentiation with neutral markers is low but ENM simulations produce non-overlapping distributions, one can conclude that gene flow is still present despite the contrasting habitats associated with the populations. On the other hand, if genetic structuring of neutral markers reflects the simulated spatial distributions, rates of gene flow must be interrupted to allow for population differentiation. Although linking ENM with genetic data is increasing rapidly in phylogeographic studies at broad scales, the potential of the ENM approach and GIS-based environmental data has rarely been explored in conjunction with population genetic variation at finer scales. Studies of landscape genetics [1,19], which investigate how environmental factors influence the spatial distribution of intra-specific genetic variation, provide a promising framework for a better understanding of the microevolutionary processes involved in population genetic differentiation. While the majority of landscape genetics studies remain focused on animals [20], the integration of environmental features to explain the genetic variation of plants lags behind.

For this study, we selected the narrow-leaved ash (*Fraxinus angustifolia* Vahl, Oleaceae), a wind-pollinated tree species with a predominantly Mediterranean distribution, but found frequently in habitats of both Continental and Mediterranean biogeographical regions of Europe. It occurs naturally throughout Southern and Eastern Europe, from Portugal in the west to the Black Sea in the east. Although the phylogeography and genetic structure of the closely related species, the common ash (*Fraxinus excelsior* L.), and the hybridisation process between the two species have been well studied [21–26], no attempts have been made to understand the genetic structure of *F. angustifolia* populations. Unlike *F. excelsior*, which is widely distributed in mixed deciduous forests all over Europe, *F. angustifolia* is a habitat specialist associated with surface and ground waters, and thus, its dispersal ability is more restricted. In Central Europe, the Pannonian Basin and the Balkans, it occurs mainly in lowlands, riparian and floodplain forests along large rivers and their tributaries (Drava, Danube, and Morava), where it forms large and continuous populations. The distribution of this species in the Mediterranean region is patchy and reduced to smaller and more isolated populations on drier sites at higher altitudes or on wetland sites [27,28].

There are several different points of view regarding the taxonomic status of this species, but in general, the prevailing opinion is that *F. angustifolia* has three subspecies restricted by geographical regions [27,29]: ssp. *angustifolia* (in the western Mediterranean), ssp. *oxycarpa* (M. Bieb. ex Willd.) Franco and Rocha Afonso (in East Central and Southeastern Europe), and ssp. *syriaca* (Boiss.) Yalt. (in Turkey and eastwards to Iran). Fukarek [30] used morphological differences to further subdivide *F. angustifolia* in Croatia and the surrounding area of the Western Balkans. Continental populations along the rivers and floodplains of the Danube River Basin were named *Fraxinus angustifolia* ssp. *pannonica* (Fuk.) Soó and Simon. This putative new taxon is morphologically closer to ssp. *oxycarpa*, whereas Mediterranean populations along rivers and wetlands of the Adriatic River Basin probably belong to the typical ssp. *angustifolia*. Such a division differs from the more accepted general geographical classification,

suggesting a possible increased within-region differentiation in the Western Balkan area.

Although located at a relatively high latitude and with a relatively small size, Croatia has been highlighted as having one of the most genetically divergent forests among 25 European forests investigated based on chloroplast genetic diversity [31]. Moreover, the area of the Western Balkans and Dinaric Alps served as an important refugium during the Pleistocene for the survival of many animal and plant species, including ash [23,32–34]. Such large genetic divergence may have originated because of the comparatively higher environmental stability of this area during Quaternary climate oscillations, complex historical demographic events, and its geographical position. In fact, Croatia is situated along the contact line of three different biogeographical regions of Europe with contrasting climates: the Continental region including parts of the Pannonian lowlands, the mountainous Alpine region including parts of the Dinaric Alps and the Mediterranean region. Such environmental, landscape and historical diversity in a small area represents a valuable opportunity to investigate the influence of these factors on genetic variation and possible differentiation within species lineages.

In this study, we combined population genetic analyses of neutral loci, landscape genetic analysis using multivariate environmental data and ENM to examine genetic variation and its ecological correlates in *F. angustifolia* populations. We focused on answering these specific questions: 1) What are the levels of genetic diversity and divergence within and among natural populations of *Fraxinus angustifolia* distributed across the Croatian Continental and Mediterranean areas? 2) Does the neutral spatial genetic variation correlate with the environmental variation? and 3) Are areas predicted as suitable by ENM concordant with patterns of population genetic divergence?

## Materials and Methods

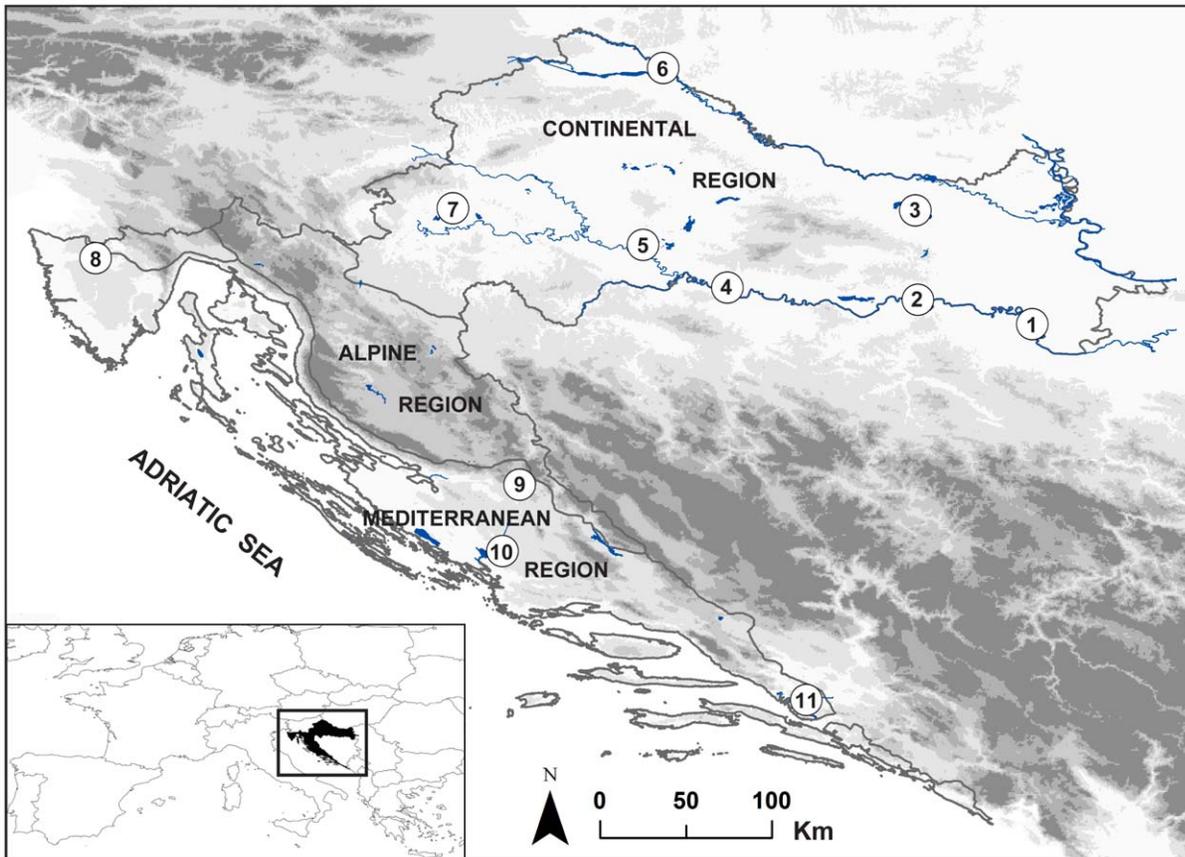
### Ethics statement

Collections of samples from protected areas were permitted by the authority of The Krka National Park and Lonjsko Polje Nature Park. For other locations no specific permits were required for the described field studies because sample collection did not involve endangered or protected plant species or privately-owned locations.

### Study site and sampling

This study was conducted across the entire species' natural distribution range in Croatia ( $\approx 56,538 \text{ km}^2$  land surface). Sampling was carried out in 11 natural populations of *F. angustifolia* (Figure 1), with about 30 individuals sampled per population (total  $n = 345$ ) (Table 1). To avoid the sampling of close relatives, the minimum distance between sampled individuals was at least 50 m. Coordinates were recorded for each sampled tree using GPS. Trees were sampled from seven Continental and four Mediterranean natural stands, comprising the whole environmental gradient in Croatia in which the species occurs (Figure 1). All populations from the Mediterranean region where stands had sufficient size to allow at least 30 individuals to be sampled were included.

About 9% of Croatian land area is frequently flooded [35], and natural floodplains of the Continental region represent the main habitat for the species. Floodplain forests in Croatia, dominated by narrow-leaved ash or mixed stands with oaks, are one of the most preserved in Europe. The majority of these forests stretch along the Sava, Kupa, Drava, and Danube rivers, all belonging to the Black Sea catchment ( $35,133 \text{ km}^2$ ). In this region, stands are



**Figure 1. Map of the study area with location of the sampled *Fraxinus angustifolia* populations in Croatia.** See Table 1 for population codes. The indicated boundaries of the biogeographical regions are based on the European Environment Agency (<http://www.eea.europa.eu>) and were adapted by the Croatian SINP (<http://www.dzzp.hr/eng>). doi:10.1371/journal.pone.0042764.g001

**Table 1. Sampling sites and statistics of genetic variation for 11 *Fraxinus angustifolia* populations in Croatia at six microsatellite loci.**

Population No.	Biogeographical Region	Population name	Lat (DD)	Long (DD)	n	$N_a$	$N_{ar}$	$N_{pr}$	$H_O$	$H_E$	$H_{Enull}$
1	Continental	Županja	18.742	45.003	30	13.00	12.67	1	0.650	0.672	0.710
2	Continental	Trnjani	18.144	45.131	29	12.67	12.56	4	0.733	0.724	0.726
3	Continental	Durdenovac	18.130	45.598	32	15.33	14.42	8	0.708	0.748	0.777
4	Continental	Stara Gradiška	17.147	45.196	31	13.33	12.81	5	0.697	0.682	0.686
5	Continental	Lonjsko polje	16.706	45.418	32	13.83	13.24	3	0.696	0.720	0.750
6	Continental	Čakovec	16.810	46.343	31	17.83	17.15	7	0.737	0.771	0.772
7	Continental	Jastrebarsko	15.711	45.611	32	15.17	14.44	2	0.754	0.715	0.727
8	Mediterranean	Mirna	13.840	45.349	32	13.33	12.67	0	0.656	0.681	0.684
9	Mediterranean	Zrmanja	16.059	44.167	32	10.67	10.20	3	0.625	0.668	0.688
10	Mediterranean	Krka	15.969	43.819	32	12.83	12.23	3	0.682	0.693	0.695
11	Mediterranean	Neretva	17.559	43.040	32	10.17	9.81	1	0.578	0.621	0.642
1–7	Continental				217	14.45	13.90	57	0.711	0.720	0.735
8–11	Mediterranean				128	11.75	11.23	9	0.635	0.666	0.677
		P†					0.03		0.02	0.03	
1–11	Overall mean					13.47	12.93	3.36	0.683	0.699	0.714

n - sample size;  $N_a$  - average number of alleles per locus;  $N_{ar}$  - allelic richness;  $N_{pr}$  - total number of private alleles;  $H_O$  - observed heterozygosity;  $H_E$  - expected heterozygosity;  $H_{Enull}$  - expected heterozygosity calculated on allele frequencies corrected for null-alleles;

†P-value of the permutation tests for differences between the putative ecotypes for  $N_{ar}$ ,  $H_O$ , and  $H_E$ .

doi:10.1371/journal.pone.0042764.t001

influenced by hot summers and cold winters with medium precipitation levels. In contrast, coastal stands are small and fragmented, influenced by the Mediterranean climate which is characterised by hot, dry summers and mild, rainy winters. These forests grow along the rivers of the Adriatic catchment area (21,405 km<sup>2</sup>), the Dinaric karst fields and wetland sites (Mirna, Krka, Zrmanja, Cetina, and Neretva).

### Microsatellite analysis

Total DNA was extracted using DNeasy 96 Plant Kit (Qiagen) from up to ten mg of dried leaf tissue following the manufacturer's protocols. We used six polymorphic microsatellite markers that had been extensively used in *Fraxinus* spp. studies: Femsat14, Femsat11, Femsat12, Femsat16, Femsat19 and M2–30 [36,37]. For Femsat12, the redefined primers of Gerard [24] were used to avoid null alleles seen with the original primer set. The PCR conditions followed those used by Morand [22]. Fluorescent labelling of the forward primers enabled the detection of PCR products by capillary electrophoresis (ABI 3100), and allele sizes were scored using GeneMapper 4.0 (Applied Biosystems).

### Population genetic diversity and structure

GENEPOP 4.0 [38] was used to estimate the following genetic diversity parameters: average number of alleles per locus ( $N_a$ ), observed heterozygosity ( $H_O$ ), and expected heterozygosity ( $H_E$ ). The program MICRO-CHECKER [39] was used to check for potential problems related to allele dropout and the presence of null alleles. The estimates of the null allele frequencies were based on the expectation-maximisation algorithm [40] and then calculated using FREENA [41]. The adjusted allele frequencies were used to recalculate the expected heterozygosity values ( $H_{Enull}$ ). GENEPOP was also used to test for Hardy-Weinberg equilibrium (HWE) for each locus in each population and to test the loci for linkage disequilibrium. The probability tests were based on the Markov chain method [42]. The sequential Bonferroni adjustment [43] was applied to correct for the effect of multiple tests using SAS (SAS Ver. 9.1; SAS Institute Inc., Cary, NC, USA). FSTAT Ver. 2.9.3.2 [44] was used to calculate allelic richness ( $N_{ar}$ ), which yields allele counts standardised to the minimum sample size, and to test the significance of the differences in average values of  $N_{ar}$ ,  $H_O$  and  $H_E$  between the Continental and Mediterranean populations. The number of private alleles ( $N_{pr}$ ) per population was assessed by MICROSAT [45].

Pairwise genetic distances between populations ( $F_{ST}$ ) and their significance were calculated using FSTAT. Pairwise  $F_{ST}$  values were also estimated after correcting for the presence of null alleles using a method implemented in FREENA [41]. The overall population genetic structure was estimated for each locus and as a multilocus estimate with Wright's  $F$ -statistics using Weir and Cockerham's method [46] implemented in FSTAT. The analysis of molecular variance (hierarchical AMOVA) was performed to examine the partition of microsatellite variation between the Continental and the Mediterranean regions, among populations within the regions, and within populations using Arlequin Ver. 3.5.1.2 [47]. The variance components were tested by non-parametric randomisation tests using 10 000 permutations.

### Association between genetic variation and environmental heterogeneity

**Species presence data.** We obtained 335 occurrence points from 11 sampled populations. Further locality records were obtained from the Flora Croatica Database (FCD, [botanic.hr/fcd/,  \$n = 60\$ \), the National Forest Inventory \( \$n = 352\$ , \[48\]\), and personal communications \( \$n = 55\$ , see acknowledgements\). In total, we compiled 802 high resolution species occurrence points.](http://hirc.</a></p>
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**Environmental data.** Climate data for current conditions were obtained from the WorldClim database with a spatial resolution close to a square km [49]. First, the correlations among all 19 WorldClim bioclimatic variables and topographic variables for all presence points were calculated to exclude the highly correlated ones ( $r > 0.75$ ), whilst keeping the variables useful in predicting the distribution limits of trees, such as climatic averages and extremes [50].

Ten environmental variables were selected to describe the ecological characteristics of the sampled stands, for the Principal Component Analysis (PCA) and for the calculation of environmental distances. The eight bioclimatic variables included averages, extremes and seasonal variation in precipitation and temperature, and the two topographic variables altitude and terrain slope (see Table 2). Three additional layers were included in the construction of the ENMs as predictors: terrain aspect, a distance-to-water variable, and habitat type (see Table 3). Because of its circular nature, terrain aspect was recalculated and presented as two variables, northness and eastness [51]. Rasterised layers of distance-to-water and habitat types were obtained from the Croatian Wetlands and Habitat Map GIS databases maintained by the State Institute for Nature Protection (SINP; [http://www.cro-nen.hr/map/index\\_en](http://www.cro-nen.hr/map/index_en)). All topographic variables were based on a 90-m spatial resolution digital elevation model (DEM) (Shuttle Radar Topography Mission; <http://www2.jpl.nasa.gov/srtm>) and prepared in ArcGIS® 9.3 (ESRI).

**Correlation between genetic, geographic and environmental distances.** To generate the environmental distance matrix, we performed a canonical discriminant analysis (CDA) based on the ten environmental variables (see Table 2) using PROC CANDISC in SAS. Squared Mahalanobis distances ( $D^2$ ) between the populations were computed to obtain a matrix of environmental distances among the populations. Mahalanobis distances are analogous to Euclidian distances but also account for covariance among variables. Mantel tests [52] were used to examine the extent to which the neutral genetic structure can be explained by the environmental heterogeneity. We computed and tested the correlations between (1) the matrix of the natural logarithm of geographical distances (in km) between pairs of populations and the matrix of pairwise  $F_{ST}/(1-F_{ST})$  ratios and (2) the matrix of environmental distances ( $D^2$ ) and the matrix of pairwise  $F_{ST}/(1-F_{ST})$  ratios. Only individuals that had information about all three parameters (genetic, geographic and environmental) were used for the correlation tests ( $n = 335$ ). In addition, a three-way Mantel test was applied between the matrix of environmental distances and the matrix of pairwise  $F_{ST}/(1-F_{ST})$  ratios while accounting for geographical distances among populations. The significance level was assessed after 10,000 permutations as implemented in NTSYS-pc Ver. 2.02 [53].

Finally, the relationships between the populations based on both genetic distances and environmental distances ( $D^2$ ) were visualised by constructing two neighbour-joining trees. Pairwise Nei's standard genetic distances [54] were calculated and an unrooted phylogenetic tree was constructed using the Neighbour-joining algorithm with 1 000 bootstrap replicates over microsatellite loci as implemented in the software PHYLIP Ver. 3.6b [55].

### Ecological niche analyses

The total set of 802 occurrence points was used to further examine the levels of ecological niche divergence between the

**Table 2.** Pearson correlation coefficients between ten environmental variables and scores of the first three principal components.

Environmental variables		Principal component					
		PC1		PC2		PC3	
BIO 01	Annual Mean Temperature	−0.978	***	−0.106	**	−0.047	ns
BIO 04	Temperature Seasonality (standard deviation*100)	0.896	***	−0.117	**	−0.288	***
BIO 05	Max Temperature of Warmest Month	−0.849	***	−0.162	***	−0.363	***
BIO 06	Min Temperature of Coldest Month	−0.954	***	−0.051	ns	0.063	ns
BIO 12	Annual Precipitation	−0.706	***	0.115	**	0.665	***
BIO 15	Precipitation Seasonality (Coefficient of Variation)	−0.779	***	0.254	***	−0.232	***
BIO 18	Precipitation of Warmest Quarter	0.744	***	−0.108	**	0.595	***
BIO 19	Precipitation of Coldest Quarter	−0.929	***	0.131	***	0.248	***
DEM 30	Digital elevation model	0.351	***	0.765	***	0.143	***
DEM S 30	Slope	0.036	ns	0.724	***	−0.263	***
	Eigenvalue	6.03		1.27		1.22	
	% of variance	60.32		12.70		12.23	

\*\*\*\*significance at the 0.1% nominal level,

\*\*\*significance at the 1% nominal level,

\*\*significance at the 5% nominal level, "ns" non-significant values.

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populations from the Continental and Mediterranean biogeographical regions. Each occurrence point was assigned to a specific region as shown in Figure 1. First, we conducted the PCA based on ten environmental variables (Table 2) that describe the ecology of all presence localities using PROC PRINCOMP in SAS. Second, we generated an overall ENM based on all 802 presence

points. In addition to the ten variables used for the PCA, terrain aspect, distance-to-water, and categorical habitat type variable were added (Table 3). All environmental layers were resampled to be used at a 100 m×100 m spatial resolution.

We applied a maximum entropy presence-only modelling technique to estimate the ecological niche of the species using

**Table 3.** Environmental variables used for *Fraxinus angustifolia* ENMs based on the Maximum Entropy (Maxent) method.

Environmental variables		Variable contributions (%)			
		Overall ENM	Continental ENM	Mediterranean ENM	Source
BIO 1	Annual Mean Temperature (°C)	1.1	1.2	38.0	www.worldclim.org
BIO 4	Temperature Seasonality (standard deviation *100)	1.0	12.7	4.1	www.worldclim.org
BIO 5	Max Temperature of Warmest Month (°C)	11.2	1.1	1.1	www.worldclim.org
BIO 6	Min Temperature of Coldest Month (°C)	0.3	0.1	0.6	www.worldclim.org
BIO 12	Annual Precipitation (mm)	2.9	0.8	0.1	www.worldclim.org
BIO 15	Precipitation Seasonality (Coefficient of Variation)	1.6	0.9	0.2	www.worldclim.org
BIO 18	Precipitation of Warmest Quarter (mm)	2.6	5.0	2.6	www.worldclim.org
BIO 19	Precipitation of Coldest Quarter (mm)	1.4	8.1	0.2	www.worldclim.org
DEM	Digital elevation model (elevation in m)	15.2	26.7	1.6	www2.jpl.nasa.gov/srtm
DEM S	Slope (degrees)	0.3	0.3	0.4	generated from DEM
DEM_ae	aspect eastness (eastness index)	0.5	0.2	0.1	generated from DEM
DEM_an	aspect northness (northness index)	0.5	0.3	0.1	generated from DEM
NKS	habitat type (as categorical variable)	21.2	26.9	1.6	www.cro-nen.hr/map
Dwater	Distance to water (m)	40.2	16.0	49.3	www.cro-nen.hr/map

Relative contributions of environmental variables to each of the three ENMs are shown as averages over ten replicate runs.

doi:10.1371/journal.pone.0042764.t003

Maxent Ver. 3.3.2 [56,57]. This method has proven robust for presence-only data [58]. We performed ten replicate runs using cross-validation with default parameters and we used a logistic output from Maxent [57]. Model performance was evaluated using the area under the curve (AUC) of a receiver-operating characteristic (ROC) plot. To depict suitable habitat maps, we used the minimum training presence threshold [56]. Finally, the relative contributions of the environmental variables to the Maxent model were recorded.

To further assess whether populations from different regions occupy divergent ecological niche space, we simulated two separated ENMs using only occurrence data from either the Continental or Mediterranean region following the same modeling procedures. To determine whether any areas of overlap between the putative ecotypes exist, we summed the probabilities of occurrence from the two regional ENMs after applying the minimum training presence threshold to each. In this way, we evaluated whether the stands present in the predicted overlap zones were congruent with low values of pairwise genetic distances. All resulting models were visualised in ArcGIS® 9.3.

## Results

### Within population genetic diversity

A total of 176 alleles were observed across the six markers, with the number of alleles per locus ranging from nine (Femsat16) to 48 (M2–30) and a mean value of 29.33.  $N_{ar}$  ranged from 9.81 to 17.15 (Table 1). High levels of both within populations  $H_O$  and  $H_E$  were found (mean values over loci and populations were 0.683 and 0.699, respectively). There was no evidence of allele dropout in the data according to MICRO-CHECKER. Null alleles were suggested in seven out of 66 locus × population combinations. Estimated null allele frequencies using FREENA ranged from 0.052 (Femsat12 in population Trnjani) to 0.146 (Femsat16 in population Lonjsko Polje). The  $H_E$  values increased slightly when recalculated using adjusted allele frequencies (Table 1), but no significant differences were observed between values of  $H_E$  and  $H_{E,null}$  in any of the analysed populations (Kruskal-Wallis test,  $P=0.52-0.81$ ). Therefore, all subsequent analyses were conducted using the original data set. Significant differences ( $P<0.05$ ) in genetic diversity (mean  $N_{ar}$ ,  $H_O$  and  $H_E$ ) were found between the Mediterranean and Continental populations, with lower values observed in the Mediterranean stands. Moreover, 57 private alleles were identified in the Continental region, whereas there were only nine in the Mediterranean region (Table 1). No significant departures ( $P<0.01$ ) from the HWE were observed at any loci in any population after applying sequential Bonferroni corrections. Finally, among a total of 165 tests for linkage disequilibrium between pairs of loci, no test was found significant after applying sequential Bonferroni corrections ( $P<0.01$ ).

### Genetic structure among populations and biogeographical regions

Although testing for HWE within each population showed no significant departures, the average multilocus inbreeding coefficient of the overall sample was slightly positive but significant ( $F_{IS}=0.024$ ,  $P=0.0025$ ). Moreover, mean multilocus values of  $F_{IS}$  in the Mediterranean region were almost four times higher and significant than that for the Continental region (Table 4). The overall multilocus differentiation among all populations ( $F_{ST}$ ) was 0.022. The within-region  $F_{ST}$ , however, was higher in the Mediterranean populations ( $F_{ST}=0.027$ ) than that among the Continental populations ( $F_{ST}=0.012$ ), showing that the coastal populations were more structured.

Pairwise  $F_{ST}$  values ranged from zero between Trnjani/Županja to 0.074 between Čakovec/Neretva population pairs (Table 5). No significant differences were observed between raw pairwise  $F_{ST}$  and pairwise  $F_{ST}$  corrected for null alleles (Kruskal-Wallis test,  $P=0.79$ ), suggesting that null alleles did not affect this analysis. Most population pairs from the Continental region had non-significant pairwise  $F_{ST}$  values, with the exception of the Čakovec population. In contrast, most population pairs within the Mediterranean were significantly differentiated. Pairwise differentiation was also detected between the Mediterranean and Continental populations, with the exception of the Istrian population Mirna, which was not significantly differentiated from several Continental populations. The AMOVA analysis (Table 6) showed that most of the genetic diversity was attributable to differences among individuals within populations (97.36%). However, a small but highly significant percentage of variation was explained by differences among populations within regions (1.72%) and by differences between regions (0.92%), confirming the geographic structuring of populations.

### Association between genetic and environmental variation

The analysed populations showed both significant levels of IBD ( $r=0.385$ ,  $P=0.026$ ) (Figure 2A) and even higher correlation between genetic divergence and environmental distance ( $r=0.549$ ,  $P=0.004$ ) (Figure 2B). The correlation between genetic and environmental distances remained significant ( $r=0.426$ ,  $P=0.002$ ) even after accounting for the effect of geographical distance in a three-way Mantel test (Figure 2C). On the other hand, the removal of the effect of environmental variation in the partial Mantel test resulted in a non-significant correlation between genetic and geographic distances ( $r=-0.025$ ,  $P=0.438$ ). Therefore, our populations show a clear “isolation by environmental distance” pattern, rather than IBD as such.

**Table 4.**  $F$ -statistics among 11 *Fraxinus angustifolia* populations and within each of the biogeographical regions.

Locus	$F_{IT}$	$F_{IS}$	$F_{ST}$
FEM11	0.028	0.011	0.017
FEM16	0.222	0.212	0.013
FEM19	−0.004	−0.053	0.047
FEM4	−0.082	−0.094	0.011
FEM12	0.165	0.145	0.024
M230	0.033	0.011	0.021
Multilocus estimates	<b>0.045</b>	<b>0.024</b>	<b>0.022</b>
Permutation test	$P<0.0001$	$P=0.0025$	$P<0.0001$
Continental region			
Multilocus estimates	0.024	0.012	<b>0.012</b>
Permutation test	$P=0.0104$	$P=0.1287$	$P<0.0001$
Mediterranean region			
Multilocus estimates	<b>0.071</b>	<b>0.046</b>	<b>0.027</b>
Permutation test	$P<0.0001$	$P=0.0024$	$P<0.0001$

$F_{IT}$  - overall inbreeding coefficient;  $F_{IS}$  - average inbreeding coefficient;  $F_{ST}$  - differentiation among populations. Significant values are indicated in bold. doi:10.1371/journal.pone.0042764.t004

**Table 5.** Pairwise  $F_{ST}$  values (lower diagonal) and null-allele corrected  $F_{ST}$  values (upper diagonal) among 11 *Fraxinus angustifolia* populations.

No	Population name	1	2	3	4	5	6	7	8	9	10	11
1	Županja		0.001	0.006	0.010	0.013	0.025	0.004	0.010	0.027	0.011	0.036
2	Trnjani	0.000 <sup>ns</sup>		0.004	0.011	0.009	0.020	0.002	0.008	0.033	0.016	0.038
3	Durdenovac	0.007 <sup>ns</sup>	0.002 <sup>ns</sup>		0.009	0.014	0.023	0.003	0.014	0.022	0.019	0.032
4	Stara Gradiška	0.010 <sup>ns</sup>	0.011 <sup>ns</sup>	0.005 <sup>ns</sup>		0.018	0.029	0.010	0.015	0.032	0.020	0.035
5	Lonjsko polje	0.013 <sup>ns</sup>	0.006 <sup>ns</sup>	0.014 <sup>ns</sup>	0.014 <sup>*</sup>		0.029	0.004	0.025	0.038	0.035	0.045
6	Čakovec	0.027 <sup>**</sup>	0.020 <sup>**</sup>	0.021 <sup>**</sup>	0.028 <sup>**</sup>	0.026 <sup>**</sup>		0.028	0.032	0.050	0.031	0.076
7	Jastrebarsko	0.006 <sup>ns</sup>	0.001 <sup>ns</sup>	0.003 <sup>ns</sup>	0.009 <sup>ns</sup>	0.003 <sup>ns</sup>	0.027 <sup>**</sup>		0.008	0.027	0.018	0.026
8	Mirna	0.009 <sup>ns</sup>	0.008 <sup>ns</sup>	0.011 <sup>ns</sup>	0.014 <sup>*</sup>	0.020 <sup>*</sup>	0.031 <sup>**</sup>	0.007 <sup>ns</sup>		0.024	0.015	0.030
9	Zrmanja	0.029 <sup>**</sup>	0.032 <sup>**</sup>	0.023 <sup>**</sup>	0.030 <sup>**</sup>	0.039 <sup>**</sup>	0.049 <sup>**</sup>	0.028 <sup>**</sup>	0.022 <sup>**</sup>		0.028	0.035
10	Krka	0.011 <sup>**</sup>	0.016 <sup>**</sup>	0.018 <sup>**</sup>	0.020 <sup>**</sup>	0.032 <sup>**</sup>	0.031 <sup>**</sup>	0.018 <sup>**</sup>	0.016 <sup>**</sup>	0.027 <sup>**</sup>		0.031
11	Neretva	0.038 <sup>**</sup>	0.037 <sup>**</sup>	0.032 <sup>**</sup>	0.033 <sup>**</sup>	0.045 <sup>**</sup>	0.074 <sup>**</sup>	0.028 <sup>**</sup>	0.027 <sup>**</sup>	0.038 <sup>**</sup>	0.032 <sup>**</sup>	

P-values as obtained by randomisations:

\*\*\*"significance at the 1% nominal level,

\*\*"significance at the 5% nominal level, "ns" non-significant values.

doi:10.1371/journal.pone.0042764.t005

## Ecological niche analyses

**PCA analyses.** The first principal component (PC1) explained 60.32% of the total variation and clearly separated the Continental and Mediterranean localities along temperature and precipitation gradients (Figure 3). Elevation and slope were highly positively correlated with PC2, explaining 12.70% of the total variation and reflecting a topographic gradient. In addition, PCA on environmental data revealed a notable environmental sub-structure within the Mediterranean region, where each of the populations clustered along a different river valley. The results show that Continental populations occupy habitats that are cooler with lower winter temperatures (below zero) and higher summer rainfall. In contrast, coastal populations are characterised by warmer habitats with higher winter temperatures and wetter winters. Generally, the range of variation in the examined environmental variables appears to be more pronounced between the Mediterranean localities than among the Continental localities.

**Ecological niche modelling.** The Maxent model performed well with high average training and test AUC values across ten replicate runs (Table 7) and was congruent with the currently known distribution of *F. angustifolia* in Croatia (Figure 4A). The highest probabilities of occurrence were in the Continental region, in lowlands along large rivers (Sava and Drava) with more or less continuous distribution. In contrast, the predicted distribution was discontinuous in the Mediterranean region with several isolated areas of high suitability associated with shorter karst river valleys

along the eastern Adriatic coast (Mirna, Krka, Zrmanja, and Neretva). The overall model did not predict suitable habitats in the Alpine region, confirming that the species distribution is strongly associated with river valleys and wetland sites (Table 3).

The overlap between the two regional modelled distributions was very low (Fig. 4B), suggesting strong regional niche differentiation between the two putative ecotypes. Despite high AUC values, each model alone predicted a highly reduced distribution of the species in comparison with the overall model. Contrary to our expectations, the variables differed in their contributions to the three distribution models (Table 3). Distance-to-water contributed most to the overall and Mediterranean ENM, whereas habitat type and elevation were most important predictors for the Continental ENM.

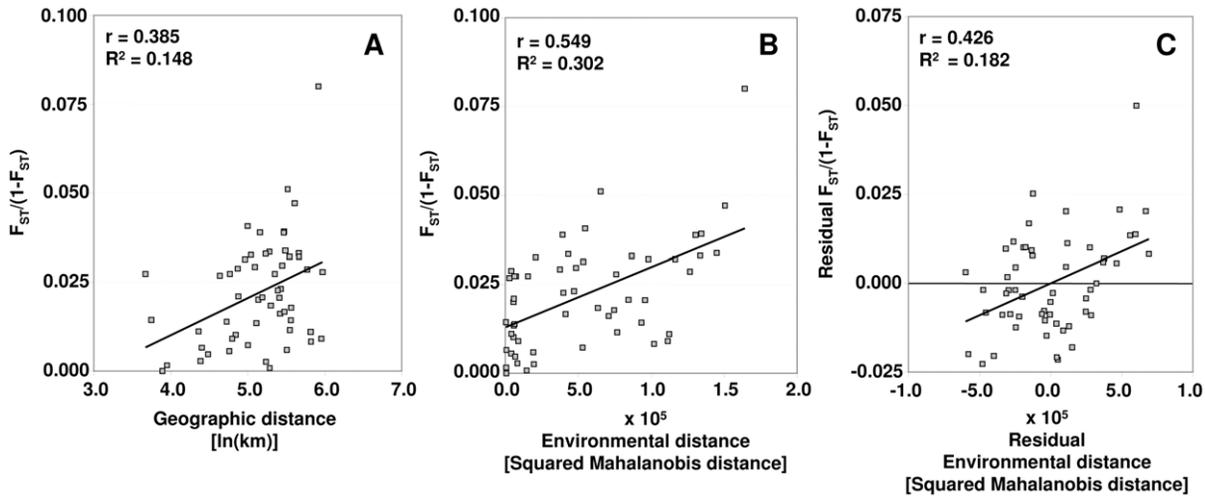
**Neighbour-joining analysis.** Trees based on either genetic distances (Figure 5A) or environmental distances (Figure 5B) were congruent in their major features, suggesting that environmental variation may promote genetic divergence of the studied populations. Moreover, a comparison of the genetic tree (Figure 5A) with the overlap map of the regional ENMs (Figure 4B) showed that the coastal population Mirna, which is situated intermediately in the genetic tree, is located in the overlap area. There are, however, some incongruencies. For example, the Jastrebarsko population belongs to the Continental region based on genetic markers but can be considered intermediate from an ecological point of view.

**Table 6.** Analysis of molecular variance (AMOVA) for the partitioning of genetic diversity among and within populations of *Fraxinus angustifolia* grouped into two biogeographical regions (Continental vs. Mediterranean).

Source of variation	df	Variance components	Percentage of variation	$\phi$ -statistics	P( $\phi$ )
Among regions	1	0.020	0.92	$\phi_{CT} = 0.009$	<0.0001
Among populations within regions	9	0.037	1.72	$\phi_{SC} = 0.017$	<0.0001
Within populations	679	2.086	97.36	$\phi_{ST} = 0.026$	<0.0001

P( $\phi$ ) -  $\phi$ -statistics probability level after 10 000 permutations.

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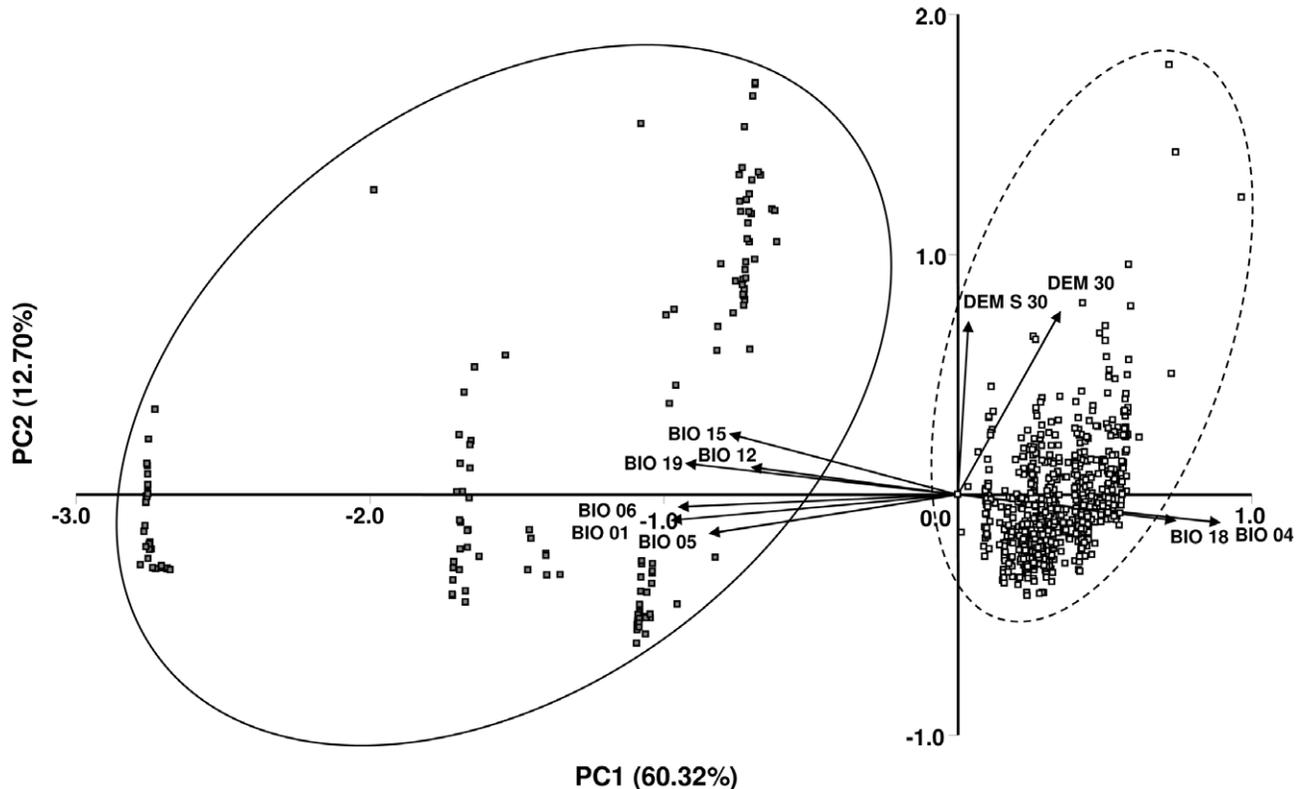


**Figure 2. Isolation-by-distance and Isolation by environmental distance.** Plots of simple and partial Mantel tests showing the relationships between A) geographic and genetic distances, B) environmental and genetic distances, and C) residual environmental and genetic distances, by taking into account the geographic distances among 11 populations of *Fraxinus angustifolia*. doi:10.1371/journal.pone.0042764.g002

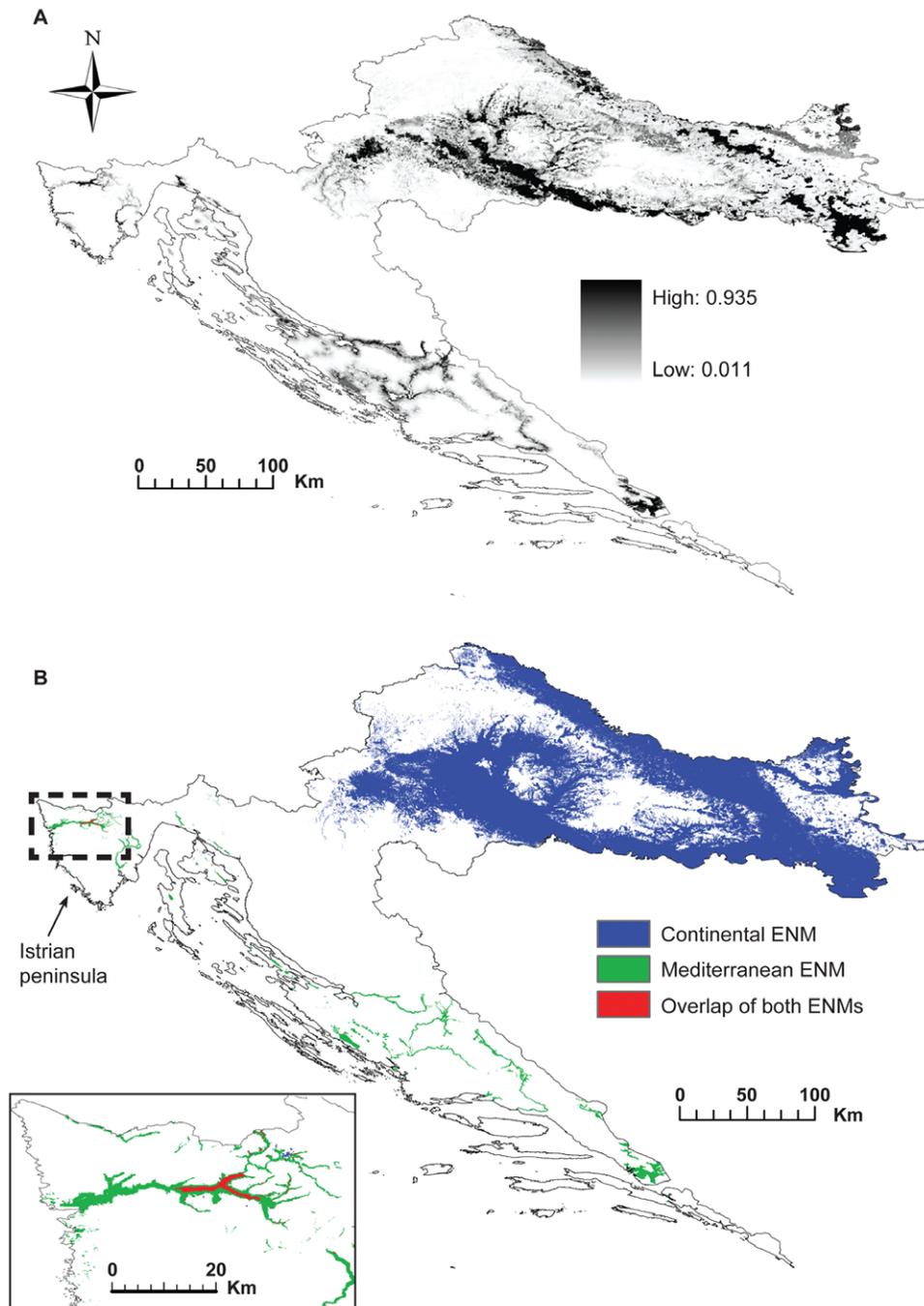
## Discussion

Our combined analysis of genetic data, multivariate statistics on environmental data and ENM suggests that current genetic variation patterns in natural *Fraxinus angustifolia* populations in Croatia may be influenced by the local ecological conditions rather than by geographic distances only. We observed an overall

pattern of significantly higher genetic diversity in the Continental region and low local differentiation that contrasts with the reduced genetic diversity and stronger structuring in the Mediterranean region. The extent of potential ecological niche overlap between the continental and coastal populations was low, suggesting that two ecologically distinct lineages of narrow-leaved ash may occur in the study area. ENM was in agreement with the genetic distance



**Figure 3. Plot of PCA based on ten environmental variables describing 802 *Fraxinus angustifolia* localities.** The Continental (white squares) and Mediterranean (grey squares) ecological lineages were separated along the PC1 and PC2. See Table 2 for environmental variable codes. doi:10.1371/journal.pone.0042764.g003



**Figure 4. Predicted Maxent Ecological niche models (ENMs) for *Fraxinus angustifolia*.** A) Overall ENM. Colour levels of shading from white (unsuitable habitat) to black (highest suitability) represent the continuous species' probability distribution after thresholding. B) Overlay of the two independently predicted regional ENMs to identify areas of environmental overlap (highlighted by the enlarged box). Minimum training presence thresholds: Continental = 0.012, Mediterranean = 0.044. doi:10.1371/journal.pone.0042764.g004

analysis, which found that most Continental and Mediterranean populations were differentiated.

#### Genetic diversity and structure

The genetic analysis results agree with the expectations of high polymorphism within populations and low genetic differentiation between populations, as observed in ashes and wind-pollinated trees in general [21,26,59,60]. However, the large and significant

local homozygote excess ( $F_{IS} > 0.15$ ) found in many European *F. excelsior* populations (22, 26, 59) was not observed in the present study. This characteristic of common ash was often attributed to the presence of null alleles, biparental inbreeding or the Wahlund effect. Unlike common ash populations studied in Europe [21,22,26,59] all of the populations studied herein were in HWE, indicating weak evidence for the presence of null alleles or of local inbreeding. Finally, the average multilocus inbreeding coefficient  $F_{IS}$ , which provides information on the cumulative

**Table 7.** Evaluation of each ENM using a threshold-independent ROC analysis with AUC.

	N presence points	Average training values	Average test values
		AUC; SD	AUC; SD
Overall ENM	802	0.948; 0.007	0.942; 0.008
Continental ENM	144	0.956; 0.006	0.951; 0.004
Mediterranean ENM	658	0.993; 0.008	0.985; 0.019

SD is the standard deviation of the average AUC values after ten replicated Maxent runs.  
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effect of inbreeding, was low but significant at the level of the overall sample (average  $F_{IS}$  of 0.024), but at the regional level only for the Mediterranean stands (average  $F_{IS}$  of 0.046). Although positive  $F_{IS}$  values may reflect the presence of null alleles, which are commonly suspected for microsatellite loci, controlled crosses carried out by Morand [22] showed that most of the loci used in this study do not show null alleles. Moreover, there were no significant differences between the  $H_E$  and  $F_{ST}$  values calculated with and without corrections for null allele frequencies. A plausible explanation for this positive inbreeding would be a Wahlund effect at the level of the overall sample population, mainly due to the differentiation between subpopulations, even though subpopulations themselves are in HW.

Local population sizes determined by suitable habitat availability may explain the contrasting genetic structures found within and among biogeographical regions. The observed higher genetic diversity in the Continental populations can possibly be maintained due to larger effective population sizes compared to their Mediterranean counterparts. Longitudinal distribution along rivers and floodplains typical for the Pannonian lowlands with no barriers to gene flow allows free pollen and seed dispersion between the populations, which explains the lack of significant genetic structure in this region based on pairwise  $F_{ST}$ , even between most distant populations. On the other hand, Mediterranean populations are reduced to a few smaller suitable sites associated with Dinaric karst fields, short karst rivers and rare natural wetlands with no apparent above-ground connections due to the limestone base. In consequence, populations are more isolated from each other and also from the Continental part of the distribution, limiting dispersion and favouring the maintenance of an intra-regional genetic structure.

### Genetic divergence and environmental heterogeneity

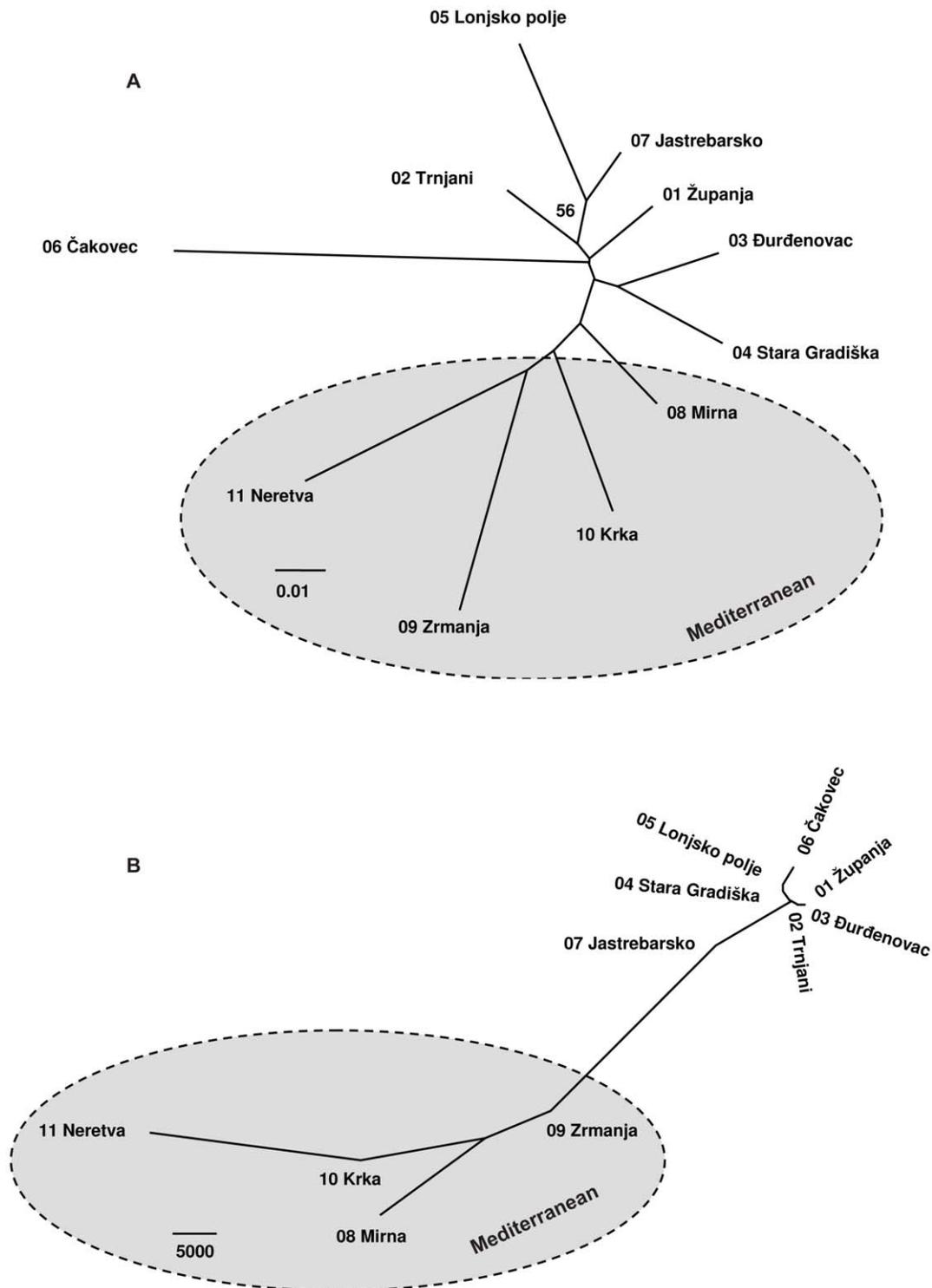
Large amounts of neutral population genetic variation were explained by environmental variation rather than by simple geographic distances, suggesting a strong role of environmental heterogeneity in the genetic divergence of populations [6–9,61]. First, pairwise  $F_{ST}$  values and multilocus estimates of  $F$ -statistics suggest that each region has different evolutionary constraints. For example, some very close Mediterranean populations (such as Zrmanja and Krka) have significant pairwise  $F_{ST}$  values although they are not geographically distant (only 40 km apart), suggesting more restricted gene flow in this region probably caused by isolation of suitable habitat and/or habitat differentiation. Environmental distinctiveness and habitat discontinuities among Mediterranean populations are apparent from the PCA and niche models, revealing a similar differentiation pattern compared to pairwise  $F_{ST}$ . The Mediterranean is known for pronounced environmental heterogeneity over very short distances because of factors such as slope, exposure, distance from sea, and rock type. In contrast, most of the Continental populations that occur in a

rather homogenous environment exhibit non-significant pairwise  $F_{ST}$  values (except for the Čakovec population), even in distant populations (>240 km). Similar trends were observed in *Taxus baccata* [62] on a broader geographical scale, in which populations located in the stronger Mediterranean climate displayed higher pairwise differentiation within regions than those from the inland areas, suggesting that the geographical and environmental features can influence population divergence of different tree species in this area.

Second, we found a significant correlation between genetic and environmental variation. IBED patterns may be arising from a neutral process of temporally disrupted gene flow among individuals living in environmentally distinct habitats, leading to phenological differences [7,22]. Gene flow should homogenise neutral genetic variation in wind-pollinated tree species at short geographical distances, but habitat differentiation can act as a barrier to gene flow, causing environmental isolation and genetic differentiation of spatially close plant populations [5], as found in our study. Finally, a significant impact of genetic drift in these populations can be discounted as drift alone would create a random genetic structure that was not observed herein; instead, ecologically similar populations were also grouped genetically. In sum, we show that the observed genetic variation pattern is associated with environmental gradients. We recognize that the observed IBED pattern does not imply causality and this correlation might have other plausible or more complex interpretations which cannot be explicitly tested using our current data, like different population ages in two regions because of independent colonization events. Future tests with candidate genes for traits of interest could also clarify the possible role of natural selection in shaping the divergence of these populations, but such markers are only recently emerging for *Fraxinus* spp. [63]. Further exploration in our study species is currently underway.

### Ecological niche models and genetic structure

Using an ENM approach, we tested whether the predicted distributions of the species corresponded with the patterns of population genetic structure. In particular, we searched for areas where the Continental and Mediterranean ENMs overlap, as they could highlight populations from different biogeographical regions with lower levels of pairwise genetic differentiation. The overall ENM detected a separation of the two putative ecotypes by the mountainous region, representing an unsuitable habitat for the survival of the species and a potential barrier to gene flow. The Continental and Mediterranean ENM barely overlapped, indicating a clear divergence in the ecological niche space occupied by the populations in each region. An overlap of independent ENMs suggests only one point of contact in the Istrian peninsula at the Mirna population site (Figure 4B). This stand is indeed the only Mediterranean population with non-significant pairwise genetic distances towards most Continental populations and is located



**Figure 5. Unrooted neighbour-joining trees for *Fraxinus angustifolia* populations.** A) Based on Nei's genetic distance. B) Based on environmental distance.  
doi:10.1371/journal.pone.0042764.g005

within an area of intermediate environmental conditions between the coast and the continent. Because one set of populations poorly predicts the distribution of the other set of populations and because their ecological niches almost do not overlap, our populations may represent two distinct evolutionary lineages, even

with the low levels of genetic divergence [12,16]. Lack of niche overlap in wide-ranging tree species may also appear to be driven by differences in abiotic conditions in different regions (soil, elevation, climate) [64]. Because our populations inhabit regions with clearly divergent climatic regimes and *F. angustifolia* has a wide

distribution, both latitudinally and altitudinally, this is a confounding factor that needs to be kept in mind.

### Stability, migration crossroads and environmental heterogeneity in refugia

From our observations, we cannot exclude the fact that historical migration processes in this refugial area could have raised the observed pattern of genetic variation in studied populations. Mediterranean tree populations have persisted in the southern refugia without significant geographical movements due to long-term stable environmental conditions in the Mediterranean [65]. Hence, the lower genetic diversity, higher genetic differentiation and higher fixation rates observed in the Mediterranean populations could result from older populations with smaller historic population sizes persisting in environmentally more stable regions over longer periods [66]. Croatia and the wider area of the Dinaric Alps have already been identified as an important refugium for ash and other temperate tree species during the Pleistocene and stand on a contact zone of their different postglacial recolonisation routes [34,67,68]. Recent studies have also confirmed the existence of a 'refugia within refugia' pattern in these areas, where differentiation of distinct lineages on a small geographical scale has been observed (see [69] and references therein). Therefore, forests in this region are expected to harbour greater regional genetic diversity and uniqueness in comparison with the rest of their range [31,65].

*F. angustifolia* is a thermophilic tree species with distinct moisture requirements that could have survived *in situ* during the last glacial period in several river valley sites along the Dalmatian coast at lower to mid-altitudes until today. These humid but not too cold sites provided continuous moisture availability and shelter for Mediterranean tree species during the Adriatic Sea level drop in the LGM, leaving the Northern half of the Adriatic Sea basin exposed and unsuitable for the survival of moisture-dependent species [33]. Northern coastal populations in Istria and the rest of the Continental populations could have been recolonised by expansion from the Dinaric Alps or from refugia in North Italy and/or Balkan Peninsula [68], most likely via the North Adriatic or along the Danube river lowlands. At least three *F. angustifolia* haplotype lineages meet at the vicinity of the investigated populations (H01, H02 and H03, *sensu* [68,27], authors' personal observations), confirming that various migration events occurred in the past within the study area and suggesting that Croatian

populations may have originated from various colonising routes that likely brought new diversity. Higher levels of genetic variation in the Continent could therefore be due to a gene flow among individuals from different glacial refugia in newly colonised regions [31] while southern coastal populations probably represent relict, genetically more divergent populations [65]. In fact, modelling of potential distribution of *Quercus robur* in Europe during the LGM [14] shows that both the Adriatic coastal and Continental lowland parts of Croatia were suitable for the survival of this ecologically similar species *in situ*. Assuming that *F. angustifolia* followed a similar distribution during the LGM, this species likely survived for long time in this area, allowing enough time for the differentiation of distinct populations to occur through a processes of ecological isolation.

### Conclusions

Overall, our results suggest that long-term stability of heterogeneous environments at regional spatial scales may explain current levels of genetic diversity and population genetic divergence in narrow-leaved ash in these ancient refugia. Environmental differences between the regions may have led to the general subdivision into two ecotypes, with the pronounced environmental heterogeneity in the Mediterranean further promoting the genetic differentiation of the coastal populations. Thus, the local genetic structure in the narrow-leaved ash is more complex than a simple allopatry divergence model as the populations are not clear-cut differentiated but rather in a complex genetic cline, probably resulting from the environmental heterogeneity over the studied geographical area.

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### Author Contributions

Conceived and designed the experiments: MT JF NFL. Performed the experiments: MT. Analyzed the data: MT JFFM ZS MG. Contributed reagents/materials/analysis tools: MT NFL. Wrote the paper: MT JFFM ZS.

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**ZNANSTVENI RAD BR. 2**

# Identifying refugia from climate change using coupled ecological and genetic data in a transitional Mediterranean-temperate tree species

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## Abstract

Populations occurring in areas of overlap between the current and future distribution of a species are particularly important because they can represent “refugia from climate change”. We coupled ecological and range-wide genetic variation data to detect such areas and to evaluate the impacts of habitat suitability changes on the genetic diversity of the transitional Mediterranean-temperate tree *Fraxinus angustifolia*. We sampled and genotyped 38 natural populations comprising 1006 individuals from across Europe. We found the highest genetic diversity in western and northern Mediterranean populations, as well as a significant west to east decline in genetic diversity. Areas of potential refugia that correspond to approximately 70% of the suitable habitat may support the persistence of more than 90% of the total number of alleles in the future. Moreover, based on correlations between Bayesian genetic assignment and climate, climate change may favour the westward spread of the Black Sea gene pool in the long term. Overall, our results suggest that the northerly core areas of the current distribution contain the most important part of the genetic variation for this species and may serve as *in situ* macrorefugia from ongoing climate change. However, rear-edge populations of the southern Mediterranean may be exposed to a potential loss of unique genetic diversity owing to habitat suitability changes unless populations can persist in microrefugia that have facilitated such persistence in the past.

**Keywords:** conservation genetics, *Fraxinus*, genetic variation, habitat suitability, niche modeling, refugia from climate change

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## Introduction

Evidence is accumulating that the current global warming is decreasing the amount of suitable habitat for plant and animal species, which can cause significant changes in their distribution patterns (Parmesan & Yohe 2003; Parmesan 2006). However, decreases in habitat suitability because of climate change can be

extremely variable, even between adjacent regions, because of latitude, topography and particular regional climates. For instance, Mediterranean ecosystems are expected to be especially affected by climate change due to water stress and desertification processes, and the adjacent temperate zone may be subject to novel climates in the future (IPCC 2007; Giorgi & Lionello 2008; Lindner *et al.* 2010). This spatial variability of climate change impacts implies that species with wide distributions may face different climatic risks in different parts of their ranges.

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Mediterranean taxa are expected to shift their distributions northwards in response to the current warming climate. As a result, peripheral populations will face an increased risk of extinction. Among these taxa, tree species are considered to be particularly vulnerable to climate change because of stronger dispersal limitations and slow evolutionary rates (Petit *et al.* 2005). Nevertheless, this threat can be overcome if populations have sufficient standing adaptive variation to respond rapidly to a changing environment (Savolainen *et al.* 2011). Temperate trees with peripheral populations located within the Mediterranean basin could lose a portion of their overall within-species genetic diversity if their southern-edge populations become extinct unless microhabitats can buffer major climate changes. Such a loss may jeopardize the long-term adaptive potential of the species to a changing environment (Hampe & Petit 2005; Eckert *et al.* 2008). Although the genetic consequences of climate changes are recognized as an important issue, how ongoing climate changes might affect within-species genetic variation is still poorly studied (Gienapp *et al.* 2008). For widespread tree populations in heterogeneous landscapes with a low seed migration capacity and high levels of standing genetic variation, habitat-driven selection may be the main mechanism structuring future populations. Under the ongoing climate change, we may expect a slow but significant decay of populations exposed to strong climatic stress and the survival of trees in areas where the landscape may buffer major climate changes. Indeed, it is probable that these mechanisms allowed survival of trees through the different climate oscillations of the Quaternary. Most likely, trees in microrefugia facilitated recolonization after the end of the last glaciation because the estimated postglacial migration rates of trees far exceed known rates of seed dispersal (Petit *et al.* 2008). Whether genomes preserved in refugia always include special genetic adaptive variation is unknown, but it is likely that certain genotypes with a selective advantage in the new environments may increase in frequency given enough time.

Patterns of genetic variation across the ranges of species have a key role in their capacity to survive in changing environments. Therefore, identifying the spatial distribution of genetic variation remains fundamental for understanding the responses of species to ongoing climate change, as well as for developing effective conservation strategies. Several hypotheses have been proposed to predict the change in the pattern of genetic variation across the range of a species. The central-marginal hypothesis predicts that a species is most abundant in the centre of its range and that populations become gradually less dense towards the range limits (Eckert *et al.* 2008). As a result, peripheral populations

are expected to be smaller and more spatially isolated, with reduced gene flow and lower within-population genetic diversity compared with central populations (Vucetich & Waite 2003; Eckert *et al.* 2008). This genetic decline towards the range margins suggests that the priority for conservation should be given to central populations because they harbour the highest genetic diversity and most likely have the greatest evolutionary potential for adaptation to a changing environment.

In contrast, for temperate species in the Northern Hemisphere that may have undergone range expansions after the end of the last glaciation, the “leading-edge expansion” theory predicts a gradual northward decrease of population genetic diversity away from the main glacial refugia located in the three Mediterranean peninsulas (Iberian, Italian and Balkan) (Hewitt 2000). This “southern richness versus northern purity” genetic pattern is an outcome of northward postglacial recolonization by founder events and has been confirmed by numerous phylogeographical studies for a variety of widespread taxa, including temperate trees (Magri *et al.* 2006). However, many species do not conform to the predicted latitudinal gradient of genetic diversity. For numerous temperate trees, the intrapopulation genetic diversity is highest in Central Europe. This pattern is explained by the merging of divergent colonizing lineages from different refugia at intermediate latitudes (Petit *et al.* 2003). In contrast, longitudinal clines in genetic diversity may be expected across the Mediterranean basin for typical Mediterranean trees (Fady & Conord 2010). These clines add complexity to the “core” versus “edge” dynamics. Finally, the “rear edge versus leading edge” concept emphasizes the importance of peripheral populations for species survival and argues that rear-edge populations may serve as an important source of unique genetic diversity, which requires a high conservation priority (Hampe & Petit 2005). This increased genetic value is inferred based on the long-term stability of rear-edge populations, which have persisted *in situ* since past climate changes and have been influenced by environments different from those affecting the central populations. Hence, these southern populations are expected to be genetically more divergent and better adapted to local, usually suboptimal environmental conditions.

Hypotheses about climate change impacts on habitat suitability are usually derived from ecological niche modelling (ENM; also known as species distribution modelling or SDM) (Thuiller *et al.* 2005). Assuming stability in niche preferences, the predicted shifts of habitat suitability by projecting species distributions in the future can give insights into which parts of the current distribution of species with low dispersal capacity are subject to low and high exposure to climate change

regimes. Of particular interest are the areas where a current suitable habitat remains stable despite anthropogenic climate change in the forecasted distributions. Such areas may signal potential *in situ* “refugia from climate change”, a concept recently proposed by Ashcroft (2010). Hence, the identification of relatively stable climate change refugia where taxa have the best opportunities to survive in the future is needed to guide decisions about *in situ* and *ex situ* conservation strategies. However, the classic ENM approach does not consider within-species genetic variation across a distribution range when developing assumptions about climate change effects. Thus, multidisciplinary approaches are strongly encouraged for the identification of refugia from anthropogenic climate change (Keppel *et al.* 2012). Merging ENM with genetic variation data provides a better view into the possible genetic response of populations to climate change and helps in developing more effective conservation guidelines for species facing changing environments (Alsos *et al.* 2009; D’Amen *et al.* 2012). For example, Collevatti *et al.* (2011) showed that the genetic diversity of an endemic tree from Brazil is expected to decrease under climate change if the habitat suitability predicted by niche modelling drops below a certain threshold. Recently, Jay *et al.* (2012) introduced ancestry distribution models to predict changes in the genetic structure of alpine plants in response to temperature increase. Although the strengths of these new approaches are similar to those of niche models, particularly in terms of the generality with which they can be applied, they may suffer from the same limitations. Modelling the correlations of genetic structure and climate and projecting these correlations onto future climates imply that dispersal is not limited and that the projected genetic variation will be the same for neutral and non-neutral genes. In a similar way niche modelling assumes that habitat preferences are not supposed to change across time (niche conservatism), one must assume that the observed genetic associations between different genotypes and specific climates will remain the same in the future.

In our study, we apply a multidisciplinary approach by combining ENM, classic population genetics and recently developed Bayesian models to explore the possible genetic consequences of current climate changes through the example of a widespread, wind-pollinated transitional Mediterranean-temperate tree species, the narrow-leaved ash. We focus here on the broad-scale impacts of rapid ongoing climate changes on genetic variation in the near future (2050–2080). We based our analysis on the assumptions of no long-distance dispersal (i.e. “no-migration”; Thuiller *et al.* 2005) in view of the short time period of our predictions (<100 years); no evolution of habitat preferences (niche conservatism);

and constancy of the correlations between genetic variation and climate. Under these assumptions, we might expect extinctions of local populations in the long term in areas of decreased habitat suitability. These extinctions may in turn cause a decrease in the overall genetic diversity of the species unless microrefugia allow the survival of local populations after the macroclimate becomes unsuitable.

We first model potential broad-scale changes in habitat suitability between current and future conditions to identify the boundaries of putative macrorefugia from 21st-century climate change for the species. We consider macrorefugia areas where the predicted current and future suitable habitats overlap. Microrefugia (Rull 2009), or areas likely to conserve favourable microclimates where climate change may be buffered locally, may occur outside or within these boundaries, but they may not be apparent at the continental scale of our study. Second, we investigate the range-wide patterns of current neutral genetic variation and test for the existence of a specific geographical gradient of genetic diversity across the European range of the species. We, then, apply recently developed Bayesian models to forecast intraspecific changes in genetic structure under climate change, based on associations between genetic variation and environmental variables (Jay *et al.* 2012). Finally, we evaluate how habitat suitability changes induced by climate change may affect the overall genetic diversity of the species in the future. To do so, we, specifically, attempt to identify populations occurring in areas of stable and decreased habitat suitability in the future and explore how the distribution of current genetic variation relates to potential climate change refugia. We, then, quantify the potential loss of genetic diversity caused by predicted local extinctions.

## Materials and methods

### *Study species*

*Fraxinus angustifolia* Vahl (the narrow-leaved ash) is a widely distributed wind-pollinated tree species naturally extending throughout Southern and Eastern Europe, from Portugal in the west to the Black Sea in the east. Despite its wide distribution, this species is a local habitat specialist restricted to humid areas and waterways throughout the Mediterranean, whereas it can be notably abundant and dominant in the temperate floodplain and riparian forests along the large rivers and wetlands in Central and South-eastern Europe (the Danube and the Pannonian basin). Natural hybridization between *F. angustifolia* and *F. excelsior* has been widely reported and occurs primarily at the northern limit of the species range in the Loire, Saône and

Danube river valleys (Gérard *et al.* 2012). The current distribution of this species is largely confined to areas of glacial refugia proposed to be located in the Balkan, Iberian and northern Apennine Peninsulas, as well as in the Dinaric Alps (Heuertz *et al.* 2006).

### Ecological niche modelling

*Species occurrences and environmental data.* We used a total of 3010 occurrence points collected from online databases, literature, personal communications and our own sampling (Table S1, Supporting Information). For current climate, we used eight WorldClim bioclimatic layers (<http://www.worldclim.org/>) previously demonstrated to be important for modelling tree distributions (Temunović *et al.* 2012; Table S2, Supporting Information). For future climate projections, we used the same bioclimatic variables for the years 2050 and 2080 derived from seven different general circulation models (GCMs) (Table S3, Supporting Information) based on the “business-as-usual” A1B emission scenario (IPCC 2007). In addition to bioclimatic variables, our current and future environmental data set included elevation calculated from a digital elevation model (obtained from <http://www.worldclim.org/>), the soil type layer (<http://webarchive.iiasa.ac.at/Research/LUC/External-World-soil-database/HTML/>) and the Euclidean distance to rivers (obtained from <http://hydrosheds.cr.usgs.gov>) (Table S2). We assumed that these variables will remain constant in the future. We also verified that the selected variables are not highly correlated ( $r \leq \pm 0.8$ ). All environmental layers were used at a resolution of 30 arc-seconds ( $\sim 1$  km). Our data set for the ENM includes the northern localities of the Loire Valley in France and neighbouring areas that are known to have a mix of hybrid populations with *Fraxinus excelsior* L. and pure *F. angustifolia*; however, because hybrid and nonhybrid populations occur in a gradient without a clear-cut separation, we restricted our genetic analyses to the southern and eastern populations that were shown to be pure *F. angustifolia* (Gérard *et al.* 2012).

*Modelling approach.* To create current and future niche models for *F. angustifolia*, we applied the maximum entropy presence-only modelling approach implemented in Maxent software version 3.3.e (Phillips *et al.* 2006). For each of the seven GCMs and for both future periods, we ran ten replicated runs using the default parameters in Maxent and cross-validation. The performances of the models were validated using a threshold-independent area under the receiver operation characteristics curve (AUC) (Fielding & Bell 1997). We used a median output for each GCM future prediction and applied a “maximum training sensitivity plus specificity” threshold to

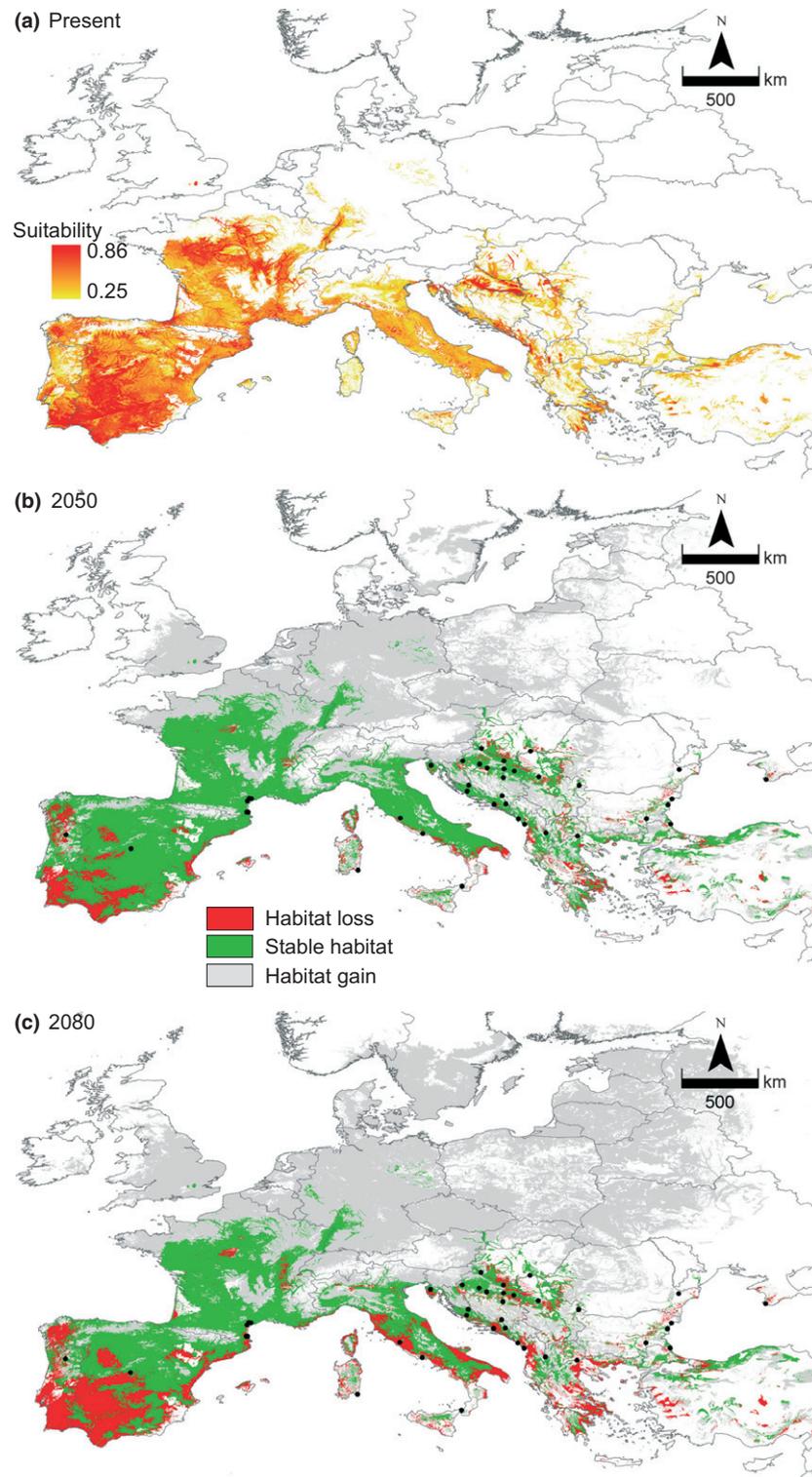
obtain suitable/nonsuitable habitat maps (Liu *et al.* 2005). To reduce the level of uncertainty arising from different GCM projections, we averaged the values of suitable pixels across the seven projections in ArcGIS 9.3 (ESRI, CA, USA) to obtain consensus niche models (Araújo & New 2007) for the years 2050 and 2080. Finally, we overlaid the consensus model for each future period with the predicted current niche model to identify putative refugia from climate change as areas where the predicted current and future suitable habitats overlap. Areas of decreased habitat suitability in the future were estimated as areas from the current predicted suitable habitat that were not present in the predictions for 2050 or 2080. We estimated the reduction of suitable habitat as the percentage of currently suitable pixels projected to be lost assuming the no-dispersal hypothesis.

### Sampling and genotyping

We sampled a total of 1006 trees of *F. angustifolia* from 38 natural populations across its European range (Fig. 1 and Table S4, Supporting Information). Total DNA was extracted with the DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA) from approximately 10 mg of dry leaves or, alternatively, with the DNeasy 96 Plant Kit (Qiagen) from approximately 20 mg of dry leaves following the manufacturer’s protocol. Six widely used and highly polymorphic *Fraxinus* spp.-specific nuclear microsatellite markers (Femsat14, Femsat111, Femsat112, Femsat116, Femsat119 and M2-30) (Brachet *et al.* 1999; Lefort *et al.* 1999) were used and genotyped as previously described in Morand *et al.* (2002). PCR products were detected on a sequencer ABI 3100 (Applied Biosystems), and allele sizes were scored using GeneMapper 4.0 (Applied Biosystems). Genotyping errors due to null alleles or allele dropouts were analysed using Micro-checker 2.2.3 (Van Oosterhout *et al.* 2004), and paternity exclusion probabilities were estimated using Identity 1.0 (available at [www.uni-graz.at/~sefck/manual.pdf](http://www.uni-graz.at/~sefck/manual.pdf)).

### Patterns of genetic diversity and divergence

The genetic diversity of each population was characterized by the average number of alleles per locus ( $N_{av}$ ), allelic richness ( $N_{ar}$ ) and private allelic richness ( $N_{par}$ ) standardized for the minimum sample size, the number of private alleles ( $N_{pr}$ ), the observed ( $H_o$ ) and expected ( $H_e$ ) heterozygosity and the multilocus inbreeding coefficient ( $F_{IS}$ ). All genetic diversity parameters were calculated using FSTAT v. 2.9.3.2 (Goudet 1995), HP-RARE (Kalinowski 2005) and GDA v. 1.0 (Lewis & Zaykin 2001) software. Deviations from Hardy–Weinberg equilibrium (HWE) for each population across all loci were



**Fig. 1** Maxent models for *Fraxinus angustifolia*. Current habitat suitability (a) and predicted changes of habitat suitability under an A1B climate change scenario by (b) 2050 and (c) 2080. Future models show consensus ENMs averaged across seven GCMs. Red areas are currently suitable habitats projected to be lost, green areas are currently suitable habitats projected to remain stable (putative climate change refugia), and grey areas are projected to become suitable in the future. Black dots represent sampled populations used for genetic analyses.

tested using Fisher's exact test in GENEPOP v. 4.0 (Raymond & Rousset 1995). To test for possible geographical gradients in genetic diversity, we explored relationships between genetic diversity estimates ( $N_{ar}$ ,  $H_E$  and  $F_{IS}$ ) and the latitude and longitude of each population site using multiple regression in SAM v.4 (Rangel *et al.* 2010). Population genetic differentiation was measured by calculating pairwise  $F_{ST}$  values (Weir & Cockerham 1984) and testing the significance between all pairs of populations using FSTAT. FSTAT was also used to compare and test the significance of differences in genetic diversity between the western and eastern Mediterranean groups of populations. Finally, we tested for a spatial genetic structure across all sampled populations in Europe using the Mantel test with 9999 permutations in GenAlEx 6.3 (Peakall & Smouse 2006).

#### *Genetic diversity under climate change*

We estimated the potential loss of genetic diversity under climate change assuming no dispersal and considering only macroclimate. These assumptions suggest that the genetic diversity of populations could be potentially compromised in areas where the habitat is no longer suitable in the future. We first calculated the total number of observed alleles and  $N_{ar}$  by pooling all the current populations and then recalculating the parameters by pooling all the populations predicted to remain in stable habitats in each future period (all standardized for eight individuals per population) using the software HP-RARE. We also calculated the expected values corrected for differences in sample size between the periods using a rarefaction method implemented in HP-RARE in which allele counts are standardized for the minimum number of individuals present in any period to allow the estimation of a count of private alleles. FSTAT was used to compare and test the differences in  $H_E$ ,  $F_{IS}$  and  $F_{ST}$  between the groups of populations pooled into three periods (current, 2050 and 2080).

#### *Bayesian population genetic structure*

To investigate the population genetic structure, we used a recently developed spatial Bayesian clustering algorithm that incorporates a hidden regression framework implemented in the software POPS (<http://membres-timc.imag.fr/Olivier.Francois/pops.html>). This new approach shares several features with the software TESS and incorporates spatially explicit information on geographic coordinates for each sampled individual (Durand *et al.* 2009). Therefore, it is useful to use POPS if genetic clines and low population differentiation are expected, because the admixture model accounts for geographical clines of genetic variation and spatial

autocorrelation residuals account for isolation-by-distance effects (Durand *et al.* 2009). In addition, POPS includes environmental covariates and predicts the individual membership coefficients based on correlations with environmental variables (Jay *et al.* 2011). The regression coefficients between membership coefficients and environmental covariates are learned during the Markov Chain Monte Carlo (MCMC) algorithm runs. Finally, based on the estimated relationship between the membership coefficients and the effects of the environmental variables, POPS is able to project population genetic structure under climate change scenarios assuming that future correlations between climate and genetic variation remain the same as today and that there is enough time and sufficient dispersal capacity for genes to expand in the neighbouring populations (Jay *et al.* 2012).

We performed an admixture model with 10 independent runs per each putative maximum number of clusters  $K_{max}$  ( $K_{max}$  ranging from 2 to 10) using a run length of 20 000 sweeps with a burn-in of 5000. We used the default value for the POPS spatial interaction parameter ( $\psi = 0.6$ ) that describes the intensity of the spatial dependence in the admixture model. We identified the optimal genetic structure when the deviance information criterion (DIC) curve began to reach the plateau (Durand *et al.* 2009), the results remained similar when higher values of  $K_{max}$  were used, and no additional clusters appeared. To check for the consistency of our results obtained with POPS, we also applied TESS 2.3 (Chen *et al.* 2007) with longer runs (200 000 sweeps, burn-in of 100 000). To determine the correlations between membership coefficients and climate covariates, we selected the same eight bioclimatic variables used for ENM. The best POPS runs were selected for projecting the population genetic structure for 2050 and 2080 under future climates averaged across seven different GCMs. The individual assignment probabilities for the most likely  $K_{max}$  value were averaged across replicated runs using CLUMPP v. 1.1.2 (Jakobsson & Rosenberg 2007). Bar plots were produced using DISTRUCT v. 1.1 (Rosenberg 2004), and maps of mean membership coefficients per population were displayed in ArcGIS 9.3.

## Results

### *Habitat suitability changes under climate change*

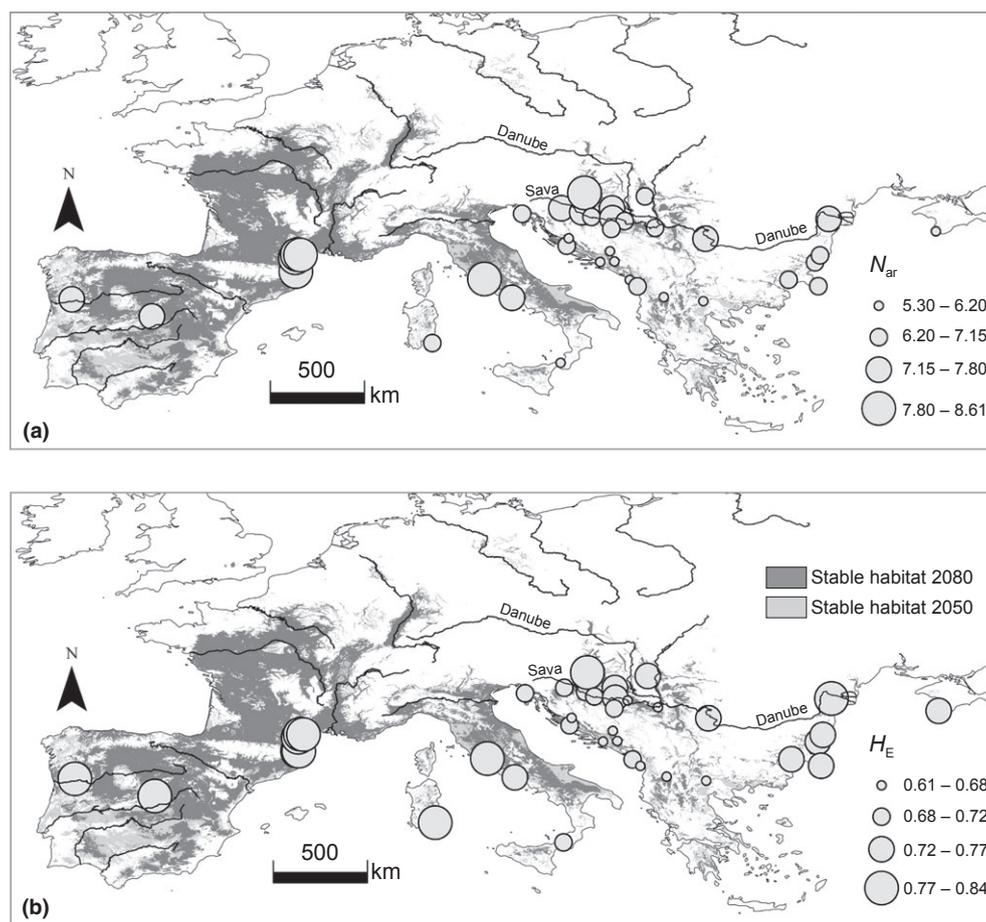
Maxent models performed well in predicting the current ecological niche of *Fraxinus angustifolia* (average training AUC = 0.91; average test AUC = 0.91 across ten replicate runs). Compared with the current niche model (Fig. 1a), future projections for 2050 and 2080

time slices predicted northward shifts of suitable habitats for *F. angustifolia*. The 2050 prediction indicated that most of the current species range will remain suitable (Fig. 1b). However, more severe reductions in habitat suitability were predicted by 2080 (Fig. 1c). Under the no-dispersal scenario (which assumes that species will persist only in areas of stable habitat where the current and future niche models overlap), 16% of the total suitable habitat was predicted to be lost by 2050 and 33% by 2080 based on the average consensus model (Fig. 1). Loss of currently suitable habitats is suggested to varying degrees in the southernmost parts of the current distribution of the species, primarily in coastal Mediterranean areas. Conversely, a substantial gain of potential suitable habitats at higher latitudes beyond the current predicted range is expected under climate change (Fig. 1). However, the average habitat suitability, calculated as the mean value across all pixels classified as suitable in the consensus ENMs, showed a decrease from 0.42 (present) to 0.25 in 2050 and to 0.2 in 2080, suggesting

that the overall habitat suitability will decrease despite the predicted habitat gain.

#### Patterns of genetic diversity and divergence

We detected a total of 223 alleles over six microsatellite loci within the whole data set that provided an exclusion probability  $>0.99$ .  $N_{ar}$  over loci ranged from 5.31 to 8.61 per population (Fig. 2a, Table S4). The mean  $N_{par}$  was 0.16, ranging from 0.02 to 0.48 per stand, and we found a total of 38 private alleles across 38 stands (Table S4). The observed ( $H_O$ ) and expected ( $H_E$ ) heterozygosity per population varied greatly (Fig. 2b, Table S4). The multilocus inbreeding coefficient per population was low to moderate ( $F_{IS} = -0.13$  up to 0.16). The HWE results showed significant deviation from HWE in half of the studied populations (Table S4). Null alleles were suggested in 29 of 228 locus  $\times$  population combinations. Pairwise population differentiation ( $F_{ST}$ ) ranged from zero (between six population pairs) to 0.19



**Fig. 2** Range-wide distribution of genetic diversity in 38 *Fraxinus angustifolia* populations: (a) allelic richness ( $N_{ar}$ ) and (b) expected heterozygosity ( $H_E$ ). Circles indicate sampling locations, and the circle size is proportional to the genetic diversity values. The potential refugia from climate change are depicted in grey.

(between populations PO and NT) and was significant for 87% of the pairwise comparisons ( $P < 0.05$ , Table S5, Supporting Information). We also detected a significant spatial genetic structure ( $r = 0.52$ ,  $P < 0.001$ ) across Europe, confirming that populations are spatially structured.

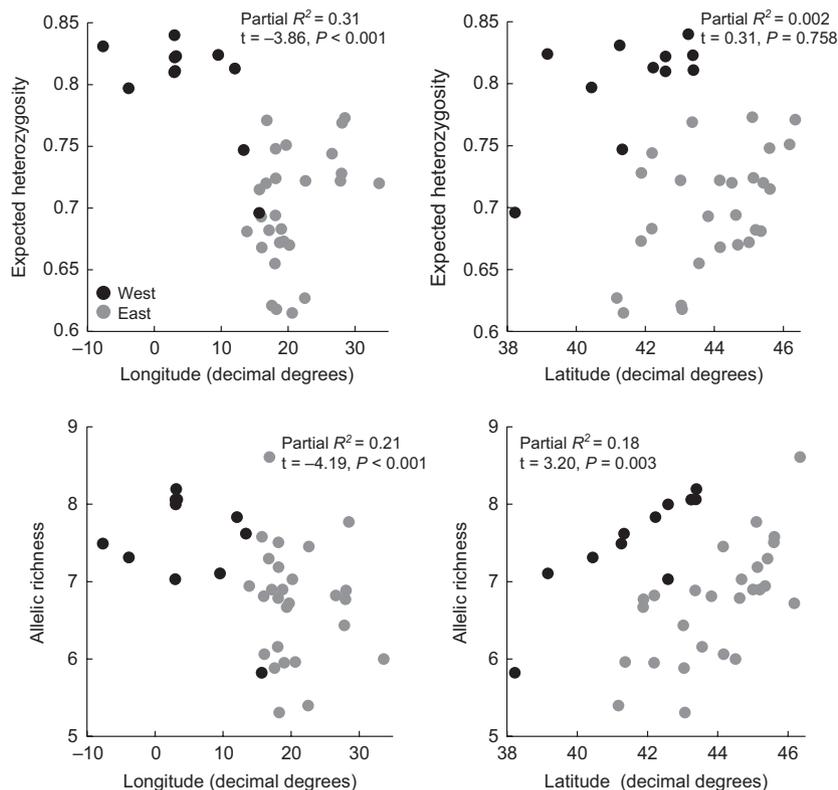
#### Geographical gradients of genetic diversity

The spatial distribution of  $H_E$  and  $N_{ar}$  was notably similar and revealed that the highest genetic diversity was typically located at the mid-latitudes, approximately in the centre of the current species distribution, compared with more peripheral populations (Fig. 2). When tested, genetic diversity was significantly higher in the western compared with the eastern Mediterranean populations ( $N_{ar}$ :  $P = 0.005$ ;  $H_E$ :  $P = 0.001$ ), but we found no significant differences in inbreeding coefficient values ( $F_{IS}$ :  $P = 0.081$ ). The highest genetic diversity values were observed in the French populations and in the northernmost Croatian population (CK). In general, the southernmost populations were the least diverse. The multiple regression of allelic richness against longitude and latitude ( $F = 11.016$ ,  $P < 0.001$ ) showed that  $N_{ar}$  decreased significantly with longitude (partial  $R^2 = 0.21$ ,  $P < 0.001$ )

and increased with latitude (partial  $R^2 = 0.18$ ,  $P = 0.003$ ), revealing a bidirectional cline across Europe (Fig. 3). Expected heterozygosity decreased significantly only with longitude (overall model  $F = 7.809$ ,  $P = 0.002$ ; partial  $R^2 = 0.31$ ,  $P < 0.001$ ), confirming a clear west–east gradient of genetic diversity at the continental level (Fig. 3), while latitude had no significant contribution on  $H_E$  patterns (partial  $R^2 = 0.002$ ,  $P = 0.758$ ). No clear geographical pattern was observed for  $F_{IS}$  (longitude:  $P = 0.709$ ; latitude:  $P = 0.673$ ).

#### Potential loss of genetic diversity under climate change

We first sought to identify populations occurring within the areas of stable habitat and decreased habitat suitability (defined by pixels predicted to be currently suitable and projected to be unsuitable in the future). Assuming no dispersal and no possibility of buffering new climates in microrefugia, seven populations were predicted to be at risk of extinction by 2050 and 16 by 2080 due to the reductions in suitable habitat. In allele terms, this habitat suitability change would result in a total loss of 17 unique alleles from the 223 currently detected (Table 1).  $N_{ar}$  for all current populations pooled together was 30.58, but it was slightly smaller if



**Fig. 3** Longitudinal and latitudinal variation of genetic diversity (allelic richness and expected heterozygosity) in 38 *Fraxinus angustifolia* populations across Europe. Western and eastern populations are marked with black and grey dots.

**Table 1** Predicted changes of overall genetic diversity of *Fraxinus angustifolia* under climate change in Europe. Values are calculated for all sampled populations pooled together (Current), for populations that are included within the predicted suitable areas of the current niche model (Current predicted) and for populations occurring in predicted suitable areas for each future period (2050 and 2080)

Period	Npop	Nind	N <sub>tot</sub>	N <sub>ar</sub>	H <sub>E</sub>	F <sub>IS</sub>	F <sub>ST</sub>
Current	38	1006	223	30.58	0.716	0.033	0.050
Current predicted	33	892	217	29.39	0.707	0.044	0.041
2050 predicted	31	859	212	28.627	0.707	0.049	0.039
2080 predicted	22	604	206	27.122	0.719	0.047	0.035

Npop, Number of populations; Nind, number of individuals; N<sub>tot</sub>, total number of alleles; N<sub>ar</sub>, allelic richness; H<sub>E</sub>, expected heterozygosity; F<sub>IS</sub>, multilocus inbreeding coefficient, F<sub>ST</sub>, overall genetic differentiation.

**Table 2** Predicted changes of *Fraxinus angustifolia* allelic richness (N<sub>ar</sub>) and private allelic richness (N<sub>par</sub>) corrected for differences in sample sizes between the periods using a rarefaction method in HP-RARE

Period	Nind	N <sub>par</sub>	N <sub>ar</sub>
Current	604	1.45	34.27
Europe 2050	604	0.26	33.38
Europe 2080	604	0.48	34.26

Values are calculated based on a minimum number of individuals (Nind) predicted to be present in all three periods.

recalculated only for populations occurring within the currently predicted suitable habitat (Table 1). When we recalculated N<sub>ar</sub> by pooling all populations remaining in the predicted stable habitats for each future period, N<sub>ar</sub> over loci decreased to 28.63 by 2050 and to 27.12 by 2080. N<sub>ar</sub> values standardized for a minimum of 604 individuals predicted to be present in all three periods were almost identical; however, N<sub>par</sub> decreased more strongly from the present to the future due to the loss of private alleles (Table 2). If we recalculated H<sub>E</sub>, F<sub>IS</sub> and F<sub>ST</sub> by omitting the populations predicted to occur in areas of future habitat loss, the values remained similar, and we found no significant differences between the present and the future (Table 1).

#### Bayesian population genetic structure

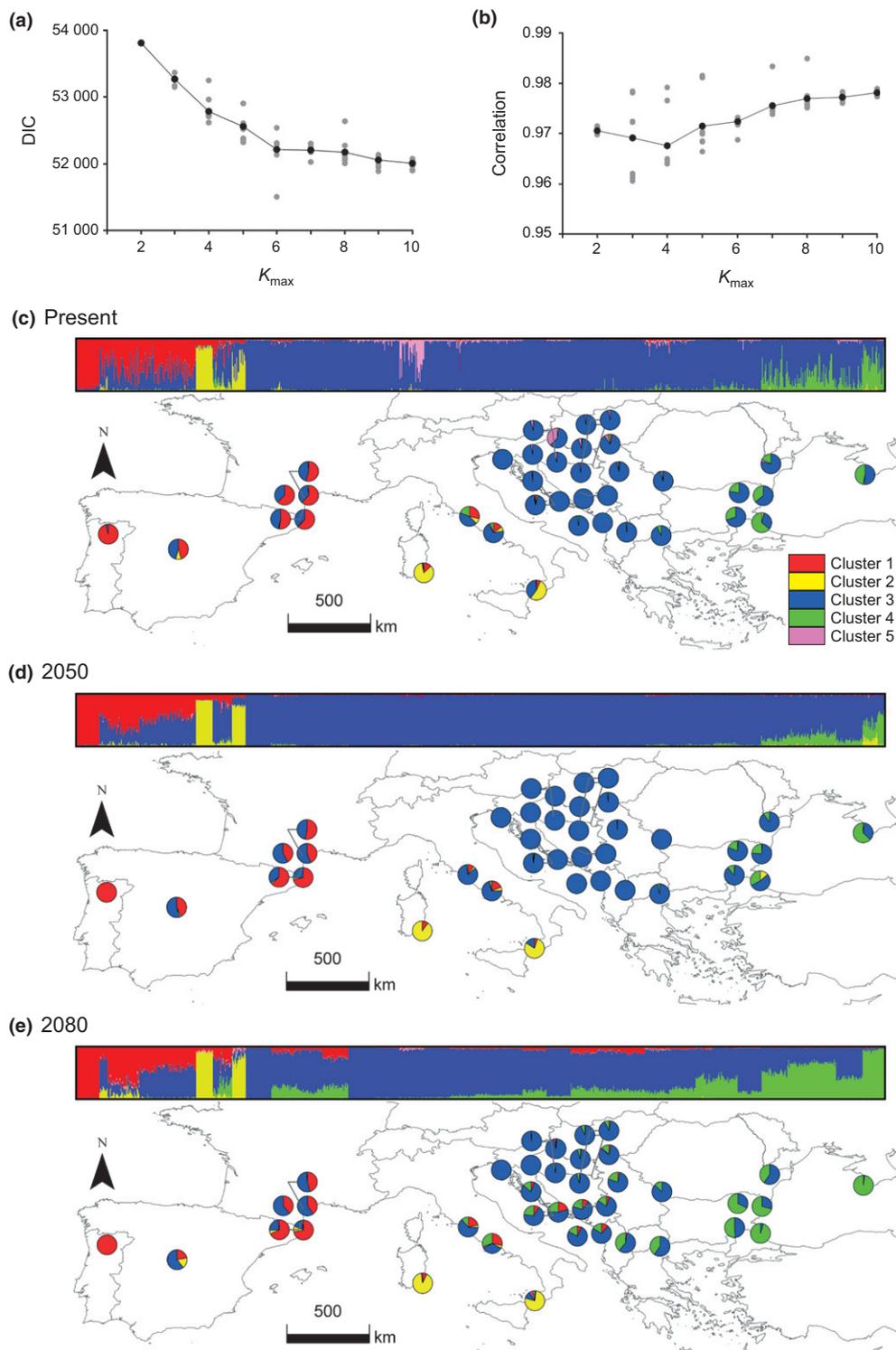
The POPS DIC curve reached a stable plateau at K<sub>max</sub> = 6, and further increases in K<sub>max</sub> did not produce new clusters or substantially change the genetic structure

(Fig. 4a). The inspection of the plotted membership coefficients showed that only five genetic clusters were in fact present. It is not unusual for DIC to select models in which K<sub>max</sub> is greater than the actual number of clusters (K) (Durand *et al.* 2009). TESS identified the same five major genetic groups based on longer runs, and for brevity, we present only the results from the POPS analyses. The first cluster (red) was dominant in the western Mediterranean, the second cluster (yellow) was primarily present within populations from Southern Italy (Sardinia and Calabria), the third cluster (blue) was the most widespread one and occurred across most of the western Balkan populations that were clustered into a single homogeneous gene pool, and the fourth cluster (green) dominated in Black Sea coastal populations (Fig. 4c). Finally, the fifth cluster (pink) was comprised of individuals from a single Croatian population (CK) (Fig. 4c).

Correlations between estimated membership coefficients and predicted membership coefficients based on current bioclimatic variables were, on average, very high (0.97), indicating the high relevance of the bioclimatic covariates relating to population genetic structure (Fig. 4b). Consequently, POPS enabled us to predict the possible changes in the population genetic structure within the current distribution limits under future climate conditions. The predicted genetic structure by 2050 remained rather similar to the current one except that the fifth genetic cluster was no longer present (Fig. 4d). Predictions for 2080 suggest that future conditions may favour the westward migration of the Black Sea genotypes towards the Balkans in the long term (Fig. 4e). Western Mediterranean populations did not show potential significant changes in their genetic structure.

#### Discussion

The potential responses of widely distributed species to ongoing climate changes largely depend on the distribution of within-species genetic variation because the opportunities for survival in changing environments are expected to be greater for populations harbouring higher levels of standing genetic variation. Thus, refugia from ongoing climate change are most probable in areas where the majority of contemporary genetic diversity is present and predicted current and future suitable environments overlap. The results for our studied species suggest that such refugia are potentially located in the northerly parts of the current distribution, where core high-diversity populations occur and suitable habitat is predicted to remain stable under future climate conditions. Rear-edge populations in the southern Mediterranean, of which some have probably served as



**Fig. 4** Spatial Bayesian clustering inferred by POPS. (a) Estimated number of clusters ( $K_{\max} = 5$ ) based on DIC curve (b) correlations between estimated membership coefficients and predicted membership coefficients based on current bioclimatic covariates. Estimated genetic structure for 1006 *Fraxinus angustifolia* individuals assuming  $K_{\max} = 5$  is shown for (c) present, (d) predicted for 2050 and (e) predicted for 2080 under averaged climate for A1B emission scenario. Bar plots show the membership coefficients of individuals. Pie charts represent the proportion of gene pools in each population.

ancient refugia from Quaternary climate changes, tend to be most exposed to extinction due to the loss of suitable habitat induced by climate change. Although the predicted decrease in the total number of alleles was comparatively low, the results suggest that a more drastic potential loss of unique alleles at the trailing edge will occur unless microhabitat availability compensates for overall degraded macroclimate conditions.

#### *Spatial patterns of genetic diversity*

The nature of the genetic diversity that could persist or be lost because of future climate change is highly dependent on the history of the species. We demonstrated the existence of a specific west–east geographical gradient of decreasing genetic diversity of *F. angustifolia* populations at the continental level and showed that the genetic diversity of peripheral populations is reduced. The stands with the highest genetic diversity typically occurred at mid-latitudes closer to the northern edge of the current distribution compared with their southern counterparts. Contrary to our expectations, the pattern observed herein was opposite to the conventional “leading-edge expansion” paradigm of the highest diversity in the southern Quaternary refugia and decreasing diversity towards northern parts of the distribution (Hewitt 2000). Instead, our genetic pattern seems to be most consistent with the central–marginal model of decreasing genetic diversity towards peripheral populations (Eckert *et al.* 2008), which may reflect the ecological marginality of contemporary southern *F. angustifolia* populations. The result that the majority of genetic diversity was found at mid-latitudes is also likely to be of historical origin as a consequence of a secondary contact of divergent lineages arriving from separate Quaternary refugia during postglacial expansion (Petit *et al.* 2003) and likely due to extensive gene flow during interglacial periods, especially during the expansion of broad-leaved forests throughout the warm Mid-Holocene (Benito-Garzón *et al.* 2007).

Furthermore, our eastern populations were significantly less diverse than the western ones. This finding is contrary to the general east–west trend of decreasing genetic diversity confirmed recently for numerous woody and tree species in the Mediterranean basin (Fady & Conord 2010), most likely due to taxon-specific ecological requirements (Conord *et al.* 2012). All studied coastal populations grow below 45°N latitude in areas recognized as suitable for the survival of temperate trees during the last glacial maximum (LGM) (Petit *et al.* 2005). If all three Mediterranean peninsulas acted as refugia, historical reductions in population sizes are expected to affect the neutral genetic diversity pattern in similar ways, and the signature that demographic

history left in contemporary populations should be genome-wide. However, populations along the eastern Adriatic coast are genetically the most depleted. This finding is highly surprising because this region is recognized as one of the most important glacial refugia for European taxa, including ash (Heuertz *et al.* 2006; Médail & Diadema 2009). In fact, the levels of genetic diversity decrease with increasing time spent in the refugia. Thus, unexpectedly, slower range contractions result in more severe reductions of genetic diversity than fast contractions (Arenas *et al.* 2012). In fact, LGM mean summer temperatures are shown to be correlated with longitude in the Mediterranean basin, increasing from west to east (Fady & Conord 2010), suggesting that eastern Mediterranean populations may have contracted more slowly towards suitable habitats, leading to a higher loss of genetic diversity. Finally, lower within-population genetic diversity can indicate relict tree populations persisting *in situ* during the LGM and through the past climate changes of the Quaternary until the present in the comparatively stable Mediterranean environment (Petit *et al.* 2005). Eastern Adriatic populations might have been restricted to sheltered, moist and deep river valleys and wetlands along the coast in this area since the glacial retreats (Médail & Diadema 2009) and may have remained relatively stable since that time. The postglacial expansion of these populations from these areas, as well as extant gene flow, may have been highly limited compared with the western Mediterranean due to the scarcity of suitable habitat in the eastern Adriatic because of limestone terrain and the linear distribution of populations along karstic river canyons (Temunović *et al.* 2012).

#### *Habitat suitability changes, climate change refugia and implications for conservation*

Although a growing body of literature aims to predict the range shifts and extinction probabilities of species populations in correlation with the environment, only a few empirical studies to date have attempted to investigate the genetic consequences of current climate changes (Collevatti *et al.* 2011; Jay *et al.* 2012; Rubidge *et al.* 2012). Our results suggest a reduction of 33% of the suitable habitat by the end of this century in the southern parts of the current distribution of the species, but a comparatively small decrease in the total number of alleles remaining in the future. In addition, the habitats predicted to remain stable under climate change harbour most of the current genetic diversity and may be considered large-scale modern refugia from climate change. Nevertheless, one has to keep in mind that dispersal limitations will have a major effect on the ability of tree populations to withstand climate changes.

Based on molecular evidence, the postglacial migration rates of trees are now estimated to be much lower than previously thought, for example, <100 m/year (McLachlan *et al.* 2005), which is largely insufficient to keep pace with current climate changes (Aitken *et al.* 2008). Under the “worst” case no-dispersal scenario that appears more likely for a long-lived tree species with poor dispersal abilities, the detected areas of habitat stability can be considered realistic *in situ* macrorefugia from 21st-century climate change (*sensu* Ashcroft 2010) for narrowed-leaved ash. In such refugia, populations have the best chance to persist in the future. Newly gained habitats could become potential *ex situ* refugia (Ashcroft 2010). However, due to habitat fragmentation, human-altered landscape and natural geographical barriers to dispersion, these areas could be reached only by human-assisted colonization or by improving the connectivity between current and newly suitable habitats (Vos *et al.* 2008).

The existence of microrefugia (Rull 2009) cannot be excluded in areas predicted to be lost by ENM, as microrefugia are known to occur in smaller areas where patches of favourable local microclimates are likely to support viable populations, even if the surrounding regional macroclimate is no longer suitable for the survival of the species. This principle may be particularly true for moisture-dependent species, such as narrow-leaved ash, because humid microsites such as coastlines, river valleys and canyons are expected to be more resistant to climatic warming (Ashcroft *et al.* 2009). However, potential microrefugia could not be identified in our study due to the relatively coarse spatial resolution of the selected environmental grids (we used 1-km pixels); therefore, the potential habitat and allele loss detected with our models may be overestimated. As the Mediterranean basin is known for its complex topography and habitat heterogeneity over small scales (Temunović *et al.* 2012), we provide here only broad-scale boundaries of future macrorefugia. Outside these boundaries, microrefugia could be located using regional fine-scale predictor variables (at scales of less than 1 km) that include habitat features not present in our study, capturing the unique environments of the coastal areas (Daly 2006). However, populations surviving in such microrefugia may experience more restricted gene flow and stronger drift in fragmented landscapes once the surrounding macroclimate is no longer suitable, thus becoming more vulnerable to future disturbances in the Mediterranean.

Our study shows that the western Mediterranean (Portugal, Spain, France and Central Italy) and Pannonian populations harbour the highest levels of present-day genetic diversity and, therefore, represent the most important reservoirs for conservation of genetic

resources for *F. angustifolia*. In fact, most of these populations are located in areas of predicted stable habitat. These areas will likely support the *in situ* survival of these populations in the future while maintaining long-term high levels of genetic diversity (>90% of alleles). It is probable that high-diversity leading-edge populations are also good potential candidates as source populations for the assisted migration or colonization of newly gained habitats at higher latitudes (Pfeifer *et al.* 2010) in the long term. Conversely, isolated southern populations are likely more endangered because of habitat fragmentation, smaller population sizes and increased human activities in the coastal areas, as well as future climate changes that are predicted to be especially pronounced in the Mediterranean areas (Giorgi & Lionello 2008). Our results confirm that rear-edge populations in this species have the highest probability of undergoing habitat loss in the future, but also have the lowest genetic diversity. Although they do not appear to be at the centre of conservation priorities if we assume the “core” population conservation guidelines, we stress the principle that the relationship between genetic diversity and conservation value is not straightforward. Although the possible loss of approximately 40% of the studied populations by the end of the century would not cause a very drastic decrease in the overall genetic diversity (only about 8% of the total number of alleles), that population loss corresponds to approximately 37% of the private alleles unique to these populations. Populations predicted to be potentially lost may thus negatively affect the overall genetic diversity and possibly the evolutionary potential of the species unless microrefugia provide buffering against predicted extinctions.

Conservation actions should be based on both leading-edge and rear-edge populations, and conservation strategies should be adapted for opposing species borders, as shown for the European orchid (Pfeifer *et al.* 2010). Common management policies support the practice of using seeds of local origin in afforestation programmes, which is reasonable, as populations are usually adapted to the local environmental conditions. However, rear-edge populations could potentially be preadapted to a warmer climate despite their low genetic diversity and are, moreover, under greater climatic stress (Aitken *et al.* 2008); therefore, one might reconsider using such populations in management and restoration programmes under climate change scenarios. In addition, POPS results suggest that the Black Sea gene pool has the greatest potential for westward gene expansion under the predicted future climate in the long term, and adaptation to future warming may be partly facilitated by gene flow from this gene pool if the necessary adaptive variation is linked in some way to the markers used in this study. Our predictions of

future genetic structure depend primarily on the ability of the species to disperse across the landscape and should be interpreted only as the upper boundaries of future long-term gene migrations (Jay *et al.* 2012). Our approach to identify refugia from climate change can be applied for other species with reasonable number of records to allow adequate niche modelling and when sufficient populations have been sampled for genetic variation, especially including populations at the distribution margins. However, our method would be less appropriate for narrow endemic species for which chances of having some sort of climate overlap between present-day conditions and expected future climates are low. Future models incorporating migration dynamics (including possible long-distance dispersal events), adaptive genetic variation and explicit climatic selection are thus needed to provide more realistic predictions of genetic responses to climate change.

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M.T. and J.F.F.M. designed the study; M.T. and J.F. collected the samples; M.T. genotyped the samples; M.T. and Z.S. performed the simulations and statistical analysis; and N.F.L. provided laboratory and molecular biology facilities and participated to discussions. M.T. and J.F.F.M. wrote the manuscript.

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### Data accessibility

Microsatellite data and Maxent input file for current niche model: Dryad doi:10.5061/dryad.435s1. *Fraxinus angustifolia* occurrence records used for the Maxent models are provided in Table S1 (Supporting Information), and sampling locations of the populations are available in Table S4 (Supporting Information).

### Supporting information

Additional supporting information may be found in the online version of this article.

**Table S1.** *Fraxinus angustifolia* occurrence records used for Maxent models.

**Table S2** Environmental variables used for niche modelling with Maxent.

**Table S3** List of General Circulation Models used for future projections of the Maxent models under A1B climate change scenario.

**Table S4** Coordinates, number of genotyped individuals, genetic diversity parameters and Maxent habitat suitability values of the sampled populations.

**Table S5** Population pairwise  $F_{ST}$  values and their significance.

**Table S2** Environmental variables used for ecological niche modelling with Maxent

Variable ID	Variable description (unit)	Variable source
BIO 1	Annual Mean Temperature (°C *10)	<a href="http://www.worldclim.org">http://www.worldclim.org</a> , <a href="http://www.ccafs-climate.org">http://www.ccafs-climate.org</a>
BIO 4	Temperature Seasonality (standard deviation *100)	<a href="http://www.worldclim.org">http://www.worldclim.org</a> , <a href="http://www.ccafs-climate.org">http://www.ccafs-climate.org</a>
BIO 5	Max Temperature of Warmest Month (°C *10)	<a href="http://www.worldclim.org">http://www.worldclim.org</a> , <a href="http://www.ccafs-climate.org">http://www.ccafs-climate.org</a>
BIO 6	Min Temperature of Coldest Month (°C *10)	<a href="http://www.worldclim.org">http://www.worldclim.org</a> , <a href="http://www.ccafs-climate.org">http://www.ccafs-climate.org</a>
BIO 12	Annual Precipitation (mm)	<a href="http://www.worldclim.org">http://www.worldclim.org</a> , <a href="http://www.ccafs-climate.org">http://www.ccafs-climate.org</a>
BIO 15	Precipitation Seasonality (CV in mm)	<a href="http://www.worldclim.org">http://www.worldclim.org</a> , <a href="http://www.ccafs-climate.org">http://www.ccafs-climate.org</a>
BIO 18	Precipitation of Warmest Quarter (mm)	<a href="http://www.worldclim.org">http://www.worldclim.org</a> , <a href="http://www.ccafs-climate.org">http://www.ccafs-climate.org</a>
BIO 19	Precipitation of Coldest Quarter (mm)	<a href="http://www.worldclim.org">http://www.worldclim.org</a> , <a href="http://www.ccafs-climate.org">http://www.ccafs-climate.org</a>
DEM	Digital elevation model (elevation in m)	<a href="http://www2.jpl.nasa.gov/srtm/">http://www2.jpl.nasa.gov/srtm/</a>
Driver	Euclidean distance to rivers (arc degree)	<a href="http://hydrosheds.cr.usgs.gov">http://hydrosheds.cr.usgs.gov</a>
SOIL	Soil type layer (categorical)	<a href="http://webarchive.iiasa.ac.at/Research/LUC/External-World-soil-database">http://webarchive.iiasa.ac.at/Research/LUC/External-World-soil-database</a>

**Table S3** List of General Circulation Models (GCMs) used for future projections of the Maxent models under A1B climate change scenario

(obtained from <http://www.ccafs-climate.org>).

Model name	Model Source
CCCMA-CGCM3.1	Canadian Centre for Climate Modelling and Analysis
CSIRO-Mk3.0	Australia
IPSL-CM4	Institute Pierre Simon Laplace (France)
MPI-ECHAM5	Max Planck Institute for Meteorology (Germany)
NCAR-CCSM3.0	National Center for Atmospheric Research (USA)
UKMO-HADCM3	Hadley Centre for Climate Prediction (UK)
UKMO-HADGEM1	Hadley Centre for Climate Prediction (UK)

**Table S4** Population ID, country of origin, sample size (n), genetic diversity parameters, coordinates, and habitat suitability values obtained with Maxent models for the 38 *F. angustifolia* populations sampled in this study: average number of alleles across loci ( $N_a$ ), allelic richness ( $N_{ar}$ ), number of private alleles ( $N_{pr}$ ), observed heterozygosity ( $H_O$ ), expected heterozygosity ( $H_E$ ), multilocus inbreeding coefficient ( $F_{IS}$ ),  $P$ -values for the exact HWE test (\*\*\*  $P < 0.001$ ; \*\*  $P < 0.01$ ; \*  $P < 0.05$ )

No	Country	PopID	n	$N_a$	$N_{ar}$	$N_{pr}$	$N_{par}$	$H_O$	$H_E$	$F_{IS}$	$P$	Lat	Long	Habitat suitability		
														Current	2050	2080
1	Portugal	PO	28	12.5	7.49	0	0.187	0.881	0.831	-0.06	ns	41.26	-7.68	0.228	0.136	0.119
2	Spain	KR	10	8	7.31	0	0.162	0.672	0.797	0.156	***	40.44	-3.85	0.359	0.297	0.095
3	France	MLO	16	9	7.03	0	0.037	0.731	0.81	0.098	**	42.58	2.96	0.605	0.450	0.000
4	France	ML	24	12.83	8	1	0.164	0.816	0.822	0.008	ns	42.59	3.03	0.182	0.000	0.000
5	France	CDA	22	12.83	8.06	1	0.259	0.802	0.84	0.046	*	43.24	3.00	0.596	0.584	0.112
6	France	CLB	24	13.17	8.2	1	0.129	0.813	0.811	-0.002	ns	43.39	3.10	0.451	0.451	0.050
7	France	BSL	24	14	8.06	1	0.247	0.763	0.823	0.073	*	43.38	3.25	0.498	0.495	0.054
8	Italy	SA	21	10.5	7.11	2	0.405	0.881	0.824	-0.069	ns	39.16	9.77	0.074	0.000	0.000
9	Italy	MT	8	7.83	7.83	0	0.213	0.813	0.813	0	ns	42.23	12.03	0.317	0.155	0.000
10	Italy	LF	16	11.17	7.62	3	0.439	0.771	0.747	-0.032	ns	41.33	13.35	0.266	0.000	0.000
11	Italy	CAL	17	7.67	5.82	1	0.231	0.784	0.696	-0.127	ns	38.22	15.67	0.255	0.000	0.000
12	Croatia	MR	32	13.33	6.94	0	0.076	0.656	0.681	0.036	*	45.35	13.84	0.609	0.422	0.000
13	Croatia	KK	32	12.83	6.81	2	0.183	0.682	0.693	0.015	ns	43.82	15.97	0.421	0.239	0.000
14	Croatia	ZR	32	10.67	6.06	0	0.075	0.625	0.668	0.065	*	44.17	16.06	0.351	0.155	0.153
15	Croatia	NT	32	10.17	5.88	1	0.071	0.578	0.621	0.069	***	43.04	17.56	0.586	0.044	0.000
16	Croatia	JA	32	15.17	7.58	0	0.161	0.754	0.715	-0.054	ns	45.61	15.71	0.696	0.518	0.441
17	Croatia	LP	32	13.83	7.3	0	0.102	0.696	0.72	0.033	*	45.42	16.71	0.711	0.490	0.445
18	Croatia	CK	31	17.83	8.61	2	0.476	0.737	0.771	0.044	ns	46.34	16.81	0.425	0.153	0.258
19	Croatia	SG	31	13.33	6.9	2	0.216	0.697	0.682	-0.022	ns	45.20	17.15	0.741	0.585	0.475

20	Croatia	DU	32	15.33	7.51	5	0.27	0.708	0.748	0.053	**	45.60	18.13	0.691	0.467	0.388
21	Croatia	TR	29	12.67	7.19	2	0.244	0.733	0.724	-0.012	ns	45.13	18.14	0.723	0.514	0.425
22	Croatia	ZU	30	13	6.9	0	0.093	0.65	0.672	0.032	*	45.00	18.74	0.629	0.377	0.303
23	BiH	BJ	30	10.17	6.16	1	0.116	0.633	0.655	0.033	ns	43.55	18.03	0.311	0.075	0.000
24	BiH	MG	30	11.5	6.79	2	0.119	0.631	0.694	0.091	**	44.62	18.09	0.359	0.139	0.044
25	BiH	DP	31	8.83	5.31	1	0.105	0.608	0.618	0.017	ns	43.06	18.25	0.502	0.222	0.092
26	Montenegro	BU	31	9.33	5.95	0	0.024	0.597	0.683	0.126	**	42.19	18.98	0.576	0.045	0.000
27	Montenegro	ADA	31	12.5	6.67	0	0.049	0.638	0.673	0.052	**	41.87	19.35	0.636	0.078	0.000
28	Serbia	SU	31	11.33	6.72	2	0.215	0.79	0.751	-0.052	ns	46.18	19.70	0.395	0.047	0.155
29	Serbia	CL	32	13.83	7.03	0	0.05	0.62	0.67	0.074	***	44.68	20.18	0.398	0.390	0.287
30	Serbia	NG	30	14	7.45	0	0.112	0.643	0.722	0.11	***	41.36	20.60	0.384	0.268	0.080
31	Macedonia	CD	22	9.67	5.96	0	0.083	0.599	0.615	0.026	ns	41.17	22.49	0.251	0.125	0.000
32	Macedonia	GG	30	8.67	5.4	1	0.124	0.598	0.627	0.047	*	44.15	22.60	0.428	0.229	0.213
33	Bulgaria	EH	32	12.5	6.82	4	0.254	0.628	0.744	0.155	***	42.19	26.56	0.251	0.113	0.077
34	Bulgaria	KC	30	11.17	6.44	0	0.046	0.629	0.722	0.129	***	43.02	27.82	0.374	0.388	0.383
35	Bulgaria	AL	31	12.33	6.89	0	0.078	0.687	0.769	0.108	***	43.36	28.06	0.336	0.362	0.353
36	Romania	TC	33	14.5	7.77	2	0.254	0.827	0.773	-0.07	ns	45.10	28.48	0.037	0.000	0.000
37	Turkey	TU	19	10.17	6.77	1	0.111	0.743	0.728	-0.021	ns	41.88	27.98	0.295	0.089	0.037
38	Ukraine	UK	8	6	6	0	0.076	0.708	0.72	0.017	ns	44.51	33.60	0.209	0.036	0.075
Mean				11.69	6.96	1	0.165	0.706	0.728	0.031				0.425	0.240	0.135

Table S5 Population pairwise  $F_{ST}$  values and their significance

	PO	KR	Mlo	ML	CdA	ClesB	BsurL	SA	MT	LF	CAL	MR	KK	ZR	NT	J	LP	CK	SG	DU	TR	ZU	BJ	MG	DP	BU	ADA	SU	CL	CD	GG	NG	EH	KC	AL	TC	TU	UK	
PO	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**
KR	0.084	ns	*	ns	ns	**	ns	ns	ns	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	ns
Mlo	0.060	0.017	ns	ns	ns	ns	ns	ns	ns	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**
ML	0.051	0.027	0.000	ns	ns	ns	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	*
CdA	0.049	0.028	0.003	0.006	ns	ns	**	*	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	
ClesB	0.058	0.024	0.010	0.002	0.001	ns	**	*	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	ns
BsurL	0.051	0.028	0.016	0.012	0.010	0.004	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**
SA	0.067	0.098	0.079	0.071	0.071	0.071	0.068	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**
MT	0.078	0.052	0.028	0.031	0.042	0.040	0.046	0.067	ns	*	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	ns
LF	0.109	0.039	0.033	0.036	0.039	0.025	0.033	0.095	0.053	**	**	**	**	**	**	*	**	**	**	*	**	**	*	**	**	*	**	ns	**	**	ns	**	**	**	**	**	**	ns	
CAL	0.109	0.109	0.083	0.068	0.080	0.067	0.067	0.091	0.093	0.066	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**
MR	0.137	0.075	0.046	0.045	0.046	0.042	0.050	0.129	0.099	0.023	0.083	**	**	**	ns	ns	**	ns	ns	ns	ns	ns	*	**	**	ns	**	ns	ns	**	ns	**	**	**	**	**	**	**	**
KK	0.136	0.076	0.053	0.052	0.053	0.048	0.056	0.121	0.103	0.031	0.084	0.016	**	**	**	**	**	*	**	**	*	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**
ZR	0.154	0.083	0.070	0.074	0.065	0.062	0.073	0.149	0.131	0.048	0.120	0.022	0.027	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**
NT	0.186	0.100	0.083	0.081	0.082	0.073	0.079	0.180	0.147	0.050	0.139	0.027	0.032	0.038	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**
J	0.133	0.052	0.039	0.039	0.042	0.034	0.045	0.125	0.085	0.019	0.072	0.007	0.018	0.028	0.028	ns	**	ns	ns	ns	ns	ns	**	ns	**	**	ns	**	ns	ns	**	ns	**	**	**	**	**	ns	
LP	0.132	0.065	0.048	0.044	0.040	0.046	0.048	0.124	0.081	0.040	0.079	0.020	0.032	0.039	0.045	0.003	**	*	ns	ns	ns	**	**	**	**	**	**	ns	**	**	**	ns	**	**	**	**	**	**	
CK	0.102	0.077	0.044	0.036	0.038	0.033	0.032	0.082	0.064	0.031	0.031	0.031	0.049	0.074	0.027	0.026	**	*	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	ns
SG	0.143	0.092	0.066	0.064	0.056	0.055	0.061	0.137	0.115	0.039	0.090	0.014	0.020	0.030	0.033	0.009	0.014	0.028	ns	ns	ns	**	**	**	**	**	**	ns	**	**	**	ns	**	**	**	**	**	**	**
DU	0.109	0.058	0.036	0.037	0.034	0.028	0.035	0.109	0.082	0.012	0.072	0.011	0.018	0.023	0.032	0.003	0.014	0.021	0.005	ns	ns	**	ns	**	**	ns	**	ns	*	**	ns	**	**	**	**	ns	**	*	
TR	0.122	0.065	0.045	0.044	0.041	0.038	0.041	0.110	0.077	0.017	0.067	0.008	0.016	0.032	0.037	0.001	0.006	0.020	0.011	0.002	ns	**	*	**	**	ns	**	ns	ns	**	ns	**	**	**	**	**	**	**	
ZU	0.147	0.080	0.060	0.056	0.055	0.050	0.060	0.137	0.106	0.024	0.086	0.009	0.011	0.029	0.038	0.006	0.013	0.027	0.010	0.007	0.000	**	ns	**	**	ns	**	ns	*	**	ns	**	**	**	**	*	**	**	
BJ	0.145	0.086	0.059	0.057	0.060	0.049	0.065	0.144	0.114	0.033	0.103	0.020	0.041	0.053	0.040	0.030	0.039	0.050	0.045	0.026	0.028	0.026	**	**	**	**	**	**	**	**	*	**	**	**	**	**	**	**	
MG	0.135	0.072	0.054	0.048	0.047	0.034	0.049	0.133	0.105	0.022	0.076	0.018	0.025	0.037	0.043	0.004	0.021	0.029	0.018	0.009	0.009	0.011	0.032	**	**	**	**	ns	**	**	ns	**	**	**	**	**	**	**	
DP	0.177	0.089	0.085	0.085	0.088	0.073	0.081	0.173	0.131	0.038	0.115	0.036	0.034	0.051	0.029	0.031	0.050	0.075	0.049	0.029	0.027	0.030	0.025	0.041	**	**	**	**	**	**	**	**	**	**	**	**	**	**	
BU	0.148	0.085	0.059	0.060	0.065	0.056	0.070	0.133	0.106	0.026	0.112	0.026	0.040	0.051	0.041	0.029	0.050	0.054	0.037	0.020	0.029	0.033	0.045	0.041	0.050	**	**	**	**	**	**	**	**	**	**	**	**	**	
ADA	0.141	0.068	0.056	0.056	0.058	0.047	0.056	0.135	0.110	0.016	0.088	0.007	0.023	0.034	0.032	0.013	0.031	0.039	0.024	0.012	0.011	0.008	0.014	0.025	0.023	0.020	**	ns	ns	**	ns	**	**	**	**	**	**	**	
SU	0.113	0.065	0.045	0.042	0.042	0.038	0.048	0.111	0.068	0.026	0.074	0.039	0.032	0.048	0.063	0.025	0.035	0.038	0.036	0.013	0.022	0.028	0.055	0.030	0.044	0.045	0.036	**	**	**	**	**	**	**	**	**	**	**	
CL	0.140	0.067	0.053	0.052	0.059	0.044	0.052	0.139	0.097	0.015	0.078	0.009	0.017	0.028	0.035	0.003	0.021	0.029	0.015	0.004	0.003	0.000	0.021	0.012	0.022	0.034	0.006	0.026	ns	**	ns	**	**	**	**	**	**	ns	
CD	0.177	0.096	0.075	0.077	0.079	0.073	0.086	0.170	0.133	0.049	0.116	0.017	0.026	0.030	0.033	0.025	0.039	0.054	0.033	0.030	0.028	0.023	0.042	0.036	0.033	0.046	0.023	0.058	0.023	**	ns	**	**	**	**	**	**	**	
GG	0.169	0.102	0.081	0.085	0.085	0.078	0.084	0.164	0.133	0.054	0.109	0.034	0.053	0.050	0.052	0.037	0.050	0.059	0.040	0.033	0.039	0.043	0.054	0.048	0.049	0.050	0.037	0.066	0.038	0.018	**	**	**	**	**	**	**	**	
NG	0.117	0.052	0.030	0.034	0.036	0.032	0.041	0.110	0.071	0.012	0.066	0.007	0.009	0.020	0.033	0.000	0.011	0.017	0.008	0.000	0.000	0.000	0.021	0.007	0.030	0.029	0.011	0.019	0.000	0.016	0.023	*	*	**	ns	**	ns		
EH	0.109	0.066	0.046	0.046	0.045	0.042	0.047	0.089	0.048	0.027	0.082	0.034	0.037	0.049	0.059	0.038	0.043	0.042	0.046	0.024	0.024	0.032	0.038	0.047	0.042	0.051	0.034	0.028	0.026	0.057	0.059	0.022	ns	ns	**	**	ns		
KC	0.120	0.061	0.046	0.050	0.040	0.044	0.049	0.111	0.083	0.035	0.091	0.024	0.032	0.030	0.039	0.021	0.028	0.040	0.025	0.015	0.018	0.026	0.047	0.027	0.044	0.039	0.026	0.035	0.025	0.027	0.031	0.015	0.016	ns	*	**	ns		
AL	0.103	0.053	0.044	0.048	0.040	0.042	0.047	0.100	0.055	0.034	0.088	0.053	0.049	0.056	0.077	0.039	0.044	0.048	0.051	0.026	0.034	0.042	0.068	0.052	0.059	0.066	0.047	0.027	0.037	0.066	0.062	0.027	0.009	0.015	**	**	ns		
TC	0.109	0.072	0.050	0.044	0.042	0.039	0.045	0.097	0.077	0.035	0.064	0.035	0.032	0.042	0.065	0.021	0.018	0.019	0.023	0.008	0.014	0.023	0.050	0.024	0.057	0.043	0.033	0.023	0.027	0.056	0.053	0.015	0.024	0.022	0.029	**	ns		
TU	0.110																																						

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# Chilled but not frosty: understanding the role of climate in the hybridization between the Mediterranean *Fraxinus angustifolia* Vahl and the temperate *Fraxinus excelsior* L. (Oleaceae) ash trees

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## ABSTRACT

**Aim** To examine mechanisms related to the formation of hybrid zones between the Mediterranean narrow-leaved ash tree *Fraxinus angustifolia* Vahl and the common ash *Fraxinus excelsior* L., a mostly temperate tree species, at the continental scale.

**Location** Temperate and Mediterranean Europe and the western part of the Black Sea basin.

**Methods** We used species distribution models to determine the potential zones of sympatry between the two species, which remain largely unknown. In addition, we analysed 58 populations and 456 samples of ash tree that spanned most of the distribution of the two species across Europe, and included both parental species and selected hybrid populations. Levels of hybridization in the 58 populations were estimated using 19 nuclear microsatellite loci, including six anonymous nuclear single sequence repeat (SSR) markers and 13 recently developed single sequence repeats from expressed RNA sequence tags (EST-SSRs).

**Results** Bayesian assignment supported the notion of two separate gene pools regardless of the type of marker used, which suggest an ancient population structure. Populations located within the predicted overlap zones had intermediate levels of admixture with a tendency for hybrid populations to occur towards temperate areas. Selection analyses indicated that six of the EST-SSRs had been subjected to stabilizing selection whereas two others had been subjected to directional selection. Results of spatial filtering on the allele frequencies of the loci under directional selection suggest that the number of days of frost and summer temperatures are both ecological factors that can limit the extent of the hybrid zone. Moreover, areas associated with known or predicted hybrid zones showed abrupt changes in allele frequencies compared with the periphery of the distributions.

**Main conclusions** Our analyses suggest that the hybrid structure in these closely related ash species is ancient and asymmetric and that climate-driven selection, in particular cold weather, can potentially limit the extent of hybrid populations.

## Keywords

Climate-driven selection, Europe, *Fraxinus*, hybrid zones, Oleaceae, species distribution models.

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## INTRODUCTION

Tree species can form hybrid zones over hundreds of kilometres or more, and the distributions of these zones are often related to ecological conditions (e.g. Dodd & Afzal-Rafii, 2004; Kamiya *et al.*, 2011; Wang *et al.*, 2012). As for many other species, the hybrid zones of trees often originate from sister lineages that are not completely isolated reproductively, are evolving independently, and come into secondary contact at the edges of their distributions (Givnish, 2010). However, the large distribution ranges of closely related tree species make it difficult to detect all possible hybrid zones, or the ecological factors that could explain the origin and maintenance of these zones.

In general, several types of hybrid zone have been identified, and their characteristics depend on the relative importance of different types of selection and the dispersal capacity of the parental species and hybrids. Classic hybrid zone theory (Endler, 1977; Barton & Hewitt, 1985, 1989) predicts that the extent and shape of hybrid zones depend upon a balance between migration and selection, with the latter involving a combination of 'exogenous' and 'endogenous' forms of selection (Barton, 2001). At finer scales, rates of hybridization in tree species are influenced largely by the mating system and by the relative frequency of each species (Field *et al.*, 2011), which can create asymmetric gene flow. However, it is difficult in large-scale analyses to infer which of these processes (gene flow or selection) is playing the major role in shaping the hybrid zone, unless evidence of signatures of selection can be demonstrated.

At least two types of hybrid zone can be inferred from the spatial patterns observed at large scales when populations are mapped. If the predicted distributions of parental species and their hybrids do not match the observed positions of the actual hybrid zone and the boundaries of the species ranges, then the spatial pattern can be compatible with the so-called 'tension zone model' (Barton & Hewitt, 1985). In this model, the combined effects of dispersal and selection against hybrids determine the location and extent of hybrid zones, in a manner that can be independent of ecological gradients (Swenson, 2008). On the other hand, when hybrids perform better than either parental species in a given environment, the predicted distributions of hybrid zones should match the observed distributions closely; this scenario is described in the 'bounded hybrid superiority model' (Moore, 1977). In this second case, the predicted distributions of one or both parental species could expand into the observed hybrid zone because the hybrids largely control parental population expansions (Swenson, 2008). In addition, if the distributions of parental species and hybrid zones are correlated strongly with different environmental conditions, then exogenous selection can be identified as an important factor that contributes to the dynamics of the hybridizing species, even if the role of endogenous selection cannot be ruled out completely. Recently, species distribution models (SDMs) have proven to be useful for characterizing such areas of hybridization. They have been used successfully to study hybrid

zones in insects, birds and reptiles (Swenson, 2006; Martinez-Freiria *et al.*, 2008; Moritz *et al.*, 2009) and can be used to predict areas of sympatry in which hybridization can then be examined in further detail using molecular and morphological markers (Swenson, 2008).

Many temperate trees in Europe have large distributions and show evidence of hybridization between closely related species, which provides a valuable opportunity to examine the ecological conditions that might allow sympatry and hybridization to occur. The genus *Fraxinus* L. (Oleaceae) is one such case. It is represented in Western Europe by two hybridizing species: the common ash *Fraxinus excelsior* L. and the narrow-leaved ash *Fraxinus angustifolia* Vahl. *Fraxinus excelsior* is distributed mainly in the continental areas of Europe, whereas *F. angustifolia* is found mainly around the Mediterranean basin and to the west of the Black Sea in the Danube basin. The latter species has been assigned different botanical names at the species and subspecies levels (Wallander, 2008), which probably reflects different levels of local adaptation (Temunović *et al.*, 2012). Chloroplast haplotypes of the two species largely overlap in Western Europe, which suggests frequent historical gene flow between the species (Heuertz *et al.*, 2006). Indeed, no clear signals about the precise location of hybrid zones are evident in the chloroplast data [see haplotype distribution maps in Heuertz *et al.* (2006), which show little variation in the known hybrid zones detailed below]. Similarly, we could not resolve the taxonomic status between the two species completely using sequence data, including data based on barcode genes, because of the long history of gene exchange (Arca *et al.*, 2012). Consequently, species boundaries and hybrid zones in these ashes might be resolved better using molecular markers that show biparental inheritance, evolve more rapidly than chloroplast genes, and can potentially show signals of selection.

Several lines of evidence suggest that the two species of *Fraxinus* have clearly divergent ecological preferences. Physiological studies have shown that *F. excelsior* and *F. angustifolia* respond differently to temperature stresses during the summer: the former relies on malate to cope with a lack of water (Marigo *et al.*, 2000), whereas the latter relies on the accumulation of mannitol (Oddo *et al.*, 2002). *Fraxinus angustifolia* is frequently located close to waterways and is largely resistant to flooding (Jaeger *et al.*, 2009), in contrast to *F. excelsior*, which is located mostly in areas with well-drained soil or on slopes. Winter temperatures are also important for the reproductive success of *F. angustifolia*, because they determine the dates of flowering in December/January and the corresponding risk of winter frosts, which often damage flowers in the most northerly regions in which the species is distributed. *Fraxinus angustifolia* can survive as an adult tree in parks and botanical gardens in temperate areas, but seldom produces seeds successfully under such conditions. Although the two species show flowering phenologies that do not overlap (Gérard *et al.*, 2006a), hybridization might be possible in years in which the flowering of *F. angustifolia* is delayed owing to a mild winter, or when *F. angustifolia* loses most of its initial flowers to frost and

starts a second but smaller flowering that overlaps with the spring flowers of *F. excelsior* (Gérard *et al.*, 2006a). Mating patterns usually result in *F. angustifolia*-like trees siring *F. excelsior*-like mothers (Gérard *et al.*, 2006b). Hence, we can hypothesize that the expansion of the Mediterranean species *F. angustifolia* and the formation of hybrid populations in temperate zones would be favoured in geographical areas in which winter frosts are rare.

To our knowledge, *Fraxinus* hybrid zones have been identified in the upper basins of the Loire and Saône rivers in France, along the Rhine in the Alsace region between France and Germany (J. Dufour, pers. obs.), and in the Danube/March river basin in Eastern Austria (B. Heinze and F. Starlinger, Institute of Forest Genetics, Federal Forest Research Centre, Vienna, Austria, pers. comm.) (see Fig. S1 in Appendix S1 in Supporting Information). In addition, hybrid populations have been observed in the central plateau of Spain (H. Sainz, Universidad Autónoma, Madrid, Spain, pers. comm.) and south of the Pyrenees (J. Dufour, pers. obs.). Possible zones have been identified in the southern Czech Republic, Hungary, and the Balkans (FRAXIGEN, 2005), and probably also exist elsewhere in Eastern Europe. In France, the extent of hybridization between the two species has been analysed using anonymous nuclear microsatellites and morphological characteristics, and results suggest that patterns of hybridization differ between the Loire and Saône valleys (Fernández-Manjarrés *et al.*, 2006). The mild climatic conditions in the Loire valley appear to promote morphological and molecular introgression of *F. angustifolia* into *F. excelsior*, whereas the more continental climate of the Saône valley appears to allow only molecular introgression between species.

In the study reported herein, we used two independent approaches to determine the degree and direction of introgression and possible mechanisms that determine the levels of hybridization between populations of the Mediterranean *F. angustifolia* and the temperate *F. excelsior* at the continental scale. First, we investigated how the geographical areas of sympatry predicted by SDM correlate with the observed levels of molecular hybridization. As molecular markers, we used anonymous single sequence repeats (SSRs), which are putatively neutral in terms of selection, and RNA-based expressed sequence tag (EST-SSR) markers, which potentially can be subjected to disruptive selection between species. Second, to obtain insights at the continental scale about the process of hybridization, we investigated whether the spatial patterns of allele frequencies for loci that were potentially subjected to selection could identify the main ecological drivers that maintain the boundaries between the parental species and the hybrid populations.

## MATERIALS AND METHODS

### Distribution data

The distribution of *F. excelsior* is well known and was obtained as a shapefile from the European Forest Genetic

Resources Program (EUFORGEN; <http://www.euforgen.org/>) and converted into a regular grid of 12,285 geographical points separated by 2.5', which is the resolution of the climate data (Fig. S1). In contrast, the geographical distribution of *F. angustifolia* is only partially known. We combined all the available records of localities of *F. angustifolia* in the Global Biodiversity Information Facility (GBIF) database (<http://data.gbif.org/>,  $n = 4438$ ), the Spanish Plant Information System (<http://www.anthos.es/>,  $n = 483$ ), and the Flora Croatica Database (<http://hirc.botanic.hr/fcd/>,  $n = 116$ ). We supplemented these records with our own sampling ( $n = 36$ ), as well as with information obtained from personal communications and other literature ( $n = 121$ ), which resulted in a total of 2567 records after discarding duplicates and dubious geographic coordinates (Fig. S1).

### Environmental data

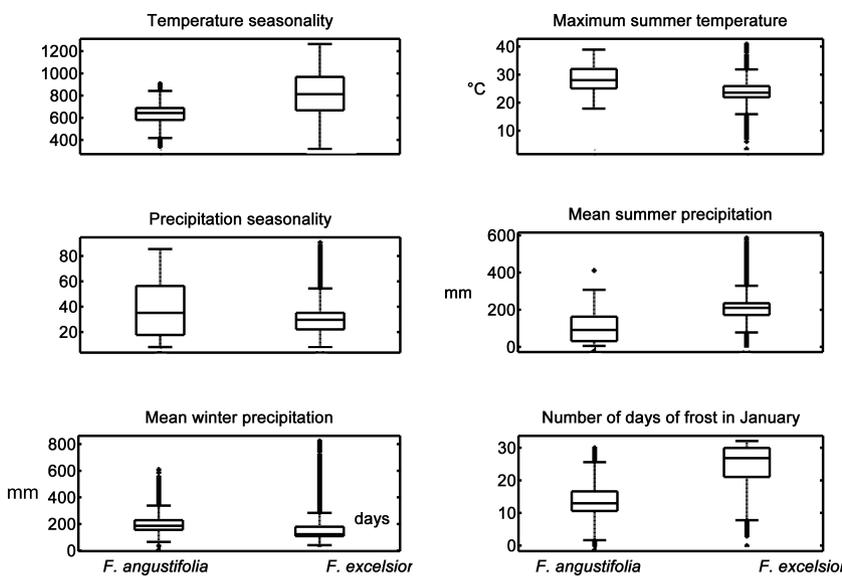
We used variables that were appropriate for continental scale analysis, and included climate variability and climate extremes (Table 1) (Zimmermann *et al.*, 2009), which were obtained from the WorldClim database at 2.5' resolution (<http://www.worldclim.org/>). In addition, we included the mean number of days of frost in January observed between 1961 and 1990 from the Intergovernmental Panel on Climate Change (IPCC) data distribution centre (New *et al.*, 2002) downloaded from <http://www.cru.uea.ac.uk/cru/data/hrg/tmc/>, as a potential limiting factor for *F. angustifolia*, which often loses flowers to frost during early winter (see Fig. 1 for climate variable differences between species). We also included data on soil type in which soils were classified into 28 major categories from the Harmonized World Soil Database 1.1. To compensate for an unequal representation of cells owing to the use of geographical projections in continental areas with a large variation in latitude, we used geographically compensated cells to create a background of 20,000 random points for the training of all SDMs (see below) using the package RASTER 1.9 in R (Hijmans & Eten, 2011), as explained by Elith *et al.* (2011). Finally, owing to the ecology of *F. angustifolia* and its dependence on waterways and flooded areas, we added a dataset for this species only by calculating an additional layer that consisted of the Euclidean distances for all cells to the vectors of major rivers in Europe (<http://hydrosheds.cr.usgs.gov>).

### Species distribution modelling

We used presence-only based modelling to estimate probability distributions from incomplete information using the maximum entropy method (Phillips *et al.*, 2006) as implemented in MAXENT 3.3.2 (<http://www.cs.princeton.edu/~schapire/maxent>). We used the cross-validation routine in MAXENT to generate a  $k$ -fold cross validation with  $k = 10$ . Output models were cut at a threshold defined by the 'maximum training sensitivity plus specificity' as recommended for exploratory analyses (Liu *et al.*, 2005). The adequacy of

Variable	<i>F. angustifolia</i>		<i>F. excelsior</i>	
	Percentage contribution	Permutation importance	Percentage contribution	Permutation importance
Temperature seasonality	16.7	<b>37.1</b>	11.3	15.5
Maximum summer temperature	16.3	<b>24.6</b>	6.6	7.0
Precipitation seasonality	6.9	7.9	1.9	11.6
Mean summer precipitation	12.2	9.1	69.3	<b>31.3</b>
Mean winter precipitation	21.6	7.5	5.0	<b>21.7</b>
Number of days of frost in January	16.0	3.6	4.0	10.0
Elevation	0.8	2.4	0.4	0.7
Soil type	4.6	3.5	1.6	2.3
Distance to rivers	4.8	4.2	–	–

**Table 1** Heuristic estimate of the relative contributions of the environmental variables that are potential limiting factors for the distribution of *Fraxinus excelsior* and *F. angustifolia* in Europe to the MAXENT models. The environmental variables that contributed the most to each group are shown in bold. Temperature seasonality is the standard deviation of monthly temperatures multiplied by 100 and precipitation seasonality is the coefficient of variation of monthly precipitation.



**Figure 1** Box plots of the climate variables used to predict the distributions of *Fraxinus angustifolia* and *F. excelsior* in Europe. The centre line within the box represents the mean, the extent of the box the standard deviation, the whiskers a confidence interval of 95%, and the small crosses beyond the whiskers the outlier records. Differences are significant for each pair of variables (paired *t* tests,  $P < 0.001$ ).

the distribution model was verified for each group using the area under the curve (AUC) of a receiver-operating characteristic plot based on test data. Finally, the gain contributed by each environmental variable (a measure of goodness of fit) to the models produced by MAXENT and the relative contributions to the gain by each variable (heuristic contribution) were recorded.

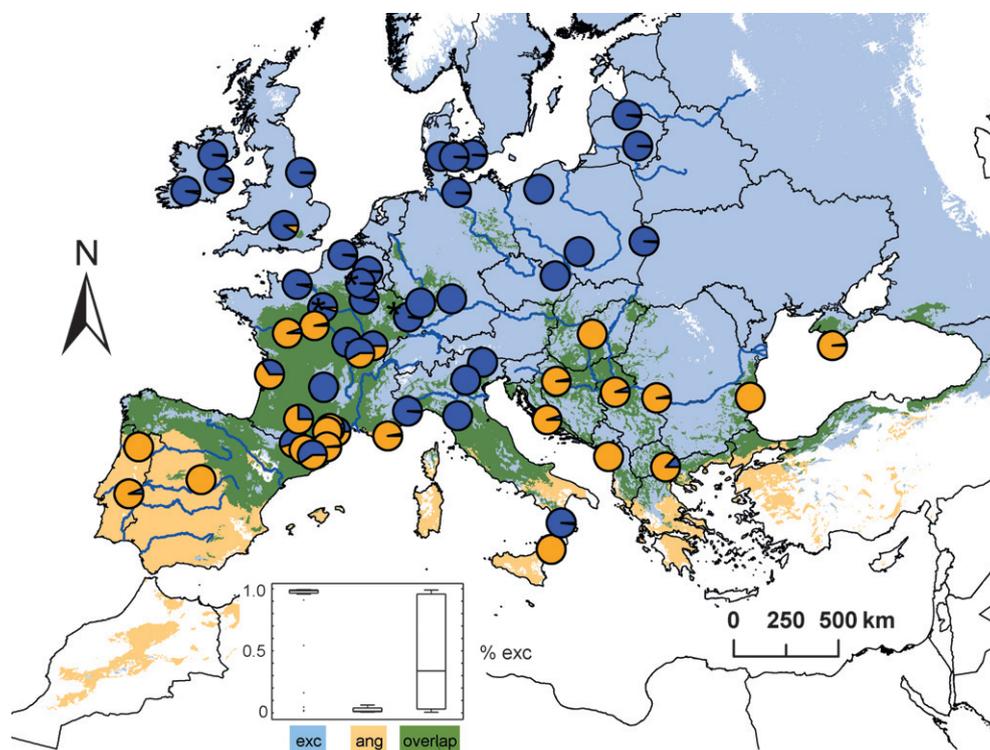
### Population data for genetic analyses

Our samples for genetic analysis consisted of 456 individuals from 58 populations (sampling sites) that corresponded to both parental species and known and putative hybrid zones. *Fraxinus excelsior* was represented by 31 populations, *F. angustifolia* by 21, and hybrids by 6 (Fig. 2, and Table S1 in Appendix S2). Hybrid status was assigned preliminarily on the basis of our previous research; however, the original assigned status of certain populations was changed according to the levels of genetic admixture because we did not have morphological data on all the samples. Here, our focus is on continental-wide patterns; thus we assume that our sample

size is adequate to detect overall trends. However, we acknowledge that it might be limited for characterization of some introgressed populations. Finally, several of the *F. excelsior* populations were obtained from a provenance test site of 33 European populations of common ash that was established in Normandy, France, in 2002 by J. Dufour. The populations were established from open pollinated seeds that were obtained from 10 to 20 unrelated individuals. The remaining populations were obtained by field collection and exchange with colleagues.

### Molecular data

We used six anonymous nuclear SSR markers that have been used widely in the European ashes and 13 new RNA-based (EST-SSR) markers that we developed recently (Aggarwal *et al.*, 2011) from EST libraries produced by the CBiB (Centre de Bioinformatique de Bordeaux). Total DNA was extracted using a NucleoSpin 96 Plant Kit (Macherey-Nagel, Düren, Germany), in accordance with the manufacturer's instructions, from 0.1 g of either fresh leaves or buds that



**Figure 2** Species distribution models for *Fraxinus excelsior* (blue), *F. angustifolia* (light orange) in Europe, and areas of overlap (green) and genetic assignment based on the proportion of gene pools (pie charts with dark blue representing *F. excelsior* and orange *F. angustifolia* gene pools). Three populations north of the Loire in France are marked with asterisks and correspond to well-known *F. excelsior* populations that occur within the overlap zone (see text for discussion). The insert box-plot depicts the proportion of *F. excelsior* gene pool in each population and whether each population occurs in parental only (exc, *F. excelsior*; ang, *F. angustifolia*) or overlap areas according to the predicted distributions. The map was drawn with the Lambert azimuthal equal-area projection.

had been desiccated previously in a 1:1 mixture of alcohol and acetone. The polymerase chain reaction (PCR) conditions complied with previously published protocols (Gérard *et al.*, 2006a) with respect to annealing and extension times, as well as temperatures, and primers were 5'-labelled with the fluorescent molecules 6-FAM<sup>TM</sup>, VIC<sup>®</sup>, NED<sup>TM</sup>, and PET<sup>®</sup>. PCR products were analysed in an ABI PRISM 7900<sup>®</sup> Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA) and allele sizes were scored on the basis of a 500-bp GIZ ladder (Life Technologies, Carlsbad, CA, USA) using GENEMAPPER 4.0 (Applied Biosystems). Allele sizes were verified twice: first, two researchers in the group carried out independent readings, and then they conducted a joint reading of the genotypes.

### Genetic diversity and structure

To determine the probable number of gene pools and the degree of hybridization, we used the Bayesian approach implemented in STRUCTURE 2.3.3 (Pritchard *et al.*, 2000). We ran three analyses with STRUCTURE, one with only six nuclear SSR markers, one with the 13 EST-SSR markers, and the last with the complete dataset. The assignment of individuals was conducted using a uniformly distributed prior for population membership, with 10,000 burn-ins and

100,000 replicates. Simulations were run for a putative number of clusters,  $K = 1$  to 10, with each value of  $K$  having 10 replicates. The most likely number of clusters was inferred by examining the rate of change in likelihood of the number of clusters (Evanno *et al.*, 2005) as implemented in STRUCTURE HARVESTER 0.6.8 (Earl & vonHoldt, 2012). Finally, the STRUCTURE results were averaged across replicate runs in CLUMPP 1.1.2 (Jakobsson & Rosenberg, 2007). Genetic diversity parameters (total and effective number of alleles, heterozygosity, and the inbreeding coefficient) were calculated with the R package GENETICSTUDIO 0.7 (Dyer, 2012).

### Selection analysis

To detect loci that were potentially subjected to selective pressures, we used LOSITAN 1.0 (Antao *et al.*, 2008), which is a workbench for the detection of selection that is constructed around the  $F_{ST}$  outlier method (Beaumont & Nichols, 1996; Vitalis *et al.*, 2001). This method evaluates the relationship between  $F_{ST}$  and  $H_E$  (expected heterozygosity) and the expected distribution of these variables in an island model. Outlier loci that have excessively high and low  $F_{ST}$  values compared with neutral expectations are considered to be directionally selected or subjected to stabilizing selection. In the present study, the program was run for 100 populations,

with a sample size of 50 individuals per population, a step-wise mutation model, and a confidence interval (CI) of 99%. Analyses of selection were run separately for the nuclear SSR and the EST-SSR markers because initial runs with the complete dataset produced a very high  $F_{ST}$  that identified several of the anonymous nuclear SSR loci as being subjected to stabilizing selection.

### Spatial analysis

Initial principal component analysis (PCA) of the joint allele frequencies for all 19 markers showed very low loadings (< 5%), which was mainly due to the frequencies of some alleles of the EST-SSR markers. Hence, we concentrated our spatial analysis on the EST-SSR loci that could potentially show signals of selection on the basis of previous analyses. We conducted an eigenvector-based spatial filtering regression (Borcard *et al.*, 2004) of allele frequencies on climatic variables. In this method, spatial trends are included in regression models by extracting eigenvectors from a matrix that expresses the spatial relationships among sampling sites based on the pairwise distances between sampling locations. One or more eigenvectors extracted from the pairwise distance matrix are added to the regression equation as a new filter variable to reduce the autocorrelation of the residuals (Rangel *et al.*, 2010). To choose significant eigenvectors, we used the option of minimizing Moran's  $I$  as the selection rule. All analyses were carried out using SAM 4 (Rangel *et al.*, 2010) using the default settings.

## RESULTS

### Species distribution models and sympatry areas

The MAXENT distribution models appeared to be robust because the distributions were largely in accordance with what is known for each species and the AUC values were high: 0.732 (SD = 0.005) for *F. excelsior* and 0.961 (SD = 0.004) for *F. angustifolia* (see Fig. S2 in Appendix S1). As expected, the AUC values for *F. angustifolia* were higher than those for *F. excelsior* because the former is a habitat specialist, whereas *F. excelsior* is a habitat generalist, and thus its distribution is more difficult to simulate, even when high-resolution data are used (Guisan *et al.*, 2007). For *F. excelsior*, the environmental variable that resulted in the highest gain when used in isolation was summer precipitation, but the variable that decreased the gain the most when it was omitted was seasonality of temperature. In contrast, for *F. angustifolia*, the number of days of frost in January was the variable that produced the highest gain, whereas summer temperature was the variable that decreased the gain the most when it was omitted. Summer temperature appeared to be highly correlated with seasonality of precipitation and inversely related to summer precipitation (Table S2 in Appendix S2), which is in accordance with the mostly Mediterranean distribution of *F. angustifolia*, because in the Medi-

terranean the hottest summers are often also dry. However, using the present data, it was impossible to test whether the distribution of *F. angustifolia* was constrained more by temperature, precipitation levels or variability in precipitation. Finally, the heuristic estimates of the variable contributions (Table 1) suggested that overall levels of winter and summer precipitation were important for the distribution of the temperate *F. excelsior*, whereas the temperature in summer and its annual variability were more important for the distribution of *F. angustifolia*.

### Genetic assignment of populations

As expected, allelic diversity was higher for the anonymous SSR loci than for the EST-SSR loci (Table 2). The anonymous SSRs had 15 to 51 alleles, whereas the EST-SSR loci had 3 to 15 alleles for the complete dataset. For the population samples, the mean numbers of alleles were lower (between two and five on average for all loci, see Table S1) owing to the sample size of  $n = 8$ . The Bayesian assignment supported a genetic structure that consisted of two main gene pools with intermediate populations regardless of the combination of loci used (Fig. 2 and Fig. S3 in Appendix S1). Nevertheless, the inclusion of the EST-SSR loci increased the accuracy of identification of hybrid populations. The Bayesian assignments with each type of marker (with and without EST-SSRs) were highly correlated ( $r^2 > 0.90$ , see Fig. S4 in Appendix S1), which suggested an ancient population

**Table 2** Overall genetic diversity of the loci analysed in 58 populations of *Fraxinus excelsior* and *F. angustifolia* in Europe. Abbreviations correspond to sample size ( $n$ ), number of alleles ( $A$ ), effective number of alleles ( $A_E$ ), observed heterozygosity ( $H_O$ ), expected heterozygosity ( $H_E$ ), and the local population inbreeding coefficient ( $F_{IS}$ ).

Locus	$n$	$A$	$A_E$	$H_O$	$H_E$	$F_{IS}$
Fem4	456	27	5.40	0.76	0.82	0.07
Fem11	452	29	6.83	0.75	0.85	0.12
Fem12	448	28	10.27	0.73	0.90	0.19
Fem16	456	15	2.07	0.45	0.52	0.13
Fem19	454	31	9.34	0.74	0.89	0.17
M230	454	51	24.29	0.84	0.96	0.12
EST-SSR 39	454	4	2.49	0.35	0.60	0.42
EST-SSR 130	451	3	2.00	0.93	0.50	-0.86
EST-SSR 203	453	7	1.13	0.11	0.12	0.10
EST-SSR 279	456	7	2.35	0.42	0.58	0.28
EST-SSR 308	456	11	3.35	0.71	0.70	-0.01
EST-SSR 326	456	4	2.94	0.53	0.66	0.20
EST-SSR 353	456	7	2.29	0.61	0.56	-0.07
EST-SSR372	456	4	1.85	0.23	0.46	0.51
EST-SSR 389	443	3	1.11	0.10	0.10	-0.01
EST-SSR427	456	7	2.18	0.38	0.54	0.29
EST-SSR 431	455	5	1.39	0.23	0.28	0.17
EST-SSR 520	454	11	3.72	0.48	0.73	0.35
EST-SSR 528	454	15	3.74	0.74	0.73	0.00
Mean	453.70	14.20	4.67	0.53	0.61	0.11
SD	3.35	13.09	5.42	0.25	0.25	0.28

structure. Incidentally, none of the markers showed any linkage disequilibrium (results not shown). Therefore, subsequent analyses and results were based on data from the combination of anonymous SSR and EST-SSR markers.

Hybrid populations, as assigned on a genetic basis, occurred frequently in areas that the SDMs predicted to pertain to the overlap between the species (Fig. 2). In contrast, no hybrid populations occurred in areas that were identified by the SDMs to be typical of *F. angustifolia* only. These results suggest a tendency for asymmetry in the occurrence of the hybrid populations, with a bias towards the areas of distribution of *F. excelsior* and the temperate region in general (Kruskal–Wallis test,  $\chi^2 = 24.92$ ,  $P < 0.001$  calculated on the percentage of *F. excelsior* gene pool grouped by predicted area as in Fig. 2). If there were no bias in the direction of the hybridization, highly admixed populations should appear randomly associated with the SDMs of both parental species, which is not the case here.

### Selection analysis

Among the 13 EST-SSR loci analysed, eight appeared to have been subjected to some type of selection; six loci appeared to have been subjected to stabilizing selection and two to directional selection (Table 3). The signals of selection are probably caused by the specific allele clines that were observed between the two parental species (alleles of sizes 388/391 and 258/

266 bp for the loci 372 and 427, respectively), with heterozygotes occurring frequently in the hybrid populations and homozygotes occurring at the extremes of the distributions of the parental species (results not shown). BLAST searches of GenBank with the loci under directional selection (Table 3) identified candidate genes that encoded proteins similar to histones found in drought-resistant clones of robusta coffee, *Coffea canephora*, (EST-SSR 372) and to one protein of unknown function in pedunculate oak, *Quercus robur*, (EST-SSR 427). One of the loci that appeared to be under stabilizing selection was also associated with water stress (EST-SSR 203). However, most were associated with floral buds, which was the tissue used to develop the library. We focused subsequent analyses on the two EST-SSR 372 and 427 loci that potentially had been subjected to directional selection.

The anonymous SSR loci showed no signs of selection, with the exception of the locus Femsat 19, which tended to have many small alleles (less than 140 bp) in *F. angustifolia* and larger alleles (more than 170 bp) in *F. excelsior*, as has been reported previously (Fernández-Manjarrés *et al.*, 2006). No homologous sequence was found for Femsat 19 when it was submitted to a BLAST search in GenBank and it is not linked either to any of the EST-SSR loci used (results not shown). These patterns of selection at a putatively neutral marker could be explained by hitchhiking of the Femsat 19 marker by a positively selected allele at a closely linked locus that remains unknown at the time of the study.

**Table 3** Results of the LOSITAN selection analysis on *Fraxinus excelsior* and *F. angustifolia* in Europe.  $H_E$  is the expected heterozygosity,  $F_{ST}$  is Wright's population structure parameter, and  $P$  is the probability that the observed  $F_{ST}$  value is too small or too large with respect to a neutral locus in an island model ( $F_{ST}^*$ ). Probabilities marked with a § sign correspond to loci subjected to stabilizing selection and those marked with a Δ sign correspond to loci possibly subjected to directional selection. Putative function corresponds to function in other species as identified by a BLAST search of the EST-SSRs in GenBank. Among the anonymous loci, only Fem19 showed signs of directional selection, but its links to any functional gene remain unknown.

Locus	GenBank accession number	$H_E$	$F_{ST}$	$P(F_{ST}^* > \text{sample } F_{ST})$	Putative function in other species (from GenBank Unigene information link)
EST-SSR 39	FR638723	0.601	0.258	0.4725	unknown
EST-SSR 130	FR640915	0.500	-0.047	0.0000§	Similar to peptidase in flower buds of <i>Mimulus guttatus</i>
EST-SSR 203	FR636736	0.117	0.059	0.0002§	Moderately similar to water stress-induced protein in <i>Populus trichocarpa</i>
EST-SSR 279	FR635387	0.579	0.340	0.9762	Unknown
EST-SSR 308	FR644535	0.706	0.162	0.0000§	Moderately similar to <i>M. lewisii</i> floral bud RNA
EST-SSR 326	FR639294	0.664	0.324	0.9554	Similar to chloroplast-binding product
EST-SSR 353	FR644953	0.565	0.165	0.0035§	Similar to 1-aminocyclopropane-1-carboxylic acid oxidase in <i>Quercus robur</i>
EST-SSR 372	FR637753	0.461	0.632	1.0000Δ	Similar to histone H1 <i>Populus trichocarpa</i> ; found also in drought resistant clones of <i>Coffea canephora</i>
EST-SSR 389	FR642190	0.106	0.054	0.0001	Moderately similar to transmembrane family protein in <i>Arabidopsis</i>
EST-SSR 427	FR638723	0.547	0.376	0.9982Δ	Similar to <i>Quercus robur</i> transcription factor expressed constitutively in roots, stems, and flowers
EST-SSR 431	FR645842	0.277	0.097	0.0001§	Similar to hypothetical protein in <i>Vitis vinifera</i>
EST-SSR 520	FR639485	0.734	0.287	0.7830	Unknown
EST-SSR 528	FR646655	0.734	0.144	0.0000§	Unknown
Femsat 4	AF006069	0.812	0.086	0.0519	–
Femsat 11	AF029882	0.858	0.077	0.0144	–
Femsat 12	AF020397	0.903	0.096	0.2378	–
Femsat 16	AF029880	0.513	0.096	0.3013	–
Femsat 19	AF020400	0.898	0.169	0.9998Δ	?
M230	AF021337	0.962	0.071	0.4212	–

### Spatial analysis of allele frequencies

The results of eigenvector filter analysis between the allele frequencies for the loci EST-SSR 427 and EST-SSR 372 and the climate data indicated that filtering improved the fitting

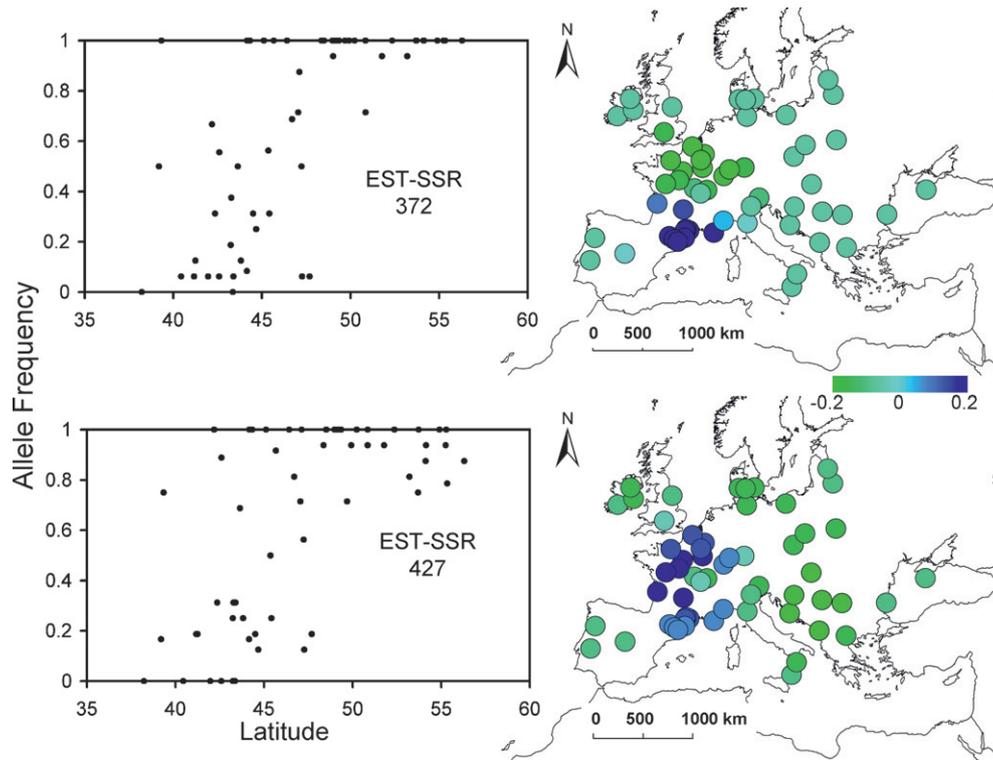
**Table 4** Results of spatial filtering for the regression between the allele frequencies of the two loci putatively under directional selection from *Fraxinus excelsior* and *F. angustifolia* in Europe.

	EST-SSR 372		EST-SSR 427	
	Coefficients	P-value	Coefficients	P-value
Intercept	3.98	< 0.001	3.245	< 0.001
Temperature seasonality	0.001	0.230	< 0.001	0.302
Maximum summer temperature	-0.138	< 0.001	-0.125	< 0.001
Precipitation seasonality	0.006	0.157	0.004	0.374
Mean summer precipitation	0.001	0.300	< 0.001	0.655
Mean winter precipitation	< 0.001	0.390	< 0.001	0.927
Number of days of frost in January	-0.029	0.048	< 0.001	0.963
Filter 1			1.246	0.020
Filter 2	-1.044	0.003		

of the regression model and suggested that summer temperatures and number of frost days in January explained the observed patterns of allele frequencies (Table 4). For these loci,  $r^2$  increased from 0.673 to 0.725 for EST-SSR 372 and from 0.646 to 0.682 for EST-SSR 427 when the most significant spatial filter variable was added to the general regression model. Akaike's information criterion values decreased from 46.767 to 39.406 and from 47.623 to 44.060, for EST-SSR 372 and EST-SSR 427, respectively. The filtering accounted for all the spatial autocorrelation of the data for both EST-SSR loci because the residuals did not contain any significant values of Moran's  $I$  at the different distance classes compared to the unfiltered regression (see Fig. S5 in Appendix S1). Moreover, the spatial filtering was generally higher in the areas in which the two species overlapped according to the SDM predictions, which suggested that changes in allele frequencies were abrupt in these regions, as expected for hybrid zones (Fig. 3).

### DISCUSSION

The results of our study show that, at the continental scale, hybrid populations between *F. excelsior* and *F. angustifolia* are found mostly in areas in which the two species are predicted to overlap by an independent SDM. However, not all areas of overlap showed significant levels of introgression, especially in more northerly areas, which suggested that dis-



**Figure 3** Latitudinal clines in the frequency of the most common allele for the stated loci EST-SSR 372 and EST-SSR 427 in *Fraxinus excelsior* and *F. angustifolia* in Europe. For each locus, the left panel shows the allele gradient in relation to latitude. The right panel depicts the values of the spatial filters calculated for each locus. Darker blue or green colours imply a higher degree of filtering, which corresponds to the populations that are known to have important admixture and are in an area of rapid change of allele frequency.

persal might be limited for *F. angustifolia* and hybrids towards northern latitudes. We found that the number of days of frost in January, summer precipitation, and summer temperature were variables that not only contributed to the MAXENT distribution model, but also explained the distribution of allele frequencies in loci potentially subjected to selective pressures. Below, we discuss these findings with regard to the insights that we gain about the pattern of hybridization, the role of climate, and the origin of a hybrid zone between close species that are ecologically divergent.

### SDM and hybrid patterns

Given the clearly different climatic preferences of the hybrids and parental species (box plots in Fig. 1) and the possibility of directional selection at two EST-SSR loci that could be explained by allele gradients that correlated with climate conditions, it seems possible that exogenous selection is the main factor that shapes the structure of the hybrid zones of these two European ash species. Moreover, the presence of populations that are clearly assigned genetically as *F. excelsior* to the north of the Loire valley (populations marked with an asterisk in Fig. 2), within the predicted region of overlap, makes it tempting to conclude that at least for this region, the pattern of hybridization fits locally the 'bounded hybrid superiority model'. As pointed out by Swenson (2008), if the predicted distributions of hybrid zones match closely the observed distributions of hybrid populations, and if the predicted distribution of at least one of the parental species extends beyond the known areas of hybridization, it could be concluded that the higher fitness of the hybrids prevents the demographic expansion of parental lineages beyond their current limits. Indeed, we have empirical evidence of some hybrid superiority in terms of the number of seeds sired by *F. angustifolia* in the Loire valley (Gérard *et al.*, 2006b), but we are cautious to deduce that the whole distribution of hybrid populations fits the 'bounded hybrid superiority model' for the following reasons.

First, the areas predicted by the SDM for *F. angustifolia* that occur beyond the hybrid areas in the north (the Loire valley for example) might simply be an artefact of the data used. The extent of the distribution of *F. angustifolia* might be overestimated because this parental species and its hybrid populations are mixed in the hybrid zones. We suspect that in numerous botanical inventories (from which we obtained most of the distribution data), many hybrids have been assigned to *F. angustifolia* because they often resemble the Mediterranean species more closely than the temperate one.

Second, the presence of non-hybrid *F. angustifolia* individuals at high latitudes suggests that the fitness advantage of the hybrids in relation to *F. angustifolia* must be relatively small. *Fraxinus angustifolia* was certainly able to colonize areas in the north of Europe without the process of hybridization, by taking advantage of microclimates associated with large river systems; however, hybridization has probably accelerated colonization.

Third, the narrow predicted areas of overlap and the almost non-existent levels of admixture in Eastern Europe towards the Black Sea suggest that at the continental scale of the analysis, no clear pattern of hybridization can be assigned. The Danube and Pannonian lowlands in south-eastern Europe host large floodplain forests in which *F. excelsior* is rare and is replaced by the flood-tolerant *F. angustifolia* as a dominant species. If the bounded hybrid superiority model were applicable here (i.e. the predicted distribution of *F. excelsior* extended beyond the predicted zone for *F. angustifolia*), the molecular marker analysis should have revealed a mixture of *F. angustifolia* and hybrid populations in this basin, but this was not the case. For the sampled populations from Eastern Europe, at least, no hybridization was detected. Hence, all other things being equal, the conditions in Eastern Europe appear to be less favourable for the formation of hybrid zones than the conditions in Western Europe. Future work should address whether the rate of hybridization between *F. angustifolia* and *F. excelsior* in Eastern Europe is effectively low at the population level because winter frosts are more common than in Western Atlantic Europe, which results in non-overlapping flowering periods.

There are two possible origins for the asymmetrical hybridization patterns identified in the present study. First, the available data indicate that it is more likely for a Mediterranean species to colonize temperate areas, in which frosts are rare, than for a temperate species to colonize the Mediterranean zones. If the latter were the case, *F. excelsior* should be found along waterways in riparian vegetation of the Mediterranean, but that has not been observed to the best of our knowledge. Second, we have evidence that in the Loire hybrid zone, *F. angustifolia* or hybrid trees that usually flower early can sire seeds from early flowering *F. excelsior* in years in which the overlap in flowering time between the two species is increased. In general, we would expect the opposite pattern, with the more abundant *F. excelsior* saturating the less abundant *F. angustifolia*. However, it seems that the Mediterranean species *F. angustifolia* is successful in taking advantage of low pollen competition at the beginning of the flowering in spring. Hence, both demographic and phenological isolation might act together to produce an overall asymmetrical pattern of hybridization towards temperate areas in these ash species in the long term.

### Historical scenario of hybridization

From our finding that the number of days of frost and the summer temperatures play an important role in the maintenance of hybrid populations, we can contend that the degree of hybridization between the two species of ash might vary in a cyclical manner during glacial and interglacial periods. If the ecological preferences of the two species diverged in allopatry during glacial maxima, as proposed for several taxa in Europe (Hewitt, 2000, 2004), the most parsimonious hypothesis is that hybridization occurred after the expansion of *F. angustifolia* and *F. excelsior* from different refugia. In

the present study, we cannot assess whether hybrid zones existed during glacial periods and whether their position changed during post-glacial recolonization. However, the conditions that are associated with stable hybrid zones today (i.e. winters with little frost and high levels of summer precipitation) were probably rare during glacial periods. The Mediterranean climate was characterized by summers that were at least 5 °C colder than at present and winters that were at least 12 °C colder than today (Wu *et al.*, 2007), a climate that we now associate more with *F. excelsior* than with *F. angustifolia*. Relatively few cold days are required to induce flowering in *F. angustifolia* (Jato *et al.*, 2004), so under colder conditions, this species would flower even earlier in the winter, just after the end of autumn. This shift would increase the phenological isolation of *F. angustifolia* from *F. excelsior*, which flowers in the spring because it requires a greater accumulation of chill hours. Fifty years of observations of *F. angustifolia* in Spain during the second half of the 20th century have shown that this species now flowers 37 days later in winter than at the beginning of the observation period, owing to milder temperatures in November and December (Peñuelas *et al.*, 2002). This finding clearly indicates that warmer winters decrease the reproductive isolation between *F. excelsior* and *F. angustifolia*. Taking these results together, we suggest that post-glacial warming might have favoured not only the expansion of *F. angustifolia* from the Mediterranean but also its hybridization with *F. excelsior*. The question of whether current global warming will increase the hybridization between the two species remains open, but we think it is likely that gene exchange will increase in the long term.

## CONCLUSIONS

Overall, the results of our study show that the temperate ash *F. excelsior*, the Mediterranean ash *F. angustifolia*, and their hybrids are distributed at a continental scale along a cline that appears to be determined largely by the number of days of frost in January and the levels of summer precipitation and summer temperature. The first variable limits the number of seeds set by *F. angustifolia*, whereas the second might be more limiting for the distribution of *F. excelsior*, which is sensitive to drought. Local studies are needed to verify whether the same processes of climate selection occur at a finer scale and genome-wide analyses would be required to verify the selection signals observed in the study. In this regard, we suggest that the overlap zones that were predicted in our modelling can be used as a starting point to look for the presence of hybrids in unexplored areas of the distribution of these two closely related ashes.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Appendix S1** Supplementary figures (Figs S1–S5), detailing the distribution data sets for *Fraxinus excelsior* and *F. angustifolia*, distribution models, assignment results, and autocorrelation tests between allele frequencies and climate data.

**Appendix S2** Supplementary tables (Tables S1–S2), summarizing coordinates and population genetic parameters of the sampled *Fraxinus* spp. populations in Europe as well as correlations of climate variables.

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## BIOSKETCH

This contribution is part of the research programme on European ash hybridization led by Juan F. Fernández-Manjarrés within the research group of ‘Gene flow and anthropic landscapes’ at the University of Paris-Sud, France.

Author contributions: P.G., N.F.-L., and J.F.F.-M. designed the study; P.G. and J.F.F.-M. performed the simulations and statistical analysis; J.D., P.B. and M.T. collected the samples; J.S. designed the EST-SSR molecular markers; P.B. genotyped the samples; and P.G., M.T. and J.F.F.M. wrote the paper. All authors read and approved the manuscript.

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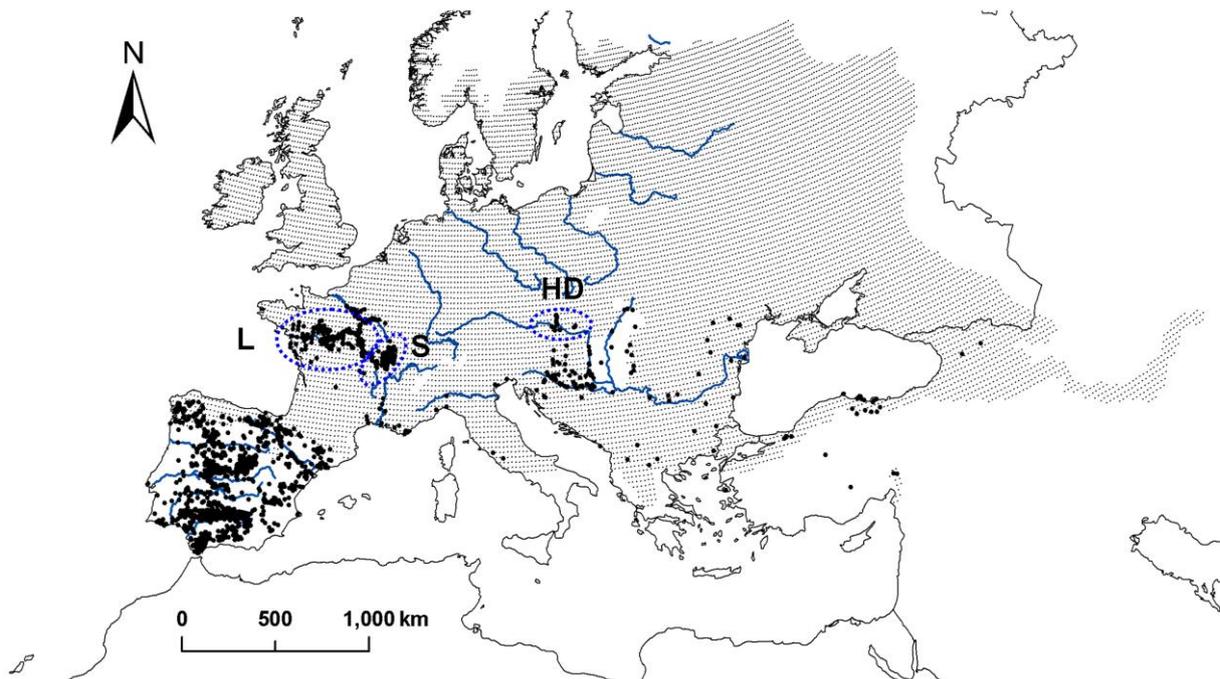
## SUPPORTING INFORMATION

### Chilled but not frosty: understanding the role of climate in the hybridization between the Mediterranean *Fraxinus angustifolia* Vahl and the temperate *Fraxinus excelsior* L. (Oleaceae) ash trees

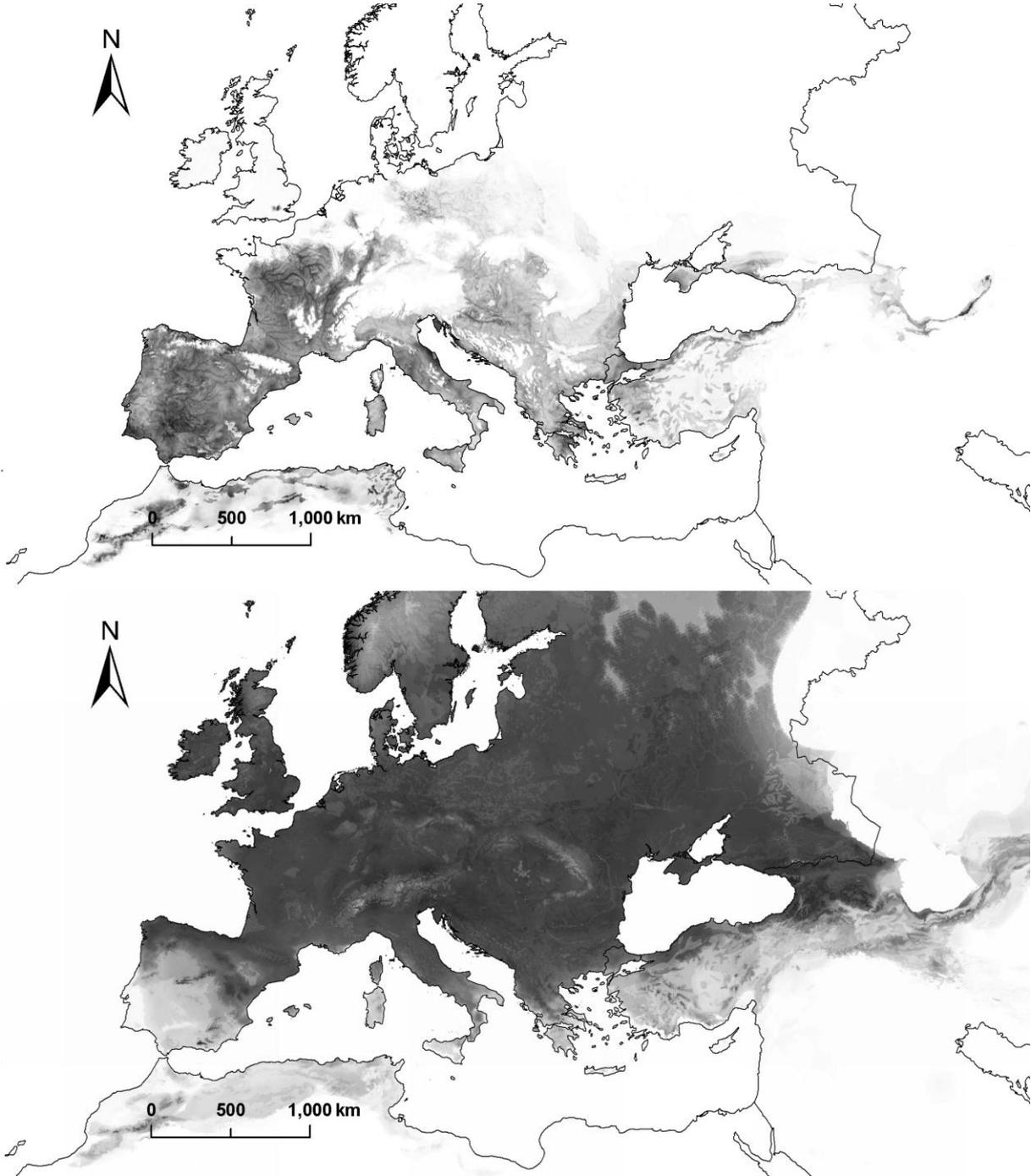
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**Appendix S1** Supplementary figures (Figs S1–S5), detailing the distribution data sets for *Fraxinus excelsior* and *F. angustifolia*, distribution models, assignment results, and autocorrelation tests between allele frequencies and climate data.

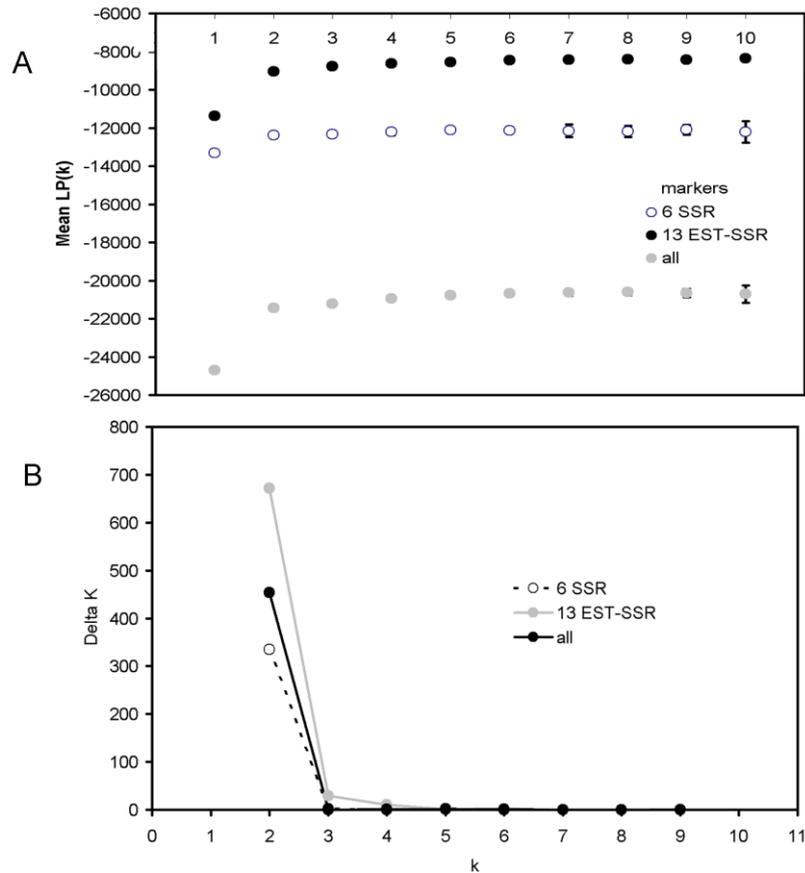
**Figure S1** Records used for the MAXENT models of *Fraxinus excelsior* (grey grid area) and *F. angustifolia* (black dots). Known hybrid areas to the north of the *F. angustifolia* distribution are depicted approximately with a dotted blue ellipse and include the Loire valley (**L**), the Saône valley (**S**), and the High Danube basin in Austria and neighbouring countries (**HD**). Other hybrid zones populations may exist in the north of the Iberian Peninsula and possible also in the Balkan areas (see main text for details).



**Figure S2** MAXENT models for the distribution of *Fraxinus angustifolia* (upper map) and *F. excelsior* (lower map) in Europe.



**Figure S3** Summary results of the Bayesian assignment performed with STRUCTURE from populations of *Fraxinus angustifolia* and *F. excelsior* in Europe for different types of combination of genetic markers. (A) Mean likelihood results with standard errors of at least 10 runs of structure for each putative number of gene pools ( $k = 1$  to 10) for each combination of markers. (B) Graph of the rate of change in likelihood from (A). According to the Evanno *et al.* (2005) method, the probable number of gene pools is close to the number of  $k$  where the rate of change stabilizes to zero, among other criteria.

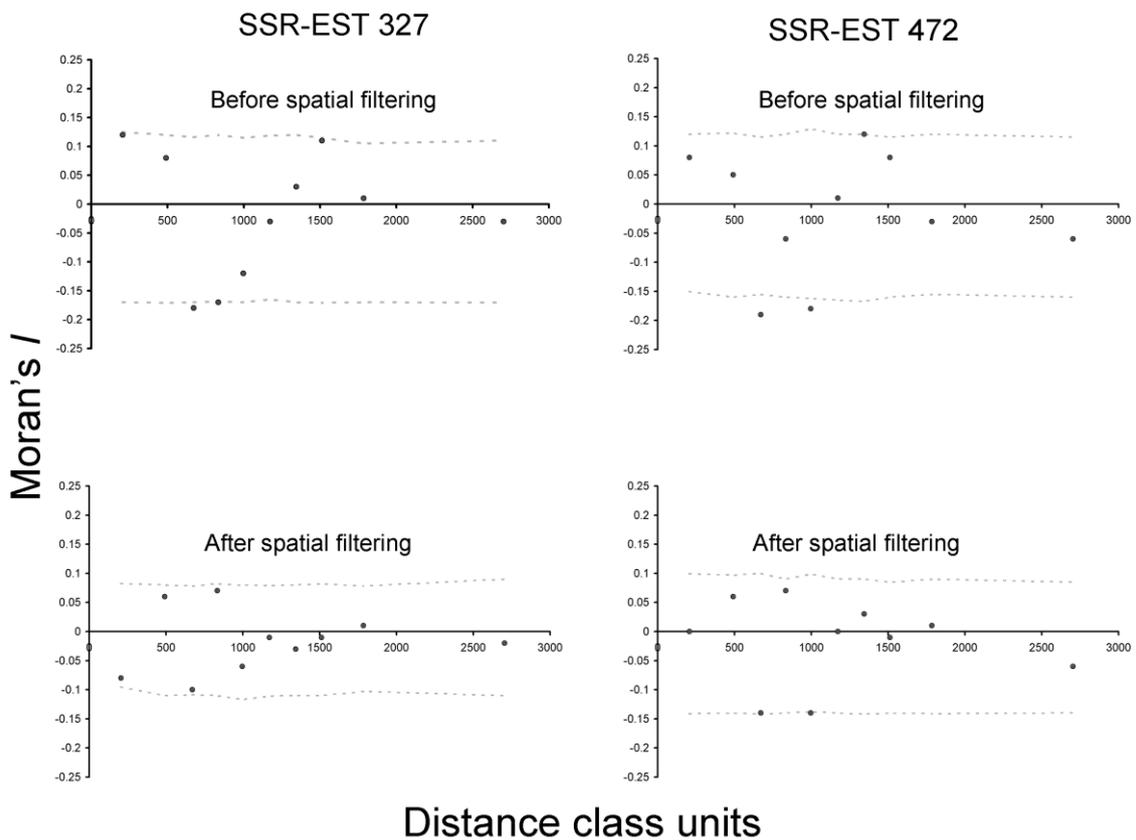


**References:**

Evanno, G., Regnaut, S. & Goudet, J. (2005) Detecting the number of clusters of individuals using the software structure: a simulation study. *Molecular Ecology*, **14**, 2611-2620.



**Figure S5** Spatial autocorrelation (Moran's  $I$ ) of the residuals of the linear model before (upper panel) and after including the spatial filter variables (lower panel) between allele frequencies and climate variables for populations of *Fraxinus angustifolia* and *F. excelsior* in Europe. None of the distance classes of the filtered model have Moran's  $I$  significantly different from a random structure for neither EST-SSR 427 nor EST-SSR 372 loci based on 1000 permutations after spatial filtering (lower panel).



## SUPPORTING INFORMATION

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**Appendix S2** Supplementary tables (Tables S1–S2), summarizing coordinates and population genetic parameters of the sampled *Fraxinus* spp. populations in Europe as well as correlations of climate variables.

**Table S1** Population name, coordinates, species and sample size and descriptive genetic parameters of the 58 European populations of *Fraxinus excelsior* and *F. angustifolia* sampled in this study. SP is the classification based on prior morphological knowledge of the populations. However, some populations were re-classified as either hybrid (HYB) or *F. angustifolia* (ANG) based on the percentage of *F. excelsior* (EXC) gene pool determined by STRUCTURE (*F. exc* %): we reclassified for further analyses into Hybrids populations with more than 25% of the *F. excelsior* gene pool and as *F. angustifolia* those exhibiting less. Abbreviations correspond to number of alleles *A*, effective number of alleles  $A_E$ , observed heterozygosity  $H_O$ , expected heterozygosity  $H_E$ , and the local population inbreeding coefficient  $F_{IS}$ .

population	longitude	latitude	SP	<i>F. exc</i> %	Sample size	<i>A</i>	$A_E$	$H_O$	$H_E$	$F_{IS}$
Abetone	10.667	44.167	EXC	0.993	8 (0)	3.5 (3)	2.33 (1.74)	0.44 (0.35)	0.42 (0.31)	-0.08 (0.43)
AdaBojana	19.348	41.866	ANG	0.007	7.9 (0.2)	3.9 (2.3)	2.65 (1.86)	0.58 (0.28)	0.53 (0.25)	-0.11 (0.29)
Athis	4.609	49.020	EXC	0.961	8 (0)	3.6 (2.4)	2.5 (1.41)	0.54 (0.39)	0.5 (0.3)	-0.07 (0.54)
Onay	5.499	47.341	EXC	0.956	8 (0)	3.6 (3)	2.63 (2.11)	0.45 (0.36)	0.46 (0.33)	-0.01 (0.46)
BoisdeRosee	4.717	50.233	EXC	0.988	7 (0)	3.7 (3.1)	2.88 (2.54)	0.45 (0.39)	0.49 (0.33)	0.09 (0.51)
Bregentved	11.960	55.341	EXC	0.984	6.9 (0.2)	3.2 (1.9)	2.19 (1.13)	0.47 (0.35)	0.46 (0.28)	-0.03 (0.41)
By	-0.840	45.369	ANG	0.338 (H)	7.9 (0.2)	4.3 (2.3)	2.95 (1.73)	0.58 (0.23)	0.6 (0.23)	0.01 (0.31)
Cadore	12.250	46.417	EXC	0.992	8 (0)	3.5 (3)	2.66 (2.23)	0.36 (0.33)	0.44 (0.35)	0.17 (0.47)
Calabria	15.672	38.222	ANG	0.006	7.9 (0.2)	3.1 (1.7)	2.29 (1.32)	0.53 (0.4)	0.45 (0.29)	-0.15 (0.4)
CapdAgde	3.485	43.280	ANG	0.161	7.9 (0.2)	4 (2.2)	3.15 (1.64)	0.7 (0.24)	0.65 (0.19)	-0.1 (0.41)
Cazouls	3.115	43.394	ANG	0.053	7.9 (0.5)	4.1 (2.2)	2.81 (1.59)	0.6 (0.25)	0.58 (0.24)	-0.09 (0.4)
CrniLug	20.183	44.678	ANG	0.057	7.9 (0.2)	4.1 (2.7)	2.97 (2.1)	0.55 (0.3)	0.54 (0.29)	-0.04 (0.34)
Currachase	-8.530	52.360	EXC	0.968	8 (0)	4 (3.4)	2.83 (2.62)	0.39 (0.36)	0.45 (0.33)	0.12 (0.47)
CuxacDAude	2.994	43.241	ANG	0.009	8 (0)	3.6 (2.1)	2.5 (1.23)	0.63 (0.25)	0.56 (0.17)	-0.12 (0.38)
DehesaDeBoh adilla	-3.852	40.440	ANG	0.010	7.9 (0.2)	3.4 (2.2)	2.6 (1.65)	0.71 (0.37)	0.52 (0.25)	-0.37 (0.4)
Donadea	-6.450	53.210	EXC	0.957	8 (0)	4.1 (3.3)	2.97 (2.9)	0.44 (0.31)	0.49 (0.32)	0.06 (0.33)
Dourdan	1.967	48.513	EXC	0.953	6.9 (0.2)	4.1 (3)	2.91 (2.56)	0.44 (0.35)	0.51 (0.32)	0.13 (0.5)
Enniskillen	-7.280	54.140	EXC	0.979	8 (0)	3.6 (2.8)	2.49 (2.29)	0.47 (0.36)	0.44 (0.29)	-0.06 (0.44)
Farchau	10.717	53.733	EXC	0.984	6.9 (0.2)	3.8 (3.4)	2.77 (2.84)	0.44 (0.39)	0.43 (0.33)	0.01 (0.47)
Feuchtwagen	10.333	49.167	EXC	0.990	8 (0)	3.2 (2.3)	2.39 (1.7)	0.51 (0.41)	0.45 (0.31)	-0.16 (0.53)
Gevgelijia	22.491	41.172	ANG	0.127	7.9 (0.2)	3.9 (2.2)	2.94 (1.53)	0.66 (0.32)	0.58 (0.28)	-0.15 (0.26)
Haderslev	9.536	55.290	EXC	0.987	8 (0)	3.3 (2.1)	2.38 (1.49)	0.49 (0.37)	0.47 (0.28)	-0.02 (0.51)
HogeBoss	2.950	50.840	EXC	0.98	8 (0)	3.6 (2.3)	2.48 (1.56)	0.52 (0.35)	0.48 (0.29)	-0.07 (0.39)
Huttenheim	7.533	48.365	EXC	0.977	7.9 (0.2)	3.9 (3.3)	2.83 (2.64)	0.49 (0.35)	0.47 (0.31)	-0.06 (0.42)

Kaisidoris	24.350	54.908	EXC	0.988	7.9 (0.2)	3.4 (2.8)	2.6 (2.15)	0.49 (0.36)	0.46 (0.32)	-0.1 (0.44)
Karlsruhe	8.298	48.984	EXC	0.990	7.9 (0.2)	3.1 (2)	2.3 (1.3)	0.49 (0.38)	0.45 (0.31)	-0.12 (0.44)
LaMolle	6.537	43.237	ANG	0.026	8 (0)	3.4 (1.5)	2.5 (1.23)	0.52 (0.31)	0.54 (0.24)	0.04 (0.42)
LaRomagne	4.286	49.692	EXC	0.983	6.9 (0.2)	3.3 (2.3)	2.33 (1.58)	0.45 (0.36)	0.45 (0.31)	0.01 (0.48)
MasLarrieu	3.033	42.586	ANG	0.017	8 (0)	4 (1.9)	2.66 (1.37)	0.58 (0.31)	0.56 (0.26)	-0.06 (0.35)
Mircze	23.500	50.850	EXC	0.985	7 (0)	3.4 (2.5)	2.67 (1.88)	0.5 (0.38)	0.51 (0.31)	0 (0.52)
Monti-Lessini	11.167	45.667	EXC	0.992	5.8 (0.5)	2.8 (1.9)	2.24 (1.64)	0.39 (0.38)	0.39 (0.35)	-0.03 (0.33)
Negotin	22.597	44.154	ANG	0.024	6 (0)	3.7 (2.3)	2.72 (1.7)	0.61 (0.34)	0.53 (0.3)	-0.17 (0.29)
OcsaHungary	19.231	47.268	ANG	0.005	7.9 (0.2)	2.3 (0.9)	1.92 (0.57)	0.7 (0.42)	0.43 (0.22)	-0.59 (0.51)
Rabstejn	17.250	49.933	EXC	0.993	7.9 (0.5)	3.7 (2.9)	2.5 (2.13)	0.43 (0.34)	0.44 (0.3)	0.04 (0.39)
Ravnholt	10.576	55.256	EXC	0.988	7.9 (0.2)	3.9 (2.6)	2.62 (1.89)	0.44 (0.36)	0.47 (0.33)	0.05 (0.38)
SaintDye	1.564	47.695	HYB	0.032 (A)	8 (0)	4 (2.3)	2.72 (1.88)	0.59 (0.28)	0.55 (0.23)	-0.08 (0.33)
SaintGatien	0.136	49.354	EXC	0.967	8 (0)	3.5 (2.3)	2.38 (1.48)	0.54 (0.36)	0.47 (0.3)	-0.19 (0.4)
SaintPauldeSa lers	2.528	45.119	EXC	0.992	7.9 (0.2)	3.5 (2.8)	2.65 (2.11)	0.52 (0.41)	0.45 (0.33)	-0.16 (0.44)
Settrington	-0.726	54.125	EXC	0.989	7.8 (0.7)	3.8 (3.3)	2.81 (2.52)	0.44 (0.35)	0.44 (0.33)	0.01 (0.42)
SilaGrande	16.333	39.333	EXC	0.980	4 (0)	2.8 (1.4)	2.17 (1.13)	0.58 (0.39)	0.49 (0.27)	-0.18 (0.5)
Szczecinek	16.683	53.700	EXC	0.992	7.9 (0.2)	3.6 (2.5)	2.63 (1.59)	0.55 (0.39)	0.5 (0.3)	-0.05 (0.49)
Tavaux	5.378	47.053	HYB	0.635	13.9 (0.2)	5.7 (4.3)	3.54 (2.82)	0.61 (0.29)	0.6 (0.25)	-0.02 (0.34)
VallePezio	7.667	44.333	EXC	0.984	8 (0)	3.6 (2.9)	2.51 (1.91)	0.37 (0.36)	0.45 (0.32)	0.21 (0.53)
Wloszczowa	19.017	50.850	EXC	0.993	7.9 (0.2)	3.7 (3)	2.55 (2.09)	0.4 (0.36)	0.42 (0.34)	-0.01 (0.42)
WythamWood	-1.330	51.780	EXC	0.909	7.9 (0.2)	3.7 (2.5)	2.51 (1.83)	0.49 (0.32)	0.49 (0.27)	-0.01 (0.32)
Zeimelis	24.033	56.293	EXC	0.975	7.8 (0.4)	4.2 (3)	2.9 (2.41)	0.48 (0.35)	0.49 (0.33)	-0.01 (0.36)
Aiguestortes	1.135	42.595	EXC	0.543 (H)	9 (0)	3.3 (1.8)	2.2 (0.99)	0.67 (0.31)	0.49 (0.21)	-0.36 (0.26)
Albena	28.062	43.358	ANG	0.007	7.8 (0.4)	3.3 (1.9)	2.35 (1.32)	0.56 (0.31)	0.51 (0.24)	-0.13 (0.43)
AlterdoChao	-7.663	39.193	ANG	0.063	6 (0)	3.6 (1.8)	2.65 (1.32)	0.55 (0.25)	0.59 (0.23)	0.06 (0.29)
Cerdanya	1.739	42.366	ANG	0.014	8 (0)	3.7 (2.3)	2.73 (1.79)	0.56 (0.33)	0.52 (0.31)	-0.12 (0.28)
Krim	33.602	44.506	ANG	0.020	7.9 (0.3)	3.5 (1.7)	2.5 (1.25)	0.63 (0.32)	0.53 (0.25)	-0.19 (0.32)
Krka	15.969	43.819	ANG	0.047	8 (0)	3.9 (2.1)	2.58 (1.59)	0.55 (0.31)	0.51 (0.27)	-0.06 (0.3)
Lonjsko_Polje	16.706	45.418	ANG	0.027	8 (0)	3.4 (1.7)	2.18 (0.92)	0.58 (0.33)	0.49 (0.23)	-0.16 (0.37)
Ripoll	2.244	42.182	EXC	0.594 (H)	9 (0)	3.6 (2.1)	2.33 (1.22)	0.61 (0.28)	0.5 (0.22)	-0.23 (0.26)
Saumur	-0.085	47.235	ANG	0.054	8 (0)	3.4 (2.1)	2.51 (1.78)	0.63 (0.32)	0.51 (0.24)	-0.26 (0.38)
St_privé	1.816	47.868	HYB	0.579	7.9 (0.2)	3.7 (2.4)	2.61 (1.47)	0.54 (0.29)	0.54 (0.27)	-0.04 (0.32)
Tanha	-7.675	41.255	ANG	0.009	7.9 (0.2)	3.3 (2)	2.38 (1.49)	0.5 (0.34)	0.48 (0.28)	-0.02 (0.42)
Toulouse	1.226	43.641	ANG	0.253 (H)	8 (0)	3.2 (1.6)	2.22 (1.02)	0.67 (0.33)	0.49 (0.21)	-0.33 (0.44)

**Table S2** Correlations between the ecological variables potentially determining species distribution of *Fraxinus excelsior* and *F. angustifolia* used to run the MAXENT simulations. Correlations greater than 0.75 are marked in bold.

Variables <sup>1</sup>	<i>F. excelsior</i>						<i>F. angustifolia</i>						Frosts-Jan
	elev	TempSD	TempSumm	PrCV	PrSumm	PrWint	elev	TempSD	TempSumm	PrCV	PrSumm	PrWint	
TempSD	-0.16						0.30						
TempSumm	-0.19	0.44					0.38	0.50					
PrCV	0.29	0.31	0.15				0.39	0.29	<b>0.90</b>				
PrSumm	0.13	-0.19	-0.65	-0.04			-0.43	-0.09	<b>-0.86</b>	<b>-0.86</b>			
PrWint	0.12	-0.64	-0.37	-0.28	0.32		0.01	-0.30	0.12	0.33	-0.17		
Frosts-Jan	0.15	<b>0.77</b>	-0.05	0.38	0.22	-0.51	0.22	0.55	-0.35	-0.43	0.59	-0.37	
Dist-rivers							0.46	0.00	0.27	0.37	-0.35	0.21	-0.10

<sup>1</sup> elev, elevation; TempSD, temperature seasonality (standard deviation of the monthly average multiplied by 100); TempSumm, maximum summer temperature; PrCV, precipitation seasonality (coefficient of variation of monthly precipitation); PrSumm, mean summer precipitation; PrWint, mean winter precipitation; Frosts-Jan, number of days of frost in January; and Dist-rivers, distance to main river (*F. angustifolia* only).

**3. RASPRAVA**

Kombinacijom populacijske genetike, analize ekoloških čimbenika i njihovog utjecaja na genetičku varijabilnost te modela ekoloških niša u ovom se radu nastojalo utvrditi osnovne principe i mehanizme mikroevolucije na primjeru česte i široko rasprostranjene vrste drveća, kao što je poljski jasen. Glavni ciljevi rada bili su utvrditi genetičku varijabilnost populacija poljskog jasena na području Hrvatske i Europe, istražiti na koji način ekološki čimbenici, krajobraz i klimatske promjene utječu na unutarvrstu genetičku varijabilnost, te istražiti razinu i mehanizme hibridizacije s običnim jasenom. Rezultati ovog istraživanja potvrdili su da u Hrvatskoj postoje značajne razlike u genetičkoj varijabilnosti između kontinentalnih i mediteranskih populacija poljskog jasena, što upućuje na mogućnost da istraživane populacije predstavljaju dva različita ekotipa. Također je utvrđeno da divergencija okoliša ima važan utjecaj na oblikovanje genetičke strukture populacija, te da je ekološka varijabilnost bolji prediktor genetičke varijabilnosti populacija u odnosu na geografsku udaljenost. Unatoč očekivanom protoku gena na relativno malom području, dugotrajna je stabilnost heterogenog okoliša vjerojatno promicala ekološko i genetičko odvajanje istraživanih populacija u Hrvatskoj.

Suprotno očekivanjima i polaznoj hipotezi, utvrđeno je da najveću genetičku raznolikost u Europi imaju sjeverne i zapadne populacije, te da postoji specifična prostorna raspodjela genetičke raznolikosti unutar areala – genetička raznolikost opada značajno od zapada prema istoku, te od sjevera prema jugu. Nadalje, klimatske promjene mogle bi pogodovati povećanju učestalosti određenih genotipova. Potencijalni budući “makrorefugiji” tijekom predstojećih klimatskih promjena identificirani su u najsjevernijim dijelovima areala. Naime, danas su u tim dijelovima areala rasprostranjene populacije s najvećom zalihom genetičke varijabilnosti za koje modeli ekološke niše predviđaju povoljno stanište unatoč budućim klimatskim promjenama. Dobiveni rezultati ukazuju isto tako da su periferne populacije, koje se nalaze na južnoj granici areala („*rear-edge*“) i koje vjerojatno predstavljaju reliktno populacije, najugroženije budućim promjenama klime, te da im prijete najveći rizik od izumiranja. Iako ove populacije imaju najmanju genetičku raznolikost, njihov gubitak mogao bi negativno utjecati na razinu ukupne genetičke varijabilnosti vrste. Naposljetku, Bayesovskom analizom populacijske strukture i modelima ekoloških niša potvrđeno je da poljski i obični jasen imaju dva jasno odvojena genska skupa, a hibridne zone se formiraju kada dvije roditeljske vrste dolaze u simpatriji. Međutim, čini se da je hibridizacija asimetrična s tendencijom da se hibridne populacije stvaraju u umjerenom pojasu na sjevernoj granici distribucije poljskog

jasena, dok su formiranje i veličina hibridnih zona ograničeni klimatskim čimbenicima kao što su broj hladnih dana u siječnju i ljetne temperature i padaline.

### 3.1. Genetička raznolikost i struktura populacija poljskog jasena u Hrvatskoj

Populacije poljskog jasena u Hrvatskoj geografski su strukturirane. Naime, utvrđena je visoka genetička raznolikost i niska diferencijacija populacija u kontinentalnoj regiji u odnosu na signifikantno nižu genetičku raznolikost i povećanu divergenciju populacija u mediteranskoj regiji. Genetička udaljenost između populacija bila je najjače korelirana s ekološkom udaljenošću, unatoč korekciji na geografsku udaljenost, što potvrđuje „*isolation by environmental distance*“ uzorak. Modeli ekološke niše i multivarijatna analiza ekoloških čimbenika pokazali su da postoje jasne razlike u okolišu dvije regije i između populacija koje se u njima nalaze, što upućuje na moguću regionalnu divergenciju ekološke niše kontinentalnih i mediteranskih populacija poljskog jasena. Ekološku divergenciju ove dvije skupine populacija odražavala je i njihova genetička struktura, što je vidljivo i iz sličnosti nezakorijenjenih srodstvenih stabala konstruiranih temeljem matrice genetičke i ekološke udaljenosti (Znanstveni rad br. 1). Stoga je moguće da hrvatske populacije predstavljaju dvije zasebne evolucijske linije međusobno odvojene nepovoljnim staništem (Raxworthy i sur. 2007; Wiens i Graham 2005). Svi ovi rezultati ukazuju na vrlo važnu ulogu divergencije okoliša na oblikovanje genetičke varijabilnosti vrsta (Pilot i sur. 2006; Parisod i Christin 2008; Sork i sur. 2010; Freedman i sur. 2010).

Utvrđeni obrazac genetičke strukture može biti posljedica nejednoliko raspoređenog povoljnog staništa i razlika u efektivnoj veličini populacija u dvije biogeografske regije. Kontinentalne populacije su veće, rasprostranjene duž velikih nizinskih rijeka (Sava, Drava, Dunav) gdje je povoljno stanište kontinuirano, homogeno i gotovo da nema fizičkih barijera za protok gena između geografski udaljenih populacija; stoga ne čudi što dobiveni rezultati ne ukazuju na značajnu genetičku diferenciranost. S druge strane, mediteranske populacije oskudijevaju povoljnim staništem koje je fragmentirano i reducirano duž kratkih krških rijeka, nižih krških polja i rijetkih močvara uz jadransku obalu, što ograničava njihovu disperziju. Tako su mediteranske populacije manje i međusobno izolirane nepovoljnim staništem, a kao posljedica javlja se smanjen protok gena i povećana genetička diferencijacija signifikantna i na vrlo malim udaljenostima (npr. 40 km između populacija uz rijeke Zrmanju i Krku). Također, heterogenost staništa koja je izražena u mediteranskoj biogeografskoj regiji može

predstavljati barijeru za protok gena. Primjerice, različita mikroklima može dovesti do razlika u fenologiji populacija koje rastu u različitom okolišu, a time do vremenske izolacije u protoku gena (Morand i sur. 2002; Parisod i Christin 2008). Kod anemofilnih vrsta drveća očekivali bi da protok gena može homogenizirati genetičku strukturu populacija na malim udaljenostima, međutim divergencija staništa može uzrokovati ekološku izolaciju i genetičku diferencijaciju geografski bliskih populacija (Bockelmann i sur. 2003).

Alternativno, smanjena genetička raznolikost i povećana divergencija mediteranskih populacija, te veća genetička raznolikost kontinentalnih populacija može biti posljedica historijskih migracijskih procesa na ovim područjima. Naime, poznato je da je Balkanski poluotok služio kao jedan od najvažnijih refugija za europsku floru u vrijeme ledenih doba, pa tako vjerojatno i za jasen, te da se Hrvatska nalazi na raskrižju nekoliko postglacijalnih rekolonizacijskih putova (Petit i sur. 2002; Magri i sur. 2006; Heuertz i sur. 2006). Moguće je stoga da južne mediteranske populacije (Krka, Zrmanja, Neretva) predstavljaju stare, reliktno populacije koje su „zapele“ na ovim prostorima još od zadnjeg ledenog doba kada je sjeverni Jadran presušio, a dalmatinska obala je mogla pružiti povoljno stanište i dovoljno vlage za preživljavanje higrofilnih vrsta kao što je poljski jasen (Hampe i sur. 2003; Tzedakis 2004; Petit i sur. 2005). Kontinentalne i istarske populacije vjerojatno su podrijetlom iz refugija koji su se nalazili u sjevernoj Italiji, uz obale Crnog mora ili negdje na području Dinarida (Heuertz i sur. 2006), a spajanje i miješanje rekolonizacijskih linija iz različitih refugija može objasniti veću razinu genetičke varijabilnosti sjevernijih populacija (usp. Sliku 1; Petit i sur. 2003).

### **3.2. Prostorna raspodjela genetičke varijabilnosti poljskog jasena u Europi**

Prostorna raspodjela genetičke varijabilnosti europskih populacija djelomično je slijedila uzorak koji je utvrđen u Hrvatskoj. Naime, u pravilu su najmanju genetičku raznolikost imale najjužnije mediteranske populacije, dok je najveća genetička raznolikost zabilježena kod kontinentalnih i najsjevernijih populacija na srednjim geografskim širinama (Znanstveni rad br. 2). Ovakva raspodjela genetičke raznolikosti u Europi suprotna je očekivanoj tzv. „*leading-edge expansion*“ hipotezi koja previđa da upravo južne populacije refugijalnog tipa imaju najveću raznolikost (Hewitt 2000). Umjesto toga, možemo pretpostaviti da distribucija genetičke raznolikosti poljskog jasena najbolje prati centralno-marginalni model prema kojem možemo očekivati da su periferne populacije manje, izoliranije s manjom genetičkom raznolikošću, te povećanom divergencijom (Eckert i sur. 2008). Ovakav rezultat sugerira da

južne populacije uz obale Mediterana žive blizu margine ekološke niše, odnosno u suboptimalnim okolišnim uvjetima, na što upućuje i izrađeni model povoljnosti staništa. Međutim, kao i kod hrvatskih populacija, ne možemo isključiti mogućnost da je opažena distribucija genetičke varijabilnosti rezultat sekundarnog kontakta divergentnih linija podrijetlom iz različitih refugija, što je moglo dovesti do značajno povećane genetičke raznolikosti populacija na sjevernoj granici areala (Petit et al. 2003). Također, tijekom toplijih interglacijala, a posebno tijekom srednjeg holocena, bilo je omogućeno intenzivno miješanje i protok gena na višim geografskim širinama zbog širenja listopadnih šuma umjerenog pojasa prema sjeveru (Benito Garzón i sur. 2007).

Osim toga, na kontinentalnoj razini zabilježen je i specifičan gradijent genetičke raznolikosti koja je opadala od zapada prema istoku (Znanstveni rad br. 2). Pritom su se posebno isticale populacije uz istočnu obalu Jadrana koje su imale najnižu genetičku raznolikost u Europi. Ovakav prostorni uzorak genetičke raznolikosti je iznenađujući i također nije bio u skladu s očekivanjima jer je kod većine mediteranskih drvenastih vrsta utvrđen upravo suprotan gradijent genetičke raznolikosti, tj. opadanje od istoka prema zapadu (Fady i Conord 2010). Osim toga, istočni Jadran i područje Dinarida istaknuti su više puta kao važna refugijalna područja za europsku floru i faunu (Tzedakis 2004; Heuertz et al. 2006; Médail i Diadema 2009; Surina i sur. 2011), a time i kao područja visoke genetičke raznolikosti. Utvrđeni gradijent raznolikosti kod poljskog jasena može biti posljedica specifičnih zahtjeva ove vrste spram staništa (Conord i sur. 2012), razlika u mikroklimi unutar mediteranske regije ili prostorne raspodjele povoljnog staništa, kao i u slučaju Hrvatske. Sve istraživane obalne populacije nalaze se južnije od 45° sjeverne geografske širine gdje je klima za vrijeme posljednjeg ledenog doba bila povoljna za opstanak termofilnih drvenastih vrsta (Petit i sur. 2005). Stoga ako su sva tri mediteranska poluotoka služila kao pribježište poljskog jasena, migracije i promjene veličine populacija u prošlosti ostavile bi jednaki trag na neutralnu genetičku varijabilnost svih populacija, što nije slučaj poljskog jasena. Nedavno je dokazano da sporije kontrakcije areala dovode do većeg gubitka genetičke raznolikosti u odnosu na brže kontrakcije (Arenas i sur. 2012). Također je utvrđeno da je srednja temperatura ljeti na Mediteranu za vrijeme posljednjeg ledenog doba bila viša na istoku, te je postepeno opadala prema zapadu (Fady i Conord 2010). Tako je moguće pretpostaviti da su se istočnojadranske populacije sporije povlačile u mikrorefugije s povoljnim staništem, što je moglo rezultirati njihovom smanjenom genetičkom raznolikošću. Naposljetku, jednom kada su se povukle u mikrorefugije koji su se potencijalno nalazili u dubokim i vlažnim dolinama krških rijeka uz

jadransku obalu (Médail i Diadema 2009), njihova ekspanzija po završetku oledbe i recentni protok gena vjerojatno su bili ograničeni zbog nedovoljno povoljnog staništa. Kako je već spomenuto, povoljno stanište za poljski jasen uz istočnu jadransku obalu oskudnije je u usporedbi sa zapadnim Mediteranom zbog krške podloge koja ograničava distribuciju populacija uz kratke riječne kanjone i rijetka močvarna područja (Znanstveni rad br. 1). Tako je još potvrđena mogućnost da populacije uz istočnu obalu Jadrana predstavljaju reliktnu populaciju koje su preživjele *in situ* od zadnjeg ledenog doba do danas u relativno stabilnoj klimi istočnog Mediterana (Petit i sur. 2005). Tome u prilog ide i činjenica da razina genetičke raznolikosti opada proporcionalno s vremenom provedenim u refugijima.

### 3.2.1. Genetička struktura populacija poljskog jasena u Europi

Genetička struktura europskih populacija poljskog jasena provedena je temeljem Bayesovske analize pomoću programa POPS. Utvrđeno je da se populacije u Europi geografski grupiraju u pet genskih skupova. U grubo to su: zapadni Mediteran (Portugal, Španjolska, Francuska), južna Italija (Sardinija i Kalabrija), zapadni Balkan, obala Crnog mora, a peti genski skup činile su isključivo jedinke uzorkovane iz hrvatske populacije na području Čakovca (Znanstveni rad br. 2). Ovakva genetička struktura populacija se približno podudara s filogeografskom strukturom poljskog jasena dobivenom temeljem kloroplastnih regija koja je potvrdila genetičku divergenciju između populacija podrijetlom iz različitih refugija (smještenih na području Iberijskog poluotoka, sjevernog dijela Apeninskog poluotoka, na Balkanskom poluotoku uz obale Crnog mora, te potencijalno na području Dinarida) (Huntley i Birks 1983; Heuertz i sur. 2006). Populacije iz središnje Italije dijele veći dio genskog skupa s istočnojadranskim populacijama, što ukazuje na određenu razinu protoka gena između istočne i zapadne obale Jadrana. Međutim, teško je za razlučiti predstavlja li ovakva strukturiranost populacija rezultat recentnog ili povijesnog protoka gena koji je bio olakšan za vrijeme posljednjeg ledenog doba kada je sjeverni dio jadranskog bazena presušio i postojala je kopnena veza između njegove istočne i zapadne obale.

Nadalje, ovakva genetička strukturiranost populacija ne odgovara granicama rasprostranjenosti podvrsta poljskog jasena (*Fraxinus angustifolia* ssp. *angustifolia* i ssp. *pannonica*) predloženima od strane Fukarek-a (1983). Zaseban genski skup zapadnog mediterana mogao bi predstavljati tipičnu podvrstu ssp. *angustifolia*, međutim populacije istočne obale Jadrana očito ne pripadaju toj podvrsti, kao što je smatrao Fukarek (1954).

Umjesto toga, Bayesovska analiza populacijske strukture ukazuje da sve populacije Balkanskog poluotoka, izuzev onih uz crnomorsku obalu, čine relativno homogeni genetički skup, iako je na području Hrvatske utvrđena značajna genetička struktura između kontinentalnih i obalnih populacija (Znanstveni rad br. 1). Prema dobivenim rezultatima temeljem jezgrinih mikrosatelitnih biljega (SSR) teško je nagađati o jasnim taksonomskim i srodstvenim odnosima unutar kompleksa *Fraxinus angustifolia* i izvoditi bilo kakve zaključke. Međutim, ovi rezultati idu najviše u prilog podjeli poljskog jasena u Europi na barem dvije podvrste – ssp. *angustifolia* (zapadni Mediteran do Jadrana) i ssp. *oxycarpa* (istočnije od Jadrana). Iako ni najnoviji rezultati molekularne filogenije roda *Fraxinus* temeljem univerzalnih DNA barkoding regija nisu dali jasan odgovor na pitanje taksonomske podjele poljskog i običnog jasena (Arca i sur. 2012), svakako bi za rješavanje taksonomske problematike poljskog jasena trebalo posegnuti za ITS/ETS sekvencama. Potrebno je još spomenuti da se vrsta *F. angustifolia* križa sa srodnom vrstom *F. excelsior*, da obje vrste imaju identičnu organizaciju rDNA i stvaraju plodne križance (Siljak-Yakovlev i sur., u tisku). To svakako dodatno komplicira traženje odgovora na ovo još uvijek otvoreno pitanje.

### **3.3. Potencijalni utjecaj klimatskih promjena na genetičku varijabilnost poljskog jasena**

Iako su moguće posljedice klimatskih promjena na genetičku strukturu populacija prepoznate kao važno i otvoreno pitanje u znanosti, dosad je vrlo mali broj znanstvenih istraživanja pokušao istražiti ovaj fenomen (Collevatti i sur. 2011; Jay i sur. 2012; Rubidge i sur. 2012). Jedan od ciljeva ove disertacije bio je izraditi prediktivne modele sadašnje i buduće rasprostranjenosti poljskog jasena uslijed klimatskih promjena, identificirati potencijalne „suvremene“ refugije za ovu vrstu u kojima će populacije moći opstati u budućnosti, te procijeniti utjecaj klimatskih promjena na genetičku varijabilnost vrste. Rezultati multidisciplinarnih analiza sugeriraju gubitak oko 30 % trenutno povoljnog staništa u južnim, obalnim dijelovima areala, ali komparativno mali gubitak broja alela do kraja stoljeća. Štoviše, preostalih 70 % povoljnog staništa može potencijalno održati 90 % od ukupnog broja zabilježenih alela (Znanstveni rad br. 2). Kao što je i predviđeno hipotezom, klimatske promjene mogle bi dovesti do pomaka distribucije poljskog jasena prema većim geografskim širinama u budućnosti, što može negativno utjecati na ukupnu razinu genetičke varijabilnosti ove vrste. Naime, dio alela jedinstvenih za južne, obalne populacije biti će izgubljen ukoliko one izumru. Sudeći po rezultatima ovog rada, od gubitka povoljnog staništa uslijed klimatskih

promjena najugroženije su južne populacije koje imaju najnižu genetičku varijabilnost, a posebice one u obalnim zonama Mediterana. Potencijalni gubitak 40% istraživanih populacija značio bi gubitak samo 8% ukupne genetičke varijabilnosti, ali čak 40% jedinstvenih alela koje smo zabilježili isključivo u ovim populacijama, što bi u konačnici moglo smanjiti adaptivni potencijal vrste.

Treba međutim imati na umu da svi dobiveni rezultati i stvaran odgovor vrste na promjene klime velikim dijelom ovise o mogućnosti disperzije vrste. Temeljem molekularnih analiza utvrđeno je da je brzina postglacijalne rekolonizacije drvenastih vrsta mnogo manja nego što se to prije pretpostavljalo, te da iznosi svega oko 100 m godišnje (McLachlan i sur. 2005). Ova brzina migracije nedovoljna je da drveće prati predviđene klimatske promjene (Aitken i sur. 2008). Prema najgorem mogućem scenariju za poljski jasen koji podrazumijeva nemogućnost migracije (*“no-migration”*; Thuiller i sur. 2005), vrsta će opstati samo u područjima stabilne klime gdje se sadašnji i budući modeli povoljnosti staništa preklapaju. Takva područja možemo smatrati najrealnijim *in situ* „makrorefugijima“ tijekom klimatskih promjena (Ashcroft 2010). U slučaju poljskog jasena, dobiveni rezultati u ovom radu upućuju da se takvi suvremeni refugiji potencijalno nalaze u sjevernijim dijelovima trenutne distribucije gdje će stanište u budućnosti ostati povoljno i gdje se ujedno nalaze populacije s najvišom genetičkom varijabilnošću (Znanstveni rad br. 2). Međutim, ne možemo isključiti mogućnost postojanja „mikrorefugija“ (Rull 2009) u područjima smanjene povoljnosti staništa, odnosno manjih lokaliteta sa specifičnom mikroklimom koja može održavati populacije nakon što regionalna makroklima više nije povoljna. To može biti slučaj i kod poljskog jasena jer higrofilne vrste često mogu preživjeti na vlažnim mikrolokalitetima poput obalnih zona, riječnih dolina i kanjona koji su otporniji na zatopljenje klime (Ashcroft i sur. 2009). Treba zato uzeti u obzir da je gubitak povoljnog staništa i broja alela možda precijenjen.

#### **3.4. Hibridizacija poljskog (*Fraxinus angustifolia* Vahl) i običnog jasena (*Fraxinus excelsior* L.) u Europi**

Bayesovska analiza populacijske strukture temeljem šest mikrosatelitnih biljega (*Simple Sequence Repeats*; SSR) i 13 novih nedavno razvijenih EST-SSR biljega (*Expressed Sequence Tags*; Aggarwal i sur. 2011) otkrila je dva jasno odvojena genska skupa koja pripadaju poljskom i običnom jasenu, te cijeli niz hibridnih populacija s jedinkama koje

posjeduju različite udjele genskih skupova roditeljskih vrsta (Znanstveni rad br. 3). Rezultati provedenih istraživanja potvrdili su da je stvaranje hibridnih populacija između poljskog i običnog jasena u Europi omogućeno na područjima gdje se modeli ekološke niše ove dvije srodne vrste preklapaju (Znanstveni rad br. 3). To su uglavnom područja s blagim zimama gdje je mraz rijedak, a padaline su ljeti obilnije. Međutim, nije u svim područjima simpatrije potvrđena introgresija gena i križanje između dvije vrste jasena. Hibridne populacije nalaze se uglavnom na zapadnom dijelu areala, u području umjerene klime blizu sjeverne granice rasprostranjenosti poljskog jasena u Europi. Utvrđeno je da su broj dana s mrazom u siječnju, te temperature i padaline ljeti glavni okolišni čimbenici koji ograničavaju nastanak hibridnih zona. Ove klimatske varijable najviše su doprinijele modelima ekološke niše i najbolje su objasnile raspodjelu frekvencija alela pod potencijalnim utjecajem selekcije (Znanstveni rad br. 3). Do nagle promjene frekvencije gena došlo je upravo u predviđenim područjima simpatrije, odnosno hibridizacije. Od ukupno 13 EST-SSR lokusa, analizom je za njih osam utvrđeno da se nalaze pod utjecajem dva različita tipa prirodne selekcije – šest pod utjecajem stabilizirajuće selekcije („*stabilizing selection*“) i dva pod utjecajem usmjerene selekcije („*directional selection*“) (Znanstveni rad br. 3).

Dvije roditeljske vrste bile su jasno povezane s različitim okolišnim uvjetima. U skladu s tim gradijent frekvencije alela dva EST-SSR lokusa, pod potencijalnim utjecajem usmjerene prirodne selekcije, jasno je pratio promjene u okolišu (Znanstveni rad br. 3). Ovi rezultati sugeriraju da je egzogeni selekcijski pritisak odgovoran za oblikovanje hibridne zone poljskog i običnog jasena. Nadalje, prisutnost genetički „čistih“ populacija običnog jasena u modeliranim područjima potencijalne hibridizacije u Francuskoj ide u prilog tzv. „*bounded hybrid superiority*“ modelu, barem na lokalnoj razini. Kao što je objašnjeno u uvodu ove disertacije, ako područja hibridne zone predviđena modelom ekološke niše približno odgovaraju stvarnoj opaženoj distribuciji hibridnih populacija i ako predviđena distribucija barem jedne roditeljske vrste zalazi u područje opažene hibridne zone, tada možemo zaključiti da veći fitness križanaca unutar hibridne zone sprječava širenje roditeljskih vrsta izvan njihovih trenutnih granica distribucije (Swenson 2008).

Međutim, ne znači nužno da distribucija svih hibridnih populacija odgovara ovom modelu jer kao i kod svakog modela koji pojednostavljuje stvarnost treba biti kritičan.

Kao prvo, moguće je da je potencijalna rasprostranjenost poljskog jasena predviđena modelom ekološke niše šira od stvarne rasprostranjenosti, posebice prema sjeveru, zbog

ulaznih podataka o prisutnosti poljskog jasena korištenih za izgradnju modela. Naime, postoji opravdana sumnja da su križanci poljskog i običnog jasena u različitim bazama podataka i herbarskim zbirkama iz kojih su preuzeti podaci krivo određeni kao čisti poljski jasen jer mu morfološki više sličje.

Drugo, utvrđena je prisutnost čistih populacija poljskog jasena prema višim geografskim širinama, pa je za pretpostaviti da križanci nemaju značajnu prednost u odnosu na roditeljsku vrstu jer je očito poljski jasen uspio kolonizirati najsjevernije dijelove svog areala bez pomoći križanja s običnim jasenom, koristeći vjerojatno povoljne mikroklimatske uvjete uz široke riječne doline koje zalaze duboko u kontinent (poput rijeke Loire u Francuskoj). Međutim nije isključeno da proces hibridizacije ubrzava introgresiju i širenje genskog skupa poljskog jasena prema sjeveru.

Treće, modeli ekološke niše predviđjeli su veća područja simpatrije i u jugoistočnoj Europi, točnije oko Panonske nizine i oko obale Crnog mora. Međutim, na ovom dijelu areala nije zabilježeno genetičko križanje poljskog i običnog jasena. Podunavlje, Posavina i Panonska nizina primarno su stanište poljskog jasena koji dominira nizinskim poplavnim šumama u ovom dijelu Europe. U srednjoj i sjevernoj Europi situacija je suprotna i obični jasen postepeno zamjenjuje poljski jasen kao dominantnu vrstu. Obični jasen vrlo je rijedak u nizinskim poplavnim šumama poljskog jasena i hrasta lužnjaka, jer u pravilu ne podnosi duže plavljenje tla i močvarna staništa. Pretpostavljamo da su ove dvije vrste na području jugoistočne Europe ekološki jasno odjeljenje i rijetko dolaze zajedno u istim šumskim zajednicama, što otežava njihovu potencijalnu hibridizaciju. Osim toga, izraženija hladnija klima s učestalijim mrazom u kontinentalnoj biogeografskoj regiji vjerojatno održava vremensku izolaciju u razdoblju cvjetanja dvije vrste, za razliku od blaže klime u atlantskoj biogeografskoj regiji koja povremeno omogućava preklapanje fenologije cvjetanja i olakšava hibridizaciju. Ukoliko bi tzv. „*bounded hybrid superiority*“ model vrijedio na području cijelog europskog areala, očekivali bi da će primijenjeni molekularni markeri otkriti križanje između poljskog i običnog jasena u predviđenim zonama simpatrije na području jugoistočne Europe, što nije bio slučaj. Može se zaključiti kako se na kontinentalnoj razini ne može utvrditi jedinstveni obrazac hibridizacije između ove dvije srodne vrste, da su ekološki uvjeti na istoku areala manje pogodni za stvaranje križanaca, te da je hibridizacija očito asimetrična. Bez obzira na to koja vrsta je dominantna, čini se da uvijek polen poljskog jasena oprašuje cvjetove običnog jasena (Gerard i sur. 2006b).

Na osnovi ovih rezultata može se očekivati da će toplije zime i sve rjeđi mraz smanjiti reproduktivnu izolaciju poljskog i običnog jasena, pa bi globalno zatopljenje moglo

pogodovati širenju hibridnih zona prema višim geografskim širinama. Primjerice, dugoročno praćenje poljskog jasena u Španjolskoj tijekom posljednjih 50 godina pokazalo je da ova vrsta danas cvjeta 37 dana kasnije, u odnosu na početak praćenja, uglavnom zbog viših temperatura tijekom studenog i prosinca (Peñuelas i sur. 2002). Također, Fernandez-Manjarrés i sur. (2006) utvrdili su već ranije da udio genskog skupa poljskog jasena negativno korelira s temperaturama zimi, pa tako vrlo hladne zime ograničavaju njegovu distribuciju. Osim toga, temperature zimi određuju vrijeme cvjetanja poljskog jasena jer početak cvjetanja inducira određen broj hladnih dana (akumulacija broja hladnih jedinica; Jato i sur. 2004). Zato hladniji dani ranije u godini mogu potaknuti cvjetanje već početkom zime. Isto tako, Jato i sur. (2004) opazili su da cvjetanje poljskog jasena može biti i odgođeno ako primjerice temperatura u prosincu padne ispod 0 °C, pa je tada posebno osjetljiv na kasni mraz. U svakom slučaju, klimatske promjene mogu značajno utjecati na fenologiju poljskog jasena, a zatopljenje će potencijalno omogućiti njegovo širenje prema sjeveru, moguće i izvan granica recentnog areala.

### **3.5. Smjernice za zaštitu i upravljanje**

Poznavanje genetičke varijabilnosti poljskog jasena izravno može doprinijeti razvoju učinkovitijih planova zaštite i gospodarenja ovom ekonomski važnom vrstom. Ovo istraživanje je od posebne važnosti za upravljanje i očuvanje genetičkih resursa uslijed klimatskih promjena i po prvi put su za neku vrstu identificirani potencijalni budući „refugiji tijekom klimatskih promjena“. Iz rezultata je jasno vidljivo da najvišu razinu genetičke varijabilnosti imaju najsjevernije populacije koje se većinom nalaze u područjima stabilne klime, što će omogućiti njihovo dugotrajno preživljavanje i time predstavljaju najvažniji rezervoar za očuvanje genofonda poljskog jasena. Osim toga, dugoročno gledano, najsjevernije populacije su potencijalni izvor jedinki za kolonizaciju sjevernijih područja izvan trenutnih granica areala (Pfeifer i sur. 2010), kao i za potencijalne translokacije. Za južne, obalne populacije situacija je upravo suprotna: one su najugroženije zbog već postojeće fragmentacije staništa, izraženog antropogenog utjecaja u obalnim zonama Mediterana, te naposljetku zbog predviđenog smanjenja ili gubitka povoljnog staništa uslijed izraženih klimatskih promjena na Mediteranu (Giorgi i Lionello 2008). Time ove populacije imaju najveći rizik od izumiranja i ujedno imaju najmanju genetičku raznolikost. Iako se iz perspektive očuvanja genetičke raznolikosti čini da nisu prioritetne za zaštitu, treba naglasiti da konzervacijska vrijednost populacija nije nužno proporcionalna s razinom genetičke

raznolikosti. Više puta je istaknuto kako periferne populacije često imaju karakter reliktnih populacija i genetički su jedinstvene, te značajno doprinose adaptivnom potencijalu vrste. Stoga zaslužuju podjednaku pažnju i zaštitu kao i centralne populacije.

Iz navedenog može se lako zaključiti kako se konzervacijske strategije i planovi gospodarenja vrstama mogu razlikovati na suprotnim granicama areala i u slučaju poljskog jasena potrebno ih je prilagoditi novostečenim spoznajama. Primjerice, trenutna je praksa u gospodarenju gospodarski važnim vrstama šumskog drveća da se za obnovu šuma koristi sjeme lokalnih populacija, što je u pravilu opravdano jer su populacije često genetički adaptirane na lokalne uvjete okoliša. Međutim, rezultati ovog istraživanja jasno ukazuju da u Hrvatskoj nema značajnih razlika u genetičkim karakteristikama između populacija poljskog jasena unutar kontinentalne regije kojima se gospodari. Iz toga proizlazi da nema potrebe za ograničavanjem korištenja reprodukcijiskog materijala unutar jedne sjemenske zone, kao što je propisano važećim Pravilnikom o provenijencijama svojti šumskog drveća (NN 147/11). Izuzetak predstavlja populacija Čakovec za koju je utvrđeno da je genetički značajno diferencirana od većine kontinentalnih populacija, da ima najveću genetičku raznolikost od svih istraživanih populacija u Europi, te da jedinke ove populacije čine jedinstveni, zasebni genski skup.

Nadalje, postoje značajne razlike u genetičkoj varijabilnosti kontinentalnih i mediteranskih populacija, a genetička varijabilnost prati varijabilnost u okolišu. Stoga, iako imaju značajno manju genetičku raznolikost, postoji realna mogućnost da su obalne mediteranske populacije bolje prilagođene na toplije i suše okolišne uvjete, te da žive u većim uvjetima stresa (Aitken i sur. 2008). Iz ovih rezultata proizlazi mogućnost da upravo takve populacije budu uključene u buduće planove gospodarenja i prometovanja šumskim reprodukcijiskim materijalom jer mogu potencijalno obogatiti genetičku raznolikost kontinentalnih populacija, povećati njihovu adaptabilnost i ublažiti utjecaj klimatskih promjena. Na osnovi vrlo visoke korelacije (0,97) između klime i genetičke strukture populacija (udjela pripadnosti pojedine jedinke pojedinom genskom skupu), predviđeno je da će buduća klima dugoročno pogodovati širenju crnomorskog genskog skupa prema zapadu (Znanstveni rad br. 2). To može dodatno amortizirati posljedice zatopljenja, ukoliko su zaista populacije s obale Crnog mora bolje adaptirane na buduće klimatske uvjete. Međutim, i ovi rezultati se trebaju pažljivo interpretirati, uzimajući u obzir stvarnu mogućnost migracije vrste. Za slabo mobilne drvenaste vrste kao što je poljski jasen, POPS modele buduće genetičke strukture treba interpretirati kao gornju granicu dugoročne migracije gena (Jay i sur. 2012). Ostaje međutim

otvoreno kontroverzno pitanje treba li napustiti dosadašnju praksu gospodarenja šumama i dopustiti antropogene translokacije i potpomognutu migraciju slabo mobilnih vrsta drveća, kako bi ovakvim mjerama barem djelomično kompenzirali predviđene negativne utjecaje klimatskih promjena.

### 3.6. Smjernice za buduća istraživanja

Što se tiče utjecaja klimatskih promjena na genetičku varijabilnost, buduća istraživanja trebalo bi usmjeriti na razvijanje što realističnijih modela koji bi trebali uključivati procjenu dinamike migracije poljskog jasena i adaptivnu genetičku varijabilnost. To uključuje istraživanja stvarne sposobnosti disperzije vrste, te neke novije molekularne metode poput genomike i razvijanja biljega za testiranje varijabilnosti gena pod utjecajem selekcije koji kodiraju svojstva od interesa (primjerice otpornost na sušu). Tako bi primjerice bilo poželjno varijabilnost lokusa koji je odgovoran za određeno kvantitativno svojstvo (QTL – „*quantitative trait loci*“) direktno povezati sa fenotipskom varijabilnošću željenog svojstva kod populacija ili jedinki („*genetic association analysis*“). Genetičku varijabilnost QTL potrebno je testirati npr. pomoću „*single nucleotide polymorphism*“ ili AFLP biljega.

Kao što je već spomenuto, za rješavanje taksonomske problematike poljskog jasena potrebno je analizirati različite jedinke poljskog jasena sa čitavog areala vrste, kao i jedinke srodnih vrsta pomoću ITS ili ETS sekvenci, te konstruirati detaljno filogenetsko stablo. Za bolji uvid u filogeografsku povijest poljskog jasena, kao i za identifikaciju glacijalnih refugija na području Balkanskog poluotoka i Dinarida potrebno je analizirati kloroplastne haplotipove populacija na ovom dijelu areala koji nije bio dovoljno zastupljen u dosadašnjim istraživanjima (Heuertz i sur. 2006). Dodatno, pomoću modela ekološke niše može se djelomično rekonstruirati rasprostranjenost poljskog jasena za vrijeme posljednjeg ledenog doba („*paleodistribution models*“). Također, upravo je završena analiza veličine i organizacije genoma (heterokromatina i rDNA) sve tri europske vrste jasena (Siljak-Yakovlev i sur., u tisku).

Što se tiče gospodarenja, potrebno je temeljem rezultata ove disertacije napisati nove smjernice za korištenje reprodukcijskog materijala i očuvanje genetičkih resursa poljskog jasena u Hrvatskoj. Naposljetku, bilo bi zanimljivo razjasniti genetičku jedinstvenost populacije Čakovec koja se nalazi blizu sjeverne granice areala poljskog jasena.

## **4. ZAKLJUČCI**

1. Ekološki čimbenici u velikoj mjeri utječu na oblikovanje genetičke varijabilnosti. Genetička varijabilnost prati varijabilnost u okolišu, a heterogeni okoliš promiče ekološku i genetičku divergenciju populacija.
2. Ekološka izolacija i raspodjela povoljnosti staništa imaju potencijalno važniju ulogu od geografske izolacije i povijesnih migracijskih procesa u formiranju recentne genetičke strukture populacija.
3. Kontinentalne i mediteranske populacije u Hrvatskoj genetički su i ekološki strukturirane na sličan način, te potencijalno predstavljaju dva ekotipa. Međutim genetički nisu strogo odvojene, već u gradijentu koji je vjerojatno rezultat heterogenosti okoliša na istraživanom području.
4. Najveću genetičku raznolikost u Europi imaju najsjevernije populacije koje se nalaze bliže središtu svog areala, a najmanju južne refugijalne populacije. Također, genetička raznolikost značajno opada od zapada prema istoku areala.
5. Klimatske promjene potencijalno će omogućiti širenje poljskog jasena i hibridnih zona prema većim geografskim širinama i pogodovat će migraciji određenih genotipova, potencijalno adaptiranih na buduće uvjete klime.
6. Od gubitka povoljnog staništa najugroženije su periferne populacije u obalnim zonama Mediterana koje imaju najmanju genetičku raznolikost, ali čije izumiranje može negativno utjecati na ukupnu razinu genetičke varijabilnosti, a time i na adaptivni potencijal vrste.
7. Potencijalni budući *in situ* refugiji tijekom klimatskih promjena nalaze se u sjevernim dijelovima recentne rasprostranjenosti vrste gdje se nalaze populacije s najvišom razinom genetičke raznolikosti i gdje će stanište u budućnosti ostati povoljno.
8. Hibridizacija između poljskog i običnog jasena je asimetrična s većom tendencijom stvaranja križanaca u zapadnim i sjevernijim dijelovima areala poljskog jasena, a klima utječe na razinu i smjer hibridizacije.
9. Stvaranje hibridnih populacija između dvije srodne vrste jasena omogućeno je u područjima simpatrije gdje im se ekološke niše preklapaju, a mraz i ljetne temperature su glavni klimatski čimbenici koji ograničavaju hibridne zone.

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**POPIS PUBLIKACIJA**

## a) Izvorni znanstveni i pregledni radovi u CC časopisima

1. **Temunović M**, Frascaria-Lacoste N, Franjić J, Satovic Z, Fernández-Manjarrés JF (2013) Identifying refugia from climate change using coupled ecological and genetic data in a transitional Mediterranean-temperate tree species. *Molecular Ecology*, 22 (8), 2128-2142.
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3. Siljak-Yakovlev S, **Temunović M**, Robin O, Raquin C, Frascaria-Lacoste N (2013) Molecular-cytogenetic studies of ribosomal RNA genes and heterochromatin in three European *Fraxinus* species. *Tree genetics & genomes* (u tisku).

## b) Znanstveni radovi u drugim časopisima

1. **Temunović M**, Franjić J, Satovic Z, Grgurev M, Frascaria-Lacoste N, Fernández-Manjarrés JF (2012) Environmental heterogeneity explains the genetic structure of Continental and Mediterranean populations of *Fraxinus angustifolia* Vahl. *PLoS ONE*, 7 (8), e42764.
2. Škvorc Ž, Sever K, Bogdan S, Krstonošić D, Alešković I, **Temunović M**, Dobraš J, Franjić J (2008) Varijabilnost fiziološko-morfoloških svojstava hrasta lužnjaka (*Quercus robur* L.) u klonskom testu – prvi rezultati. *Radovi - Šumarski institut Jastrebarsko*. 43 (2), 79-92.
3. **Temunović M**, Šerić Jelaska L, Durbešić P (2008) Diversity of water beetles (Hydradephaga, Coleoptera) in temporary ponds of Nature park "Lonjsko polje", Croatia. *Entomologia Croatica*. 8 (1), 1-15.

## c) Drugi radovi u zbornicima skupova s recenzijom

1. Šerić Jelaska L, **Temunović M**, Durbešić P (2008) Popis vodenih kornjaša podreda Adephaga iz zbirke Entomološkog odjela Gradskog muzeja Varaždin. U: Zbornik radova sa znanstvenog skupa „Franjo Košćec i njegovo djelo 1882.-1968.“ Vargović E, Bregović A (ur.) 163-172. HAZU-Zavod za znanstveni rad u Varaždinu, Gradski muzej Varaždin, Gimnazija Varaždin, Hrvatsko entomološko društvo, Zagreb – Varaždin.

## d) Sudjelovanja i sažeci u zbornicima međunarodnih i domaćih skupova

## - Usmena priopćenja:

1. **Temunović M** (2013) Decision-making under uncertainty - Decision making with incomplete data in forest adaptation to climate change: management perspective. European Climate Change Adaptation Conference. Hamburg, Njemačka. (pozvano predavanje)
2. **Temunović M**, Franjić J, Satović Z, Grgurev M, Frascaria-Lacoste N, Fernández-Manjarrés JF (2012) Genetička varijabilnost populacija poljskog jasena u zavisnosti o ekološkim čimbenicima u heterogenom okolišu. U: Zbornik sažetaka, 11. Hrvatski biološki kongres s međunarodnim sudjelovanjem. Jelaska SD, Klobučar GIV, Šerić Jelaska L, Leljak Levanić D, Lukša Ž (ur.) 144-145. Hrvatsko biološko društvo 1885, Zagreb, Hrvatska.
3. Turić N, Vignjević G, **Temunović M**, Vručina I, Merdić E (2012) Usporedba metoda uzorkovanja, brojnosti i populacijske dinamike vrste *Graphoderus bilineatus* De Geer, 1774 (Coleoptera, Dytiscidae) u Parku prirode Kopački rit tijekom 2010. i 2011. godine. U: Zbornik sažetaka, 11. Hrvatski biološki kongres s međunarodnim sudjelovanjem. Jelaska SD, Klobučar GIV, Šerić Jelaska L, Leljak Levanić D, Lukša Ž (ur.) 67-68. Hrvatsko biološko društvo 1885, Zagreb, Hrvatska.
4. Mikulić K, **Temunović M**, Budinski I (2012) Konzervacijska strategija za novootkrivenu populaciju bjelonokte vjetruše (*Falco naumanni*) u Hrvatskoj. U: Zbornik sažetaka, 11. Hrvatski biološki kongres s međunarodnim sudjelovanjem. Jelaska SD, Klobučar GIV, Šerić Jelaska L, Leljak Levanić D, Lukša Ž (ur.) 193-194. Hrvatsko biološko društvo 1885, Zagreb, Hrvatska.
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6. **Temunović M**, Frascaria-Lacoste N, Šatović Z, Fernández-Manjarrés JF, Škvorc Ž, Franjić J (2009) Genetska raznolikost populacija poljskoj jasena (*Fraxinus angustifolia* Vahl) u Hrvatskoj. U: Zbornik sažetaka, 10. Hrvatski biološki kongres s međunarodnim sudjelovanjem. Besendorfer V, Kopjar N, Vidaković Cifrek Ž, Tkalec M, Bauer N, Lukša Ž (ur.) 126-127. Hrvatsko biološko društvo 1885, Osijek, Hrvatska.

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3. Dražina T, **Temunović M**, Šerić Jelaska L (2012) Saproksilna zajednica kornjaša starih gradskih parkova: primjer iz parka Maksimir (Zagreb, Hrvatska) U: Zbornik sažetaka, 11. Hrvatski biološki kongres s međunarodnim sudjelovanjem. Besendorfer V, Kopjar N, Vidaković Cifrek Ž, Tkalec M, Bauer N, Lukša Ž (ur.) 79-80. Hrvatsko biološko društvo 1885, Zagreb, Hrvatska.
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6. **Temunović M**, Turić N, Merdić E, Csabai Z (2012) Distribution, Habitat and Conservation Status of the Endangered Water Beetle *Graphoderus bilineatus* in Croatia. In: Book of Abstracts, 3rd European Congress of Conservation Biology. P24.1. Society for Conservation Biology, Glasgow, Scotland.
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11. Krstonošić D, Škvorc Ž, Franjić J, Sever K, Alešković I, **Temunović M** (2010) Suhi travnjaci slavonskoga gorja. U: Knjiga sažetaka, Treći hrvatski botanički kongres. Jasprica N, Pandža M, Milović M (ur.) 116-116. Hrvatsko botaničko društvo, Murter, Hrvatska.
12. Sever K, Škvorc Ž, Krstonošić D, Alešković I, **Temunović M**, Seletković I, Potočić N, Franjić J (2010) Sadržaj klorofila i sezonska dinamika dušika u listovima hrasta lužnjaka (*Quercus robur* L.). U: Knjiga sažetaka, Treći hrvatski botanički kongres. Jasprica N, Pandža M, Milović M (ur.) 178-178. Hrvatsko botaničko društvo, Murter, Hrvatska.
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14. Sever K, Škvorc Ž, Krstonošić D, Alešković I, **Temunović M**, Seletković I, Potočić N, Franjić J (2010) Use of portable chlorophyll meter for assessment of nitrogen nutrition of pedunculate oak (*Quercus robur* L.). In: Book of Abstract, 5<sup>th</sup> Slovenian symposium on plant biology. Dolenc-Koce J, Vodnik D, Pongrac P (ur.) 28-28. Ljubljana, Slovenia.
15. **Temunović M**, Šerić Jelaska L (2010) Seasonal changes in water beetle assemblages in a temporary pond of Lonjsko polje Nature Park. In: Programme and Book of Abstracts of the 9th European Congress of Entomology. Vásárhelyi, Tamás (ur.) 96-96. Budapest, Hungary.
16. Šerić Jelaska L, **Temunović M** (2010) Assessing the conservation status of the lower Una river basin using records of Aedeopagan Coleoptera. In: Programme and Book of Abstracts of the 9th European Congress of Entomology. Tamás Vásárhelyi (ur.) 99-99. Budapest, Hungary.
17. Sever K, Škvorc Ž, Bogdan S, Franjić J, Krstonošić D, **Temunović M**, Alešković I (2009) Varijabilnost fiziološko-morfoloških značajki klonova hrasta lužnjaka (*Quercus robur* L.) iz pokusa „Brestje“ – prvi rezultati. U: Zbornik sažetaka, 10. Hrvatski biološki kongres s međunarodnim sudjelovanjem. Besendorfer V, Kopjar N, Vidaković Cifrek Ž, Tkalec M, Bauer N, Lukša Ž (ur.) 158-158. Hrvatsko biološko društvo 1885, Osijek, Hrvatska.
18. **Temunović M**, Frascaria-Lacoste N, Šatović Z, Fernández-Manjarrés JF, Škvorc Ž, Franjić J (2009) Genetic diversity of *Fraxinus angustifolia* Vahl populations in Croatia assessed by nuclear microsatellites. In: Book of Abstracts, International scientific conference „Balkans - hot spots of ancient and present genetic diversity“. 57-57. Sofia, Bulgaria.
19. **Temunović M**, Šerić Jelaska Lucija (2009) First record of diving beetle *Cybister tripunctatus africanus* Laporte, 1835 in the Croatian Hydradeopagan fauna. In: Communications and abstracts, SIEEC 21 Symposium Internationale Entomofaunisticum Europae Centralis. Soldán T, Papáček M, Boháč J (ur.) 85-85. Biological Centre, Academy of Sciences of the Czech Republic, Institute of Entomology, České Budějovice, Czech Republic.
20. **Temunović M**, Šerić Jelaska L, Durbešić P (2006) Privremene lokve kao staništa vodenih kornjaša (Coleoptera) na području Parka prirode Lonjsko polje. U: Zbornik sažetaka, 9. hrvatski biološki kongres. Besendorfer V, Klobučar IVG (ur.) 238-239. Hrvatsko biološko društvo 1885, Rovinj, Hrvatska.