Integrative biodiversity research of pseudoscorpions (Pseudoscorpiones, Arachnida, Chelicerata) and stoneflies (Plecoptera, Insecta, Hexapoda) in Croatia

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INTEGRATIVE BIODIVERSITY RESEARCH OF PSEUDOSCORPIONS (PSEUDOSCORPIONES, ARACHNIDA, CHELICERATA) AND STONEFLIES (PLECOPTERA, INSECTA, HEXAPODA) IN CROATIA

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DOCTORAL DISSERTATION

Supervisors:

Professor Mladen Kučinić, PhD

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Zagreb, 2023

This doctoral dissertation was made at the University of Zagreb, Faculty of Science, Department of Biology, under the supervision of Professor Mladen Kučinić, PhD and Assistant Professor Martina Podnar Lešić, PhD, as part of the Doctoral programme of Biology at the University of Zagreb, Faculty of Science, Department of Biology. The research presented in this doctoral thesis was supported by the Croatian Science Foundation (CSF) and the project *DNA barcoding of Croatian faunal biodiversity* (project number IP-2016-06-9988). The work of Dora Hlebec was fully funded by the *Young Researchers' Career Development Project – Training of Doctoral Students* of the CSF, which is financed by the European Union from the European Social Fund (project number DOK-2018-09-1417). The experimental parts of this thesis were carried out at the Croatian Natural History Museum (Croatia), Slovenian Museum of Natural History (Slovenia), Museum of Nature Hamburg - Zoology (Germany), and Western Australian Museum (Australia).

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University of Zagreb Doctoral dissertation

Faculty of Science

Department of Biology

Integrative biodiversity research of pseudoscorpions (Pseudoscorpiones, Arachnida, Chelicerata) and stoneflies (Plecoptera, Insecta, Hexapoda) in Croatia

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A key step in conserving biological diversity is to understand biodiversity patterns based on multiple independent lines of evidence. The aims of this dissertation were to investigate the morphological variability, and by applying DNA barcoding, provide first insights into genetic diversity of two invertebrate lineages: pseudoscorpions and stoneflies, both characterized by high degree of endemism and cryptic diversity. Generated DNA barcodes, 499 for pseudoscorpions and 355 for stoneflies, revealed DNA barcoding as an effective tool for specimen identification. Integrative approach identified 47 putative new species of pseudoscorpions and six species of stoneflies, including recently described stenoendemic *Isoperla popijaci*. Several species and generic hypotheses in both groups need to be re-tested when additional data become available. Phylogenetic reconstructions confirmed high genetic diversity in both groups, while this diversity in pseudoscorpions correlates with geomorphological features and consists of evolutionarily young lineages. Highlighting localities with emphasized biodiversity in fragmented karstic microhabitats, which promote speciation and endemism, creates preconditions for further conservation management.

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Keywords: freshwater and subterranean biology, morphology, phylogeny, taxonomy

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Integrativni pristup u istraživanju bioraznolikosti lažištipavaca (Pseudoscorpiones, Arachnida, Chelicerata) i obalčara (Plecoptera, Insecta, Hexapoda) u Hrvatskoj

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Ključni korak u očuvanju biološke raznolikosti je razumijevanje njenih obrazaca uz korištenje višestrukih neovisnih pokazatelja. Ciljevi ove doktorske disertacije bili su utvrditi morfološku varijabilnost i, primjenom metode DNA barkodiranja, pružiti prvi uvid u genetsku raznolikost dviju skupina beskralješnjaka: lažištipavaca i obalčara, obje karakterizirane visokim stupnjem endemizma i kriptičnom raznolikošću. Generirani DNA barkodovi, 499 za skupinu lažištipavaca i 355 za skupinu obalčara, potvrdili su mogućnost primjene DNA barkodiranja u svrhu identifikacije uzoraka. Integrativnim pristupom otkriveno je postojanje 47 potencijalno novih vrsta lažištipavaca i šest vrsta obalčara, uključujući nedavno opisanu stenoendemsku vrstu *Isoperla popijaci*. Za razrješavanje taksonomskih statusa i odnosa među nekoliko vrsta i rodova u obje skupine, potrebno je prikupiti dodatan materijal. Filogenetska rekonstrukcija potvrdila je veliku genetsku raznolikost i kod lažištipavaca i kod obalčara, pri čemu je genetska raznolikost lažištipavaca u korelaciji s geomorfološkim značajkama Dinarskog krša i sastoji se uglavnom od evolucijski mladih genetskih linija. Isticanjem lokaliteta s izraženom bioraznolikošću u fragmentiranim krškim mikrostaništima, koja potiču specijaciju i endemizam, stvaraju se preduvjeti za daljnje upravljanje očuvanjem vrsta i staništa.

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Ključne riječi: biologija slatkih voda i podzemnih staništa, morfologija, filogenija, taksonomija

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List of Publications

This doctoral dissertation is based on three publications, which are referenced in the text using their assigned Roman numerals.

I. **Hlebec D**, Sivec I, Podnar M, Skejo J, Kučinić M (2021) Morphological and molecular characterisation of the Popijač's Yellow Sally, *Isoperla popijaci* **sp. nov.**, a new stenoendemic stonefly species from Croatia (Plecoptera, Perlodidae). ZooKeys 1078: 85–106.

II. **Hlebec D**, Sivec I, Podnar M, Kučinić M (2022) DNA barcoding for biodiversity assessment: Croatian stoneflies (Insecta: Plecoptera). PeerJ 10: e13213.

III. **Hlebec D**, Podnar M, Kučinić M, Harms D (2023) Molecular analyses of pseudoscorpions in a subterranean biodiversity hotspot reveal cryptic diversity and microendemism. Scientific Reports 13: 430.

1 Introduction

1.1 Biodiversity and biodiversity loss

Human actions pose a significant threat to biological diversity. Habitat degradation and fragmentation, pollution, overexploitation of species, the introduction of invasive species, and climate change are leading to biodiversity loss in various ecosystems. This loss includes the decline of genetic and cryptic diversity (Bálint et al., 2011). The reduction in species richness and abundance is occurring simultaneously in both freshwater (Dudgeon et al., 2006) and terrestrial (Leclère et al., 2020) ecosystems, which has garnered significant attention in our society. Of particular concern is the fact that areas with exceptionally high alpha taxonomic diversity and endemism are not spared from these threats, such as the Mediterranean Basin, recognized as one of the 36 biodiversity hotspots in the world (Médail & Myers, 2004). In the postglacial period, numerous terrestrial and aquatic species spread from the Iberian, Apennine, and Balkan Peninsula to the northern parts of Europe (Hewitt, 2000). Therefore, these regions are considered principal glacial refugia for various taxa (Pauls et al., 2006; Deharveng et al., 2012), often following "refugia within refugia" pattern in terrestrial (Kryštufek et al., 2007; Jug-Dujaković et al., 2020) and freshwater biota (Previšić et al., 2009, 2014a). Cryptic diversity, probably the result of convergent evolution, recent speciation, and/or isolation in microhabitats (Struck et al., 2018a, 2018b), has already been observed in many groups, including marine amphipods (Hupało et al., 2019), fishes (Buj et al., 2019), and mayflies (Bisconti et al., 2016).

Freshwater ecosystems in the Mediterranean Basin exhibit high genetic diversity, primarily influenced by past climatic conditions, as well as geomorphological and hydrogeographic processes such as sea level changes, tectonic activities, and glaciation (Hewitt, 2004). Several phylogeographic studies have emphasized that area played a significant role in the genetic differentiation of taxa (Klobučar et al., 2013; Sket, 1999). Due to its paleogeomorphological features, Croatia possesses abundant water resources across its three biogeographical regions: Continental, Alpine and Mediterranean. Moreover, the region is characterized by a multitude of specific habitats, including intermittent rivers and streams, which are renowned for harboring exceptionally diverse animal communities (Culver & Sket, 2000).

Anthropogenic pressure on habitats is most evident in the alteration of water flows, including the construction of dams and reservoirs, the canalization of riverbeds, and land drainage, which result in the fragmentation and degradation of natural habitats. Freshwater biodiversity is particularly vulnerable to climate change due to dependence on specific hydrological regimes and limited dispersal capacities of many freshwater organisms, such as stoneflies (Fochetti & Tierno de Figueroa, 2008) and mayflies (Schröder et al., 2021). Additionally, the cumulative impact of various anthropogenic stressors further exacerbates the threats to freshwater ecosystems (Woodward et al., 2010).

1.1.1 Dinaric Karst

Nested within the Mediterranean Basin, the Dinaric Karst is widely recognized as a biodiversity hotspot, harbouring a plethora of endemic and/or cryptic lineages. The region is primarily characterized by a substrate composed of carbonate rocks, namely limestone, dolomite, and sandstone (Velić et al., 2015). These rocks are highly soluble in water, and due to the region's high levels of precipitation, they undergo extensive dissolution, resulting in the formation of numerous underground channels and caverns. The area is treated as the world´s primary subterranean hotspot (Sket, 2012), boasting more than 20,000 caves (with approximately 10,000 of them explored) and hosting a highly adapted subterranean fauna (Zupan Hajna, 2012; Gottstein Matočec et al., 2002). Among these exceptional taxa are first scientifically described cave-dwelling animal *Proteus anguinus* Laurenti, 1768; first cave insect *Leptodirus hochenwartii* Schmidt, 1832; first cave spider *Stalita taenaria* Schiödte, 1847; and *Eunapius subterraneus* Sket & Velikonja, 1984 and *Congeria kusceri* Bole, 1962, which belong to groups of only a few known stigobiotic sponges and bivalves in the world.

The Dinaric Karst region is home to some of the most complex cave systems, including Postojna Cave in Slovenia, Lukina jama-Trojama Pit in Croatia, and Vjetrenica Cave in Bosnia and Herzegovina. These caves not only serve as geological wonders but also play a vital role as habitats for a wide range of rare and endemic species. Caves can be regarded as perfect “natural laboratories” for investigating speciation mechanisms and drivers of adaptive evolution due their stringent environmental constraints, such as limited or modified food resources, restricted light availability, high humidity, shallow temperature fluctuations, and barriers to dispersal. Life in perpetual darkness often leads to reduced gene flow, limited dispersal (Pavlek et al., 2021) and the development of troglomorphic adaptations, such as pallid coloration, elongated appendages, and loss of eyes.

The biodiversity of subterranean fauna is highest in two “hotspots within the hotspot“: the northwestern and southwestern parts of the Dinarides (Zagmajster et al., 2008; Bregović & Zagmajster, 2016). Additionally, the northern-central Dinaric region represents a hotspot for freshwater fauna (Klobučar et al., 2013). Furthermore, karstic river springs within the Dinaric Karst region serve as vital connections between underground and aboveground habitats, enabling the exchange of organisms. The biodiversity found in these habitats is the result of intricate interactions between the geological characteristics of the karst landscape and the hydrological dynamics of the river systems (Sket, 1999).

Dynamic climatic-ecological fluctuations and tectonic-palaeogeographic history of the Dinarides have resulted in high habitat complexity for terrestrial, aquatic, and subterranean ecosystems (Klobučar et al., 2013; Popov et al., 2004; Sket, 2012; Zagmajster et al., 2014). The isolation of habitat patches stimulates a high diversification rate (Zakšek et al., 2007), and cryptic diversity has been observed in many range-restricted taxa (Trontelj et al., 2009; Previšić et al., 2016; Delić et al., 2017a, 2017b). Cryptic species are typically identified as a single nominal species because they are, at least superficially, morphologically identical (Bickford et al., 2007). They are often found as island and/or short-range endemics with limited dispersal abilities (Pfingstl et al., 2021), but the evolutionary processes leading to phenotypic stasis have not been thoroughly investigated. Moreover, large-scale integrative analyses of taxa characterized by high degree of endemism and cryptic diversity, such as highly adapted subterranean pseudoscorpions and range-restricted stoneflies, remain relatively rare.

More than 500 animals have been described from approximately 331 different caves and pits in Croatia (Jalžić et al., 2022). Caves, pits, and other subterranean habitats, along with their biota, are susceptible to biodiversity loss (Raven & Miller, 2020), but they are generally less studied due to their inherent inaccessibility. Subterranean radiations in the area have only been extensively studied in a few groups, such as springtails (Lukić et al., 2020), amphipods (Borko et al., 2021), and isopods (Bedek et al., 2019). Monitoring and protecting these species and their type localities should be one of the main priorities in conservation biology.

However, despite the long history of research in the area and advancements in the application of molecular tools over the past 30 years, the patterns of genetic and taxonomic diversity, speciation, phylogenetic relationships, and distribution of particular species of pseudoscorpions and stoneflies in Croatia are still unknown. This situation is somewhat disappointing, as simultaneous research of all groups of organisms, not just flagship taxa like *Proteus anguinus* Laurenti, 1768 (Recknagel, 2023), is crucial for cataloging biodiversity and understanding how this diversity has been generated and maintained *in situ*. Globally, studies aiming to explain the evolutionary history and biodiversity patterns of pseudoscorpions and stoneflies remain scarce, with most focused on higher-level phylogeny (Murienne et al., 2008; Benavides et al., 2019; Ding et al., 2019). The results of this thesis fill an important gap in the biodiversity assessment of pseudoscorpions and stoneflies across regions.

1.2 DNA barcoding

DNA barcoding, initially proposed as a *COI*-based identification system (Hebert et al., 2003a), enables rapid specimen identification when accurate, high-quality, and comprehensive reference sequences with Linnaean binomials are available. Nowadays, DNA barcoding is utilized in integrative taxonomy routines for species delineation (Hubert & Hanner, 2015). Mitochondrial DNA was selected due to its small size, simple organization, maternal inheritance, and lack of introns. Specifically, the 658-bp cytochrome *c* oxidase subunit I (*cox1* or *COI*) gene region was chosen because of the availability of universal primers applicable to nearly all animal phyla (Folmer et al., 1994) and its wide phylogenetic signal (Hebert et al., 2003a). The concept is based on the notion that species represent distinct lineages (De Queiroz, 2007) and are considered reliable if interspecific variation in *COI* exceeds intraspecific variation, known as the DNA barcoding gap (Hebert et al., 2004a; Meyer & Paulay, 2005; Puillandre et al., 2012a, 2012b). DNA-based taxon delimitation has revolutionized systematic and integrative taxonomy, as well as research in evolution, ecology, and conservation biology (Scheffers et al., 2012; Kress et al., 2015). It is particularly effective in revealing new species and cryptic diversity (Hebert et al., 2003a, 2004a; Yin et al., 2022). However, DNA barcodes alone should not be relied upon for species description, as suggested by the "minimalist approach" proposed by Meierotto et al. (2019). DNA barcoding is often used as a tool for assessing biodiversity (Barcode Index Number (BIN) counts are considered proxies for species), although relying solely on *COI* genetic divergence for species delimitation can lead to over-splitting of population lineages into putative species (Kekkonen & Hebert, 2014; Muster & Michalik, 2020; Meier et al., 2021).

The success of specimen identification depends on the availability and completeness of the DNA barcode library for the specific group. Special cases arise with “dark taxa“ (Page, 2016), which are taxonomically challenging yet species-rich organisms lacking sufficient taxonomic expertise, such as Diptera (Chimeno et al., 2022), Hymenoptera (Jafari et al., 2023) and Pseudoscorpiones (Muster et al., 2021). Consequently, most species in these groups remain undescribed, resulting in a limited allocation of Linnean names for the sequences in databases. This disparity between the substantial number of species and the limited number of researchers available to study them is commonly referred to as the ”taxonomic impediment” (Engel et al., 2021).

Despite its advantages, the single-locus approach fails to capture the complex evolutionary history of species, and the use of a short DNA region can often limit the accurate quantification of taxonomic and genetic diversity. Furthermore, the method’s efficiency can be influenced by various factors, including changes in pathways of mitochondrial DNA inheritance caused by *Wolbachia* infections (Werren et al., 1995; Smith et al., 2012), heteroplasmy (Frey & Frey, 2004), incomplete lineage sorting (Petit & Excoffier, 2009), pseudogene co-amplification (Song et al., 2008) and introgressive hybridization and recent speciation (Raupach et al., 2014).

1.3 Pseudoscorpions (Pseudoscorpiones, Arachnida, Chelicerata)

1.3.1 Diversity

Pseudoscorpions, a meso-diverse arachnid order, exhibit remarkable diversity in morphology and behavior despite their small size (1–10 mm in length). Currently, there are 4032 species belonging to 456 genera and 24 families that have been described (WPC, 2022).

Pseudoscorpions are distributed worldwide and inhabit various terrestrial habitats, including soil, leaf litter, tree bark, tree hollows, and animal nests, as well as the subterranean realm (Weygoldt, 1969). They play important roles as predators in many ecosystems and have been observed preying on a variety of arthropods such as mites, springtails, and small insects. Due to their limited dispersal abilities, most species have small distribution ranges, leading to the occurrence of spatially structured lineages (Harrison et al., 2014; Harvey et al., 2016; Harms et al., 2019). However, the phenomenon of phoretic behavior, which involves the passive use of other organisms for dispersal purposes, is common in the superfamily Cheliferoidea and allows for wider distribution (Poinar et al., 1998; Opatova & Šťáhlavský, 2018).

Many species are known only from isolated species descriptions, and studies investigating intra- and interspecific morphological variability using multi-variate morphometrics are scarce (Christophoryová et al., 2016).

Research on pseudoscorpions, one of the most diverse invertebrate lineages in the Dinaric Karst, began in the mid-19th century (Schiödte, 1847; Schmidt, 1848) followed by descriptions of 143 species from this area (e.g., Beier, 1939; Ćurčić, 1988; WPC, 2022). Many of them are highly adapted to cave environments (troglobitic). Troglobitic species possess specific morphological features associated with their cave-dwelling lifestyle, such as full eyes regression, elongated appendages, and lack of body pigmentation. These species tend to be endemic to specific caves or have a limited range (Harrison et al., 2014).

The last checklist of Croatian pseudoscorpions, published by Ozimec (2004), counted 99 species and 10 subspecies, including unique endemic genera such as *Insulocreagris* Ćurčić, 1987, found on the island of Vis, the relict genus *Troglochthonius* Beier, 1939 with two species, the genus *Microchthonius* Hadži, 1933 with nine described species, the genus *Protoneobisium* Ćurčić, 1988 found at Biokovo Mountain, and the subgenus *Neobisium* (*Pennobisium*) Ćurčić, 1988 found at Velebit Mountain. Species richness generally increases towards lower latitudes (Fischer, 1960), which is why it is not surprising that most pseudoscorpion taxa are found in the Mediterranean ecoregion of Croatia. Many new species and genera have been described from caves (Beier, 1939; Ćurčić, 1988), while knowledge of the epigean fauna remains incomplete.

1.3.2 Taxonomy and evolutionary systematics

Accurate identification of pseudoscorpion species to the species level requires advanced taxonomic expertise due to their cryptic nature and the potential presence of homoplasy in morphological characters, such as the number of teeth on the chelal fingers and trichobothria patterns. The current taxonomy of Croatian pseudoscorpions poses several challenges. Many species were inaccurately described, often based solely on morphology and small sample sizes with incomplete distributional data. Additionally, taxonomic characters for generic classification are not well-defined, and the availability of holotypes, is limited as they are mostly held in private collections outside of Croatia. The majority of species belong to two families: Chthoniidae (43 species) and Neobisiidae (79 species), both of which are taxonomically complex and highly adapted to subterranean habitats. Prior to this doctoral dissertation, no DNA sequence data existed to serve as a baseline for taxonomic revisions.

Although pseudoscorpions constitute a large and diverse group, there is still limited knowledge about sequence variation within this taxon. Previous research has primarily focused on the classification, taxonomy, and systematics of pseudoscorpions, relying predominantly on morphological features. The first phylogenetic tree was constructed by Chamberlin (1931). Molecular systematics began with a broader multi-locus phylogeny published by Murienne et al. (2008). This study demonstrated the monophyly of the order and several of its superfamilies, but not of Neobisioidea or Garypoidea. Chthonioidea was found to be the sister group to the Iocheirata, which includes pseudoscorpions with venom glands according to Harvey's classification (1992). This result suggests that venom glands evolved only once within the order. Due to their ancient origins (fossils are known from the Middle Devonian period (Harms & Dunlop, 2017), high diversity, global distribution, (micro)endemism and limited dispersal abilities, pseudoscorpions represent good model for historical biogeography analyses and can reflect evolutionary history and geological patterns (Johnson et al., 2022). Subsequent studies on evolutionary pathways have confirmed slight population structuring among geographically distant localities (Ranius & Douwes, 2002; Harvey et al., 2015), deep structuring in non-vagile pseudoscorpions (Harrison et al., 2014; Cosgrove et al., 2016) and the presence of cryptic diversity within several widely distributed taxa, such as *Chernes hahnii* (C.L. Koch, 1839) (Opatova & Šťáhlavský, 2018), *Neobisium carcinoides* (Hermann, 1804) and *Dinocheirus panzeri* (C.L. Koch, 1837) (Muster et al., 2021). A more comprehensive analysis that includes 41 pseudoscorpion transcriptomes was published by Benavides et al. (2019). This study resulted in several new classifications, including the proposal of new nomenclatural and taxonomic acts. Pseudoscorpion library generated by Benavides et al. (2019) was later used by Ontano et al. (2021) to investigate the phylogenetic relationships between pseudoscorpions and scorpions, as well as the placement of pseudoscorpions within the newly defined group of Arachnopulmonata. In addition, three DNA barcode reference libraries for pseudoscorpions have been developed using specimens from Canada (Cameron & Buddle, 2019), South Korea (Ohira et al., 2018), and Germany (Muster et al., 2021).

1.4 Stoneflies (Plecoptera, Insecta, Hexapoda)

1.4.1 Diversity

Freshwater insects represent a key link between aquatic and terrestrial ecosystems. Among these insects, stoneflies (Plecoptera), which encompass 17 families and have around 3800 described species (South et al., 2021), are particularly susceptible to environmental changes. Stoneflies, hemimetabolous and stenothermic organisms, have experienced declines in their distributional ranges due to environmental shifts (Fochetti & Tierno de Figueroa, 2008). The Mediterranean region with many freshwater ecosystems, is especially vulnerable to climate change due to temperature increase and changes in precipitation regime (Markovic et al., 2014). Stoneflies are closely associated with clean running water and surrounding terrestrial areas (William & Felmate, 1992) and are considered indicators of water quality. Given their limited ability to disperse, regional endemism among stoneflies is relatively high. Southeast Europe, in particular, is recognized as a biodiversity hotspot for stoneflies, with the presence of 21 *Isoperla* Banks, 1906 species alone (Murányi, 2011; Graf et al., 2018a).

Until 2005, and prior to comprehensive field research, only 28 stonefly species were recorded in Croatia (Sivec, 1980). This number was surprisingly low considering the abundance of suitable habitats (Illies, 1966), and in comparison to neighboring countries such as Slovenia (100 species) (Sivec, 2001), Bosnia and Herzegovina (73 species) (Kaćanski, 1976), Montenegro (57 species) (Murányi, 2008), Hungary (61 species) (Andrikovics & Murányi, 2001), and Serbia (90 species) (Petrović et al., 2014). Subsequent studies were limited to specific areas such as Plitvice Lakes National Park (Ridl et al., 2018), Cetina River (Popijač & Sivec, 2009b), and the lower reaches of the Una River (Popijač & Sivec, 2011), which resulted in the recording of a total of 50 stonefly species. High morphological variability was observed in several species, including *Isoperla grammatica* (Poda 1761), *Isoperla inermis* Kacanski & Zwick, 1970, and species of the genus *Taeniopteryx* Pictet, 1841 (Popijač & Sivec, 2009b, 2011). The importance of resolving phylogenetic relationships in the Balkan Peninsula has already been emphasized (Murányi, 2011; Graf et al., 2018a, 2018b).

Previous research on stoneflies in Croatia provided data on species distribution, identified localities with high diversity, and noted a significant reduction in species richness and abundance. It has also been observed that some species now only exist as museum specimens, leading to the publication of The Red List of Croatian Plecoptera in 2007 (Popijač, 2007).

According to the International Union for Conservation of Nature (IUCN) Red List Categories, 17 stoneflies are classified as endangered: two are considered as extinct in the wild, one as critically endangered, three as endangered and 11 as vulnerable (Popijač, 2007).

1.4.2 Taxonomy and evolutionary systematics

Despite their importance in aquatic ecosystems worldwide, fully resolved phylogeny of stoneflies has remained elusive. The classification proposed by Zwick (2000) is currently the most widely accepted system. The advent of genomics and transcriptomics has provided new opportunities for testing phylogenetic hypotheses in stoneflies and overcoming the limitations of morphology-based identification. In a study by South et al. (2020), transcriptome data from 94 species, encompassing all families, subfamilies, and tribes from North America, provided the most comprehensive molecular dataset for the North American fauna. This study built upon previous research with limited taxon sampling (Thomas et al., 2000; Chen et al., 2018; Wang et al., 2018; Ding et al., 2019). The phylogenetic analysis revealed that family Perlidae is the first branch-off within the clade composed of the monophyletic Perloidea. However, the positions of Taeniopterygidae and Leuctridae, which are among the most speciose families in Croatia, remain unresolved. Several DNA barcode libraries have already been generated, and DNA barcoding has proven to be a valuable tool for specimen identification within the EPT (Ephemeroptera, Plecoptera, and Trichoptera) group (Gill et al., 2014; Ball et al., 2005; Webb et al., 2012; Morinière et al., 2017).

To date, sequencing of the *COI* gene fragment has been utilized in stonefly systematics and phylogeography (Fochetti et al., 2009, 2011; Weiss et al., 2011), species delineation (Boumans & Murányi, 2014; Graf et al., 2018a; Pelingen & Freitag, 2020; South et al., 2019) and revisionary systematics (Fochetti et al., 2011). Molecular phylogenetic studies conducted on other freshwater organisms collected in Croatia have indicated significant genetic diversity (Bogutskaya et al., 2020) and allopatric speciation (Previšić et al., 2009, 2014a; Vitecek et al., 2017a). These findings serve as motivation to investigate whether stonefly species follow similar patterns of genetic differentiation and speciation.

1.5 Integrative taxonomy

Traditional taxonomy has been the primary approach for discovering, describing, and classifying species for nearly three centuries.

However, over the past two decades, the limitations of relying solely on morphology for studying biodiversity have become increasingly apparent. The emergence of DNA-based delimitation procedures has shed light on the significant challenges faced by morphology-based approaches in understanding and accurately identifying species. Relying solely on the traditional approach is insufficient for resolving relationships in diverse or morphologically conserved groups like pseudoscorpions (Muster et al., 2021; Christophoryová et al., 2023) or for detecting cryptic diversity (Bickford et al., 2007). The scarcity of skilled taxonomists and the lack of funding and training in taxonomy (Khuroo et al., 2007) exacerbate the problem. Moreover, the use of morphology alone can not provide a comprehensive understanding of evolutionary processes and adaptations at the species and population levels. Conversely, relying solely on Molecular Operational Taxonomic Units (MOTUs) or BINs for estimating species richness, assuming they can be treated as biodiversity units, akin to species (Blaxter, 2004), often leads to over-splitting (Ahrens et al., 2016; Ranasinghe et al., 2022).

The synergistic use of both morphological and molecular approaches, known as “integrative taxonomy” (Dayrat, 2005; Zhang et al., 2013) should be a fundamental step in any biodiversity research to achieve efficient species delimitation (Vitecek et al., 2017a; Cicero et al., 2021). This approach is crucial for resolving any discrepancies between DNA barcoding and traditional taxonomy. By combining knowledge of species' natural history, rarity, endemism, ecology, behavior, and intra- and interspecific morphological and genetic variability, a comprehensive understanding can be gained, which is essential for the development of effective long-term conservation strategies (Cristescu, 2014).

1.6 Conservation biology

The protection of biodiversity become one of humanity's most important goals. An essential preliminary step is to encourage research in conservation biology (Primack, 2006) through the integration of multiple approaches. The development of conservation genetics highlights the significance of understanding genetic diversity at the population and species levels. Genetic diversity directly reflects the adaptive potential of populations or species to respond to environmental changes and their ability to survive (Frankham et al., 2002; Toro & Caballero, 2005).

Higher genetic diversity increases their chances of survival. Conversely, populations and species with limited ability to adapt (low genetic diversity) may experience distribution shifts (migration towards more favorable habitats), and the worst-case scenario is extinction (Aitken et al., 2008).

As global temperatures rise due to anthropogenic climate change, many subterranean species that lack the ability to adapt to environmental changes may become extinct before they are even discovered and scientifically described (Mammola et al., 2019). In fact, a Protected Area Gap Analysis in the Dinaric Karst region has indicated that hypogean habitats, which are largely understudied, require comprehensive sampling and detailed research to identify biodiversity hotspots before effective conservation policies can be implemented (WWF Mediterranean Programme Office, 2010).

Stoneflies are recognized as biological indicators of well-oxygenated water in freshwater ecosystems, and their absence can indicate negative environmental changes (Fochetti & Tierno de Figueroa, 2008). Given the critical threats facing stoneflies, studying their distribution, morphological variability, and genetic diversity should be one of the priorities in conservation biology. Haubrock et al. (2023) investigated the response of freshwater insects, including stoneflies, to climate change in Central Europe over the past three decades. Their findings revealed increases in species richness (10.6%) and abundance (9.5%), with summer temperature and precipitation identified as driving factors. These results contradict previous studies that indicated a rapid decline in stonefly abundance in Central Europe (Fochetti & Tierno de Figueroa, 2006; Bojková et al., 2012).

1.7 Research aims and hypotheses

The aims and hypotheses of this doctoral dissertation focus on addressing biodiversity knowledge gaps for pseudoscorpions and stoneflies through an integrative approach. The research aims were to investigate morphological variability, gain insights into the presence of distinct lineages in pseudoscorpions and stoneflies through DNA barcoding, determine intra- and interspecific phylogenetic relationships, and to identify and describe new species using integrative taxonomy. In addition to morphological descriptions that include illustrations and photographs of key taxonomic features, studies will provide molecular characterization within the genus, distributional data and the ecological characteristics of the type locality. To achieve these goals, this dissertation encompasses three scientific publications (**I–III**).

The specific aims of the research were:

1. to determine the morphological variability and intra- and interspecific phylogenetic relationships within pseudoscorpions and stoneflies, based on the results of field research, comparative morphology, and molecular biology techniques, with the emphasis on the DNA barcoding, as well as to identify and describe new species using integrative approach (Publications I, II and III)
2. to estimate the time of separation of lineages, and to identify centres of diversification, as well as areas with the highest conservation value (Publications I, II and III)
3. to determine the taxonomic position and phylogenetic relationships of pseudoscorpion species, and compare the time of divergence of species inhabiting epigean and hypogean habitats, as well as species with populations on mainland and islands (Publication III)

Hypotheses:

1. Among the species of stoneflies and pseudoscorpions in Croatia exists great morphological and genetic diversity (Publications I, II and III).
2. The fauna of stoneflies and pseudoscorpions of Croatia contains some not yet described species (Publications I, II and III).
3. Pseudoscorpion species from island and mainland areas, as well as those from underground and aboveground habitats show different temporal patterns of divergence (Publication III).

Publication I contributed to the first and second aims, as well as the first and second hypotheses, by providing morphological and molecular characterization of new stenoendemic stonefly species. The study highlighted the importance of conserving intermittent rivers. Publication II addressed the first and second aims, as well as the first and second hypotheses, focusing on stoneflies. The study established a DNA barcode reference library consisting of 355 sequences from 74 morphospecies. Additionally, the research provided valuable species records, including those thought to be locally extinct, and presented the first molecular characterization of several species. Publication III directly contributed to all aims and hypotheses related to pseudoscorpions. The study generated the most comprehensive DNA barcode reference library, which will facilitate future taxonomic work and revisions. Study generated 499 *COI* barcodes from 128 morphospecies and a high proportion of endemic, rare and elusive taxa. The research also estimated optimal identification thresholds and identification efficiencies for pseudoscorpions.

2 Scientific publications

Publication I

ZooKeys 1078: 85–106 (2021)
doi: 10.3897/zookeys.1078.66382
https://zookeys.pensoft.net

RESEARCH ARTICLE

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Morphological and molecular characterisation of the Popijač's Yellow Sally, *Isoperla popijaci* sp. nov., a new stenoendemic stonefly species from Croatia (Plecoptera, Perlodidae)

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Abstract

A new species of the Yellow Sally genus (*Isoperla* Banks, 1906) is described, based on morphological (males and females adults, larval and egg) and molecular (the barcode region of the cytochrome c oxidase subunit I gene (*COI*)) features. Popijač's Yellow Sally, *I. popijaci* Hlebec & Sivec, **sp. nov.** inhabits two karstic sources of the Krasulja rivulet in Croatia. Male and female of the new species are characterised by colouration patterns of the head and pronotum; the dimensions of the female subgenital plate; the medial penial armature and oval-shaped egg without collar and anchor. The larvae differ from their congeners by the uniquely coloured head and pronotum. Based on morphological characteristics *I. popijaci* **sp. nov.** belongs to the *I. tripartita* species group. Phylogenetic and taxonomic relationships were reconstructed using three methods of phylogenetic inference and three species delimitation methods. As *I. popijaci* **sp. nov.** occurs at a narrow area of the Krasulja rivulet in Krbava field, the study puts emphasis on the conservation and hotspot importance of the temporary rivers in the Dinaric karst. Furthermore, the study accentuates the necessity for further research on the genetic diversity of Plecoptera in Croatia.

Keywords

Conservation, Dinaric karst, DNA barcoding, *Isoperla popijaci* sp. nov., karstic source, species delimitation

Introduction

Predominantly regarded as a biological indicator of well oxygenated water in freshwater ecosystems (Illies and Schmitz 1980; Hamid and Rawi 2017; Morinière et al. 2017; DeWalt and Ower 2019; Ferreira et al. 2020), stoneflies (Plecoptera) and their absence can indicate pollution, changes in habitat conditions, habitat destruction and climate changes (Urbanič and Toman 2007; Fochetti and Tierno de Figueroa 2008; Bálint et al. 2011). In total, 50 Plecoptera species are reported from Croatia and, due to the many suitable habitats, it is assumed that this number is higher (Popijač and Sivec 2009a, 2009b). Members of the subfamily Perlodinae are, in general, vividly coloured, medium to large-sized, show high genetic diversity and are often microendemic (Zwick 1973, 2004; Li and Murányi 2015). The genus *Isoperla* Banks, 1906 is represented by 188 species worldwide and 60 species in Europe (DeWalt et al. 2020). The genus has a Holarctic and Oriental distribution (Zwick 1973; Szczytko and Stewart 1979; Sandberg and Kondratieff 2013; Szczytko and Kondratieff 2015) and represents the most diverse genus of the family Perlodidae in Europe (Graf et al. 2009, 2018). Thereby, the area of the Balkan stands out as a diversity hotspot with 21 species, of which 12 are endemic to the Peninsula and often restricted to specific habitats (Murányi 2011; Murányi et al. 2016).

Basic characteristics by which the species within the genus *Isoperla* are distinguished are penial morphology, head and pronotal pattern, egg structures and drumming signals (Despax 1936; Illies 1952, 1954, 1966; Sivec and Stark 2002; Murányi 2011; Michalik et al. 2017). In the last few years, a considerable number of new Plecoptera taxa have been described, especially from China (Li et al. 2013; Ji et al. 2014; Li and Murányi 2015; Chen et al. 2019; Cao et al. 2020), but also in Europe, like *Isoperla pesici* Murányi, 2011; *I. autumnalis* Murányi, 2011; *I. citrina* Murányi, 2011 (Murányi 2011; Murányi et al. 2016); *I. vjosae* Graf et Vitecek, 2018 (Graf et al. 2018); *I. claudiae* Graf et Konar, 2014 (Graf et al. 2014) and *I. nagyi* Murányi, Kovács et Graf, 2020 (Murányi et al. 2020).

During fieldwork research since 2004, ten *Isoperla* species were recorded in Croatia. An additional one is here described as *Isoperla popijaci* sp. nov., which shares morphological characteristics of the penial armature with species from the *I. tripartita* species group.

The following study provides a morphological description of the new species: illustrations of the main taxonomical characters (in males, females, larvae and eggs); as well as its phylogenetic placement within the genus based on the mitochondrial cytochrome c oxidase subunit I (*COI*) barcode region as a marker. Moreover, the conservation importance of the intermittent Krasulja rivulet and its watercourse, as well as Dinaric karst (Western Balkan region) is discussed.

Materials and methods

Material collection and preparation.

Adults of *I. popijaci* sp. nov. were collected in June 2019 at the entrance to the Ševerova Cave (karstic source of the intermittent Krasulja rivulet in Krbava field).

A subsequent collecting trip upstream of the Krasulja rivulet (in June 2021), near the karstic source adjacent to the village of Mirići, resulted in finding more specimens of *I. popijaci* sp. nov.

A total of 42 specimens (34 adults and 8 larvae) belonging to *Isoperla popijaci* sp. nov., were collected. Adult specimens were collected using sweep nets, while larval specimens were collected by handpicking. The aedeagus was everted in the field and specimens were fixed and stored in 96% ethanol for morphological and molecular analysis. Morphological characteristics of male terminalia were examined after potassium hydroxide (KOH) treatment.

Type material depository and museum acronyms.

The holotype and part of the paratypes series are deposited in the Croatian Natural History Museum, Zagreb, Croatia (CNHM), Collection of Plecoptera Sivec & Hlebec, while other paratypes are kept in the Slovenian Museum of Natural History, Ljubljana, Slovenia (PMSL).

Photography and drawings.

Photographs, diagnostic characterisation and comparative morphological examination of specimens were made using a ZEISS SteREO DiscoveryV.20 stereomicroscope. Pencil drawings were produced with a camera lucida and then digitally edited and inked. Figures 3A, B, 4A–D (SEM images) were made using a JEOL JSM-7000F scanning electron microscope. The penis (one of paratype specimen) for the SEM study was critical-point dried (Figure 4A–D).

Nomenclature is in accordance with the International Code of the Zoological Nomenclature (ICZN 1999). The species is proposed by following the rules of the Code. Abbreviations for the type specimens are HT–holotype, PT–paratype and PTs–paratypes.

Comparative analysis.

Comparative study on the morphology of penial structures was conducted using ten species belonging to the genus *Isoperla*, collected in Croatia: *I. bosnica* Aubert, 1964; *I. inermis* Kaćanski et Zwick, 1970; *I. rivulorum* (Pictet, 1841); *I. lugens* (Klapálek, 1923); *I. illyrica* Tabacaru, 1971; *I. tripartita* Illies, 1954; *I. grammatica* (Poda, 1761); *I. difformis* (Klapálek, 1909); *I. oxylepis* (Despax, 1936) and *I. albanica* Aubert, 1964. Morphological taxonomic classifications follow the traditional system (Poda 1761; Pictet 1841; Klapálek 1909, 1923; Despax 1936; Illies 1952, 1954, 1966; Aubert 1964; Tabacaru 1971; Kaćanski and Zwick 1970, Murányi 2011; Murányi et al. 2016).

DNA extraction, amplification, and sequencing.

One male, one female and one larva of *Isoperla popijaci* sp. nov. were used in molecular analyses and mutually associated. DNA was extracted from the single leg of specimens using QIAamp DNA Micro Kit (Qiagen, Germany) according to the manufacturer's specifications and eluted in 50 µl of elution buffer. The 5' fragment of the mitochondrial cytochrome c oxidase subunit I gene (*COI*) was amplified using standard PCR-protocols and four sets of primers: LCO-1490/HCO-2198 (Folmer et al. 1994) or C_LepFolF/C_LepFolR (as was used in Hebert et al. 2004) or a combination of MLepF1/LepR1 and MLepR1/LepF1 (yielding two shorter, overlapping fragments as was used in Hajibabaei et al. 2006) in 20 µl reactions. Polymerase chain reactions (PCRs) for all primer sets were carried out using: 1 x DreamTaq reaction buffer with 2 mM $MgCl_2$ (Thermo Fisher Scientific Inc., US), 0.2 mM dNTPs, 0.4 µM of each primer, 0.025 U/µl of DreamTaq polymerase (Thermo Fisher Scientific Inc., US) and 1 µl of eluted DNA. For the first mentioned primers set (LCO-1490/HCO-2198) the following PCR cycling conditions were applied: initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 1 min, followed by a final extension step at 72°C for 10 min. PCR products were purified using Exonuclease I (0.05 U/µl), FastAP Thermosensitive Alkaline Phosphatase (0.025 U/µl) enzymatic system (Thermo Fisher Scientific Inc., US). The reaction was carried using the protocol: 1 h at 37°C followed by 20 min at 80°C. Sequencing was performed by Macrogen Inc. (Amsterdam, The Netherlands) using the amplification primers. Sequences obtained in the study were deposited in the BOLD database (Ratnasingham and Hebert 2007) and GenBank (under the accession numbers MW907977–MW907980, MW907982–MW907988 and MW907990–MW907993).

Sequence data and phylogenetic analysis.

In total, 15 obtained *Isoperla* sequences were checked, edited, assembled from both directions and inspected manually for base-pair ambiguities, as well as stop codons, indels or double peaks in chromatograms (as indicators for the possible erroneous amplification of nuclear mitochondrial pseudogene) in Geneious R6 (https://www.geneious.com). All available *Isoperla* sequences were retrieved from the GenBank and BOLD databases (accessed 10/01/2021) and aligned with sequences from this study using MAFFT v.7 (Katoh and Standley 2013). Any length variants were excluded from the final alignments. Sequences were collapsed into 456 unique *COI* haplotypes using the online tool FaBox v.1.5 (Villesen 2007) and, from all species, the most diverse haplotypes from *I. tripartita* and *I. rivulorum* species group, as well as species *I. lugens*, were retained for further analysis. The final dataset for phylogenetic analysis and species delineation comprised 27 sequences, including 10 haplotypes observed in this study (see Table 1). *Isoperla obscura* (INTAP055-17) and *Taeniopteryx burksi* (08INHSP-002) were selected as outgroups according to the North American Plecoptera phylogeny published by South et al. (2020). Amongst morphologically-defined species, evolutionary divergence was estimated using the pairwise comparison of the uncorrected genetic distances (*p*-distances) in MEGA-X (Kumar et al. 2018). For *p*-distances, a colour heat map was drawn using the Python data visualisation library Seaborn (version 0.11.1, Waskom 2021). Phylogenetic relationships were estimated

Table 1. Collection details and geographical origin of the specimens used in phylogenetic analysis. Haplotypes obtained in this study, marked with asterisk. Paratypes of *Isoperla popijaci* sp. nov. used in molecular analysis marked in bold (male, female and larval). Abbreviations: AL (Albania), AT (Austria), C (Croatia), F (France), G (Germany), M (Montenegro), S (Switzerland). Outgroups (INTAP055-17 and 08INHSP-002) are not shown. Specimen identifier: I. Sivec.

Specimen ID	BOLD/GenBank Process ID	Taxon	Locality	Legit	Coordinates	Publication
*DH71	CROPL066-21	*Isoperla rivulorum*	C: Kupa River, spring	I. Sivec	45°29.47'N, 14°41.36'E	this study
GBOL01391	GBCOU1198-13	*Isoperla rivulorum*	F: Rhone-Alpes, Hauteville	Balke, Morinière, Toussaint, Taenzler, Bellanger, Hoch	45°29.52'N, 6°35.04'E	Morinière et al. (2017)
PE219	INTAP187-17	*Isoperla rivulorum*	AT: Flexenpass	W. Graf	47°09.17'N, 10°09.91'E	–
PE268	INTAP226-17	*Isoperla rivulorum*	AT: Flexenpass	W. Graf	47°09.17'N, 10°09.91'E	–
GBIFCH00280047	PLEAA237-20	*Isoperla rivulorum*	S: Effluent, Pont de Nant	Sartori Michel & Derleth Pascale	46°15.07'N, 7°06.43'E	–
GBOL01390	GBCOU1197-13	*Isoperla rivulorum*	F: Rhone-Alpes, Hauteville	Balke, Morinière, Toussaint, Taenzler, Bellanger, Hoch	45°29.52'N, 6°35.04'E	Morinière et al. (2017)
*DH107	CROPL097-21	*Isoperla illyrica*	C: Trilj, Grab, spring	I. Sivec	43°38.93'N, 16°45.74'E	this study
*DH482	CROPL197-21	*Isoperla illyrica*	C: Trilj, Grab, spring	I. Sivec	43°38.93'N, 16°45.74'E	this study
*DH123	CROPL109-21	*Isoperla tripartita*	C: Cetina River, spring	I. Sivec	43°58.54'N, 16°25.81'E	this study
*DH478	CROPL195-21	*Isoperla tripartita*	C: Cetina River, spring	B. Horvat	43°58.54'N, 16°25.81'E	this study
*DH551	CROPL225-21	*Isoperla tripartita*	C: Papuk, Gospin potok	I. Vučković	45°34.47'N, 17°41.76'E	this study
*DH137	CROPL122-21	*Isoperla tripartita*	C: Trilj, Grab, spring	I. Sivec	43°38.93'N, 16°45.74'E	this study
Itri0101M	VJOSA001-17	*Isoperla tripartita*	AT: Lainzer Tiergarten	O. Zweidick	48°09.57'N, 16°12.83'E	Graf et al. (2018)
Itri0102M	VJOSA002-17	*Isoperla tripartita*	AT: Lainzer Tiergarten	O. Zweidick	48°09.57'N, 16°12.83'E	Graf et al. (2018)
MT348738	GBMNC47893-20	*Isoperla tripartita*	Macedonia	D. Murányi	41°16.07'N, 20°31.24'E	Murányi et al. (2020)
MT348735	GBMNC47896-20	*Isoperla tripartita*	Macedonia	D. Murányi	42°03.14'N, 20°46.92'E	Murányi et al. (2020)
MT348732	GBMNC47899-20	*Isoperla tripartita*	Macedonia	D. Murányi	40°58.78'N, 21°15.22'E	Murányi et al. (2020)
DH129	CROPL115-21	*Isoperla popijaci* sp. nov.	C: Ševerova Cave	I. Sivec	44°40.78'N, 15°37.87'E	this study
DH130	CROPL116-21	*Isoperla popijaci* sp. nov.	C: Ševerova Cave	I. Sivec	44°40.78'N, 15°37.87'E	this study
***DH926**	CROPL249-21	*Isoperla popijaci* sp. nov.	C: Ševerova Cave	D. Hlebec	44°40.78'N, 15°37.87'E	this study
DH142	CROPL127-21	*Isoperla* PL	C: Plitvice Lakes, Drakulića River	I. Sivec	44°46.87'N, 15°39.47'E	this study
DH143	CROPL128-21	*Isoperla* PL	C: Plitvice Lakes, Drakulića River	I. Sivec	44°46.87'N, 15°39.47'E	this study
*DH538	CROPL214-21	*Isoperla* PL	C: Plitvice Lakes, Drakulića River	M. Kučinić, I. Vučković	44°46.87'N, 15°39.47'E	this study
DH541	CROPL217-21	*Isoperla* PL	C: Plitvice Lakes, Drakulića River	M. Kučinić, I. Vučković	44°46.87'N, 15°39.47'E	this study
*DH629	CROPL230-21	*Isoperla* PL	C: Plitvice Lakes, Drakulića River	M. Kučinić, I. Vučković	44°46.87'N, 15°39.47'E	this study
Itri0201M	VJOSA003-17	*Isoperla vjosae*	AL: Vjosa River, Kutë	S. Vitecek, W. Graf	40°28.35'N, 19°44.94'E	Graf et al. (2018)
Itri0202M	VJOSA004-17	*Isoperla vjosae*	AL: Vjosa River, Kutë	S. Vitecek, W. Graf	40°28.35'N, 19°44.94'E	Graf et al. (2018)
Itri0301M	VJOSA005-17	*Isoperla vjosae*	AL: Vjosa River, Kutë	S. Vitecek, W. Graf	–	–
Itri0302L	VJOSA006-17	*Isoperla vjosae*	AL: Vjosa River, Kutë	S. Vitecek, W. Graf	40°28.35'N, 19°44.94'E	Graf et al. (2018)
Ipe0101M	VJOSA007-17	*Isoperla pesici*	M: Redice	W. Graf	42°53.02'N, 19°18.95'E	Graf et al. (2018)
Ipe0102F	VJOSA008-17	*Isoperla pesici*	M: Redice	W. Graf	42°53.02'N, 19°18.95'E	Graf et al. (2018)
GBOL17507	GBMIX2517-15	*Isoperla lugens*	G: Nationalpark Berchtesgaden	R. Gerecke	47°33.48'N, 12°48.24'E	Morinière et al. (2017)
PE031	INTAP025-17	*Isoperla lugens*	AT: Koerbersee	W. Graf	47°16.09'N, 10°07.66'E	–
PE269	INTAP227-17	*Isoperla lugens*	AT: Flexenpass	W. Graf	47°09.17'N, 10°09.91'E	–

by three different optimality criteria: Neighbour Joining (NJ), Maximum Likelihood (ML) and Bayesian Inference (BI). NJ and ML were performed in MEGA-X (Kumar et al. 2018), while BI in MrBayes 3.2.7. (Ronquist et al. 2012). For ML and BI, the optimal model of nucleotide evolution (Hasegawa-Kishino-Yano model with gamma distributed rate variation amongst sites and a significant proportion of invariable sites: HKY+I+G) was selected under the Bayesian Information Criterion (BIC) using jModelTest 2.1.5 (Darriba et al. 2012). Nodes in the phylogenetic trees with bootstrap values P ≥ 70 in NJ and ML and posterior probabilities values pp ≥ 0.90 in BI were considered well supported. NJ was made using the Kimura-2-parameter (K2P) model of nucleotide substitution with the pairwise deletion option. Bootstrap support was inferred using the fast bootstrap algorithm, based on 5000 replicates. Nearest-Neighbour-Interchange (NNI), a heuristic method using the fast bootstrap algorithm, was used in ML with 1000 replicates.

For BI, the dataset was partitioned by codon positions. Two separate runs with four Metropolis-coupled Monte Carlo Markov chains (MMCM) were performed for 10 million generations while trees were sampled every 1000 generations with the first 25% of sampled trees discarded as burn-in. The remaining trees were used to create a 50% majority rule consensus tree. TRACER v.1.7.1 (Rambaut et al. 2018) was used to check the convergence between the two runs. The phylogenetic trees were visualised using FigTree v.1.4.3. (Rambaut 2009) and iTOL v.5 (Letunic and Bork 2021). Several methods of species delimitation were applied: the Automatic Barcode Gap Discovery (ABGD) method (Puillandre et al. 2012), the Bayesian implementation of the Poisson Tree Processes (bPTP) method (Zhang et al. 2013) and the multi-rate Poisson Tree Process (mPTP) method (Kapli et al. 2017). The ABGD was performed at the web server by using the K2P model. All values were set to default, except the value of relative gap width, which was set to 1, while the default gap width of 1.5 resulted in a single group. The bPTP method was performed on the web server at http://species.h-its.org, while the mPTP method was run on the web server at http://mptp.h-its.org/. Both methods were applied using default parameters, outgroups have been removed from the analysis and the same ML input tree was used.

Results

Taxonomic part

New species description

Isoperla popijaci **Hlebec & Sivec sp. nov.**
http://zoobank.org/60B76C3E-14C2-4D5D-9587-C1931C87952B
Figures 1A–E, 2A–G, 3A, B, 4A–D

Material examined (1♂ HT, 10♂♂ PTs, 23♀♀ PTs and 8 larvae PTs): 1♂HT (96% ethanol) Original label: Croatia, Lika, Krbava field, Krasulja rivulet, karstic source Ševerova Cave; 44°40.78'N, 15°37.87'E, 21 June 2019, I. Sivec leg. (CNHM: CPSH);

6♂♂ **PTs** and 11♀♀ **PTs** (96% ethanol) same data as for the holotype; 5 larvae **PTs** (96% ethanol) 09 April 2015, I. Sivec leg.; 3 larvae **PTs** (96% ethanol) 22 February 2021, D. Hlebec leg.; 1♂**PT** and 3♀♀ **PT**s (96% ethanol) 2 June 2021, I. Sivec leg.; 3♀♀ **PTs** (96% ethanol) 18 June 2021, D. Hlebec leg.; 3♂♂ **PTs** and 6♀♀ **PTs** (96% ethanol) karstic source nearby village Mirići, 44°43.14'N, 15°38.09'E, 2 June 2021, I. Sivec leg.

Type material depository. HT (1♂) and 31 PTs (7♂♂+18♀♀+6 larvae) in Zagreb, Croatia (CNHM), Collection of Plecoptera Sivec & Hlebec, under accession number CPSH 1–32; and 10 PTs (3♂♂+5♀♀+2 larvae) in Ljubljana, Slovenia (PMSL).

Type locality. Croatia, Lika, Krbava field, Krasulja rivulet, karstic source Ševerova Cave, 44°40.78'N; 15°37.87'E; 640 m a.s.l.

Diagnosis. The new species *I. popijaci* sp. nov. belongs to the *I. tripartita* species group, with divided medial penial armature into upper and lower coloured portions. It has, however, a specific penial armature on the ventral lobe of the penis, different from all known *Isoperla* species. The upper medial armature is subdivided, and the lower medial armature is present in two scale spike-like areas. The proximal part has a pair of drop-shaped areas armoured with spines, longer at the tip and shorter at the base. The medial penial armature with a field of shorter spines as in Figure 4C. Only a few irregular spines on the lateral side of the penis in the area of the upper armature of the penis.

Description. Macropterous in both sexes, medium-sized species with yellow head and pronotum.

Adult. Body length: **HT** male 18.5 mm; **PTs**: males 17–19 mm (n = 10), females 16.5–18 mm (n = 23).

Forewing length: **HT** male 12 mm; **PTs**: males 11–13.5 mm, females 11.5–14 mm.

Colouration. General colour uniformly brownish (Figure 1C), slightly paler ventrally and laterally.

Head. The central part of the head pale yellowish; darker at the lower part and between ocelli; slightly darker in the frontal and lateral part. M-line and tentorial callosities weakly expressed and inconspicuous. Pale spot positioned centrally between the ocelli, paler in the central distal part of the head. Eyes slightly smaller than the area delimited by the three ocelli. Scape and pedicel dark brown. Palpi uniformly cream coloured. The distal part of the antennae pale and the proximal segments darker (Figures 1A, 2A).

Wings. Wings translucent brownish, venation dark brown.

Pronotum. Pronotum yellowish, rectangular with angled edges. Medial and lateral parts of the pronotum pale; central part on both sides slightly darker and with dark brown textured surface (Figures 1A, 2A).

Mesothorax and metathorax. Ventral surface of thorax uniformly brownish; dorsal side slightly darker, lateral part lighter. Mesonotum and metanotum predominantly dark brown.

Legs. Femora and tibia brownish, same as body colouration. Tarsi slightly darker than femora and tibia on the dorsal side and pale ventrally.

Male abdomen. Mesobasisternum and metabasisternum brown in the middle and darker laterally. Ventral surface of male abdomen uniformly brownish, slightly darker dorsally. A few proximal segments of cerci pale, with rest dark brown.

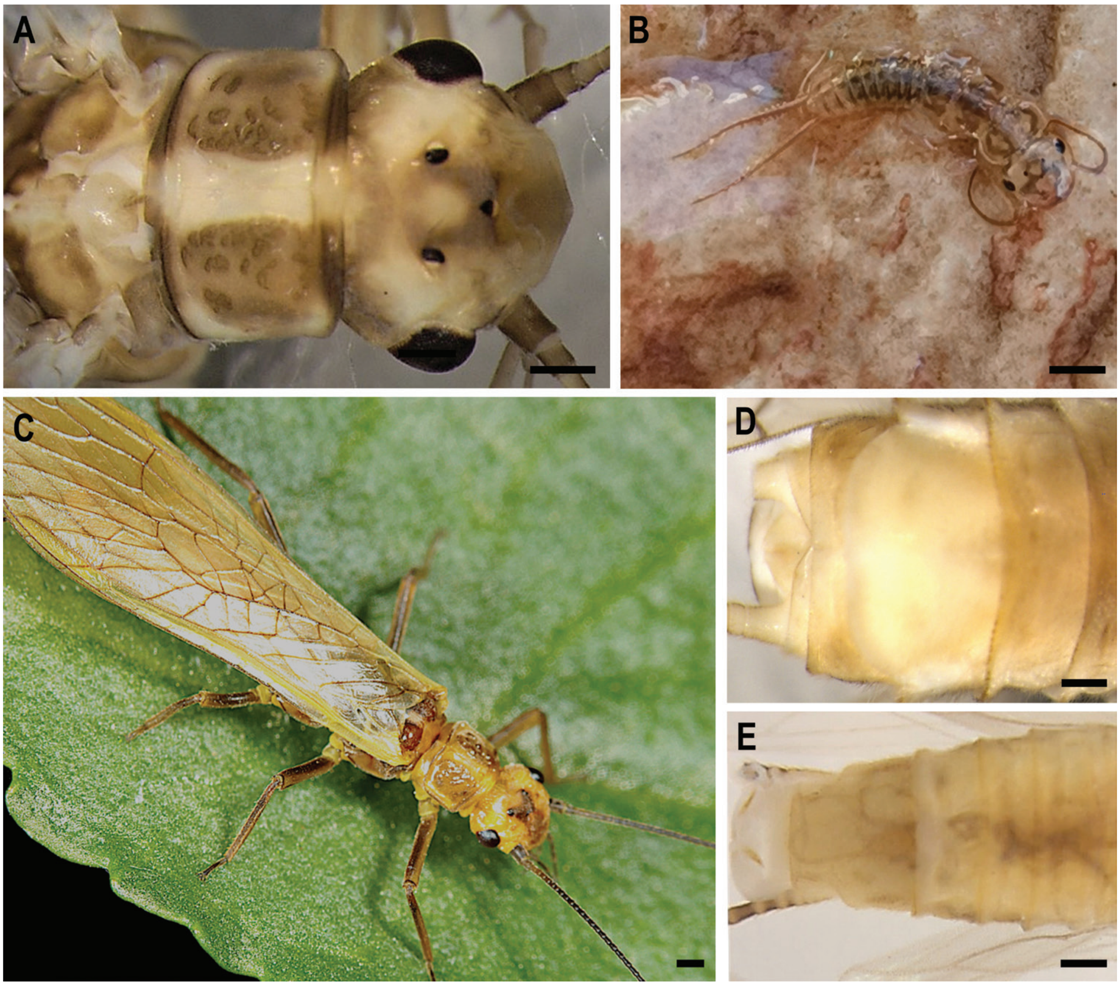

Figure 1. Morphology of *Isoperla popijaci* sp. nov. **A** head and pronotum in dorsal view (adult male HT) **B** habitus (larval PT) **C** habitus (adult male PT) **D** female terminalia in ventral view (PT) **E** everted male copulatory organ (HT). Scale bar: 0.5 mm **A–E**.

Penis (everted). Divided into four lobes, with a basal section in everted position. The medial penial armature on the ventral surface of the penis divided into an upper and a lower part, both are coloured (Figures 2C, G, 4A), upper part rather pale. The upper medial penial armature is further subdivided into left and right arms, elongated, delimited from scales of the lateral lobes (Figure 4C). Length of the arms is 200–250 μm, width 100–120 μm. Scales of the upper medial penial armature forming a drop-shaped area, spike-like, with longer scales at the tip and shorter ones at the base. Length of the scales 25–37 μm, width 7–9 μm at the base. The lower part of the medial penial armature subdivided, with an irregular upturned V-shaped area and bearing very short spines (Figure 4D). Length of the areas 220–250 μm, width 100–140 μm. The scales are spike-like, thinner than in the upper medial armature. The ventral lobe hemispherical, covered with hair-like scales, in some places ciliated scales. The medial lobe small with diverse scales. Lateral penial armatures located on the lateral lobes, above the basal section, small and indistinct with only a few spines. Detail of the lateral lobe as in Figure 4B.

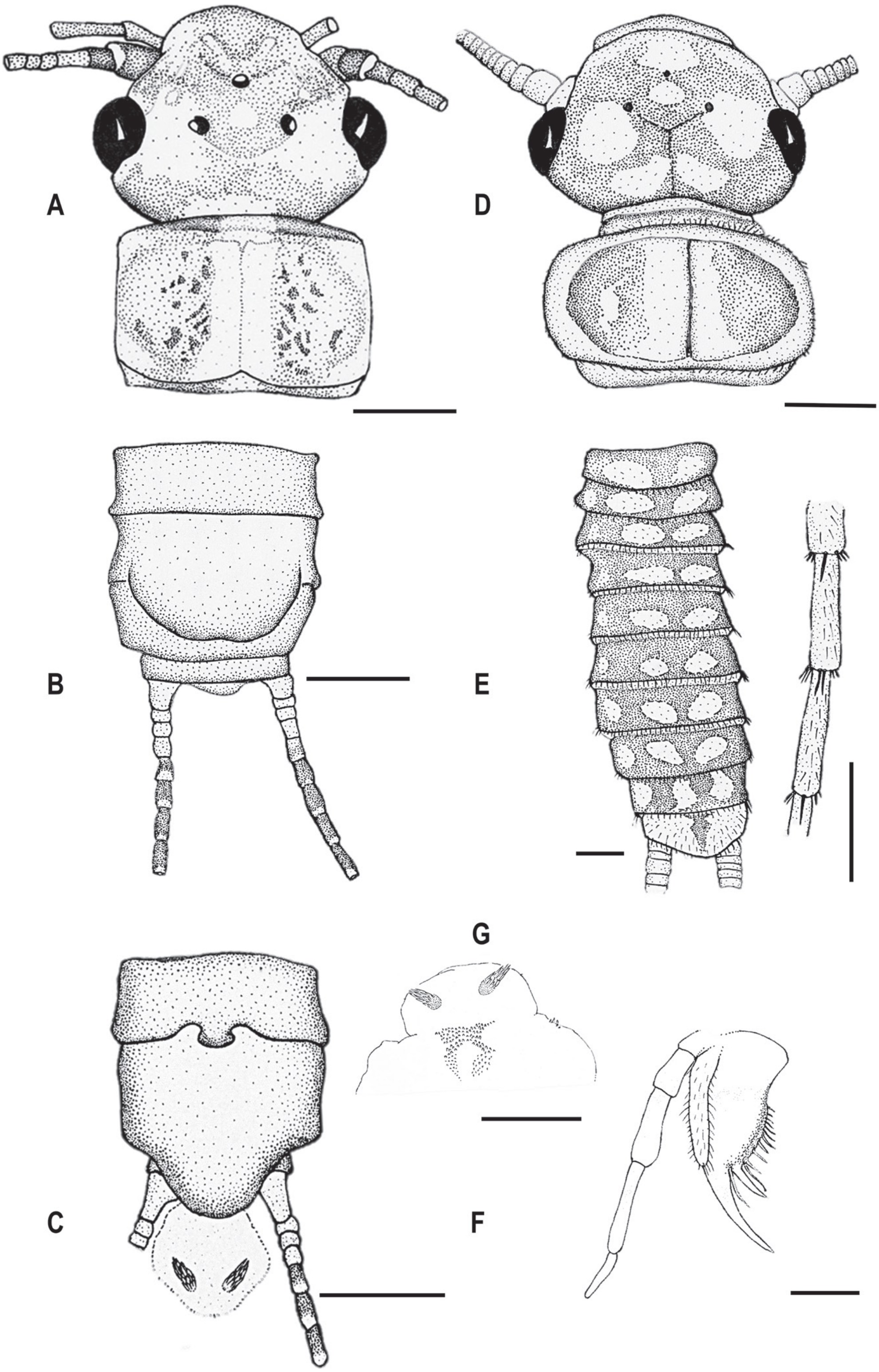

Figure 2. Morphology of *Isoperla popijaci* sp. nov. **A** head and pronotum in dorsal view (adult female PT) **B** terminalia in ventral view (adult female PT) **C** terminalia in ventral view (adult male HT) **D** head and pronotum in dorsal view (larval PT) **E** abdomen in dorsal view and detail of a distal segment of a cercus (larval PT) **F** right maxilla in dorsal view (larval PT) **G** penial armature (adult male HT). Scale bars: 1 mm (**A–D**); 0.5 mm (**E–G**).

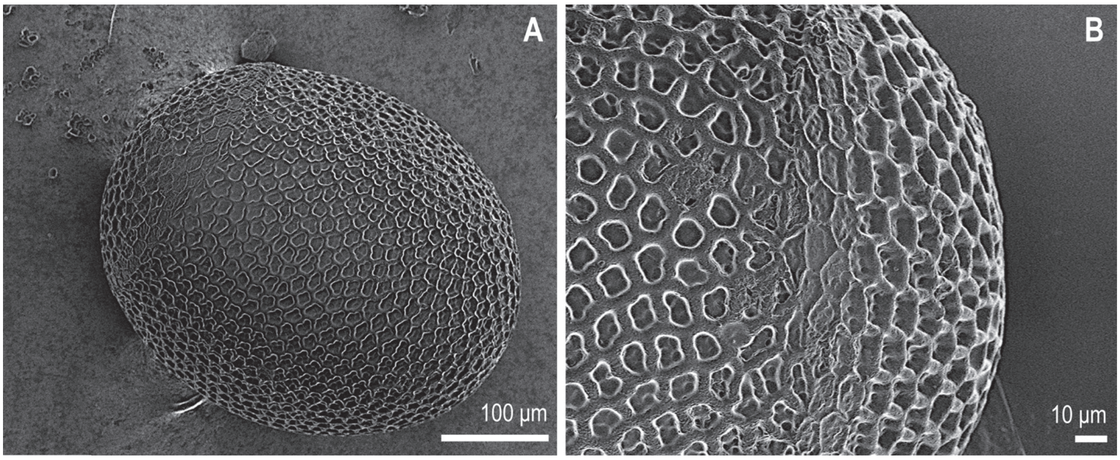

Figure 3. Egg of *Isoperla popijaci* sp.nov. **A** whole egg, lateral view **B** detail of hatching line, lateral view

Female abdomen. All tergites uniformly brownish. Sternites slightly paler brownish. A few basal segments of cerci pale, rest of cerci dark brown. Subgenital plate large and wide reaching near the end of sternite IX (widely concave in the middle) (Figure 2B).

Egg. Chorion light brown, 0.34–0.38 mm long and 0.29–0.33 mm wide (n = 22). Chorion with marked ornamentation of irregular round shape. Follicular cell impressions with finer inner punctations. Hatching line distinct. Micropyles not well recognisable. Collar and anchor missing (Figure 3A, B).

Larva. Body length of not-completely-mature larva 14–16 mm (n = 8). General colour pale brownish; with darker markings on head and abdomen. Body and legs typically pilose. Swimming hairs present on femora, tibiae and tarsi. Posterior abdominal fringe short and cercal fringe no longer than width of cercal segment. General colour of the head brownish, with a darker transversal mask connecting eyes and ocelli (Figure 2D). M line indistinct. Eyes well developed. Mouth parts and basal parts of antennae pale coloured; distal part of antennae dark brownish. Lacinia bidentate; inner margin with 4–5 stout setae and a row of short thin setae below subapical tooth. Pronotum rounded; brownish; with indistinct darker pattern centrally and distinctly paler laterally (Figure 2D). Pronotal setal fringe with short bristles and bearing only a few longer setae at posterior margin. Ventral side of the body and leg pale coloured. Abdominal tergites darker, brown with a pair of relatively large drop-shaped pale spots in the middle of the abdomen (Figure 2E). Paraprocts and cerci uniformly pale. Setation on distal section of cercal segments with rather uniform setae and single larger dorsal setae.

Etymology. The specific name is the genitive singular of the Latinised version of the surname Popijač (Popiacus, -i, m.), given in honour of colleague Dr Aleksandar Popijač and his achievements in field research and knowledge of the Plecoptera fauna in Croatia.

Distribution and ecology. The species was collected at the entrance to the Ševerova Cave, occasional karstic source of the intermittent Krasulja rivulet in Krbava field and two year later (on 2 June 2021) near the karstic source of the same rivulet,

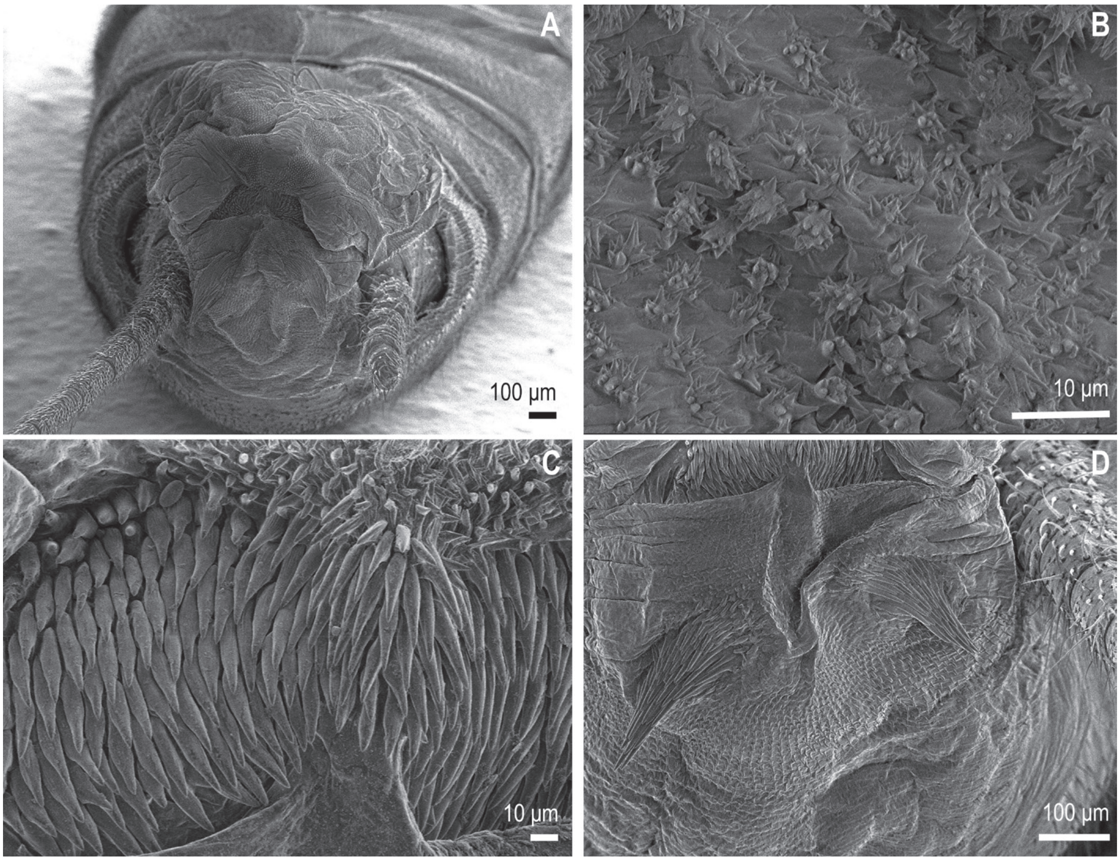

Figure 4. Extruded penis of *Isoperla popijaci* sp. nov. **A** male abdomen with extruded penis, ventral view **B** detail of penial armature on the lateral lobe, dorsal view **C** scales of the upper medial penial armature, dorsal view **D** pair of the scales spike-like on the ventral lobe, dorsal view.

near the village of Mirići. The Ševerova Cave (old name Hrnjakova Cave) is located on the northern edge of the Krbava field (karst field located near settlement Krbavica in the vicinity of the Plitvice Lakes National Park). The temporary Krasulja rivulet is part of the hydrogeological system of the Krbavica River (Figure 5E). For several months a year, the water runs from the cave and forms the Krasulja rivulet, which flows into the Krbavica River and sinks on the south side of the field. When the discharge of the Krasulja falls below 60 l/sec, the water-flow ceases from Ševerova Cave (Malinar and Čepelak 2009). The stream does not have a rich stonefly fauna and the species found at this locality, except the newly-described species of *Isoperla*, are *Amphinemura standfussi* (Ris, 1902) and *Nemoura cinerea* (Retzius, 1783). The substrate at the collection site of larvae was mainly composed of larger fractions.

Conservation status

The new species should probably be regarded as Critically Endangered (CR) or Vulnerable (VU) by the IUCN Criteria. Up to now, it is known only from the areas nearby two karstic sources.

Figure 5. Type locality of the Popijač's Yellow Sally, *Isoperla popijaci* sp. nov.**:** Ševerova Cave in Croatia **A** and **B** photographs in wet phase **C, D** photographs in dry phase **E** map (blue circle indicates type locality).

Phylogenetic part

The alignment of *COI* gene sequences was 658 bp in length and comprised of 202 variable sites, of which 139 were parsimony informative. Three implemented criteria of phylogenetic reconstruction (NJ, ML and BI) resulted in congruent topologies with highly similar support values (Figure 6), characterised by the presence of two deeply divergent lineages, *I. popijaci* sp. nov. and "*Isoperla* PL", which did not cluster with any of the currently defined taxa.

Mitochondrial *COI* sequences, obtained from *I. popijaci* sp. nov. (adults and larva), were identical (a single unique haplotype). The monophyly of the newly-described species is highly supported (Figure 6). This species represents the first branch-off within the clade comprised of monophyletic *I. lugens* and *I. rivulorum* subclades, as well as another tentative new taxon obtained in this study (clade designated as "*Isoperla* PL" with representatives CROPL214-21 and CROPL230-21). The designation "PL" denotes the abbreviation Plitvice Lakes, nearby where a specimen was found. Five sequences of "*Isoperla* PL" represent 2 haplotypes (CROPL214-21 and CROPL230-21) with low intraspecific uncorrected *p*-distance (0.0096).

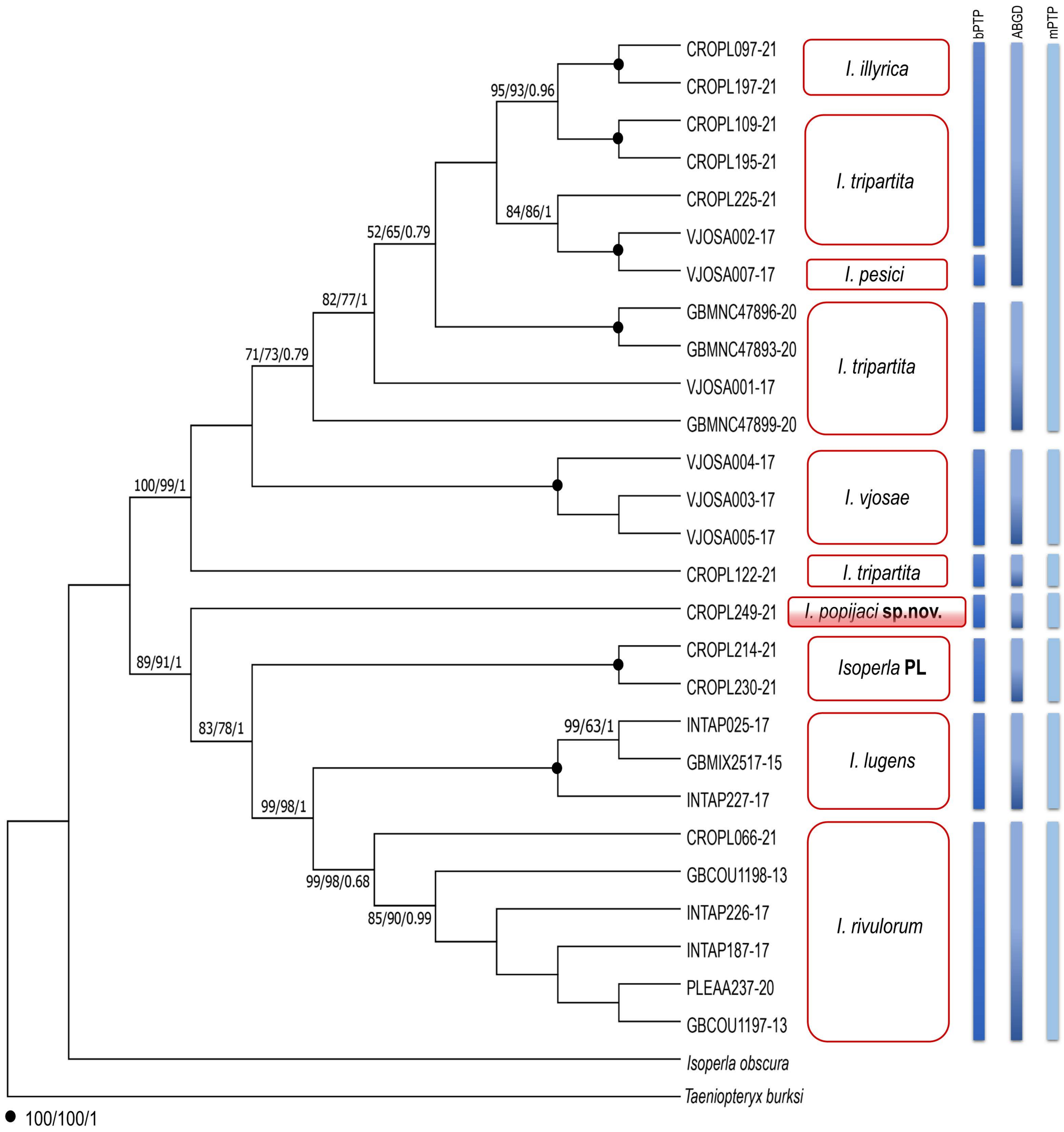

Figure 6. Maximum Likelihood cladogram, based on the analysis of the *COI* haplotypes of *Isoperla* species. Numbers at the nodes indicate Neighbour-Joining (NJ), Maximum Likelihood (ML) bootstrap support values (BS) and Bayesian posterior probabilities (BPP), respectively. The results of species delimitations are represented with the vertical bars, from left to right, indicate the OTUs inferred by bPTP, ABGD and mPTP. "*Isoperla* PL" indicates additional separate lineage obtained in this study. Terminal codes present BOLD/GenBank Process ID, as in Table 1.

Intraspecific uncorrected *p*-distances are as follows for the following species: 0.32–1.59% in *I. rivulorum*, 0.16–0.48% in *I. lugens*, 0.01–7.82% in *I. tripartita*, 0.32% in *I. vjosae* and 0.16% in *I. illyrica*. Interspecific uncorrected *p*-distances for *I. popijaci* sp. nov. ranged from 6.69–12.59%; specifically, 6.69–7.17% to *I. rivulorum*, 8.15–8.45% to *I. lugens*, 9.99–10.22% to *I. vjosae*, 10.4–12.6% to *I. tripartita*, 10.38–12.61% to *I. illyrica*, 10.69% to *I. pesici* and 8.12% to the "*Isoperla* PL" (Figure 7). Overall, observed intraspecific genetic distances within the genus ranged from 0.01–7.82%.

Within the *I. rivulorum* clade, Croatian sample CROPL066-21 appeared as a separate lineage, subdivided from Alpine specimens (Figure 6). The uncorrected *p*-distances for sample CROPL066-21 are in the range 1.28–1.59% to other *I. rivulorum* samples.

A well-supported clade comprised two newly-discovered lineages (*Isoperla popijaci* sp. nov. and "*Isoperla* PL"), together with *I. lugens* and *I. rivulorum*, and was recovered in all three tree-building algorithms.

According to the results of the first molecular characterisation of *I. illyrica* obtained in this study, specimens clustered in a within the monophyletic clade with intraspecific uncorrected *p*-distances of 0.16%. Interspecific *p*-distances between *I. illyrica* and *I. tripartita* ranged from 0.96–5.91%.

All species delimitation analyses (bPTP, ABGD and mPTP) for mtDNA (*COI*) have delineated two well-separated lineages *Isoperla popijaci* sp. nov. and "*Isoperla* PL" as tentative species. Applied methods resulted in various numbers of delineated groups. In the ABGD analysis, initial partitioning identified eight, while recursive partitioning showed the existence of nine putative species for the majority of prior intraspecific divergence values (P). The mPTP method delimited seven operational taxonomic units (OTUs) and, according to these results, is the most conservative approach, while the bPTP recognised 9 OTUs.

Contrary to ABGD and bPTP, the mPTP analysis shows *I. illyrica*, *I. tripartita* and *I. pesici*, morphologically assigned to *I. (tripartita)* species group, as a single OTU. These species are completely separated into three OTUs in the bPTP analysis. The separation of sample CROPL122-21 as a distinct species (*I. tripartita*) was supported by all three species delimitation methods.

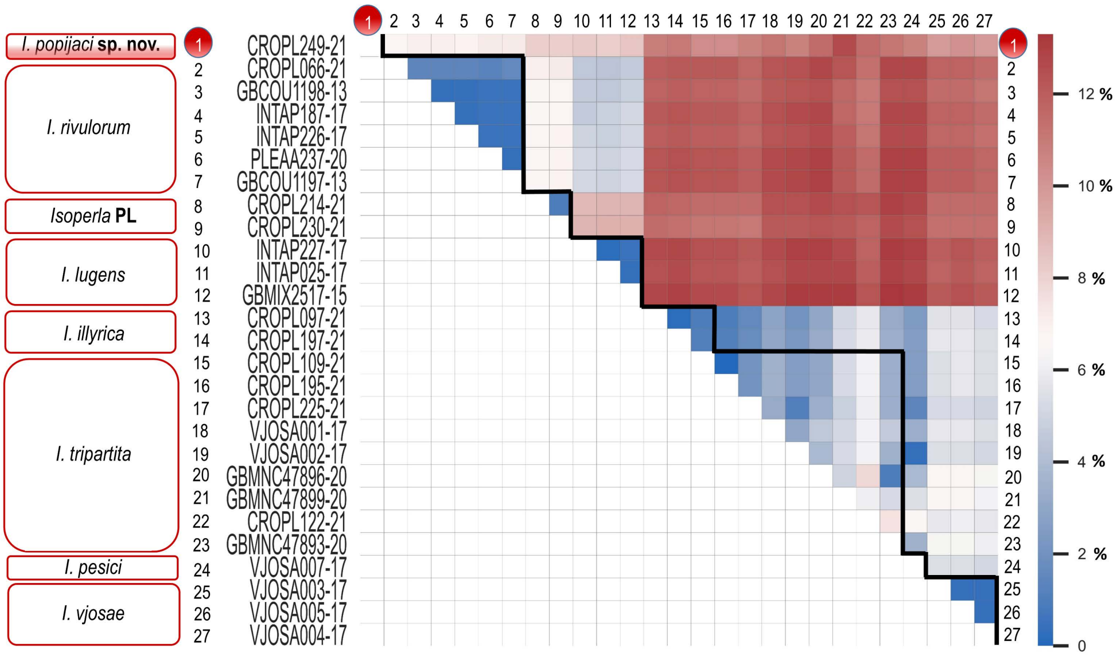

Figure 7. Colour heat map showing inter- and intraspecific uncorrected *p*-distances of the mitochondrial cytochrome oxidase subunit I (*COI*) barcode region. *Isoperla popijaci* sp. nov. and *Isoperla* PL appear as highly divergent. Intraspecific *p*-distances are outlined by the black line.

Discussion

Phylogeny and genetic diversity

The lowest interspecific *p*-distance between *I. popijaci* sp. nov. and *I. rivulorum* was found to be 6.69%, indicating distinct species. This exceeds intraspecific divergences (ISD ≥ 2%) commonly used as one of the criteria for a delimitation of closely-related species in aquatic insects: Ephemeroptera, Plecoptera and Trichoptera (Ball et al. 2009; Zhou et al. 2009). Values above 2% have already been reported amongst Plecoptera (Zhou et al. 2010, Gill et al. 2015), which was probably caused by poor mobility of some Plecoptera species (Boumans and Baumann 2012) and, consequently, geographical isolation among populations.

The finding of the second well-separated lineage ("*Isoperla* PL"), most closely related to species *I. rivulorum* (interspecific *p*-distance from 6.54–7.19%) implies existence of another new species of the genus *Isoperla* (unpublished data). Taxa obtained in this study (*Isoperla popijaci* sp. nov. and "*Isoperla* PL") are separated by a large interspecific *p*-distance of 8.12%. Future research will seek to determine whether this value has repercussions to the geographical isolation and specificity of the (micro-) habitats in which the taxa were found.

Based on the occurrence of *I. lugens* (alpine species) and *I. rivulorum* (alpine, central European species) in the Dinaric karst and their appearance as the most recently diverged lineages within *I. popijaci* + "*Isoperla* PL" + *I. lugens* + *I. rivulorum* clade (Figure 6), it can be assumed that the Dinaric karst might represent the area of origin of those alpine species as well as the diversification centre from where they spread northwards. However, to test this hypothesis, data across the whole distributional range and use of other molecular markers (mitochondrial and nuclear as well) are necessary.

To establish a final phylogenetic relationship in the monophyletic *I. tripartita* species group, it is necessary to collect specimens from its entire range and use a multigene molecular approach as well.

Previous research showed the wide range of variability in intraspecific divergence within the order Plecoptera (Zhou et al. 2009; Gill et al. 2015; Stark et al. 2015) and uncorrected intraspecific *p*-distances from our study (0.01–7.82%) are consistent with the previously reported values.

Systematic implications

Based on the morphological characteristics, the new species can be assigned to the *Isoperla tripartita* species group. The *I. tripartita* species group is characterised by the divided medial penial armature (into upper and lower coloured portions, divided or subdivided) and lateral penial armatures (Illies 1954; Murányi 2011; Murányi et al. 2016). Popijač's Yellow Sally is characterised by divided medial penial armature, with the distal part bearing short spines, but with indistinct lateral penial armature. The genetic distinction, in combination with morphological features, is significantly different from all other species and promotes *I. popijaci* sp. nov. as a new species.

Phylogenetic reconstructions support the monophyly of the *I. tripartita* species group, which is, together with *I. grammatica*, notable by the high morphological variability of certain species (Zwick 1978; Murányi 2011). In Croatia, significant morphological variability has been also observed in *I. inermis* from different localities (personal observation), of which some are very similar to *I. difformis* (Central European species) in the penial armature. Therefore, future studies should investigate relationships between and within *Isoperla* populations from the Balkan Peninsula (e.g. Cetina River, National Park Plitvice Lakes, Kupa River and nearby springs in Slovenia) by applying a multi-gene approach.

Other species are somewhat less variable and occupy smaller distributional areas (as recently described species from Europe and Asia). Those endemics are of special interest to our study because it is assumed that more endemics species are likely to be discovered, especially in poorly-explored areas with high biodiversity like the Balkans. More new species are expected to be found in Croatia, as the majority of the country's territory has not been studied yet regarding Plecoptera.

Anthropogenic activities have already resulted in the reduction of population size (especially larger species from the genera *Perla*, *Dinocras* and *Perlodes*) (personal observation). All the above-mentioned calls for more detailed studies of species distributional patterns, as well as of genetic diversity of populations. Emphasis should also be put on the isolated habitats (karst areas) as they can have the highest conservation value as refugium and the maintenance of genetic diversity.

Cave-dependent stoneflies?

Until now, Popijač's Yellow Sally is known to inhabit the parts of the rivulet close to two karstic sources, of which one is a cave entrance. Although there are no true troglobionts within the order Plecoptera, several species have been found to inhabit stream sources around the openings of caves (for example *I. inermis*) and there are no records of these species from the downstream part of the same stream. Another example is *Brachyptera tristis* (Klapálek, 1901), a species that spends its entire life cycle underground (the stream of Krupa River) (personal observations). It is, hence, important to pay special attention to the research of caves, pits, underground and temporary rivers and streams that abound in the Dinaric karst geology. These habitats host some of the most complex and diverse faunas (Culver and Sket 2000) as a consequence of composite geological history and the intensive process of karstification (Sket 1999). The Balkan Peninsula is known for its high biodiversity (Sket et al. 2004), especially of aquatic species (Kryštufek et al. 2007, Previšić et al. 2009, 2014; Murányi 2011; Vitecek et al. 2015; Kučinić et al. 2017). It can be expected that future research will contribute to the discovery of biodiversity patterns as well as new species, especially microendemic species (Graf et al. 2009, 2012; Kučinić et al. 2013; Vitecek et al. 2017). Karst habitats, such as Ševerova Cave, represent some of the most dynamic freshwater habitats, especially in terms of biological-geological interactions (Ridl et al. 2018). With the alternation of wet and dry phases and temporal dynamics of water flow, temporary rivers have a great influence on local ecological interactions, both in aquatic and terrestrial habitats (Larned et al. 2010). It is a significant assumption that climate change will increase the duration and frequency of dry phases, so it is expected that this will lead to the disappearance of taxa whose entire life cycle (or at least part of it) is related to aquatic environments (Larned et al. 2010).

Conclusions

Isoperla popijaci sp. nov. is probably a stenoendemic Yellow Sally species found at two karstic sources of the intermittent Krasulja rivulet in Lika (Croatia), which has morphological characteristics similar to species from the *I. tripartita* species group. Phylogenetic analysis revealed the well-supported sister-group relationship of *I. lugens* and *I. rivulorum* and a basal position of *I. popijaci* sp. nov. relative to this clade. Considering its restricted distribution, *Isoperla popijaci* sp. nov. should have the highest priority in conservation efforts.

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Publication II

DNA barcoding for biodiversity assessment: Croatian stoneflies (Insecta: Plecoptera)

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ABSTRACT

Background: The hemi-metabolous aquatic order Plecoptera (stoneflies) constitutes an indispensable part of terrestrial and aquatic food webs due to their specific life cycle and habitat requirements. Stoneflies are considered one of the most sensitive groups to environmental changes in freshwater ecosystems and anthropogenic changes have caused range contraction of many species. Given the critical threat to stoneflies, the study of their distribution, morphological variability and genetic diversity should be one of the priorities in conservation biology. However, some aspects about stoneflies, especially a fully resolved phylogeny and their patterns of distribution are not well known. A study that includes comprehensive field research and combines morphological and molecular identification of stoneflies has not been conducted in Croatia so far. Thus, the major aim of this study was to regenerate a comprehensive and taxonomically well-curated DNA barcode database for Croatian stoneflies, to highlight the morphological variability obtained for several species and to elucidate results in light of recent taxonomy.

Methods: A morphological examination of adult specimens was made using basic characteristics for distinguishing species: terminalia in males and females, head and pronotum patterns, penial morphology, and egg structures. DNA barcoding was applied to many specimens to help circumscribe known species, identify cryptic or yet undescribed species, and to construct a preliminary phylogeny for Croatian stoneflies.

Results: Sequences (658 bp in length) of 74 morphospecies from all families present in Croatia were recovered from 87% of the analysed specimens (355 of 410), with one partial sequence of 605 bp in length for *Capnopsis schilleri balcanica* Zwick, 1984. A total of 84% morphological species could be unambiguously identified using *COI* sequences. Species delineation methods confirmed the existence of five deeply divergent genetic lineages, with monophyletic origin, which also differ morphologically from their congeners and represent distinct entities. BIN (Barcode Index Number) assignment and species delineation methods clustered *COI* sequences into different numbers of operational taxonomic units (OTUs). ASAP delimited 76 putative species and achieved a maximum match score with morphology (97%). ABGD resulted in 62 and mPTP in 61 OTUs, indicating a more conservative approach. Most BINs were congruent with traditionally recognized

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species. Deep intraspecific genetic divergences in some clades highlighted the need for taxonomic revision in several species-complexes and species-groups. Research has yielded the first molecular characterization of nine species, with most having restricted distributions and confirmed the existence of several species which had been declared extinct regionally.

Subjects Biodiversity, Entomology, Evolutionary Studies, Taxonomy, Zoology
Keywords DNA barcode library, Plecoptera, Morphology, Species delimitation, Bioindicators, COI, Mitochondrial DNA, Water quality

INTRODUCTION

With 3,800 described species across 17 families (*Fochetti & Tierno de Figueroa, 2008*; *DeWalt et al., 2021*; *South et al., 2021*), the ancient (*Béthoux et al., 2011*) hemi-metabolous insect order Plecoptera, commonly known as stoneflies, represents an important component of freshwater ecological systems and terrestrial and aquatic food webs (*Fochetti & Tierno de Figueroa, 2008*; *South et al., 2020*). Stoneflies are widely distributed on all continents except Antarctica (*Zwick, 2000*) and their range and abundance have declined rapidly in the last 30 years in Central Europe (*Fochetti & Tierno de Figueroa, 2006*; *Bojková et al., 2012*), mainly due to anthropogenic influences (*i.e.*, habitat destruction and pollution) (*Fochetti & Tierno de Figueroa, 2008*; *Bálint et al., 2011*). Typical stonefly habitat is a lotic system characterized by cold, fast flowing and well-oxygenated water (*Sivec & Yule, 2004*) in the mountains of temperate regions. Typically, stoneflies are macropterous or brachypterous, but several species, especially males at higher elevations are apterous or micropterous (*Illies, 1966*). Due to their poor dispersal capacity (*Lee et al., 2022*), stoneflies are ideal organisms for biogeographical studies (*Murányi, 2011*; *Graf et al., 2012*; *Pessino et al., 2014*; *Stevens, Bishop & Picker, 2018*; *Gamboa et al., 2019*).

Since Plecoptera taxa exhibit high degrees of endemism and morphological diversity (*Fochetti & Tierno de Figueroa, 2006*; *Murányi, 2011*; *Murányi, Kovács & Orci, 2016*), regional field research has both local and global value. Prior to 2009 when concerted efforts in field research began, only 28 species of stoneflies were recorded in Croatia (*Sivec, 1980*, *1985*), a surprisingly low number considering the many suitable habitats (*Illies, 1978*) and compared to checklists for neighbouring countries: Slovenia with more than 100 species (*Sivec, 2001*), Bosnia and Herzegovina with 73 species and subspecies (*Kaćanski, 1976*), Montenegro with 57 species (*Murányi, 2008*), Hungary with 61 species (*Andrikovics & Murányi, 2001*) and Serbia with 90 documented species (*Petrović et al., 2014*). Greater efforts in Croatia resulted in 50 species (*Popijač, 2008*; *Popijač & Sivec, 2009a*), but still studies were limited to narrow areas such as Plitvice Lakes National Park (*Popijač & Sivec, 2009b*; *Ridl et al., 2018*), Cetina River (*Popijač & Sivec, 2009b*), Čabranka and Gerovčica Rivers (*Popijač & Sivec, 2009c*) and lower reaches of the Una River and its tributaries (*Popijač & Sivec, 2011*). In Plitvice Lakes National Park during the above-mentioned studies, some specimens were recorded that could not be assigned with certainty to known species: *Perlodes* cf. *intricatus*, (Pictet, 1841) *Isoperla* cf. *lugens* (Klapálek, 1923), *Leuctra* cf. *pusilla* Krno, 1985, *Leuctra* sp., *Nemoura* sp., *Protonemura* sp., *Isoperla* sp. and *Perlodes* sp.

Also, during these studies, several remarkable species were documented: *Marthamea vitripennis* Burmeister, 1839, which was re-discovered again after one century (*Sivec, 1985*; *Popijač & Sivec, 2011*), *Perla burmeisteriana* Claassen, 1936 (*Popijač & Sivec, 2009a*), *Besdolus imhoffi* Pictet, 1841 (*Popijač & Sivec, 2010*) and *Protonemura julia* Nicolai, 1983 (*Popijač & Sivec, 2009c*).

Currently, the most widely accepted system of stonefly classification is by *Zwick (2000)*, with two recognized suborders: Arctoperlaria and Antarctoperlaria. To resolve deeper phylogenetic relationships, research has highlighted the need for molecular data, which in the last years, at least in part, has helped overcome morphology-based identification limitations. *South et al. (2020)* produced the most complete molecular phylogenetic study of stoneflies based on the North American fauna, following several studies with limited taxon sampling (*Thomas et al., 2000*; *Chen et al., 2018*; *Wang et al., 2018*; *Ding et al., 2019*). Allopatric diversification, driven mostly by glaciation and orogenesis, has been the main contributor to current diversity patterns in Plecoptera (*Zwick, 2000*; *Weiss, Stradner & Graf, 2011*; *Theissinger et al., 2013*).

DNA barcoding, which uses sequence diversity in the standardized 658-bp region of the mitochondrial cytochrome c oxidase subunit I (*COI*) gene to aid in species identification and circumscription, is a useful tool for associating immature life stages and to identify cryptic species. Such an approach is extremely important for biodiversity assessment (*Hebert et al., 2003a*, *2004*; *Hebert, Ratnasingham & deWaard, 2003b*; *Valentini, Pompanon & Taberlet, 2009*; *Morinière et al., 2017*). The application of DNA barcoding has aided species delimitation in various groups of organisms and has pointed to divergent haplotypes and hybridization (*Van Velzen et al., 2012*; *Sziválk et al., 2017*; *Zangl et al., 2019*, *2021*). Once a set of barcodes for a group of organisms is established, examination of previously unidentified specimens is greatly facilitated (*DeSalle, 2006*; *Ratnasingham & Hebert, 2007*). However, it has been observed that the results of DNA barcoding could be confounded (*Havemann et al., 2018*) due to *Wolbachia* infections (*Werren, Zhang & Guo, 1995*), incomplete lineage sorting (*Petit & Excoffier, 2009*), pseudogenes (*Ribeiro Leite, 2012*), introgressive hybridization and recent speciation (*Raupach et al., 2014*). DNA barcoding has proved to be a great tool for identification of species from the EPT (Ephemeroptera, Plecoptera and Trichoptera) group (*Gill et al., 2014*; *Ball et al., 2005*; *Webb et al., 2012*; *Morinière et al., 2017*; *Kučinić et al., 2020*). Furthermore, the analysis of DNA barcoding results is often difficult due to deposited barcode sequences without scientific species names, known as “dark taxa”, which represent groups of organisms characterized with a lack of taxonomic expertise or undescribed species (*Page, 2016*; *Ryberg & Nilsson, 2018*). Additionally, results from DNA barcoding and the traditional taxonomic approach can be incongruent and this is not surprising given the process of evolution itself (*Hendrich et al., 2010*, *2014*). Therefore, for efficient species delimitation, it is necessary to analyze multiple character systems and use integrative taxonomy (*Vitecek et al., 2017a*; *Zhang et al., 2013*).

Conducted in the framework of the project *DNA barcoding of Croatian faunal biodiversity*, the present study aims at: (i) developing a DNA reference barcode library for the Croatian stonefly fauna with macrophotographs of 26 species, (ii) getting first insight into inter- and intraspecific genetic diversity, (iii) assessing morphological variability of stoneflies in Croatia, (iv) highlighting localities with high biodiversity, especially in isolated habitats of the Dinaric Karst area to assist conservation planning and strategies for protecting the genetic diversity of stoneflies, and (v) filling the gaps in the Barcode of Life Data System database (BOLD). Furthermore, the study will contribute to knowledge about species distribution, species complexes and systematic and phylogenetic relationships.

MATERIALS AND METHODS

Taxon sampling

Specimen collection was conducted with approval from the Ministry of Economy and Sustainable Development of the Republic of Croatia (UP/I-612-07/21-48/73). A total of 337 stonefly specimens (Data S1, Table S1) from 95 different localities in Croatia and eighteen specimens from seventeen localities in Slovenia (Fig. S1) were used in phylogenetic analysis. Information regarding species determinations and details about sampling sites can be found in Table S1. Adult specimens were collected using sweep nets and beating sheets, while larval specimens were collected by handpicking. The aedeagus of males was everted in the field and specimens were fixed and stored in 96% ethanol for morphological and molecular analysis. Morphological characteristics of male terminalia were examined after KOH treatment (*Stark & Gaufin, 1978*).

Comparative study on morphology was made using specimens kept in the Collection of stoneflies in the Slovenian Museum of Natural History, Ljubljana, Slovenia (PMSL). Newly collected specimens are deposited in the Croatian Natural History Museum, Zagreb, Croatia (CNHM), under the Collection of Plecoptera Sivec & Hlebec (CPSH). Voucher information for individuals used in phylogenetic analysis are publicly accessible in BOLD (dx.doi.org/10.5883/DS-CROPL) and GenBank under the accession numbers as listed in Table S1.

Individuals were sorted and identified using a Leica Wild M3Z stereomicroscope. Macrophotographs were taken using a Canon EOS 5D Mark II. Morphological examination was made using descriptions and identification keys: *Illies, 1955*; *Kaćanski & Zwick, 1970*; *Raušer, 1980*; *Ravizza, 2002*; *Sivec & Stark, 2002*; *Graf & Schmidt-Kloiber, 2003*; *Zwick, 2004*; *Murányi, 2011*. The most reliable diagnostic characters for species determination were terminalia in males and females, head and pronotum patterns, penial armatures and egg structures for species within the genus *Perla*.

DNA extraction, gene amplification and sequencing

The number of specimens per species for *COI* marker amplification ranged from one to 24 (*Isoperla inermis* Kaćanski & Zwick, 1970; see Table S1). Tissue was dissected from a single leg for each specimen and genomic DNA was extracted from recently collected samples using the Sigma GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, MI, USA) and from specimens older than 10 years using the QIAamp DNA Micro Kit (Qiagen, Hilden, Germany) and eluting in 50 µl of elution buffer to increase DNA yield. A partial region of the mitochondrial cytochrome c oxidase subunit I gene (*COI*, the DNA barcode region, *Hebert et al., 2003a*) was amplified using two sets of primers: (a) LCO-1490/HCO-2198 (*Folmer et al., 1994*) or (b) C_LepFolF/C_LepFolR (*Folmer et al., 1994*; *Hebert, Ratnasingham & deWaard, 2003b*). For samples older than 10 years, the DNA barcode region was amplified as shorter overlapping fragments with primer set: (c) MLepF1/LepR1 and MLepR1/LepF1 (*Hajibabaei et al., 2006*). All polymerase chain reactions (PCRs) were carried out in a total volume of 20 µl with the PCR mixture prepared following *Hlebec et al. (2021)*. Thermocycling conditions are given in Table S2. PCR products were purified using Exonuclease I (0.05 U/µL) and FastAP (0.025 U/µL) enzymatic system (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The reaction was carried out using the following conditions: 37 °C for 1 hr followed by 80 °C for 20 min. Bi-directional sequencing was done by Macrogen Inc. (Amsterdam, Netherlands), using amplification primers.

Sequence editing and phylogenetic analyses

In total, 355 obtained sequences were checked and inspected manually for base pair ambiguities, indels and stop codons in Geneious Prime 2021.2 (Biomatters, Auckland, New Zealand) to confirm overall sequences quality. Sequences were aligned using MAFFT ver. 7 (*Katoh & Standley, 2013*). The final alignment for the *COI* gene fragment was 658 bp in length (Data S2). Sequences were collapsed into 268 *COI* haplotypes using the online tool FaBox ver. 1.5 (*Villesen, 2007*). Evolutionary divergence was estimated using uncorrected pairwise genetic distances (*p*-distances) in MEGA-X ver. 10.2.6 (*Kumar et al., 2018*) (Figs. 1A–1D, mean values are shown in Table S3). Phylogenetic analyses were performed using Maximum likelihood (ML) optimality criteria in IQ-TREE2 (*Minh et al., 2020*) under a GTR + I + G optimal model of nucleotide evolution (as determined by jModelTest ver. 2.1.10 (*Darriba et al., 2012*) under the Bayesian information criterion (BIC)) and bootstrapping with 2,000 ultrafast bootstrap replicates (*Hoang et al., 2018*) (Fig. 2). ABMAY005-09 (Heptageniidae) and ABMAY015-09 (*Stenacron interpunctatum* Say, 1839) were selected as outgroups. Additionally, phylogenetic relationships between haplotypes were inferred using the Neighbour joining (NJ) method in MEGA-X ver. 10.2.6 (*Kumar et al., 2018*) (Fig. S2) and Bayesian inference (BI) in MrBayes ver. 3.2.7 (*Ronquist et al., 2012*) *via* the online CIPRES Science Gateway ver. 3.3 (*Miller, Pfeiffer & Schwartz, 2010*) (Fig. S3). The NJ analysis was performed using the Kimura-2-parameter (K2P) model with the pairwise deletion option. Bootstrap support was inferred using the fast bootstrap algorithm, based on 5,000 replicates. For the BI, two separate runs with four Markov chain Monte Carlo (MCMC) simulations were performed for 10 million generations, sampling every 1,000 generations, using ABMAY005-09 (Heptageniidae) as the root and discarding the initial 25% of the trees as burn-in. Remaining trees were used to create a 50% majority rule consensus tree, with nodal values representing the posterior probabilities. TRACER ver. 1.7.1 (*Rambaut et al., 2018*) was used to check the convergence between the two runs. Phylogenetic trees were visualized using FigTree ver. 1.4.3 (*Rambaut, 2009*) and iTOL ver. 5 (*Letunic & Bork, 2021*). Existence of a barcoding gap (distance between the mean intraspecific sequence variability and interspecific variability for congeneric *COI* sequences) was ascertained using the "Barcode Gap

Analysis" tool, provided in BOLD, using the Kimura-2-Parameter (K2P) distance metric (*Puillandre et al., 2012b*) (Figs. 1A–1D, Table S4). To visualize phylogeographic relationships among specimens from the largest observed lineage, a median joining (MJ) network (*Bandelt, Forster & Röhl, 1999*) among 12 haplotypes (Fig. 3, Data S3) was generated using the program PopART ver. 1.7 (*Leigh & Bryant, 2015*) with default settings. Six different alignments were created for additional phylogenetic (ML in IQ-TREE2 with settings as above, under the optimal model of nucleotide evolution as is listed in Table S5) and species delineation analysis in order to interpret the results and to check the plausibility. We applied these analyses to: (a) species with high levels of intraspecific morphological variability (*Perlodes intricatus* (Pictet, 1841) and *Isoperla inermis*), (b) specimens that differed from the described morphospecies (*Isoperla* cf. *lugens*, *Protonemura hrabei* (Raušer, 1956) and *Taeniopteryx* n.sp. CRO-1) and (c) species, which were taxonomically interesting (*Besdolus imhoffi* (Pictet, 1841) and *B. illyricus* Kovács & Zwick, 2008). Analyses were performed with sequences of these species and their closely related congeners from BOLD (*Ratnasingham & Hebert, 2007*, http://www.boldsystems.org) and GenBank (https://www.ncbi.nlm.nih.gov/) databases (accessed 20 August 2021). Accession numbers of all sequences used in additional analysis are listed in Datasets 1–6, Table S5. Molecular species delineation was achieved through four different methods: the BIN (Barcode Index Number) assignment tool on the BOLD server using the refined single linkage (RESL) algorithm (*Ratnasingham & Hebert, 2013*), ABGD (Automatic Barcode Gap Discovery) (*Puillandre et al., 2012a*), ASAP (Assemble Species by Automatic Partitioning) (*Puillandre, Brouillet & Achaz, 2021*) and mPTP (Multi-rate Poisson Tree Processes) (*Kapli et al., 2017*). All methods clustered *COI* sequences into Operational Taxonomic Units (OTUs) based on sequence similarity.

Members of a BIN usually belong to a species recognized using traditional morphological analysis and taxonomy (*Hendrich et al., 2014*) and species assignment is based on a universal upper threshold for intraspecific distances (*e.g.*, 2.2%) (*Ratnasingham & Hebert, 2013*). BIN counts are usually used for species richness (*Hebert et al., 2016*), but single-locus delineation methods tend to oversplit by mistaking different lineages within populations as putative species (*Muster & Michalik, 2020*; *Meier et al., 2021*). Use of the "BIN Discordance" tool on BOLD, provides insight into the concordance between barcode sequence clusters and species designations.

Automatic Barcode Gap Discovery (ABGD) was carried out on the web server (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html), applying the K2P model and using default parameters, except for the relative gap width, which was set to X = 1.0. ASAP was also carried out on the web server (https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html) using *p*-distances with default settings. The mPTP method, run on the web server (http://mptp.h-its.org/), was implemented using default settings and inputting the ML tree from IQ-TREE2 (*Minh et al., 2020*), as the starting tree.

Validity and reliability of the generated DNA barcode library was evaluated by comparing classical taxonomy with the counts of OTUs from the various species delineation methods.

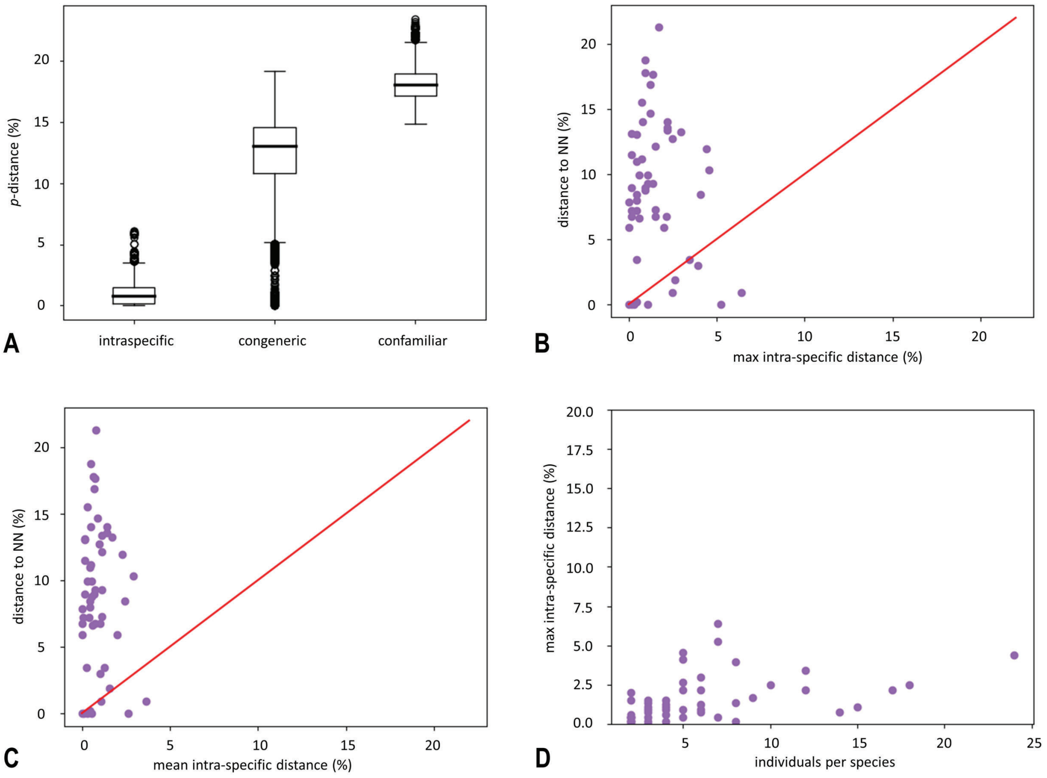

Figure 1 Box plot of uncorrected pairwise genetic distances (*p*-distances) (A) and results of "Barcode Gap Analysis" (B–D). (A) Sorted by distance category: intraspecific (specimens that belong to the same species), congeneric (specimens belonging to different species, but to the same genus) and confamiliar (specimens that belong to the same family). Boxes indicate interquartile range (IQR: between upper (Q3) and lower (Q1) quartile). Black bars designate medians, whiskers indicate values within 1.5 × IQR beneath Q1 or 1.5 × above Q3. Circles depict outliers (above or below 1.5 × IQR). (B) The barcode gap for 74 species of Croatian stoneflies shown by plotting maximum intraspecific distance against interspecific (nearest-neighbour) distance. Dots above the diagonal indicate species with a barcode gap. (C) Scatterplot plots the mean intraspecific distances against the minimum interspecific distances. (D) Scatterplot plots the number of individuals in each species against their maximum intraspecific distances.

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RESULTS

Field sampling and morphological identification produced 74 species of stoneflies in seven families and nineteen genera. Four species (*Capnopsis schilleri balcanica*, *Zwicknia rupprechti* Murányi, Orci & Gamboa, 2014, *Zwicknia bifrons* (Newman, 1838) and *Nemoura sciurus* Aubert, 1949) were identified by comparing with available sequences in the BOLD and GenBank databases.

Barcode sequencing was successful for 355 of 410 (87%) individuals. All *COI* sequences were 658 bp in length, except for one sequence of *Capnopsis schilleri balcanica*, which was 605 bp in length.

Molecular results were largely congruent with morphological assessment such that 62 of the 74 morphological species (84%) could be unambiguously identified using *COI* sequences alone. The median number of barcodes per species was four, and seventeen predominantly rare species were known only from single specimens.

The average confamilial *p*-distance was 20.8% (ranging from 18.2–23.4%) while the average congeneric distance was 15.6% (ranging from 12.1–19.2%). The mean intraspecific *p*-distance was 1.3% (ranging from 0–6.8%) (Fig. 1A). The maximum intraspecific distance of 6.8% was obtained for *Isoperla illyrica* Tabacaru, 1971. Most individuals were above the barcoding gap, meaning that for each individual the difference between the distance to the NN (Nearest Neighbour) and the distance to the furthest conspecific is above zero (Figs. 1B and 1C).

The mean intraspecific *p*-distance distribution is partially overlapping with distance to the nearest neighbour distribution (Fig. 1C), but for most species, nearest-neighbour distances were on average several times higher than maximum intraspecific distances (Fig. 1B). Maximum intraspecific *p*-distance was positively correlated with the number of individuals per species (Fig. 1D) (the correlation coefficient (ρ) = 0.447). For several neighbour species pairs, maximum intraspecific *p*-distance values were higher than their nearest-neighbour distance: *Leuctra albida* Kempny, 1899/*Leuctra mortoni* Kempny, 1899; *Leuctra fusca* (Linnaeus, 1758)/*Leuctra albida*; *Perla pallida* Guérin-Méneville, 1838/*Perla marginata* (Panzer, 1799) and *Isoperla illyrica*/*Isoperla tripartita*. For the following neighbour species pairs, *p*-distance to the nearest neighbour was below 2%: *Leuctra albida*/*Leuctra mortoni*, *Nemoura* cf. *rivorum* Ravizza & Ravizza Dematteis, 1995/*Nemoura flexuosa* Aubert, 1949, *Perla illiesi* Braasch & Joost, 1973/*Perla burmeisteriana*, *Perla* sp./*Perla marginata*, *Perla pallida*/*Perla marginata* and *Isoperla illyrica*/*Isoperla tripartita*.

This study resulted in five entities, which morphologically differ from their congeners and genetically appeared as separate lineages. These species therefore represent candidates for new, previously undescribed species: *Leuctra* cf. *prima* Kempny, 1899 (clade No. 7 in Fig. 2, distance to NN = 9.3%), *Leuctra* cf. *inermis* Kempny, 1899 (clade No. 4 in Fig. 2, distance to NN = 3.6%), *Protonemura* cf. *autumnalis* Raušer, 1956 (clade No. 72 in Fig. 2, distance to NN = 8.7%), *Isoperla* cf. *lugens* (clade No. 37 in Fig. 2, distance to NN = 6.7%) and *Taeniopteryx* n.sp. CRO-1 (clade No. 47 in Fig. 2, distance to NN = 7.1%) (Fig. 2, Table S4). Another separate lineage was recently described *Isoperla popijaci* Hlebec & Sivec, 2021 (clade No. 35 in Fig. 2, distance to NN = 6.7%) (*Hlebec et al., 2021*).

All methods used for phylogenetic reconstruction (ML (Fig. 2), NJ (Fig. S2) and BA (Fig. S3)) recovered the same, well-supported topology. All methods grouped phenotypically defined species in distinct, highly supported monophyletic species clusters (ultrafast bootstrap support >99). Phylogenetic relationships above the species level are in concordance with morphology-based hypotheses. All genera were monophyletic, while analyses resulted in unresolved phylogenetic relationships between families Capniidae and Taeniopterygidae.

Obtained sequences were allocated to 85 BINs (of which 29 were unique to BOLD), and delimited OTUs were mostly consistent with the clustering pattern observed in the ML tree, which was also concordant with morphological identification. Twenty-six BINs were represented by a single individual (singletons).

One BIN often belongs to a single species delineated by traditional taxonomy (*Hausmann et al., 2013*), and every different case can be an incentive for re-evaluation of

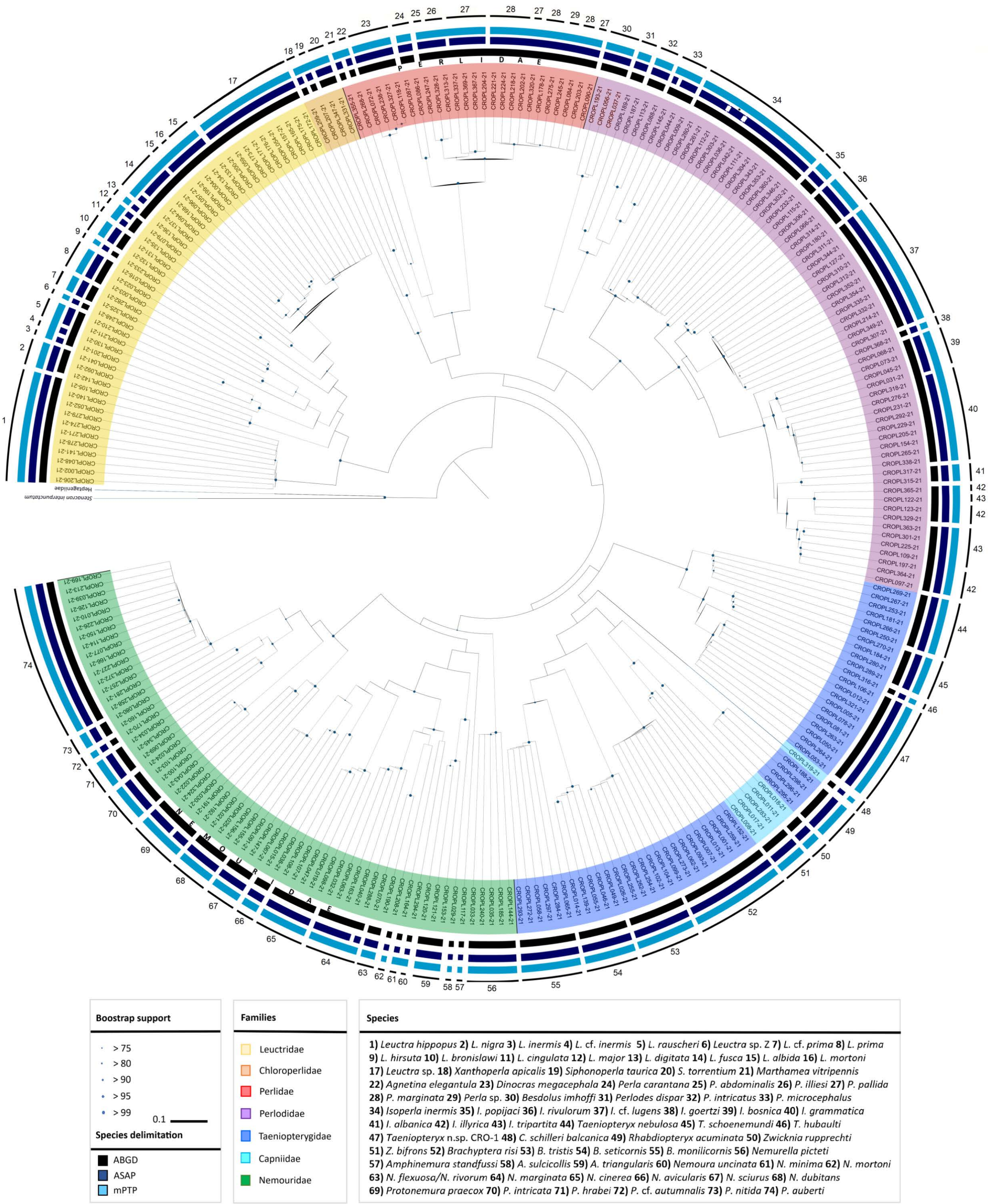

Figure 2 Circular maximum-likelihood (ML) phylogram from analysis of the released dataset and results of species delineation methods. Maximum likelihood phylogeny based on the DNA barcoding region (5′ fragment of the mitochondrial *COI* gene). Species are colour-coded by family. Dots on nodes represent ultrafast bootstrap support values (BS) categories and the size of dots is proportional with the value. The results of species delineations are represented with the bars in different colours and indicate the OTUs inferred by ABGD, ASAP and mPTP methods. Terminal codes present BOLD IDs, as in Table S1. An asterisk indicates two tentative species within *Isoperla inermis* specimens inferred by ASAP method. The tree was annotated in FigTree ver. 1.4.3 (*Rambaut, 2009*) and iTOL ver. 5 (*Letunic & Bork, 2021*) and finished in Adobe Illustrator.

Full-size DOI: 10.7717/peerj.13213/fig-2 morphological and molecular data (*Hendrich et al., 2014*). Specimens of several species showed deep *COI* divergence resulting in multiple BINs within a species: *Protonemura praecox* (Morton, 1894) (BOLD:AEH4111 and BOLD:AEH7722), *Perlodes microcephalus* (Pictet, 1833) (BOLD:AAL2343 and BOLD:AEH5507), *Nemoura marginata* Pictet, 1836 (BOLD:AAN1631, BOLD:AEH3564 and BOLD:AEK9273), *Isoperla illyrica* (BOLD: AEH3875 and BOLD:AEH7030), *Leuctra fusca* (BOLD:AAE6442 and BOLD:ACY3863), *Leuctra albida* (BOLD:AAM4011 and BOLD:AEH5504), *Leuctra hippopus* Kempny, 1899 (BOLD:ACL7184 and BOLD:AEH4770), *Isoperla tripartita* (BOLD:AEH3875, BOLD: AEH3876, BOLD:AEG6510 and BOLD:AEH7030), *Isoperla grammatica* (Poda, 1761) (BOLD:AEH6396 and BOLD: AEG4373) and *Isoperla inermis* (BOLD:ACS6073, BOLD: AAZ7905 and BOLD:AEH8653). Intraspecific *p*-distances were as follows for the following species: *P. praecox* (0–3.2%), *P. microcephalus* (0–4.2%), *N. marginata* (0–4.8%), *Isoperla illyrica* (0.002–6.8%), *Leuctra fusca* (0–5.8%), *Isoperla tripartita* (0–6.6%), *Isoperla grammatica* (0–5.0%) and *Isoperla inermis* (0–4%).

A shared BIN assignment was obtained within three genera: *Isoperla, Perla* and *Nemoura*, and species *Isoperla illyrica/Isoperla tripartita, Nemoura flexuosa/Nemoura* cf. *rivorum* and *Perla pallida/Perla* sp./*Perla marginata*.

Overall, use of different species delineation algorithms resulted in a different number of putative species. ASAP, with the best ASAP-score (6.00) which was achieved at a distance threshold of 2.5%, delimited 76 putative species and achieved a maximum match score with morphology (97%). The partition with the second-best ASAP-score (8.50, distance threshold 3.6%) delimits 70 species and the third partition (9.00, distance threshold 5.0%) delimits 64 species. The ABGD method delineated 62 putative species, while mPTP delineated 61 putative species. ABGD and mPTP represent conservative estimates of DNA species. Results of all species delineation methods are shown in Fig. 2.

Median joining (MJ) network depicted relatedness and distribution within the newly obtained divergent lineage (also unique to BOLD) named as *Isoperla* cf. *lugens*. The MJ network among 12 unique haplotypes which were separated by a different number of mutational steps is shown in Figs. 3A and 3B. The MJ network also revealed low haplotype sharing among sampling sites. Haplotypes CROPL311-21, CROPL344-21, CROPL127-21, CROPL310-21, CROPL312-21 and CROPL352-21 grouped together in a well-supported subclade (Fig. 2, Fig. 3B). Haplotype CROPL352-21 (discovered in Vitunjčica River) was separated by five mutational steps from the closest haplotype that was recorded in Drakulić rijeka, Crna rijeka and Čabranka River. The remaining seven haplotypes also grouped together in a well-supported subclade (Fig. 2, Fig. 3B).

DISCUSSION

The present study represents the first comprehensive research combining morphological and molecular identification of stonefly species in Croatia and establishes DNA barcoding as an effective tool for reliable species identification. Such an approach enhances taxonomic resolution and assists in the quality of faunal research and can be used in discovering cryptic diversity and species complexes (*Zangl et al., 2021*). A taxonomically, well-curated, DNA barcode library can democratize species level work for non-experts

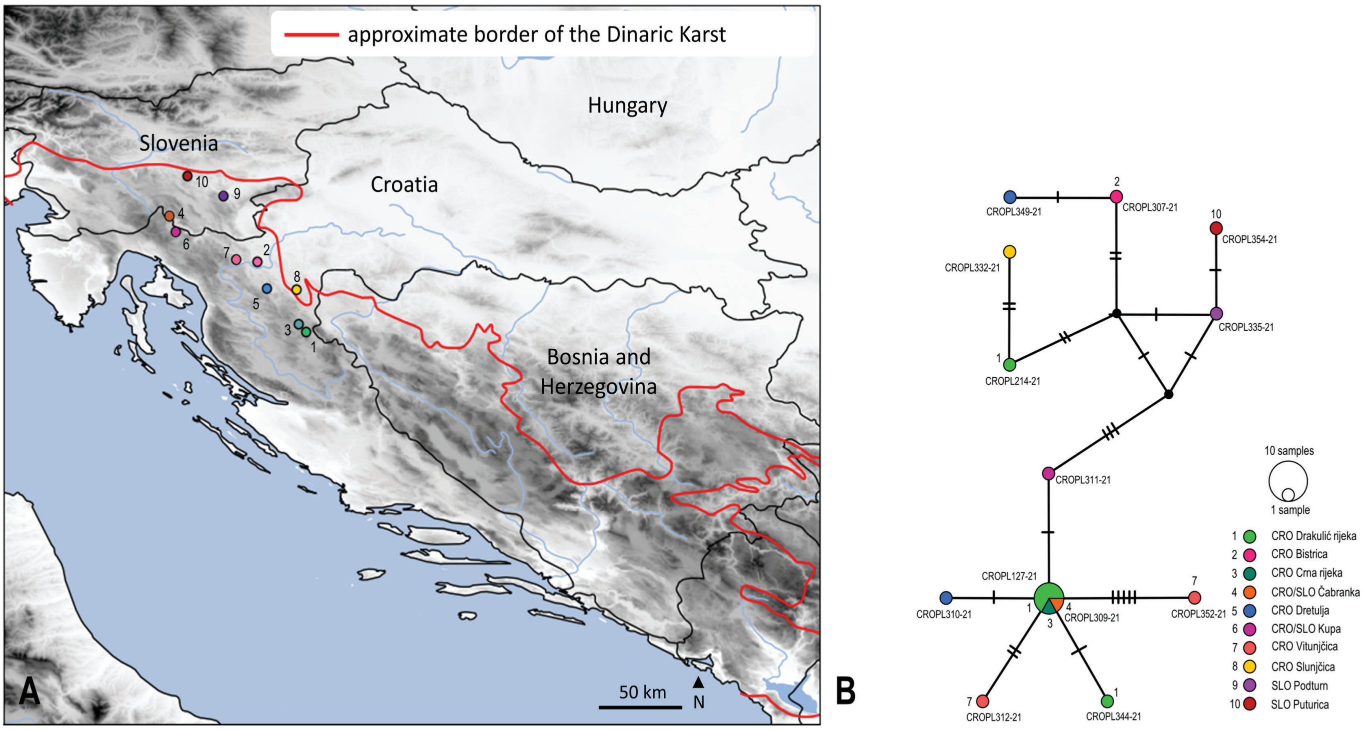

Figure 3 **Sampling sites and median-joining network of 658 bp long *Isoperla* cf. *lugens COI* sequences.** (A) Map of Croatia and neighbouring countries with sampling localities (colour coding matches insert in 3B). (B) MJ network of *COI* sequences. Colours and numbers indicate different sampling localities. Numbers of mutational steps are given as hatch marks. The black dots indicate the extinct ancestral or unsampled haplotypes. Frequencies of the haplotypes are proportional to the size of the circles. Haplotypes are labelled with BOLD IDs, as in Table S1. Approximate border of the Dinaric Karst according to *Gams (2004)*. Map is produced with Cartopy package 0.19 in Python with use of elevation data from *European Union, Copernicus Land Monitoring Service (2016)*. In order to distinguish similar colours we included additional number coding.

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with application in ecological research and in conservation biology across taxa, communities, and ecosystems. Other studies with specimens from Croatia that focus on mollusks (*Buršić et al., 2021*), mosquitoes (*Bušić et al., 2021*) and caddisflies (*Kučinić et al., 2013*; *Valladolid et al., 2020*) have obtained similar results, which support the efficacy of DNA barcoding for species determination.

So far, sequencing of the *COI* gene fragment has been used to elucidate the systematics and phylogeography of Plecoptera (*Fochetti et al., 2009*; *Fochetti et al., 2011*; *Weiss, Stradner & Graf, 2011*), and identify new species (*Boumans & Murányi, 2014*; *Graf, Pauls & Vitecek, 2018*; *Pelingen & Freitag, 2020*; *South et al., 2019*) as part of DNA barcoding initiatives (*Morinière et al., 2017*; *Gattolliat et al., 2016*; *Ferreira et al., 2020*) and revisionary systematics (*Fochetti et al., 2011*). Distinct DNA lineages obtained within morphospecies indicate the need for re-examination of morphological characters (*Muster & Michalik, 2020*; *Wachter et al., 2015*).

Within this study, all methods for phylogenetic reconstructions show that most species can be distinguished using *COI* barcodes. Exceptions are the following pairs: *Isoperla illyrica*/*Isoperla tripartita*, *Perla pallida*/*Perla sp.*, *Perla pallida*/*Perla marginata*, *Perla burmeisteriana*/*Perla illiesi*, *Leuctra albida*/*Leuctra mortoni*, *Leuctra albida*/*Leuctra* *fusca*, *Nemoura* cf. *rivorum*/*N. flexuosa*, which possessed identical or overlapping *COI* sequences. For the above-mentioned species, identification is sometimes difficult due to high levels of intraspecific morphological variability between closely related species (*Ravizza & Ravizza-Dematteis, 1995*; *Sivec & Stark, 2002*; *Murányi, 2011*) and often both sexes and eggs are necessary for identification. BIN sharing reported within the species of the four genera: *Isoperla, Perla*, *Leuctra* and *Nemoura* potentially indicates introgression or hybridization.

High numbers of intraspecific BINs (obtained for ten species) can indicate discrete geographical populations of a species or overlooked cryptic species (*Hawlitschek et al., 2017*; *Morinière et al., 2017*), *e.g.*, *Dinocras cephalotes* (Curtis, 1827) (*Elbrecht et al., 2014*), so the number of 85 BINs cannot be a proxy for the total number of stonefly species in Croatia. Nevertheless, the underlying RESL algorithm is based on a distance threshold of 2.2%, so it is expected to have a larger number of BINs in the dataset. *Meier et al. (2021)* made a claim that the cytochrome oxidase I (*COI*) barcode region cannot be used as the only/main data source for describing or delimiting species (*Sharkey et al., 2021*) and stressed the importance of additional species delimitation methods as well as examination of morphological characters for justifying the validity of a given species. *COI* barcode clusters ("BINs") as a basis for species descriptions, given the assumption that a BIN equals a species, is often not consistent with the results of other species delineation methods (*Meier et al., 2021*) due to theoretical and empirical reasons (*Puillandre, Brouillet & Achaz, 2021*; *Zhang et al., 2013*).

Intra- and interspecific distances overlapped for some species (Fig. 1). Existence of a barcoding gap allows the use of DNA barcoding to identify species (*Meyer & Paulay, 2005*). Overlapping intra- and interspecific *p*-distances can be represented against a universal cut-off value (*Collins & Cruickshank, 2013*). Also, they can be a consequence of inaccurate taxonomy, indicating oversplit or cryptic species, especially for species within the genera *Isoperla* and *Perla* (Fig. 1).

In the present study, the distance to the NN is predominantly higher than the maximum intraspecific distance, confirming the clear local barcoding gap, enabling successful use of DNA barcoding for Croatian stonefly identification (Table S4). Furthermore, the congeneric average distance among species was five times higher than the average distance within species.

Nevertheless, the observed overlap between intra- and interspecific *p*-distances may be related to the presence of cryptic species or species complexes, which would not be surprising given the results of the morphological study.

Geographic morphological variation

Genus *Perlodes*

Previous research of the stonefly fauna in Croatia, based exclusively on morphological analysis, have established the presence of specimens, which could not be identified with certainty to known species (*Popijač & Sivec, 2009b*). *Perlodes intricatus* (clade number 32, Fig. 2) from Plitvice Lakes, in which morphological differences were observed with respect to the typical *Perlodes intricatus*, was also found during this study. Molecular methods confirmed identification, whereas DNA barcoded specimens were grouped with sequences of *P. intricatus* retrieved from BOLD database, into a highly supported monophyletic clade (Dataset 1, Table S5), but with high intraspecific *p*-distances (5.2–5.6%), indicating the need for further field research across the entire range of the species. For another species, from the same genus, *P. microcephalus*, we obtained high intraspecific *p*-distance, and it is accompanied by increasing morphological variability from type specimens (Dataset 1, Table S5).

Genus *Isoperla*

The genus *Isoperla* is characterized by several poorly circumscribed West Palearctic species (*Zwick, 2004*) and *Murányi (2011)* argued for the need of taxonomic revision. Within *Isoperla inermis* (clade number 34, Fig. 2), we found great morphological variability as observed in previous research (*Popijač & Sivec, 2009b*). Individuals from Plitvice Lakes are almost double in size compared to specimens from Cetina River (*Popijač & Sivec, 2009b*), which may be a result of different climates and a longitudinal gradient. Colour variation is found in the abdomen, head, and pronotum, which vary from brown to black. Phylogenetic analysis of sequences from all *Isoperla* species and from *I. difformis* (Klapálek, 1909) from central Europe, resulted in a highly supported monophyletic clade *I. inermis-I. difformis*, which could ultimately result in the synonymy of these species (Dataset 2, Table S5).

Isoperla cf. *lugens* (clade No. 37, Fig. 2) was recorded in the area of the Plitvice Lakes (*Popijač & Sivec, 2009b*), and was determined based on similarity of the penial armature. During comprehensive field research in this study, specimens were found associated with several headwaters of karst rivers. The species differs morphologically from the alpine species, *I. lugens*, by having a lighter coloured head and pronotum and different penial armatures. In addition to these morphological characteristics, the species is also characterized by exceptional genetic distinctiveness. The lowest interspecific *p*-distance value from *I.* cf. *lugens* compared to other congeners from the *I. tripartita* and *I. rivulorum* species-groups is 6.7% and it represents a separate genetic lineage within the clade consisting of typical *I. lugens* and *I. popijaci* (clade No. 37, Fig. 2) (Dataset 3, Table S5). Due to the above-mentioned characteristics, *Isoperla* cf. *lugens* most probably represents a new species. The MJ network (Fig. 3B) for *Isoperla* cf. *lugens* revealed low haplotype sharing among sampling sites, which may be due to the small number of specimens per sampling site. Additional specimens would be useful to test this hypothesis.

Genus *Protonemura*

Protonemura hrabei (clade No. 71, Fig. 2) from the Cetina and Zrmanja Rivers emerges at the beginning of summer, mostly due to climatic conditions, in contrast populations from Central Europe, which emerge in autumn (*Popijač & Sivec, 2009b*). Molecular analysis of *P. hrabei* sequences and sequences of closely related *Protonemura* species, confirmed morphological identification and individuals from the Cetina River form a highly supported, monophyletic clade with *Protonemura hrabei* from Central Europe with an intraspecific *p*-distance of 2.5% (Dataset 4, Table S5).

Genus *Taeniopteryx*

In a comprehensive study of the genus *Taeniopteryx* in the framework of this study, morphological differences were identified among newly collected individuals of *Taeniopteryx* n.sp. CRO-1 (clade number 47, Fig. 2), *Taeniopteryx hubaulti* (Aubert, 1946) (clade number 46, Fig. 2) and *Taeniopteryx auberti* Kis & Sowa, 1964, as well as museum specimens in Croatia, Slovenia, Bosnia and Herzegovina, Montenegro and Germany. The morphological differences between *Taeniopteryx* n.sp. CRO-1 and other species are accompanied by genetic distinctiveness, making *Taeniopteryx* n.sp. CRO-1 a candidate for a new species (interspecific *p*-distances ranged from 7.8–9.5%) as suggested by *Popijač & Sivec (2009b)*.

Morphological analysis of *T. hubaulti* confirmed the variability of the femoral thorn on the hind legs, present in some individuals but varying in size, even though this character was originally described as absent (*Aubert, 1946*). This morphological character should be clearly visible in *T. auberti* (*Kis & Sowa, 1964*). Genetic analyses of our sequences and those retrieved from BOLD and GenBank (Dataset 5, Table S5) resulted in the unclear taxonomic status of *T. hubaulti* and *T. auberti*, so there is the suspicion that *T. hubaulti* may be a junior synonym of *T. auberti*, and morphological variability is a consequence of geographical distribution. This is also similar for the species, *T. stankovitchi* Ikonomov, 1978 and *T. schoenemundi* (Mertens, 1923), for which it has already been pointed out that additional research is needed to clarify their distinction (*Fochetti & Nicolai, 1996*). As has been noted, the genus *Taeniopteryx* is, from the taxonomic point of view, complicated, and oftentimes only females show reliable characters, so the whole genus needs revision.

Genus *Leuctra*

Within the *Leuctra inermis* species-group, congruence of morphospecies concepts and phylogenetic relationships among taxa was not studied until 2017 (*Vitecek et al., 2017b*). *Vitecek et al. (2017b)* found that relationships among species remained unresolved, suggesting sister taxon relationships between morphologically similar species and potential subspecies-level diversity. Morphological variability was observed within geographically isolated populations and the same was confirmed by the present study. As many species from the *L. inermis* species-group have overlapping geographical ranges, appearance of morphological variability within several species is expected (*Fochetti et al., 2011*) and mitochondrial introgression has already been confirmed within the *Leuctra* species pair, *L. fusca* and *L. digitata* (*Boumans & Tierno de Figueroa, 2016*). Thus, it can be assumed that assessment of drumming call variations could be helpful in resolving taxonomic relationships within this species-group (*Vitecek et al., 2017b*).

Few individuals collected in this study appeared as a distinct lineage (Fig. 2), which possessed morphological features resembling already known species: *Leuctra* cf. *inermis* (CROPL130-21) collected in the Plitvice Lakes National Park, *Leuctra* sp. collected at Cetina River (clade number 17, Fig. 2), *Leuctra* sp. Z (CROPL248-21) collected at Žumberak Hills and *L.* cf. *prima* (CROPL282-21, CROPL325-21 and CROPL326-21) collected at Papuk Mountain and near Plitvice Lakes National Park. Due to the unavailability of specimens of *L. carphatica* Kis, 1966, only found in the Carphatian

Mountains, Slovenia and Austria (*Andrikovics & Murányi, 2001*), the comparison of collected individuals with this species was omitted. Morphological differences were also observed among individuals of *L. mortoni* and *L. fusca*, which requires further research with a more comprehensive sampling, which would contribute to a more precise taxonomy of these species.

Dinaric Karst as a biodiversity hotspot

The Dinaric Karst system of the Dinaric Mountains, represents one of the most diverse European freshwater habitats in terms of biological, geological and hydrological interplay, including many available microhabitats, which has resulted in speciation and endemism (*Bonacci, 2009*). Considering the results of earlier studies, high genetic diversity could be a result of the specific habitat requirements and biological characteristics of individual taxa, which promoted speciation and played an important role in the genetic differentiation of freshwater taxa (*Previšić et al., 2009*, *2014b*, *2014a*; *Klobučar et al., 2013*; *Jelić et al., 2016*; *Szivák et al., 2017*; *Lovrenčić et al., 2020*). The same goes for taxa restricted to particular microhabitats (caves, pits, underground and intermittent rivers and streams) within the Dinaric Karst (*Bilandžija et al., 2013*; *Bedek et al., 2019*; *Pavlek & Mammola, 2021*), which in general, can be considered as refugia from which taxa re-colonise Europe following glacial periods (*Hewitt, 2000*), often showing a pattern "refugia within refugia" (*Kryštufek et al., 2007*; *Ursenbacher et al., 2008*; *Previšić et al., 2009*; *Jug-Dujaković et al., 2020*).

The diversity of the stonefly fauna in Croatia is a probable consequence of the different climatic conditions in a variety of regions (Continental, Alpine and Mediterranean), a substantial altitudinal gradient, and an immense number of protected habitats. Within this study, we identified hotspots of elevated stonefly species. The highest levels of species richness were primarily located in the northwest Dinarides (Fig. 4): the border rivers, Kupa and Čabranka; Plitvice Lakes National Park; the Cetina and Una Rivers; and Mt. Papuk where several localities have five or more species. Hotspots of species richness are coincident with protected areas (*e.g.*, Plitvice Lakes National Park, Mt. Papuk, Mt. Medvednica), which is not surprising, given that these areas have suitable conditions and mostly include fast streams with high oxygen saturation. Current research shows a significant decrease in the number of species from the northern part of the Dinaric Karst (Gorski kotar and Lika) to the Cetina River and the headwater of the Una River, which is consistent with the biological features of stoneflies. Great species richness is also confirmed by *Ridl et al. (2018)*, who recorded 7–18 species across different study sites (in total 31 species) in the Plitvice Lakes National Park. Furthermore, *Popijač & Sivec (2010)* found significant species richness (14) within the Cetina River. The Croatian fauna shows great species richness, not only for stoneflies but also for other aquatic insects (*Ivković & Plant, 2015*; *Vilenica et al., 2015*; *Vilenica et al., 2016*; *Kučinić et al., 2017*; *Vilenica, Ternjej & Mihaljević, 2021*). Still, many parts of the Dinaric Karst are not well studied, due to the size of area and inaccessibility of the habitats. Species richness patterns are likely incomplete, especially from the aspect of the stonefly fauna.

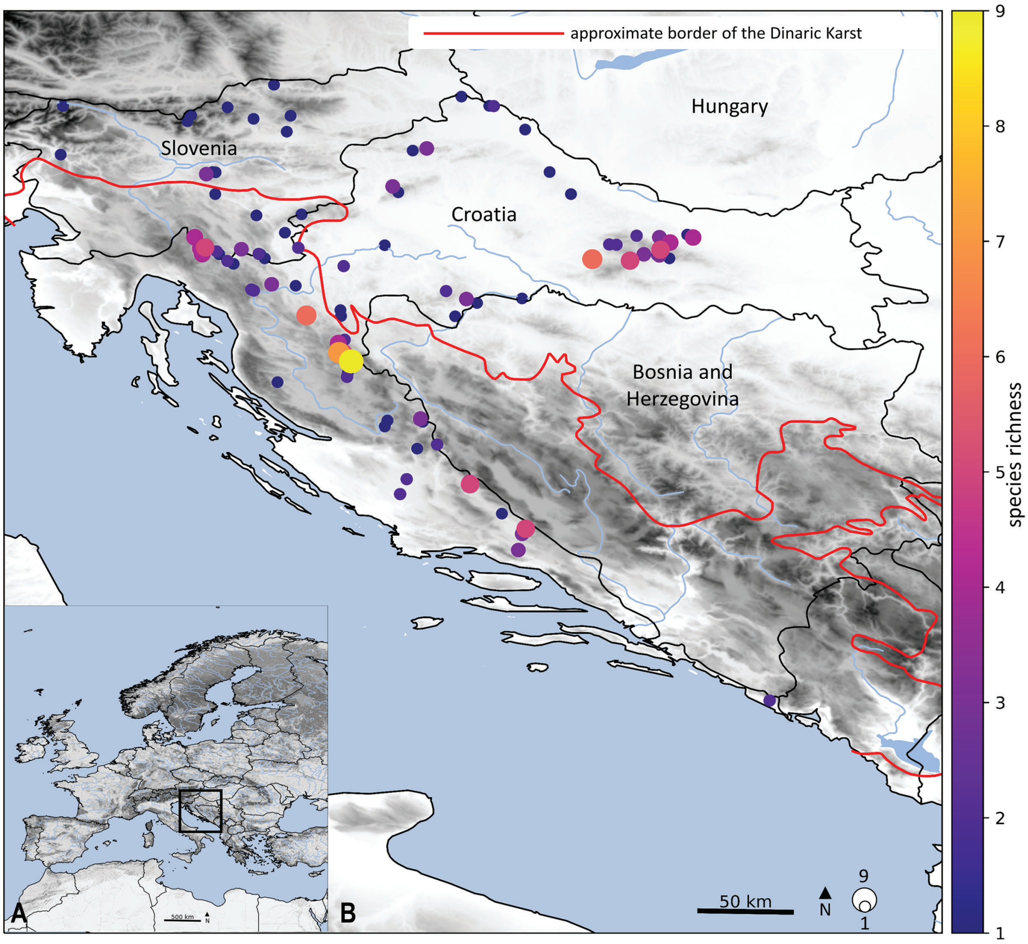

Figure 4 **Geographical location of the studied stonefly species.** Colours of dots represent the species richness in each locality and size of dots is proportional with the number. Main map (B) is an enlarged framed area in the bottom left corner (A). Approximate border of the Dinaric Karst according to *Gams (2004)*. Map is produced with Cartopy package 0.19 in Python with use of elevation data from *European Union, Copernicus Land Monitoring Service (2016)*. Full-size DOI: 10.7717/peerj.13213/fig-4

Database's enrichment and systematic implications

This study provides the first molecular characterization of nine species: *Brachyptera tristis, Perlodes dispar, Leuctra bronislawi* Sowa, 1970, *Isoperla bosnica* (Aubert, 1964), *Isoperla illyrica, Isoperla albanica* (Aubert, 1964), *Perla carantana* Sivec & Graf, 2002, *Perla illiesi* and *Agnetina elegantula* (Klapálek, 1905).

Genus *Protonemura*

Our molecular characterization of *Protonemura auberti* (Illies, 1954), *P. hrabei*, *P. intricata* (Ris, 1902), *P. nitida* (Pictet, 1836) and *P. praecox* should be considered in future revision of the genus, a need that has already been emphasized (*Wagner et al., 2011*; *Vinçon, Reding & Ravizza, 2021*). The discovery of specimens from the *P. auberti* species subgroup (determined as *P.* cf. *autumnalis*, clade number 72, Fig. 2) at the Plitvice Lakes National Park with morphological characteristics similar to *P. aestiva* Kis, 1965, which emerges throughout the spring and seems to be restricted to the Carpathian Mountains (*Kis, 1974*; *Graf et al., 2009*), suggests a hybrid of these two species, with range expansion of *P. aestiva* (*Vinçon, Reding & Ravizza, 2021*). Nevertheless, the production of hybrids with intermediate morphological characters has already been observed in the newly described *P. bispina* Vinçon, Ravizza & Reding, 2021, which is often vicariant with specimens of *P. auberti* (*Vinçon, Reding & Ravizza, 2021*). Therefore, it is necessary to pay additional attention to the genus also from that point of view. DNA barcoded specimens of *P. auberti* (CROPL257-21, CROPL258-21, CROPL281-21) collected during the spring at the Una River appeared as a separate clade among other specimens of *P. auberti* (clade number 74, Fig. 2). Establishing phylogenetic relationships with a multi-gene approach is necessary to unravel the taxonomy of this group.

Genus *Dinocras*

DNA barcoding of the species *Dinocras megacephala* (Klapálek, 1907) (clade number 23, Fig. 2), the only *Dinocras* species in Croatia, which is widely distributed in its northern and central part, emphasizes the need for revision of the BOLD database due to the noticeable number of erroneous determinations of this species. It often appears to be misidentified as *D. cephalotes*. Males of *D. cephalotes* differ from *D. megacephala* by having patches of stronger sensilla basiconica on the ventral side of the abdomen. Furthermore, brachypterous males of *D. megacephala* also occur at higher elevations (*Illies, 1966*), so brachyptery cannot be a characteristic to distinguish this species from *D. cephalotes* (usually at higher elevations).

Genus *Zwicknia*

Morphological examination of individuals from the *Capnia bifrons* Zhiltzova, 2001 species-group, following *Murányi, Gamboa & Orci (2014)* determined the presence of two species in Croatia: *Zwicknia bifrons* (clade No. 51, Fig. 2) and *Zwicknia rupprechti* (clade number 50, Fig. 2). Within the family Capniidae, we also discovered one of the smallest European species, *Capnopsis schilleri balcanica* (CROPL319-21) from only a single locality, a surprising find given that this was 17 years since the first finding (*Murányi, 2004*).

Genus *Nemoura*

Within the genus *Nemoura*, ten species were DNA barcoded (clades numbers 60–68, Fig. 2): *N. avicularis* Morton, 1894, *N. cinerea* (Retzius, 1783), *N. dubitans* Morton, 1894, *N. marginata*, *N. minima* Aubert, 1946, *N. sciurus*, *N. flexuosa*, *N.* cf. *rivorum*, *N. mortoni* (Ris, 1902) and *N. uncinata* Despax, 1934. The *Nemoura flexuosa-marginata* complex is one of the most enigmatic assemblages of species within European stoneflies and requires revision (*Ravizza & Ravizza-Dematteis, 1995*).

The *flexuosa-marginata* species-group is composed of widely distributed European species (*N. flexuosa*, *N. marginata* Pictet, 1836 and *N. uncinata*) and six Italian and French Alps endemic species: *N. hesperiae* Consiglio, 1960, *N. lucana* Nicolai & Fochetti, 1991, *N. oropensis* Ravizza & Ravizza Dematteis, 1980, *N. pesarinii* Ravizza & Ravizza Dematteis, 1979 (all occurring in Italy), *N. palliventris* Aubert, 1953 and *N. rivorum* Ravizza &

Ravizza-Dematteis, 1995 (occurring in Italy and further north in the French Alps) (*Fochetti & Vinçon, 2009*). Descriptions of two species within that complex: *Nemoura rivorum* and *Nemoura sabina* Fochetti & Vinçon, 2009 helped in understanding morphological variation among individuals within this species complex. *Nemoura rivorum*, which is endemic to the northern section of the Apennines and exhibits a variable apical and arched sclerite of the epiproct, is often erroneously identified as *N. flexuosa*, especially when only females are available for morphological analysis. Pregenital plate shape is similar in all species belonging to the *N. flexuosa*-*N. marginata* species-group and separating nymphs among species is almost impossible. Specimens collected as part of this study (CROPL075-21, CROPL076-21 and CROPL162-21) have morphological characteristics similar to *N. rivorum* (determined as *N.* cf. *rivorum*). However, based on the similarity of the sequences, these specimens clustered with *N. flexuosa* (CROPL070-21, CROPL095-21, CROPL190-21), which further emphasizes the importance of species-group revision, and revision of the sequences in BOLD and GenBank databases, respectively.

Furthermore, high morphological variability has been observed even among specimens of *Nemoura marginata* Pictet, 1836 (clade number 64, Fig. 2) (*Popijač & Sivec, 2009b*). The mean intraspecific *p*-distance was 3.1%. Future research should assess whether *N. marginata* represents a single species with large intraspecific distances and high morphological variability or a species-complex, as stated earlier (*Ravizza & Ravizza-Dematteis, 1995*).

Genus *Leuctra*

This study also provides the first molecular characterization of *Leuctra bronislawi* (CROPL131-21 and CROPL132-21). This autumnal species, which is relatively rare and a relict species with disjunct distribution in the Balkan and the Carpathians, was recently found in the Czech Republic (*Kroča, 2010*) and the first early spring records were reported from the Republic of Macedonia (*Murányi, Kovács & Orci, 2014*). Considering the limited knowledge of the stonefly fauna in countries which can include potential distribution areas of *L. bronislawi*, it is anticipated that many more populations remain to be discovered and recorded.

Genus *Isoperla*

Within the mostly endemic genus *Isoperla*, three species were DNA barcoded for the first time: *Isoperla bosnica* (clade number 39, Fig. 2), *Isoperla illyrica* (clade number 42, Fig. 2) and *Isoperla albanica* (clade number 41, Fig. 2). *Isoperla bosnica* is, based on morphology, a member of the *Isoperla oxylepis* species-group, which was redescribed based on SEM studies of the penis and egg structure (*Murányi, 2011*). *I. bosnica* was hitherto reported only from type locality (SE Bosnia-Herzegovina), NW Macedonia and Montenegro (*Murányi, 2011*) and the medial penial armature, a basic diagnostic characteristic, is like the armature of *Isoperla oxylepis* (*Murányi, 2011*). *Isoperla albanica* has an Eastern Alpine-Illyrian distribution and is characterized by an undivided medial penial armature (*Murányi, 2011*). *I. illyrica*, described as an endemic species to the Postojna Cave entrance, is now common across a wide area of the Dinaric Karst. Our phylogenetic analysis does not resolve the placement of this species (Dataset 2, Table S5). A multi-gene approach across the entire range is a priority. A similar approach is warranted for the *I. grammatica* species-complex (*Murányi et al., 2021*).

Genus *Perla*

The taxonomy of *Perla* species is unresolved and constitutes a big challenge, and the most recent revision of the genus suggests using characters in the egg chorionic (*Sivec & Stark, 2002*) as reliable for species recognition. To revise such a problematic genus, the inclusion of genomic data is required. For some species, it is already considered that they represent a species-complex, such as *P. pallida* (distributed in the Caucasus, Anatolia, the Balkans, and the Carpathians) (*Sivec & Stark, 2002*), often erroneously identified as *P. marginata*. Furthermore, the taxonomic status of some species is uncertain, such as *P. bipunctata* Pictet, 1833 (*Sivec & Stark, 2002*) and specimens found within this study on Ruda River (marked as *Perla* sp., clade No. 29, Fig. 2), which morphologically differed from congeners but genetically represent one lineage and one BIN. *Perla burmeisteriana* was recorded for the first time in 1908 and a few larvae were found at the northern foot of Papuk Mountain (*Popijač & Sivec, 2009a*). *Perla illiesi* was found in several localities in Croatia (Kupa and Čabranka Rivers). Within the present study, this species was recorded at two localities in Lika and DNA barcoded for the first time (clade No. 26, Fig. 2), as well as *Perla carantana* (clade No. 24, Fig. 2), which was reported at several localities in Slovenia and Austria (*Sivec & Graf, 2002*).

Genus *Besdolus*

Interspecific *p*-distances between newly obtained sequences of *B. imhoffi* and *B. illyricus* retrieved from GenBank (10.6–12.4) do not support the synonymy of *B. illyricus* and *B. imhoffi*, as stated before (*Fochetti et al., 2011*) (Dataset 6, Table S5).

Extirpation and conservation

High sensitivity of stoneflies to abiotic changes may lead to local or global extirpation of taxa (*Fochetti & Tierno de Figueroa, 2006*; *Graf et al., 2018*). Extensive field research that began in Europe about twenty years ago, however, found the presence of taxa that were considered extinct, but with range reduction. The local and regional extinction rate of stoneflies would be the highest across the Animal Kingdom, according to IUCN criteria (*Sánchez-Bayo & Wyckhuys, 2019*). In neighbouring countries, such as Italy, several species can be considered already extirpated: *Brachyptera trifasciata* (Pictet, 1832), *Isogenus nubecula* Newman, 1833, *Taeniopteryx nebulosa* (Linnaeus, 1758) and *Perla abdominalis* Burmeister, 1839 (*Fochetti et al., 1998*; *Fochetti, 2020*), while even more species can be considered threatened with extinction. The situation is, as usual, also critical for endemic species (known only from their type locality or a few populations) and all microendemic taxa (*Fochetti, 2020*).

Despite the relatively large effort invested in researching the stonefly fauna in Croatia (*Popijač, 2008*; *Popijač & Sivec, 2009a*, *2009b*), several species are preserved only in museum collections: *Perla bipunctata, Perla grandis*, Rambur, 1842 *Isoperla obscura* (Zetterstedt, 1840) and *Isogenus nubecula* (*Popijač & Sivec, 2009a*). It is questionable whether these species have gone locally extinct, or their populations have decreased so much that it is difficult to detect them. In future systematic research of the stonefly fauna, discovering these species and their distributions will be one of the priorities.

But in spite of that, we recorded many species that have been declared extirpated in other European countries. *Brachyptera monilicornis* (Pictet, 1841) has long been considered extinct because it does not occur in Central Europe (*Zwick, 1992*). In the border rivers of Croatia and Slovenia (Kupa and Čabranka), as well as in the streams and rivers of Papuk Mountain, *B. monilicornis* is very common. Furthermore, the rare and endangered lowland species, *Taeniopteryx nebulosa* and *Rhabdiopteryx acuminata* (Klapálek, 1905), have been recorded at several localities in the foothills of Papuk Mountain. *Besdolus imhoffi* was re-discovered in Croatia in 2005 (Plitvice Lakes National Park) (*Kovács & Murányi, 2008*) after a one-hundred-year-old record (*Popijač & Sivec, 2009a*). This finding was confirmed with molecular analysis in the present study at the Una and Cetina Rivers. Species of the genus, including *B. imhoffi*, have relictual distributions (*Zwick & Weinzierl, 1995*) and are sensitive to environmental perturbations (*Fochetti et al., 2011*). *Marthamea vitripennis*, a species lost from most of Europe (*Zwick, 2004*) due to destruction of river potamon, was found in the Rába River in Hungary (*Kovács & Ambrus, 2000*), but also in Croatia in 2011 (*Popijač & Sivec, 2011*), and during field research in this study in 2021, on the rapids of the Una River. Another rare plecopteran, found in the Rába River in Hungary (*Kovács & Ambrus, 2000*) is *Agnetina elegantula*, which we recorded during this study at Papuk Mountain.

CONCLUSIONS

The current study generated a validated national reference DNA barcode library for stoneflies in Croatia, which can support the implementation of cost-efficient DNA-based identifications and assessments to ecological status. DNA barcoding proved to be an effective tool for the identification and delimitation of some closely related species. Furthermore, this study provides several findings of species thought to be extirpated from Croatia and neighbouring regions, as well as the first molecular characterization of species with restricted distributions. For some genera (*e.g.*, *Isoperla, Taeniopteryx* and *Perla*) an integrative revisionary examination based on more comprehensive geographic sampling and application of a multi-gene approach, especially on type specimens, is necessary for resolving taxonomic relationships. Identifying areas with high biodiversity, including both morphological variability and genetic diversity, will allow further protection of stoneflies and their habitats.

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Competing Interests

The authors declare that they have no competing interests.

Author Contributions

- Dora Hlebec conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Ignac Sivec conceived and designed the experiments, performed the experiments, analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Martina Podnar conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Mladen Kučinić conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft.

Field Study Permissions

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

The specimen collection was conducted in concordance with the approval of the Ministry of Economy and Sustainable Development of the Republic of Croatia (UP/I-612-07/21-48/73).

DNA Deposition

The following information was supplied regarding the deposition of DNA sequences:

The COI sequences are available at GenBank: OK316149 to OK316486 and MW907977 to MW907993.

The data are also available in the Barcode of Life Data System (BOLD): http://dx.doi.org/10.5883/DS-CROPL.

Data Availability

The following information was supplied regarding data availability:

The data are available in the Supplemental Files.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.13213#supplemental-information.

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Molecular analyses of pseudoscorpions in a subterranean biodiversity hotspot reveal cryptic diversity and microendemism

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Nested within the Mediterranean biodiversity hotspot, the Dinaric Karst of the western Balkans is one of the world's most heterogeneous subterranean ecosystems and renowned for its highly diverse and mostly endemic fauna. The evolutionary processes leading to both endemism and diversity remain insufficiently understood, and large-scale analyses on taxa that are abundant in both subterranean and surface habitats remain infrequent. Here, we provide the first comprehensive molecular study on Croatian pseudoscorpions, a lineage of arachnids that is common and diverse in both habitats. Phylogenetic reconstructions using 499 *COI* sequences derived from 128 morphospecies collected across the Dinaric Karst show that: (i) occurrence in karstic microhabitats boosters speciation and endemism in the most diverse genera *Chthonius* C.L. Koch, 1843 (37 morphospecies) and *Neobisium* Chamberlin, 1930 (34 morphospecies), (ii) evidence for ongoing diversification is found in many species and species complexes through low optimal thresholds (OTs) and species delineation analyses, and (iii) landscape features, such as mountain ranges, correlate with patterns of genetic diversity in the diverse genus *Neobisium*. We present two synonymies: *Protoneobisium* Ćurčić, 1988 = *Neobisium*, syn. nov., and *Archaeoroncus* Ćurčić and Rađa, 2012 = *Roncus* L. Koch, 1873, syn. nov. Overall, our study suggests that karstic microhabitats promote diversification in soil- and cave-dwelling arthropods at all taxonomic levels, but also provide important refugia for invertebrates in past and present periods of environmental change.

The Dinaric Karst of the western Balkans is one of the world's largest karstic areas and located in Mediterranean Basin biodiversity hotspot. This karstic area covers about 60,000 km^2 and extends from north-eastern Italy through Slovenia, Croatia, Bosnia and Herzegovina, Serbia, the Kosovo, and Montenegro to Albania in the south-east, and comprises a network of more than 20,000 caves and pits[1]. The dynamic geo-climatic history of the Dinaric Karst has led to high habitat complexity in terrestrial[2], freshwater[3], and subterranean ecosystems[4], thereby boosting *in-situ* speciation and lineage isolation[5,6]. With more than 900 obligate subterranean species and many short-range endemics, the region hosts a unique assemblage of subterranean fauna[7] and is considered a primary hotspot for subterranean fauna[4]. Diversity in the Balkans is generally very high because the region has acted as a major refugium for both the flora and fauna during the Pleistocene glacial cycles[8], supporting relict species[9] and recently evolved karstic lineages[10]. Most studies on the karstic biota in the Balkans have focused on flagship aquatic taxa such as world's largest cave amphibian, the olm *Proteus anguinus* Laurenti, 1768[11], and subterranean amphipod crustaceans[12]. The status of cryptic taxa[13,14], especially terrestrial invertebrate fauna[15] is often unclear and hampers a deeper understanding of evolutionary processes that lead to high diversity in this biodiversity hotspot.

Pseudoscorpions (Arachnida: Pseudoscorpiones de Geer, 1778) represent such a lesser-known terrestrial invertebrate lineage[16], despite their extraordinary diversity[17], with more than 100 described endemics in subterranean habitats across the Dinaric Karst. Many pseudoscorpions have poor dispersal abilities, small distributional

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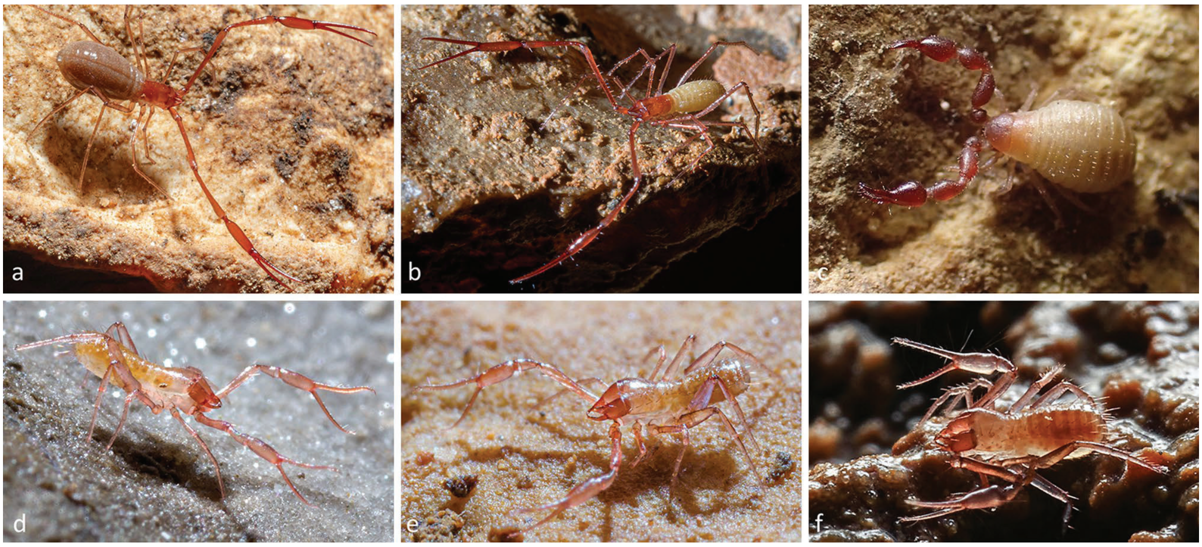

Figure 1. Life habitus of species within the most speciose families in the Dinaric Karst. (**a**, **b**) Neobisiidae Chamberlin, 1930; (**c**) Chernetidae Menge, 1855; (**d**, **e**, **f**) Chthoniidae Daday, 1889. Photos are courtesy of Petra Bregović (**a**, **b**, **d** and **e**), Branko Jalžić (**c**), and Tin Rožman (**f**).

ranges, and specific habitat requirements[18]. The order has a high proportion of subterranean endemics across the world and these species often show strong troglomorphic adaptations such as full-eye regression, elongated appendages, and loss of body pigment (Fig. 1). Some species can even be found in both subterranean and surface habitats[19], thereby providing ideal systems to study aspects of subterranean diversification and processes leading to endemism.

The most recent checklist of Croatian pseudoscorpions[20] lists 99 species and 10 subspecies. Since 2004 additional species have been described, and presently there are 126 species and 21 subspecies in 29 genera listed in the World Pseudoscorpiones Catalog[21], of which 84 species and subspecies have their type locality in Croatia. The taxonomic literature pertaining to Croatian pseudoscorpions is fragmentary and contains incomplete distributional data, is biased towards single-specimen descriptions, and is burdened with taxonomic redundancy and radical changes in generic classification schemes[17,22]. The type specimens of many Dinaric species are either part of private collections, lost, or have not been deposited in the designated depositories as stated in their original descriptions[23–25], thereby impeding or even completely preventing taxonomic work. However, the application of DNA barcoding in conjunction with species descriptions offer an avenue towards reevaluating species diversity in the Dinaric Karst both morphologically and genetically.

While DNA barcoding is known to facilitate rapid identification of specimens in the presence of accurate, high quality, and deep coverage reference sequences of described species[26], there are obvious limitations when dealing with genetically understudied groups such as pseudoscorpions. Currently, the BOLD database contains more than 2700 pseudoscorpion sequences and of these only 1523 (representing 356 species) have been identified to species level (accessed 04 June 2022). The unidentified sequences are classified as "dark taxa"[27] and do not correspond to any current morphospecies. Although DNA barcoding is often applied for biodiversity assessment using BOLD's unique identifiers called BINs (Barcode Index Numbers)[28], the use of a single (*COI*) gene also often leads to over-splitting of species[29].

In the present study, we provide the first insights into patterns of genetic (inter- and intraspecific) diversity and endemism for pseudoscorpions from the Dinaric Karst using *COI* barcodes and concatenated datasets with the *28S* nuclear ribosomal RNA gene. We hypothesize that: (i) isolation in karstic microhabitats across the Dinaric Karst promotes diversification and speciation in pseudoscorpion lineages, (ii) genetic diversity might be higher than the current morphological taxonomy suggests, and (iii) patterns of endemism and species distribution correspond to geomorphological features. To test these, we created an extensive DNA barcode reference library comprising 499 lineages (455 hypogean and 44 epigean) to: (i) test current morphological species concepts and known distributional ranges using a combination of morphology and genetics, (ii) estimate optimal identification thresholds for species delineation in Dinaric pseudoscorpions, and (iii) identify refugia with high genetic and taxonomic diversity for conservation management.

Materials and methods

Sampling and taxonomic assignment.

A total of 2015 pseudoscorpion specimens from 435 localities in Croatia (including 249 specimens representing type localities of 20 species), and 181 specimens from 49 localities in Bosnia and Herzegovina, and Montenegro were collected and preserved in 96% ethanol. Specimens were collected from both surface and subterranean habitats, including 1985 specimens from the Dinaric Karst. For morphological identification, adult specimens were cleared in 70% lactic acid if necessary and examined to morphospecies level using Leica M205 C stereomicroscope and Leica DM2500 compound microscope and

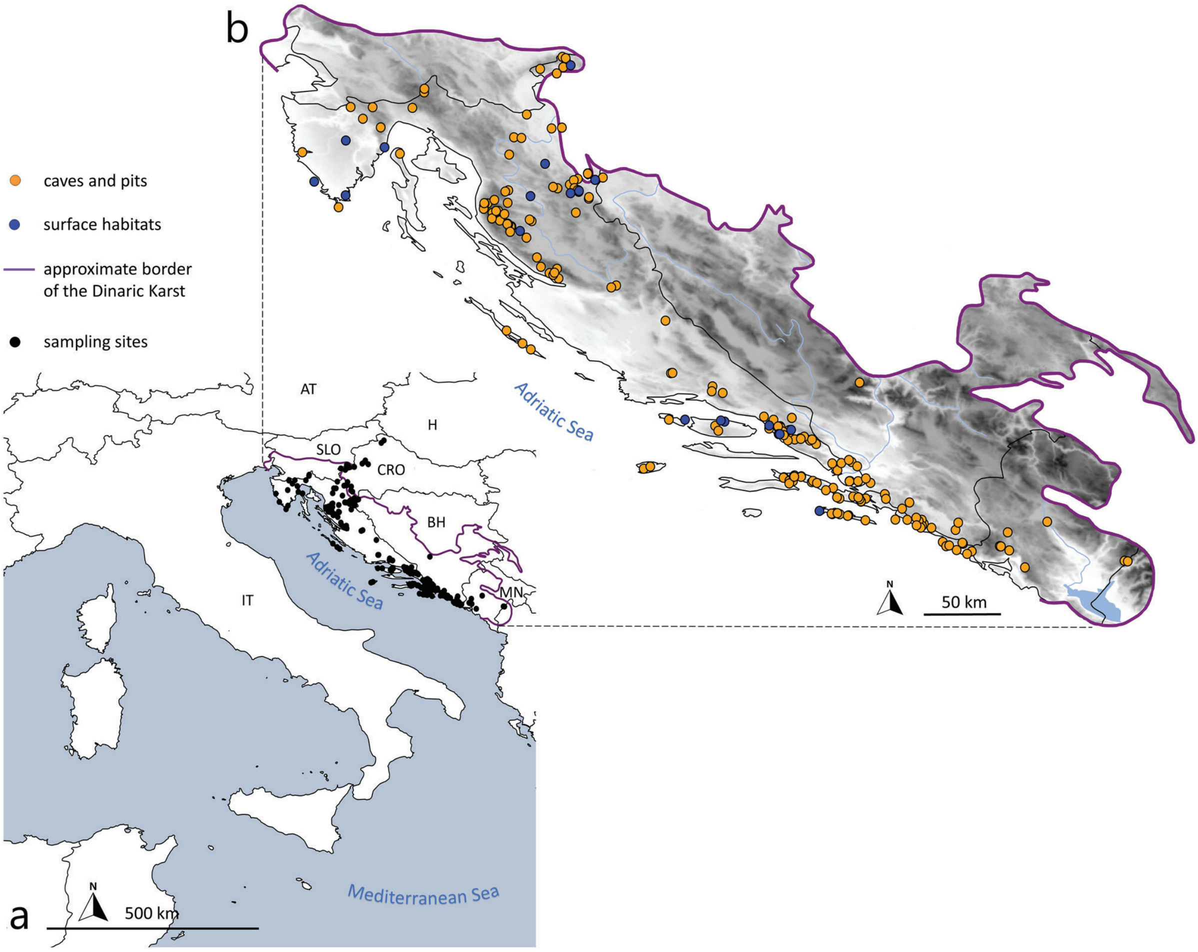

Figure 2. Distribution of georeferenced DNA barcode records is represented with black dots on the map (a). The main map (**b**) is an enlarged Dinaric Karst area marked in the bottom left corner (**a**). For locality references see Supplementary Table 1 online. Approximate border of the Dinaric Karst according to[35]. Map is produced with Cartopy package ver. 0.19 (https://scitools.org.uk/cartopy/docs/v0.18/index.html) in Python ver. 3.8 with use of elevation data from[36]. Abbreviations: IT Italy, AT Austria, SLO Slovenia, CRO Croatia, H Hungary, BH Bosnia and Herzegovina, MN Montenegro.

relevant taxonomic literature[30–34]. Whenever possible, specimens were compared with topotypic vouchers (those collected from or near the type locality). Specimens were also re-checked against the literature if conventional morphology and DNA barcoding results mismatched. The abbreviation aff. was used for specimens that were morphologically close to described species but did not entirely match the species diagnoses. Images were taken using Canon EOS 7D Mark II attached to a BK PLUS Lab System by Dun, Inc., with the software Capture One Pro 9.3 64 Bit ver. 9.3.0.85 and stacked using Zerene stacker (Zerene Systems LLC 2016). Details about the specimens are listed in Supplementary Table 1 online.

Molecular methods. For DNA extraction, we selected a total of 550 individuals from 219 localities (Fig. 2), including 61 specimens from 20 type localities, together with 23 specimens from Bosnia and Herzegovina, and Montenegro, representing morphological variability between specimens and multiple collection events. Genomic DNA was extracted using the QIAamp DNA Micro Kit (Qiagen, Hilden, Germany) following the standard protocol, except that 50 µl of elution buffer was used to increase DNA yield. DNA was extracted from 1 or 2 legs, and additional legs were used if samples were old or in poor condition. Polymerase chain reactions (PCRs) were performed to amplify the 658 base pairs (bp) barcode region of the mitochondrial gene cytochrome *c* oxidase subunit I (hereafter "*COI*")[26]. Additionally, ca. 900 bp of the domain I region of the *28S* nuclear ribosomal RNA gene (hereafter "*28S*") was amplified for closely related *COI* lineages within the most diverse families Chthoniidae (41 individuals) and Neobisiidae (48 individuals) to test the monophyly of recognized morphospecies using a non-mitochondrial marker. For details on PCR protocols see Supplementary Table 2 online. Bi-directional sequencing was done by Macrogen Inc. (Amsterdam, The Netherlands).

Sequence data authentication. All sequence chromatograms were manually edited in Geneious Prime 2022.1 (Biomatters, Auckland, New Zealand). Quality control included checking the sequences for double peaks, stop codons, amino acid translations using Mesquite ver. 3.61[37], and BLAST searches to check for contamination. Sequences were aligned using MAFFT ver. 7[38] with the "auto" strategy for *COI* datasets, while the E-INS-I algorithm with a 1PAM/k = 2 scoring matrix and the highest gap penalty was used for *28S* datasets. A dataset consisting of 499 successfully obtained *COI* sequences was compiled and will heretofore be referred to as the "Dinaric dataset" (Alignment 1). The Dinaric dataset was collapsed into haplotypes using the online tool FaBox ver. 1.61[39].

Phylogenetic inference. Phylogenetic analyses were performed using a maximum likelihood (ML)[40] approach in IQ-TREE ver. 2.0.3[41] with 5000 ultrafast bootstraps[42] for: (i) the Dinaric dataset and additional sequences from central Europe[43] to check species-level determination (Supplementary File 1 online), and ii) Dinaric *COI* haplotypes only to select taxonomically relevant sequences for detailed analyses (Fig. 3). Ultimate outgroups for both datasets included two scorpions: *Pandinus imperator* (C.L. Koch, 1841) (AY156582) and *Euscorpius italicus* (Herbst, 1800) (AMSCO005-10), considering the sister-group relationship between scorpions and pseudoscorpions[44]. All datasets were rooted against the harvestman *Platybunus pinetorum* (C. L. Koch, 1839) (GBBSP1395-15).

To resolve phylogenetic relationships among closely related *COI* lineages, we further generated two concatenated (*COI-28S*) alignments: one for the family Chthoniidae ("concatenated Chthoniidae subset": 41 individuals, Alignment 2) and one for the Neobisiidae ("concatenated Neobisiidae subset": 48 individuals, Alignment 3), including sequences available in GenBank (Supplementary Table 3 online). We removed gap-rich regions from the *28S* sequences in Gblocks ver. 0.91b[47] using settings for less stringent selection and generated concatenated alignments 2a for Chthoniidae and 3a for Neobisiidae. For these four datasets (Alignments 2, 3, 2a and 3a), we constructed phylogenetic trees with both ML and Bayesian inference (BI) methods. To determine node support for ML trees within IQ-TREE, we used 5000 ultrafast bootstraps and 2000 Shimodaira-Hasegawa-like (SHL) approximate likelihood ratio test replicates[48]. BI was performed using MrBayes ver. 3.2.7[49] with the optimal substitution models determined using PartitionFinder ver. 2.1.1[50] (Supplementary Table 2 online). The complete dataset was partitioned by gene and codon positions (for *COI*). Two parallel runs each comprising four Markov chain Monte Carlo (MCMC) were run simultaneously for 30 million generations, with every 1000th tree sampled. Resulting log-files were analyzed in Tracer ver. 1.7.1[51] to check for stationarity of parameters, and the first 25% of sampled trees were discarded as burn-in. All analyses were executed on the CIPRES Science Gateway[52].

Efficiency of DNA barcoding for identification. Two additional *COI* alignments for the most diverse families (Alignment 4 for Dinaric Chthoniidae and Alignment 5 for Dinaric Neobisiidae) were generated to calculate pairwise nucleotide distance matrices using the Kimura's 2-parameter (K2P)[53] in the package *ape*[54] in R ver. 3.5.2[55]. Matrices were further used for the estimation of optimal threshold (OT) using *threshold optimization* analysis in the library *spider* ver. 1.4–2[56]. After preliminary analyses we explored a range of threshold values (3–11%) that minimized cumulative error (false positive + false negative). The estimated OT was then used in the *Best Close Match* (BCM) analysis[57] and implemented in *spider* library to evaluate identification efficiency of Dinaric Chthoniidae and Neobisiidae at the genetic level. All singletons were removed from these analyses.

Inter- and intraspecific diversity. BOLD's *Barcode Gap Analysis* was used to ascertain the existence of a barcoding gap, analyze mean and maximum intraspecific variation, and identify minimum genetic distances to the nearest-neighbour, using the *p*-distance model and pairwise deletion option (Fig. 4a–d, Supplementary Table 4 online) and excluding species represented by singletons. Concordance between *COI* sequence clustering and morphospecies concepts within the Dinaric dataset and the two concatenated datasets (Chthoniidae and Neobisiidae subsets) was explored using three distance-based and one tree-based delimitation approaches: Automatic Barcode Gap Discovery (ABGD)[58], Assemble Species by Automatic Partitioning (ASAP)[59], BIN assignments[28], and the Bayesian implementation of the Poisson tree process (bPTP)[60]. For details, see Supplementary Methods online.

Based on the Chthoniidae (Alignment 4) and Neobisiidae (Alignment 5) *COI* alignments, we analyzed the spatial patterns of mitochondrial genetic diversity (Supplementary Fig. 1a–b online) using uncorrected pairwise distances (*p*-distances) as calculated in MEGA-X ver. 10.2.6[61] with the pairwise deletion option. Six sequences (shorter than 500 bp) were excluded from the distance calculation. For geodesic distance calculation, we used an algorithm described in[62], as implemented in GeoPy Python module ver. 2.2.0.

To visualize haplotype relatedness for species with genetic structuring across their distributional ranges, median joining networks (MJ) were reconstructed using PopART ver. 1.7[63]. These networks included *Chthonius* aff. *occultus* Beier, 1939 (Fig. 5a,b) and *Neobisium stygium* Beier, 1931 (Fig. 6b,c). Additionally, we examined relationships among populations of *Neobisium sylvaticum* (C.L. Koch, 1835) from Croatia and Germany, using published sequences[43] (Supplementary Fig. 2 online).

Quantification of biodiversity patterns. Taxonomic diversity (species richness) was plotted onto a raster of the study area by overlaying the sum of identified morphospecies (Fig. 7a) and the sum of molecular operational taxonomic units (MOTUs) obtained by BOLD (Fig. 7b). We used a grid-based approach and quadratic cells with 20 × 20 km resolution, as in previous studies of the Dinaric subterranean fauna[64]. Graphs and maps were made in Matplotlib ver. 3.5.2.[65] and the Cartopy package ver. 0.19 in Python ver. 3.8.

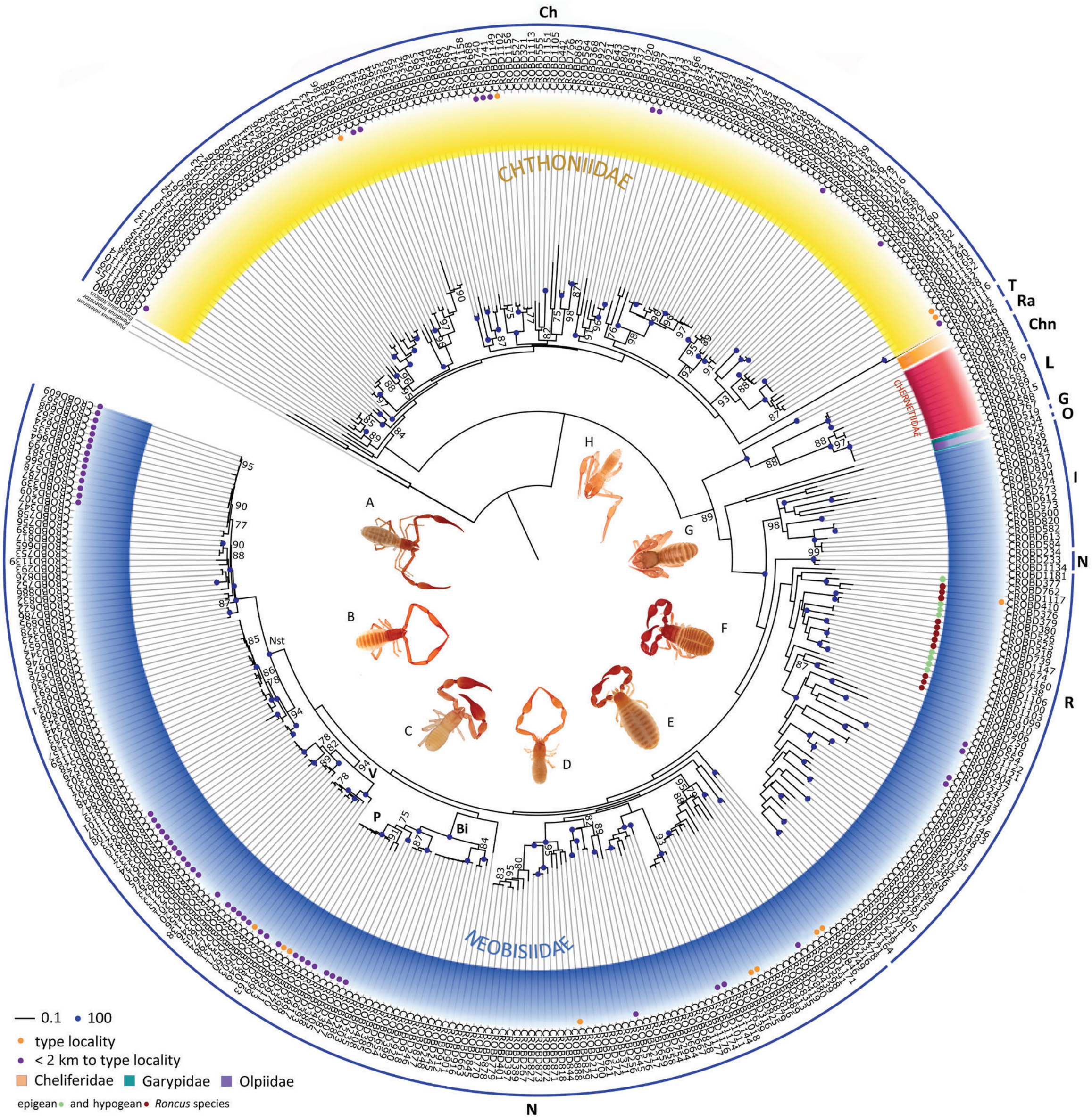

Figure 3. Circular ML tree. Species are colour-coded by family. Numbers on nodes represent ultrafast bootstrap support values (BS). Terminal codes present Sample IDs in BOLD, as in Supplementary Table 1 online. The tree was annotated in FigTree ver. 1.4.3[45] and for the circular visualization finished in iTOL ver. 5[46] and Adobe Illustrator. Representative pseudoscorpions of most recognized genera from the Dinaric Karst are shown in the middle: (**a**) *Neobisium vjetrenicae* Hadži (Neobisiidae), (**b**) *Neobisium staudacheri* Hadži (Neobisiidae), (**c**) *Roncus ragusae* Ćurčić (Neobisiidae), (**d**) *Insulocreagris* sp. (Neobisiidae), (**e**) *Pselaphochernes litoralis* Beier (Chernetidae), (**f**) *Lasiochernes siculus* Beier (Chernetidae), (**g**) *Chthonius* sp. (Chthoniidae), (**h**) *Chthonius magnificus* Beier (Chthoniidae). Different genera are represented with blue bars and labelled as: Ch (*Chthonius*), T (*Troglochthonius*), Ra (*Rhacochelifer*), Chn (*Chernes*), L (*Lasiochernes*), G (*Garypus*), O (*Olpium*), I (*Insulocreagris*), R (*Roncus*) and N (*Neobisium*). Abbreviations: P (*Protoneobisium*), Nst (*Neobisium stygium*), V (Velebit Mountain) and Bi (Biokovo Mountain).

Results

Conventional taxonomy.

Using a combined approach of morphology and genetics, we identified 128 morphospecies in 15 genera and six families, collected from 219 localities (Fig. 2). A total of 282 specimens matched the original descriptions of 50 known species and subspecies, but 111 specimens (31 morphospecies) were assigned with the prefix "aff." since they deviated slightly from the morphological species diagnoses. An additional 106 specimens did not match any morphological description and are treated here as 47 putative new

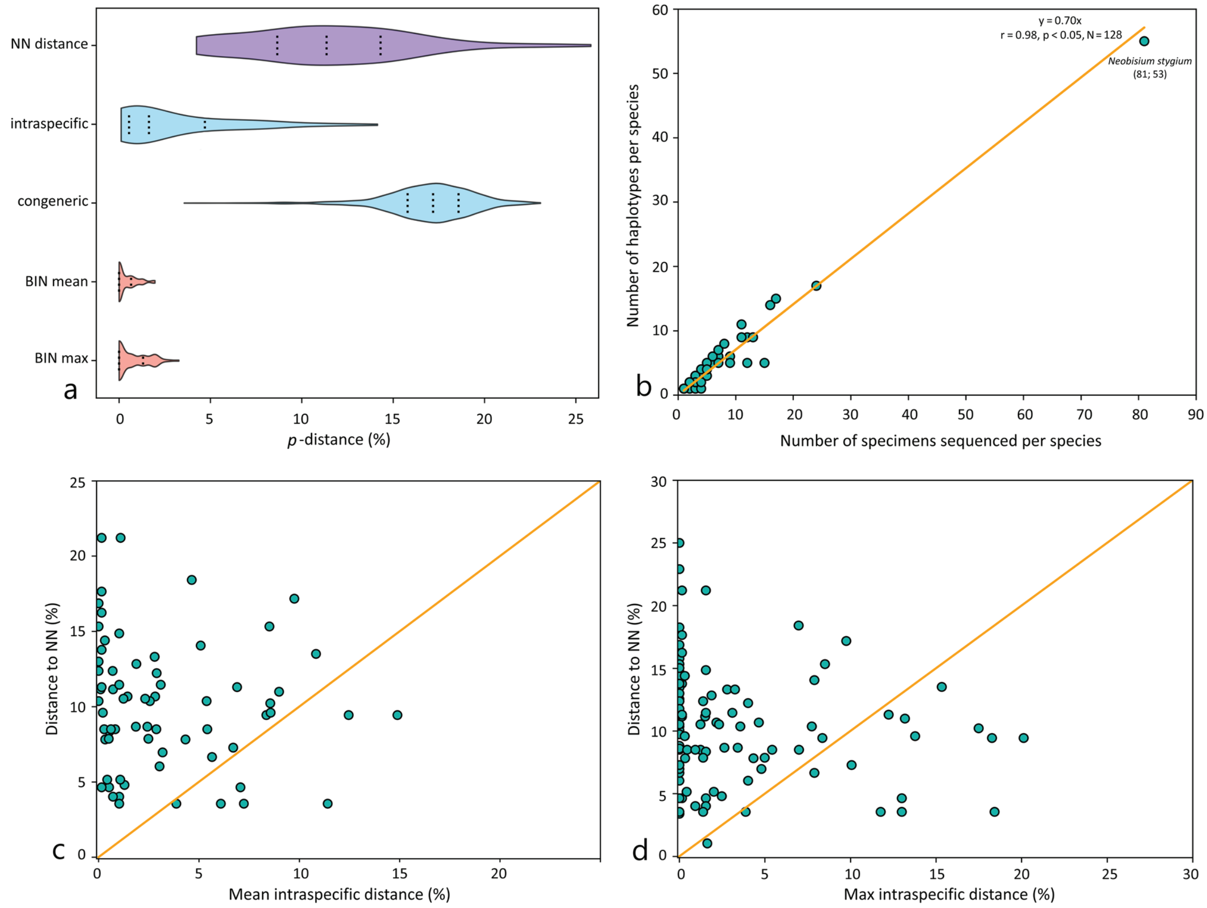

Figure 4. Intra- and interspecific diversity of pseudoscorpion species. Missing data was treated with pairwise deletion option. (**a**) Violin plot representing nearest-neighbour (NN), mean intraspecific and congeneric genetic distances, and BIN mean and maximal distances across all species in the dataset. The dashed lines inside indicate the first, middle, and third quartiles of the data. (**b**) Number of distinct haplotypes per species vs. specimens' sequences per species. Correlation equation, Pearson correlation coefficient, probability (p), and species number are shown above graph. *Neobisium stygium*, most common species is indicated in the graph (number of specimens; number of haplotypes); (**c**) Mean intraspecific distances vs. minimum interspecific distances; (**d**) Maximal intraspecific distances vs. minimum interspecific distances.

species from 74 sampling localities (32 of them being single-site endemics). For 40 described species and subspecies (251 samples), we provide the first molecular characterization. The most diverse genera were *Chthonius* (37 morphospecies) and *Neobisium* (34 morphospecies). Thirty-three specimens represented five genera endemic to the Dinaric Karst: *Archaeoroncus* Ćurčić and Rađa, 2012, *Insulocreagris* Ćurčić, 1987, *Microchthonius* Hadži, 1933, *Protoneobisium* Ćurčić, 1988, and *Troglochthonius* Beier, 1939. There were two new records for Croatia: species *Chernes hahnii* (C.L. Koch, 1839) and genus *Lasiochernes* Beier, 1932.

Sequence statistics. The Dinaric dataset was collapsed to 396 haplotypes, contained no ambiguous positions or internal stop codons, and had a median length of 655 bp (range 349–658 bp). Sequences belonging to the families Neobisiidae and Chernetidae possessed a single amino acid deletion, as previously reported[66]. Haplotype diversity was correlated with the number of individuals sequenced per species (Fig. 4b), with an average of 3.2 haplotypes per species.

The *28S* alignment had no base polymorphisms and was 1192 bp (including gaps) and 919 bp (soft trimmed) in length for Chthoniidae, whereas it was 1183 bp and 941 bp in length, respectively for Neobisiidae.

Phylogenetic analyses. Phylogenetic analyses of the *COI* gene resulted in well-resolved trees, with most clades supported by bootstrap values ≥ 95, and generally supporting family, generic, and species hypotheses (Supplementary File 1 online, Fig. 3). Preliminary analyses revealed distinct mitochondrial lineages that could not be assigned to described morphospecies (Supplementary File 1 online). In the Dinaric *COI* tree, two unresolved phylogenetic relationships were observed: polytomy consisting of the *Troglochthonius* and *Chthonius* species (see clade T, Fig. 3), and unresolved species hypotheses within the genus *Insulocreagris* (see clade I, Fig. 3).

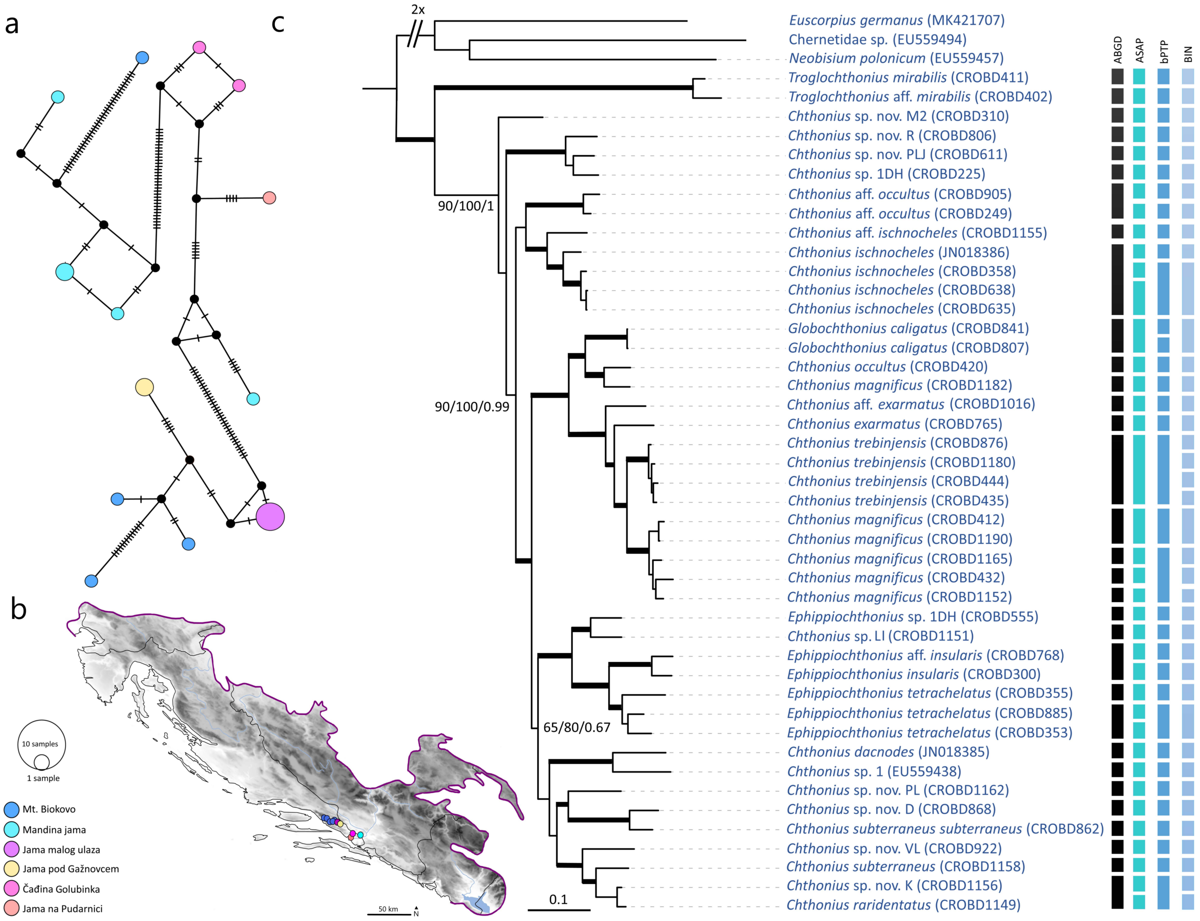

Figure 5. Median-joining haplotype network for *Chthonius* aff. *occultus* and ML phylogram of the concatenated *COI-28S* Chthoniidae subset with species delineation results. (**a**) Median-joining haplotype network for *Chthonius* aff. *occultus* haplotypes based on the *COI* gene. Haplotype circles are colour-coded according to localities and circle size is proportional to overall haplotype frequency. Black dots indicate the extinct ancestral or unsampled haplotypes. Numbers of mutational steps are given as hatch marks. (**b**) Sampling localities (colour coding matches insert in Fig. 5a). Map was made using Cartopy package ver. 0.19 (https://scitools.org.uk/cartopy/docs/v0.18/index.html) in Python ver. 3.8. (**c**) Node support is reported as ultrafast bootstrap resampling frequencies (BS), followed by the Shimodaira-Hasegawa-like (SHL) approximate likelihood ratio and posterior probabilities (PP). Branches with ultrafast bootstrap support value (100), SHL approximate likelihood ratio (100) and posterior probabilities (1) were shown in boldface. Morphospecies were mapped onto the terminals of the ML along with the Sample IDs as in Supplementary Table 1 online. Colour strips refer to species delimitation results (MOTUs) as indicated by ABGD, ASAP, bPTP, and BIN assignments.

Most genera and species were recovered as monophyletic, except for the genus *Globochthonius* Finnegan, 1932 (polyphyletic) and the species *Roncus lubricus* L. Koch, 1873 (polyphyletic).

The use of the concatenated *COI-28S* subsets helped in resolving deeper relationships within the diverse families Chthoniidae and Neobisiidae and resolved polytomies from the Dinaric *COI* tree. Since the tree topologies obtained from the BI and ML analyses of both the Chthoniidae and Neobisiidae concatenated subsets (untrimmed and soft trimmed) were mostly consistent, we present only the ML results of the untrimmed subsets (Figs. 5c, 6a).

Interspecific genetic variability and *COI* species delineation. Based on the BOLD's *Barcode Gap Analysis*, a DNA barcoding gap was obtained in 51, but not found in 15 species pairs (Fig. 4d), representing morphologically diagnosed but genetically closely related *Chthonius*, *Neobisium* and *Roncus* species.

An OT of 4.7% K2P nucleotide distance was estimated as an optimal identification threshold for the Dinaric Chthoniidae (based on 106 sequences), and 3.6% for the Dinaric Neobisiidae (285 sequences), respectively. The BCM analysis correctly identified 104 Chthoniidae sequences, yielding an efficiency of 98.1%. For Neobisiidae, the BCM analysis correctly identified 277 sequences and produced an efficiency of 97.2%. For both families, the *COI* gene showed high genetic diversity even at a small spatial scale (Supplementary Fig. 1 online).

None of the molecular species delimitation methods inferred the same number of species as morphospecies assignment. For the Dinaric dataset, ABGD delimited 155 putative species, while ASAP (with the best

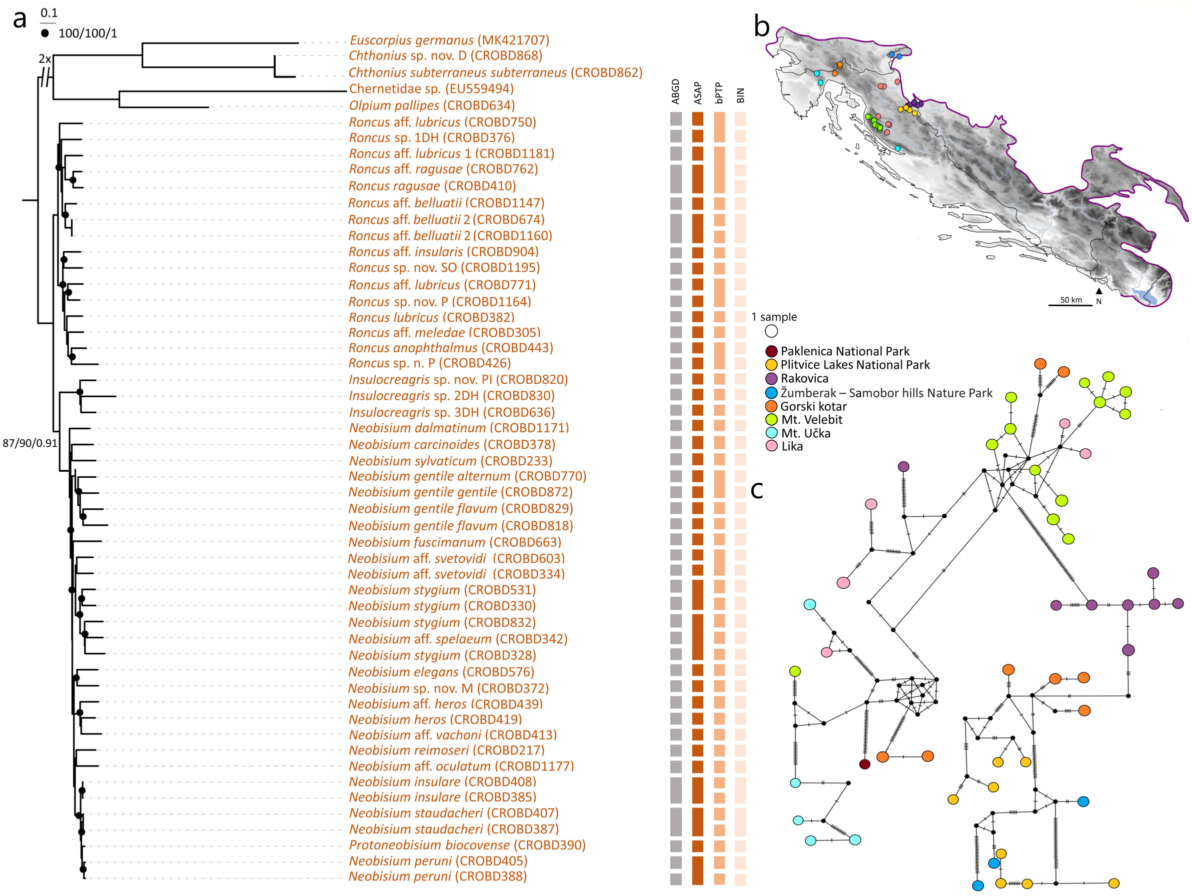

Figure 6. ML phylogram of the concatenated *COI-28S* Neobisiidae subset with species delineation results, and median-joining haplotype network for *Neobisium stygium*. (**a**) Node support is reported as ultrafast bootstrap resampling frequencies (BS), followed by the Shimodaira-Hasegawa-like (SHL) approximate likelihood ratio and posterior probabilities (PP). Branches with ultrafast bootstrap support value (100), SHL approximate likelihood ratio (100) and posterior probabilities (1) were marked with black dots. Morphospecies were mapped onto the terminals of the ML along with the Sample IDs as in Supplementary Table 1 online. Colour strips refer to species delimitation results (MOTUs) as indicated by ABGD, ASAP, bPTP, and BIN assignments. (**b**) Sampling localities (colour coding matches insert in Fig. 6c). Map was made using Cartopy package ver. 0.19 (https://scitools.org.uk/cartopy/docs/v0.18/index.html) in Python ver. 3.8. (**c**) Median-joining haplotype network for *Neobisium stygium* based on the *COI* gene. Haplotype circles are colour-coded according to localities and circle size is proportional to overall haplotype frequency. Black dots indicate the extinct ancestral or unsampled haplotypes. Numbers of mutational steps are given as hatch marks.

ASAP-score 11.50) inferred 130 putative species and agreed most with morphological identifications. The bPTP method inferred the highest with 199 putative species. Using the BOLD's BINs, sequences were allocated to 236 BINs of which 232 were unique, while BIN counts were 1.8 times higher than traditionally recognized morphospecies counts. One case of BIN sharing was observed (*Chthonius* sp. ZB and *Chthonius* sp. LI). In total, 133 BINs represented single individuals (singletons) and there is clearly a need for examining additional specimens to estimate true BIN diversity within the Chthoniidae and Neobisiidae. ABGD, bPTP and BIN assignments altogether split the species *Neobisium stygium*, *Roncus lubricus* and *Chthonius magnificus* Beier, 1938, mostly according to collection sites. For detailed results of applied species delineation methods, see Supplementary Table 1 and Supplementary Table 5 online.

For *COI* sequence clustering within the concatenated Chthoniidae subset (29 morphospecies), the ABGD method delineated 32 putative species that were mostly congruent with morphological identifications (Fig. 5c). ASAP, with the best ASAP-score (1.50), indicated 38 putative species, bPTP recovered 34 putative species, and the BOLD's assignment resulted in 39 BINs. For the concatenated Neobisiidae subset (39 morphospecies), ABGD delimited 44 putative species, ASAP, with the best ASAP-score (3.50) predicted 40 putative species, bPTP resulted in 41 species, and the BOLD assignment revealed 45 BINs (Fig. 6a). Overall, for all datasets, ABGD and ASAP provided conservative estimates of putative species, while bPTP and BIN assignments indicated potential oversplitting when compared with morphology.

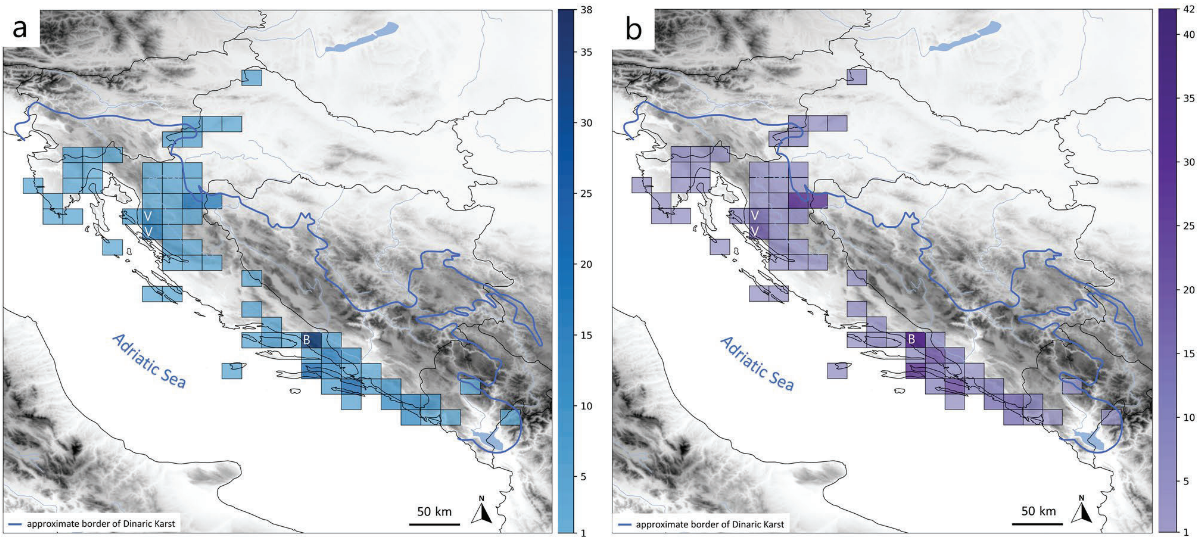

Figure 7. Species richness. (**a**) Species richness as sum of morphospecies. (**b**) Species richness as number of MOTUs in each cell. Areas without cells represent missing data. Abbreviations: V and B (Velebit and Biokovo Mountains). Map was made using Cartopy package ver. 0.19 (https://scitools.org.uk/cartopy/docs/v0.18/index.html) in Python ver. 3.8.

Intraspecific genetic variability. Within the Dinaric dataset, the mean intraspecific *p*-distance was 3.5% and overlapped slightly with the distance to the nearest-neighbour (NN) (Fig. 4a). The highest values of intraspecific *p*-distances were obtained for seven cave-dwelling species: *Chthonius* aff. *absoloni* Beier, 1938 (11.7%), *Chthonius* sp. ZB (14.4%), *Neobisium* aff. *spelaeum* (6.1%), *Neobisium* aff. *svetovidi* Ćurčić, 1988 (7.0%), *Neobisium stygium* (7.2%), *Roncus* aff. *lubricus* (14.9%), and *Roncus lubricus* (12.6%). Large intraspecific distances generally corresponded with high haplotype diversity within these species, indicating geographical isolation of localized populations. The most common species, *Neobisium stygium*, was represented by 81 specimens and 53 haplotypes (Fig. 6b,c).

The two Dinaric species with broader distributions, *Chthonius* aff. *occultus* and *Neobisium stygium*, both show deep intraspecific splits and complex phylogeographic structuring across their range, without haplotype sharing between sampling localities (Figs. 5a,b, 6b,c). Deep intraspecific splits were also observed in eight cave-dwellers (*Chthonius* aff. *alpicola* Beier, 1951, *Chthonius* aff. *occultus*, *Chthonius magnificus*, *Ephippiochthonius insularis* (Beier, 1938), *Globochthonius caligatus* (Beier, 1938), *Neobisium gentile alternum* Beier, 1939, *Neobisium gentile gentile* Beier, 1939, *Neobisium reimoseri* (Beier, 1929)) and 5 surface-dwellers (*Roncus italicus* (Simon, 1896), *Ephippiochthonius tetrachelatus* (Preyssler, 1790), *Rhacochelifer maculatus* (L. Koch, 1873), *Chthonius raridentatus* Hadži, 1930 and *Chernes hahnii*). Phylogeographic structuring was also observed in the epigean species, *N. sylvaticum* (Supplementary Fig. 2 online), which is widely distributed in Europe[21].

Discussion

This study represents the first comprehensive DNA barcode reference library of pseudoscorpions from the Dinarides, including 499 *COI* barcodes from 128 morphospecies and a high proportion of endemic, rare and elusive taxa, thereby filling an important gap in biodiversity assessment across this region. The very high identification efficiencies (98.1 and 97.2% for Dinaric Chthoniidae and Neobisiidae, respectively), as estimated from BCM analyses, suggest that DNA barcoding is an effective approach to identify many morphospecies based on *COI* divergences. The high success rate of this technique us comparable to other studies involving arachnid groups globally, including eriophyoid mites (99%;[67]), feather mites (100%;[68]) and spiders (98%;[69]).

Our analyses resulted in low optimal identification thresholds for Dinaric pseudoscorpions (4.7 and 3.6% for Chthoniidae and Neobisiidae, respectively), suggesting that the Dinaric lineages represent evolutionarily young and morphologically distinguishable sister-taxa[70]. DNA barcoding, however, is known to fail in species delineation when a high proportion of species are either closely related allopatric or parapatric taxa, or are known to hybridize[70,71]. This might also have affected the Dinaric dataset to some degree since the DNA barcoding gap was not obtained for 15 pairs of morphospecies (Fig. 4d). On the contrary, a significant DNA barcoding gap was obtained for 51 species-pairs that can be the result of insufficient sampling, at both the interspecific and intraspecific level. Sampling the subterranean matrix and cryptic taxa such as pseudoscorpions is difficult and collecting biases are almost inevitable because the entire distribution of most taxa is either unknown or cannot be covered by field collections.

This study further shows that the high levels of taxonomic diversity and endemism in the Dinarides, both at the generic and species level, are also recovered by genetic data and not the result of taxonomic over-splitting. The ASAP method, which is not based on *a priori* defined maximal genetic intraspecific divergences, was in general agreement with established morphospecies concept and revealed 130 MOTUs compared with 128 identified morphospecies. Conversely, the ABGD, bPTP and BIN assignments showed over-splitting in species such as *Neobisium stygium*, *Chthonius* aff. *absoloni*, *Chthonius magnificus* and *Roncus lubricus.* It splitted morphospecies according to their sampling localities. The ABGD method, which assumes that interspecific genetic distances are higher than intraspecific divergences, also encountered difficulties in differentiating among species. It recovered *Neobisium insulare* Beier, 1938, *Neobisium staudacheri* Hadži, 1933, *Protoneobisium biocovense* (Müller, 1931) and *Neobisium maderi* Beier, 1938 in one group, and *Neobisium* aff. *vachoni* Heurtault, 1968, *Neobisium dinaricum* Hadži, 1933, *Neobisium vjetrenicae* Hadži, 1932 in a second group, while all other delineation methods were able to delineate them as distinct species. Lumping these species indicates a major limitation of this method, which is known to perform poorly on datasets comprising large numbers of evolutionarily young species and taxa with high diversification rates[72], both of which are applicable to the Dinaric dataset.

Extensive subterranean radiation and speciation among Dinaric pseudoscorpions at a relatively narrow geographic scale can be at least partly attributed to vicariance in subterranean habitats, i.e., the presence of caves and pits and their colonization by a soil-dwelling fauna such as chthoniid and neobisiid pseudoscorpions. This is evident in the Dinaric dataset in which epigean *Roncus* species were sister to *Roncus* cave-dwellers (Fig. 3). Further, haplotype networks indicated population isolation for species with broad distributions in the Dinarides such as for *Chthonius* aff. *occultus* (Fig. 5b) and *Neobisium stygium* (Fig. 6b), which both have high numbers of mutational steps between lineages and lack shared haplotypes among localities. The presence of an extraordinarily rich karstic landscape resulted in high *COI* divergences in 15 cave-dwellers and 5 surface-dwellers, and karstification is obviously a major trigger for diversification in the Dinaric Karst. This agrees with previous studies of many other invertebrate taxa that show the Dinarides as a center of remarkable allopatric diversification[6,73,74]. Additionally, various zoogeographical divisions along the Adriatic coast, including coastal islands, and mountain ranges, can also drive speciation and act as potential microrefugia during periods of environmental change[15]. In our study, species richness was highest in the caves of the Velebit and Biokovo Mountains, and at the margin of karstic areas and alluvial plains in southern Croatia (Fig. 7). The highest number of morphospecies and MOTUs were 38 and 42 per cell, and were found in Biokovo Mountain, which is well-known for high endemism and the presence of a specialized subterranean fauna[75,76]. Both the Velebit and Biokovo Mountains are protruding ridges of limestone on subordinate dolomites near the coastline, and harbor two genetically well-supported clades of the genus *Neobisium* (see clades V and Bi, Fig. 3). Both areas are highly karstified with erosional processes and glacial activity during the Late Pleistocene[77] and potentially earlier, providing new microhabitats for colonization and diversification during and since the European ice ages. Furthermore, these two massifs support neobisiid species (*Neobisium peruni* Ćurčić, 1988, *Neobisium staudacheri*, *Neobisium stribogi* Ćurčić, 1988 and *Protoneobisium biocovense*), which are amongst the largest pseudoscorpions in the world with a body length above 10 mm. The high intraspecific *COI* divergences obtained for 15 subterranean and 5 surface lineages found in isolated karstic microhabitats indicate incipient speciation and overlooked cryptic diversity[78], driven at least in parts by natural selection in subterranean habitats[79] or by genetic drift[78].

Two "widespread" *Neobisium* species have complex phylogeographic signatures. *Neobisium stygium* (Fig. 6b) has a broader, north Dinaric distribution[80], but falls into two geographically isolated clades (see Nst in Fig. 3), 53 haplotypes and 14 BINs. In this region, karstic substrates are porous and therefore not a limiting factor for species distribution within MSS (*Milieu Souterrain Superficiel*) habitat. Similar patterns in this area have been observed for other taxa such as the millipede, *Haasia stenopodium* (Strasser, 1966)[81] and the beetle *Leptodirus hochenwartii* (Schmidt, 1832)[82]. The diversity of *Neobisium sylvaticum* at the genetic level is also remarkable and this species presently has a broad distribution in Europe but is almost certainly a cryptic-species complex. Although our geographic sampling did not cover the complete range of this species, we identified at least three genetically diverse phylogroups that are geographically isolated across Central Germany, Southern Germany, and Croatia (Supplementary Fig. 2 online). However, it can be predicted that more comprehensive sampling would produce an even more complex phylogeographic structure.

None of the Dinaric types were available for sequencing for the present study and most of them are lost in private collections that cannot be located or accessed. Fortunately, re-collections from several type localities were possible as part of this study, and established species hypotheses could be re-tested, thereby allowing us to comment on the present taxonomy. In Neobisiidae, the monophyly of the troglobiotic genus *Insulocreagris*, which exhibits a disjunct distribution on the island of Vis and the coastal area of southern Croatia, is highly supported in both the *COI* and concatenated datasets (see clade I, Fig. 3). Conversely, the genus *Protoneobisium*, known from two species on Biokovo Mountain, was neither supported genetically (6% interspecific *p*-distance to the closely related *Neobisium* species, see clade P, Fig. 3) or morphologically. The type species *Protoneobisium biocovense* nests within *Neobisium* and we transfer this species to the latter genus, resulting in the new combination *Neobisium biocovense* (Müller, 1931) comb. nov., and with *Protoneobisium* becoming a junior synonym of *Neobisium* (*Protoneobisium* = *Neobisium*, syn. nov.). Further, *Protoneobisium basilice* Ćurčić, Dimitrijević, Rađa and Rađa, 2008 becomes *Neobisium basilice* (Ćurčić, Dimitrijević, Rađa and Rađa, 2008) comb. nov. The type species of *Archaeoroncus*, *A. dalmatinus* (Hadži, 1933), nests within *Roncus* and we reclassify this species as *Roncus dalmatinus* (Hadži, 1933) comb. nov., thereby establishing the junior synonymy of *Archaeoroncus* under *Roncus* (*Archaeoroncus* = *Roncus*, syn. nov.). Further, *Archaeoroncus tenuis* (Hadži, 1933) becomes *Roncus tenuis* (Hadži, 1933) comb. nov., *Archaeoroncus salix* Ćurčić and Rađa, 2012 becomes *Roncus salix* (Ćurčić and Rađa, 2012) comb. nov., and *Archaeoroncus aspalathos* Ćurčić and Rađa, 2012 becomes *Roncus aspalathos* (Ćurčić and Rađa, 2012) comb. nov.

In the *COI* dataset of the family Chthoniidae, the troglobiotic genus *Troglochthonius* nested within a polytomy of other *Chthonius* species (see clade T in Fig. 3), while in the concatenated dataset it was placed as the most basal branch of Chthoniidae, and thus should be re-tested when additional data become available. The genera *Ephippiochthonius* Beier, 1930 and *Globochthonius*, that were elevated from subgeneric to generic rank by previous authors[34], nested within *Chthonius* in the *COI* and concatenated datasets. The type species have type localities in Czech Republic and France and were not examined in this study. These genera are maintained for now but require further investigation.

In Chernetidae, both morphological examination and genetic analyses revealed three putative new species of the genus *Lasiochernes* that will be described elsewhere (see clade L in Fig. 3).

Although 17 *Roncus* species from Croatia are included in published identification keys[24,25], many of these remain difficult to identify to the species level based on the available genetic data and morphology. Generally, the genus is characterized by several species-complexes and the diagnostic boundaries between species are often unclear[83].

The Dinaric Karst of the Western Balkans is the global subterranean biodiversity hotspot[84], nested within a key area for long-term conservation[85] that support extraordinary radiations in many taxa[86], relict species[9], and short-range endemics. The importance of the area is often missed in biodiversity protection[87]. Results of the present study highlight the significant diversity of pseudoscorpions in the subterranean realm at both the generic and species level thereby warranting conservation management.

Populations of many morphospecies defined in this study represent evolutionarily significant units (ESUs)[88] and were supported with different species delineation approaches, so we recognize the Dinaric Karst as a global hotspot for this ancient arachnid order. Of the 50 identified valid species and subspecies, 32 Dinaric Karst endemics (65%) merit priority conservation measures, both at the regional and national level. Subterranean habitats at Velebit and Biokovo Mountains, islands and areas around the alluvial plain in southern Croatia were identified as centers of taxonomic diversity and endemism and can be considered as “hotspots within hotspot”, hosting at least 18 Dinaric endemics of which all occur at small spatial scales (single-site endemics or species with ranges less than 10 km from the type locality). *COI* divergences in 15 cave-dwellers and 5 surface-dwellers indicated isolation of karstic populations and ongoing speciation that should be given the highest conservation priority.

Future species descriptions and assignment of species boundaries should provide rigorous and unbiased analysis of morphological, molecular, and ecological data, thereby accelerating conservation efforts in Croatia, which hosts 87 endemics[21], including 61 (70%) single site-endemics and 20 (23%) with a linear distribution of less than 20 km^2. Using both molecular and morphological data to document pseudoscorpion diversity in Croatia will also prevent an increase in taxonomic anarchy.

Data availability

Specimens are deposited in the following collections: Croatian Biospeleological Society Collection (CBSS), Collection of Scorpiones and Pseudoscorpiones at the Croatian Natural History Museum (CNHM), and Collection at the Museum of Nature Hamburg - Zoology (formerly Zoological Museum Hamburg (ZMH)). Museum voucher numbers are listed in Supplementary Table 1 online, while sequences and metadata for specimens are publicly accessible in BOLD database under https://doi.org/10.5883/DS-CROPS and GenBank under the accession numbers ON841793–ON842291 for *COI* and ON950244–ON950333 for *28S*.

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Author contributions

D.H. designed study, performed research, analysed, and visualised data. M.K. and D. Harms acquired funding. D.H. wrote the main manuscript, and M.P., M.K. and D. Harms contributed to further writing and editing.

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The authors declare no competing interests.

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3 Discussion

3.1 Filling the biodiversity knowledge gap

Doctoral dissertation has provided first insights into the evolutionary history of pseudoscorpions and stoneflies in southeastern Europe. The aims and hypotheses have been successfully addressed through the scientific publications included.

Taxonomy serves as a vital key to unlocking our understanding of the intricate world of biodiversity, and alpha- taxonomy remains crucial in its studying (Mayo et al., 2008). Taxonomists nowadays face the new challenge of harmonizing morphological and molecular species taxonomies (Will et al., 2005). Consequently, there is a growing demand for the development of data collection and analytical tools that can provide more comprehensive and user-friendly approaches for comparing taxonomic patterns derived from diverse data sources. In that regard, and following good practice (e.g., Graf et al., 2018a; South et al., 2019), Publication I provides a description of stenoendemic stonefly species from Croatia, incorporating both morphological and molecular data. Based on morphological characteristics, specifically the divided medial penial armature in males, *Isoperla popijaci* Hlebec & Sivec, 2021 belongs to the *I. tripartita* Illies, 1954 species group, which contradicts the results of phylogenetic analyses. A similar discrepancy was observed in other genera and species, such as in genus *Leuctra* Stephens, 1835 (Vitecek et al., 2017b). In the phylogenetic tree, *I. popijaci* appeared as the first branch-off within the clade consisting of monophyletic *I. lugens* (Klapálek, 1923) and *I. rivulorum* (Pictet, 1841) subclades, along with another tentative new taxon identified as *Isoperla* PL (species description in preparation). Considering the occurrence of *I. lugens* (an alpine species) and *I. rivulorum* (an alpine and central European species) as the most recently diverged lineages within the *I. popijaci* + "*Isoperla* PL" + *I. lugens* + *I. rivulorum* clade, it can be hypothesized that the Dinaric region of the Western Balkans may serve as a center for their diversification (Gaston & David, 1994), as well as the area from which they dispersed northwards. This aligns with the significant diversity of the genus *Isoperla* in the Balkans (Murányi, 2011). However, to test this hypothesis, comprehensive data across the entire distributional range and the utilization of other molecular markers are required.

Tierno de Figueroa et al. (2010) noted that at least 324 stonefly taxa in Europe (62.79%) could be categorized as vulnerable to climate change. Among these, 43 taxa are highly threatened, as they fall into three or more vulnerability categories. Since the described species *Isoperla popijaci* is only known from areas adjacent to two karstic springs of an intermittent river, it should be classified as Critically Endangered (CR) or Vulnerable (VU) by the IUCN Criteria.

In Publication II, full length (658-bp) *COI* sequences (355) were successfully recovered from 87% of the analyzed specimens, representing 74 morphospecies from all families present in Croatia. This contrasts with Gattolliat et al. (2016), where the recovery percentage was significantly lower (61%). PCR amplification failed only for samples older than 10 years, possibly due to a high rate of DNA degradation (Mullin et al., 2022). *COI* sequences allowed for the unambiguous identification of 84% of the morphospecies, although Morinière et al. (2017) reported a higher percentage. Deep intraspecific genetic divergences in certain clades highlighted the need for taxonomic revision in various species-complexes and species-groups (Morinière et al., 2017). Uncommon for stoneflies, BIN sharing was observed within species of four genera: *Isoperla*, *Perla* Geoffroy, 1762, *Leuctra* Stephens, 1835 and *Nemoura* Latreille, 1796 potentially indicating hybridization, and/or introgression (Hawlitschek et al., 2017). Mitochondrial introgression has already been confirmed within the *Leuctra* species pair, *L. fusca* and *L. digitata* (Boumans & Tierno de Figueroa, 2016). The research has provided the first molecular characterization of nine species, most of which have restricted distributions, and has confirmed the rediscovery of several species that had been declared regionally extinct (Fochetti & Tierno de Figueroa, 2008).

Publication III revealed the phylogenetic relationships among pseudoscorpions using a diverse range of collected specimens. The study focused on the Dinaric Karst and hypogean habitats, encompassing samples representing morphological diversity. A total of 499 *COI* barcodes were generated from 128 morphospecies, including a significant proportion of endemic, rare, and elusive taxa. This study represents the most comprehensive DNA barcode reference library for pseudoscorpions to date. In comparison, other studies included 64 specimens from South Korea (Ohira et al., 2018) and 459 specimens mostly from Germany (Muster et al., 2021). Overall, the study demonstrated that the high levels of taxonomic diversity and endemism observed in the Dinarides, at both the generic and species levels, are supported by genetic data and not the result of taxonomic over-splitting. This outcome was suspected due to the chaotic state of pseudoscorpion taxonomy in the Balkans region.

The existing taxonomic literature is incomplete and fragmented, lacking sufficient distributional data. It also exhibits a bias towards single-specimen descriptions and suffers from taxonomic redundancy and significant shifts in generic classification schemes (Ćurčić, 1988; Ćurčić et al., 2021b). The low optimal identification thresholds for Dinaric pseudoscorpions (4.7% and 3.6% for Chthoniidae and Neobisiidae, respectively), suggest that the Dinaric lineages represent evolutionarily young sister-taxa that can be morphologically distinguished. Young & Hebert (2015) noted the largest BIN distance (mean divergence among BINs within families) for pseudoscorpions among all arachnids, possibly indicating undocumented species diversity (Muster et al., 2021). The karstification of the Dinaric Karst, along with the biological characteristics of pseudoscorpions such as limited dispersal capacities, appears to have played a major role in triggering speciation and promoting endemism. Similar characteristics are shared by other organisms, including cave-dwelling bivalves (Bilandžija et al., 2013).

3.2 Pseudoscorpions (Pseudoscorpiones, Arachnida, Chelicerata)

3.2.1 Biodiversity

After conducting comprehensive field research in both hypogean and surface habitats, the analysis of pseudoscorpion taxonomic diversity in Croatia identified a total of 128 morphospecies. Among them, 50 species and subspecies have been previously described, and 32 species are endemic to the study area. This represents only a small fraction of the global pseudoscorpion diversity, which currently stands at 4,032 known species according to the World Pseudoscorpion Catalog (WPC, 2022), as well as the expected diversity in the region (Publication III). Nevertheless, this doctoral dissertation represents a significant contribution to our understanding of the morphological variability of pseudoscorpions at a local scale. In addition to the morphological characterization, this study also investigated the genetic diversity and evolutionary relationships of Croatian pseudoscorpions for the first time. De novo DNA barcode reference library provided the initial molecular characterization of 40 described species and subspecies, including five endemic genera. Furthermore, the study revealed the existence of 47 putative new species, which will be described using an integrative taxonomic approach. It is worth noting that this approach is still uncommon for various groups, including pseudoscorpions, and studies utilizing it are relatively rare (Christophoryová et al., 2023). Generated sequences were allocated to 236 BINs, indicating potential over-splitting of morphospecies.

Importantly, 131 BINs consisted of single individuals (singletons), indicating the necessity for a more comprehensive examination of specimens to accurately determine the actual BIN diversity. Significant genetic differentiation within conspecific lineages was observed in several species from the highly diverse genera *Neobisium* Chamberlin, 1930 and *Chthonius* C. L. Koch, 1843. Both genera are characterized by a high rate of diversification, with biological features such as active ongoing dispersal, and karstification emerging as significant triggers for this diversification. These findings align with previous studies on various invertebrate taxa, highlighting the Dinarides as a region of remarkable allopatric speciation (Culver et al., 2009; Polak et al., 2016; Lukić et al., 2020). Using the Barcode Gap Analysis provided by BOLD (Barcode of Life Data System; Ratnasingham & Hebert, 2007), a DNA barcoding gap was identified in 51 species pairs, indicating distinct genetic distances between them. However, in 15 species pairs, despite being morphologically distinguishable, a barcoding gap was not observed, suggesting close genetic relatedness between them. These species pairs belong to the genera *Chthonius*, *Neobisium* and *Roncus* L. Koch, 1873.

The molecular data generated in this study not only contribute to our understanding of pseudoscorpion diversity but also provide a baseline for future molecular dating and estimation of divergence times of subterranean and surface lineages. However, molecular clock analysis was not conducted in this study due to the lack of calibration points, as well as the unavailability of certain lineages that were not collected during the planned fieldtrips. Calibration points, typically derived from fossil records, geological events, biogeographic events, or other well-documented historical events, play a crucial role by providing temporal constraints and anchoring the molecular clock (Ho & Duchêne, 2014). The selection of appropriate calibration points is essential as their accuracy and reliability directly influence the accuracy of estimated divergence times. Given the increasing frequency of new pseudoscorpion fossil discoveries (Harvey et al., 2018; Wriedt et al., 2021), the application of molecular dating methods is expected to become more reliable soon. This will likely lead to more precise estimates of divergence times for pseudoscorpion lineages.

Like previous studies on subterranean diversity in the Dinaric Karst (Bregović et al., 2019; Borko et al., 2022), a grid-based approach with quadratic cells measuring 20 x 20 km resolution was employed in this study to investigate taxonomic (species richness) and molecular (number of MOTUs) diversity across space.

The highest diversity was found at Velebit and Biokovo Mts., and at the margin of karstic areas and alluvial plains in southern Croatia. Velebit and Biokovo are prominent mountain ridges primarily composed of limestone and subordinate dolomites, characterized by high annual precipitation levels and renowned for their elevated endemic subterranean fauna (Lohaj & Delić, 2019).

Both areas are highly karstified due to erosional processes and glacial activity during the Late Pleistocene (Velić et al., 2017), which created new microhabitats that facilitated colonization and diversification during and since the European ice ages. Geographically isolated populations of numerous morphospecies can be considered evolutionarily significant units (ESUs) (Moritz, 1994) and were supported by two molecular markers and various species delineation approaches. The Dinaric Karst region deserves recognition as a global hotspot for pseudoscorpions.

An exceptionally large number of different haplotypes (53) were discovered in *Neobisium stygium* Beier, 1931, making this species an outstanding candidate for future phylogeographic investigation. Unlike typical troglobitic species *Neobisium stygium* exhibits a broader geographic distribution in the northern part of the Dinarides. This occurrence can be attributed to the highly porous nature of the karst substrates in the region, which facilitates dispersal between subterranean habitats. Similar distribution patterns have been observed in other taxa within this area, such as the millipede, *Haasia stenopodium* (Strasser, 1966) (Antić et al., 2015) and the beetle *Leptodirus hochenwartii* (Schmidt, 1832) (Pretner, 1973).

3.2.2 Taxonomic remarks

The most species-rich genera in the Dinaric Karst region are *Chthonius* (37 morphospecies) and *Neobisium* (34 morphospecies), both of which present challenges in terms of their morphology. Meristic and morphometric characteristics traditionally used for describing new species within the *Neobisium* genus (such as pedipalpal length, pedipalpal chela and femur length to breadth ratio, length of whole leg IV, and carapace breadth) have proven to be insufficient for establishing the distinctiveness of new candidate species (Gardini, 2020). These mentioned characters have shown correlations with other body parts and, unfortunately, they are frequently relied upon when describing new species in the Dinaric region (Ćurčić, 1988).

While the *Roncus* species found in Croatia are included in published identification keys (Ćurčić et al., 2021a, 2021b), it is often challenging to definitively assign specimens to the described species (Publication III). Generally, genus is characterized by several species complexes, and the diagnostic boundaries are often unclear. One of the characters that exhibits sufficient variability to differentiate between species is the chelal microsetal pattern proximal to trichobothria *eb* and *esb*, while the tibia IV length to breadth ratio can provide insight into the degree of adaptation to the subterranean ecosystem (Zaragoza & Šťáhlavský, 2008).

Šťáhlavský et al. (2013) emphasized the necessity of conducting independent phylogenetic analyses to clarify the species status and distributional areas of widespread *Roncus* species: *Roncus alpinus* L. Koch, 1873, *Roncus lubricus* L. Koch, 1873, *Roncus transsilvanicus* Beier, 1928.

To prevent the proliferation of taxonomic confusion and to facilitate conservation efforts, all future species descriptions should incorporate rigorous and unbiased analyses of morphological, ecological, biogeographic, and molecular data. Describing new species without a transparent system of peer review can lead to significant challenges in understanding the systematics and taxonomy of a particular group, and rectifying such issues can take decades (Godfray & Knapp, 2004).

3.3 Stoneflies (Plecoptera, Insecta, Hexapoda)

3.3.1 Biodiversity

An integrative analysis of stonefly diversity in Croatia identified a total of 74 morphospecies. The molecular results largely aligned with the morphological assessments, allowing for unambiguous identification of 62 out of the 74 morphological species (84%) using the *COI* sequence alone. The obtained sequences were assigned to 85 Barcode Index Numbers (BINs), with 29 of them being unique to BOLD. Additionally, a significant number of intraspecific BINs (observed in ten species) may indicate discrete geographical populations or cryptic diversity that was previously overlooked (Hawlitschek et al., 2017; Morinière et al., 2017). The presence of a barcoding gap was observed in almost all species pairs, indicating the potential utility of DNA barcoding for species delineation.

However, previous studies, including Publication I and II, have demonstrated considerable intraspecific genetic divergence within stonefly populations (Zhou et al., 2009; Gill et al., 2015; Stark et al., 2015). Overlapping intra- and interspecific *p*-distances within certain genera further emphasize the need for taxonomic revisions in various species complexes, species groups, and genera.

The highest levels of species richness were predominantly observed in the northwest region of the Dinarides (e.g., river Kupa), where several localities exhibited five or more species. These hotspots of species richness coincided with protected areas such as Plitvice Lakes National Park, Papuk, and Medvednica Mountains. This observation is not surprising, as these areas offer suitable habitats, including fast-flowing streams with high oxygen saturation, which are favorable conditions for stonefly populations (Sivec & Yule, 2004). Interestingly, the current research indicates a significant decrease in the number of species from the northern part of the Dinaric Karst, encompassing Gorski kotar and Lika, towards the Cetina River and the headwater of the Una River. This pattern aligns with the known biological characteristics of stoneflies and their distribution patterns.

3.3.2 Taxonomic remarks

Detailed morphological examination and phylogenetic analyses have highlighted the need for future revisions in several genera. Notably, certain species, such as *Perlodes intricatus* (Pictet, 1841), exhibited high levels of intraspecific genetic divergence, ranging from 5.2% to 5.6%. The genera *Isoperla*, *Leuctra*, *Nemoura*, *Perla*, *Protonemura* Kempny, 1898 and *Taeniopteryx* have also demonstrated the necessity for re-evaluating species hypotheses. These findings indicate the complexity and potential inaccuracies in the current classification and taxonomy of these genera, warranting further investigation and testing.

3.4 Integrative approach in biodiversity assessment

The investigation of genetic diversity and phylogenetic relationships, utilizing mitochondrial marker for stoneflies and an additional nuclear DNA marker for pseudoscorpions, has confirmed the presence of significant genetic diversity in both groups (as discussed in Publications I, II and III). These studies have provided evidence for the existence of numerous geographically isolated and phylogenetically divergent morphotypes within both stoneflies and pseudoscorpions.

The remarkable biodiversity and endemism observed in these groups, particularly among the pseudoscorpions inhabiting the subterranean realm, can be attributed to specific biological characteristics, such as their dispersal capacities (as highlighted for other taxa by Kučinić et al., 2014). Furthermore, the past geological and climatic processes have played a crucial role in shaping the high levels of endemism and diversity. The fragmented microhabitats found in the Dinaric Karst region have further contributed to restricted dispersal and limited gene flow between populations. The results obtained from species delineation methods, including ABGD, ASAP, bPTP, and mPTP, have generally supported delineated morphospecies. Although tree-based methods (bPTP and mPTP), tend to over-split the MOTUs (Dellicour & Flot, 2018), these results can also indicate the ongoing speciation and undiscovered cryptic diversity (Publication III).

Using DNA barcodes and uniform genetic distance thresholds for the purpose of species delimitation is a subject of controversy, as there is no universally applicable threshold that can be used across different taxonomic groups (Collins & Cruickshank, 2013). The use of the first proposed universal threshold (2%) (Hebert et al., 2003a), typically employed for aquatic insects (Ball et al., 2005; Zhou et al., 2009), or 10 times the average intraspecific divergence (Hebert et al., 2004b), can lead to significant error rates due to overlapping intra- and interspecific divergence (Meier et al., 2006). Caution must be exercised when considering these thresholds, as high intraspecific sequence divergence is not uncommon within Pseudoscorpions (Muster et al., 2021) and Plecoptera (Boumans & Baumann, 2012; Gill et al., 2015). Optimal threshold values for species delineation within a specific family or genus can be estimated using extensive datasets to improve molecular identification (Meyer & Paulay, 2005). For this purpose, two *COI* alignments were generated for two most speciose families of pseudoscorpions in the Dinaric Karst: Chthoniidae and Neobisiidae. Matrices of pairwise nucleotide distances were employed in *threshold optimization* analysis. The resulting optimal thresholds were determined to be 4.7% for Chthoniidae and 3.6% for Neobisiidae, respectively. These thresholds were subsequently utilized in *Best Close Match* analysis to evaluate the identification efficiency. The identification efficiency of the *de novo* barcode reference library of Croatian pseudoscorpions, comprising 106 Chthoniidae sequences and 285 Neobisiidae sequences identified at the species level, was found to be 98.1% for Chthoniidae and 97.2% for Neobisiidae. Low optimal threshold values and species delineation methods indicate ongoing diversification in hypogean habitats, suggesting that the Dinaric lineages represent evolutionarily young distinguishable sister-taxa (Publication III).

Rare instances of overlapping pairwise intra- and interspecific *p*-distances between morphospecies found in pseudoscorpions and stoneflies indicate that DNA barcoding is a valuable tool for the delineation of morphospecies in both invertebrate lineages. Furthermore, the analysis of inconsistencies between morphological and molecular identification has resolved two taxonomic issues within pseudoscorpions. Two synonymies have been proposed: *Protoneobisium* Ćurčić, 1988 = *Neobisium*, **syn. nov.**, and *Archaeoroncus* Ćurčić and Rađa, 2012 = *Roncus* L. Koch, 1873, **syn. nov.** (Publication III), and several other cases requiring in-depth taxonomic investigation have been observed in both pseudoscorpions and stoneflies (Publication II and III).

Unique cases involving cryptic species, which are morphologically identical but genetically distinct, require particular consideration. These cases likely represent rarer lineages compared to nominal species and may necessitate different conservation strategies for each (Schönrogge et al., 2002).

3.4.1 Integration of taxonomic knowledge in DNA barcoding databases

The DNA barcoding method, as designed, enables the identification of unknown animal specimens through similarity comparison of *COI* sequences with sequences available in the main barcoding repositories (Hebert et al., 2003a, 2004b): NCBI (National Center for Biotechnology Information) and BOLD. Only the BOLD database offers the possibility to verify voucher specimens by comparing taxon labels with specimen photographs and assessing sequence quality using sequence chromatograms. The DNA taxonomy *sensu stricto* approach, where sequences serve as a reference system and distinguishing characters, is only applicable if the databases have good coverage of reference sequences associated with currently recognized morphospecies. In terms of taxonomic coverage in BOLD, there is a difference in coverage and conspecific counterparts for the two invertebrate lineages included in this dissertation. For pseudoscorpions, the results of these searches showed that there are 502 unique taxon labels (3387 barcodes) covering 12.2% of the recognized biodiversity. The remaining described species and subspecies (see WPC, 2022) are still not linked to *COI* sequences, rendering DNA barcoding largely ineffective for most taxa in the order. For stoneflies, there are 1390 unique taxon labels (19227 barcodes) that cover 36.6% of the recognized biodiversity. The coverage for this group is higher compared to the number of recognized species (see South et al., 2021).

However, in comparison to other taxa, the number of sequences available for both groups in the databases is significantly lower, indicating the need for further studies using these groups as models.

The results emphasize the importance of integrating taxonomic expertise into DNA barcoding practice and establishing comprehensive taxonomic reference libraries specific to each barcoding campaign (Kwong et al., 2012). Without such integration, current barcoding efforts may prove ineffective or inaccurate, limiting identification only to the genus level and falling short of their intended purpose of assisting in the resolution of species diversity (Kvist, 2013).

3.5 Conservation implications

This thesis establishes a fundamental framework for future studies in conservation genetics and the investigation of evolutionary histories of unique, mostly endemic, and elusive taxa. Two invertebrate lineages used as models, pseudoscorpions and stoneflies, require appropriate management due to the presence of many divergent lineages with limited distributional ranges (Fochetti & Tierno de Figueroa, 2008; Lee et al., 2022). The initial step in implementing any conservation strategy is to identify area characterized by high intra- and interspecific genetic differentiation and taxonomic diversity (Keppel et al., 2015). DNA barcoding was employed to gain preliminary insights into genetic diversity, particularly in groups with limited knowledge on DNA barcoding and phylogenetic relationships (Muster et al., 2021; Morinière et al., 2017). Taxonomically well-curated public DNA barcode libraries play a crucial role as foundational resources for metabarcoding and genomic approaches (Theissinger et al., 2023). These libraries provide a solid basis for accurately identifying and cataloging species, enabling more comprehensive assessments of biodiversity, and facilitating targeted conservation strategies.

Subterranean habitats and freshwater ecosystems are among the most fragile and vulnerable habitats, often overlooked in biodiversity assessments and conservation efforts (Fišer et al., 2022; Bagella et al., 2013). Furthermore, numerous Dinaric pseudoscorpions and stoneflies exhibit a high level of micro-endemism, being restricted to one or a few specific localities. For instance, the stonefly species *Isoperla popijaci* has only been documented in areas surrounding two karstic springs of an intermittent river in the Dinaric Karst. This species can be classified as a troglophile, possessing the ability to inhabit both surface and hypogean habitats and successfully completing its life cycle in either environment.

During the dry season, the larvae survive in the water inside the cave, which is an extremely rare occurrence among stoneflies.

In some cases, despite extensive field research, only a single specimen has been found, underscoring the rarity of certain species and the challenges involved in studying them (e.g., *Marthamea vitripennis* (Burmeister, 1839), *Agnetina elegantula* (Klapálek, 1905)) (Publication II). This highlights the urgency of conservation efforts as stoneflies have the highest local and regional extinction rate across the Animal Kingdom according to IUCN criteria (Sánchez-Bayo & Wyckhuys, 2019). Recent field trips in Croatia have revealed several species that have been declared extirpated in other European countries, such as *Brachyptera monilicornis* (Pictet, 1841) (Zwick, 1992), *Taeniopteryx nebulosa* (Linnaeus 1758), *Rhabdiopteryx acuminata* (Klapálek, 1905), *Besdolus imhoffi* (Pictet, 1841), *Agnetina elegantula* (Klapálek, 1905). *Marthamea vitripennis* (Burmeister, 1839) has become extinct in most of Europe due to river potamon destruction (Zwick, 2004), but it was found in Croatia on the rapids of the Una River (Popijač & Sivec, 2011). The situation is particularly critical for endemic species, known only from their type locality or a few populations, and all microendemic taxa, such as *Isoperla popijaci*. Furthermore, the increasing impact of climate change, leading to longer and more frequent dry periods, poses a significant concern as it is expected to result in the disappearance of taxa relying on aquatic environments for their entire life cycle or at least a part of it (Larned et al., 2010).

Croatia is home to 87 endemic pseudoscorpion species, of which 61 (70%) are single site-endemics and 20 (23%) have a linear distribution of less than 20 km (Publication III). Given the rarity of certain genera in the Dinaric Karst, such as *Troglochthonius* Beier, 1939 and *Lasiochernes* Beier, 1932, it is advisable to limit further collection of representatives from these genera. *Troglochthonius* species exhibit a disjunct distribution and appear as long, isolated species branches that had basal position within the phylogenetic tree. Moreover, out of the more than 2000 specimens that were morphologically examined, only four of them belonged to the genus *Troglochthonius*. These results indicate the relict origin and rarity of *Troglochthonius* in the Dinaric Karst.

All the findings emphasize the need for further fieldwork and in-depth studies on the phylogeny, phylogeography, and taxonomy of pseudoscorpions and stoneflies. Additionally, it is crucial to establish permanent biomonitoring programs for these groups and prioritize the conservation of freshwater and subterranean habitats.

4 Conclusion

- The inclusion of understudied taxa, such as pseudoscorpions at the global level and stoneflies at the local level, in specific barcoding campaigns will enhance species coverage in barcoding databases overall. This doctoral dissertation provided initial insights into the evolutionary history of both groups in Southeast Europe.

- DNA barcoding has proven to be an effective tool for specimen identification when accurate, high-quality, and extensively covered reference sequences of described species are available. Despite the limitation of using a single gene barcode, its utility in detecting previously overlooked cryptic lineages has been demonstrated.

- The barcode region cannot serve as the primary data source for describing or delineating species. Additional species delimitation methods, along with the examination of morphological characters, must be employed to substantiate the validity of a given species.

- The level of *COI* divergence indicates that barcodes are valuable for species delimitation among closely related congeners in both pseudoscorpions and stoneflies. In particular, the application of BCM analysis on pseudoscorpions demonstrated high identification efficiencies: 98.1% and 97.2% for Dinaric Chthoniidae and Neobisiidae, respectively. Extensive datasets should be used for the estimation of optimal threshold values, while taxon-specific thresholds increase the identification efficiency.

- Subterranean habitats within the Dinaric Karst appeared both as a museum and a cradle of diversification, supporting radiations within pseudoscorpion genera *Chthonius* and *Neobisium*. These habitats also preserve relict lineages (e.g., genus *Troglochthonius*) in localised micro-refugia. Overall, the biological characteristics of stoneflies and pseudoscorpions, combined with their occurrence in karstic microhabitats, contribute to speciation and endemism in both lineages.

- Phylogenetic analyses have confirmed the high genetic diversity among pseudoscorpions and stoneflies in Croatia. Additionally, landscape features, such as mountain ranges, have been found to correlate with genetic diversity in the megadiverse pseudoscorpion genus *Neobisium*.

- Two synonymies were proposed: *Protoneobisium* Ćurčić, 1988 = *Neobisium*, **syn. nov.**, and *Archaeoroncus* Ćurčić and Rađa, 2012 = *Roncus* L. Koch, 1873, **syn. nov.** Additionally, several other cases requiring further taxonomic investigation were observed in both pseudoscorpions and stoneflies. In order to resolve taxonomic relationships, certain genera, such as *Chthonius* and *Neobisium* in pseudoscorpions and *Isoperla*, *Taeniopteryx*, and *Perla* in stoneflies, require an integrative revisionary examination that includes more extensive geographic sampling and the application of a multi-gene approach.
- In pseudoscorpions, a high degree of endemicity at a narrow geographic scale was observed, along with the identification of putative cryptic lineages and the discovery of 47 potential new species. Genetic data also support the presence of significant taxonomic diversity and endemism at both the genus and species levels. It can be presumed that more endemic species are yet to be discovered, particularly in poorly explored areas with high biodiversity, such as the Dinaric Karst.
- An integrative approach has revealed the existence of six potential new species of stoneflies, including the recently described stenoendemic *Isoperla popijaci*. Furthermore, the study has made significant findings of stonefly species that were previously believed to be extirpated from Croatia and neighboring countries. The research also provided the first molecular characterization of several species with restricted distribution.
- The identification of areas exhibiting high alpha-taxonomic diversity and evolutionary significance, encompassing both morphological variability and genetic diversity, creates preconditions for further protection of species and their habitats. The isolation of localized, often small, and highly fragmented populations, as well as ongoing speciation processes, should be prioritized for conservation efforts. Integrative taxonomy and barcodes should serve as the foundation for preserving the rich biological heritage in the Mediterranean Basin, with particular focus on dark taxa, such as species-rich, but taxonomically challenging pseudoscorpions.
- The results presented in this study establish a foundation for future taxonomic revisions and the description of new species, as well as investigations into biogeography and evolutionary patterns. These findings also highlight the importance of implementing conservation genetics at both local and global levels.

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Curriculum Vitae

Dora Hlebec was born in Čakovec, Croatia, on March 29th, 1994. She attended elementary school in Nedelišće and Josip Štolcer Slavenski Gymnasium in Čakovec. In 2012, she enrolled in Integrated program in biology and chemistry at the Faculty of Science, University of Zagreb where she earned a master's degree (mag. educ. biol. et chem.) in 2017. During her studies, she received the Medal of the Department of Chemistry and an Award of the Department of Biology for outstanding achievements. She also was also granted three scholarships, including Scholarships from the University of Zagreb and Scholarship from the Private Foundation Mikec. After completing her studies, she began working as a biology and chemistry teacher at a vocational school in Zagreb. In 2019, she started working as a research assistant in the project *DNA barcoding of Croatian faunal biodiversity*, funded by Croatian Science Foundation. She also enrolled in the PhD program in biology at the Faculty of Science, University of Zagreb. She has authored three first-author scientific publications and has co-authored seven more. She has presented her research at 9 conferences, including seven oral presentations and five posters. She has received five research and training grants that have allowed her to establish collaborations with institutions around the world, including the Slovenian Museum of Natural History, Museum of Nature Hamburg - Zoology, and the Western Australian Museum. Her grants include *Research Grant: One-Year Grant for Doctoral Candidates* from German Academic Exchange Service (DAAD), *Oscar and Jan Francke Student Research Fund* and the *ISA Travel Grant* from International Society of Arachnology (ISA), *AraGes Student Grant* from Arachnologische Gesellschaft, and *LinnéSys: Systematics Research Fund* from the Linnean Society of London and the Systematics Association. During her PhD, she has supervised students for the Rector's Award at the University of Zagreb. She has participated in ten inventories of the subterranean fauna in collaboration with the Croatian Biospeleological Society. Additionally, she was teaching assistant for undergraduate courses of Zoology and Field Course in Botany and Zoology. Since 2019, she has been a member of the National Committee for the National Biology Competition. She has also contributed to scientific popularization events such as the Night of Biology and the European Researchers' Night, as well as communication events like the documentary show “Scientific Circles” with a focus on pseudoscorpions.

Scientific Publications

1. Hlebec D, Podnar M, Kučinić M, Harms D (2023) Molecular analyses of pseudoscorpions in a subterranean biodiversity hotspot reveal cryptic diversity and microendemism. Scientific Reports 13: 430.

2. Hlebec D, Sivec I, Podnar M, Kučinić M (2022) DNA barcoding for biodiversity assessment: Croatian stoneflies (Insecta: Plecoptera). PeerJ 10: e13213.

3. Ibrahimi H, Bilalli A, Kučinić M, **Hlebec D**, Gashi A, Grapci-Kotori L, Stojanović K, Živić I (2022) *Potamophylax idliri* sp.nov. (Trichoptera: Limnephilidae), a new species from the Jastrebac Mountains in Serbia, with molecular and ecological notes. Zootaxa 5116: 373–392.

4. Ćuk R, Kučinić M, Kladarić L, **Hlebec D**, Đanić V, Miličić M (2021) First record of *Cyrnus crenaticornis* (Kolenati, 1859) (Insecta, Trichoptera, Polycentropodidae) in Croatia: morphological determination and DNA barcoding. Natura Croatica 30: 405–416.

5. Kučinić M, Šalinović-Steinbacher A, Žalac S, Gumhalter D, **Hlebec D**, Ćukušić A, Vučković I, Šašić M, Mihoci I, Hađina J, Vajdić M (2021) Faunal features of caddisflies (Insecta, Trichoptera) in Konavle region (Croatia) with notes on DNA barcoding and conservation biology. Natura Croatica 30: 331–350.

6. Ibrahimi H, **Hlebec D**, Bilalli A, Musliu M, Previšić A, Gashi A, Grapci-Kotori L, Cerjanec D, Geci D, Kučinić M (2021) *Rhyacophila siparantum* sp. nov. (Trichoptera: Rhyacophilidae), a new species of the *R. philopotamoides* species group from the Republic of Kosovo with molecular and ecological notes. Ecologica Montenegrina 49: 12–22.

7. Hlebec D, Sivec I, Podnar M, Skejo J, Kučinić M (2021) Morphological and molecular characterisation of the Popijač's Yellow Sally, *Isoperla popijaci* sp. nov., a new stenoendemic stonefly species from Croatia (Plecoptera, Perlodidae). ZooKeys 1078: 85–106. [Cover article]

8. Kladarić L, Popijač A, **Hlebec D**, Previšić A, Ćuk R, Vučković I, Kučinić M (2021) A surprising finding of *Ecclisopteryx asterix* Malicky, 1979 (Insecta, Trichoptera) in Croatia with notes to DNA barcoding and new distributional data of the subfamily Drusinae. Ecologica Montenegrina 48: 71–85.

9. Kolar A, **Hlebec D**, Dolina K, Franjević M, Kučinić M (2021) First DNA barcoding of a new alien species *Glycaspis brimblecombei* Moore, 1964 (Hemiptera: Aphalaridae) in Croatia with a distribution note. Ecologica Montenegrina 43: 59–68.

10. Juranović Cindrić I, Zeiner M, **Hlebec D** (2018) Mineral Composition of Elements in Walnuts and Walnut Oils. International Journal of Environmental Research and Public Health 15: 2674–2685

Conference Proceedings

1. Hlebec D (2023) Karstification as a major trigger for diversification of pseudoscorpions in Dinaric Karst. 22nd International Congress of Arachnology, Montevideo, Uruguay – oral presentation

2. Hlebec D, Harms D (2022) Biodiversity patterns of pseudoscorpions in Dinaric Karst. European Congress of Arachnology 2022, Greifswald, Germany – oral presentation

3. Hlebec D, Harms D, Podnar M, Kučinić M (2022) DNA barcoding sheds light on hidden biodiversity of pseudoscorpions in Croatia. International Conference on DNA Barcoding and Biodiversity 2022, Sofia, Bulgaria – oral presentation

4. Kermek D, Pischiutta N, **Hlebec D**, Kučinić M. (2022) Inventory and analysis of diversity of stoneflies (Plecoptera), caddisflies (Trichoptera) and scorpionflies (Mecoptera) in Medvednica Nature Park using classical taxonomy and DNA barcoding. International Conference on DNA Barcoding and Biodiversity 2022, Sofia, Bulgaria – poster

5. Hlebec D, Harms D (2022) Explosive evolution of pseudoscorpions in Croatia. 3rd Dinaric Symposium on Subterranean Biology 2022, Trebinje, Bosnia and Herzegovina – oral presentation

6. Dražina T, Šepčević M, Kermek D, Bedek J, Čupić I, **Hlebec D**, Pavlek M, Kuharić N, Kirin A (2022) Recent biospeleological research in the Žumberak – Samobor hills Nature Park. 3rd Dinaric Symposium on Subterranean Biology 2022, Trebinje, Bosnia and Herzegovina – poster

7. Bregović P, Mišerić I, Sudar N, Bedek J, Ćukušić A, Čupić I, Dražina T, **Hlebec D**, Pavlek M, Rožman T (2022) An overview of the biospeleological research in wider area of Barać caves. 3rd Dinaric Symposium on Subterranean Biology 2022, Trebinje, Bosnia and Herzegovina – poster

8. Hlebec D, Sivec I, Podnar M, Kučinić M (2021) Integrative taxonomy, biogeography, and biodiversity conservation of the Plecoptera fauna in Croatia. Symposium for European Freshwater Sciences 2021, Dublin, Ireland – oral presentation

9. Hlebec D, Sivec I, Podnar M, Kučinić M (2021) Use of integrative taxonomy in description of two new stenoendemic stonefly species (Plecoptera) from Croatia. Symposium of doctoral students 2021, Zagreb, Croatia – poster

10. Hlebec D, Podnar M, Sivec I, Jalžić B, Kučinić M (2020) First results of DNA barcoding of Plecoptera (Insecta) and Pseudoscorpiones (Arachnida) in Croatia. Symposium of doctoral students 2020, Zagreb, Croatia – poster

11. Hlebec D, Podnar M, Sivec I, Jalžić B, Kučinić M (2019) First results of DNA barcoding of Plecoptera (Insecta) and Pseudoscorpiones (Arachnida) in Croatia. 6th ABOL meeting 2019, Innsbruck, Austria – oral presentation

12. Hlebec D, Podnar M, Sivec I, Jalžić B, Kučinić M (2019) DNA barcoding of biodiversity of Croatian fauna. Symposium of Biology Students in Europe 2019, Glasgow, Scotland – oral presentation

Books

Bregović P, Mišerić I, Bedek J, Ćukušić A, Čupić I, Delić T, Dražina T, **Hlebec D**, Komerički A, Lukić M, et al. (2022) The hidden animal life of Barać Caves). Karlovac: Javna ustanova za upravljanje zaštićenim prirodnim vrijednostima na području općine Rakovica (monograph)

Workshops & Trainings

2023 – Scientific training at Western Australian Museum, Australia (3 months; supervision: Dr. Mark S. Harvey)

2022 – Scientific training at Leibniz Institute for the Analysis of Biodiversity Change, Museum of Nature Hamburg – Zoology, Germany (1 year; supervision: Dr. Danilo Harms)

2022 – “Using R in basic statistical data analysis” course at University of Hamburg, Germany

2021 – Scientific training at Slovenian Museum of Natural History, Slovenia (3 months; supervision: Dr. Ignac Sivec)

2020 – “Molecular phylogeny and evolution” course at Johannes Gutenberg University of Mainz, Germany

2019 – “Introduction to computational biology” course at University of Glasgow, Scotland

Activities

since 2019 – Member of State Commission for the Biology Competition

2019 – Educational workshop organizer at European Researchers’ Night, Zagreb, Croatia

2017 – Technical team member at the 20th European Congress of Lepidopterology, Podgora, Croatia

2015–2016 Education workshop organizer and presenter at the science popularization event “Night of Biology” at the Faculty of Science, Zagreb, Croatia

Awards and Grants

2023 – ISA Travel Grant – 22nd International Congress of Arachnology travel grant sponsored by International Society of Arachnology

2022 – Rector’s Award (University of Zagreb) – supervision

2022 – LinnéSys: Systematics Research Fund – Linnean Society of London and the Systematics Association

2022 – AraGes Student Grant – European Congress of Arachnology 2022 travel grant sponsored by Arachnologische Gesellschaft

2021 – Oscar and Jan Francke Student Research Fund – International Society of Arachnology

2021 – Research Grant: One-Year Grant for Doctoral Candidates – DAAD (German Academic Exchange Service)

2017 – Medal of the Department of Chemistry for outstanding success in the studies – Faculty of Science, Zagreb

2017 – Award of the Department of Biology for outstanding achievements during studies – Faculty of Science, Zagreb)

2016 – Scholarship of private Foundation Mikec

2015–2016 Scholarship of the University of Zagreb

Prošireni sažetak

Područje Hrvatske obuhvaća dio Sredozemlja, jednog od žarišta biološke raznolikosti koje obiluje specifičnim ekosustavima s različitim klimatskim, geološkim i hidrološkim uvjetima. Dodatno se dijelom Hrvatske proteže planinski masiv Dinarida koji osigurava veliku raznolikost mikrostaništa, te je ujedno dom mnogih reliktnih i endemskih vrsta, potičući radijaciju rodova brojnih skupina organizama u podzemnim i slatkovodnim staništima. Iz tog razloga, područje je poznato kao najistaknutije žarište bioraznolikosti podzemnih staništa na svjetskoj razini i ključno je središte za dugotrajno očuvanje bioraznolikosti. Klimatske promjene i fragmentacija staništa nisu zaobišle ni centre biraznolikosti, što izravno ugrožava opstanak najosjetljivih skupina organizama, poput troglobiontskih vrsta lažištipavaca u podzemnim staništima i obalčara u slatkovodnim ekosustavima. Obje skupine karakterizira visoka stopa endemizma i kriptična raznolikost, i pomalo je razočaravajuće što nikada nisu bile model sveobuhvatnih studija unatoč dugoj povijesti istraživanja i napretku molekularno-genetičkih metoda u posljednjih 20 godina.

Ciljevi istraživanja ove doktorske disertacije bili su: utvrditi morfološku varijabilnost i upotrebom mitohondrijskog (*COI*) i dodatnog nuklearnog markera za skupinu lažištipavaca, istražiti unutar- i međuvrsne filogenetske odnose te objasniti evolucijske odnose među vrstama lažištipavaca i obalčara u Hrvatskoj. Primjenom različitih metoda filogenetske rekonstrukcije i metoda razgraničavanja vrsta kritički su uspoređeni rezultati detaljne morfološke obrade i molekularno-genetičkih analiza. Također, dobiven je prvi uvid u postojanje divergentnih linija te je primjenom integrativne taksonomije opisana nova vrsta obalčara, *Isoperla popijaci*.

Lažištipavci predstavljaju jedan od najraznolikijih redova razreda paučnjaka u Hrvatskoj. U posljednjih 20 godina s područja Hrvatske opisano je 35 novih vrsta, bez detaljnije komparativne morfološke analize srodnih vrsta, od čega ni jedan holotip nije deponiran u Hrvatskoj. Globalno gledajući, skupina pripada u „dark taxa“ kategoriju koju karakterizira nedostatak taksonomske ekspertize pri čemu veliki broj prikupljenih jedinki ostaje nedeterminiran, a vrste neopisane. Sekvence upisane u baze podataka nisu povezane sa specifičnim taksonima zbog čega se primarna svrha DNA barkodiranja - identifikacija biološkog materijala, ne može ostvariti.

Obalčari su hemimetabolni kukci, među kojima većina vrsta svoj životni ciklus završava u jednoj sezoni. Stenotermni su organizmi, izuzetno osjetljivi na okolišne promjene i predstavljaju jednu od najvažnijih skupina indikatorskih organizama za procjenu stupnja onečišćenja voda. Dosadašnja istraživanja provedena na području Hrvatske, posebno na zaštićenim područjima, pružaju podatke o rasprostranjenosti vrsta i ukazuju na značajno smanjenje bogatstva vrsta i veličine populacija na određenim staništima. Važnost primjene molekularno-genetičkih metoda naglašena je u svrhu razrješavanja taksonomskih i filogenetskih odnosa brojnih vrsta na području Balkanskog poluotoka. Studije provedene na drugim organizmima u slatkovodnim ekosustavima Hrvatske ukazuju na veliku genetsku raznolikost, postojanje alopatrijske specijacije i mikrorefugija, što je potaknulo pitanje slijede li obalčari jednake obrasce.

Doktorska disertacija se sastoji od tri znanstvene publikacije i predstavlja prvo istraživanje evolucijske povijesti vrsta iz redova ležištipavaca i obalčara na području jugoistočne Europe.

Publikacija **I** predstavlja opis endemske vrste obalčara primjenom integrativne taksonomije. Filogenetski i taksonomski odnosi rekonstruirani su korištenjem različitih molekularno-filogenetičkih analiza i metoda za razgraničavanje vrsta. Prema morfološkim obilježjima, vrsta pripada u *Isoperla tripartita* grupu. Filogenetsko rekonstruiranje nije potvrdilo taj status, što ukazuje na sistematsku kompleksnost roda *Isoperla*. Istraživanje je ukazalo na visoke vrijednosti genetskih udaljenosti između pojedinih vrsta roda *Isoperla*. Novoopisana vrsta se ističe svojom genetskom divergentnošću, a primjerci su pronađeni na vrlo uskom geografskom području (tek dva lokaliteta uz izvorišno područje povremene rječice Krasulje u Krbavskom polju). Zbog navedenih karakteristika, vrsta bi, kao i njeno stanište trebali biti prioritet u konzervacijskim planovima.

Publikacija **II** obuhvaća filogenetsku rekonstrukciju svih prikupljenih primjeraka obalčara. Utvrđena je prisutnost 74 vrste i generirano je 355 *COI* sekvenci. Za 84 % vrsta utvrđeno je da se mogu identificirati na temelju razlika u barkod fragmentu. Visoka genetska divergencija utvrđena je za nekoliko vrsta i kompleksa vrsta, naglašavajući potrebu za taksonomskom revizijom određenih rodova. Istraživanje uljučuje prvu molekularnu karakterizaciju 9 vrsta, a u fauni Hrvatske potvrđen je nalaz nekoliko vrsta koje se na području Europe smatraju izumrlima.

U publikaciji **III** rekonstruirani su evolucijski odnosi među svim prikupljenim uzorcima lažištipavaca objedinjujući morfološku varijabilnost, geografsku distribuciju i specifičnost staništa (44 epigejska i 455 uzoraka iz podzemnih staništa). Generirano je 499 *COI* sekvenci uz veliki udio endemskih i rijetkih vrsta. Istraživanje je pokazalo da područje Hrvatske karakterizira velika morfološka i genetska raznolikost, kao i visok stupanj endemizma. Po prvi su puta izračunate vrijednosti za optimalno razgraničavanje vrsta na temelju genetskih udaljenosti (4.7 % za Chthoniidae i 3.6 %, za Neobisiidae) kao i efikasnost identifikacije metodom DNA barkodiranja. Izračunate niske vrijednosti ukazuju da genetske linije na području Dinarskog krša nemaju veliku evolucijsku starost. Kao najistaknutiji poticaji visokoj stopi specijacije i endemizma istaknute su biološke značajke obiju skupina te okršavanje. Provedeno istraživanje predstavlja temelj za buduća biogeografska istraživanja, kao i za primjenu metode molekularnog datiranja.

Provedeno znanstveno istraživanje, temeljeno na integrativnom pristupu, rezultiralo je prvim uvidom u morfološku i genetsku raznolikost dviju skupina beskralježnjaka. Cjelovito terensko istraživanje doprinijelo je poznavanju rasprostranjenosti vrsta i genetskih linija unutar vrsta, te omogućilo prvu rekonstrukciju filogenetskih odnosa. Generirane DNA sekvence (499 *COI* sekvenci lažištipavaca i 355 sekvenci obalčara) upisane su u međunarodne baze te su kao takve primjenjive u analizama, čime se podacima dobivenim na lokalnoj razini daje globalna vrijednost. Prisutnost 47 neopisanih vrsta lažištipavaca i pet vrsta obalčara, s rasprostranjenošću na uskom području, potvrđena je detaljnom morfološkom analizom uz primjenu molekularno-genetičkih metoda, pa će sve nove vrste biti opisane primjenom integrativne taksonomije uz naglasak na važnost zaštite mikrostaništa. Dobiveni molekularni podaci bit će temelj pri uvođenju promjena na taksonomskoj razini. Također, istaknuti su centri diversifikacije te područja s najvećom konzervacijskom vrijednošću.