UNIVERSITY OF LJUBLJANA
BIOTECHNICAL FACULTY

Špela BORKO

ADAPTIVE RADIATION OF *Niphargus* (CRUSTACEA: AMPHIPODA) AND ITS CONTRIBUTION TO THE DIVERSITY OF DINARIC SUBTERRANEAN FAUNA

DOCTORAL DISSERTATION

Ljubljana, 2022

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ADAPTIVNA RADIACIJA SLEPIH POSTRANIC (CRUSTACEA: AMPHIPODA: *Niphargus*) IN NJEN PRISPEVEK K PESTROSTI DINARSKE PODZEMNE FAVNE

DOKTORSKA DISERTACIJA

Ljubljana, 2022

Based on the Statute of the University of Ljubljana and the decision of the Biotechnical Faculty senate, as well as the decision of the Commission for Doctoral Studies of the University of Ljubljana adopted on 24th September 2019 it has been confirmed that the candidate meets the requirements for pursuing a PhD in the interdisciplinary doctoral programme in Biosciences, Scientific Field Biology. Doc. Dr. Cene Fišer is appointed as supervisor.

Doctoral dissertation was conducted at the Department of Biology, Biotechnical Faculty, University of Ljubljana.

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TI	ADAPTIVE RADIATION OF *Niphargus* (CRUSTACEA: AMPHIPODA) AND ITS CONTRIBUTION TO THE DIVERSITY OF DINARIC SUBTERRANEAN FAUNA
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AB	Speciation and dispersal are among key processes that drive biodiversity. Adaptive radiation (AR), a monophyletic species proliferation accompanied by ecological diversification, may be generating much of Earth's biodiversity, but it has never been tested in subterranean environment. We reconstructed deep evolutionary history of amphipods. Next, we comprehensively analysed the speciation and ecomorphological diversification of the subterranean genus *Niphargus*, on continental scale, within a subterranean hotspot, and within one community. Amphipods evolved in several diversification pulses that correspond to ecological opportunities. *Niphargus* followed the course of AR that unfolded in the time of emerging mountain ranges. Several clades independently radiated, adaptively and nonadaptively. The clades adapted to distinct sets of adaptive optima with limited convergence. The speciation and dispersal contributed differently within the Dinarides. The diversity of the south-eastern Dinarides emerged mostly through local diversification of several clades. They acted as a donor area. The north-western hotspot arose through a combination of dispersal and local diversification. Although the general patterns support the AR hypothesis, explicit tests for ecological speciation are lacking. A community in Melissotrypa Cave is a potential case of ecological speciation of *Niphargus*. AR is thus a universal phenomenon, present also in subterranean environment, but more research is needed to understand the contribution of different processes to biodiversity, especially on microevolutionary level.

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ŠD	Dd
DK	UDK 575.8:551.44:595.371(043.3)
KG	Evolucijske radiacije, *Niphargus*, podzemlje, Dinaridi, filogenije, biodiverziteta
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IN	ADAPTIVNA RADIACIJA SLEPIH POSTRANIC (CRUSTACEA: AMPHIPODA: *Niphargus*) IN NJEN PRISPEVEK K PESTROSTI DINARSKE PODZEMNE FAVNE
TD	Doktorska disertacija
OP	VIII, 116 strani, 5 prilog, 130 virov
IJ	en
JI	en/sl
AI	Speciacija in disperzija sta ključna procesa, ki poganjata biotsko pestrost. Adaptivna radiacija (AR), hitro vznikanje vrst iz skupnega prednika, pospremljeno z ekološko diverzifikacijo, bi lahko bila ključen proces nastanka biotske pestrosti na Zemlji, a še ni bila testirana v podzemlju. Najprej smo rekonstruirali globoko evolucijsko zgodovino postranic. Nato smo celovito analizirali vzorce speciacije in ekomorfološke diverzifikacije slepih postranic iz rodu *Niphargus*, na kontinentalnem nivoju, znotraj vroče točke podzemne biotske pestrosti in znotraj ene združbe. Postranice so se razvile v več diverzifikacijskih pulzih, ki sovpadajo z ekološkimi priložnostmi. Slepe postranice so diverzificirale v procesu AR, na območju dvigajočih se kraških gorstev. Več kladov je neodvisno radiiralo, adaptivno in neadaptivno. Čeprav deloma konvergirajo, so se prilagodili na različne adaptivne optimume. Prispevek speciacije in disperzije se vzdolž Dinaridov spreminja. Biotska pestrost jugovzhodnih Dinaridov je večinoma posledica lokalne diverzifikacije več kladov in deluje kot prispevno območje vrst. Severozahodna vroča točka pa je posledica kompleksne kombinacije disperzije in lokalne diverzifikacije. Čeprav splošni vzorci pritrjujejo hipotezi AR, pa ekološka speciacija še ni bila primerno testirana. Združba slepih postranic v jami Melissotrypa bi lahko predstavljala primer ekološke speciacije. AR je tako univerzalni fenomen, prisoten tudi v podzemlju. Za razumevanje prispevka različnih procesov, še posebej na mikroevolucijskem nivoju, pa bodo potrebne dodatne raziskave.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

ANNEXES

TABLE OF CONTENTS OF SCIENTIFIC WORKS

Copilaş-Ciocianu D., Borko Š., Fišer C. 2020. The late blooming amphipods: Global change promoted post-Jurassic ecological radiation despite Palaeozoic origin. Molecular Phylogenetics and Evolution, 143: 106664, https://doi.org/10.1016/j.ympev.2019.106664: 12 p.

Borko Š., Trontelj P., Seehausen O., Moškrič A., Fišer C. 2021. A subterranean adaptive radiation of amphipods in Europe. Nature Communications, 12: 3688, https://doi.org/10.1038/s41467-021-24023-w: 12 p.

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LIST OF ANNEXES

ABBREVIATIONS AND SYMBOLS

MOTU: Molecular Operational Taxonomic Unit

1 INTRODUCTION

Biodiversity can be measured in different ways: as number of species (species diversity), phenotypic or ecological disparity (phenotypic diversity), or phylogenetic relatedness (phylogenetic diversity) (Devictor et al., 2010). Understanding the origins of the various facies of biodiversity is a primary goal of evolutionary biology and macroecology. The current biodiversity patterns emerged through an interplay of speciation, dispersal, and extinction (Davies et al., 2007).

Speciation, the evolution of two or more descendant species from a common ancestor, is the ultimate mechanism that generates biodiversity. Speciation can be driven by geographic distance. When a geographic barrier arises between populations, reproductive isolation will eventually follow regardless of the type or strength of selection (allopatric speciation). However, selection accelerates speciation. If speciation is driven by selection, it can be ecological or nonecological. In the first case, reproductive isolation evolves as a side product of the adaptation to different ecological niches. In the second case, reproductive isolation develops through the fixation of different advantageous mutations in separate populations experiencing similar selection pressures (Rundell and Price, 2009; Schluter, 2009).

Phenotypic diversification commonly goes hand in hand with the speciation, either in response to local selection or simply due to random evolution of phenotype. If phenotypic diversification is due to exploitation of different ecological niches (ecological speciation), morphological diversification will relate to ecological diversity (Michaud et al., 2018; Schluter, 2000). In these occasions, phenotypic diversification may be causally linked to speciation. However, the link between form and function is not always clear and is sometimes difficult to establish (Guillerme et al., 2020).

The second process that influences the biodiversity is dispersal. Dispersal is a process of movement by which individuals shift their geographic position. It has implications on gene flow, population dynamics, resource competition, and species distribution. Despite its importance, it is often poorly understood (Duarte and Mali, 2018).

Disentangling how speciation and dispersal contributed to biodiversity patterns is a challenging task. Combining different metrics of biodiversity, such as species richness and phylogenetic diversity, can elucidate the relative contributions of the two respective processes to overall biodiversity patterns (Davies et al., 2007; Fritz and Rahbek, 2012; Li and Yue, 2020).

Phylogenetic diversity is a measure of diversity that incorporates the information on shared ancestry of species (Faith, 1992). It can unravel the biogeographic history and interactions among species in an assemblage, especially if combined with other diversity measures. For example, species-rich areas with low phylogenetic diversity may emerge through rapid recent speciation or high temporal turnover of lineages and rare dispersal events. In contrast, species-rich areas with high phylogenetic diversity may emerge through long periods of steady speciation or high dispersal. Individual dispersal events may have a small impact on species richness, but elevate phylogenetic diversity.

High phylogenetic diversity can also be a result of extinction, a process of disappearance of a species (Davies et al., 2007). However, the extinction rates of clades without fossil evidence, such as soft tissue invertebrates, are difficult to reconstruct from phylogenies alone.

Biodiversity hotspots form through high diversification, low extinction, high immigration, or a combination of these processes (Wiens and Donoghue, 2004). It has been proposed that most of the extant diversity in many major clades across the globe emerged through sudden bursts of speciation, often accompanied by extensive ecological diversification – evolutionary radiations (Stroud and Losos, 2016). We tested this view in an ecologically simple, permanently dark, and nutrient-deprived subterranean realm.

1.1 EVOLUTIONARY RADIATIONS

The tempo and mode of speciation vary among clades, in space and in time. Speciation can be slow or fast, gradual or abrupt. Evolutionary radiations, rapid proliferations of species within a single lineage, have been recognised as important drivers of biodiversity. Evolutionary radiations may or may not be accompanied by relevant niche differentiation (Rundell and Price, 2009). Adaptive radiations are species proliferations accompanied by ecological divergence among descendants, i.e. differentiation of relevant ecological niches (Czekanski-Moir and Rundell, 2019; Rundell and Price, 2009; Schluter, 2000), while nonadaptive radiations are not accompanied by relevant niche differentiation and descendant species do not exhibit ecological divergence (Gittenberger, 1991). Clades may contain elements of both adaptive and nonadaptive diversification, i.e. ecologically differentiated sympatric lineages, as well as ecologically similar allopatric or parapatric lineages. Moreover, ecological differentiation is sometimes difficult to define. Consequently it can be difficult to draw a line between these two phenomena (Rundell and Price, 2009).

1.1.1 Adaptive radiations

Adaptive radiation is a monophyletic species proliferation accompanied by ecological diversification (Rundell and Price, 2009; Schluter, 2000). Adaptive radiation causally integrates species and phenotypic components of biodiversity (Losos and Mahler, 2010; Stroud and Losos, 2016). The adaptive radiation model uses eco-evolutionary dynamics operating on a local scale such as local adaptation and interspecific interactions, to explain macroevolutionary processes and global biodiversity patterns, including speciation, clade diversification, and latitudinal gradients of species richness (Schluter, 2000). According to the strict definition of adaptive radiation, ecology-based divergent selection among populations leads to barriers to gene flow and consequent ecological speciation. Strong divergent natural and sexual selection in different environments results in rapid ecological divergence and hence rapid speciation. However, sometimes it is hard to reconstruct the sequence of processes. It is possible that reproductive isolation evolves as a result of nonecological processes and ecological differentiation happens later. If these species later enter into sympatry the resulting pattern would resemble the ecological speciation (Rundell and Price, 2009).

The classic definition of adaptive radiation incorporates four criteria: common ancestry of species, phenotype-environment correlation, trait utility, and rapid speciation (Schluter, 2000). Common ancestry can be inferred from phylogenies. A clade that has undergone adaptive radiation does not need to be monophyletic, i.e. it does not need to include all the descendants of the ancestral lineage. In fact, many classic examples of adaptive radiation, such as anoles exhibit old species that dispersed into environments that did not promote diversification. Phenotype-environment correlation is necessary for ecological specialization. This means that differences in phenotype are associated with features of the environment, such as the use of different resources. Trait utility is the advantage of a trait value in its environment. To prove trait utility in the strict sense, i.e. that a trait value improves performance in a given environment or task, experimental evidence is needed, which is often difficult to achieve (Gillespie et al., 2020). Finally, an initial rapid diversification followed by a slowdown in net diversification through time is described by an early-burst model (Harmon et al., 2010). Rapid speciation often begins after a period of steady pace of evolution, triggered by newly emerged ecological opportunity.

The adaptive radiation process depends on intrinsic and extrinsic factors. Internal factors of a clade include sexual selection, hybridization and developmental plasticity. An external prerequisite is an ecological opportunity, "a wealth of unexploited ecological resources" (Schluter, 2000). The relative importance and interaction of intrinsic and extrinsic factors are poorly understood. It is also not clear whether these factors also have predictive power or if they can only be used for post-hoc explanation of the adaptive radiation (Losos and Mahler, 2010; Stroud and Losos, 2016).

Adaptive radiations have primarily been studied in discrete, replicated habitats, such as on islands or in lakes, where the study design can be relatively easily framed in space and time (Grant and Grant, 2008; Losos, 2009; Seehausen, 2006). In this type of settings the dispersal from source areas is limited, the onset of diversification coincides with initial colonisation, and the ecological niches for the incoming species can be better defined and quantified (Losos, 2009; Seehausen, 2006). Famous examples are anole lizards in the Greater Antilles islands, Darwin's finches in Galapagos, or cichlids in the African Great Lakes (Grant and Grant, 2008; Losos, 2009; Seehausen, 2006). Less attention has been paid to continental adaptive radiations (Blom et al., 2016), or radiations of invertebrates. Habitat-specific diversification and allopatric speciation have been shown as drivers of radiation of shrimps in Sulawesi's ancient lake (Von Rintelen et al., 2010). Competition and heterogeneity of resources were recognised as ecological opportunities in the adaptive radiation of Galápagos land snails (Parent and Crespi, 2009). Similar sets of ecomorphs arose through both dispersal and diversification in Hawaiian spiders (Gillespie, 2004). Studies of radiation of amphipods in Lake Baikal were mainly descriptive (Macdonald et al., 2005; Naumenko et al., 2017; Sherbakov, 1999). No study explicitly asked whether adaptive radiation could unfold in the subterranean environment. Although ecologically harsh, subterranean habitats are both ecologically heterogeneous and geographically fragmented (Culver and Pipan, 2019) and as such a candidate environment for it (Naciri and Linder, 2020).

1.1.1.1 Ecological opportunity

Adaptive radiation requires empty or underutilised adaptive zone, a multitude of ecological resources that are free of competitors (Schluter, 2000). Ecological opportunities can arise from different key events. The first is the colonization of a new area. The Hawaiian archipelago was colonised by several groups of animals that radiated within new area: birds, insects and arachnids (Gillespie, 2004; Lovette et al., 2002). The aquatic equivalent of islands are ancient lakes with multitude of cichlid radiations (Seehausen, 2015). Next ecological opportunity is the emergence of new resources. The uplift of new mountain ranges like the Andes or the Miocene appearance of grasslands in North America were such events (Stroud and Losos, 2016). The extinction of the ecologically dominant group also opens an ecological opportunity within ancestral range of taxon. Mass extinction events were usually followed by rapid radiations of surviving species, such as the rise of birds and mammals after the Cretaceous-Paleogene extinction (Hull, 2015). Finally, a taxon can evolve a feature that enables new interaction with the environment without specific change of the environment: a key innovation. The origin of wings in birds and bats opened a way to the aerial realm. New study even suggests that anoli adaptive radiation was driven by a key innovation rather than colonisation of islands, specifically the adhesive toe pads that facilitated the exploitation of arboreal niches (Burress and Muñoz, 2022). The diversification of one group of organisms can also in turn create ecological opportunity for another unrelated group of species, a phenomenon that is described by the "diversity begets diversity" model. In this case, new species increase the number of available ecological niches (Losos and Mahler, 2010).

1.1.1.2 Independent replicated radiations

Large adaptive radiations within discrete ecosystems often comprise of several parallel radiations that result in similar suites of ecomorphs. Ecomorphs are morphologically and behaviourally similar species that occupy similar microhabitats but are not necessarily phylogenetically closely related (Mahler et al., 2013). Such cases are above mentioned anole lizards on islands (Mahler et al., 2013) and cichlid fishes in lakes (Elmer et al., 2014) or South American rivers (Burress et al., 2018), where clades repeatedly and independently evolved into a similar sets of habitat specialists that cluster in functional morphological space. These parallel radiations are not necessarily replicates. The anole radiation comprises of convergent phenotypes and also of species with unique phenotypes that have evolved a morphology and ecology not found on other islands. It has been shown, that anole lizards exhibit significant pairwise phenotypic similarity between species on different islands, even when the full ecomorphological diversity of the lineages is taken into account (Mahler et al., 2013). The repeated occurrence of not closely related ecomorphs has also been reported from subterranean environments, within amphipod genus *Niphargus* (Trontelj et al., 2012).

1.1.2 Nonadaptive radiations

A counterpoint to adaptive radiation, nonadaptive radiation is a lineage diversification with minimal ecological differentiation, resulting in related and ecologically similar species. Most often, the speciation in nonadaptive radiation would be allopatric or parapatric, where gene flow reduces due to geographic isolation (Rundell and Price, 2009), at times fortified by intensified genetic drift in smaller populations (Nürk et al., 2020). The process is affected by the similarity of environments of the diverging populations. It may also be possible for ecologically similar species to coexist in sympatry. One such case is sexual selection leading to reproductive isolation (Rundell and Price, 2009).

The main difference between adaptive and nonadaptive radiation is that species proliferation from common ancestor is due to nonecological speciation. However, the definition of nonadaptive radiation is even vaguer than that of adaptive radiation and published cases of nonadaptive radiation often do not exhibit exceptionally fast proliferation of species, but are nevertheless referred to as such. Nonadaptive radiations often involve organisms with limited dispersal abilities or those living in highly subdivided environments with similar habitats and conditions on either side of barriers (Czekanski-Moir and Rundell, 2019). However, at larger spatial scales, organisms with intermediate dispersal abilities may diversify fastest, because they can colonize new areas but cannot maintain gene flow (Agnarsson et al., 2014). One optional mechanism underlying nonadaptive radiation could be also phylogenetic niche conservatism, the tendency of species to retain ancestral ecological characteristics. In spatially and temporally fluctuating environment, this mechanism would promote vicariant isolation and speciation (Kozak et al., 2006).

The first case of a highly diversified clade, where diversification cannot be associated with its environment, was recognised already in 1872 in land snails in Hawaii (Gulick, 1872). Sexual selection has been identified as a driver of nonadaptive radiation in several genera of damselflies, either as nonadaptive divergence in colouration, behaviour, or genital structures differentiation (Wellenreuther and Sánchez-Guillén, 2016), and also as an early driver of radiation of electric fish (Arnegard et al., 2010). A high degree of speciation but low phenotypic specialization was found in sigmodontine rodents (Maestri et al., 2017) and cold-climate lizards (Reaney et al., 2018). Evidence of cryptic species of amphipods in Lake Baikal suggests a possible combination of adaptive and nonadaptive events (Schön and

Martens, 2004). A similar case reveals in subterranean realm, where highly diversified clades on one hand, and eco-morphologically similar or even cryptic clades of *Niphargus* on the other (Delić et al., 2017a; Delić et al., 2017b; Trontelj et al., 2012) hint that a combination of nonadaptive and adaptive processes may be at work in this subterranean hyper-speciose clade.

1.2 ORIGINS OF BIODIVERSITY IN THE SUBTERRANEAN ENVIRONMENT

The subterranean environment is harsh, compared to the surface. Due to the complete absence of light and consequently photosynthetic production, subterranean ecosystems are among the most resource-limited environments on Earth. Only a few species have successfully colonized them. The subterranean environment has long been perceived as an evolutionary dead end, whose inhabitants are doomed to extinction before they can diversify further (Barr and Holsinger, 1985; Culver and Pipan, 2019; Poulson and White, 1969). However, the dead-end hypothesis has soon been challenged by some authors (Stoch, 1995), and studies in past two decades have shown that evolutionary processes continue after the colonisation of the subterranean environment, including speciation within the subterranean environment (see below). Yet, the hypothesis of entirely subterranean adaptive radiation has never been tested.

A considerable number of subterranean species derived from subterranean ancestor, apparently in both, nonecological and ecological speciation. Some species retained similar morphology due to similar environmental conditions, and some of them are morphologically indistinguishable (morphologically cryptic) (Esposito et al., 2015; Faille et al., 2013; Fišer et al., 2015). It has been shown that species diversity of European groundwater isopods and amphipods is probably two to three times greater than expected due to cryptic species (Eme et al., 2018). Most cryptic species probably emerged in allopatry, by geographic fragmentation. Sometimes they later came into sympatry, examples can be found among amphipod genus *Niphargus* or cave shrimps (Fišer et al., 2018; Zakšek et al., 2009).

On the other hand, a number of species have further diversified morphologically and ecologically, apparently in a process of specialization to different subterranean habitats and trophic niches in both, terrestrial and aquatic environment. Within the group of Australian groundwater dytiscid beetles multiple sympatric pairs or triplets of sister species evolved, that exhibit high morphological disparity (Leijs et al., 2012; Vergnon et al., 2013). Another case come from subterranean spiders. Two sister species from the genus *Troglohyphantes,* ecomorphologically adapted to different subterranean habitats, apparently evolved through spatial niche partitioning (Mammola et al., 2018). Several sympatric sister species of the subterranean Leiodidae beetles were hypothesised to evolve adaptively (Njunjić et al., 2018). Adaptation to different habitats has been shown in the genus *Niphargus* (Delić et al., 2016; Trontelj et al., 2012). There are other examples of subterranean diversification, such as in freshwater crayfishes (Stern et al., 2017), anchialine cave shrimps (Jurado-Rivera, Pons, et al., 2017) or Carabidae beetles (Faille et al., 2013). The amount of evidence, albeit indirect, suggests that evolutionary processes in subterranean environment closely resemble those on the surface and that the phenomenon of adaptive radiation cannot be ruled out.

Although mostly viewed as ecologically simple, stable and extreme compared to surface counterpart, subterranean environment is also diverse and variable. The emerging alternative view on subterranean environment is that it should at least theoretically promote diversification and adaptive radiation. It offers various habitats, both terrestrial and aquatic, with spaces of different sizes, with different flow velocity and variously distant from the surface. It is also highly fragmented (Culver and Pipan, 2019). Subterranean environment thus provides a complex landscape for lineages to ecologically differentiate and also barriers for gene flow that promote allopatric speciation (Naciri and Linder, 2020). Karst massifs represent discrete natural replicates and offer an interesting model system analogous to lakes and islands. To date, no comprehensive analysis of the speciation-diversification of the exclusively subterranean group has been made. The aim of this PhD programme is to fill this gap.

1.3 STUDY MODEL

Our study model is the amphipod genus *Niphargus*, the largest genus of freshwater amphipods in the world (Väinölä et al., 2008). It is distributed through the Western Palearctic, from Ireland to Iran, with the highest diversity in south-eastern Europe, area of the modern South-Eastern Alps, the Dinarides, and the Carpathians. With more than 420 described species, and a high number of undescribed species, this mega-diverse genus importantly contributes to biodiversity patterns in the aquatic subterranean habitats of the Western Palearctic (Fišer, 2019; Horton et al., 2021; Zagmajster et al., 2014). The genus presumably evolved from a subterranean ancestor, and the diversification of this monophylum apparently took place entirely in the subterranean environment (Fišer et al., 2008b). Nowadays, members of the genus are found in virtually all subterranean aquatic habitats from the surface to great depths. Their morphology is related to habitat properties, suggesting that species adapted to subterranean habitats (Delić et al., 2016; Trontelj et al., 2012). *Niphargus* thus comprises three key elements defining adaptive radiation: monophyly, massive speciation, and ecological disparity, making it an excellent candidate to test the model of subterranean adaptive radiation.

Second, *Niphargus* species constitute a major part of the European groundwater fauna and importantly contribute to the biodiversity patterns in the region. As such, it is also a suitable model system for studying groundwater biodiversity patterns of the global subterranean hotspot, the Dinarides. The Dinarides are a 166,000 km^2 large mountain range in south-eastern Europe. The carbonate part of the Dinarides is the Dinaric karst. This 650 km long and up to 150 km wide area with more than 2,000 m of elevation span and more than 20,000 known caves, is the paramount karst region in Europe (Hajna, 2019). The area is a global biodiversity hotspot for surface and subterranean ecosystems (Myers et al., 2000; Sket, 2012; Zagmajster et al., 2014). More than ¼ of all described *Niphargus* species have been found here, and several large monophyletic clades occur only in this region (Fišer et al., 2008b).

1.4 AIMS AND HYPOTHESES

The aim of this dissertation was to comprehensively analyse the speciation and ecomorphological diversification of the genus *Niphargus* across different spatio-temporal scales, and to evaluate its contribution to the origin of a subterranean biodiversity hotspot. We followed five specific aims.

First, we aimed to reconstruct phylogenetic relationships and major diversification patterns of order Amphipoda and solve *Niphargus* position within the order.

Second, we quantified the tempo and mode of diversification patterns of *Niphargus* and explored if they corroborate with adaptive radiation model expectations. We explored whether shifts in speciation and diversification spatially and temporally concur with geologic and paleogeographic events that might have grounded the ecological opportunity. We expected that the evolutionary history of the genus consist of multiple independent adaptive and nonadaptive radiation events in the areas of the emerging mountain ranges in south-eastern Europe.

Third, we in analysed in detail a possible case of ecological speciation within *Niphargus* on a level of a single community, that can be considered as an adaptive radiation in a narrow sense. We analysed a monophylum of three species occupying three distinct habitats within the Melissotrypa Cave in Greece, forming a possible case of ecological speciation.

Fourth, we explored in what extent speciation, either through adaptive or nonadaptive radiations, and dispersal from neighbouring regions contributed to the overall biodiversity of *Niphargus* in Dinarides, a global hotspot of a subterranean fauna. Given the exceptionally high diversity and morphological disparity in the Dinaric Karst, a combination of processes seems to be involved.

Finally, we evaluated the importance of Dinarides for the development of subterranean fauna in Southern Europe. We questioned whether the Dinaric region, where intense speciation took place, acted as a donor of species for the neighbouring areas, especially Apennine Peninsula.

2 SCIENTIFIC WORKS

2.1 THE LATE BLOOMING AMPHIPODS: GLOBAL CHANGE PROMOTED POST-JURASSIC ECOLOGICAL RADIATION DESPITE PALAEOZOIC ORIGIN

Copilaş-Ciocianu D., Borko Š., Fišer C. 2020. The late blooming amphipods: Global change promoted post-Jurassic ecological radiation despite Palaeozoic origin. Molecular Phylogenetics and Evolution, 143: 106664, https://doi.org/10.1016/j.ympev.2019.106664: 12 p.

The ecological radiation of amphipods is striking among crustaceans. Despite high diversity, global distribution and key roles in all aquatic environments, little is known about their ecological transitions, evolutionary timescale and phylogenetic relationships. It has previously been proposed that the amphipod ecological diversification began in the Late Palaeozoic. By contrast, due to their affinity for cold/oxygenated water and absence of pre-Cenozoic fossils, we hypothesized that the ecological divergence of amphipods arose throughout the cool Late Mesozoic/Cenozoic. We tested our hypothesis by inferring a large-scale, time-calibrated, multilocus phylogeny, and reconstructed evolutionary patterns for major ecological traits. Although our results reveal a Late Palaeozoic amphipod origin, diversification and ecological divergence ensued only in the Late Mesozoic, overcoming a protracted stasis in marine littoral habitats. Multiple independent post-Jurassic radiations took place in deep-sea, freshwater, terrestrial, pelagic and symbiotic environments, usually postdating deep-sea faunal extinctions, and corresponding with significant climatic cooling, tectonic reconfiguration, continental flooding, and increased oceanic oxygenation. We conclude that the profound Late Mesozoic global changes triggered a tipping point in amphipod evolution by unlocking ecological opportunities that promoted radiation into many new niches. Our study also provides a solid, time-calibrated, evolutionary framework to accelerate research on this overlooked, yet globally important taxon.

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The late blooming amphipods: Global change promoted post-Jurassic ecological radiation despite Palaeozoic origin

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ABSTRACT

The ecological radiation of amphipods is striking among crustaceans. Despite high diversity, global distribution and key roles in all aquatic environments, little is known about their ecological transitions, evolutionary timescale and phylogenetic relationships. It has previously been proposed that the amphipod ecological diversification began in the Late Palaeozoic. By contrast, due to their affinity for cold/oxygenated water and absence of pre-Cenozoic fossils, we hypothesized that the ecological divergence of amphipods arose throughout the cool Late Mesozoic/Cenozoic. We tested our hypothesis by inferring a large-scale, time-calibrated, multilocus phylogeny, and reconstructed evolutionary patterns for major ecological traits. Although our results reveal a Late Palaeozoic amphipod origin, diversification and ecological divergence ensued only in the Late Mesozoic, overcoming a protracted stasis in marine littoral habitats. Multiple independent post-Jurassic radiations took place in deep-sea, freshwater, terrestrial, pelagic and symbiotic environments, usually postdating deep-sea faunal extinctions, and corresponding with significant climatic cooling, tectonic reconfiguration, continental flooding, and increased oceanic oxygenation. We conclude that the profound Late Mesozoic global changes triggered a tipping point in amphipod evolution by unlocking ecological opportunities that promoted radiation into many new niches. Our study also provides a solid, time-calibrated, evolutionary framework to accelerate research on this overlooked, yet globally important taxon.

1. Introduction

Global environmental changes shaped biodiversity patterns throughout Earth's history (Condamine et al., 2013; Hannisdal and Peters, 2011; Roelants et al., 2007). Understanding the historical factors that triggered large-scale evolutionary radiations or extinctions remains a central tenet in evolutionary biology. Investigating the effects of these past changes at the planetary level requires suitable model systems, which can be represented by species rich taxonomic groups with a global distribution and high ecological diversity.

The Amphipoda is among the most ecologically diverse and speciose crustacean orders, encompassing over 10,000 species (Arfianti et al., 2018; Horton et al., 2019) inhabiting all aquatic environments worldwide, from hadal depths to alpine freshwater streams, from lightless groundwater to tropical forests, and from sea bottom sediments to the entrails of gelatinous plankton (Barnard and Karaman, 1991; Bousfield, 1983; Lowry and Myers, 2017). Amphipods are highly abundant and have an important function in structuring aquatic communities (Best and Stachowicz, 2014; Duffy and Hay, 2000; González et al., 2008; Oliver et al., 1982). Furthermore, due to their omnivorous diet and intermediary trophic position, they represent a key link between trophic levels, thus playing an essential role in nutrient recycling (Dangles and Malmqvist, 2004; Machado et al., 2019; Piscart et al., 2011). The dispersal abilities of amphipods are poor due to egg brooding, lack of free-swimming larvae and extended parental care (Barnard and Karaman, 1991; Thiel, 1999; Väinölä et al., 2008). Consequently, populations can easily become genetically isolated, leading to high species diversity and biogeographical patterns which accurately reflect ancient historical events (Bauzà-Ribot et al., 2012; Copilaş-Ciocianu et al., 2019; Copilaş-Ciocianu and Petrusek, 2017; Finston et al., 2007; Hou et al., 2011). Lastly, amphipods are emerging model organisms for research on development, regeneration, ecotoxicology and evolutionary biology (Fišer, 2012; Fišer et al., 2018; Kao et al., 2016; Naumenko et al., 2017; Weston et al., 2013).

Despite global distribution, high abundance, ecological significance, and importance as emerging model organisms, only little is known about the evolutionary history of amphipods and the factors that triggered their impressive radiation. This scarcity of knowledge is due to

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several critical factors. Deep evolutionary relationships within the Amphipoda are uncertain. The most comprehensive phylogenetic studies were based on morphology (Lowry and Myers, 2017, 2013), which is known to be highly homoplastic in amphipods (Berge et al., 2000). Indeed, molecular phylogenies at lower taxonomic levels do not fully agree with the morphology-based systematics (Copilaş-Ciocianu et al., 2019; Esmaeili-Rineh et al., 2015; Havermans et al., 2010; Hou and Sket, 2016; Hurt et al., 2013; Macdonald et al., 2005; Mamos et al., 2016; Verheye et al., 2016). The age of the order is puzzling as well. Bousfield (1977, 1978) and Schram (1986) suggested that amphipods already appeared in the Late Palaeozoic, when the lineages of the superorder Peracarida split. Yet, unlike the rest of peracaridan orders with a pre-Cenozoic fossil record (Schram, 1986; Wolfe et al., 2016), the handful of amphipod fossil taxa are dated no earlier than Eocene and usually bear a modern appearance (Bousfield and Poinar, 1994; Coleman, 2006; Derzhavin, 1927; Kupryjanowicz and Jażdżewski, 2010). Hence, the temporal origin and main cladogenetic events of modern amphipods are not known and cannot be paralleled to main global environmental changes.

The present view on the modern diversity of amphipods can be summarised by two competing hypotheses. The first hypothesis, stating that most of the modern diversity has been attained by the end of the Palaeozoic or Early Mesozoic, is based on the current distribution of superfamilies in relationship to geochronology, cladistic relationships, or on patterns observed in related malacostracans (Barnard and Barnard, 1983; Lowry and Myers, 2013). The alternative hypothesis suggests that the diversity of amphipods could be much younger due to morphological continuity among higher taxa, and due to lower taxonomic diversity in terrestrial and deep-sea habitats in comparison to the closely related isopods (Bousfield, 1978). Molecular phylogenetic studies tend to support this view because they indicate that the onset of diversification of several major amphipod clades dates to the Cretaceous/Palaeogene (Copilaş-Ciocianu et al., 2019; Corrigan et al., 2014; Hou et al., 2014; McInerney et al., 2014; Verheye et al., 2017).

Apart from the recent fossil record and molecular timetrees, several other independent lines of evidence point out to a more recent radiation of amphipods. They are particularly cold adapted animals, exhibiting an inverse latitudinal richness gradient in marine and freshwater habitats (Barnard, 1976; Barnard and Barnard, 1983; Barnard and Karaman, 1991; Copilaş-Ciocianu et al., 2019; Rivadeneira et al., 2011; Väinölä et al., 2008), and high diversity and dominance in the cold, deep-sea benthic assemblages (Brandt, 2005, 2000; De Broyer et al., 2004; Havermans and Smetacek, 2018; Verheye et al., 2017). This pattern is probably related to their generally low tolerance to hypoxia, given that warmer water has a lower concentration of dissolved oxygen (Modig and Ólafsson, 1998; Vaquer-Sunyer and Duarte, 2008; Wiklund and Sundelin, 2001; Wu and Or, 2005). As such, it seems unlikely that amphipods could have attained most of their current ecological disparity during the Early to Middle Mesozoic (Triassic to Early Cretaceous), a period characterized by warm temperatures even in the deep-sea (> 1000 m), by weakly stratified oceans and frequent anoxic events that caused major extinctions (Jacobs and Lindberg, 2002; Lear et al., 2000; McClain and Hardy, 2010). Therefore, we hypothesize that amphipods ecologically radiated in the Late Mesozoic/Cenozoic, when large-scale continental reconfiguration induced global climatic cooling, causing the oceans to transition to a thermohaline (two-layered) circulation which, in turn, increased productivity and oxygenation levels (Donnadieu et al., 2016; McClain and Hardy, 2010; Mills et al., 2019). To test our hypothesis, we generated the first large-scale, time calibrated molecular phylogeny of the Amphipoda and reconstructed the course of diversification and ecological transitions.

2. Materials and methods

2.1. Data collection and sequence alignment

As a taxonomic backbone for data collection we used the classification on the World Register of Marine Species database (WoRMS; http://www.marinespecies.org/) which is mainly based on Lowry and Myers (2017). All the data used in the present study is publicly available in GenBank (www.ncbi.nlm.nih.gov/genbank) and originated from 63 published and 13 unpublished studies (a list of the data sources is found in Appendix 1 and Table S1; data collection ended in January 2018). Taxa were included in a way that we would cover as much phylogenetic and ecological diversity as possible. Maximizing phylogenetic diversity diminishes the effect of long-branch attraction and increases topological accuracy by dispersing homoplasy across the tree (Heath et al., 2008). In most cases, we included one representative species per genus. We selected four molecular markers based on their abundance and representativeness for all main clades: the mitochondrial cytochrome *c* oxidase subunit I (COI), the nuclear ribosomal RNA for the large and small subunits (28S and 18S), and the nuclear histone 3 (H3). All sequences were screened for contamination, presence of stop codons and homology. Preliminary gene trees were constructed for each marker to identify and eliminate unreliable sequences. To properly root the phylogeny, we included nine outgroup representatives: the sister order Ingolfiellida, as well as other members of the Peracarida and Decapoda. The dataset contained 210 (201 ingroup) terminals, representing 102 of the 226 recognized families (45%) (Table S1).

The PhyRe python script (Plazzi et al., 2010) was used to assess the phylogenetic representativeness of our dataset. The analysis was run at the genus level and the reference taxonomy was obtained from the WoRMS database. Confidence intervals for the average and the variation in taxonomic distinctness were calculated using 1000 random replicates.

The protein coding COI and H3 sequences were aligned with MUSCLE (Edgar, 2004) in MEGA 6 (Tamura et al., 2013) and amino acid translated to check for premature stop codons (indicating pseudogenes). Following Copilaş-Ciocianu et al. (2018), we separately aligned the 18S and 28S rRNA sequences with SATé 2.2.7 (Liu et al., 2012). SATé simultaneously co-estimates the alignment and phylogenetic tree, which makes it more accurate than other alignment methods (Mirarab et al., 2015). MAFFT 6.7 (Katoh et al., 2005) was used as the aligner and OPAL 1.0.3 (Wheeler and Kececioglu, 2007) as the merger because this combination provides the highest phylogenetic accuracy (Liu and Warnow, 2014). For tree inference we used the maximum-likelihood method implemented in FastTree 2.1.4 (Price et al., 2010) with the GTR + G20 substitution model. The cycle of alignment and tree building was repeated ten times for each marker. The alignments with the best likelihood score were used as input for final tree estimation and statistical support analyses (see *Phylogenetic reconstruction*). Gblocks 0.9 (Talavera and Castresana, 2007) was used to remove poorly aligned regions with questionable homology in the 18S and 28S alignments. Minimum restrictive settings were applied and regions with gap positions were allowed in the final alignment. The final alignment length was 1741 bp for 18S, 883 bp for 28S, 436 bp for COI (third codon position removed; see next section), and 327 bp for H3, totalling 3387 bp. Individual marker alignments were concatenated using Sequence Matrix (Vaidya et al., 2011). The degree of missing data was 29%. The alignment in NEXUS format is available on Figshare (doi:https://doi.org/10.6084/m9.figshare.8241401) and the Mendeley Data platform.

Ecological data regarding habitat (marine, freshwater and terrestrial; benthic vs. pelagic), mode of life (free vs. symbiotic), depth (littoral/epipelagic, shelf/mesopelagic, bathyal/bathypelagic, abyssal/abyssalpelagic and hadal/hadalpelagic) and temperature (cold vs. warm) were gathered from the relevant literature at the genus level (Barnard and Karaman, 1991; Bazikalova, 1945; De Broyer et al., 2007; Laval, 1980; Vinogradov et al., 1996; Barnard and Barnard, 1983). Note that "symbiotic mode of life" encompasses different types of symbiosis (commensalism, parasitism, and amensalism). The depth zone was attributed on the mean depth value, obtained from the minimum and maximum values for depth ranges of each taxon. A taxon was considered as cold water distributed when its representatives occurred at high to temperate latitudes or deeper than 1000 m. Similarly, a taxon was classified as warm water when its representatives were distributed in tropical to temperate waters above 1000 m depth. All ecological data can be found in the supplementary information (Table S2). To provide an overview of the geographic distribution of clades, we also gathered distribution data which was obtained from Barnard and Karaman (1991). Terminals were assigned to 17 geographical areas (Table S2).

2.2. Phylogenetic reconstruction

We evaluated the level of substitution saturation of each marker using the index of Xia et al. (2003) implemented in DAMBE 5.3.10 (Xia and Xie, 2003). Significant levels of saturation were detected at the COI 3rd codon position ($I_{ss} > I_{ss.c}$, $p = 0.0001$), and, as such, these sites were not included in the phylogenetic analyses. Variable and parsimony informative sites were calculated in MEGA. The concatenated alignment contained 1917 parsimony informative out of 2333 variable sites (18S: 1006/1273; 28S: 575/667; COI: 209/251; H3: 127/142). Best-fitting evolutionary models were selected using PartitionFinder 2 (Lanfear et al., 2017) under the Bayesian Information Criterion and greedy search option.

Phylogenetic relationships were inferred using maximum likelihood (ML), Bayesian inference (BI) and maximum parsimony (MP) methods. The ML analyses were conducted with IQTREE 1.6 (Nguyen et al., 2015) and RAxML HPC 8.2.10 (Stamatakis, 2014). The IQTREE search was performed under an edge-linked partitioned model, using the GTR model (applied to each gene partition) with free rate heterogeneity ($+R$) which relaxes the assumption of Gamma distributed rates and has a better fit to large and complex datasets (Soubrier et al., 2012; Yang, 1995). Statistical support for branches was assessed using 1000 ultrafast bootstrap replicates (UFBS; Hoang et al. 2018) and the Shimodaira-Hasegawa approximate likelihood ratio test (SH-aLRT; Shimodaira and Hasegawa 1999, Guindon et al. 2010). The RAxML analysis was run with the $GTR + \Gamma$ model applied to each gene partition. A thorough ML search was performed and 1000 rapid bootstrap replicates (RBS) were used to assess branch support. Bayesian analyses were performed with ExaBayes 1.5 (Aberer et al., 2014) under the $GTR + \Gamma$ model applied to each gene partition. All parameters (except branch length) were unlinked and rates were allowed to vary independently. The analysis was run for 10^7 iterations, with a thinning of 500 and 50% burn-in. The value for the parsimony subtree pruning and regrafting (SPR) radius parameter was set to 50 and the number of swaps per generation to 10. Maximum parsimony was performed with PAUP* 4.0a164 (Swofford, 2002), using heuristic searches with TBR branch swapping and 1000 random taxon additions. Only phylogenetically informative sites were retained, gaps were treated as missing data, and all characters were unordered and equally weighted. Nodal support was estimated with 500 jackknifing replicates (JK) with 50% character removal. All phylogenetic analyses were performed on the CIPRES Science Gateway v3.3 (Miller et al., 2010).

2.3. Molecular dating

Molecular dating was performed in BEAST 1.8.2 (Drummond et al., 2012) using the $GTR + I + \Gamma$ model for COI and H3, and $SYM + I + \Gamma$ for 18S and 28S (as selected with PartitionFinder). The ML phylogram from the IQTREE analysis was used as a starting tree in order to reduce computational time. We preferred this topology since it had the highest agreement with the current systematic view. An uncorrelated relaxed clock with a lognormal distribution was applied to each partition and speciation was modelled using the Birth-Death process. The MCMC chain was run for 10^8 generations, with a sampling frequency of 2000. Convergence of parameters and effective sample size were assessed with Tracer 1.6 (Rambaut et al., 2014) after discarding 20% of trees as burn-in. We performed three independent runs, which gave the same result. As such, all runs were combined using LogCombiner 1.8.2 and the maximum clade credibility tree was produced with TreeAnnotator 1.8.2, both part of the BEAST package.

For divergence times estimation we employed the fossil calibration scheme described in detail by Copilaş-Ciocianu et al. (2019), to which we added one more calibration point. Only fossil taxa that are well studied and represented by several specimens were used for calibration. All calibration nodes were assigned exponential prior distributions since they require fewer parameters and are more appropriate when the fossil record of the focal group is poorly known (Ho and Phillips, 2009). Briefly, we used five calibration points: the youngest (node 1), representing the putative origin of the Ponto-Caspian gammarid amphipod radiation, was set to a minimum age of 9 Ma (million years) (mean = 25, offset = 8, 95% HPD = 9–83) based on Caucasian fossil specimens of late Miocene age (Derzhavin, 1927). The Niphargidae/Pseudoniphargidae and Crangonyctidae/Pseudocrangonyctidae splits (nodes 2 and 3, respectively) were set at a minimum of 35 Ma (mean = 60, offset = 35, 95% HPD = 38–215) based on Eocene Baltic amber fossils (Coleman, 2006, 2004; Coleman and Myers, 2000; Kupryjanowicz and Jażdżewski, 2010). The additional calibration point (node 4) that we use in this study is based on Miocene amber specimens of the family Talitridae from Central America (Bousfield and Poinar, 1995, 1994). The minimum age was set to 25 Ma (mean = 30, offset = 21, 95% HPD = 22–111) and was applied to the stem of the Talitridae because this family has an uncertain phylogenetic position within the Talitroid clade of our phylogeny. Finally, the oldest calibration point (node 5) was placed as close to the root as possible (following Duchêne et al. 2014), and represents the oldest known member of Eumalacostraca, *Palaeopalaemon newberry* Whitfield, 1880 (minimum = 358 Ma, mean = 55, offset = 355, 95% HPD = 358–514) (Schram et al., 1978). The nodes do not seem misdated, given that inconsistency between fossil ages and lineage history was not significant (Shapiro-Wilk normality test, $W = 0.91$, $p = 0.51$, see Marshall 2008). We acknowledge that there are fossil Isopoda and Tanaidacea that could be used as calibration points. However, their inclusion would have little effect on the Amphipoda ingroup because of the poorly understood phylogenetic relationships among Peracarids (Wilson, 2009; Wolfe et al., 2016) which were reflected as low branch support in our phylogeny. In such cases the best strategy is to use calibration points placed close to the root (Duchêne et al., 2014).

Due to the notable difference between the recent amphipod (ingroup) calibration points (9–35 Ma) and the old outgroup (358 Ma) calibration, we also ran two additional analyses, one only with ingroup calibrations, and one only with the outgroup calibration to assess if they produce compatible results. Additionally, for the complete calibration scheme we performed a BEAST run without sequence data to determine whether the results of the divergence times are dominated by signal from the data rather than the prior.

2.4. Diversification through time and ancestral state reconstruction

A sliding window analysis was performed according to Meredith et al. (2011) to visually inspect the tendency of diversification rates through time. The period between 180 and 10 Ma was divided into sliding windows of 10 Ma, with a frequency of 2.5 Ma. The period prior to 180 Ma was not considered due to the low number of lineages which would indicate an artificially inflated diversification rate. The number of lineages originating in a particular sliding window was divided by the number of lineages occurring prior to the start of that respective sliding window.

We reconstructed ancestral states for the five above mentioned ecological traits with the aim of evaluating the temporal framework of the amphipod ecological transitions. As such, we used the time-calibrated tree from the BEAST runs in subsequent analyses. Traits were treated as discrete and analysed with the re-rooting method using maximum likelihood (ML) (Yang et al., 1995) as well as stochastic character mapping (SCM) using Bayesian inference (Bollback, 2006), both implemented in R (v.3.5.2) package *phytools* (v.0.6–60) (R Core Team, 2018; Revell, 2012). ML ancestral state reconstruction was performed with the *rerootingMethod* function, while SCM with the *make.simmap* function (Revell, 2012). In order to estimate the possible ancestral character states on internal nodes, we ran 1000 simulations of stochastic character histories, using continuous-time reversible Markov model parameters of trait evolution, estimated using default settings and the character states on the tips of the phylogeny.

In order to evaluate the tempo of ecological disparification through time, we divided the phylogenetic history in 10 MY time bins, and calculated the average number of all possible transitions between states per time bin, as well as for the whole phylogeny. For the sake of clarity, we refer the term “diversification” to the process of speciation, while we use the term “disparification” to the process of ecological divergence.

3. Results

3.1. Dataset

The phylogenetic representativeness analysis indicated a highly comprehensive taxon sampling. The Average Taxonomic Distinctiveness (AvTD) and Variation in Taxonomic Distinctiveness (VarTD) were above the highest AvTD and below the mean VarTD respectively (Fig. S1). Furthermore, von Euler’s index of imbalance ($I_E = 0.102$) was well below the recommended 0.25 threshold value, indicating unbiased sampling (Plazzi et al., 2010) (Fig. S1).

3.2. Phylogenetic reconstruction and molecular dating

All five phylogenetic reconstruction methods yielded congruent results by recovering the same major clades. All model based methods recovered similar topologies and discordance was observed mainly at poorly supported nodes (Fig. 1, Figs. S2–S5). Altogether, we identified nine major clades which we named informally: Gammaroids–mainly Holarctic taxa with freshwater proclivity, Lysianassoids–ecologically diverse deep-sea species, Crangonyctoids–Holarctic freshwater species, Corophioids–tube-building epifaunal/infaunal marine species, Eusiorids/Iphimedioids–ecologically diverse cold-water marine species, Physosomatans and Physocephalatans–exclusively commensal and parasitic warm-water marine species, most of them pelagic, Talitroids–shallow water, ecologically diverse containing the only terrestrial lineage, and Atylids–a morphologically plesiomorphic and cosmopolitan marine group. The following groups were either weakly supported or polyphyletic, but we retained them for the sake of brevity: the Miscellaneous clade was weakly supported and comprised morphologically and ecologically disparate families, while the Hadzioids were morphologically consistent but polyphyletic. The order Ingolfiellida was recovered as a sister to Amphipoda only in the IQTREE analysis. All remaining analyses (ML with RAxML, BI, and MP) recovered the order Spelaeogriphacea as a sister to amphipods with high support, while the order Ingolfiellida was sister to Amphipoda + Spelaeogriphacea clade (Figs. S3–S5). The ML IQTREE tree is available on Figshare (doi:https://doi.org/10.6084/m9.figshare.8241401) and the Mendeley Data platform.

Molecular dating using all the calibration points or only the root calibration resulted in similar estimations (10 to 20 Ma differences; Table S3). The calibration scheme that included only the recent ingroup fossils resulted in expectedly younger estimates. However, the 95% HPD intervals of all calibration schemes overlapped to some extent despite the discrepancy in the ages of the calibration nodes (Table S3). Furthermore, the BEAST run without sequence data (prior only) indicated that most of the signal of node ages is coming from the data itself since the prior distributions were much younger than the posterior ones (Fig. S6). Altogether, these results contradict the previous views that the modern diversity of the Amphipoda was already established by the Late Palaeozoic/Early Mesozoic (Barnard and Barnard, 1983; Lowry and Myers, 2013). Given the paucity of the amphipod fossil record, we considered the complete calibration scheme for evolutionary, ecological and biogeographical interpretations since it is the most balanced and informed (Warnock et al., 2015). Accordingly, Amphipoda has split off from Ingolfiellida during the Permian (~281 Ma) and started radiating shortly after the Permo-Triassic mass extinction (~240 Ma). Crown ages of all major clades lie between the Early Cretaceous and Early Palaeogene (Fig. 2, Table S3). A fully annotated chronogram is available as a supplementary figure (Fig. S7). The chronogram in nexus format is available on Figshare (doi:https://doi.org/10.6084/m9.figshare.8241401) and the Mendeley Data platform. An additional tree with the geographical distribution of taxa mapped onto it is also available as supplementary information (Fig. S8).

3.3. Diversification through time and ancestral states reconstruction

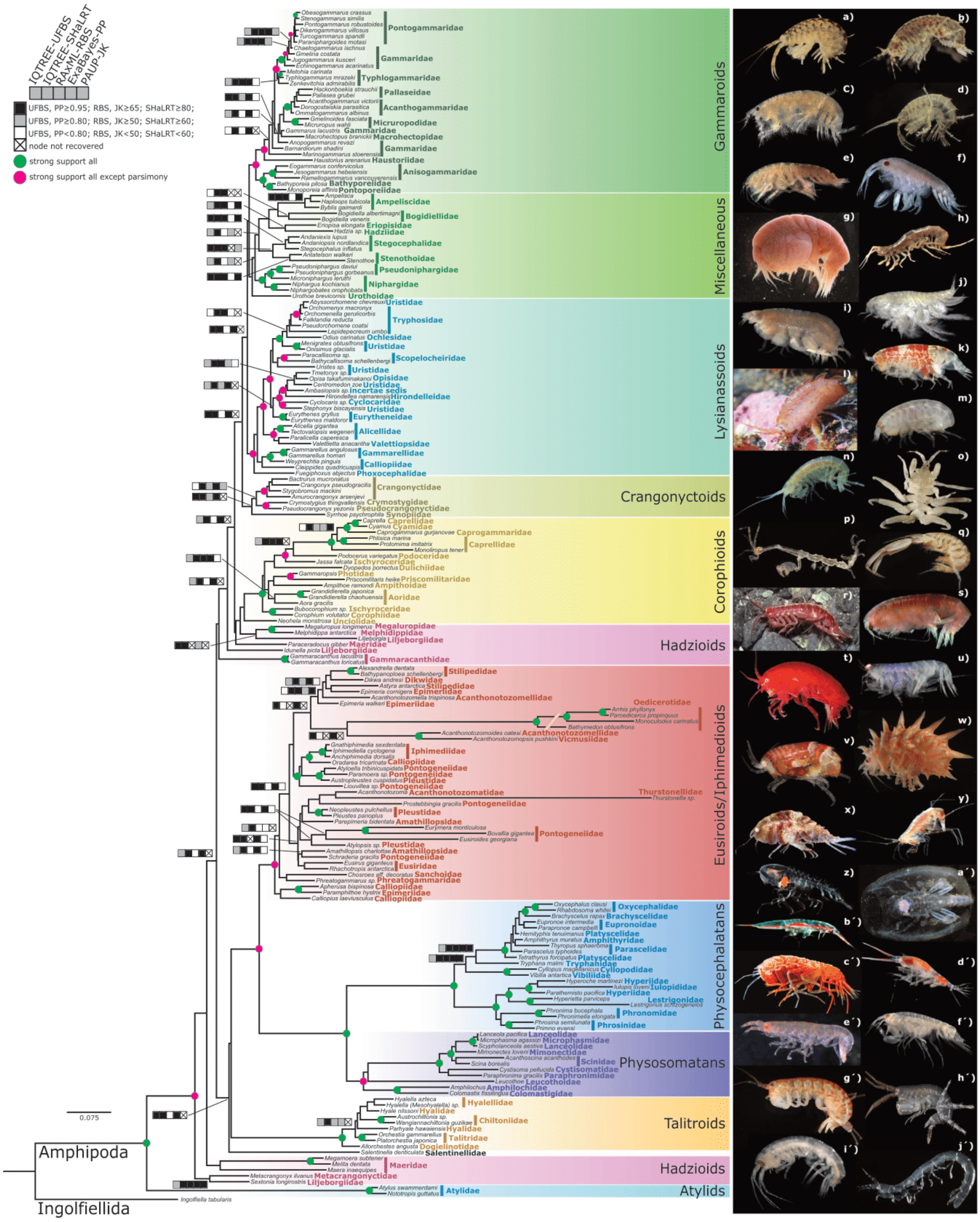
The sliding window analysis identified four main pulses of diversification: the first occurred during the middle Jurassic, the second during the Late Jurassic/Early Cretaceous, the third during the mid-Cretaceous, and the fourth in the late Cretaceous (Fig. 2).

The SCM (Fig. 3) and ML (Fig. S9) reconstructions of ancestral states were highly congruent and revealed a dynamic evolution of Amphipod ecology. The analyses based on 1000 simulations suggested that all ecological changes, except the shift to semiterrestriality, happened multiple times: on average 5.7 times from free-living to symbiotic lifestyle, 7.4 times from marine to freshwater and 2.6 times vice versa, 12.9 times from cold to warm waters and 5.3 times back to cold water, and 9.5 times from benthic to pelagic habitat (Table 1). At least 100 changes of depth zones were estimated, mostly from shallow waters to deep sea (> 1000 m) (Table 1). There was a presumably single shift to semiterrestrial life from ancestors with uncertain salinity preference (Table 1, Fig. 3) (see Discussion).

All these ecological changes took place relatively late in the amphipod evolutionary history, no earlier than 170 Ma. The periods of intense ecological disparification corresponded to peaks of lineage diversification. The ancestral amphipod was reconstructed as a cold-water, free-living, marine animal, inhabiting shallow benthos. The first ecological shifts date to middle Jurassic, when the ancestor of the Hyperiid clade (Physosomatans + Physocephalatans) likely switched from a cold-water and free-living benthic lifestyle to a warm-water and mostly pelagic symbiotic mode of life (Table 1, Fig. 3-S9). The ancestral salinity of Talitroids is uncertain because the ML analysis suggested a marine ancestry while the SCM indicated a freshwater ancestry (see Discussion for details). The next pulse of ecological divergence took place in Late Cretaceous, when the caprellid lineage of the Corophioid clade switched from cold-water free-living animals to warm water-symbionts. At the same time, at least two transitions to freshwater occurred in Crangonyctoids and Niphargidae/Pseudoniphargidae clades. Gammaroids presumably transitioned to fresh waters at least twice during the Palaeogene. Similarly, transitions to deep-sea niches (> 1000 m) occurred predominantly during the Palaeogene, mainly in the Lysianassoids, the Physosomatans and several lineages of the Eusiroid/Iphimediod clade. The third pulse of ecological divergence took place during the last 50 Ma, with multiple ecological shifts all over the phylogeny (Table 1, Fig. 3-S9).

4. Discussion

Our study reveals an ancient Permian origin of amphipods, and their delayed diversification during the Late Jurassic-Early Cretaceous



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followed by ecological radiation during the Cretaceous-Palaeogene. These results refute the view that most of the modern amphipod diversity already existed since the Late Palaeozoic-Early Mesozoic (Barnard and Barnard, 1983; Lowry and Myers, 2013) and help reconcile their old history with their absence in the pre-Cenozoic fossil record. Below we discuss the main global events that may have affected their diversification and ecological radiation. We focus on large-scale patterns and emphasize that although many nested radiations likely occurred, it is beyond the scope of this study to discuss them in detail.

Fig. 1. Maximum likelihood phylogram obtained from the concatenated dataset (COI, H3, 28S and 18S) using IQTREE. Nodes receiving strong support in all analyses are indicated with green circles, while nodes that received strong support in all analyses except parsimony are indicated with purple circles. Bars indicate the strength of the support that each node received in each analysis. Nodes that were weakly supported in all analyses are not annotated. Abbreviations for statistical support: UFBS–ultrafast bootstrap replicates, SHaLRT–Shimodaira-Hasegawa approximate likelihood ratio test, RBS–rapid bootstrap replicates, PP–posterior probabilities and JK–jackknife resampling. Families are indicated with coloured font. Outgroups are omitted for clarity. Photographs on the right indicate the extent of morphological diversity of the Amphipoda. Photographs: Denis Copilas Ciocianu: a) *Dikerogammarus villosus*, b) *Chaetogammarus warpachowskyi*, d) *Gammarus roeselii*, h) *Niphargus pannonicus*; Dante Fenolio (used with author's permission, photos available at www.anotheca.com/photograph): n) *Stygobromus* sp., z) *Cystisoma* sp., a') *Phronima* sp., b') *Streetsia* sp., c') *Scypholanceola* sp., d') *Scina* sp.; David Fenwick (used with author's permission, photos available at www.aphotomarine.com): m) *Lysianassa ceratina*, p) *Caprella tuberculata*, q) *Corophium volutator*, u) *Pontocrates arenarius*, g') *Orchestia gammarellus*, h') *Melita hergensis*, j') *Ingolfiella britannica*; Hans Hillewaert (licensed under Creative Commons, photos available at www.flickr.com/photos/bathyporeia/albums/72157639365477036): c) *Bathyporeia pilosa*, e) *Ampelisca brevicornis*, f) *Stenothoe marina*, i) *Lepidepecreum longicorne*, j) *Urothoe brevicornis*, o) *Cyamus boopis*, s) *Megaluropus agilis*, f') *Leucothoe incisa*, i') *Nototropis swammerdamei*, v) *Iphimedia nexa*; Russ Hopcroft (licensed under Creative Commons, photo available at https://en.wikipedia.org): y) *Eusirus holmi*; Joanna Legeżyńska (licensed under Creative Commons, photo available at www.marinespecies.org): g) *Stegocephalus inflatus*; Gustav Paulay (licensed under Creative Commons, photo available at https://calphotos.berkeley.edu/): e') *Colomastix* sp.; Martin Rauschert (licensed under Creative Commons, photo available at www.marinespecies.org): r) *Paraceradocus* sp.; Alexander Semenov (used with author's permission, photos available at www.coldwater.science): k) *Aristias timidus*, l) *Gammarellus homari*, t) *Acanthonotozoma* sp., x) *Pleustes panopla*; Cédric d'Udekem d'Acoz (licensed under Creative Commons, photo available at www.marinespecies.org): w) *Epimeria oxicarinata*;

4.1. Global evolutionary and biogeographical patterns

Our results indicate that amphipods split off from ingolfiellids during the Permian (~280 Ma). This is in good agreement with previous studies which suggested a Late Palaeozoic age of the Amphipoda based on the fact that peracarids went through an extensive phase of radiation and dispersal during this time (Bousfield, 1983; Schram, 1986; Wolfe et al., 2016). Furthermore, the Permian is also known for its peak in the diversification of marine invertebrates (Alroy et al., 2008). Extant amphipod lineages started radiating after the major Permo-Triassic extinction, a catastrophic event that wiped out up to 95% of marine taxa (Benton and Twitchett, 2003). During this dramatic extinction, the Panthalassic Ocean was largely anoxic, especially at higher latitudes or depths, and only the Palaeotethys Ocean retained suitable levels of oxygenation (Penn et al., 2018), where hypoxia-sensitive amphipods could survive and from where they subsequently radiated. This hypothesis is concordant with the tethyan distribution of the basal, shallow-water Hadziod lineages in our phylogeny (Barnard, 1976; Bauzà-Ribot et al., 2012; Stock, 1993).

Most of the deep splits in the phylogeny leading towards the extant major clades took place during the Triassic and Early Cretaceous (Fig. 2). Also, three of the four main lineage diversification peaks took place in this period. Assuming that amphipods were littoral inhabitants throughout this time-frame, it is likely that the breakup of Pangaea followed by subsequent fragmentation of the resulting Laurasia and Gondwana supercontinents (Seton et al., 2012) played an important vicariant role, as seen in the Holarctic freshwater crangonyctoids (Copilaş-Ciocianu et al., 2019) or thalassoid lineages with an amphi-Atlantic distribution (Bauzà-Ribot et al., 2012). We consider that more detailed inferences are speculative due to the cosmopolitan distribution of many marine lineages (obscuring the biogeography history) and the lack of pre-Cenozoic fossils. Exploring this issue further requires more data and in-depth phylogenetic and biogeographical analysis of the cosmopolitan clades. Similarly, a detailed analysis and discussion of patterns of diversification rates through time was not realistic due to the extremely scarce amphipod fossil record and our incomplete dataset lacking half of the known families (Diaz et al., 2019; Marshall, 2017).

The last major spike in the amphipod diversification history occurred ca. 90 Ma ago (Fig. 2). This peak of diversification could be related to the globally high sea-level stands which resulted in vast shallow epicontinental seas that provided a lot of habitat suitable for amphipods (Scotese, 2014). This is particularly visible in the warm, shallow-water Corophioid, Physocephalatan and Physosomatan clades which started proliferating in this period. Furthermore, this diversification peak coincides with a peak in diversity of marine invertebrates (Alroy et al., 2008) and also to the highest levels of atmospheric oxygen during the last 250 Ma (Berner, 2002), which seems to be a critical factor for amphipod diversification.

Our estimated time-frame of overall amphipod diversification agrees well with recent molecular studies which consistently indicate a Cretaceous-Palaeogene diversification of several major clades, despite different approaches to calibrate divergence times (Copilaş-Ciocianu et al., 2019; Corrigan et al., 2014; Hou et al., 2014; McInerney et al., 2014; Verheye et al., 2017). Therefore, these studies provide strong evidence to indicate that the diversity of extant amphipods is indeed relatively recent, despite the old Permian origin of the group.

4.2. Ecological radiation

Our ancestral state reconstructions suggested that ancestral amphipods were free-living, marine, littoral animals with an affinity for cold water for a large part of their evolutionary history (Permian to Late Jurassic), and are in agreement with previous hypotheses (Bousfield, 1983). The littoral, which is the most oxygenated part of the ocean (Keeling et al., 2010) possibly acted as a long-term refugium for hypoxia sensitive animals such as amphipods (Modig and Ólafsson, 1998; Vaquer-Sunyer and Duarte, 2008; Wiklund and Sundelin, 2001; Wu and Or, 2005). This would suggest that the long Permian-to-mid Mesozoic ecological stasis in littoral habitats was a result of environmental constraints, reflecting the suboptimal conditions present in non-littoral parts of the ocean, such as high global temperatures (especially in the deep sea), weak oceanic circulation, hypoxic conditions and frequent deep-sea anoxia (McClain and Hardy, 2010; Mills et al., 2019). Considering that the coastal environment is prone to erosion rather than deposition (Wilke et al., 2016), our results support the view that the absence of a pre-Cenozoic amphipod fossil record (Bousfield 1978; Schram 1986) is best explained by a combination of long evolutionary confinement to active littoral habitats, small size and weakly fossilizable cuticle (Bousfield, 1983).

Most of the freshwater radiations emerged relatively recently (Late Cretaceous/Cenozoic) after the substantial Late Cretaceous temperature drop (Figs. 2 and 3) (Mills et al., 2019). All of these freshwater clades (especially the Holarctic Crangonyctoids, Gammaroids and Niphargids) descended from shallow-water marine ancestors and often exhibit a peculiar biogeographic pattern with older lineages and higher phylogenetic diversity occurring in the northern part of their ranges (Copilaş-Ciocianu et al., 2019, 2017; McInerney et al., 2014). This is likely a relict pattern which reflects their more northwards pre-Pleistocene distribution, and consequently their affinity for cold water. This hypothesis is supported by amber fossils clearly belonging to the Niphargidae clade, found in the Baltic region, which lies northwards of the contemporary range of the group (Coleman and Myers, 2000; Kupryjanowicz and Jażdżewski, 2010). Noteworthy, the ancestral salinity preference of the Talitroid clade is uncertain in our analyses. The Bayesian reconstruction indicated a freshwater ancestry (Fig. 3) while the ML reconstruction suggested a marine ancestry (Fig. S9). These

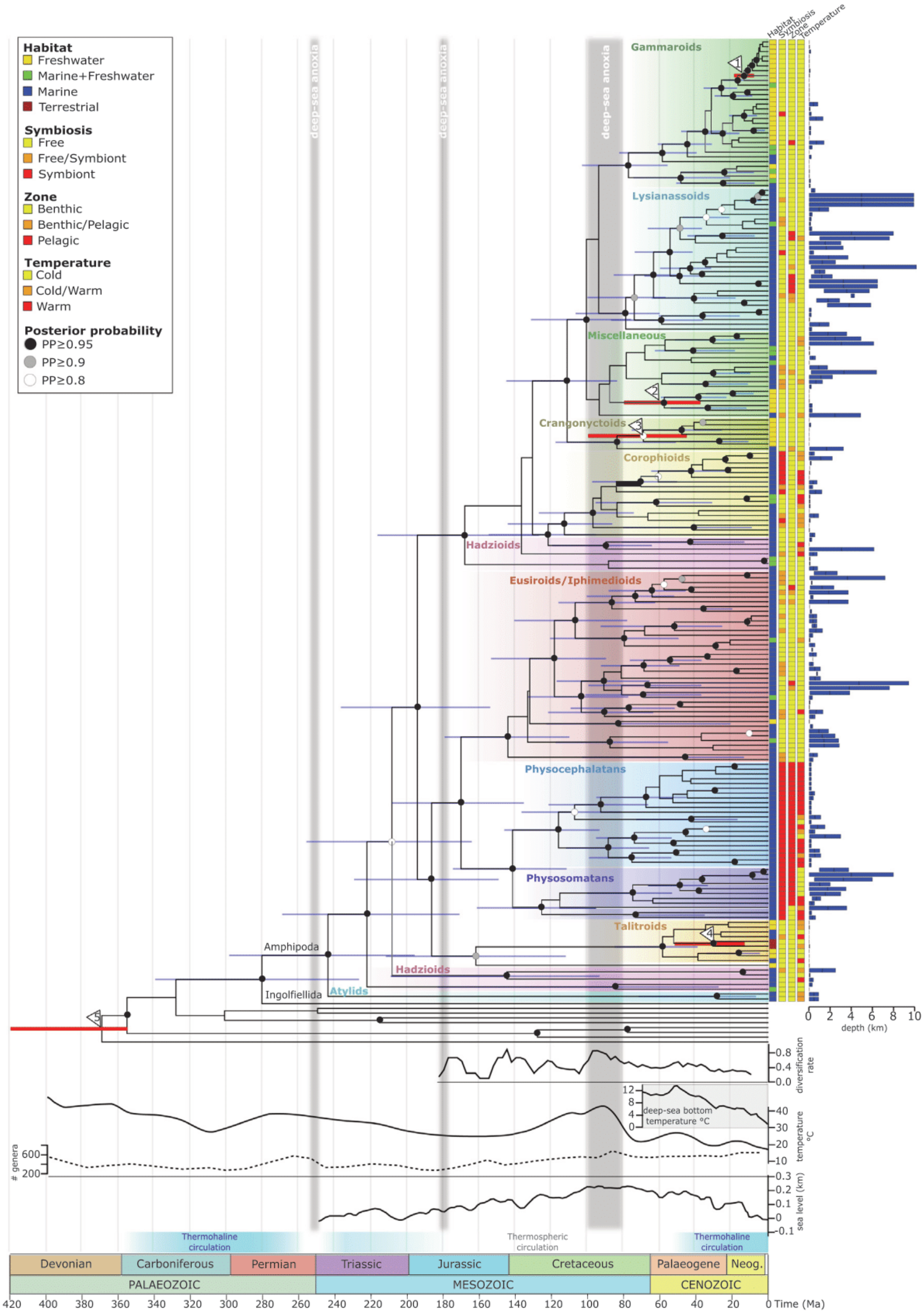

Fig. 2. Evolutionary timescale of the amphipod radiation. The time-calibrated phylogeny was obtained with BEAST using the concatenated dataset (COI, H3, 28S and 18S). Circles at nodes indicate posterior probabilities (black ≥ 0.95, grey ≥ 0.9, white ≥0.8, not shown if < 0.8) and blue bars indicate the 95% HPD intervals of clade age (shown only for main and/or strongly supported nodes). Red bars accompanied by numbers indicate node ages that were constrained using fossils. The fully annotated chronogram can be found in the supplementary information (Fig. S7). Coloured boxes on the right summarize the ecological traits of each terminal in the tree (see legend in the top left) and bars indicate depth ranges. The line graphs below the phylogeny indicate (from top to bottom) the amphipod diversification rate through time, the evolution of Cenozoic deep-sea bottom temperature (Lear et al. 2000), the evolution of Phanerozoic surface temperature (Mills et al., 2019), the evolution of Phanerozoic invertebrate diversity (Alroy et al., 2008), and the evolution of the global Mesozoic-Cenozoic sea level (Miller et al., 2005). The gradient between turquoise and white colours indicates the transitions between thermohaline (two-layered ocean) and thermospheric (weakly stratified) circulation (McClain and Hardy, 2010).

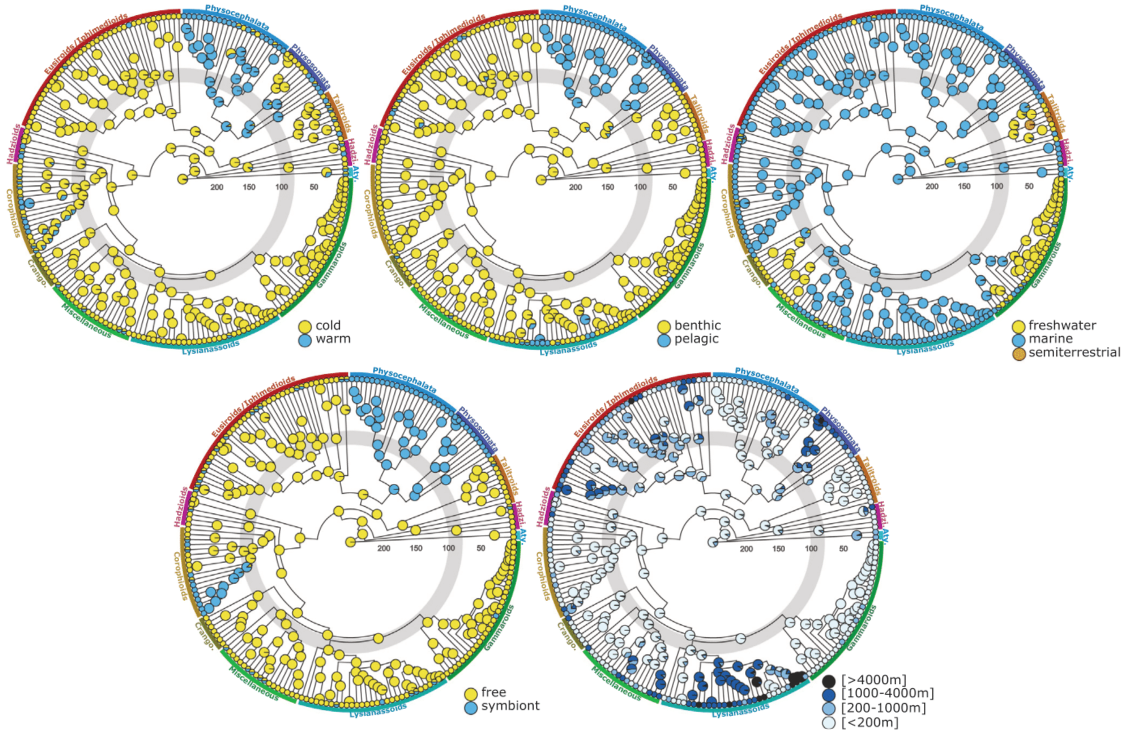

Fig. 3. Ecological transitions of Amphipoda through time. Ancestral state reconstruction was estimated using stochastic character mapping. Pie-charts at nodes indicate the probability of ancestral states. The maximum likelihood results of the ancestral states are presented in the supplementary information (Fig. S9). The grey circle in the 80–100 Ma interval indicates the Cenomanian-Turonian oceanic anoxic event.

contrasting results are most likely an artefact of low sampling of marine Talitroid lineages due to limited sequence availability. Most likely the group has a marine origin (Serejo, 2004) and more fine scale studies also showed a saline origin of some inland Talitrids (Yang et al., 2013).

All the colonizations of the deep-sea (> 1000 m) also took place throughout the cooling of the Late-Cretaceous/Cenozoic, and postdate the Cenomanian-Turonian oceanic anoxic event (80–100 Ma) which triggered global faunal extinctions (Figs. 2 and 3) (Arthur et al. 1987). This time frame also corresponds with the opening and deepening of the North Atlantic and Southern Oceans (Seton et al., 2012) which are thought to have played an important role in the diversification of the Lysianassoid and Eusiroid/Iphimedioid clades (Corrigan et al., 2014; Verheye et al., 2017). These relatively recent deep-sea radiations agree well with the prevailing view that most of the deep-sea fauna is of Cenozoic origin due to the hypoxic conditions during most of the Mesozoic (Herrera et al., 2015; Lindner et al., 2008; McClain and Hardy, 2010; Vrijenhoek, 2013). At least for deep-sea amphipods, the tectonic-induced climatic cooling, leading to a better ventilated ocean (Donnadieu et al., 2016), coupled with the Cenomanian-Turonian extinction not only dwindled the competitors but also created suitable environmental conditions for amphipods to thrive and ecologically expand. These circumstances are believed to have triggered adaptive radiations in other deep-sea lineages (Brandt, 2005). Moreover, the inverse latitudinal richness gradient observed in contemporary freshwater and marine amphipods (Barnard and Barnard, 1983; Rivadeneira et al., 2011; Väinölä et al., 2008) further emphasizes that low temperature is somehow critical for the colonization of freshwater and deep-sea niches. However, we lack a precise understanding of these processes, which are probably related to the generally low tolerance of amphipods to hypoxic conditions (Modig and Ólafsson, 1998; Vaquer-Sunyer and Duarte, 2008; Wiklund and Sundelin, 2001; Wu and Or, 2005).

Transitions to a pelagic life-style occurred numerous times as well, and are especially prevalent in deep-sea lineages (Fig. 3). In freshwater, this transition occurred only once, in the highly specialized Baikal species *Macrohectopus branickii*. Like in the previous cases, the majority of these pelagic transitions are relatively recent (Late Cretaceous to Cenozoic), mainly because these lineages occur in the deep-sea. Exceptionally, the diverse Physocephalatan clade colonized warm shallow epipelagic waters during the Early Cretaceous, earlier than all of the other pelagic lineages. Our results reveal that Hyperiids (Physocephalatans + Physosomatans) colonized pelagic niches two times independently and contradict the prevailing view of a single colonization of pelagial (Lowry & Myers 2017).

The expansion into symbiotic niches seems to not have been influenced that much by climatic cooling. Both the large symbiotic Hyperiid clade as well as the caprellid lineage of the Corophioid clade switched to this life-style during the warm Late Jurassic/Cretaceous (Figs. 2-3). These clades are associated with an ancestral preference for warmer temperatures (Fig. 3). Therefore, it is likely that the Cretaceous high sea-level which led to the formation of numerous shallow and warm epicontinental seas (Scotese, 2014) created vast amounts of suitable habitats which promoted diversification and facilitated the formation of symbiotic relationships. The crown age of the exclusively symbiotic Hyperiid clade (ca. 140 Ma) corresponds well with a peak of reef forming coral diversity at 150 Ma (Simpson et al., 2011), an important habitat for the basal members of this clade (Barnard and Karaman, 1991; Lowry and Myers, 2009). These symbiotic clades often contain taxa with extreme ecological specialization, and exhibit a highly modified morphology (Fig. 1) (Hurt et al., 2013; Ito et al., 2011; Laval,

Table 1
Average number of total transitions (first column) and average number of ecological transitions per time bin, from 1000 trees with mapped discrete character states. Cells with average changes ≥0.5 per time bin or average total change ≥1 are highlighted in bold font.

		Triassic	Jurassic		Cretaceous			Palaeogene		Neog.
Time bins	Mean	> 200	200–170	170–140	140–110	110–80	80–60	60–40	40–20	20–0
Mode of life										
free - symbiont	**5.9**	0	0	**1**	0.1	**0.9**	0.3	**0.5**	**0.6**	**2.6**
symbiont - free	0.2	0	0	0	0	0	0	0	0	0
Habitat										
freshwater - marine	**2.6**	0	0	0	0	0	0	0.2	**1.5**	**0.8**
freshwater - semiterrestrial	**1**	0	0	0	0	0	0	**0.5**	**0.5**	0
marine - freshwater	**7.4**	0	0.4	0.3	0.2	**0.9**	**1.8**	**1.3**	**1.8**	**0.6**
marine - semiterrestrial	0	0	0	0	0	0	0	0	0	0
semiterrestrial - freshwater	0	0	0	0	0	0	0	0	0	0
semiterrestrial - marine	0	0	0	0	0	0	0	0	0	0
benthic - pelagic	**9.5**	0	0	0.2	**1**	**0.7**	**0.5**	**1**	**3.1**	**3.1**
pelagic - benthic	0.7	0	0	0	0.1	0.2	0.1	0.1	0.1	0.2
Temperature										
cold - warm	**12.9**	0.1	0.1	**1**	**0.7**	**0.8**	**1.3**	**1.6**	**3.1**	**4.2**
warm - cold	**5.3**	0.1	0	0.1	0.1	0.4	**0.9**	**1**	**1.2**	**1.5**
Depth										
[< 0.2 km] - [0.2–1 km]	**35.5**	0.3	0.4	**0.9**	**1.9**	**3.4**	**4.5**	**5.6**	**7.4**	**11**
[< 0.2 km] - [1–4 km]	**5**	0	0	0.1	0.2	0.4	**1.1**	**1**	**0.9**	**1.3**
[< 0.2 km] - [> 4 km]	0	0	0	0	0	0	0	0	0	0
[0.2–1 km] - [1–4 km]	**19.6**	0.1	0.1	0.1	**0.5**	**1.4**	**2.7**	**3.7**	**4.5**	**6.5**
[0.2–1 km] - [> 4 km]	**1.5**	0	0	0	0	0	0.2	0.2	0.2	**0.9**
[1–4 km] - [> 4 km]	**5.5**	0	0	0	0	0	0.1	0.4	**1.9**	**2.9**
[0.2–1 km] - [< 0.2 km]	**19.1**	0.2	0.2	0.4	**0.8**	**1.6**	**2.4**	**3.4**	**4.4**	**5.8**
[1–4 km] - [< 0.2 km]	**1.7**	0	0	0	0	0.1	0.1	0.3	**0.5**	**0.7**
[1–4 km] - [0.2–1 km]	**12.5**	0	0	0.1	0.2	**0.6**	**0.8**	**2**	**3.2**	**5.6**
[> 4 km] - [0.2–1 km]	**1.2**	0	0	0	0	0	0	0	0	**1.1**
[> 4 km] - [1–4 km]	**1**	0	0	0	0	0	0	0.1	0.3	**0.5**
[> 4 km] - [< 0.2 km]	0	0	0	0	0	0	0	0	0	0

1980).

The colonization of terrestrial habitats occurred only in the lineage of the family Talitridae of the Talitroid clade. Although this is possibly the most radical ecological transition among amphipods (Spicer et al., 1987), it is also one of the most recent, occurring during the Palaeogene (Figs. 2 and 3). It has been previously proposed that the terrestrial transition of talitrids was dependent on the availability of angiosperms (main food source), and thus, should not be older than the Cretaceous radiation of this group of plants (Barba-Montoya et al., 2018; Bousfield, 1983). Nevertheless, some authors suggest a Pangaean origin of talitrids given their global distribution (Lowry and Myers, 2019). Additional evidence supporting a young age for this clade are its less advanced stage of terrestrial specialization and far lower taxonomic diversity in comparison with the related oniscoidean isopods (Bousfield, 1983; Spicer et al., 1987), which have colonized this habitat at least since the Early Cretaceous, possibly even the Late Palaeozoic (Broly et al., 2013). The young age of the Talitridae implies that the switch to semiterrestrial life likely took place multiple times within the Talitroid lineage, a hypothesis that remains to be tested using a more comprehensive taxon sampling.

It is important to note that our dataset contained about half of the known families. As such, it is possible that inclusion of additional data could change the outcome of the ancestral state reconstructions and divergence times. However, we consider that most of the remaining families would fall into the clades already recovered herein because there is a relatively good agreement between morphological and molecular clades (see next section).

4.3. Systematic implications

The main molecular clades recovered in our phylogeny correspond well in most cases with the major morphological groups (Lowry and Myers, 2017). Due to some unresolved basal nodes and incomplete taxon sampling, the topology of our molecular phylogeny cannot refute the current systematic view. Although we had data on four of the six recognized suborders (Hyperiopsidea and Pseudoingolfiellidea were missing), our phylogeny generally supports the three main suborders, Amphilochidea, Hyperiidea and Senticaudata, but suggests some reshuffling. For example, the parvorders Lysianassidira, as well as the polyphyletic Haustoriidira and Synopiidira should be placed into the Senticaudata rather than Amphilochidea. Similarly, the suborder Colomastigidea along with the amphilochidean families Amphilochidae and Leucothoidae should be placed within Hyperiidea, reinforcing a previously proposed close relationship between Hyperiidea and Amphilochidae (Kim and Kim, 1993). Due to missing data we consider that a thorough systematic discussion is beyond the scope of this paper, and suggest that a phylogenomic approach could prove invaluable for a better understanding of amphipod evolution, as evidenced in a recent study on decapod crustaceans (Wolfe et al., 2019). However, any evolutionary hypothesis needs to be tested by independent lines of evidence, and, as such, we consider that phenotypic data plays a crucial role even in the modern era of phylogenomics (Lee and Palci, 2015).

5. Conclusion

The Late Mesozoic is notable for its dramatic global changes which saw the rise and demise of many organismal groups, leading towards the modern biota (Alroy et al., 2008; Barba-Montoya et al., 2018; Meredith et al., 2011; Roelants et al., 2007; Schulte et al., 2010; Scott, 1995; Varga et al., 2019). In the case of the Amphipoda, these changes brought an important turning point in their evolution. The fortuitous coupling of several critical circumstances such as extinction of deep-sea competitors followed by climatic cooling, oceanic deepening and increased oxygenation, created ecological opportunities which allowed hypoxia-sensitive amphipods to overcome a long period of ecological stasis and to radiate into many new niches. Such patterns of protracted stasis followed by extensive ecological radiation due to global changes seem to be common throughout the tree of life (Halliday et al., 2019; Mitchell and Makovicky, 2014). Our results also emphasize the importance of molecular phylogenetics in illuminating the evolutionary history of highly diverse clades with an extremely poor fossil record.

6. Research data

Trees and alignments are available at FigShare (doi: https://doi.org/10.6084/m9.figshare.8241401) and the Mendeley Data Platform.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2019.106664.

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2.2 A SUBTERRANEAN ADAPTIVE RADIATION OF AMPHIPODS IN EUROPE

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Adaptive radiations are bursts of evolutionary species diversification that have contributed to much of the species diversity on Earth. An exception is modern Europe, where descendants of ancient adaptive radiations went extinct, and extant adaptive radiations are small, recent and narrowly confined. However, not all legacy of old radiations has been lost. Subterranean environments, which are dark and food-deprived, yet buffered from climate change, have preserved ancient lineages. Here we provide evidence of an entirely subterranean adaptive radiation of the amphipod genus *Niphargus*, counting hundreds of species. Our modelling of lineage diversification and evolution of morphological and ecological traits using a time-calibrated multilocus phylogeny suggests a major adaptive radiation, comprised of multiple subordinate adaptive radiations. Their spatio-temporal origin coincides with the uplift of carbonate massifs in South-Eastern Europe 15 million years ago. Emerging subterranean environments likely provided unoccupied, predator-free space, constituting ecological opportunity, a key trigger of adaptive radiation. This discovery sheds new light on the biodiversity of Europe.

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A subterranean adaptive radiation of amphipods in Europe

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Adaptive radiations are bursts of evolutionary species diversification that have contributed to much of the species diversity on Earth. An exception is modern Europe, where descendants of ancient adaptive radiations went extinct, and extant adaptive radiations are small, recent and narrowly confined. However, not all legacy of old radiations has been lost. Subterranean environments, which are dark and food-deprived, yet buffered from climate change, have preserved ancient lineages. Here we provide evidence of an entirely subterranean adaptive radiation of the amphipod genus *Niphargus*, counting hundreds of species. Our modelling of lineage diversification and evolution of morphological and ecological traits using a time-calibrated multilocus phylogeny suggests a major adaptive radiation, comprised of multiple subordinate adaptive radiations. Their spatio-temporal origin coincides with the uplift of carbonate massifs in South-Eastern Europe 15 million years ago. Emerging subterranean environments likely provided unoccupied, predator-free space, constituting ecological opportunity, a key trigger of adaptive radiation. This discovery sheds new light on the biodiversity of Europe.

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Adaptive radiations are large bursts of speciation from single ancestors, accompanied by extensive ecological diversification[1–5]. They have played a significant role in the origin of extant diversity in many major clades across the globe[3,5,6]. Europe, after Antarctica, stands out at the low end of global biological diversity[7,8]. This part of the world, whose natural history has been most thoroughly explored, has shown little evidence of extensive adaptive radiations. Most modern species assemblages of continental Europe seem to have assembled by immigration from elsewhere rather than by in situ adaptive radiations, with minor exceptions among flowering plants, butterflies, and fish in large lakes[9,10]. Albeit interesting because of their high rates of speciation, most of these are geographically narrowly confined and limited in the number of species[11,12]. Notwithstanding the lack of large extant faunal radiations, the European landmass contributed opulently to global species richness during Earth's deeper geological epochs. Fossil records and reconstructions suggest that European adaptive radiations unfolded between Eocene and Miocene[4,13], but these taxa either moved south- and eastward or went extinct due to tectonic changes and paleoclimate perturbations[4,10,13]. The temporary disappearance of the Mediterranean Sea 6–5 million years ago (Mya)[14] and the desiccation of paleo lakes[4] likely eliminated most descendants of older marine and lacustrine adaptive radiations.

There is scattered evidence suggesting that the legacy of Europe's prolific pre-Pleistocene faunal history is not entirely lost but that some groups have survived in subterranean environments that were isolated and protected from the turbulent geological and paleoclimatic history. Indeed, the global species richness of caves and groundwater is the highest in European karstic areas of the Pyrenees, Southern Alps, and the Dinaric Karst. This pattern is well-documented but insufficiently understood[15,16]. A second hint comes from the finding that even environments extremely poor in energy and ecological variation such as deep karstic caves can support considerable adaptive evolutionary diversification[17]. Finally, modern speleological techniques have enabled an advance of biological knowledge in a habitat that is as challenging to explore as the deep sea[18]. Morphological and molecular data from thousands of individuals and hundreds of new species is now available for several subterranean taxa[19–21]. The front-runner of them all is the subterranean amphipod genus *Niphargus*, the largest genus of freshwater amphipod crustaceans in the world[16,22,23].

Here, we present and test an entirely new view on the evolution of subterranean biodiversity and on the origins of major extant European faunal components. We do so by demonstrating that *Niphargus* is a mega-diverse genus with hundreds of species that has not only evolved and diversified entirely on the European continent but has survived here from the times when the landmasses emerged from the sea (Fig. 1). We analyse the tempo and mode of diversification and ecological disparification of this exclusively subterranean clade and show that diversification patterns satisfy the predictions of evolution by adaptive radiation. Next, using spatio-temporal correlations between diversification events and the geological uplift of large carbonate massifs, we suggest that the formation of caves and subterranean habitats created a multitude of ecological opportunities, the quintessential condition for adaptive radiation[24].

Results

Mega-diversification of *Niphargus*. We reconstructed the most complete multilocus phylogeny of *Niphargus*[25] (Fig. 2), containing more than twice as many species as the next largest subterranean clade analysed so far[19]. The analyses of 377 Molecular Operational Taxonomic Units (MOTUs) are based on DNA sequences of one mitochondrial and seven nuclear genes (7067 bp in total, with a mean value per MOTU 3017 ± 2146 (standard deviation)) (Supplementary Data 1). MOTUs also include species discovered in recent studies but not formally named yet (see 'Methods' section).

We found that the genus *Niphargus* originated in the Middle Eocene (mean value 47 Mya, highest posterior density (HPD) interval 56–39 Mya) in the region that is contemporary Western Europe (Fig. 3 and Supplementary Data 2, Supplementary Fig. 1). Lineage through time analysis[26] identified an increase in net diversification rate that occurred at ~15 Mya with subsequent slowdown starting at 5 Mya (Fig. 2a), implying dynamics consistent with adaptive radiations, a so-called 'early burst' ($\gamma = -5.1719$, p-value = 0). Testing alternative models of diversification, we identified an increase in diversification between 15 and 16 Mya corresponding to increased speciation (best model), or increased speciation together with increased carrying capacity (suboptimal model), rather than decreased extinction rates. The impact of extinction is inferred to be negligible in all models (Table 1).

Niphargus species fall into six broad categories by aquatic subterranean habitat: (1) unsaturated fissure system, (2) interstitial groundwater, (3) cave lakes, (4) cave streams, (5) shallow subterranean[27] and (6) groundwater with specific chemistry (brackish, sulfidic, acidic, or mineral) (Supplementary Data 3). The reconstruction of ancestral states using stochastic habitat mapping under Brownian motion suggested that during the first 20–30 million years *Niphargus* species predominantly lived in and dispersed via interstitial coastal and alluvial habitats (interstitial groundwater and shallow subterranean habitat categories). This period was followed by colonisation and adaptation to new subterranean habitats that took place repeatedly in different clades (Figs. 2, 4 and Supplementary Fig. 2). The ecological diversification was analysed using changes through time plots (CTT), which estimates the mean number of character changes per sum of branch lengths in a given period and compares it against a null model of constant evolution[26]. The CTT plot suggested ecological diversification consistent with a neutral scenario at the onset, followed by a substantial drop, and a late phase that begins with a sudden steep increase of ecological diversification at 15 Mya and further increase to the present (Fig. 2). The onset of increased diversification agrees in time with the onset of increased speciation rates. This suggests that ecological diversification and speciation took place hand in hand, coinciding with the orogenesis and karstification of South-Eastern Europe in the Early Miocene (see section 'Geographical origin of adaptive radiations').

The functional morphology of *Niphargus* species (body size, appendage length, body shape) corresponds to environmental conditions to which these species are adapted and can be used to predict components of the ecological niche such as water flow, size of subterranean spaces, mode of locomotion and to some extent feeding habits[17,28]. There is no simple one-to-one correspondence between the morphotypes (Fig. 1) and the habitats listed above. Different morphotypes sometimes share the same habitat but may differ in their trophic niches. Such examples are species of different body size co-inhabiting cave lakes[17], or species of different body shapes coexisting in interstitial groundwater[23] (see Fig. 2b for an example). Conversely, some species with generalist morphology can be found in more than one subterranean habitat[29].

In the next step, we explored the evolutionary dynamics of morphological disparity within *Niphargus*, using disparity through time plots (DTT)[30]. Eleven morphological quantitative traits served as a proxy for the ecological function of species[17,23] (Supplementary Data 4). The DTT approach enables the investigation of

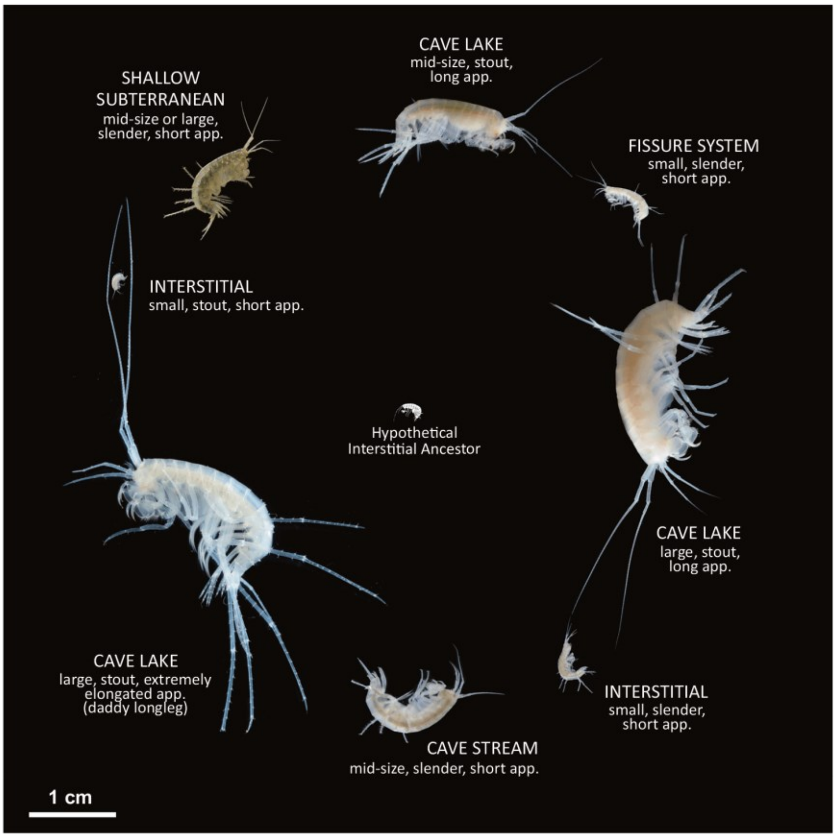
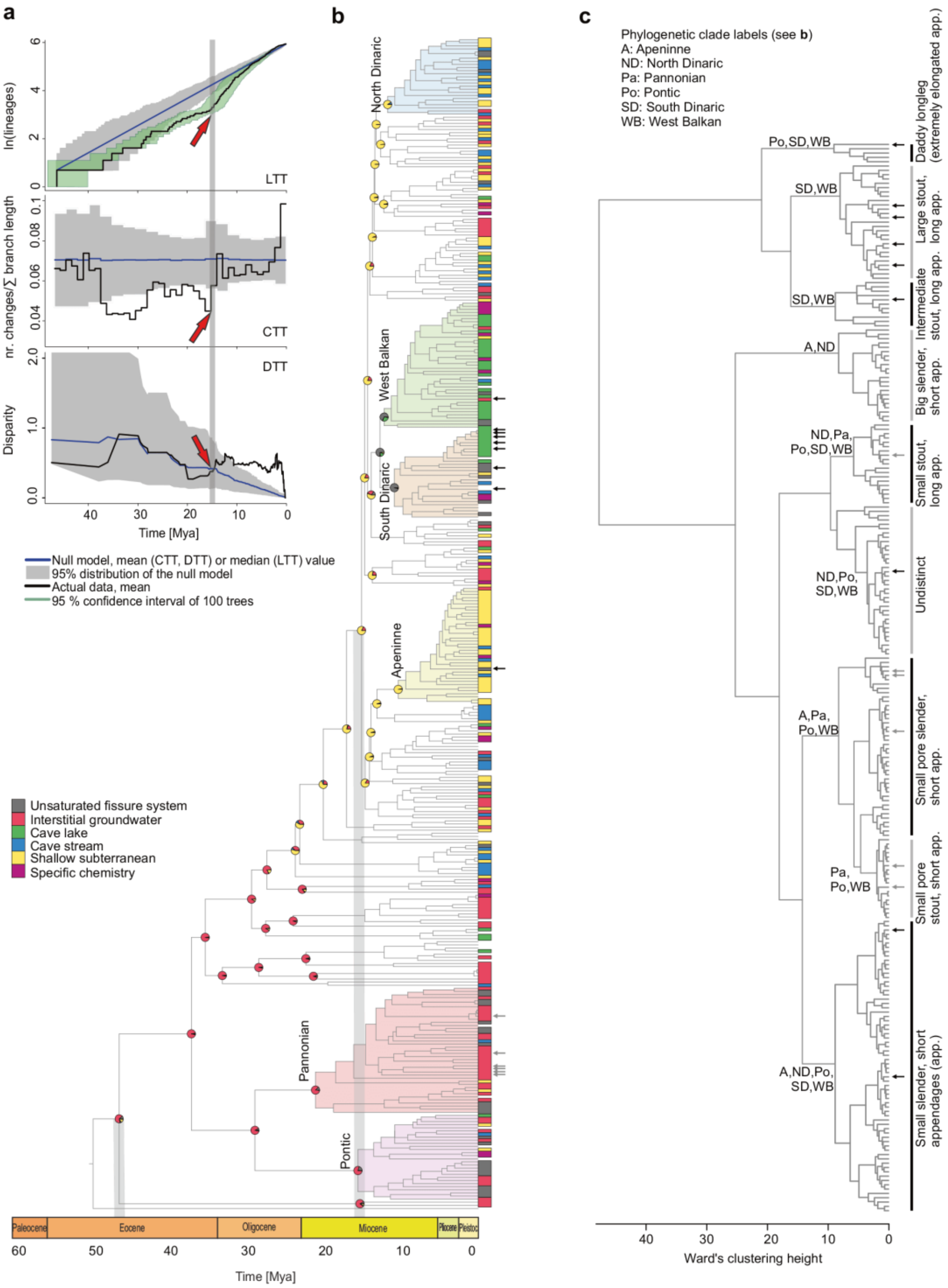


Fig. 1 Morphological and ecological diversity of *Niphargus* species. Adaptive radiation of *Niphargus* produced several morphotypes that inhabit distinct subterranean habitats and niches. Different-sized morphotypes can occur together in cave lakes, and differently shaped morphotypes can occur together in interstitial habitats[17]. These morphotypes evolved in at least five adaptive radiation events from hypothetical small-bodied ancestors from the shallow interstitial habitat[20,23]. Appendages are abbreviated as *app*. Photographs used with author's permission: Denis Copilaș-Ciocianu (shallow subterranean), Teo Delić (all others).

disparity patterns in conjunction with clade age. For each divergent event (i.e. each node) we calculated the average of the disparities of all subclades whose ancestral lineages were present at that time, standardised against the overall disparity. Disparity values near 0 imply that subclades contain relatively little variation present within the entire clade, and most variation is partitioned as differences between subclades. Values near 1 imply that subclades contain a substantial proportion of the total variation, indicating that species within subclades have independently evolved to occupy similar regions of the ecomorphological space[30,31]. The observed DTT curve was compared to the null hypothesis of neutral evolution of morphology in which we simulated the relative disparities obtained from Brownian motion model[30]. The DTT plot suggested an early burst of disparity when the two major lineages arose ~35 million years ago, followed by 15 million years of continuous disparity decline that is in accord with neutral models of evolution. This decline is sharply ended by a significant recovery of morphological disparity at 15 Mya, pointing towards independent diversification of ecological traits within subclades. This phase of phenotypic diversification coincides with the increase in the rates of species accumulation and their ecological diversification (Fig. 2: LTT and CTT). The morphological disparity index (MDI)[31] that measures the overall difference in the relative disparity of a clade against the disparity expected under the null model, showed insignificantly higher disparity than expected by the null model ($\mathrm{MDI} = 0.028$, $p = 0.4$). We attribute this insignificant result to the overall DTT dynamics where low disparities in early history cancelled out higher values during the last 15 Mya. The rank envelope test that tests how extreme the reconstructed DTT curve is compared to the simulated curves, showed that the DTT curve falls outside the expected 95% confidence intervals of the null model simulations (p-interval 0.0009–0.0270). Visual inspection of the DTT plot (Fig. 2) showed that this deviation happened from 15 Mya onward. We also tested whether the evolutionary rates of the eleven studied traits changed in time using the univariate node height test[31,32]. The results were significant for all eleven traits, showing that the rate of their evolution indeed systematically increased during the evolutionary history of the genus (Supplementary Table 1 and Supplementary Fig. 3). Further exploration of multivariate evolutionary models applied to morphological traits[33] suggested that morphological diversification of the genus can be best explained by a switch from the neutral Brownian motion model of morphological diversification to an early burst model at the time of presumed increase in diversification (15.5 Mya). This shift suggests increased morphological diversification that finally slowed down again at 2 Mya, possibly reflecting the emergence of



ecological opportunity associated with the formation of karstic massifs, and the subsequent filling of the new ecological niches as adaptive radiation progressed (Table 2).

In summary, all diversification analyses suggested sudden increases in the rates of species accumulation, morphological evolution and ecological disparification at ~15 Mya—the time when novel habitat emerged at a grand scale[27,34,35] (Fig. 3). These results are consistent with predictions from the ecological theory of adaptive radiation, whereby the emergence of a large volume of novel and diverse habitat triggers the evolutionary diversification of a lineage suited to this habitat.

Fig. 2 Three different aspects of the adaptive radiation of *Niphargus*. a Lineage diversification, ecological and morphological disparification notably accelerated ~15 Mya. The pattern is well visible on LTT, CTT and DTT plots. The time of an evolutionary shift is indicated by red arrows and grey line. **b** Calibrated phylogeny, species' habitats and ancestral habitat reconstructions of *Niphargus*. Tips are labelled according to extant habitat. Pies on selected nodes represent reconstructed ancestral habitat (for complete reconstructions see Supplementary Fig. 2). Clades that were subjected to further analyses are coloured and named. **c** Cluster dendrogram based on eleven functional morphological traits. The same groups were partially recovered by PCA (Supplementary Fig. 8). The phylogenetic composition of morphological groups is labelled on the dendrogram. Cluster analysis shows that unrelated species evolved similar morphology (see clade's acronyms on basal nodes of dendrogram). High morphological disparity presumably allowed high levels of syntopy and the formation of species-rich communities of closely related species. As an example, black and grey arrows on **b** and **c** point to species from a cave (Vjetrenica Cave System, Bosnia and Hercegovina) and interstitial (Sava river close to Medvode, Slovenia) communities, respectively. Note that many community members are closely related (same clade) but belong to different morphological clusters.

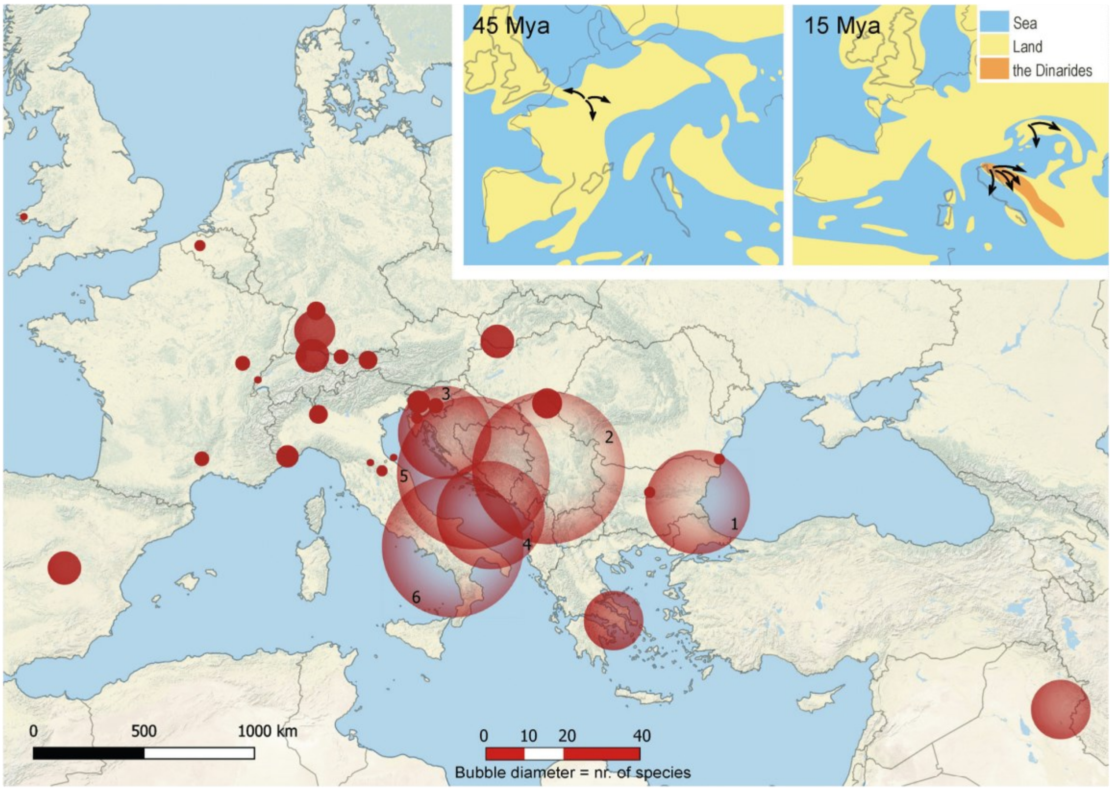


Fig. 3 Geographical extent of the adaptive radiation of *Niphargus*. Bubbles represent the geographic position of subclades; their size reflects species numbers. Clades are numbered as follows: 1. Pontic, 2. Pannonian, 3. North Dinaric, 4. South Dinaric, 5. West Balkan and 6. Apennine clade. Inset: Phylogeny-based reconstruction of ancestral areas and directions of colonisation at 45 and 15 Mya, plotted on corresponding paleo maps, adapted after[39,40]. The geographic origin of species-rich subclades that arose through adaptive radiation corresponds to emerging karstic regions in South-Eastern Europe at about 15 Mya. The contemporary map was produced using QGIS[77] and Esri World Physical Map[78].

Multiple independent radiations. The genus *Niphargus* comprises several large, geographically well-defined clades. In exceptional settings, large adaptive radiations can be the sum of several independent radiations occurring in closely related lineages[3,36]. We selected six well-supported reciprocally monophyletic clades that were geographically well-defined and sufficiently large ($N \geq 25$ species) to be explored for patterns of adaptive radiation by repeating LTT, CTT and DTT analyses on each clade separately. They are distributed mostly in the karstic regions of Italy and South-Eastern Europe and spatially overlap to a various extent (Table 3, Fig. 2 and Supplementary Fig. 4). The Pontic and the Pannonian clade diverged from the rest of *Niphargus* early (38 Mya, HPD 40–35 Mya), and split 29 Mya (HPD 34–24 Mya). The South Dinaric, West Balkan, North Dinaric and the Apennine clade are younger, they started to diversify at 15–11 Mya (Supplementary Fig. 1).

The analyses of diversification (LTT plots and γ-test) suggested that all clades display the pattern of an early burst of species accumulation with onset between 15 and 5 Mya. CTT plots did not deviate from null models, but DTT analyses imply adaptive radiation patterns in the Pontic, Pannonian, West Balkan and the North Dinaric radiations (Table 4 and Fig. 4). Dynamics of species accumulation and ecological disparification among these four clades differ (Table 4 and Fig. 4), possibly reflecting differences in habitat diversity among regions where these radiations unfolded or differences in the time of arrival of these lineages in the novel habitats. The Apennine clade is composed mainly of morphologically similar, still undescribed species.

Table 1 Comparison of evolutionary models tested in order to identify the diversification pattern and rate shifts in the phylogeny of *Niphargus*.

Model	la1	mu1	K1	la2	mu2	K2	t-shift	AIC	AICw
Shift in speciation rate	0.09	0	505.10	0.26	mu_1	K_1	15.70	2214.64	0.57
Shift in speciation rate and carrying capacity	0.12	0	45.15	0.25	mu_1	523.01	15.52	2217.02	0.17
Shift in speciation rate, extinction rate and carrying capacity	0.14	0.01	35.20	0.26	0	501.18	15.70	2217.21	0.16
Shift in extinction rate and carrying capacity	0.24	0.07	25.08	la_1	0	552.42	15.49	2217.97	0.11
Shift in carrying capacity	0.20	0	1.56	la_1	mu_1	666.39	37.51	2238.11	0
Diversity dependent model without shift	0.21	0	609.67	/	/	/	/	2241.14	0

Optimised parameters: la = speciation, mu = extinction, K = carrying capacity, t-shift = time of shift in Mya. The number denotes parameters before (1) or after (2) the shift. All optimising functions reached convergence.

Because of the lack of morphological data, we could not derive conclusions about the nature of this radiation.

Finally, we explored whether these clade-level radiations show signs of between- or within clade convergent evolution. We used SURFACE, a method that tests whether lineages have converged in phenotype without using a priori designations of ecomorphs[37]. It applies Hansen's model of adaptive peaks[38] modelled onto the phylogeny and assumes that in the case of convergence similar phenotypes in different clades could be attributed to the same adaptive peaks. Three models that best explained the evolution of ecomorphological traits predicted 14–16 different adaptive peaks, of which 11–12 were found to be convergent whereas only three to four were unique. These peaks partially correspond to clusters from the morphological analysis (Fig. 2). Of 11 convergent peaks, two evolved multiple times within one clade, whereas nine peaks evolved in two or more focal or non-focal clades. The results of the best model are illustrated in Supplementary Fig. 5.

We tentatively conclude that at least four out of six large and phylogenetically distinct clades could be considered as adaptive radiations. Although showing some degree of convergence, the radiations overall adapted to distinct sets of adaptive optima, especially so among the South-Eastern Europe clades.

Geographic origin of adaptive radiations. The increase in diversification and disparification around 15 Mya corresponds to the emergence of karstic regions in South-Eastern Europe in the area of the modern South-Eastern Alps, the Dinarides and the Carpathians[39,40]. This area underwent a complex geological history. The collision of the European and Adriatic slabs during the Eocene (66–34 Mya) caused vivid tectonic movements that resulted in drastic topographic changes[41]. In the Oligocene (32–23 Mya), the South-Eastern Alps and the Dinarides emerged from the surrounding seas, and the uplift of the Carpathians begun. The exposure of carbonate rocks of the Alpine-Carpathian-Dinaridic orogenic system to atmospheric processes triggered karstification, i.e. the formation of caves and a variety of other subterranean habitats[17,27]. This process began in the Oligocene and peaked in the Early Miocene (23–16 Mya). In that period, mountain uplift continued and the three mountain ranges acted as islands in the Paratethys Sea, occasionally connected during marine regressions. During the later Miocene (16–14 Mya), a mosaic of saline and freshwater aquatic environments and new emerging carbonate islands replaced the Paratethys, which completely regressed from Late Miocene onwards (11 Mya)[34,35].

The dynamic orogenesis of South-Eastern Europe opened a new unpopulated ecological space. These vast new freshwater environments, initially free of predators and competitors, constituted an ecological opportunity for lineages preadapted to freshwater subterranean lifestyles to colonise and undergo subterranean adaptive radiations. We tested this hypothesis by reconstructing ancestral habitats and ancestral areas of the six *Niphargus* clades at 20 and 15 Mya. The evolution of ancestral ranges reconstructed under the Brownian motion model within a Bayesian framework suggested that *Niphargus* originated in what is now a part of Western Europe (Fig. 3). According to ancestral habitat reconstruction these species lived in and dispersed via interstitial and shallow subterranean water systems to South-Eastern Europe. The ancestral ranges of the six clades coincide with emerging carbonate islands of the Tethys Ocean and later Paratethys Sea, nowadays representing the South-Eastern Alps, the Dinaric Karst, and parts of the Carpathian arch (Fig. 3 and Supplementary Data 2).

These results suggest that Miocene karstification on island-like parts of the continent apparently provided an ecological opportunity for groundwater-dwelling *Niphargus* that was able to colonise and subsequently exploit the subterranean realm. The newly colonised empty space possibly triggered ecological divergence from the ancestral interstitial and shallow subterranean forms, followed by morphological diversification and speciation within each independent radiation (Table 3).

Discussion

The recovered patterns are concordant with predictions of adaptive radiation theory: a lineage with heritable ecological versatility to diversify that finds its ecological opportunity, will rapidly proliferate into ecologically different species, until the available niches fill up[5]. We applied different methods which conservatively pinpointed that tree-wide shifts in evolutionary dynamics spatially and temporally correspond to the onset of ecological opportunity.

Niphargus originated from marine ancestors[20] 56–39 Mya, in Western Europe as an interstitial amphipod. For the first 20–30 million years, speciation and ecological diversification followed or even lagged behind neutral expectations. The genus presumably dispersed via coastal or brackish interstitial aquatic habitats (Fig. 2) and accumulated genetic variation, which supported rapid speciation that followed[2].

Uplift of several carbonate platforms in South-Eastern Europe as islands in the Parathetys and subsequent karstification in the Early Miocene, 23–16 Mya, created new, ecologically diverse subterranean habitats. *Niphargus* lineages colonised these new predators- and competitor-free habitats and underwent several evolutionary radiations. At least five of these radiations exhibit patterns of lineage diversification and trait evolution consistent with adaptive radiation, and the available information from the undersampled clades in Greece and Iran[42] hint there may be more such clades. Early habitat diversification, detected in tree-wide CTT analysis but not at the level of an individual clade, may have constrained further clade-level morphological

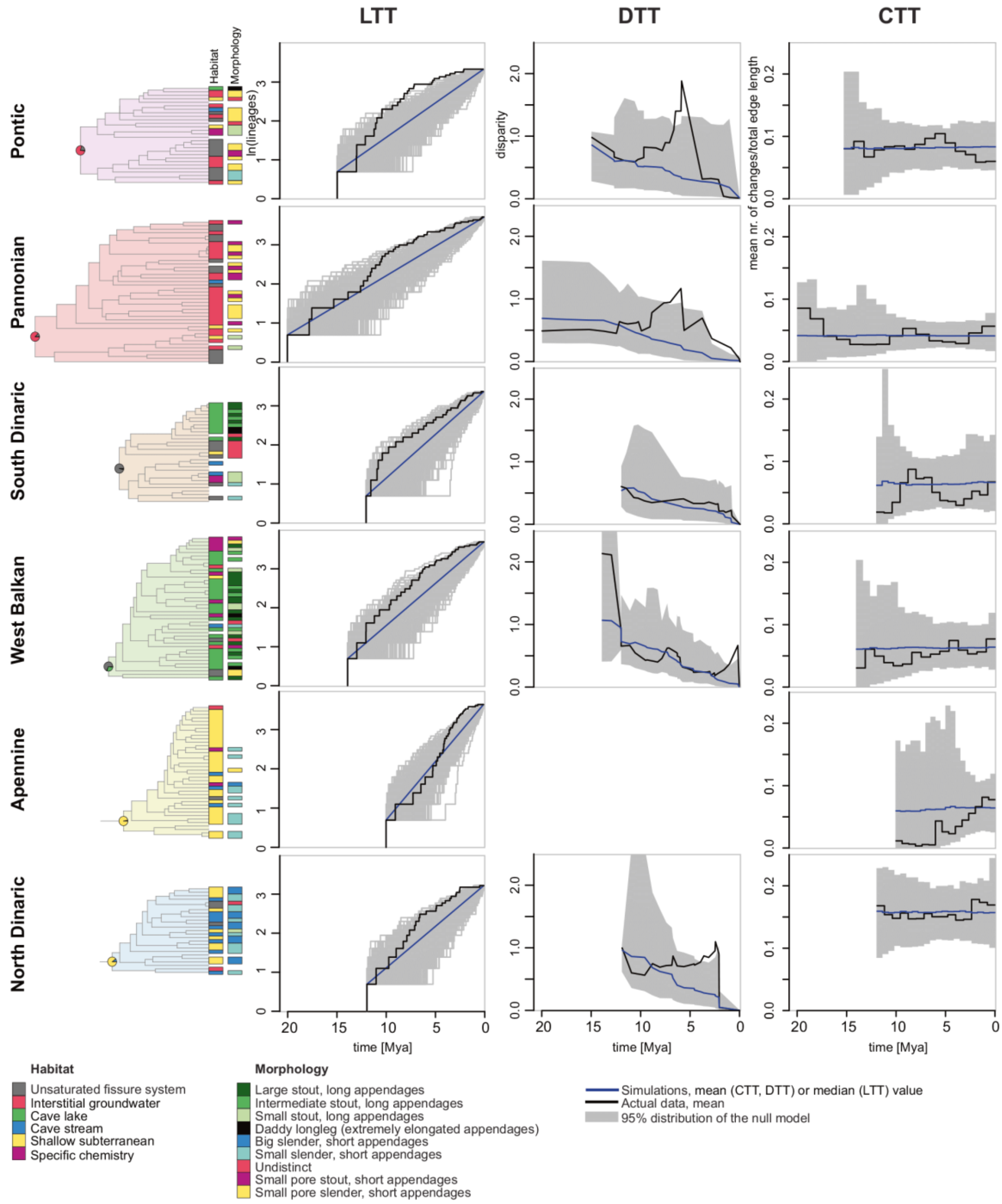


Fig. 4 Evolution of six major *Niphargus* subclades. Zoomed phylogenies with plotted habitats and morphotypes (missing data not available), lineage through time (LTT), changes through time (CTT) and disparity through time (DTT) plots. The DTT analysis could not be performed for the Apennine clade due to too much missing data.

diversification. Subsequent morphological diversification of clades predominantly unfolded within one or few habitat types, like cave lakes (West Balkan and South Dinaric clade) or interstitial groundwater (Pontic and Pannonian clade). This within- and between-habitat diversification prompted high levels of sympatry and allowed for uniquely species-rich communities, counting up to nine species per site[17,23] (Fig. 3). High local diversity combined with multiple independent radiations can explain the extraordinarily high number of species in the Dinaric Karst, a global subterranean biodiversity hotspot[16]. The mega adaptive radiation

Table 2 Comparison of multivariate evolutionary models with or without shift, tested to identify the morphological diversification pattern of the radiation of *Niphargus*.

Model	logLik	params	AIC	diff	AICw
Brownian motion to early burst with independent drift	−1420	9	2859	0	0.555
Ornstein-Uhlenbeck to early burst	−1422	9	2862	2.78	0.138
Brownian motion to Ornstein-Uhlenbeck with independent drift	−1420	11	2862	3.02	0.123
Brownian motion to early burst	−1425	6	2862	3.36	0.104
Early burst to Ornstein-Uhlenbeck with independent drift	−1420	12	2864	5.02	0.045
Ornstein-Uhlenbeck to early burst with independent drift	−1420	12	2865	5.88	0.029
Ornstein-Uhlenbeck	−1426	8	2868	9.4	0.005
Early burst to Ornstein-Uhlenbeck	−1427	9	2871	12.31	0.001
Brownian motion to Ornstein-Uhlenbeck	−1454	8	2925	65.8	0
Early burst to Brownian motion	−1490	6	2991	132.6	0
Ornstein-Uhlenbeck to Brownian motion	−1489	8	2994	134.8	0
Early burst to Brownian motion with independent drift	−1489	9	2996	137.5	0
Ornstein-Uhlenbeck to Brownian motion with independent drift	−1890.18	21	3822.36	250.55	0
Brownian motion	−1915.91	9	3849.82	278.02	0
Early burst	−1915.91	10	3851.82	280.02	0

Table 3 Summary of diversification patterns of the genus *Niphargus* and its major subclades.

Clade	Nr. of species analysed	Distribution	Nr. of habitats occupied	Nr. of morphotypes	Diversification pattern
Niphargus genus	377	From Ireland to Iran	6	9	Adaptive radiation
Pontic	28	Eastern Europe to Crimean Peninsula	6	6	Adaptive radiation
Pannonian	41	Northern Italy, Pannonian Basin and Carpathian arch	4	3	Adaptive radiation
South Dinaric	29	Southern Dinaric Region, Adriatic Islands, southern parts of Apennine Peninsula	5	6	Adaptive radiation
West Balkan	41	North-Western Balkan	6	8	Adaptive radiation
Apennine	38	Apennine Peninsula, Eastern coasts of Adriatic Sea	5	3	Non-adaptive radiation
North Dinaric	25	Northern Dinaric Region, Adriatic Islands, central parts of Apennine Peninsula	4	4	Adaptive radiation

Table 4 Lineage through time (LTT), changes through time (CTT) and disparity through time (DTT) statistics for six major Niphargus subclades.

Clade	LTT statistics	DTT statistics (MDI; *p*-interval of ranked envelope test)	CTT
Pontic	$\gamma = -3.9$, p-value $= 0$	0.15; 0.001-0.025	Random
Pannonian	$\gamma = -2.6$, p-value $= 0.009$	0.17; 0.001-0.026	Random
South Dinaric	$\gamma = -1.8$, p-value $= 0.076$	0.06; 0.364-0.371	Random
West Balkan	$\gamma = -3.1$, p-value $= 0.002$	0.14, 0.001-0.033	Random
Apennine	$\gamma = -3.9$, p-value $= 0$	n.a.[a]	Recent increase
North Dinaric	$\gamma = -2.9$, p-value $= 0.004$	0.15; 0.001-0.028	Random

[a] The DTT analysis could not be accurately performed for the Apennine clade due to missing data.

of the amphipod genus *Niphargus* that generated 20–25% of all freshwater amphipods in the World, can be seen as an exception to the widespread pattern where the fauna of present-day Europe contributes relatively little to global biological diversity[8,43].

At a first glance, it seems paradoxical that what might be the largest surviving pre-Pleistocene adaptive radiation on the European continent took place in an environment as desolate as the karstic underground. The environment is ecologically simple, permanently dark, and nutrient-deprived[27]. As it lacks primary producers, ecological opportunity—an environmental prerequisite for adaptive radiation[24]—does seem unlikely to be fulfilled. However, adaptive radiations have been reported from other oligotrophic habitats, including the abyssal zone of deep lakes[44], the deep sea[45] and the Antarctic[46]. Along with studies from major clades in the tree of life and large biomes[1,3], these studies support Simpson's idea that adaptive radiations are a universal phenomenon and an important global generator of biodiversity[5].

We noted a pattern of independent regional adaptive radiations in several clades. This mechanism likely contributed to the outstanding species richness of *Niphargus*, across different geographic scales[22]. Consequently, the *Niphargus* radiation is similar in size to the radiations of amphipods from the Baikal lake[44,47]. Evolution of distinct phenotypes in independent adaptive radiations as already reported in plants and vertebrates[1,3,48] may be more common than previously thought, yet overlooked in studies that were narrow in taxonomic focus[49].

In the Western Palearctic, ancient in situ adaptive radiations that predate the climate chaos of the Pleistocene do not reveal themselves readily. The case of *Niphargus* suggests that one must search for them in environments that are insulated against the effects of climate fluctuations. Climate history clearly had a strong impact on the rise and loss of biodiversity in Europe[4]. Climatic perturbations wiped out faunas that had evolved in ancient adaptive radiations[4]. Notably, these Pleistocene extinctions created ecological opportunity[24] for multiple Holocene adaptive radiations of limited extent[11,12]. Only in ecosystems that were shaded from the devastating effects of climatic fluctuations could the full historical richness of older radiations be preserved. If this hypothesis is correct, further relic adaptive radiations should be expected among taxa from environments that were so far neglected, such as the subterranean realm and deep soil[21,50]. Whether or not we shall be able to discover them depends also on the progression of adverse anthropogenic impacts: while resilient and buffered from ecological fluctuations throughout geological history, these species are not immune to habitat destruction and may be particularly vulnerable to pollution[51].

Methods

Dataset. The molecular dataset consisted of all available genetic data for described and undescribed *Niphargus sensu lato* species. Several species formally included in different niphargid genera (*Carinurella*, *Haploginglymus*, *Chaetoniphargus*, *Niphargobates*) are nested within the *Niphargus* radiation. Some of them are monophyletic, and others are not. Although the monophyly of *Niphargus sensu lato* was established multiple times[25,29,52] the nested genera were never formally synonymized, and thus we use the valid nomenclature. Sequences included newly acquired and previously unpublished sequences (SubBio Database[53], accessed on 20. 2. 2018) and existing GenBank records[54]. To account for cryptic species, we substituted nominal species with MOTUs, molecularly delimited in the previous works[25,29,55,56]. Most of these species were delimited using multilocus species delimitations; only a smaller fraction was delimited using only mitochondrial COI gene sequences. In these cases, we relied on delimitations using the most conservative delimitation approach, i.e. a 16% patristic distance treshold[20]. In total, our dataset counted 377 MOTUs of *Niphargus*, rooted with five outgroup species from the genera *Niphargellus, Microniphargus* and *Pseudoniphargus*[57]. Of these, 223 MOTUs corresponded to nominal species (50% of the 447 nominal *Niphargus* species (WORMS[58])); 154 MOTUs are not yet formally named.

Ecological information on the studied species was extracted from literature compiled in the European Groundwater Crustacean Database (EGCD)[16], from own data and from databased field notes (SubBio Database[53], accessed on 20. 2. 2018). We assigned 331 species to six habitat categories ((1) unsaturated fissure system, (2) interstitial, (3) cave lakes, (4) cave streams, (5) shallow subterranean and (6) groundwater with specific chemistry (brackish, sulfidic, acidic or mineral)). Species for which ecological data are lacking and MOTUs identified from published GenBank sequences were left uncharacterised. Morphometric traits included 11 morphological continuous traits known to correspond to habitat properties[17,23]. The traits were measured using standard protocol[59]. Briefly, we partially dissected the specimens, mounted them on glycerol slides, and photographed them using a ColorView III camera mounted on an Olympus SZX stereomicroscope. We measured the photographs using the programme CellB (Olympus, 2008) to the precision of 0.01 mm. Depending on the availability of material, we measured between 1 and 20 individuals per species. In many species, samples were heavily damaged. In such cases, we supplemented the measurements with information available in species descriptions. For 26 species we had no appropriate samples, but we could extract measurements from the original descriptions. In total, we compiled a morphometric dataset for 256 species. Ethical issues are not applicable to this study.

DNA sequences. Genomic DNA was extracted using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, United States). We amplified eight phylogenetically informative markers: the mitochondrial cytochrome oxidase I (COI) gene, two segments of the 28S rRNA gene, the histone H3 gene (H3), a part of the 18S rRNA gene, as well as partial sequences of the phosphoenolpyruvate carboxykinase (PEPCK), glutamyl-prolyl-tRNA synthetase gene (EPRS), heat shock protein 70 (HSP70) and arginine kinase (ArgKin) genes. Oligonucleotide primers and amplification protocols are given in Supplementary Table 4. Markers that were sequenced from several specimens of the same MOTU are marked as chimaeras in Supplementary Data 1. To exclude misidentification, chimeric specimens were from the same or nearby localities and with identical sequences of overlapping markers of high resolution (e.g. COI). In the dataset, 301 MOTUs were represented by at least two fragments. For 76 MOTUs only the COI fragment was available.

Nucleotide sequences were obtained by the Macrogen Europe laboratory (Amsterdam, Netherlands) using amplification primers and bi-directional Sanger sequencing. We edited and assembled chromatograms in Geneious 11.0.3. (Biomatters Ltd, New Zealand).

Phylogeny and molecular clock. The sequences were aligned in Geneious with MAFFT v7.388[60] plugin, using E-INS-i algorithm with scoring matrix 1PAM/k = 2 with the highest gap penalty. Alignments were concatenated in Geneious. We used Gblocks[61] to remove gap-rich regions from the alignments of non-coding markers prior to phylogenetic analysis, with settings for less stringent selection.

We used Partition Finder 2 for determining the optimal substitution model for each partition[62,63] under the corrected Akaike information criterion (AICc). The selected optimal substitution models are listed in Supplementary Table 5.

Backbone phylogenies (301 *Niphargus* MOTUs with at least two fragments and five outgroups MOTUs) were reconstructed using Bayesian inference (BI) and maximum-likelihood (ML) in MrBayes 3.2.6[64] and IQ-TREE 1.6.6[65], respectively (Supplementary Fig. 6 and 7). In MrBayes we run two simultaneous independent runs with eight chains each for 200 million generations, sampled every 20,000th generation. We discarded the first 25% of trees as burn-in and calculated the 50% majority-rule consensus tree. Convergence was assessed through average standard deviation of split frequencies, LnL trace plots, potential scale reduction factor (PSRF), and the effective sample size. Results were analysed in Tracer 1.7[66]. The ML tree was calculated in IQ-TREE using the same evolutionary models as in BI, with ultrafast bootstrap approximation (UFBoot)[67], H-like approximate likelihood ratio test[62] and -bnni function to reduce the risk of overestimating branch supports with UFBoot. Phylogenetic analyses were run on the CIPRES Science Gateway (http://www.phylo.org). For the molecular clock analysis, we built a time-calibrated chronogram of all 377 *Niphargus* MOTUs with BEAST 2[68] (Supplementary Fig. 1).

We set four internal calibration points. (1) Fossil: 'modern-looking' *Niphargus* fossils from Eocene Baltic amber estimated to be 40–50 million years old[69]. Their morphology resembles modern *Niphargus* species with distinct pectinate dactyls. We placed the calibration point on the node where this particular morphological character occurs for the first time (*N. ladmiraulti*). (2) European niphargids: the final submergence of the land bridges between Eurasia and North America presumably prevented the dispersal of niphargids towards Greenland and North America. If so, niphargids are most probably younger than the three natural bridges connecting North Europe, Greenland and North America, which existed between 57 and 71 Mya[70]. (3) Crete clade: species from Crete form a highly supported monophyletic group with closest relatives on mainland Greece. The calibration point relies on the isolation of Crete Island from the mainland that happened at the end of the Messinian salinity crisis (5.3–5 Mya)[71]. (4) Middle East clade: species from Lebanon to Iran form a highly supported monophyletic group of eastern *Niphargus*. With rising sea levels and the opening of the connection between Paratethys and the Mediterranean basin 11–7 Mya, the Dinarides and eastern mainland became separated[39,40]. Technical details of calibration points are given in Supplementary Table 6.

To assess the degree of incongruence among the calibration points, we run analyses using each calibration point separately and compared age distributions for main nodes (Supplementary Table 2). For each gene partition we used a set of priors as followed: linked birth-death tree model, unlinked site models as in previous analyses, with fixed mean substitution rate and relaxed clock log-normal distribution with estimated clock rate. We used default settings of distributions of all estimates. We run the analyses for 200 million generations, sampled every 20,000 generations. The first 25% of trees were discarded as burn-in.

All subsequent analyses were run on the clock-calibrated trees of *Niphargus* (377 MOTUs). To account for phylogenetic uncertainty, we used a random subset of 100 calibrated trees and maximum clade credibility (MCC) tree. To assess the potential influence of missing sequence data in the dataset, we repeated the BEAST 2 and through time analyses (LTT, CTT, DTT and subsequent statistics) on the subset of 301 *Niphargus* MOTUs with at least two markers (Supplementary Fig. 8). The results were congruent with the results obtained from the extended dataset and showed a negligible effect of missing data on the final phylogeny reconstruction and downstream analyses.

General strategy in diversification analyses. The theory of adaptive radiation predicts an initial burst of speciation and disparification rates followed by a decline as the available niches fill. We tested this prediction using two alternative strategies. First, we analysed species diversification and ecological as well as morphological disparification through time against simulated null models. Secondly, we tested which evolutionary models of diversification and morphological disparification best fit to our data.

The analyses were run on the whole dataset and repeated on six selected clades that satisfied three conditions: high node support, a high number (>25) of species and geographically well-defined distribution within limited areas (Pontic, Pannonian, North Dinaric, South Dinaric, West Balkan and Apennine clade). MOTUs with missing morphometric data were excluded from the DTT and multivariate modelling analyses. The clades are marked on the chronogram (Fig. 2). All analyses were run in *R v.3.6.0*[72], using packages *vegan v.2.5-5*, *phytools v.0.6-60*, *geiger v.2.0.6.1*, *DDD v.4.0*, *surface v.0.5*, *mvMorph v.1.1.0*, *readxl v.1.3.1*, *RColorBrewer v.1.1-2*, *ggplot2 v.3.3.3*, *grid v.4.0.3*, *gridExtra v.2.3*, *dplyr v.1.0.2* and *plyr v.1.8.6*.

Estimation of speciation dynamics. The diversification dynamics was assessed from the lineage through time (LTT) plot on 100 randomly chosen trees[26], together with the γ-test[73]. The γ-test asks whether per-lineage speciation and extinction rates have remained constant through time. A deviation from 0 indicates a change in speciation rates. As a null model, we simulated 1000 trees under the pure birth assumption, since the analyses under the birth-death assumption did not differ and the estimated extinction rates were negligible.

A change in diversification rate may be related to differences in rates of extinction or speciation. We searched for the best model that describes the course of diversification. To do this, we first fitted different models that account for shifts in diversification parameters: speciation rate (λ), extinction rate (μ) and clade-carrying capacity (K)[74]. Specifically, we tested whether our data is best described by a simple birth-death model with constant λ and μ, diversity dependent models with incorporated K, or models with a shift in time: shift in one or more parameters λ, K and μ. We also estimated the time of the shift. The fit of the models was compared using Akaike weights.

Analysis of ecological diversification. The ecological diversification of *Niphargus* species was estimated using changes through time plots (CTT)[26]. First, discrete habitat traits were mapped onto a phylogenetic MCC tree using stochastic character mapping, assessed from 1000 simulated stochastic character histories using the tip states on the tree and a continuous-time reversible Markov model of trait evolution, fitted on the phylogeny[75]. The evolutionary rates of transition between different ecologies (i.e. transition matrix Q) were estimated from the data. We plotted the mean number of character changes per sum of branch lengths in a given time period from a set of stochastic map trees against evolutionary time. The empirical CTT was tested against the assumption of constant evolution inferred from a null model. The null Brownian-motion model was generated from 1000 simulated stochastic character maps using the tree and the observed transition matrix Q[26].

Analysis of morphological diversification. Morphological diversification was examined using disparity through time plots (DTT), the morphological disparity index (MDI), the ranked confidence envelope test, the node height test, and by searching for the best-fitting model of trait evolution. We used eleven continuous morphological traits (see Dataset). Morphological traits were treated as follows. First, for each trait, we calculated mean values per species. In subsequent analyses, body length was used as a raw variable, whereas other traits were corrected for the body length. We regressed all traits onto body length and calculated phylogenetically corrected residuals using phylogenetic generalised least squares[26]. DTT plots were calculated to examine the course of morphological diversification[30]. The disparity value at each node represents a variance-related estimate of the dispersion of points in multivariate space insensitive to sample size, measured as average squared Euclidean distance among all pairs of species of given subclade[30]. Disparity values of subclades were divided by the overall disparity of the clade. For each divergent event, the average of the relative disparities of all subclades whose ancestral lineages were present at that time was calculated and the empirical DTT curve was compared to null model expectations, generated from 1000 simulated trait distributions obtained from a model of evolution by Brownian motion. We tested the significance of deviation from expected disparity by two methods, pairwise and ranked confidence envelope tests (only results for more stringent, ranked confidence envelope are shown). Second, we calculated the morphological disparity index (MDI) that measures the overall difference in the relative disparity of a clade compared with that expected under the null model[31].

We also performed the univariate node height test that searches for a significant correlation between phylogenetically independent contrasts and the heights of the nodes at which they are generated[32]. The height of a node is defined as the absolute distance between the root and the most recent common ancestor of the pair from which the contrast is generated. Significant correlation indicates that the rate of trait evolution is changing systematically through the tree[32]. We performed a node height test for each of the 11 traits separately.

Finally, we explored which evolutionary model best describes trait evolution within a multi-trait framework that accounts for trait covariances[33]. To reduce the number of parameters, we calculated phylogenetically corrected PCA, and used the first two axes (cumulative variance 97.5%, Supplementary Table 3). We fitted different multivariate models of continuous trait evolution to our tree: Brownian Motion (BM), Early Burst (EB), ACcelerate-DeCelerate (ACDC), Ornstein–Uhlenbeck and models with a shift in time from one regime to another. As a shifting point, we took the estimated time of change of diversification rates (see Species diversification). The fit of the models was estimated using Akaike weights[33].

To illustrate morphological variability and parallel evolution of morphotypes we used hierarchical clustering using Ward's method, using the same dataset as for DTT. We identified nine different clusters, well segregated also when plotted onto the first two PCA axes (see above) (Supplementary Fig. 9).

Analysis of convergence. To test for convergence on the macroevolutionary adaptive landscape we used SURFACE (acronym for 'SURFACE Uses Regime Fitting with AIC to model Convergent Evolution'). SURFACE tests for lineages' convergence in phenotype without using a priori designations of ecomorphs[37]. Briefly, within adaptive radiation, different lineages may evolve into distinct ecological niches, each occupying its own adaptive peak. These adaptive peaks are explicitly modelled in Hansen's model of trait evolution using two parameters, trait value and strength of stabilising selection[38]. The assessment of convergence is made in two steps. In the first step, SURFACE successively fits a series of adaptive peaks onto a phylogeny using stepwise AIC. In turn, it simplifies the model by merging similar adaptive peaks using stepwise AIC. These adaptive peaks may point to convergent evolution[37]. The adaptive peaks were inferred using the first two axes of phylogenetically corrected PCA (explaining 97.5% of total variance) as a surrogate for morphological data, and the MCC phylogeny. We ran two analyses. First, we used the entire genus phylogeny. This analysis was not sensitive enough to capture evolutionary dynamics in the Pannonian and Pontic clade. These species are generally small-pore inhabitants and on average an order of magnitude smaller than in other clades. Relative morphological variation within these two clades is smaller as compared to variation among larger species and traits that vary in these clades explain only the fourth PCA axis, which was not used in the analysis. The genus-level analysis identified one and two adaptive peaks for Pontic and Pannonian clade, respectively. To account for a potential lack of sensitivity, we repeated the analysis on a pruned tree, composed of only two clades. Indeed, this analysis identified additional adaptive peaks (not reported in the main text). The results of both analyses are presented in Supplementary Fig. 5.

Ancestral habitat and ancestral area reconstruction. Ancestral habitat was estimated from the species habitats that were mapped onto a phylogenetic MCC tree using stochastic character mapping. We ran 1000 simulated stochastic character histories using the tip states on the tree and a continuous-time reversible Markov model of trait evolution, fitted on the phylogeny[75]. The evolutionary rates of transition between different habitats (i.e. transition matrix Q) were estimated from the data. Species with unknown habitat were given equal probability for each state. We reconstructed ancestral geographical areas for six analysed clades using the longitude and latitude of our samples mapped onto phylogeny. The evolution of ancestral ranges was mapped on the MCC tree under the Brownian motion model within a Bayesian framework implemented in Geo Model in BayesTraits V3.0.1[76]. The Geo model maps longitude and latitude onto a three-dimensional Cartesian coordinates system, and thus eliminates error in reconstructions due to the spherical nature of the Earth. We ran 11 million MCMC generations, sampled every 10,000th, discarded the first million as a burn-in, and checked the trace for convergence. The 95% confidence intervals for regions of origin were determined such that we discarded 5% of the reconstructed sites that were farthest away from the centroid. The remaining estimated points of origin were plotted onto paleo maps, adapted from the available literature[39,40]. Maps were produced using QGIS[77], and Esri World Physical Map[78].

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Sequence data have been deposited in GenBank. Vouchers, GenBank accession numbers with hyperlinks to GenBank, spatial coordinates, morphometric data, and ecological data of samples are listed in Supplementary Data 1, 3 and 4. Newly obtained sequences are available in GenBank under accession numbers MT191378–MT192029, MZ270543 and MZ295224. Alignments and settings for phylogenetic analyses are available on Zenodo (https://doi.org/10.5281/zenodo.4779097)[79]. European Groundwater Crustacean Database (EGCD) used for compiling ecological information of species is accessible on http://www.freshwatermetadata.eu/metadb/bf_mdb_view.php?entryID=BF73.

Code availability

All code used in analyses is available on Zenodo (https://doi.org/10.5281/zenodo.4779097).

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Author contributions

C.F., P.T. and Š.B. conceived and designed the study. A.M., Š.B. and C.F. collected the data. Š.B. collected the data, conducted analyses, and prepared the results. C.F., Š.B., P.T., O.S. and A.M. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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2.3 AMPHIPODS IN A GREEK CAVE WITH SULPHIDIC AND NON-SULPHIDIC WATER: PHYLOGENETICALLY CLUSTERED AND ECOLOGICALLY DIVERGENT

Borko Š., Collette M., Brad T., Zakšek V., Flot J.-F., Vaxevanopoulos M., Sarbu S. M., Fišer C. 2019. Amphipods in a Greek cave with sulphidic and non-sulphidic water: phylogenetically clustered and ecologically divergent. Systematics and Biodiversity, 17, 6: 558–572

We characterized taxonomically, ecologically, and phylogenetically the amphipod community of Melissotrypa Cave (Central Greece), which comprises both freshwater and sulphidic lakes. We found four amphipod species: *Niphargus jovanovici*, *Niphargus lindbergi*, *Niphargus gammariformis* sp. nov. and an unknown species of *Bogidiella*. The three *Niphargus* species form a well-supported monophylum but differ in their ecology and morphology: *N. jovanovici* is a small and slender species inhabiting small freshwater voids, *N. lindbergi* is a large and stout species living in freshwater lakes, whereas *N. gammariformis* sp. nov. is a small and stout species found predominantly in a sulphidic lake. Available evidence suggests that diversification may have happened in a geographically restricted area and was driven by ecological differentiation. *Niphargus gammariformis* sp. nov. shows morphological convergences in diagnostic traits with two species hitherto classified into the genus *Pontoniphargus*. As molecular phylogenies show *Pontoniphargus* nested within *Niphargus*, we synonymize here *Pontoniphargus* with *Niphargus*. The species originally named *Pontoniphargus ruffoi* needed to be renamed into *Niphargus pontoruffoi*.

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Article

Amphipods in a Greek cave with sulphidic and non-sulphidic water: phylogenetically clustered and ecologically divergent

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We characterized taxonomically, ecologically, and phylogenetically the amphipod community of Melissotrypa Cave (Central Greece), which comprises both freshwater and sulphidic lakes. We found four amphipod species: *Niphargus jovanovici*, *Niphargus lindbergi*, *Niphargus gammariformis* sp. nov. and an unknown species of *Bogidiella.* The three *Niphargus* species form a well-supported monophylum but differ in their ecology and morphology: *N. jovanovici* is a small and slender species inhabiting small freshwater voids, *N. lindbergi* is a large and stout species living in freshwater lakes, whereas *N. gammariformis* sp. nov. is a small and stout species found predominantly in a sulphidic lake. Available evidence suggests that diversification may have happened in a geographically restricted area and was driven by ecological differentiation. *Niphargus gammariformis* sp. nov. shows morphological convergences in diagnostic traits with two species hitherto classified into the genus *Pontoniphargus.* As molecular phylogenies show *Pontoniphargus* nested within *Niphargus*, we synonymize here *Pontoniphargus* with *Niphargus*. The species originally named *Pontoniphargus ruffoi* needed to be renamed into *Niphargus pontoruffoi.*

http://www.zoobank.org/urn:lsid:zoobank.org:pub:4F5EED3B-DEB4-4402-BCE8-6AE2B65CCC3A

Key words: amphipoda community, convergence, ecological divergence, *Niphargus*, phylogenetic clustering, sulphidic cave

Introduction

Subterranean ecosystems encompass a variety of habitats sharing three ecological generalities: the absence of light, strongly buffered short-term climate fluctuations, and limited food resources (Culver & Pipan, 2009, 2014). Most of the food is allochthonous, being produced at the surface and transported into the subterranean environment as diluted or particulate organic matter, where it serves as a substrate for bacterial growth and food for subterranean shredders (Hutchins, Schwartz, & Nowlin, 2014; Venarsky, Huntsman, Huryn, Benstead, & Kuhajda, 2014). Chemolithoautotrophic bacteria, however, contribute to net production. Although the significance of primary production by bacteria depends on the availability of electron donors and is usually negligible (Hutchins, Summers Engel, Nowlin, & Schwartz, 2016; Schmidt, Schwientek, & Cuthbert, 2017), chemolithoautotrophy has been documented to support most, if not all of the food chain in some cave ecosystems (Por, 2007).

Primary production in caves has been most thoroughly studied in sulphidic caves. In these ecosystems, sulphide-oxidizing, methanotrophic, and nitrifying bacteria provide the trophic basis for the entire local community (Flot et al., 2014; Hutchins et al., 2016; Kumaresan et al., 2018; Sarbu, 2000; Sarbu, Galdenzi, Menichetti, & Gentile, 2000; Tobler, Passow, Greenway, Kelley, & Shaw, 2016; Tobler et al., 2011).

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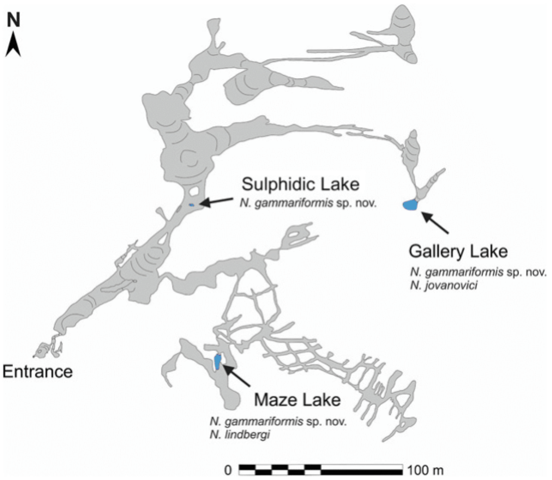

Fig. 1. Map of Melissotrypa Cave – Plan View. Kefalovriso, Elassona, Greece. Coordinates (WGS 84): N 39°52′40″, E 22°02′57″, alt. 299 m. Modified from Vaxevanopoulos (2006).

Although sulphide-rich waters are challenging environments given the toxicity of H_2S (Bagarinao, 1992; Beauchamp et al., 1984; Jiang et al., 2016), acting as a strong ecological filter to many freshwater invertebrates (Galassi et al., 2017; Tobler et al., 2016), sulphide-oxidizing bacteria may produce substantial amounts of organic matter, making food no longer a limiting factor. Among the best-studied sulphidic caves are Movile Cave in Romania, the Frasassi Cave system in central Italy, and Ayyalon Cave in Israel (Brad, Fišer, Flot, & Sarbu, 2015; Fišer, Luštrik, Sarbu, Flot, & Trontelj, 2015; Flot, Wörheide, & Dattagupta, 2010; Flot et al., 2014; Konec, Prevorčnik, Sarbu, Verovnik, & Trontelj, 2015; Por, Dimentman, Frumkin, & Naaman, 2013). Biological investigations have recently been initiated in Melissotrypa Cave in central Greece, another cave comprising both sulphidic and non-sulphidic lakes (Falniowski & Sarbu, 2015; Prevorčnik, Konec, & Sket, 2016). This hypogenic cave is formed in marble (Vaxevanopoulos, 2006). Its main components are the North-Western main corridor, with a 5-metre deep lake containing sulphidic water (named Sulphidic Lake), and the Southern and Eastern maze areas with non-sulphidic freshwater in Maze Lake and Gallery Lake, respectively (Fig. 1). Crustaceans such as isopods are found in all of these lakes (unpublished data, see also Prevorčnik et al., 2016).

Amphipods of the genus *Niphargus* commonly comprise communities of three or more species in one cave (Fišer, Blejec, & Trontelj, 2012; Fišer, Konec, Alther, Švara, & Altermatt, 2017; Trontelj, Blejec, & Fišer, 2012). Sulphidic caves are not an exception: in at least two large sulphidic cave systems, the genus *Niphargus* is represented by two or more species (Brad et al., 2015; Fišer et al., 2015; Flot et al., 2014, 2010). We found three species in Melissotrypa Cave, one mainly in Sulphidic Lake and the other two exclusively in the freshwater lakes. The purpose of this study is to clarify the evolutionary origin of the amphipod fauna from Melissotrypa Cave. First, we characterize all species taxonomically, ecologically, and phylogenetically. We show that the Melissotrypa community differs from all hitherto reported communities (Fišer et al., 2017) as it comprises three phylogenetically closely related species. Finally, we describe a new species, *Niphargus gammariformis* sp. nov. that successfully colonized all three lakes of the cave, and clarify the taxonomic status of the genus *Pontoniphargus*.

Materials and methods

Sampling methods

We visited Melissotrypa Cave (Fig. 1) on six sampling trips from 2012 to 2018: Sulphidic Lake was sampled in September 2012, May 2013, June 2015, May 2017, August 2017, and July 2018; Maze Lake was sampled in September 2012, May 2013, June 2015, May 2017; and Gallery Lake was sampled in May and August 2017, and July 2018. During all these subsequent field trips, samples were collected using hand nets or large pipettes, and were stored in 96% ethanol. We found amphipods during all visits.

Morphometric analyses

We performed three analyses of morphology: an analysis of taxonomic details that is a basis of species description, an analysis of sexual dimorphism of the newly described species, and an analysis of functional traits, in which we compared species' morphology of the focal community with other *Niphargus* species.

We first examined all samples under the stereomicroscope and separated the three species. We selected and dissected five individuals (see below) of the new species, *N. gammariformis* sp. nov. (sample June 2016) that we scrutinized for a long list of characters on which we based the species description. In addition, we dissected and measured another five males and five females for the analysis of sexual dimorphism. We analysed two individuals of *N. lindbergi* (sampled in August 2018) for morphological traits, whereas *N. jovanovici* was left intact.

In order to clear the soft tissues for detailed examination, we heated selected individuals in an approximately 10% KOH solution. We then briefly rinsed the animals in HCl, washed them in distilled water, and coloured them using chlorazol black in glycerol. Coloured individuals were partially dissected and mounted onto semi-permanent slides in glycerin-gelatine medium. The slides were studied using a microscope Zeiss Primostar and a stereomicroscope Olympus SZX. Details were photographed using camera Colour View III (Olympus) mounted onto the stereomicroscope. All measurements were performed on photos, using landmarks discussed and illustrated in previous work (Fišer, Trontelj, Luštrik, & Sket, 2009). In addition, the photographs were used as a basis for illustrations.

In *N. gammariformis* sp. nov., we observed remarkable sexual dimorphism on gnathopod articles 5–6. We quantified sexual dimorphism in order to supplement the species description. For this reason, we measured body length, carpus length, and propodus width. Due to allometric relationships, the studied traits were corrected for body size, and the differences between sexes were estimated using ANCOVA, with the relative lengths of the studied article as response variables, the body length as a co-variable, and sex as a factor. Given the small sample size, we re-checked the results using the more robust non-parametric Wilcoxon–Mann–Whitney test. The analyses were done in R (R Development Core Team, 2016).

In order to illustrate the ecological diversity of *Niphargus* in Melissotrypa Cave, we performed cluster analysis using 45 *Niphargus* species with known ecology (data on material and raw measurements are available in Table S1, see online supplemental material, which is available from the article's Taylor & Francis Online page at http://dx.doi.org/10.1080/14772000.2019.1670273). The functional morphological traits, including body length, the lengths of antennae I–II, pereopods V–VII and coxal plates II–III, and the width of bases V–VII, were measured as described above. The function of the traits has been discussed in details elsewhere (Delić, Trontelj, Zakšek, & Fišer, 2016; Fišer, Zagmajster, & Zakšek, 2013; Trontelj et al., 2012). Briefly, body length relates to the trophic position of the species, positively correlates with fecundity, and is constrained by the size of the voids the species inhabits. Antennae I–II are sensory appendages: longer antennae enhance the detection of environmental cues, chemical and mechanical. Pereopods are involved in locomotion; water currents control pereopod and antennae lengths. Coxal plate lengths and basis widths are key elements of the amphipod ventral channel, a structure involved in locomotion, breathing, and litter care. In order to eliminate the impact of body length, we regressed all traits onto body length and calculated residuals that were used in cluster analyses. Clustering was performed using the Ward agglomeration algorithm based on squared Euclidean distances standardized between −1 and +1 using SPSS 20 (IBM SPSS Statistics).

Molecular phylogenetic analyses

We ran molecular phylogenetic analyses to assess the phylogenetic position of the three Melissotrypa species with respect to the rest of *Niphargus* species. The dataset comprised 96 species that well cover the phylogenetic diversity of *Niphargus* (Esmaeili-Rineh, Sari, Delić, Moškrič, & Fišer, 2015), three species from Melissotrypa, three additional species from Crete, and one species from Sifnos (Greece). The list of the species investigated, the origin of the samples, and the GenBank accession numbers are available in Table S2 (see supplemental material online).

First, we extracted genomic DNA from seven individuals from Melissotrypa and four individuals from Crete and Sifnos using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, USA), following the mammalian tissue preparation protocol. The rest of the specimens were deposited in the zoological collection (University of Ljubljana, Biotechnical Faculty, Department of Biology).

We amplified three gene fragments: two parts of the 28S rRNA gene (28S lev2des2 and 28S lev3des5), the histone 3 subunit A (H3), and the mitochondrial cytochrome oxidase I (COI) gene. The 28S lev2des2 fragment of 28S rRNA gene was amplified using primers 28Slev2 and 28Sdes2 (Verovnik, Sket, & Trontelj, 2005), and 28S lev3des5 with primers 28Slev3 and 28Sdes5 (Fišer et al., 2013). The histone H3 gene was amplified using primers H3AFR and H3AR2 (Colgan et al., 1998). PCR cycler settings are described in Fišer et al. (2013). The mitochondrial gene was amplified using standard primers LCO1490 and HCO2198 (Folmer, Black, Hoeh, Lutz, & Vrijenhoek, 1994) with the PCR protocol after Švara, Delić, Rađa, and Fišer (2015). PCR products amplified in Ljubljana were purified using Exonuclease I and Fast AP Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific Inc., USA) and then sequenced at Macrogen Europe (Amsterdam, the Netherlands) using the same primers as for amplification; the resulting chromatograms were assembled and edited in Geneious 11.0.3. (Biomatters Ltd, New Zealand). PCRs performed in Brussels were directly sent out for sequencing (without purification) to Genoscreen (Lille, France) using the same primers as for amplifications (+ two internal primers for 28S lev2des2 as in Flot et al., 2010); the resulting chromatograms were assembled and edited in Sequencher 4.1.4 (GeneCodes, Michigan).

We aligned the sequences in MAFFT 7.388 (Katoh & Standley, 2013) using the E-INS-I algorithm with the scoring matrix 1PAM/k = 2 and with the highest gap penalty. Alignments were concatenated and the optimal substitution model for each partition was chosen using Partition Finder 2 (Guindon et al., 2010; Lanfear, Frandsen, Wright, Senfeld, & Calcott, 2016) under the corrected Akaike information criterion (AICc; Hurvich & Tsai, 1989). The optimal substitution models were GTR + I + Γ for COI, 28S lev2des2 and H3 fragments and SYM + I + Γ for 28S lev3des5 fragment.

We reconstructed the phylogenetic relationships with Bayesian inference (BI) in MrBayes v3.2.6 (Ronquist et al., 2012) and Maximum likelihood (ML) in IQ-TREE 1.6.6 (Nguyen, Schmidt, von Haeseler, & Minh, 2015).

In MrBayes we ran two simultaneous independent runs with four chains each for 50 million generations, sampled every 1000th generation. Convergence was assessed through average standard deviation of split frequencies, LnL trace plots and PSRF, and the effective sample size. Results were analysed in Tracer 1.7 (Rambaut, Drummond, Xie, Baele, & Suchard, 2018). We discarded the first 50% of trees, and from the remaining trees we calculated the 50% majority rule consensus tree. In the ML analysis we used the same evolutionary models as in BI, with ultrafast bootstrap approximation (UFBoot; Minh, Nguyen, & von Haeseler, 2013). Phylogenetic analyses were run on the CIPRES Science Gateway (http://www.phylo.org; Miller, Pfeiffer, & Schwartz, 2010).

Finally, we calculated the average uncorrected pairwise genetic differences (e.g. p-distance) for the fragments COI and 28S lev2des2 between the three species from Melissotrypa Cave using Geneious 11.0.3.

Results

Amphipod community: taxonomic structure, phylogenetic origin, and ecological diversification

Four amphipod species were collected in Melissotrypa Cave, one belonging to the genus *Bogidiella* and three to the genus *Niphargus*. All individuals of *Bogidiella* were damaged and a bogidiellid specialist (R. Vonk, the Netherlands) could not identify them beyond the genus level. We identified the three *Niphargus* species as *N. jovanovici* S. Karaman, 1931, *N. lindbergi* S. Karaman, 1956 and a new species, *N. gammariformis* sp. nov. (see next section). *N. jovanovici* is distributed from northern Macedonia to as far south as Athens (Greece), *N. lindbergi* is found southward from Melissotrypa Cave, whereas *N. gammariformis* sp. nov. is known only from this locality (Fig. 2).

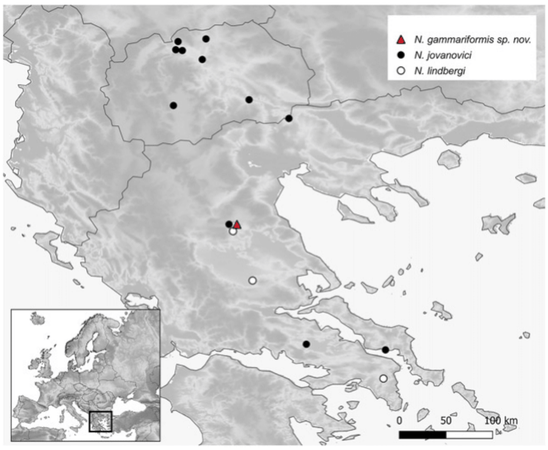

Fig. 2. Geographic distribution of the Melissotrypa clade. The distributions of *N. jovanovici* and *N. lindbergi* are from Karaman (2017, 2018).

The three *Niphargus* species showed spatial segregation. *Niphargus jovanovici* was collected in Gallery Lake, *N. lindbergi* in Maze Lake, while *N. gammariformis* sp. nov. was collected in all of the three lakes of Melissotrypa Cave. The latter species however, showed an impressive abundance of 200 individuals per m^2 in Sulphidic Lake, but was scarce in the non-sulphidic Maze and Gallery Lakes.

All three *Niphargus* species from Melissotrypa Cave are phylogenetically closely related to each other. They form a well-supported monophylum (Fig. 3, Fig. S1, see supplemental material online), whereas the position of the clade in a *Niphargus* phylogeny is not clear. It is noteworthy, that *N. gammariformis* sp. nov. is not related to any of the other *Niphargus* species previously found inhabiting sulphidic caves (Figs 3, S1). All three species are genetically distinct. The COI gene fragment of *N. gammariformis* sp. nov. differs on average by 17.55% (uncorrected *P*-values) from *N. jovanovici.* Amplification of COI fragment for *N. lindbergi* was unsuccessful, thus we also compared fragment of 28S rRNA (lev2des2 part). The fragment 28S of *N. gammariformis* sp. nov. differs on average by 9.48% and 8.42% from *N. jovanovici* and *N. lindbergi*, respectively. The latter two species differ from each other on average by 2.65%.

The three species differ notably in habitat ecology. Their ecological divergence can be summarized in a 2 × 2 ecological matrix: sulphidic *versus* nonsulphidic

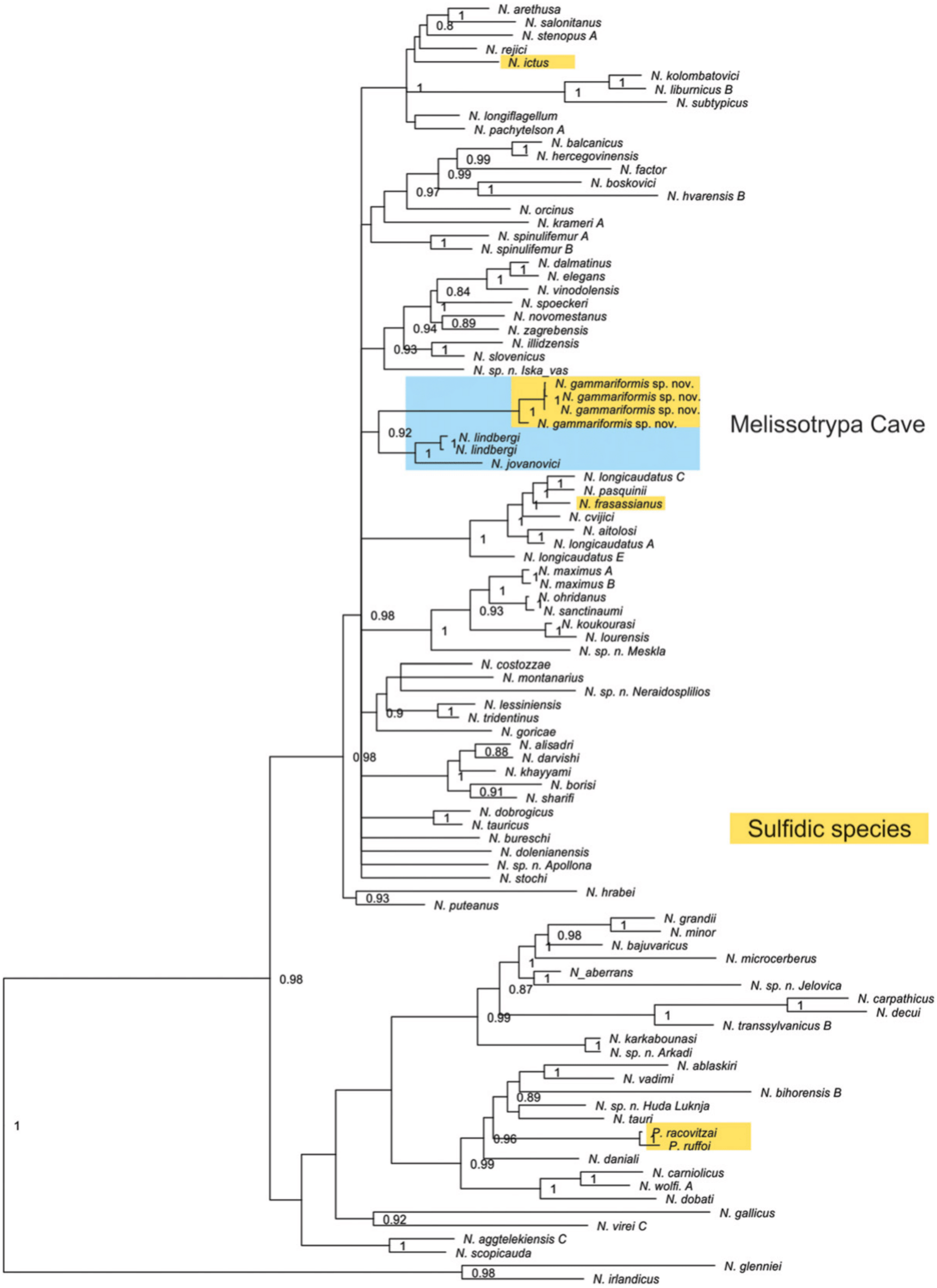

Fig. 3. Phylogenetic hypothesis from Bayesian inference. Species from sulphidic caves are labelled in yellow whereas the additional two species studied here are highlighted in blue. Nodes are labelled with posterior probabilities when higher than 0.80.

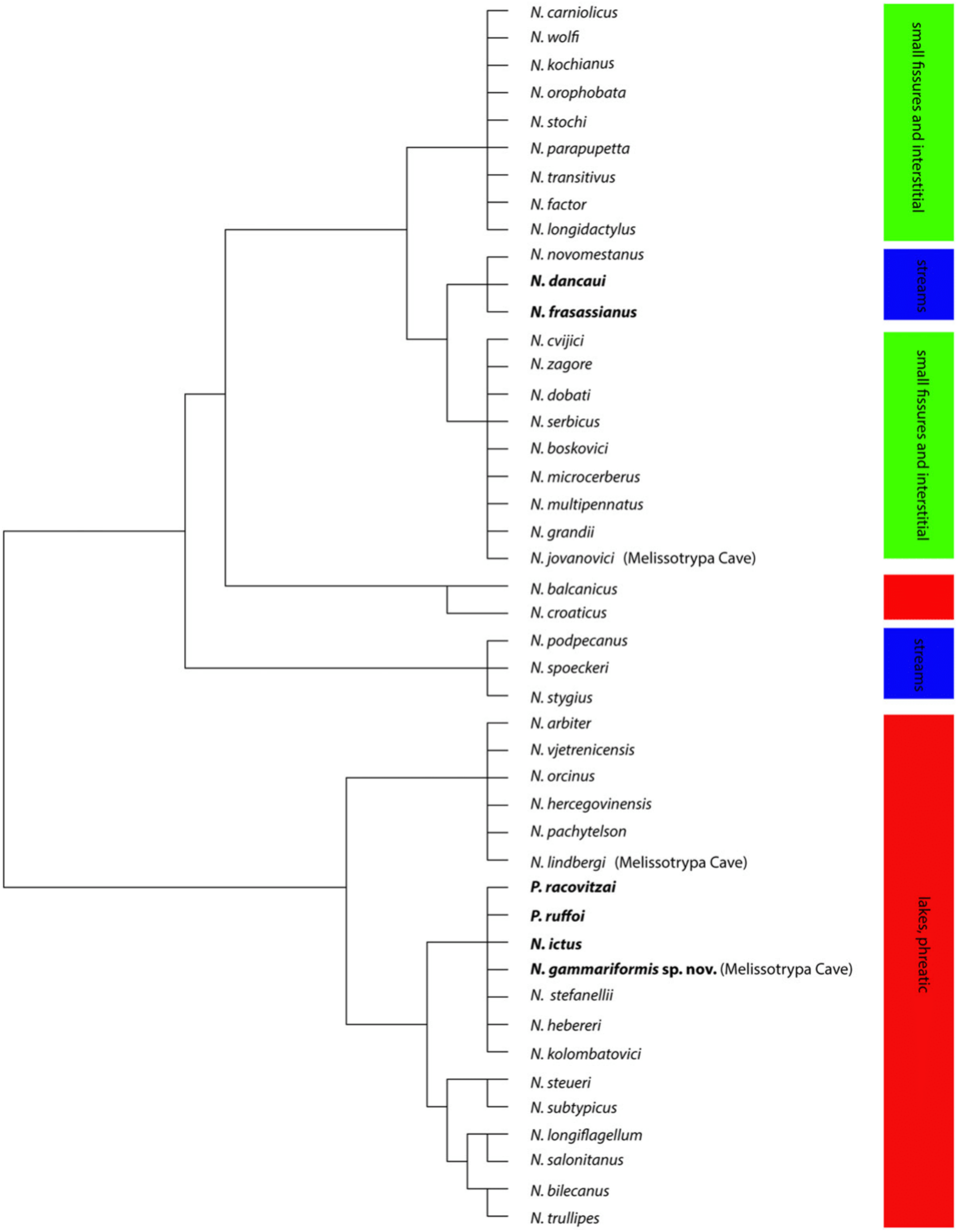

Fig. 4. Cluster analysis of functional morphological traits. The principal habitat (lake, stream, small pores) is indicated with colour bars. Note that stream species and lake species differ in sizes. All species from sulphidic water, labelled in boldface, belong to relatively small species, possibly indicating limitations in oxygen (Chapelle & Peck, 1999).

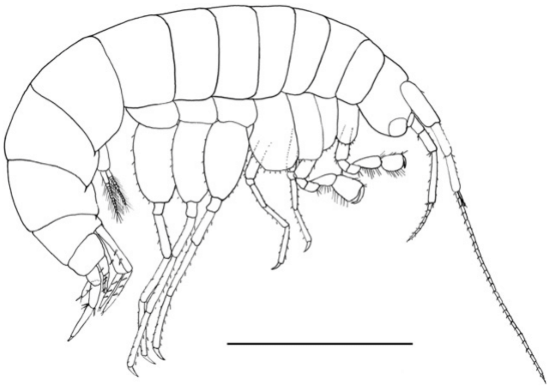

Fig. 5. General appearance of male *Niphargus gammariformis* sp. nov. Scale bar represents: 2 mm.

and tiny crevices *versus* open lakes. Field data strongly suggest that *N. gammariformis* sp. nov. prefers sulphidic water, whereas the other two species avoid hydrogen sulphide. Moreover, *N. jovanovici* was found in between rocks and sand at the bottom of the lake and seems to be constrained to tiny crevices. Conversely, *N. lindbergi* and *N. gammariformis* sp. nov. live in open water. An analysis of their functional morphology is in agreement with the field observations. *Niphargus jovanovici* clusters with other 'tiny crevice' species (epikarst, interstitial), *N. lindbergi* clusters with large species from open lakes, and *N. gammariformis* sp. nov. clusters with species from sulphidic and anchialine lakes (Fig. 4).

Description of the new species

Niphargus gammariformis sp. nov.

Holotype. An intact male. The sample is deposited in the collection of the Department of Biology, Biotechnical Faculty, University of Ljubljana under voucher number NC341 (OBBFUL-NC341).

Material examined. Four males and one female of lengths 8.8, 9.0, 9.2, 9.3, 9.6 mm; specimens are partially dissected and mounted on slides with voucher numbers NB934-NB938 (OBBFUL-NB934-NB938).

Type locality. Melissotrypa Cave, Greece (N39°52′40″, E22°02′57″ UTM 34 N, WGS 84, altitude: 299 m).

Habitat and distribution. Known only from its type locality. It is abundant in Sulphidic Lake, but rare in the freshwater lakes (where it co-occurs with *N. jovanovici*, *N. lindbergi*, and *Bogidiella* sp.).

Etymology. The name is derived from the name of amphipod genus *Gammarus* and refers to its *Gammarus*-like traits, notably its biramous 3rd uropods.

Diagnosis. Small *Niphargus* of stout appearance due to extremely deep coxal plates and very broad bases of pereopods V–VII. Telson with a pair of plumose setae subapically, 3–5 spiniform setae apically, 0–1 lateral, 0–1 mesial, and 1–2 individual dorsal spiniform setae per lobe. Maxilla I with 7 pectinate spines on outer lobe and numerous setae along mesial-apical margins of inner lobe and palpus; maxilla II with slightly visible oblique facial row of setae along inner lobe; maxilliped flattened with numerous facial setae on inner and outer lobe. Gnathopods I–II propods with multiple palmar spines, gnathopod dactyli with single seta on outer margin. Uropod III rami flattened; endopodite as long as exopodite with spiniform and plumose setae along both margins; exopodite biarticulate, distal article strongly diminished.

Description (based on dissected specimens)

Head and trunk (Fig. 5). Body length up to 9.6 mm. Head length 8.6–10.0% of body length; rostrum absent. Pereonites I–VI without setae, pereonite VII with a facial seta close to ventro-posterior corner. Pleonites I–III with up to 6, 10, and 11 setae along the entire respective dorso-posterior margins. Epimeral plate II inclined, posterior and ventral margins concave and convex, respectively; ventro-postero-distal corner distinct; along ventral margin 0–2 spines or setae; along posterior margin 1-2 setae. Epimeral plate III inclined, posterior and ventral margin concave and convex, respectively; ventro-postero-distal corner distinct but not produced; along ventral margin 0–2 spiniform setae; along posterior margin 2–4 thin setae. Urosomite I postero-dorso-laterally with 1 strong spiniform seta; urosomite II postero-dorso-laterally with 2–3 strong spiniform setae; urosomite III without setae. At the base of uropod I single strong spiniform seta. Telson length: width ratio is 1: 0.75–0.85; telson cleft is 0.73–0.78 telson length; telson margins straight and narrowing apically. Telson spiniform setae (per lobe, left-right lobe asymmetry commonly observed): 3–5 apical, 0–2 mesial, 0–1 lateral and 1–2 individual dorsal spiniform setae. Apical spiniform setae up to 0.25 telson length. Pairs of plumose setae sub-apically.

Antennae (Fig. 6). Antenna I 0.45–0.55 of body length. Flagellum with 22–29 articles; each article with 1 aesthetasc. Peduncle articles in ratio 1: 0.67–0.70: 0.57–0.59. Proximal article of peduncle dorso-distally

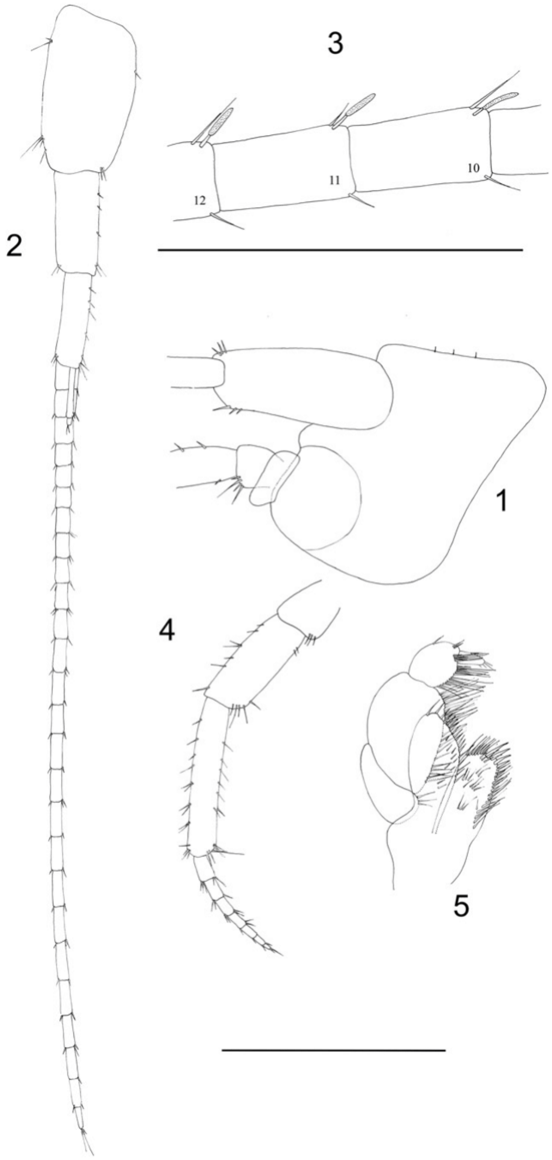

Fig. 6. *Niphargus gammariformis* sp. nov. 1. Male head detail. 2. Antenna I. 3. Antenna I detail with asthetascs. 4. Antenna II. 5. Maxilliped. Scale bars represent: Figs 1–2, 4–5, 1 mm; Fig. 3, 0.5 mm.

slightly produced. Accessory flagellum biarticulated; proximal article longer than the first two articles of the main flagellum, distal article shorter than one-quarter of proximal article length. Length ratio antenna I: antenna II as 1: 0.32–0.34. Antennal gland cone small and poorly visible. Flagellum of antenna II with 7–8 articles; each article with setae and elongate sensilla of unknown function. Peduncle articles lengths 4: 5 as 1: 1.21–1.58; flagellum 0.47–0.55 of length of peduncle articles 4 + 5.

Mouthparts (Fig. 7). Labrum typical; inner lobes of labium hardly visible. Left mandible: incisor with 5 teeth, lacinia mobilis with 4 teeth; between lacinia and molar a long row of 12–13 thick serrated setae, few spatulate setae and long seta at the base of molar. Right mandible: incisor processus with 4 teeth, lacinia mobilis with several small teeth, between lacinia and molar a long row of 13 thick serrated setae. Ratio of mandibular palp article 2: article 3 (distal) as 1: 1.2–1.5. Proximal palp article without setae; the second article with 3–5 setae; distal article with a group of 2–4 A setae; 1–2 B setae; 15–18 D setae and 4–5 E setae. Maxilla I distal palp article with 17–20 setae along mesial to apical margin. Outer lobe of maxilla I with a row of 7 stout spiniform comb-like setae; inner lobe with 10–17 setae along medial and apical margin. Maxilla II inner lobe slightly smaller than outer lobe; both of them setose apically and medially; one row of submarginal setae. Maxilliped (Fig. 6) with broad and flattened articles. Palp article 2 with rows of setae along inner margin; distal article with a dorsal seta, and setae at the socket. Maxilliped outer lobe with 9–11 stout setae mesially to subapically, 1–7 setae apically and several facial setae; inner lobe with 4 stout setae apically and more than 10 serrated setae along latero-apical margins and on the surface of the lobe.

Coxal plates and gills (Figs 5, 8, 10-11). Coxal plate I narrow and subrounded apically; anterior and ventral margin of coxa I with 6–10 setae. Coxal plates II–IV width: depth ratios as 0.64–0.67: 1, 0.55–0.59: 1, and 0.75–0.79: 1; anterior and ventral margins with 8–12, 8–11, and 23–30 setae, respectively. Coxal plate IV posteriorly with large, distal lobe. Coxal plates V–VI: deep, anterior lobe small; posterior large with 1, rarely 2 setae. Coxal plate VII half-pear shaped with 4–5 posterior setae. Gills II–VI ovoid.

Gnathopod I (Figs 8-9). Ischium with 1 group of 4–5 postero-distal setae. Carpus 0.51–0.62 of basis length and 0.85–1.31 of propodus length; broadened distally. Carpus with 1–3 groups of setae (including distal group) anteriorly; 3–5 transverse rows of setae along posterior margin and a row of setae postero-laterally. Propodus elongated with short palm. Along posterior margin 8–10 rows of setae. Anterior margin with 2–3 setae in 1–2 groups in addition to antero-distal group counting 4–5 setae. Several groups of short setae on the inner surface present. Palmar corner armed with 4–5 non-serrated spiniform setae descending in lengths towards palmar

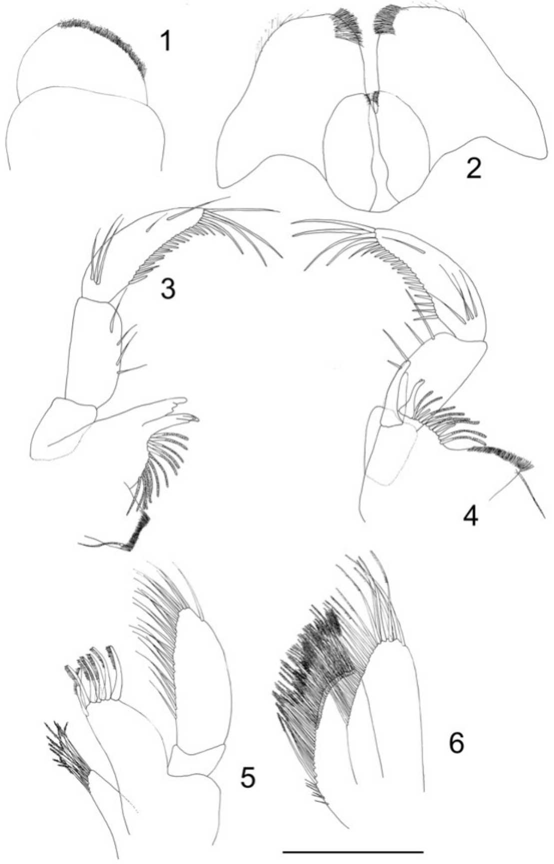

Fig. 7. *Niphargus gammariformis* sp. nov. Male mouthparts. 1. Labrum. 2. Labium. 3. Left mandible. 4. Right mandible. 5. Maxilla I. 6. Maxilla II. Scale bar represents: Figs 1–6, 0.50 mm.

corner, single supporting spiniform seta on inner surface; no denticulated thick spiniform setae were observed. No distinct groups of long setae below palmar spines was observed. Palm setose. Nail length 0.31–0.33 of total dactylus length; along anterior margin 1 seta; along inner margin a row of short setae.

Gnathopod II (Figs 8-9). Basis width: length as 0.30–0.32: 1. Ischium with 3–4 postero-distal setae. Carpus 0.59–0.73 of basis length and 1.05–1.55 of propodus length, distally broadened. Carpus with 1–2 groups of setae (including distal group) anteriorly; 5–7 transverse rows of setae along posterior margin and a row of setae postero-laterally. Propodus small (circumference up to 0.13–0.17 of body length) but larger than propodus of gnathopod I (I: II as 0.79–0.87: 1). Posterior margin with 10–11 rows of setae. Anterior margin with 1–3 individual setae in addition to 5–6 antero-distal setae. Individual surface setae present. Palmar corner with 4–5 strong palmar spiniform setae, descending in length towards lateral margin; single supporting spiniform seta on inner surface and no denticulated thick-spiniform setae on outer side. Palm setose, a group of long setae below spiniform palmar setae not observed. Nail length 0.29–0.32 of total dactylus length. Along anterior margin 1 seta; along inner margin few short setae.

Pereopods III-IV (Fig. 10). Lengths of pereopods III and IV equal. Dactylus IV 0.38–0.45 of propodus IV; nail length 0.27–0.34 of total dactylus length. Dactyli III–IV with dorsal plumose seta; at the base of nail 1 tiny seta and one spiniform seta.

Pereopods V-VII (Fig. 11). Lengths of pereopods V: VI: VII as 1: 1.07–1.1: 1.05; pereopod VII 0.42 of body length. Bases V–VII broad, respective length: width ratios as 1: 0.7–0.75, 1: 0.71–0.75, and 1: 0.85; posterior margins straight to convex; bases V–VI with moderate and basis VII with large posterior lobes; posteriorly 20–23, 19–25, and 17 setae, respectively; anteriorly 9–13, 10–13, and 8 groups of spines, respectively. Dactyli V–VII with dorsal plumose seta; at the base of nail 1 tiny seta and 1 spinifom seta.

Pleopods and uropods (Fig. 12). Pleopods I–III with 2 hooked retinacles. Pleopod II rami with 15–17 and 13–14 articles. Uropod I protopodite with 4–5 dorso-lateral spiniform setae and 3–4 dorso-medial spiniform setae. Exopodite: endopodite lengths as 1: 1.07–1.14; rami straight. Endopodite with 3–6 individual spiniform setae laterally and 5 spiniform setae apically. Exopodite with 7–11 spiniform single setae or setae in groups; apically 5 spiniform setae. Uropod II exopodite: endopodite lengths as 1: 1.07–1.18. Uropod III flattened and short, 0.12–0.13 of body length. Protopodite with 4–9 apical spiniform setae. Endopodite almost as long as exopodite and 1.46–1.63 of protopodite length, laterally armed with 3–4 groups of spiniform and plumose setae. Exopodite of uropod III flattened, distal article 0.09–0.11 of the proximal article length. Proximal article with 12–13 plumose setae along inner margin and 6–7 groups of thin-flexible and spiniform setae along outer margin. Distal article with 3 spiniform setae and 1–3 plumose setae apically, no lateral setae were observed.

Sexual dimorphism. Females are in general similar to males. We noticed a sexual dimorphism in the shape of gnathopods (Figs 9, 13). The relative width and length of the propodus of gnathopods I and II is larger in males, respectively (ANCOVA, width gnathopod I:

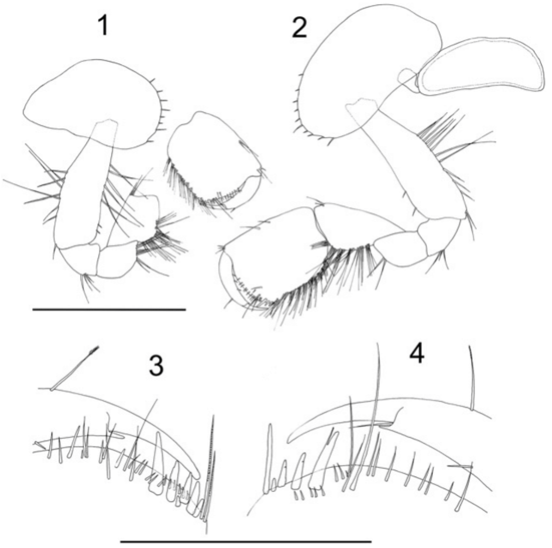

Fig. 8. *Niphargus gammariformis* sp. nov. male 1. Gnathopod I. 2. Gnathopod II. 3. Propodus palmar corner detail of gnathopod I. 4. Propodus palmar corner detail of gnathopod II. Scale bars represent: Figs 1–2, 1 mm; Figs 3–4, 0.5 mm.

$p = 0.018$, length gnathopod I: $p = 0.015$, width gnathopod II: $p = 0.023$, length gnathopod I: $p = 0.003$). The relative length of the carpus article of gnathopod I is longer in females (ANCOVA, $P < 0.001$), but the relative lengths of carpal article of gnathopod II are not sexually dimorphic (ANCOVA, $P = 0.22$). Those results were confirmed with a Wilcoxon–Mann non-parametric test (all three tests yielded $P < 0.05$).

Field notes. The newly described species, *Niphargus gammariformis* sp. nov., was found in large numbers in Sulphidic Lake, occurring in densities of approximately 200 individuals per square metre (Fig. S2, see supplemental material online). They swam close to the water surface with their ventral side facing upwards (Fig. 12). By contrast, they were less abundant in both freshwater lakes, where they tended to hide under rocks or walk on the cave walls and on sediments.

Remarks and affiliation. The diagnostic traits are a combination of unique (autapomorphic) traits and traits rarely occurring in *Niphargus*. A trait unique within *Niphargus* is a series of descending palmar spines. There are few species with double palmar spine, such as *N. hosseiniei* Esmaeili-Rineh, Sari, Fišer, & Bargrizaneh, 2017, *N. grandii* Ruffo 1936, and *N. dobrogicus* Dancau 1964 (Esmaeili-Rineh et al., 2017; Karaman, 1993), but we are not aware of a species having a row of four or more such spines. The second trait, apparently unique within *Niphargus*, is an oblique row of setae along the inner lobe of maxilla II. Such a row is common in *Gammarus* but was never reported for *Niphargus* until now (Karaman, 1993). The most distinctive properties are shared with two species of *Pontoniphargus* inhabiting the sulphidic aquifers near Mangalia (Romania): one on the western side (near the village of Hagieni), and the other one on the eastern side (near and around the town of Mangalia). These include a stout body appearance, subapical plumose setae on telson, flattened uropods III with subequal rami, and filtering mouthparts with numerous setae. However, the endopodites of *N. gammariformis* sp. nov.'s uropods III

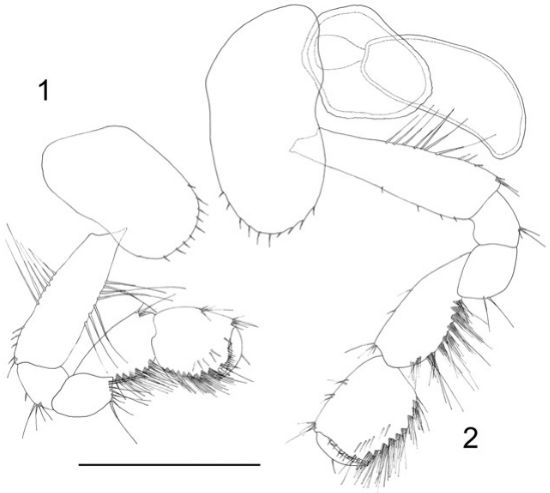

Fig. 9. *Niphargus gammariformis* sp. nov. female. 1. Gnathopod I. 2. Gnathopod II. Scale bar represents: Figs 1–2, 1 mm.

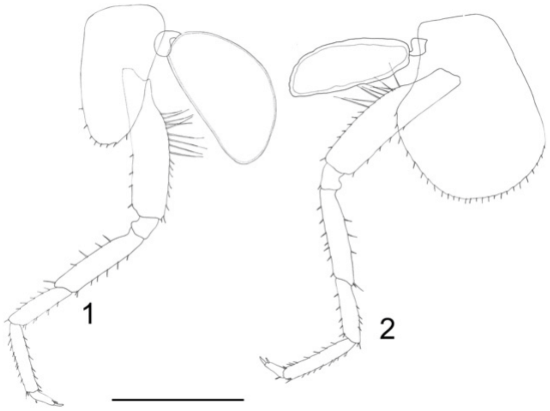

Fig. 10. *Niphargus gammariformis* sp. nov. male. 1. Pereopod III. 2. Pereopod IV. Scale bar represents: Figs 1–2, 1 mm.

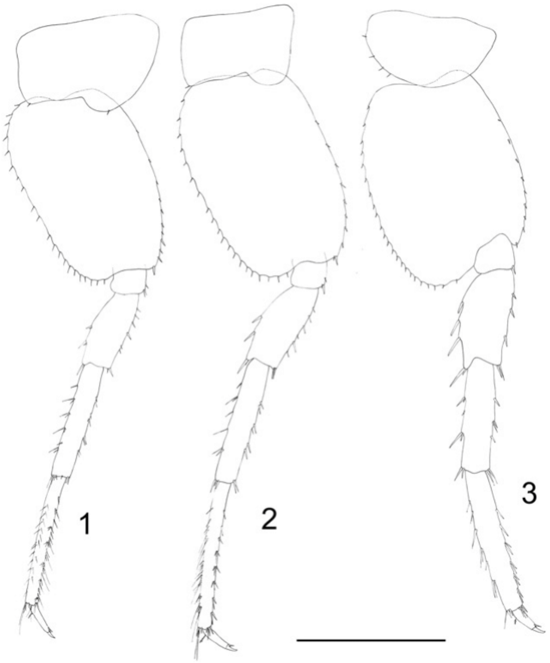

Fig. 11. *Niphargus gammariformis* sp. nov. male. 1. Pereopod V. 2. Pereopod VI. 3. Pereopod VII. Scale bar represents: Figs 1–3, 1 mm.

consistently exceed one half of the length of its exopodites, whereas they are shorter and more variable in *Pontoniphargus* (Dancău, 1968; Karaman & Sarbu, 1996). Secondly, the mouthparts are more setose than the mouthparts of *Pontoniphargus*, in particular with respect to the high number of setae along the inner margin of palpus and the inner lobe of maxilla I. In addition, the propodi of the gnathopods of *N. gammariformis* sp. nov. are wider and bear multiple palmar spines, whereas its telson is less spiny than the one of *Pontoniphargus* species (Dancău, 1968; Karaman & Sarbu, 1996).

Interestingly, *N. gammariformis* sp. nov. seems to have established an interaction with bacterial genus *Thiotrix* (Colette, in prep.), as all other *Niphargus* species inhabiting sulphidic waters (Flot et al., 2014). The function of this interaction is not yet known (Bauermeister, Assig, & Dattagupta, 2013).

Discussion

Evolutionary origin of the *Niphargus* community from Melissotrypa Cave in relation to taxonomic uncertainty

Phylogenetic structure and ecological-morphological diversity make this community unique among

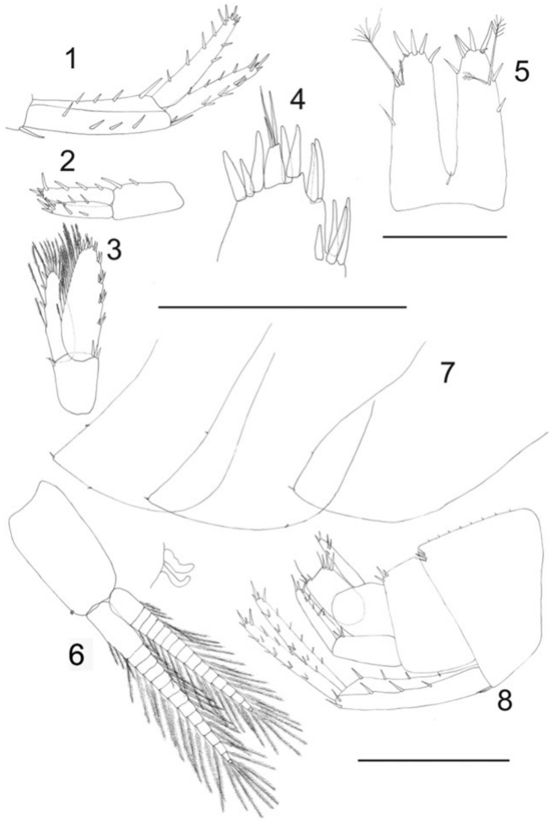

Fig. 12. *Niphargus gammariformis* sp. nov. 1. male. Uropod I. 2. Uropod II. 3. Uropod III. 4. Uropod III detail. 5. Telson. 6. Pleopod I. 7. Epimeral plates. 8. Urosoma. Scale bars represent: Fig. 4, 0.5 mm; Fig. 5, 0.5 mm; Figs 1–3, 6–8, 1 mm.

Niphargus communities. Closely related species comprising a community, a pattern called 'phylogenetic clustering' (Ackerly, 2003), can emerge through multiple scenarios. The first possibility is so-called ecological filtering, when severe environmental conditions strongly constrain colonization of a particular habitat patch (Kraft et al., 2015). Only phylogenetically related species with inherited pre-adaptations can successfully colonize such patches. This scenario, however, predicts also that species will be ecologically similar, which is not true in Melissotrypa Cave.

Phylogenetic clustering, however, might also indicate poor dispersal, and – in combination with ecological divergence – also *in situ* ecological speciation (Emerson & Gillespie, 2008; Rundle & Nosil, 2005). The theory of ecological speciation states that an ancestral population exploits different ecological resources and diverges. A side effect of ecological divergence is the emergence of reproductive barriers, leading to distinct species (Rundell & Price, 2009). This hypothesis appears

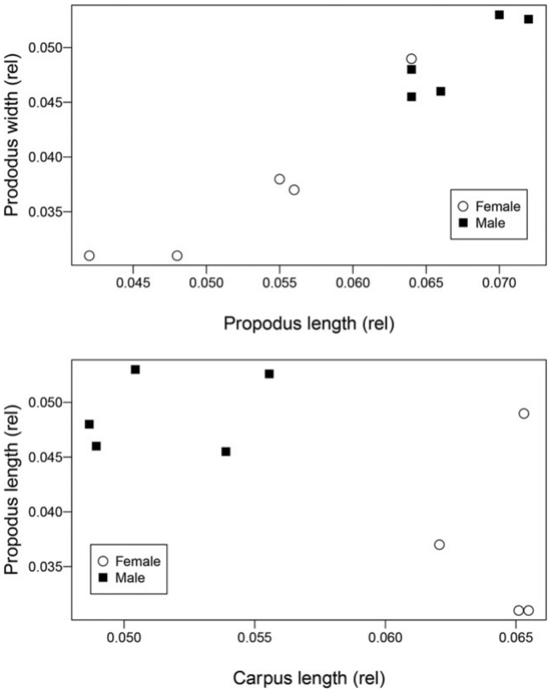

Fig. 13. Sexual dimorphism of gnathopod I. Upper plot shows that males have both wider and longer propodus (ANCOVA, $P < 0.05$ for both dimensions). Lower plot shows that females have longer carpus on gnathopod 1 (ANCOVA, $P < 0.001$). All values are relative to body length.

compatible with our data, that is, the common ancestry and ecological divergences of the three *Niphargus* could be causally linked. In this case, the *Niphargus* triplet from Melissotrypa Cave would represent a rare case of ecological speciation in the subterranean realm (Leijs et al., 2012; Mammola et al., 2018).

There is however a third possibility, in which speciation and ecological divergence would not be causally linked. For instance, one might imagine that the three species first speciated in allopatry, then diverged ecologically, and finally dispersed into Melissotrypa Cave. This scenario is as likely as the previous one, given that *N. jovanovici* and *N. lindbergi* are also broadly distributed outside Melissotrypa Cave (Fig. 2; G. S. Karaman, 2017, 2018). To resolve the issue, one will require additional insights such as detailed phylogeography and reconstruction of past dispersal.

Convergent evolution challenges the validity of the genus *Pontoniphargus*

The *Niphargus* species from the three sulphidic caves where the genus was found to date (Melissotrypa, Frasassi, and Movile), do not form a clade but are dispersed in four lineages of the *Niphargus* phylogeny (Fišer et al., 2015; Flot et al., 2014, 2010). Hence, it appears likely that *Niphargus* colonized sulphidic waters at least four times (Fig. 3), possibly because they are less sensitive to hydrogen sulphide toxicity (Bauermeister et al., 2013) and hypoxia (Hervant, Mathieu, Garin, & Fréminet, 1995) than other amphipods. Species thriving in sulphidic caves are of two types, stout and slender, respectively predominant in stagnant water and streams (Fišer et al., 2015). *Niphargus gammariformis* sp. nov. is a stout species, and it shares several traits with *N. ictus* (found in the Frasassi Cave system), and even more with *Pontoniphargus racovitzai* (Movile Cave) and *Pontoniphargus ruffoi* (Hagieni Spring, close to Movile Cave) (Fig. 4). The similarities between *Pontoniphargus* species and *Niphargus gammariformis* sp. nov. include deep coxal plates, broad bases of pereopods V–VII, flattened uropods with long endopodite, and densely setose mouthparts in particular pectinate spines on outer lobe of maxilla I. Noteworthy, part of these traits comprised a diagnostic combination of *Pontoniphargus*, the shape and proportions of uropods III with setal patterns of the mouthparts. In addition, both *Pontoniphargus* spp. and *N. gammariformis* sp. nov exhibit a similar resting behaviour with their ventral side close to surface and their dorsal side oriented towards the bottom. The functional connection between these traits and the sulphidic environment is elusive. A possible explanation could be that the morphology and behaviour co-evolved in response to the hypoxia concomitant to the presence of hydrogen sulphide, that is, in sulphidic water, dissolved oxygen is present only in the uppermost layers near the surface, and the resting position might enhance the uptake of oxygen. A stout, boat-shaped body might facilitate the exchange of water across ventral gills (Dahl, 1978; Fišer et al., 2015), while the setose mouthparts filter bacterial plankton. Possibly these traits, or a combination of them, evolved convergently in response to environmental pressures.

The discovery of *N. gammariformis* sp. nov. and its description requires taxonomic changes in the family *Niphargidae*. Given its morphological traits, *N. gammariformis* sp. nov. should be included into the genus *Pontoniphargus*. Phylogenetically, both *Pontoniphargus* species are nested within the *Niphargus* clade (Flot et al., 2014), and thereby make *Niphargus* paraphyletic (Fig. 2). Assignment of the newly described species into *Pontoniphargus* would result in a polyphyly of *Pontoniphargus*, whereas its assignment into *Niphargus* falsifies the diagnosis of *Pontoniphargus*. Hence, the alternative classifications of the newly described species would lead either to one paraphyletic and one polyphyletic genus, or to one paraphyletic genus and to a genus without diagnosis. We argue that the best solution to this unfavourable situation is to consider *Pontoniphargus* as a junior synonym to *Niphargus*.

Discarding *Pontoniphargus*, however, introduces an additional taxonomic complexity. *Pontoniphargus ruffoi* should be named *N. ruffoi*. Yet, a species with identical spelling (*N. ruffoi* Karaman, 1976) was described from Italy (Karaman, 1976). According to the International Code of Zoological Nomenclature (International Commission on the Zoological Nomenclature, 2018), this case suits the definition of 'Homonyms in the species group' (Code Art. 53.3), where only the senior homonym, as defined by the Principle of Priority (Code Art. 52.3), can be used as a valid name (Code Art. 53.2). Given that the rejected name has no valid synonym, a new substitute name needs to be established (Code Art. 23.3.5.) with its own author and date (Code Art. 60.3).

To summarize, the description of *N. gammariformis* sp. nov. introduces the following taxonomic changes: (1) *Pontoniphargus* is synonym junior to *Niphargus* and should no longer be used. (2) The species described as *Pontoniphargus racovitzai* Dancau, 1968 should be treated as *Niphargus racovitzai* (Dancau, 1968). (3) The species described as *Pontoniphargus ruffoi* Karaman & Sarbu, 1993, has been given a substitute name *Niphargus pontoruffoi* Sarbu, 2019.

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Supplemental data

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2.4 A HOTSPOT OF GROUNDWATER AMPHIPOD DIVERSITY ON A CROSSROAD OF EVOLUTIONARY RADIATIONS

Borko Š., Altermatt F., Zagmajster M., Fišer C. 2022. A hotspot of groundwater amphipod diversity on a crossroad of evolutionary radiations. Diversity and Distributions, 00: 1–13

Groundwater harbours an exceptional fauna and provides invaluable ecosystem services, yet is among the least explored and consequently least protected ecosystems. Successful protection of its biodiversity depends on complete species' inventories, knowledge of species' spatial distribution, and quantification of biodiversity patterns, as well as disentanglement of the processes that shaped biodiversity patterns. We studied the hyper-speciose amphipod genus *Niphargus* as a model system within a global subterranean biodiversity hotspot, the Western Balkans. We linked the biodiversity patterns with possible underlying processes and discuss the needs to include information on different origins of biodiversity into conservation approaches. We analysed biodiversity patterns of *Niphargus* using two biodiversity metrics, species richness and phylogenetic diversity, on a grid-based approach. To account for high cryptic diversity, we replaced nominal species with taxonomic units identified in unilocus delimitations (MOTUs). We built a time-calibrated multilocus phylogeny of 512 *Niphargus* MOTUs from within and outside the study area, and calculated Faith's phylogenetic diversity, standardized effect sizes of phylogenetic diversity, and residual of phylogenetic diversity regressed onto species richness. Within the study area, we recognized 245 MOTUs, belonging to different *Niphargus* clades. Species richness is highest in a north-western hotspot, although some species-rich cells were detected also in the southeast. High phylogenetic diversity coincides with high species richness in the northwest, while in the southeast it is lower than expected. We have shown that species richness does not predictably correlate with phylogenetic diversity. This difference suggests that different processes have led to the formation of species-rich areas in the Western Balkans: through a combination of dispersal and speciation in the northwest, and local radiation in the southeast, respectively. This calls for caution in conservation strategies relying solely on number of species and may change the view on conservation priorities within this region.

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RESEARCH ARTICLE

A hotspot of groundwater amphipod diversity on a crossroad of evolutionary radiations

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Abstract

Aim: Groundwater harbours an exceptional fauna and provides invaluable ecosystem services, yet is among the least explored and consequently least protected ecosystems. Successful protection of its biodiversity depends on complete species inventories, knowledge of species spatial distribution, and quantification of biodiversity patterns, as well as disentanglement of the processes that shaped biodiversity patterns. We studied the hyper-speciose amphipod genus *Niphargus* as a model system within a global subterranean biodiversity hotspot. We linked the biodiversity patterns with possible underlying processes and discuss the needs to include information on different origins of biodiversity into conservation approaches.

Location: Europe, Western Balkans.

Methods: We analysed biodiversity patterns of *Niphargus* using two biodiversity metrics, species richness and phylogenetic diversity, on a grid-based approach. To account for high cryptic diversity, we replaced nominal species with taxonomic units identified in unilocus delimitations (MOTUs). We built a time-calibrated multilocus phylogeny of 512 *Niphargus* MOTUs from within and outside the study area, and calculated Faith's phylogenetic diversity, standardized effect sizes of phylogenetic diversity, and residual of phylogenetic diversity regressed onto species richness.

Results: Within the study area, we recognized 245 MOTUs, belonging to different *Niphargus* clades. Species richness is highest in a north-western hotspot, although some species-rich cells were detected also in the south-east. High phylogenetic diversity coincides with high species richness in the north-west, while in the south-east it is lower than expected.

Main conclusions: We have shown that species richness does not predictably correlate with phylogenetic diversity. This difference suggests that different processes have led to the formation of species-rich areas in the Western Balkans: through a combination of dispersal and speciation in the north-west, and local radiation in the south-east, respectively. This calls for caution in conservation strategies relying solely on number of species and may change the view on conservation priorities within this region.

KEYWORDS
amphipods, evolutionary radiations, groundwater, *Niphargus*, phylogenetic diversity, species richness, Western Balkans

1 | INTRODUCTION

Groundwater is the largest reservoir of liquid freshwater globally that is of direct and major interest to humans (Gibert & Deharveng, 2002; Mammola et al., 2019). Despite its vastness, groundwater is also one of the most endangered habitats. Habitat loss, overexploitation, contamination and climate change threaten its inhabitants (Mammola et al., 2019). Groundwater fauna is unique in many aspects. It comprises phylogenetic and geographic relicts (Humphreys, 2000), disproportionally high number of endemic species (Bregović et al., 2019) and plays a key role in ecosystem services (Griebler & Avramov, 2015). However, conservation biologists have largely neglected groundwater biodiversity, even in comparatively well-studied regions such as Europe and North America (Mammola et al., 2019). Species inventories and mapping of groundwater biodiversity have been lagging behind initiatives on surface systems (Ficetola et al., 2019). Among the main reasons for this are limited access to groundwater hindering sampling (Mammola et al., 2021), deficiency in taxonomic expertise combined with high morphological similarity (i.e., cryptic species) (Delić et al., 2017), and limited availability of robust phylogenies. Consequently, and due to contemporary human activities, many species may be doomed before even being discovered (Niemiller et al., 2013). To reverse this trend, increasing scientific knowledge is crucially needed for the protection of groundwater (Wynne et al., 2021).

Successful conservation management must be based on solid scientific evidence, which itself depends on sufficient empirical knowledge and understanding of the distribution of biodiversity. The first step in assessment of biodiversity are species inventories at an appropriate spatial and taxonomic resolution (Guralnick et al., 2018). The second step is quantification of biodiversity itself. Because of phylogenetic relatedness and ecological specialization, species cannot be treated as independent or functionally equivalent units. For this reason, quantification of biodiversity has been increasingly relying on combining complementary metrics, such as species richness and phylogenetic diversity (Faith, 1992). Phylogenetic diversity arguably indirectly captures also other aspects of biodiversity, such as functional diversity (Cadotte et al., 2011; Isaac et al., 2007; Mazel et al., 2018) and has been incorporated into identification of hotspots and assessments of processes that contributed to high biodiversity (Devictor et al., 2010; Isaac et al., 2007; Purvis et al., 2000). In groundwater, biodiversity patterns were hitherto mainly inferred using species richness (Bregović et al., 2019; Eme et al., 2018; Zagmajster et al., 2014), while other metrics of biodiversity have not been applied yet. Eventually, the assessment and understanding of patterns of biodiversity using different metric may also reveal the underlying processes that shaped these patterns.

Deficiency in knowledge and incomplete application of biodiversity measures is especially problematic in areas of exceptional biodiversity, as it may lead to incorrect yet highly influential conclusions with respect to conservation. Such an area is the Western Balkans, which covers 166,000 km^2 in south-eastern Europe, including the Dinaric Karst and the Eastern parts of the Southern Calcareous Alps. The region is a global biodiversity hotspot for surface and subterranean ecosystems (Myers et al., 2000; Sket, 2012; Zagmajster et al., 2014). Importantly, groundwater biodiversity of this region is locally highly endangered due to planned construction of dams, roads and other infrastructure, and agricultural developments, which lead to habitat destruction (Schwarz, 2015). Despite a rich speleobiological history in the region, much of the groundwater diversity is still being discovered (Zagmajster et al., 2014), and its biodiversity has not yet been assessed using metrics beyond simple species counts.

Here, we studied groundwater biodiversity patterns within the Western Balkans using the subterranean amphipods of the genus *Niphargus*. With more than 400 known species and still a high number of undescribed species, *Niphargus* is an exceptionally diversified genus that importantly co-shapes biodiversity patterns in aquatic subterranean habitats of the Western Palearctic (Borko et al., 2021; Fišer, 2019; Horton et al., 2021; Zagmajster et al., 2014). Phylogenetic relationships within the genus are relatively well understood, and the evolutionary history of the genus precedes the origin of the Western Balkans as a geologic unit (Borko et al., 2021). Extensive field explorations over the past decade resulted in a large number of fresh *Niphargus* samples. This offered an opportunity to conduct a study entirely based on molecular data to assess biodiversity patterns of this region using species richness and phylogenetic diversity. We used these metrics to link biodiversity patterns with historical processes that shaped the patterns, and to discuss how these processes should be considered in conservation planning. We first assessed diversity of *Niphargus* using molecular unilocus delimitations (Eme et al., 2018) and analysed the spatial and taxonomic coverage of the molecular dataset by comparison with nominally described and published species distribution records (Bregović et al., 2019; Zagmajster et al., 2014). Then we constructed a time-calibrated phylogeny to quantify phylogenetic diversity. A comparison of species richness patterns with phylogenetic diversity allowed us to assess whether species in the richest cells assembled through local diversification (low phylogenetic diversity) or colonization of unrelated species (high phylogenetic diversity). Finally, we discuss the importance of different historical processes in conservation biology.

2 | METHODS

2.1 | Overview of the dataset

We examined the collection of *Niphargus* from SubBio Lab, Department for Biology, Biotechnical Faculty, University of Ljubljana, Slovenia, containing >11,000 individuals, and being one of the largest collections of *Niphargus* worldwide. We identified the specimens using morphological diagnosis and cross-examined them with existent molecular and morphological data in SubBio Database. We then selected 864 specimens that were sequenced for the standard barcoding marker cytochrome C oxidase subunit I (COI), to cover all existent localities and as much morphological variability as possible. Combined with already published genetic material from the Western Balkans and 280 representatives of species outside the study area, the final COI delimitation dataset counted 1492 specimens. After cross-examination of delimitations and quality checking, the dataset used for diversity patterns assessment was comprised of 1212 individuals that were aligned to 245 MOTUs, present in altogether 598 localities within the study area, of which 297 localities did not have previous published molecular data for *Niphargus* (see Appendix S1 in Supporting Information). Although this is the most extensive dataset with the highest coverage for any subterranean aquatic group in this region and well beyond (Zagmajster et al., 2014), it is based on a broad number of studies and datasets, as is common for larger faunistic databases. In order to check for consistency in sampling completeness within the studied region, we thus plotted species accumulation curves (Gotelli & Colwell, 2001) and rarefaction and extrapolation sampling curves (Chao et al., 2014) for the two main subregions, namely the north-west and south-east (see Appendix S5, Figure S5.1). While none of the curves reached a plateau, the result suggests relative homogeneity in sampling. We conclude that results and conclusions are thus comparable and conservative, as, if any, the south-eastern part is closer to the saturation of sampled biodiversity.

2.2 | Molecular analysis and delimitation procedures

We extracted genomic DNA from 864 specimens using MagMAX DNA Multi-Sample Kit (Thermo Fisher Scientific). Oligonucleotide primers and amplification protocols were the same as in (Borko et al., 2021). All nucleotide sequences were obtained by Macrogen Europe laboratory (Amsterdam, Netherlands), using amplification primers and bidirectional Sanger sequencing. We edited and assembled chromatograms and aligned sequences in Geneious 11.0.3. (Biomatters Ltd).

First, we amplified the mitochondrial cytochrome oxidase I (COI) gene for all 864 specimens, for purposes of delimitation. We built a dataset of COI sequences of all known *Niphargus* species, regardless their origin (Borko et al., 2021), combined with all previously and de novo sequenced Western Balkans specimens from SubBioDB database, yielding COI sequences for 1492 individuals (1082 COI haplotypes) (Appendix S1). We used the alignment to delimit MOTUs, using distance- and tree-based unilocus delimitation methods: Automatic Barcode Gap Discovery (ABGD) (Puillandre et al., 2012), Assemble Species by Automatic Partitioning (ASAP) (Puillandre et al., 2021) and Poisson Tree Processes (PTP) (Zhang et al., 2013), respectively. In previous studies, we detected poor performance of multi-rate PTP (Delić et al., 2020) and discarded this method for *Niphargus* dataset.

Automatic Barcode Gap Discovery (Puillandre et al., 2012) assigns the sequences to the specific MOTUs without *a priori* species hypotheses, based on the assumption that intraspecific genetic distances are smaller than interspecific ones. We ran two ABGD analyses: with Kimura two-parameter substitution model, prior for maximum value of intraspecific divergence between 0.001 and 0.1, 20 recursive steps and two gap widths of 1.0 and 1.5. We used ABGD web server (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html). For ABGD, we trimmed the alignment to 467 base pairs and excluded 16 sequences that were too short.

Similarly to ABGD, Assemble Species by Automatic Partitioning (ASAP) (Puillandre et al., 2021) is based on pairwise genetic distances, but provides a score for each defined partition and overcomes the challenge of a priori defining maximal genetic intraspecific divergence P. We ran ASAP with Kimura two-parameter substitution model, on ASAP web server (https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html).

An alternative approach is a phylogeny-based method PTP (Zhang et al., 2013) that delimits species at nodes where presumed intraspecific nucleotide substitution rates switch to interspecific substitution rates. The two nucleotide substitution rates are modelled using two different Poisson processes. For this analysis, we removed duplicate sequences, using subset of 1082 unique haplotypes. First, we inferred phylogenetic relationships with maximum likelihood approach in IQ-TREE 1.6.7 (Minh et al., 2020; Trifinopoulos et al., 2016), using automatic best fit substitution model search with ModelFinder (Kalyaanamoorthy et al., 2017). We used the resulting tree to run the PTP analysis within the Bayesian and maximum likelihood framework, using the species delimitation server http://species.h-its.org/, and running 500,000 generations, sampling every 100 generations, and discarding the first 20% of the samples as a burn-in.

The delimitation results of the 1212 individuals from study area suggested between 212 and 474 MOTUs in the study area (see Results and Table 1). In order to reconcile the alternative results of the species delimitations for the downstream phylogenetic and spatial analyses, we proceeded as follows. We assigned five putative MOTUs statuses to each specimen. These results were crosschecked and compared with previous taxonomic studies. These studies employed multilocus species delimitations and/or additionally analysed morphological variation (Borko et al., 2021; Delić et al., 2017, 2022; Delić, Trontelj, et al., 2017; Fišer et al., 2015; Šet & Borko, 2020; Zakšek et al., 2019), and we deem taxonomic conclusions of these studies more reliable than unilocus delimitations alone. We first determined unproblematic MOTUs that were recognized by all

TABLE 1 Delimitations based on COI marker, using ABGD, ASAP and PTP, reported for subset of Dinaric COI sequences

Delimitation method	Number of MOTUs	% same delimitations as final
ABGD 1.0[a]	467	45
ABGD 1.5[a]	247	79
ASAP	215	87
mlPTP	275	75
bPTP	282	78
Final determination based on crosschecking with multilocus/integrative approaches	227	

Note: ASAP appeared to perform the best (87% specimens delimited correctly, based on our criteria (see Methods section)).

[a] The two ABGD analyses differ in pre-set gap width. We tested two settings that proved useful in different clades in previous analyses.

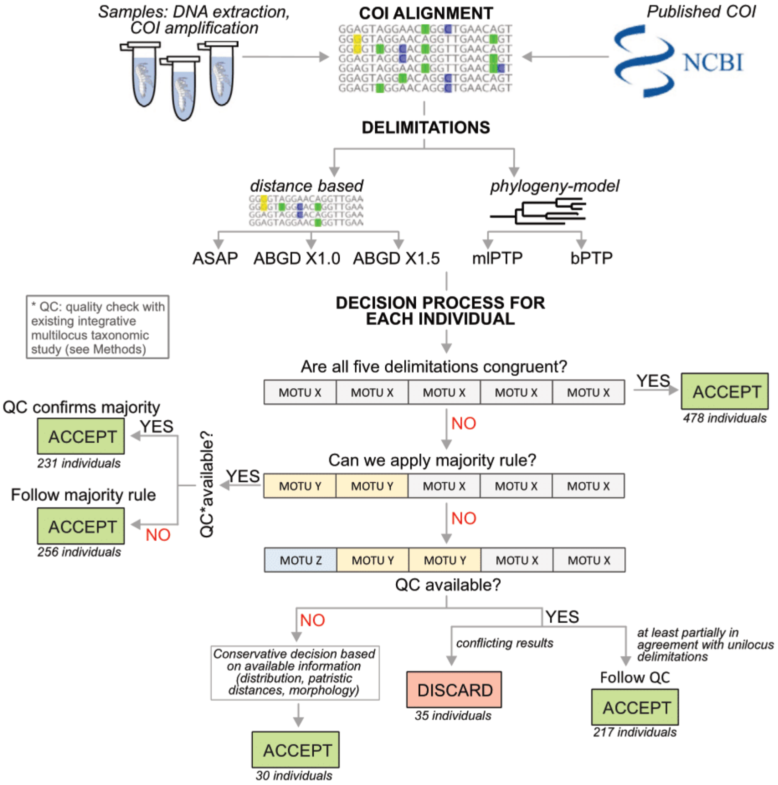

FIGURE 1 Workflow of the applied delimitation procedure. We combined existing and newly obtained COI sequences into an alignment. We applied five delimitation methods: ASAP, ABGD with two settings, mlPTP and bPTP, and assigned respective MOTU statuses to each individual. The results were compared with previous integrative taxonomic studies (Quality Check, QC: multilocus species delimitations and/or studies combining multiple molecular markers with morphology and spatial information). We first determined MOTUs that were recognized by all five delimitation methods unanimously (478 individuals). Next, we revised the individuals that were assigned to different MOTUs, and one assignment prevailed. 231 of them were confirmed with QC. For 256 QC was not possible, and majority rule prevailed. In the remaining conflicting cases without majority rule to follow, we either followed QC whenever possible (217) or we case-by-case examined additional information on morphology, geographical distribution and patristic distance (30 individuals). The 35 individuals that could not be reconciled with QC were excluded from analyses

five delimitation methods and, if applicable, agreed with previous works (478 out of 1212 specimens). Next, we revised the conflicting results. Whenever available, we chose delimitation results that corroborated with previous taxonomy-focused studies (433 out of 1212 specimens). If no such previous study existed, we assigned the specimens to a MOTU that was prevalent in the five species delimitation methods (271 out of 1212 specimens) or to more conservative MOTU in cases where two MOTUs were equally likely (30 out of 1212 specimens). MOTUs that could not have been reconciled with previous multilocus delimitations were conservatively excluded from the dataset and are not accounted for in the summary report (35 specimens). Figure 1 summarizes the workflow of delimitation procedure. This procedure yielded 227 MOTUs.

This dataset was complemented with species for which we had DNA fragments other than COI (mostly 28S), but were genetically clearly distinct (i.e. independent lineages on the phylogeny). The final dataset was comprised of 245 MOTUs from the Dinaric Region, present in altogether 598 localities (see Appendix S2 in Supporting Information).

For purpose of phylogenetic inference, we selected specimens for additional sequencing in a way that each MOTU was covered with at least one specimen. For this subset, specimens representing MOTUs were amplified for seven additional markers: two segments of 28S rRNA gene, the histone H3 gene (H3), a part of the ITS rRNA gene, as well as partial sequences of the glutamyl-prolyl-tRNA synthetase gene (EPRS), heat shock protein 70 (HSP70) and arginine kinase (ArgKin) genes. Sequencing, editing and assemblage procedures were the same as described above. Oligonucleotide primers and amplification protocols were the same as in (Borko et al., 2021).

2.3 | Phylogeny and molecular clock

To assess phylogenetic diversity patterns, we built a multilocus time-calibrated phylogeny of all *Niphargus* MOTUs (see Appendix S3 in Supporting Information). For each marker, we aligned the sequences in Geneious, using MAFFT v7.388 plugin (Katoh & Standley, 2013), with E-INS-i algorithm with scoring matrix 1PAM/k=2 and the highest gap penalty. Alignments were concatenated in Geneious. To remove gap-rich regions from the alignments of non-coding markers, we used Gblocks (Talavera & Castresana, 2007) with less stringent selection set. To determine optimal substitution model for each partition, we used Partition Finder 2 (Guindon et al., 2010; Lanfear et al., 2012) under the corrected Akaike information criterion (AICc).

We built a time-calibrated chronogram of all known *Niphargus* MOTUs with BEAST 2. We used the same four calibration points that proved to be congruent with each other (details in Borko et al., 2021). Briefly, we assigned 1) a "modern-looking" *Niphargus* fossils from Eocene Baltic amber to the node where fossil's characters evolved for the first time; 2) the age of final submergence of the land bridges between Eurasia and North America to a root node given that *Niphargus* does not live in Nearctics; 3) the age of the last land-bridge between Crete and Greek mainland to the node in which monophyletic clade on Crete split from Greek taxa; 4) the opening of the connection between Paratethys and the Mediterranean basin to the node of the monophyletic Middle East clade, which derived from Eastern European taxa.

For each gene partition, we used a set of priors as followed: linked birth-death tree model, unlinked site models as in previous analyses, with fixed mean substitution rate and relaxed clock log-normal distribution with estimated clock rate. We used default settings of distributions of all estimates. We run the analyses for 200 million generations, sampled every 20,000 generation. The first 25% of trees were discarded as burn-in.

2.4 | Diversity patterns

To assess the groundwater biodiversity patterns within the Western Balkans, we explored taxonomic and phylogenetic diversity of *Niphargus* in space. We used a grid of 415 quadratic cells with 20 × 20 km resolution that has been evaluated as most suitable according to procedure in Zagmajster et al. (2008) (results not shown) and has been used in previous studies of subterranean diversity in the region (Bregović et al., 2019; Bregović & Zagmajster, 2016; Zagmajster et al., 2008), with the Lambert Conformal Conical Projection (central meridian 18°, parallels 42° and 46°).

Taxonomic diversity was calculated as sum of different MOTUs occurring within each grid cell, i.e. species richness (SR). We plotted SR on a raster of study area.

Phylogenetic diversity was computed using Faith's phylogenetic diversity (FPD), which is the sum of the branch lengths of a phylogenetic tree connecting all the species of a given assemblage. For calculation, the distance from the deepest connecting node to the root was excluded, resulting in single-species cells with zero FPD. The alternative approach, which takes into account also branches of shared history, is more useful for phylograms which reflect individual species' accumulation of genetic changes, rather than chronograms where all species have same "time" distance to the root. We plotted FPD on a raster of study area.

Given that FPD positively correlates with number of species, we additionally analysed the pattern of standardized effect size of phylogenetic diversity (sesPD), a metrics of phylogenetic diversity that takes into account number of species per cell. sesPD compares empirical FPD against expected FPD that is estimated from a null model. Null model was generated in 9999 randomizations, in which species were randomly rearranged among the cells, while maintaining species' occurrence frequency and species richness, i.e. number of species per cell (independent swap algorithm (Gotelli, 2000)). Phylogenetic diversity of a cell expected under the null model is a mean FPD calculated from 9999 randomly obtained FPDs. This procedure allows also an estimation of *p*-value, saying in which cells the observed FPD significantly deviate from expected FPD in the assemblage under null model of species distribution. Low sesPD is expected when two or more closely related species occupy the same cell, and might point to local speciation. High sesPD detects co-occurrence of distantly related species and may be pointer of dispersal causing cross-sections of clade's ranges. We calculated standardized effect size of phylogenetic diversity (*sesPD = (observed PD – mean null PD)/standard deviation of null PD*) and *p*-value as a quantile of observed PD vs. null communities (pd.obs.p = mpd.obs.rank/runs +1). We plotted sesPD on a raster of study area and marked cells with significant *p*-value. Additionally, we applied another method to identify global variation in the PD controlling for the SR: For each cell with more than one species, we regressed PD against SR (see Appendix S4 for summary of linear regression) and calculated the residuals (the difference between observed and predicted PD, further on referred as residual PD). Cells harbouring more PD than expected for the observed richness are considered as regions of disproportionately phylogenetically diverse species compositions and vice versa (Gumbs et al., 2020). We plotted the residual PD on the 20 × 20 km grid covering the study area. Last, we compared the relationship between SR and sesPD. We plotted sesPD against SR and marked species-rich cells (seven or more species) based on their standardized phylogenetic diversity, to distinguish cells with lower and higher phylogenetic diversity than the mean value of null models, respectively. We plotted those cells on a raster of the study area.

The analyses of diversity patterns were run in R v.4.1.1 (R-core team, 2018), using packages "rgdal" (Bivand et al., 2021), "raster" (Hijmans, 2020), "fuzzySim" (Barbosa, 2015), "phytools" (Revell, 2012), "vegan" (Oksanen et al., 2020), "iNEXT" (Hsieh et al., 2020) and "picante" (Kembel et al., 2010).

3 | RESULTS

3.1 | Taxonomic structure of the dataset and phylogenetic analyses

The results of five delimitation methods suggested that our Western Balkans dataset comprised between 212 and 474 molecular operational taxonomic units (hereafter MOTUs) (Table 1). The difference in MOTU number appeared to be mostly due to different degrees of splitting, and in lesser extent due to incongruent lumping of different individuals into a single MOTU. After crosschecking with published multilocus species delimitations, we designed the dataset containing 227 MOTUs which can be considered as distinct species. After addition of 18 MOTUs without available COI marker, the final count was 245 MOTUs from Dinaric Region. ASAP outperformed other unilocus delimitation methods for large *Niphargus* dataset with 87% specimens classified to MOTUs that we consider as distinct species (Table 1).

To evaluate the molecular coverage of nominal species, we compared checklists of nominal species from that area (World Amphipoda Database (Horton et al., 2021)) with our data. Of the 123 nominal species listed from the area, we sequenced 97 (79%) species. Conversely, molecular delimitations indicate that the true species richness of the region may be 2 to 2.6 times higher.

A time-calibrated multilocus phylogeny of the whole genus included 512 MOTUs (Figure 2, Appendix S2). Of those, 377 were already recognized in previous studies (Borko et al., 2021) and 135 that were identified for the first time. Phylogeny comprised several geographically distinct, well-supported and MOTU-rich clades. Most of the species in the study region belonged to nine of these clades that started to diversify approximately 15 million years ago (Figure 2).

3.2 | Spatial analyses

To assess the biodiversity patterns in the study region, we mapped taxonomic and phylogenetic diversity of *Niphargus* using a grid of

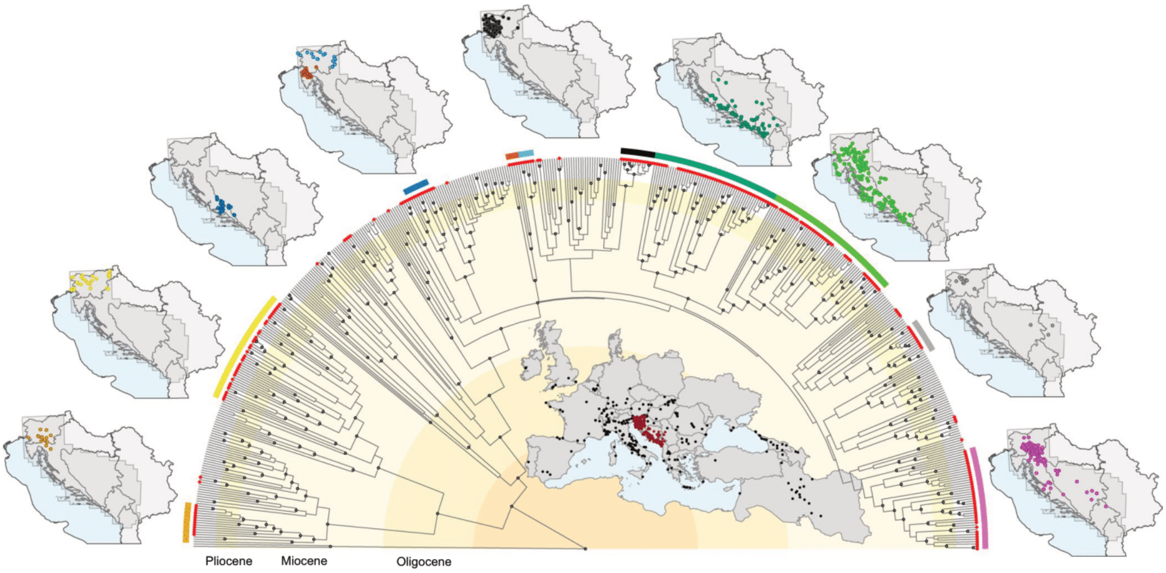

FIGURE 2 Chronogram of *Niphargus*, obtained with BEAST2. Grey dots mark nodes with posterior support >0.9. Red dots on tips are MOTUs present in West Balkans. On the map in the middle localities of MOTUs used for phylogenetic inference are marked with either red (within West Balkans) or black dots (outside study area). Clades with more than five MOTUs from Western Balkans are highlighted, and maps at the outer side of the chronogram show their distributions inside the study area (study area is marked with dark grey)

415 cells with a 20 × 20 km resolution. *Niphargus* specimens with molecular data were collected from 164 cells, compared to Bregović et al. (2019) with confirmed *Niphargus* species in 191 cells. All cells with missing data in our study were species poor in Bregović et al. (2019). Taxonomic diversity was assessed through species richness (SR) (Figure 3a). Number of species varied between zero and 14 species per cell, with median and mean being two and three species per cell, respectively. A single species per cell was detected in 58 cells (35%). Cells with highest SR are situated in the northern part; however, few species-rich cells were detected also in the south.

The pattern of Faith's phylogenetic diversity (FPD) is roughly similar to pattern of SR (Figure 3b). Cells with high FPD are more common in the north-west of the area. Two cells with the highest FPD had also the highest SR.

Patterns of SR, FPD and standardized PD (sesPD and residual PD) only partially overlap (Figure 3). The sesPD and residual PD exhibit highly similar pattern (Figure 3c,d). Most of the cells had lower phylogenetic diversity than expected under the null model. This pattern is present all over the region but dominates in the south-east. In the north-west, sesPD is generally equal to or higher than expected, although not significantly. Noteworthy, cells with high sesPD/residual PD may be species poor.

Among 16 cells with at least seven species (half of the maximum SR), seven cells had sesPD higher than zero and nine cells had low sesPD lower than zero. All but one cells are situated in the north-west (Figure 4).

4 | DISCUSSION

The contemporary biodiversity crisis, with the number of endangered species far exceeding available conservation resources, requires knowledge of exceptionally biodiverse areas to maximize the impact of conservation acts (Myers et al., 2000). Here, using an exceptionally biodiverse system of subterranean amphipods, we assessed how historical processes may explain the discrepancy among biodiversity patterns of different metrics and how these processes could be important for conservation-oriented activities.

We identified different biodiversity patterns of *Niphargus* when comparing species richness and phylogenetic diversity within the region. The pattern of species richness, inferred from MOTUs, resembles the pattern reconstructed from the distributional data of morpho-species (Bregović et al., 2019). Although the datasets differ in overall number of data records and taxonomic units used for assessment of the biodiversity patterns, the analyses of both datasets agree that species richness peaks in the north-west, but few species-rich cells can also be found in the south-east. Noteworthy, similar patterns of higher and lower diversity in the north and south were detected also on the level of individual *Niphargus* clades (Delić, Švara, et al., 2017; Zakšek et al., 2019). Comparable species diversity patterns derived from different datasets of different hierarchic levels indicate that our results are solid, even though we acknowledge that both parts of the region might unveil additional, not yet detected species, or extend the geographic coverage to additional (yet empty) cells. It is noteworthy that past studies showed that hotspots in subterranean biodiversity remain hotspots even with additional sampling and that new species can be expected in both species-poor and species-rich areas (Culver et al., 2004; Zagmajster et al., 2008, 2010). The pattern of phylogenetic diversity, however, is different: Phylogenetic diversity is low over most of the region except in the north-west, on the junction of the Dinaric Karst, South-Eastern Alps and Pannonian lowlands, at the crossroad of karstic areas and alluvial plains (Figure 3). The increase in phylogenetic diversity in the north-west can be attributed to the presence of species from two clades, Pontian and Pannonic clades that are distributed between Northern Italy and Pannonian lowlands, and cross Dinaric region in the north (Figure 2, see also Borko et al., 2021). Europe-wide sampling (Borko et al., 2021) suggests that it is highly unlikely that members of either of these two clades would be found in the south-eastern part of the region. Likewise, we did not detect some clades from the south-east in the north-west; hence, both subregions contain some exclusive clades. We conclude that phylogenetic diversity results can be considered robust.

The patterns of species and phylogenetic richness seem to be decoupled. Spatial distribution of both, sesPD and residual PD, implies that species-rich and species-poor cells may have high PD (Figures 3c,d and 4). The contrasting differences among patterns of species richness and phylogenetic diversity of *Niphargus* in the Western Balkans allow inferences of the processes behind the pattern. Here we limit our discussion to cells with higher species richness in the north-western and south-eastern parts of the region. Species-rich areas can emerge either through dispersal from neighbouring regions or from on-site speciation (Rosindell & Phillimore, 2011). The pronounced roles of speciation and dispersal can be detected on patterns of phylogenetic diversity. Local species proliferation only little increases phylogenetic diversity and results in low phylogenetic diversity relative to species richness. Conversely, immigration of unrelated phylogenetic lineages increases phylogenetic diversity relative to species richness (Davies et al., 2007; Fritz & Rahbek, 2012; Li & Yue, 2020). Therefore, we hypothesize that speciation can be considered as a universal generator of species richness patterns along the entire Western Balkans and that dispersal acted mostly in the north-west (Figure 3). This view is consistent with the observation that distributional ranges of *Niphargus* species rarely exceed 200 km in length (Bregović et al., 2019; Trontelj et al., 2012). We recognize that the differences in species and phylogenetic richness could be also an outcome of extinctions. While we cannot directly evaluate the latter's contribution with the data available, we assume that this mechanism was negligible and random in space, given that subterranean environment acts as a refugium from major catastrophic disturbances on the surface (Borko et al., 2021). The most recent driver of extinctions in Europe were Pleistocene glaciations, but at that time the entire Balkan Peninsula acted as a southern refugium (Hewitt, 2000). Even more, Alpine glaciers in the north-west of the studied area prompted *Niphargus* speciation rather than extinction (Delić et al., 2022).

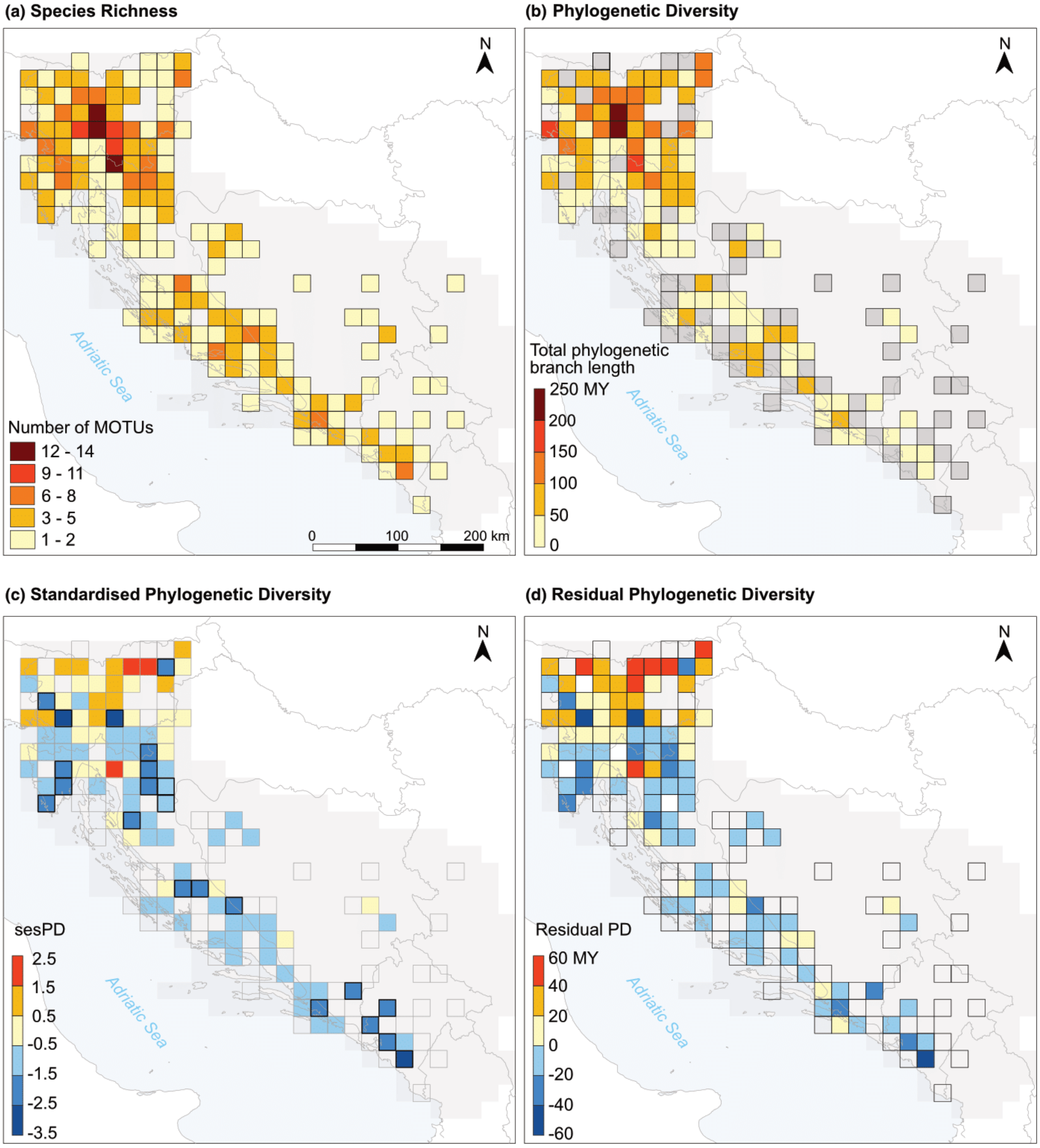

FIGURE 3 Species richness, Faith's phylogenetic diversity and standardized phylogenetic diversity. (a) Species richness as number of MOTUs in each cell. (b) Phylogenetic diversity as a sum of total branch lengths (FPD). Grey cells have only one species (FPD not calculated). (c) Standardized effect size of phylogenetic diversity (sesPD) for cells with more than one species, calculated using independent swap algorithm. Blue squares have lower sesPD and orange/red squares have higher sesPD than mean value of PD in null communities. Cells with *p*-values <.025 or >.975 are bordered. (d) Residual phylogenetic diversity. Blue squares have lower residuals than expected value of PD given SR, and orange/red squares have higher residuals. Regions without cells were either not sampled for *Niphargus*, or we could not obtain fresh material for molecular analyses

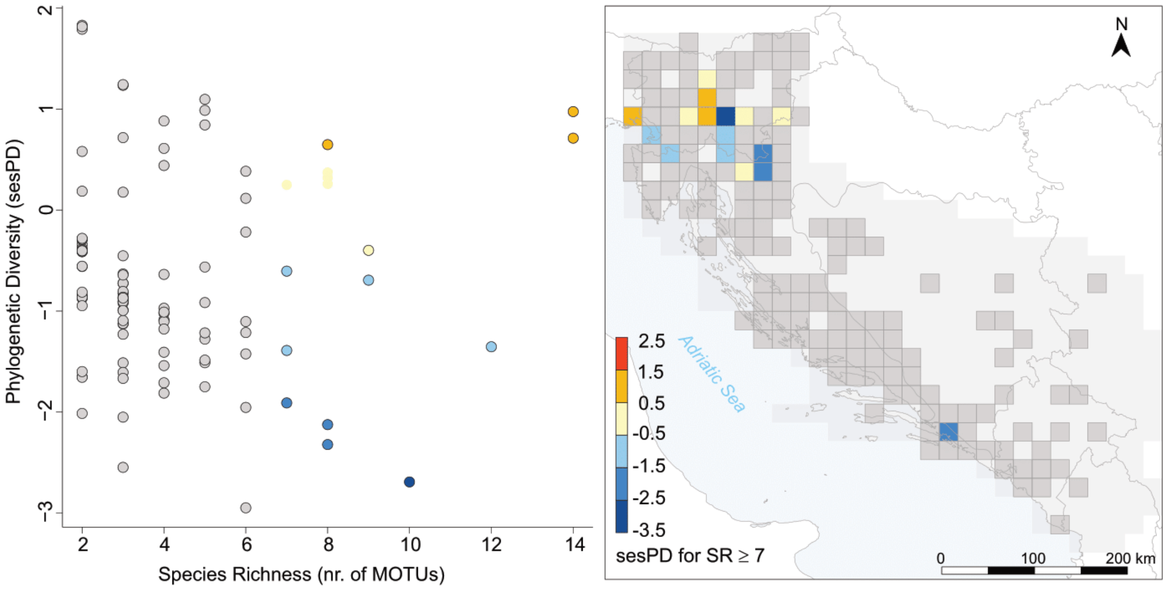

FIGURE 4 Standardized phylogenetic diversity (sesPD) vs. species richness (number of MOTUs, abb. SR). Left: scatter plot of sesPD vs. SR. Right: cells on the map with seven or more species are coloured according to their corresponding values of sesPD. The colour scheme for sesPD is the same as in Figure 3c. Grey cells on the map have less than seven species

This view is consistent with historical biogeography. The observed biodiversity patterns can be attributed to spatial overlap of unrelated clades that diverged from each other already in the Eocene (Figure 2). The Balkan Peninsula has a dynamic geological history (Handy et al., 2015; Popov et al., 2004), which directly shaped the evolutionary trajectory of *Niphargus* in the area. An uplift of carbonate mountain ranges—including the South-Eastern Alps and the Dinaric Karst (Park, 2014; Popov et al., 2004)—opened up a variety of new, unpopulated habitats, acting as an ecological opportunity for rapid ecological diversification of several *Niphargus* clades (Borko et al., 2021). Highly fragmented subterranean environment also generated dispersal barriers that might have promoted allopatric speciation, resulting in cryptic species (Delić, Trontelj, et al., 2017; Zakšek et al., 2019). These adaptive and non-adaptive local radiations resulted in cells with low standardized phylogenetic diversity, especially in the south-east. Nevertheless, some *Niphargus* species from non-Dinaric clades have large ranges and are good dispersers (Copilaş-Ciocianu et al., 2018). Those species migrated in the north-western Dinarides (Borko et al., 2021). The north-western part of the studied region was occasionally submerged (Kováč et al., 2018). Aquatic connections between Adriatic basin and Paratethys Sea apparently opened migration routes, resulting in congregation of species from several phylogenetically and geographically independent lineages (Figure 2). The established sympatries nowadays result in high species richness and high standardized phylogenetic diversity (Figure 4).

Inference of the processes driving the biodiversity patterns can be integrated into conservation implications. Our study unveiled that not all species-rich cells can be considered equivalent to each other, as species richness does not directly correlate with phylogenetic richness. This calls for caution in conservation strategies. Species richness in cells with high phylogenetic diversity in the north-west possibly originated mainly through dispersal. Their surrounding regions may have been acting as donors of species. In the worst case, species-rich areas may be an assembly of sink populations rather than regions with high functional diversity that stabilizes ecosystem functioning (Funk & Burns, 2019). If so, proper conservation strategy should not protect only the species-rich focal region, but also contributing areas.

A completely different issue is found for cells with low phylogenetic diversity, for which we hypothesize that gained species richness mainly by speciation, e.g. through local adaptive radiations (Borko et al., 2021). Such cells with high species richness and low phylogenetic diversity can be found in the south-east (Figure 4). Anthropogenic disturbance in these areas may negatively affect whole lineages, ranging from loss of diversity by reverse speciation (Seehausen, 2006), to extinction of entire clades and irreversible loss of their genetic diversity. Such locally distributed and closely related species complexes can be more threatened than unrelated species complexes simply because they are confined to a threatened environment (Funk & Burns, 2019; Tonini et al., 2016) or also because of inherited sensitivity (phylogenetic niche conservatism) (Wiens et al., 2010). Potential threats could damage the habitat template that had prompted diversification in the past, and thus make recovery of the local ecosystems less likely. Noteworthy, in the south-eastern part of the Western Balkans hundreds of hydropower plants are planned. These hydropower plants present a direct threat to the enigmatic biodiversity of this region and Europe as a whole (Schwarz, 2015).

Identification and comprehensive analysis of contrasting biodiversity patterns sheds a new light on the origin of species-rich areas of the Western Balkans. We here identify several issues that should be addressed when planning conservation measures: the hidden biodiversity (undescribed, sometimes morphologically cryptic species); the discrepancy between species richness and phylogenetic diversity patterns; and possible implications of different origins of hotspots on conservation planning. There are some additional aspects that may be relevant for conservation biology. Despite massive efforts in the past decades, we can expect additional species records (Appendix S5, Figure S5.1) and undescribed species (Table 1). Second, although *Niphargus* is an important faunistic element of the studied region, it is merely one among many taxa, many of which are still understudied. It would be beneficial to include other taxa, aquatic and terrestrial, which may reveal different relationships between species richness patterns and phylogenetic diversity. However, identification of robust biodiversity patterns is only the first step, preceding a challenging task how such science backed knowledge should be integrated into overarching conservation strategy in the region spanning across four countries. Finally, this study implies that similar approaches should be applied also in other regions, at least where species richness is high (Eme et al., 2018; Zagmajster et al., 2014). However, the generally scarce sampling of groundwater, with only few studies having similarly dense spatial resolution (Alther et al., 2021; Fišer et al., 2018; Flot et al., 2014; McInerney et al., 2014), makes such inclusions unlikely to happen in the near future. Thus, while we are still far from an optimal strategy on how to protect groundwater habitats, the numerous threats to groundwater (Mammola et al., 2019), such as hydropower dams or extensive water extraction, put an extra urge to strengthen and systematically organize future research on this topic, to make it implementable and effective.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Sequence data have been deposited in GenBank, under accession numbers OK149784–OK150101 and OK156503–OK157282. Vouchers, GenBank accession numbers with hyperlinks to GenBank used in delimitations and phylogenetic inference, spatial coordinates of samples, delimitation results and summary of regression analysis are listed in Appendices S1, S2, S3 and S4, respectively. Alignments, settings for phylogenetic analyses and R code used in analyses are available on Dryad (https://doi.org/10.5061/dryad.w0vt4b8sm).

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BIOSKETCHES

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Author Contribution: Š. B., M. Z., F. A. and C. F. conceptualized the study. Š. B. and M. Z. did the field work. Š. B. performed laboratory work and analysed the data. Š. B. prepared the first draft. Š. B., M. Z., F. A. and C. F. wrote and approved the paper.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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2.5 HOW DID SUBTERRANEAN AMPHIPODS CROSS THE ADRIATIC SEA? PHYLOGENETIC EVIDENCE FOR DISPERSAL–VICARIANCE INTERPLAY MEDIATED BY MARINE REGRESSION–TRANSGRESSION CYCLES

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Freshwater subterranean amphipods with low dispersal abilities are known from both sides of the impermeable barrier, the Adriatic Sea. We tested the hypothesis that historical marine regression–transgression cycles shaped the distribution patterns of subterranean amphipods through repeated cycles of dispersal and vicariance against the hypothesis that subterranean amphipods colonized both sides of the Adriatic Sea independently. Our study model was genus *Niphargus*, a clade of freshwater subterranean amphipods (Crustacea: Amphipoda). The taxonomic structure of the studied clade was revised using unilocus species delimitation methods. The timeframe of cladogenetic events was inferred using a multi-locus time-calibrated phylogeny and compared to the main regression–transgression events in the Miocene and Pleistocene. The geographical origin of the studied clade, species range expansions and contractions, as well as vicariance events were assessed through modelling of historical biogeography. Subterranean amphipods of the genus *Niphargus*, found on both sides of the Adriatic Sea, form a monophylum. The reconstructions of ancestral ranges suggest that the clade emerged in the Balkan Peninsula, dispersed three times independently to the Apennine Peninsula and once back to the Balkans. Adriatic Islands were colonized multiple times, predominantly from the Balkan Peninsula. The dispersal–vicariance events correspond to historical regression–transgression cycles in Miocene and Pleistocene. Marine regression–transgression cycles apparently shaped the distribution patterns of subterranean amphipods while the alternative hypothesis received no support. The actual distribution of subterranean faunas apparently reflects old biogeographical events.

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RESEARCH PAPER

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How did subterranean amphipods cross the Adriatic Sea? Phylogenetic evidence for dispersal–vicariance interplay mediated by marine regression–transgression cycles

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Abstract

Aim: Freshwater subterranean amphipods with low dispersal abilities are known from both sides of the impermeable barrier, the Adriatic Sea. We tested the hypothesis that historical marine regression–transgression cycles shaped the distribution patterns of subterranean amphipods through repeated cycles of dispersal and vicariance against the hypothesis that subterranean amphipods colonized both sides of the Adriatic Sea independently.

Location: Western Balkan Peninsula, Adriatic Sea Islands and Apennine Peninsula, Europe.

Taxon: Genus *Niphargus*, a clade of freshwater subterranean amphipods (Crustacea: Amphipoda).

Methods: The taxonomic structure of the studied clade was revised using unilocus species delimitation methods. The timeframe of cladogenetic events was inferred using a multi-locus time-calibrated phylogeny and compared to the main regression–transgression events in the Miocene and Pleistocene. The geographical origin of the studied clade, species range expansions and contractions, as well as vicariance events were assessed through modelling of historical biogeography.

Results: Subterranean amphipods of the genus *Niphargus*, found on both sides of the Adriatic Sea, form a monophylum. The reconstructions of ancestral ranges suggest that the clade emerged in the Balkan Peninsula, dispersed three times independently to the Apennine Peninsula and once back to the Balkans. Adriatic Islands were colonized multiple times, predominantly from the Balkan Peninsula. The dispersal–vicariance events correspond to historical regression–transgression cycles in Miocene and Pleistocene.

Main conclusions: Marine regression–transgression cycles apparently shaped the distribution patterns of subterranean amphipods while the alternative hypothesis received no support. The actual distribution of subterranean faunas apparently reflects old biogeographical events.

KEYWORDS
amphipod, Apennine Peninsula, Dinaric Karst, dispersal, land bridge, *Niphargus*, subterranean, transadriatic, vicariance

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*Teo Delić and Fabio Stoch contributed equally to this article.

This paper is dedicated to the memory of Augusto Vigna Taglianti (1943–2019), the first researcher to highlight the transadriatic distribution within the genus *Niphargus*, inspiring the conceptual framework of our study.

1 | INTRODUCTION

Marine regressions and transgressions have shaped many biodiversity patterns of terrestrial and freshwater faunas (Chamberland et al., 2018; Desiderato et al., 2019; Hou & Li, 2018; Woodruff, 2003). During marine regressions, when sea levels drop, new dispersal routes emerge connecting islands with each other and the mainland (Gargani & Rigollet, 2007; Woodruff, 2003). By contrast, marine transgressions with deep incursions of salty water into coastal lowlands terminate dispersal routes, reshape island sizes and archipelago structures, modify local climates and alter marine currents (Fernández-Palacios et al., 2016). From a biogeographical perspective, marine regression–transgression cycles correspond to respective periods of dispersal and vicariance, which can plausibly explain the distribution patterns of some clades along the coastal regions (Cánovas et al., 2016; Desiderato et al., 2019; Liu, Li, Ugolini, Momtazi, & Hou, 2018; White, Reimer, & Lorion, 2016; Yang, Hou, & Li, 2013).

However, biologists studying subterranean species have proposed another, different role of marine regression–transgression cycles on faunas. Fluctuations of sea level presumably play an essential role in the colonization of the continental groundwater from ancient shallow seas. Briefly, ancestral marine benthic species colonized the marine interstitial, spread into the continent during transgressions, established contact with, and became stranded in subterranean freshwater during marine regressions (Notenboom, 1991; Stock, 1980). This scenario, as elaborated with several alternatives (Boutin & Coineau, 1990; Holsinger, 1994, 2000), has been used as an explanation for the origin and distribution of many subterranean taxa (Baratti, Filippelli, Nardi, & Messana, 2010; Boulanouar, Yacoubi, Messouli, & Coineau, 1995; Coineau, 1994; Kupriyanova et al., 2009; Page et al., 2016; Sket & Zakšek, 2009). By contrast, the more classical view of marine regressions–transgressions as an alternation between processes of dispersal and vicariance has received little attention in subterranean biology, most likely due to the presumed low dispersal capacity of subterranean organisms (Bregović, Fišer, & Zagmajster, 2019; Trontelj et al., 2009). Few studies to date have invoked this scenario to explain species distributions (Bauzà-Ribot, Jaume, Fornós, Juan, & Pons, 2011; Cánovas et al., 2016; Guy-Haim, Simon-Blecher, Frumkin, Naaman, & Achituv, 2018; Stokkan et al., 2018), and none of these studies explicitly modelled biogeographical patterns using iterative cycles of regression and transgression.

In this study, we investigated whether regression–transgression cycles might explain the distribution of clades of subterranean amphipods in the Mediterranean region. We studied the amphipod genus *Niphargus*, a species-rich group distributed in the Western Palearctic (Fišer, 2019). As a freshwater taxon, *Niphargus* represents an appropriate model system for studying the role of the marine regression–transgression cycles. In North-Western Europe, *Niphargus* colonized freshwater during the Cretaceous, and the Mediterranean clades presumably derived from freshwater ancestors (McInerney et al., 2014). Today, virtually all *Niphargus* species live in subterranean freshwater, with few exceptions found in anchialine caves (Karaman & Sket, 1989; Sket & Karaman, 1990; but see also Copilaş-Ciocianu, Fišer, Borza, & Petrusek, 2018). The closest relative of *Niphargus* is the freshwater and anchialine genus *Pseudoniphargus* (Copilaş-Ciocianu, Borko, & Fišer, 2020; Jurado-Rivera et al., 2017), whereas their putative marine relatives are not known. Early taxonomic works and phylogenies identified some species groups distributed on both sides of the Adriatic Sea on the Apennine and Balkan Peninsulas as well as on islands in the Adriatic Sea (Delić, Švara, Coleman, Trontelj, & Fišer, 2017; Fišer, Sket, & Trontelj, 2008; Ntakis, Anastasiadou, Zakšek, & Fišer, 2015; Ruffo & Vigna Taglianti, 1967), making it likely that these clades experienced dispersal and/or vicariance due to regression–transgression cycles.

The Balkan Peninsula is the older of the two peninsulas. The western part of the Balkans is a carbonate mountainous region, the Dinaric Karst. The uplift of the Dinaric Karst started approximately 30 million years ago (hereafter referred as Ma), resulting in a major landmass in South-Eastern Europe (Park, 2014; Popov et al., 2004; Rögl, 1998). By contrast, the Apennine Peninsula emerged as a group of islands approximately 20 Ma. The shape of the modern Apennine Peninsula was first attained some 10 Ma (Popov et al., 2004; Rögl, 1998). The first land bridges between the two peninsulas were ephemeral archipelagos that connected the central part of the Dinaric Karst with Apulia about 13–15 Ma (Mazza & Rustioni, 2008). Later on, the two peninsulas were connected with at least two extensive land bridges. The older land bridge dates back to the Miocene, when the orogenic uplift of Gibraltar arc seaway triggered the so-called Messinian Salinity Crisis (5.96–5.33 Ma) (Garcia-Castellanos & Villaseñor, 2011). As a consequence, large parts of the Mediterranean, including the entire Adriatic basin, completely dried up (Gargani & Rigollet, 2007). The younger and the last existing land bridges connecting both sides of the Adriatic Sea, date to the Pleistocene, when glacial maxima caused drying of the northern half of the Adriatic Sea (Correggiari, Roveri, & Trincardi, 1996).

The distribution of *Niphargus* species on both sides of the Adriatic Sea can be explained by two hypotheses. The first one is based on the generally low dispersal capacity of subterranean organisms. Species on both sides of the Adriatic Sea originated through single colonization of the ancient landmasses, with no subsequent dispersal across the Adriatic basin. If so, we would expect that these species originated at the contact of both peninsulas, nowadays Northern Italy. Furthermore, lineages found on the Apennine Peninsula cannot be older than 10 million years, and – because dispersal is unlikely – present a single, monophyletic lineage, sister to the lineages from the Balkans. The alternative hypothesis states that the distribution of *Niphargus* species on both sides of the Adriatic Sea was shaped by dispersal and vicariance, corresponding to regression–transgression cycles. This hypothesis relaxes the prediction of a maximum clade age of 10 Ma and allows these species to emerge in the older Dinaric Karst. In addition, this hypothesis predicts that the ancestors of the modern species crossed the Adriatic basin and colonized the Apennine Peninsula during one or more marine regressions, when the land bridges connecting the two peninsulas were formed. Hence, it can be expected that species from both sides of the Adriatic Sea belong to several clades. To test these predictions, we performed the present study in three steps. First, we revised the taxonomic structure of the so-called transadriatic clade using molecular species delimitation methods. Second, we assessed the number and the timeframe of cladogenetic events among molecularly defined species by reconstructing a multi-locus time-calibrated phylogeny. Finally, using spatial information and the calibrated phylogeny, we assessed where the studied species originated from and analysed the dispersal–vicariance events. We employed reconstruction methods and modelled the historical biogeography of these species using a series of models, which accounted for anagenetic (dispersal) and cladogenetic (vicariance) processes. We identified four major dispersal events, three from the Dinaric to the Apennine region and one in the reverse direction.

2 | MATERIALS AND METHODS

2.1 | Taxon sampling and DNA isolation

Taxon sampling aimed to include a wide range of *Niphargus* species, distributed from Ireland to Iran, with comprehensively sampled species distributed on the both sides of the Adriatic Sea. The ingroup sampling included also the genera nested within the genus *Niphargus*, namely *Carinurella* Sket, 1971; *Chaetoniphargus* Karaman & Sket, 2019; *Haploginglymus* Mateus & Mateus, 1958 and *Niphargobates* Sket, 1981. The outgroup comprised five species of *Pseudoniphargus* Chevreux, 1901 (based on Copilaş-Ciocianu et al., 2020). We sampled altogether 214 localities and 494 individuals, and stored them in 96% ethanol. The remaining parts of the samples are deposited in the Zoological collection of the Department of Biology, Biotechnical Faculty, University of Ljubljana, Slovenia, in the collection of the Evolutionary Biology and Ecology Unit of the Université libre de Bruxelles (ULB), Belgium, and in the collection of the Natural History Museum of Verona, Italy. All the information on the specimens used in the analyses, collection sites, DNA vouchers and museum collections are available in Supporting Information 1.

Each specimen was identified to the closest morphologically resembling morphospecies using original species descriptions. Then, genomic DNA was isolated from one of the pereiopods (thoracic appendages) using the GenElute Mammalian Genomic DNA (Sigma-Aldrich, USA). The remaining part of the specimen was retained for further morphological studies. We amplified nuclear DNA – two parts of the 28S rRNA gene (28S rRNA I and 28S rRNA II), histone H3 (H3) and the mitochondrial cytochrome *c* oxidase subunit I (COI). A list of primers and PCR amplification protocols used is available in Supporting Information 2 and 3. PCR products were purified using Exonuclease I and FastAP (Thermo Fisher Scientific Inc., USA) according to the manufacturer's instructions, and sequenced in both directions by Macrogen Europe (Amsterdam, The Netherlands), using the amplification primers; the resulting chromatograms were assembled and edited using GENEIOUS 8.1.9. (Biomatters, New Zealand). Ambiguous nucleotide bases were coded using IUPAC nucleotide ambiguity characters.

2.2 | Taxonomic structure and molecular species delimitation

To account for only partially resolved taxonomy and the possible presence of morphologically cryptic species, we revised the taxonomic structure of the study dataset prior to the main analyses. We assembled a dataset containing altogether 463 COI sequences of *Niphargus*, the genera nested within it (*Carinurella*, *Haploginglymus*, *Niphargobates*) and *Pseudoniphargus* (Supporting Information 1). The sequences were acquired in this study (120 sequences) or retrieved from GenBank. Putative species structure, represented by the so-called molecular taxonomic operational units (hereafter MOTUs), was inferred using distance- and tree-based delimitation methods, Automatic Barcode Gap Discovery (hereafter ABGD) (Puillandre, Lambert, Brouillet, & Achaz, 2012) and Poisson Tree Processes (hereafter PTP) (Zhang, Kapli, Pavlidis, & Stamatakis, 2013), respectively.

ABGD (Puillandre et al., 2012) assigns the sequences to the specific MOTUs based on the assumption that intraspecific genetic distances are smaller than interspecific ones, and without a priori species hypotheses. We ran this analysis on the complete COI dataset, using the ABGD web server http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html with default parameters, namely Kimura two-parameter substitution model, prior for maximum value of intraspecific divergence between 0.001 and 0.1, 10 recursive steps and a gap width of 1.0.

An alternative taxonomic structure was assessed using PTP (Zhang et al., 2013), a phylogeny-based method that delimits species at nodes where presumed intraspecific nucleotide substitution rates switch to interspecific substitution rates. The two nucleotide substitution rates are modelled using two different Poisson processes. For the PTP analyses, we removed duplicate sequences using a custom Perl script (Eme, Malard, Konecny-Dupré, Lefébure, & Douady, 2013), and ran the analysis using a dataset of 300 unique haplotypes. Phylogenetic relationships were estimated in two separate maximum likelihood analyses. The first analysis was performed using PhyML 3.1 (Guindon et al., 2010), relying on the GTR substitution model with estimated gamma shape parameter and proportion of invariant sites. To assess the robustness of maximum likelihood tree, we repeated the analysis in IQ-TREE 1.6.7 (Nguyen, Schmidt, von Haeseler, & Minh, 2015), using the invertebrate mitochondrial codon model (GY+F+R7) selected in ModelFinder (Kalyaanamoorthy, Minh, Wong, von Haeseler, & Jermiin, 2017). The resulting trees were then used to run the PTP analysis on the species delimitation server http://species.h-its.org/ within the Bayesian and maximum likelihood framework. Bayesian posterior probabilities for tentative species were acquired after running 500,000 generations, sampling every 100 generations, and discarding the first 20% of the samples as a burn-in. The putative species were also delimited using multi-rate PTP (mPTP, Kapli et al., 2017), which accounts for multiple evolutionary rates. This analysis lumped geographically and ecologically distant species as well as sympatric species that were separated in the multi-locus framework of recent studies (Delić et al., 2017). For these reasons, the results were discarded from further analyses.

2.3 | Phylogenetic analyses

Phylogenetic structure of the studied dataset was inferred using 195 Niphargidae species (*Niphargus* and the genera nested within), and were rooted with five species of *Pseudoniphargus*, which was shown to be a sister group in two previous studies (Copilaş-Ciocianu et al., 2020; Jurado-Rivera et al., 2017). The dataset included 169 MOTUs delimited in the PTP analysis (see above and Results section) and supplemented with 31 described species (excluded from the above-described delimitation analyses because of the missing COI fragment). For the phylogenetic analysis, we assembled a multi-locus molecular dataset that included two fragments of 28S rDNA, H3 and COI sequences. All sequences were aligned using the Q-INS-I algorithm implemented in MAFFT 7 (Katoh & Standley, 2013). The best partitioning scheme and the optimal substitution models for the codon positions were obtained using PartitionFinder 2.1.1 (Lanfear, Frandsen, Wright, Senfeld, & Calcott, 2017) and are available in Supporting Information 2.

Phylogenetic relationships were reconstructed using two alternative approaches: (a) maximum likelihood with partition-specific setting and ultrafast bootstrapping in IQ-TREE 1.6.7 (Nguyen et al., 2015) and (b) Bayesian inference with partition-specific settings in MrBayes 3.2.6 (Ronquist et al., 2012). The node supports for the maximum likelihood analysis were assessed in 1,000 ultrafast bootstrap replicates (Hoang et al., 2018). Alternatively, the Bayesian Markov chain Monte Carlo (MCMC) tree was inferred after running two independent runs with four chains for 30 million generations each, and sampled every 400 generations. After reaching the stationarity phase, the first 25% of trees were discarded as burn-in and the remaining trees were used to calculate a 50% majority rule consensus tree.

2.4 | Estimation of divergence times

To define the timeframe of splits between the lineages distributed in the Dinaric Karst and the Apennine Peninsula, we reconstructed a time-calibrated multi-locus phylogeny using the package BEAST 2.5.1 (Bouckaert et al., 2018). The dataset comprising 200 individuals (same as above) and four molecular markers were analysed using partitioning schemes with distinct parameters settings, following the best-fit models of evolution proposed by bModelTest 1.2.1 (Bouckaert & Drummond, 2017). Due to the initial issues with the over parametrisation, we reduced the initial number of partitions (seven) to three, which corresponded to the three genes sequenced. The data on the partitioning schemes and the substitution models used in BEAST analyses are available in Supporting information 2. Substitution and clock models were unlinked for all partitions, while the tree partitions were linked to generate a single dated phylogeny. The marginal likelihood of Birth death and Yule speciation tree priors were compared in BEAST's extension Path Sampler. Based on the marginal likelihood, we used a Yule speciation tree prior for the final analyses (results not shown). To account for lineage-specific rate heterogeneity, we employed a lognormal relaxed clock (Drummond, Ho, Phillips, & Rambaut, 2006). The tree was calibrated based on two lines of evidence, derived from fossilized specimens and biogeography. Two fossilized *Niphargus* from Baltic amber are of presumed age 35–50 Ma (Coleman & Myers, 2000; Jażdżewski & Kupryjanowicz 2010); hence, the calibration point (the node where morphologically similar species evolved for the first time) was set to a lognormal distribution with a 95% confidence interval between 34 and 95 Ma, and a mean value of 45 Ma. Furthermore, based on the absence of *Niphargus* from North America and Greenland, we assumed that *Niphargus* cannot be older than the initial opening of the Atlantic Ocean, and that this genus most likely evolved after the final submergence of the land bridges between Eurasia and North America between 57 and 71 Ma (Brikiatis, 2014). Therefore, the second calibration point (root of the family Niphargidae) was set to a lognormal distribution with a 95% confidence interval between 35 and 154 Ma, and a mean value of 64 Ma. Four independent runs of 100 million generations, sampling every 10,000 steps, were performed and combined using LogCombiner 2.6.1. The stationarity of each single run was checked in Tracer 1.7 (Rambaut, Drummond, Xie, Baele, & Suchard, 2018) and the first 10% trees were discarded as burn-in. All the remaining samples from the posterior distribution were summarized using TreeAnnotator 1.8 in the so-called maximum clade credibility (MCC) tree, which was visualized in FigTree 1.4. Calibration points were cross-validated in two alternative analyses in which either of the calibration points was excluded.

2.5 | Modelling past dispersal, vicariance and reconstruction of ancestral ranges

To elucidate the historical biogeography of the main transadriatic clade, we modelled dispersal, extinction, vicariance, cladogenesis and ancestral ranges (Figure 1) using the R 3.5.5 (R Core Team 2019) package 'BioGeoBEARS' 1.1.2 (Matzke, 2013).

The modelling requires a time-calibrated phylogeny and the distributions of the extant species. To avoid the effects of biogeographical events outside the study region on parameter estimation of our models, we pruned the tree and ran the analyses on the focal clade (see Results). To account for phylogenetic uncertainty, we ran all the analysis on a sample of 100 random time-calibrated trees drawn from the stationary phase in the MCMC analysis. Species distributions were extracted from the European Groundwater Crustacean Dataset (Zagmajster et al., 2014), distributional database SubBio (http://subbio.net/db/) and our own, unpublished data. In turn, the distributions were categorized into three regions defined with respect to their palaeogeographical origin: The Dinaric Karst, the Apennine Peninsula and the Adriatic Islands (Figure 2).

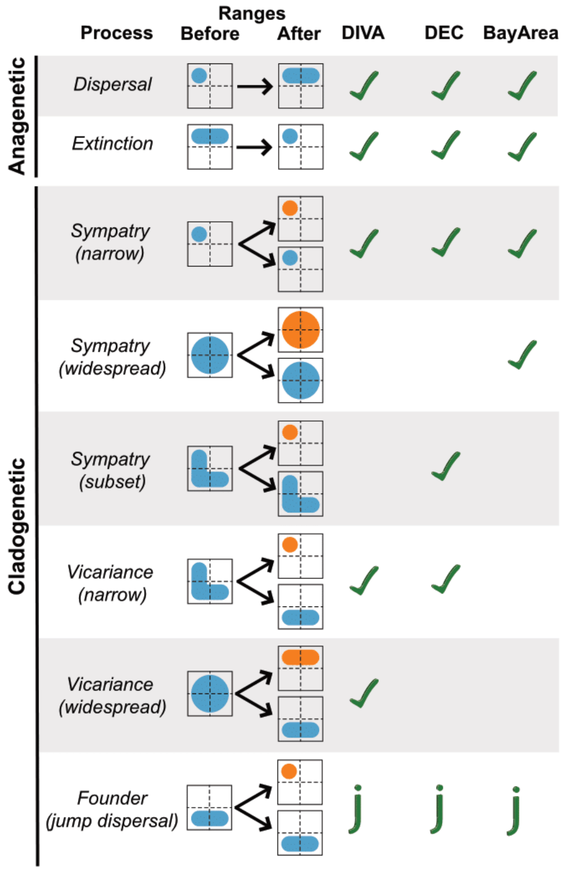

FIGURE 1 A summary of biogeographic models as implemented in the R package 'BioGeoBEARS': hypothetical anagenetic and cladogenetic processes. Redrawn from Matzke (2013). All implemented models allow for founder event (jump dispersal) speciation by addition of parameter "j" [Colour figure can be viewed at wileyonlinelibrary.com]

To explore historical biogeographical events, we ran several biogeographical models. Using a stochastic continuous-time Markov process, these models decompose range evolution at the time of cladogenesis (at the nodes) and anagenesis (along the branches), and test their fit using the Akaike information criterion (AIC) or likelihood values. Biogeographical models we used are illustrated in Figure 1. Briefly, all models assume anagenetic range extension and contraction, technically called dispersal (parameter *d*) and extinction (parameter *e*), respectively. Range evolution at cladogenetic events is treated differently between the three basic models (DEC, DIVA, BAYAREA, see Figure 1 for comparison). All the basic models, however, assume that all descending species inherited parts of the ancestral ranges (Figure 1). An important extension of basic models is the so-called jump dispersal speciation (labelled as 'basic model+J'), modelled with the parameter *j* that is estimated simultaneously with the other parameters (Matzke, 2013). Jump dispersal assumes colonization of a novel biogeographical area at the time of cladogenesis (founder event speciation) and is, arguably, especially important in island systems (Matzke, 2014; see also Ree & Sanmartín, 2018 for criticism of the jump dispersal models). In 'BioGeoBEARS', all the basic models can be extended with the jump dispersal parameter, and the fit of these models can be assessed using likelihood values or AIC.

We ran all possible basic models (DEC, DIVA and BAYAREA), and models with additional jump dispersal speciation (+J). This ended in altogether six models. Biogeographical models were tested using AIC and Akaike weights to interpret how well different models fit the geographical distributions and the proposed phylogeny. However, given that jump dispersal models (+J) may increase the number of alternative cladogenetic events and disproportionally inflate their explanatory power against anagenetic events, we did not compare the basic and +J models directly (Ree & Sanmartín 2018). The relative contribution of dispersal and vicariance at the point of cladogenesis in the diversification of the transadriatic *Niphargus* was obtained by running 100 biogeographical stochastic mappings, based on the two models with the best fit (the best basic model and the best+J extended model), the calibrated phylogeny and the geographical distribution.

3 | RESULTS

3.1 | Species delimitations and phylogenetic analyses

Both species delimitations yielded similar results. ABGD hypothesized that 463 COI sequences should be classified into 162 MOTUs, while both PTP analyses suggested that the dataset comprised 169 MOTUs (Supporting Information 4). The difference in MOTU numbers indicated different levels of splitting in three broadly distributed and genetically diverse Dinaric species (*N. subtypicus*, *N. kolombatovici* and *N. hebereri*), where the distinction between population and species level was not clear. However, the alternative structure of MOTUs did not yield conflicting results, that is, alternative haplotype segregations. Given that biogeographical events (dispersal, extinction and cladogenesis) took place already in the early stages of speciation, sometimes even at a population level, we based all subsequent analyses on MOTUs delimited by the PTP analysis.

Both maximum likelihood and Bayesian inference resulted in similar trees (Figure 2, Supporting Information 5). Species from both sides of the Adriatic Sea were nested within a large clade (ultrafast bootstrap support/posterior probabilities = 97/0.69) distributed predominantly in the Dinaric Karst and altogether including 76 MOTUs. Hereinafter, this clade is referred to as the main transadriatic clade (red clade in Figure 2).

Within the main transadriatic clade are nested three strongly supported subclades (ultrafast bootstrap support/posterior probabilities = 100%/1), with species distributed on both sides of the Adriatic Sea and on the Adriatic Islands (Figure 3). For convenience, we named them after the species comprising the clades as follows: The *N. hebereri* – *N. ictus* clade, the *N. steueri* – *N. patrizii* clade and

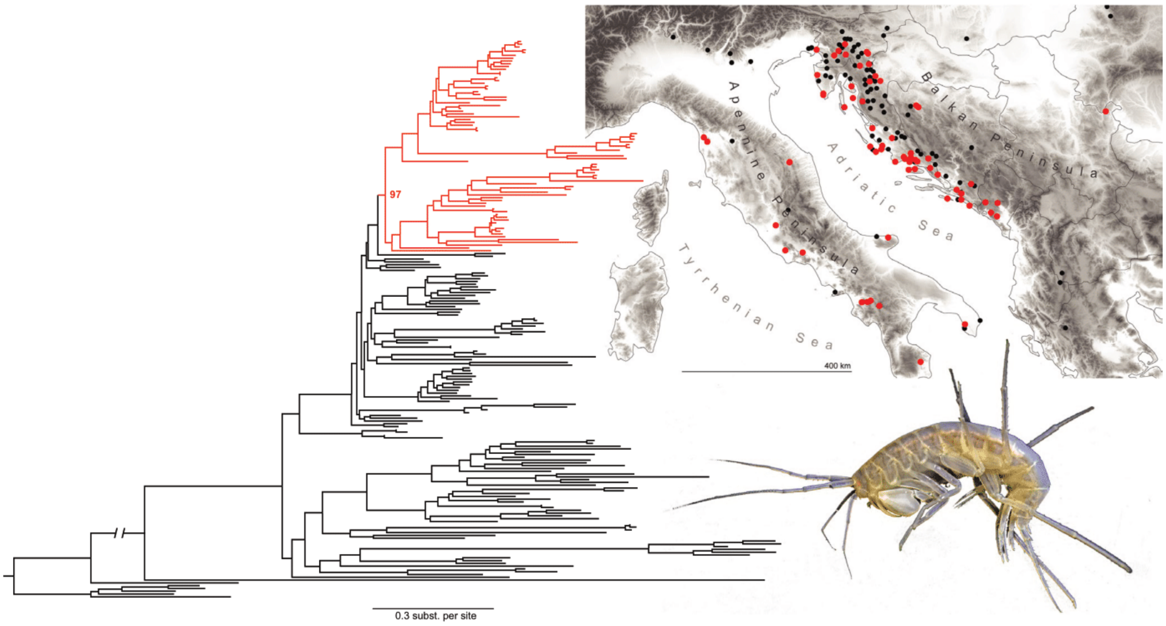

FIGURE 2 Maximum likelihood (IQ-TREE) molecular phylogeny of the genus *Niphargus*, with the representatives of the so-called main transadriatic clade (ultrafast bootstrap support = 97%) indicated in red (left). The tree was built using 28S rRNA I and II, H3 and COI gene sequences. The map on the right shows the distribution of the main Transadriatic clade representatives (red circles) and other *Niphargus* species (black circles) used in the species delimitation analyses. Bottom right: *Niphargus pectencoronatae*, an iconic species known from only a few anchialine caves in the Kornati archipelago of the Adriatic Sea (Photo courtesy of Vedran Jalžić) [Colour figure can be viewed at wileyonlinelibrary.com]

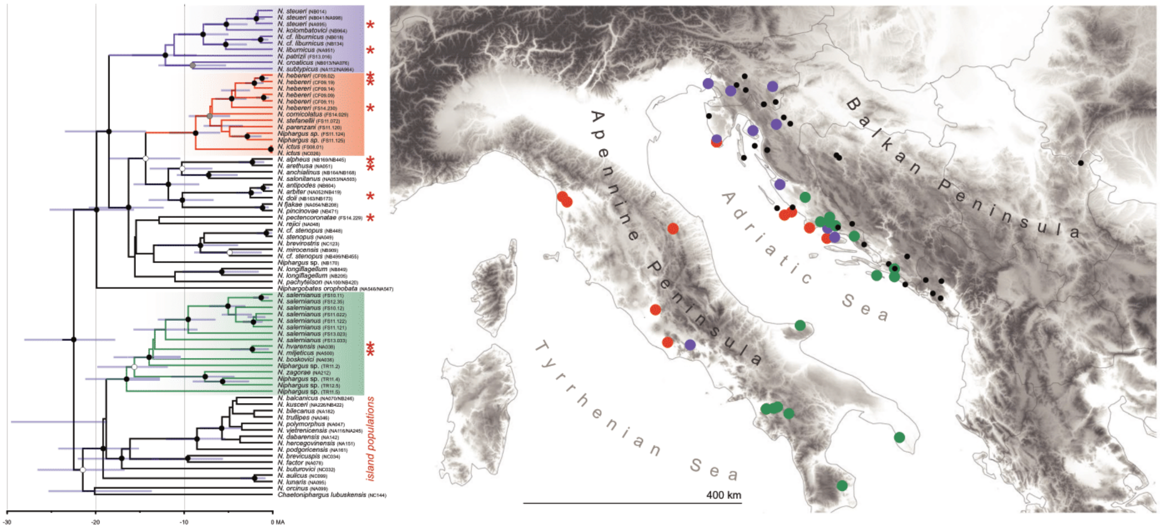

FIGURE 3 Time-calibrated maximum clade credibility tree of the main transadriatic clade derived from BEAST analysis, using 28S rDNA I and II, H3 and COI gene sequences (full phylogeny is available in Supplement Information 6). The three clades nested within the main transadriatic clade that are distributed on the both sides of the Adriatic Sea are indicated in purple (*Niphargus steueri* – *N. patrizii*), orange (*N. hebereri* – *N. ictus*) and green (*N. hvarensis* – *N. salernianus*). Island populations are indicated as red stars. The posterior probabilities of the node supports are denoted as white >0.90, grey >0.95 and black >0.99 circles and the 95% confidence intervals are shown as blue node bars (left). Geographical distribution of the main transadriatic clade representatives, with the three clades distributed on both sides of the Adriatic Sea are indicated with purple (*N. steueri* – *N. patrizii*), orange (*N. hebereri* – *N. ictus*) and green circles (*N. hvarensis* – *N. salernianus*). The rest of the main transadriatic clade MOTUs are labelled with black circles (right) [Colour figure can be viewed at wileyonlinelibrary.com]

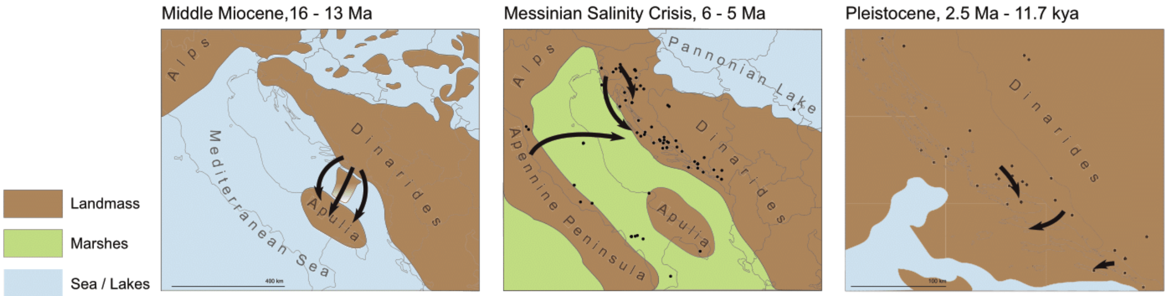

FIGURE 4 Biogeographic reconstruction of the peri-Adriatic region through time; (a) Middle Miocene, (b) Messinian Salinity Crisis and (c) Pleistocene (enlarged view on the Dinarides). The main hypothetical dispersal routes of subterranean faunas, as reconstructed using the R package 'BioGeoBEARS', are indicated as black arrows and the present geography is outlined in background. The reconstructed land-bridges are based on Correggiari et al. (1996), Popov et al. (2004) and Mazza and Rustioni (2008) [Colour figure can be viewed at wileyonlinelibrary.com]

the *N. hvarensis* – *N. salernianus* clade (Figure 3). In addition, several species pairs (e.g. *N. pachytelson* – *N. pectencoronatae*, *N. hvarensis* – *N. miljeticus*, *N. doli* – *N. arbiter*, [*N. arethusa*+*N. alpheus*] – *N. salonitanus*, *N. steueri* islands – *N. steueri* mainland, *N. liburnicus* – *N.* sp.) were distributed in the Dinaric Karst and on the Adriatic Islands (Figures 3 and 5).

3.2 | The age of splits

The estimated ages of the main nodes between the alternative calibration schemes varied ±2 Ma, implying concordance between the calibration points used. Here we report the results using both calibration points, whereas detailed results of cross-validation analyses are available in Supporting Information 6. The age of the common ancestor of the main transadriatic clade was dated to the late Oligocene – early Miocene, with a mean estimate of 23.8 Ma (95% confidence interval 18.7–29.6 Ma), likely falling into the time window of the Dinaric Karst uplift, which started 30 Ma. The splits between the species distributed in the Dinaric Karst, Adriatic Islands and Apennine Peninsula fell into three time windows (Figures 3 and 5).

The earliest splits took place between 10 and 20 Ma, hence predating the Messinian Salinity Crisis. The ancestor of the *N. hebereri* (Dinaric Karst and Adriatic Islands) – *N. ictus* (Apennine Peninsula) clade split from its Dinaric relatives approximately 14.4 Ma (95% confidence interval 10.4–18.5 Ma). Roughly in the same period, the *N. salernianus* clade (Apennine Peninsula) split from the *N. hvarensis* clade (Dinaric Karst) (13.3 Ma, 95% confidence interval 9.1–15.6 Ma). Somewhat younger is the split between the *N. steueri* clade (Dinaric Karst) and *N. patrizii* (Apennine Peninsula), dating to 11 Ma (95% confidence interval 7.2–14.2 Ma, Figure 4).

Later on, and roughly corresponding to the Messinian Salinity Crisis, the *N. hebereri* clade (Adriatic Islands and Dinaric Karst) split from the *N. ictus* clade (Apennine Peninsula) (6.9 Ma, 95% confidence interval 4.4–8.9 Ma, Figure 4).

The time of the most recent series of splits coincided with Pleistocene glaciations. These include splits among cryptic species of the *N. hebereri* complex (Adriatic Islands and Dinaric Karst), and six splits between species distributed on the Adriatic Islands and the Dinaric Karst (Figures 3 and 4).

3.3 | Biogeographical models and their spatial implications

Regardless of the model of ancestral range reconstruction used, the main transadriatic clade originated in the Dinaric Karst with high probability (Figure 5). The biogeographical models which included jump dispersal had higher likelihood than the basic models. Among the basic models, DIVA received the highest support, whereas among the models with jump dispersal (+J), DEC+J received the highest support (Table 1). These two models suggest different biogeographical histories of the main transadriatic clade. According to DIVA, the main process explaining species distribution was range extension, modelled by the parameter *d* (technically termed as dispersal), which was estimated to be an order of magnitude higher than range contraction (technically termed as extinction, parameter *e*) (Table 1). By contrast, the DEC+J model suggested that range evolution was predominantly shaped by jump dispersal, and that founder event speciation was the main driver of the clade's biogeographical history. These two contrasting models were additionally supported by 100 stochastic models. According to this, the DIVA and DEC+J models estimated that dispersal and jump dispersal had the largest contribution to the existing distribution patterns, respectively. The simulations estimated on average 6.33 (DIVA)/6.17 (DEC+J) transitions from the Dinaric Karst to the Adriatic Islands, 3.03 (DIVA)/2.95 (DEC+J) events from the Dinaric Karst to the Apennine Peninsula, 0.51 (DIVA)/0.61 (DEC+J) events from the Apennine Peninsula to the Dinaric Karst and 0.54 (DIVA)/0.57 (DEC+J) events from the Apennine Peninsula to the Adriatic Islands.

4 | DISCUSSION

The results unveil a complex historical biogeography of the main transadriatic clade, reflecting the major palaeogeographical events

TABLE 1 Comparison of the ancestral range estimation models for the main transadriatic clade of *Niphargus*, as estimated in BioGeoBEARS (Matzke, 2013)

Model name	LnL[a]	P[b]	Dispersion	Extinction	Jump dispersal	AIC[c]	AICc[d]
DEC+J	−45.5	3	1.00E−12	1.00E−12	4.47E−02	96.99	97.3
DIVAlike+J	−45.51	3	1.00E−12	1.00E−12	4.47E−02	97.03	97.35
BAYAREAlike+J	−45.51	3	1.00E−07	1.00E−07	4.34E−02	97.03	97.35
DIVAlike	−64.72	2	7.06E−03	2.39E−04	0.00E + 00	133.40	133.6
DEC	−66.00	2	7.06E−03	2.39E−04	0.00E + 00	136.0	136.16
BAYAREAlike	−96.60	2	1.15E−02	2.59E−02	0.00E + 00	197.36	197.36

[a] Log-likelihood.
[b] Number of parameters.
[c] Akaike information criterion.
[d] Akaike information criterion corrected for small sample size.

in the Adriatic basin (Figures 4 and 5). The clade apparently originated on the emerging Dinaric Karst, about 19–29 Ma. Following the emergence of land bridges between the Dinarides and Calabria/Apulia ca. 10–15 Ma (Mazza & Rustioni, 2008), the ancestral representatives of all three transadriatic subclades, namely the *N. hebereri* – *N. ictus* clade, the *N. steueri* – *N. patrizii* clade and the *N. hvarensis* – *N. salernianus* clade, spread across the sea. Subsequent submergence of the land bridge gave rise to *N. patrizii*, the complex of *N. salernianus* and to the ancestor of a clade comprising *N. stefanellii*, *N. ictus*, *N. cornicolanus*, *N. parenzani* and *N. hebereri*. During the Messinian Salinity Crisis (Krijgsman, Hilgen, Raffi, Sierro, & Wilson, 1999), approximately 5.96–5.33 Ma, the ancestor of the *N. hebereri* species clade likely spread from the Apennine Peninsula and colonized the Adriatic Islands as well as the mainland of the Dinaric Karst. Finally, during the Pleistocene (2.5 Ma–11.7 Ka) (Correggiari et al., 1996), at least six lineages colonized the Adriatic Islands from the Dinaric Karst (Figures 3–5). The timing of the marine regressions corresponded to the periods of dispersal. The details on the dispersal remain unclear, given that models incorporating jump dispersal cannot be statistically compared with the models without this parameter (Ree & Sanmartín, 2018). The results of DIVA imply longer periods of dispersal during marine regressions, followed by transgression-driven vicariance. By contrast, jump dispersal models imply that the dispersal took place in narrow time windows during regressions, simultaneously with the cessation of gene flow between ancestral and descending populations (i.e. founder event speciation) (Matzke, 2013, 2014). In spite of these uncertainties, all the presented results favour the hypothesis that marine regression–transgression cycles shaped the biogeographical history of the main transadriatic *Niphargus* clade. By contrast, the results provide no evidence for the hypothesis that the main transadriatic clade originated through a single colonization of the ancient landmasses with no subsequent dispersal across the Adriatic basin. This is because we estimated the age of this clade as older than 10 Ma, and inferred multiple bidirectional dispersal events across the Adriatic basin.

The biogeographical role of marine regression–transgression cycles proposed herein also makes sense from a palaeogeographical perspective. The first colonisations are a logical follow-up of the geological evolution of the wider area in that the origin of the Dinaric Karst preceded the origin of the Apennine Peninsula (Carminati, Lustrino, & Doglioni, 2012; Rosenbaum, Lister, & Duboz, 2002) and that the older landmass was a source of Apennine peninsular faunas.

The robustness of our conclusions depends on the accuracy of the molecular clock. We recognize that the ages of the genus and the focal clade are younger than suggested in previous genus-wide studies (Esmaeili-Rineh, Sari, Delić, Moškrič, & Fišer, 2015; McInerney et al., 2014). However, the calibrations in McInerney et al. (2014) relied on external calibration points that reflected biogeographical splits within the distantly related epigean genus *Gammarus* (Hou et al., 2011). Application of a molecular clock calibrated on epigean and distantly related species is potentially problematic given the longer life span and slower generation turnover of subterranean species (Saclier et al., 2019). For this reason, we calibrated the phylogeny using exclusively internal calibration points and suggest that our scheme more accurately reflects the timing of cladogenetic events.

The question remains how subterranean species, predominantly massive bodied and cave dwelling, crossed such long distances in relatively short temporal periods. Despite the enhanced connectivity of surface freshwaters, subterranean species hardly disperse across them, presumably due to competitive interactions and predation from surface species (Romero, 1985; Tobler, Schlupp, & Plath, 2007). We can think of two, mutually non-exclusive explanations. First, in early phases of marine regressions, the communities in emerging freshwater habitats underwent dramatic changes (Hou, Sket, Fišer, & Li, 2011). In the transitional phase, these habitats were similar to salty marshes (Popov et al., 2004), where both freshwater and marine faunas were depauperate and the competitive and predatory pressures were less severe than in fully assembled communities. Some members of the main transadriatic clade tolerate brackish water (Delić et al., 2017; Gottstein et al., 2012; Sket, 1994) or sulphide-rich waters (Fišer, Luštrik, Sarbu, Flot, & Trontelj, 2015; Flot, Wörheide, & Dattagupta, 2010). Hence, within a relatively narrow time window, the ancestors of the modern clades were possibly competitive enough to spread across species-poor areas, with crustacean communities inhabiting the moderately saline bottom of the Adriatic basin. Second, we cannot rule out subterranean

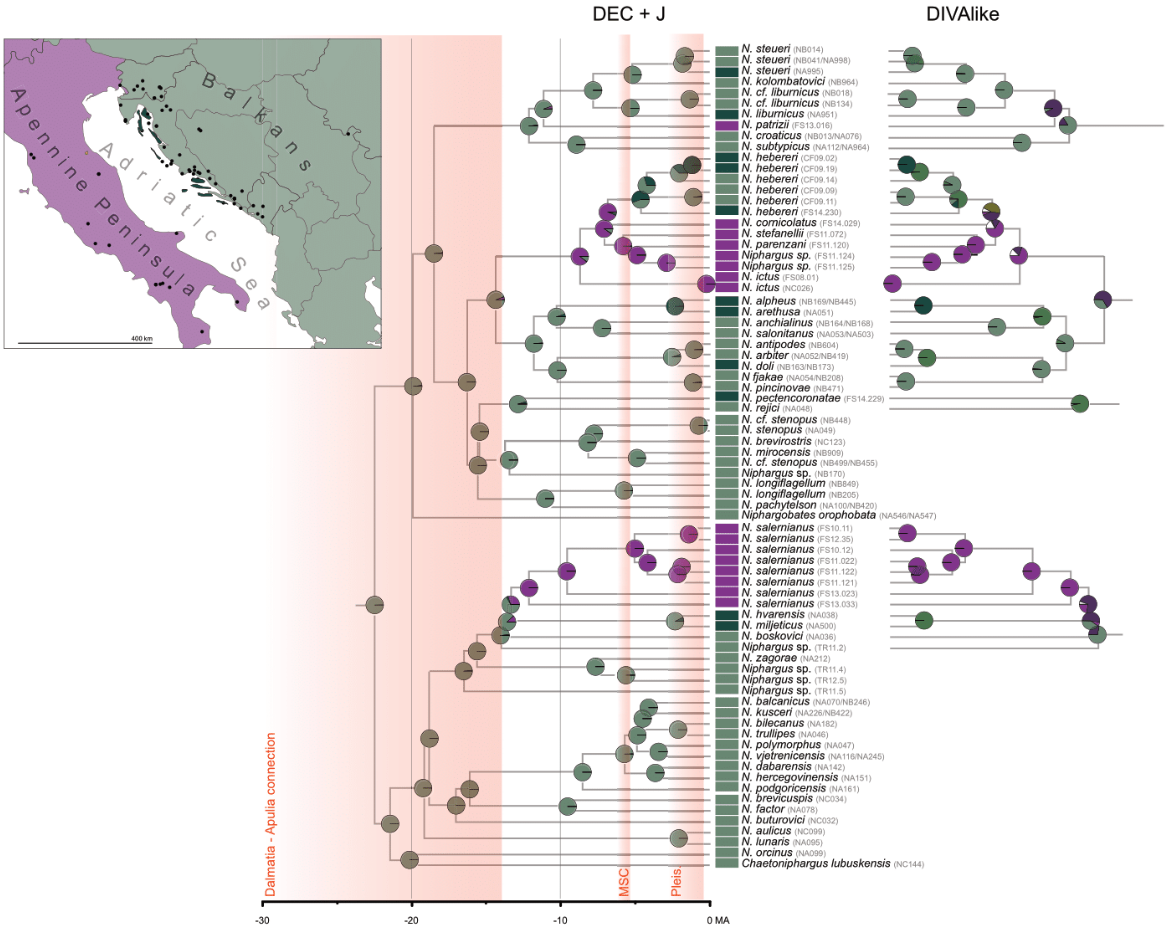

FIGURE 5 Distribution of the main transadriatic clade categorized into three regions, Dinaric Karst (light green), Adriatic Islands (dark green) and Apennine Peninsula (purple), used in the ancestral range reconstructions (left). Ancestral ranges for the main *Niphargus* transadriatic clade were estimated using the DEC+J and DIVAlike models (right). The DIVAlike model shows only sections of ancestral range reconstructions, differing from the DEC+J model. Pie charts indicate relative probabilities of all possible ancestral ranges. Bars in the middle indicate the present distribution of the main transadriatic clade representatives. The periods when the three historical land-bridges between the Dinaric Karst and the Apennine Peninsula existed are labelled as orange bars on the chronogram. The earliest land-bridge emerged in the Oligocene (Mazza & Rustioni, 2008), followed by the Messinian Salinity Crisis (ca. 5.96–5.33 Ma; Garcia-Castellanos & Villaseñor, 2011;Gargani & Rigollet, 2007) and the Pleistocene glacial periods (Correggiari et al., 1996) [Colour figure can be viewed at wileyonlinelibrary.com]

connections across the major parts of the Adriatic basin, either as patches of shallow subterranean habitats (Culver & Pipan, 2014), or as deep subterranean habitats that developed during a marine regression and filled with sediments during a transgression. Such transitional development of freshwater subterranean connections is supposed to exist due to the palaeo-hydrology of Adriatic basin rivers and the evolution of the peri-Mediterranean region during the Neogene (Audra et al., 2007; de Leeuw, Mandic, Krijgsman, Kuiper, & Hrvatović, 2011; Mocochain et al., 2009; Pavelić et al., 2014).

Our study emphasizes the importance of marine regression–transgression cycles in shaping the extant biogeography patterns of subterranean freshwater fauna. In the same area, time-calibrated phylogenetic studies found similar biogeographical patterns among epigean invertebrate and vertebrate taxa (decapods, gastropods and mammals), most of them presumably shaped during the Pleistocene or Messinian Salinity Crisis (Castiglia, Annesi, Amori, Solano, & Aloise, 2017; Jelić et al., 2016; Jesse et al., 2009; Korábek, Juřičková, & Petrusek, 2014). By contrast, only few of those studies inferred biogeographical patterns shaped by the presence of the ancient Middle Miocene land bridge between the central part of the Dinaric Karst and Apulia (Mazza & Rustioni, 2008). This paucity of data for more ancient biogeographical events in the epigean taxa may be due to the region's vivid geological history that wiped out many ancestral species living in the epigean environments.

It has been suggested that subterranean faunas, especially crustaceans, living in stable and physically remote environment, comprise relict and rare species that bear testimony to past faunas (Humphreys, 2000). We agree with this view and suggest that the value of subterranean species in historical biogeography is currently underestimated, and we argue that life in remote environments makes subterranean species an interesting model for the study of past biogeographical events.

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CONFLICT OF INTEREST

The authors declare they do not have any conflict of interest.

DATA AVAILABILITY STATEMENT

All the relevant data have been archived in the Dryad Digital Repository and are reachable at https://doi.org/10.5061/dryad.0k6djh9wp.

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BIOSKETCH

The authors are broadly interested in the biogeography of the Dinaric karst and evolutionary mechanisms underlying phylogeographical and diversification patterns of subterranean animals. They collaborate on questions of subterranean amphipod biodiversity based at SubBio Lab, University of Ljubljana, Slovenia (http://subbio.net/) and Evolutionary Biology & Ecology, Université libre Bruxelles (http://ebe.ulb.ac.be/).

Author contributions: C.F., F.S. and T.D. conceived the ideas, T.D., Š.B, F.S. and J.F.F collected the data, T.D. analysed the data and C.F and T.D led the writing.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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3 DISCUSSION AND CONCLUSIONS

3.1 DISCUSSION

We are living in a time of global biodiversity crisis, where biodiversity loss is occurring at speed and magnitude that is unprecedented in human history. It is therefore of utmost importance to understand the origins and drivers of the various facets of biodiversity, not only for the sake of knowledge, but also to be able to successfully protect them (Barnosky et al., 2011; Myers et al., 2000). Speciation, dispersal, and extinction are key processes that shape biodiversity patterns. Adaptive radiation has been recognised as a major generator of the extant diversity (Stroud and Losos, 2016). We tested this view in subterranean realm – invariant, if compared to variability of surface habitats, yet inhabited by species that exhibit a variety of forms and functions (Culver and Pipan, 2019).

In this dissertation, we explore diversification and diversity patterns of the groundwater amphipod genus *Niphargus* on continental scales, in a global subterranean hotspot, the Dinarides, and within a cave community. The diversification patterns of *Niphargus* indeed are consistent with those expected under the adaptive radiation hypothesis. Sudden independent bursts of speciation and ecomorphological diversification within multiple *Niphargus* clades unfolded in the area of emerging karst massifs (Borko et al., 2021). Nevertheless, *Niphargus* is only one of many groups within the mega radiation of amphipods, where several diversification bursts resulted in more than 10,000 extant species (Copilaş-Ciocianu et al., 2020). On one hand, we will question the generality of adaptive radiations in extreme environments, and on the other hand, we will place the radiation of *Niphargus* in the context of a broader amphipod radiation.

Next, we question ecological speciation, a key process of adaptive radiation, within a *Niphargus* community (Borko et al., 2019). We discuss the challenging and sometimes elusive distinction between adaptive and nonadaptive radiations, especially when analysing individual speciation events.

Finally, we focus on the origins of biodiversity in subterranean biodiversity hotspot, the Dinarides. We compare different biodiversity patterns across the region and discuss the imprints and implications of the evolutionary processes behind them (Borko et al., 2022). We evaluate the contribution of speciation and dispersal to the formation of Dinaric subterranean hotspot. Several adaptive radiations have unfolded in this region. However, even though dispersal is less likely in a fragmented subterranean environment where species generally have small ranges, massive dispersal events did take place (Delić et al., 2020). While the south-eastern part of the region acted as a donor of species, the situation in the north-western part is more complex.

3.1.1 Adaptive radiations in the subterranean environment

Adaptive radiations are a universal phenomenon and an important global generator of biodiversity (Schluter, 2000). Simpson (1953) even claimed that adaptive radiations could explain the entire diversity of life on Earth. Although the importance of adaptive radiations is widely recognised, case studies remain limited and the processes and mechanisms behind them are poorly understood (Gillespie et al., 2020). Despite growing numbers of studies of adaptive radiations in recent years, the generality of the adaptive radiation hypothesis has never been tested in an extreme environment.

The recovered patterns of speciation and ecological and morphological diversification of the genus *Niphargus* were consistent with the adaptive radiation theory (Borko et al., 2021). We have shown for the first time that adaptive radiation can unfold in subterranean environment, traditionally considered as ecologically harsh and highly resource-limited environment (Culver and Pipan, 2014). The genus *Niphargus* underwent massive subterranean speciation and ecomorphological diversification composed of nested patterns of adaptive and nonadaptive radiations (Borko et al., 2021). Although we did not specifically question ecological versus nonecological speciation, it is plausible to expect that *Niphargus* radiation contains both elements.

Our results support the hypothesis of the generality of adaptive radiations (Schluter, 2000), which can apparently unfold even in ecologically simple and resource-limited environments such as the subterranean one, and contrary to the evolutionary dead-end hypothesis (Culver and Pipan, 2019). However, *Niphargus* is only one of many subterranean taxa and for now remains a rare exception of adaptive radiation in subterranean environment, rather than the rule. We suggest that further studies of subterranean radiations in various clades are needed to better understand the origins of subterranean biodiversity. Such potential candidate groups would be aquatic or terrestrial clades with known ecomorphological diversification, such as isopods of genus *Monolistra* (Prevorčnik et al., 2010) or beetles from the family Leiodidae (Njunjić et al., 2018).

Subterranean adaptive radiation seems even less likely if we consider a degree of morphologically cryptic species. Two to three times greater molecular species diversity than expected by nominal species in crustaceans (Eme et al., 2018), and even seven time greater in collembolans (Lukić et al., 2020) suggest massive allopatric speciation within similar habitats. On the other hand, it is possible that ecological differentiation exists within seemingly similar species but has not been recognised as such; the differences could manifest in physiology (Delić et al., 2017a) or life histories (Cieslak et al., 2014). Diversification patterns of the genus *Niphargus* suggest a complex combination of adaptive radiations, dispersal, and nonadaptive radiations (Borko et al., 2021). Further studies are needed to assess the contribution of colonisation, dispersal, and speciation within the subterranean realm.

Not only that subterranean environment can sustain adaptive radiations, it can also protect radiated clades from climatic perturbations at the surface. The extensive adaptive radiations in Europe were mostly wiped out by tectonic changes or climatic perturbations, and most modern European fauna arose through immigration (Hewitt, 2000; Neubauer et al., 2015). In contrast, we have shown that *Niphargus* originated in north-western Europe, dispersed and radiated in the south and east, and survived major extinction events (Borko et al., 2021). Our results suggest that more radiations may be expected and preserved in environments that are insulated from the effects of climate fluctuations but have been so far overlooked due to their apparent simplicity, such as the subterranean realm and deep soil (Von Saltzwedel et al., 2016).

3.1.2 The genus *Niphargus*: adaptive radiation within the amphipod radiation

With more than 10,000 species, amphipods are among the most ecologically diverse and speciose crustaceans inhabiting all aquatic and semiaquatic habitats worldwide (Horton et al., 2021). Analysis of the entire order revealed a major eco-evolutionary radiation that occurred in pulses, with multiple convergent shifts in ecology (Copilaş-Ciocianu et al., 2020).

Amphipods diversified in several pulses that followed the periods of delayed speciation. Although their ancestors originated already in the Permian, the first diversification was delayed until the Jurassic-Cretaceous, followed by a second pulse of speciation and ecological radiation during the Cretaceous-Paleogene (Copilaş-Ciocianu et al., 2020). The pattern of delayed burst of speciation was also observed in *Niphargus* (Borko et al., 2021). These pulses can be associated with environmental conditions and possible key events that triggered diversification, i.e., an ecological opportunity: a newly available abundance of unexploited ecological resources (Schluter, 2000). Although it is impossible to explicitly test the hypothesis of which key event triggered adaptive radiation (Stroud and Losos, 2016), the temporal concurrence of the onset of radiation and the event may indicate possible ecological opportunity.

Amphipods are generally cold-adapted animals with low tolerance to hypoxia. We hypothesise that the first ecological opportunity for amphipods emerged after the Permo-Triassic mass extinction event, and when oxygenation of marine environments was restored (Copilaş-Ciocianu et al., 2020). From the theory of ecological opportunity, we can assume that the extinction emptied the space of predators and paleoclimatic changes made it hospitable again (Schluter, 2000). The second pulse of diversification may have been driven by vicariance after the breakup of Pangea. The last major spike of speciation and ecological diversification in the deep history of amphipods occurred during a period of high sea level, with a variety of shallow marine habitats and highly oxygenated environments. Multiple transitions into freshwaters or deep sea occurred (Copilaş-Ciocianu et al., 2020). Although plausible, our hypotheses are mostly speculative in nature. Deep-time reconstructions are accompanied with multitude of uncertainties, especially when it comes to poorly fossilised groups such as amphipods. Without extensive fossil data and lacking a good share of molecular data from extant groups, detailed reconstructions are impossible.

Within amphipods, many nested radiations likely occurred. Transition to freshwater was recognised as ecological opportunity that promoted diversification in amphipod genus *Gammaraus* (Hou et al., 2011). Multiple colonisations and convergent adaptations were shown for deep sea lysianassoid amphipods (Corrigan et al., 2014). Occurrence of gelatinous plankton and adaptation to symbiotic lifestyle in hyperiid amphipods resulted in one mega-radiation and several smaller radiations (Copilaş-Ciocianu et al., 2020). Multiple radiations unfolded also in ancient lakes of temperate climates. The amphipods in Lake Baikal appear to be among the largest adaptive radiations of extant amphipods, although their studies were mainly descriptive and adaptive radiation was never explicitly tested (Macdonald et al., 2005; Naumenko et al., 2017; Sherbakov, 1999). A recent study suggests that the Pontocaspian amphipods, with more than 80 species that have convergently evolved into at least four ecomorphs, may meet adaptive radiation criteria (Copilaş-Ciocianu and Sidorov, 2022). Less studied are smaller potential radiations in Lake Titicaca, Lake Ohrid, and lake Fuxian (Copilaş-Ciocianu and Sidorov, 2022; Jaume et al., 2021). *Niphargus* can thus be viewed as a case of nested radiation, albeit the largest one among freshwater amphipods, generating 20–25% of all freshwater and 5 % of all amphipods in the world (Horton et al., 2021; Väinölä et al., 2008). Interestingly, *Niphargus* radiation unfolded in a very different environment than ancient lakes, although some parallels can be drawn, such as diversity of habitats and geographic size of the area in which these radiations unfolded.

Niphargus ancestors were shallow-water marine amphipods (Copilaş-Ciocianu et al., 2020) in western Europe in the early Eocene (Borko et al., 2021), with slow pace of speciation for the first 20 to 30 million years. During this time, the genus dispersed through interstitial, probably in coastal or brackish waters. We hypothesise that the genus accumulated genetic variation during this time that supported subsequent rapid diversification (Seehausen, 2015). The timing of the diversification burst in *Niphargus* coincides with the uplift of karst massifs in south-eastern Europe, subsequent karstification and favourable hydrogeological conditions (e.g. Miocene Dinaric lakes (de Leeuw et al., 2012)). The South-Eastern Alps, Dinarides, and Carpathians uplifted in south-eastern Europe as islands in the Parathetys. Exposure of the carbonate rocks to atmospheric processes triggered karstification and consequently the formation of new, diverse subterranean habitats (Culver and Pipan, 2019). The vast new freshwater environments, initially free of predators and competitors, likely constituted an ecological opportunity for the *Niphargus* ancestors. Early habitat diversification from interstitial to cave lakes and streams and to unsaturated fissure system and shallow subterranean begun, followed by within-habitat diversification in several clades in the area. We developed a plausible explanation for the ecological opportunity, however the intrinsic factors of *Niphargus* that promote diversification were not explored yet (but see Fišer et al., 2008a). The next interesting question would be to investigate the evolvability and possible drivers, such as hybridization and phenotypic and behavioural plasticity, of *Niphargus* and comparably among subclades (Stroud and Losos, 2016).

Although the molecular and morphological data of *Niphargus* are well resolved, they are still limited. We acknowledge that we were not able to directly evaluate the contribution of extinctions to the recovered diversification patterns. However, we assume that extinctions were negligible and randomly distributed under assumption that subterranean environment acts as a refugium from major catastrophic disturbances on the surface (Borko et al., 2021). Second, many areas are still under sampled and many species have not yet been discovered. It is possible that other emerging karst areas have supported similar events, but we have not detected them – recent discoveries hint that the Zagros or Caucausus mountain ranges may be possible candidate areas (Esmaeili-Rineh et al., 2015; Rendoš et al., 2021).

In exceptional settings, large adaptive radiations can be the sum of independent radiations occurring in closely related lineages. As noted above, *Niphargus* is likely just one of many amphipod radiations. Furthermore, this pattern of nested radiations is present also within *Niphargus*. The genus consists of several large monophyletic and geographically well-defined clades. All six major clades displayed an early-burst speciation, but without increased habitat diversification, that unfolded earlier in the evolutionary history of the genus. Four clades exhibited significantly high morphological disparification, namely the West Balkan, North Dinaric, Pontic, and Pannonian clades. The South Dinaric clade was not significant, but the high number of morphotypes and newly obtained data suggests adaptive radiation. Finally, the youngest Apennine clade has only few morphotypes. We hypothesise that this clade could be a predominantly nonadaptive radiation that onset after the final formation of the Apennine Peninsula 10 million years ago (Popov et al., 2004), with morphologically similar species living predominantly in one habitat type. We hypothesise that the diversification may have been driven by allopatric speciation in a highly fragmented karst environment. Since one lineage from the Apennine clade dispersed also into the Dinarides, this is consistent with nonadaptive radiation hypothesis of intermediate dispersers (Agnarsson et al., 2014). However, due to the lack of morphological and distributional data, we have not been able to reliably estimate the course of diversification of this clade (Borko et al., 2021).

We found that these clade-level radiations show evidence of between and within clade convergent evolution. Although showing some degree of convergence, the radiations overall adapted to distinct sets of adaptive optima. We hypothesise that the early habitat diversification detected in the tree-wide analysis may have constrained further clade-level morphological diversification, which mostly unfolded within one or a few habitat types, such as cave lakes (West Balkan and South Dinaric clade), cave streams (North Dinaric), or interstitial groundwater (Pontic and Pannonian clade). We may expect that diversification analysis of other traits, such as gnathopods that are associated with trophic ecology, would reveal additional level of diversification within habitats (Premate et al., 2021). These within-and between-habitat diversifications prompted high levels of sympatry, resulting in uniquely species-rich communities, counting up to nine *Niphargus* species per site (Fišer et al., 2019; Trontelj et al., 2012). Alternative constraining force could be a competition among *Niphargus* lineages. It is possible that radiations have evolved within limited habitat types because others were already occupied. One such example is competing cichlid genera in Lake Mweru that do not overlap in morphospace (Meier et al., 2019).

We have shown that parallel and partially convergent adaptive radiations have unfolded within several *Niphargus* clades. Available information from the under sampled clades in Greece and Iran, or a lineage in the Iberian Peninsula morphologically described as separate genus *Haploginglymus* suggests that there may be more radiation events (Borko et al., 2019; Esmaeili-Rineh et al., 2015; Jurado-Rivera, Álvarez, et al., 2017).

3.1.3 Distinguishing between ecological and nonecological speciation

Although overall macroevolutionary patterns of the group imply adaptive nature of radiation, the distinction between adaptive and nonadaptive processes becomes difficult on the level of individual speciation events. Not only that many clades contain elements of adaptive and nonadaptive radiations, ecological and nonecological speciation cannot be separated from each other in many cases (Rundell and Price, 2009). For example, species can first speciate in allopatry, then diverge ecologically, and only later come into sympatry (Zakšek et al., 2019). The resulting pattern of ecomorphologically distinct species living in sympatry would imply ecological speciation.

At the level of population genetics, one must thoroughly analyse the evolution of lineages on a case-by-case basis to gain insight into the underlying processes. Studied individual speciation events show complex scenarios. For example, within a West Balkan clade one lineage evolved in a series of habitat fragmentations and parapatric ecological divergence (Delić et al., 2017a), while another lineage exhibited allopatric speciation due to dispersal, ecomorphological differentiation in allopatry, and only later post-speciation dispersal that resulted in sympatry (Zakšek et al., 2019).

We studied a *Niphargus* community in a Greek cave that at a first glance fits the adaptive radiation hypothesis. We have shown that three closely related and morphologically highly dissimilar sympatric species occupy different aquatic habitats within a cave – small pores in the lake bottom, phreatic waters of freshwater lake and phreatic waters of a sulphidic lake. Phylogenetic clustering and ecological divergence indicate ecological speciation of the species triplet within one cave. Moreover, morphological adaptation to sulphidic water is a showcase of convergent evolution, that evolved at least four times in *Niphargus* (Borko et al., 2019). However, similarly as in studies on dytiscid beetles (Vergnon et al., 2013) we did not specifically test whether reproductive isolation evolved due to ecological differentiation and thus cannot rule out an alternative hypothesis. In the alternative scenario, a speciation and ecomorphological differentiation happened in allopatry, followed by secondary contact. This view is supported by the fact that two of the studied species have a wide distribution outside the studied cave, although the occurrences were mostly not confirmed by molecular methods.

To answer this question, one would first need a reliable distribution and molecular data to infer a detailed phylogeography and reconstruct past dispersal. Inadequate sampling is a common problem in subterranean biology (Culver and Pipan, 2019). Second issue is a well-resolved molecular phylogeny, a key tool for successful disentangling of evolutionary history. Relationships in times of abrupt speciation often remain unclear, as is the case in *Niphargus*. While the overall pattern inferred from the available data is unlikely to be challenged, higher resolution of individual splitting events would overcome the gap between macro- and microevolutionary processes in *Niphargus*. The use of next-generation sequencing technologies, such as ddRADseq (Parker et al., 2022), is therefore a plausible next step in *Niphargus* studies.

3.1.4 Processes that have shaped a subterranean biodiversity hotspot

The Dinarides are a global subterranean biodiversity hotspot, a region with the highest diversity of groundwater amphipods and subterranean fauna in general (Zagmajster et al., 2014). Most of the species found here are endemic to the region. 123 nominal species of *Niphargus* have been recorded in the Dinarides (Horton et al., 2021). We were able to obtain molecular data for 79% of them. However, the exceptionally high number of new, previously unrecognised Molecular Operational Taxonomic Units (MOTUs) suggests that the number of species is still underestimated and in reality 2 to 2.6 times higher than acknowledged (Borko et al., 2022). A somewhat surprising result of the massive sequencing was 148 new MOTUs that do not correspond to any of nominal species and were not recognised in previous studies. Despite the lack of reliable ecological and morphometric data for most of this vast, previously hidden diversity, we were able to disentangle main processes that have shaped the Niphargus' diversity in the region.

The species found in this area belong to several clades. The central and south-eastern Dinarides are mainly occupied by the West Balkan, South Dinaric, North Dinaric and Apennine clade. The Aegean clade is only marginally present in southeast and a clade of broadly distributed shallow subterranean species is present in the eastern alluvial planes (*N. sphagnicolus* lineage), but they do not spatially overlap with Dinaric clades. In the north-western Dinarides, two of the Dinaric clades occur, the West Balkan and the North Dinaric clade. The first is distributed only within the inner boundary of the Dinarides, while the second penetrates into the South-Eastern Alps. Additionally, also species of the Pannonian and Pontic clades are present in this region. Another four lineages with five or more species occur in the north-western Dinarides: shallow subterranean or interstitial species from lineages *N. sphagnicolus*, *N. julius* – *N. kenki* and *N. spinulifemur*, and predominantly stream species from lineage *N. stygius s. str*. Lastly, 18 species from various non-Dinaric clades or with unclear phylogenetic position occur here (Borko et al., 2022).

The distribution and phylogenetic relations of species along the Dinarides suggest that speciation and dispersal contributed differently in different parts of the region, with predominant local diversification within the south-eastern Dinarides and a combination of dispersal and local diversification in the north-western Dinarides and surrounding regions. However, more work will be needed to reliably evaluate the contribution of adaptive and nonadaptive speciation events within the region, mostly due to lack of ecomorphological data for newly discovered MOTUs. The second insight is that Dinaric lineages importantly contributed to the niphargid fauna of neighbouring regions.

In the south-eastern Dinarides local fauna derives predominantly from three Dinaric clades, whereas other clades are lacking and occupy only boundary regions. The ancestors of the West Balkan, South Dinaric and North Dinaric clades have diversified entirely within the region (Borko et al., 2021), to a high number of 143 MOTUs, which is probably the largest *Niphargus* radiation within areas of similar size. The overall diversification patterns of three clades are consistent with the adaptive radiation hypothesis (Borko et al. 2021), but more data is needed be able to reliably reconstruct the diversification course of each clade.

Three Dinaric clades were an important source of niphargid fauna of the Apennine Peninsula. Dispersal from the Dinarides to the Apennine Peninsula took place along land bridges that emerged during marine regressions: during the connection between the Dinaric Karst and Apulia 10–15 million years ago, during the Messinian salinity crisis, and most recent during the Pleistocene glaciations (Delić et al., 2020). Conversely, only one, the ancestral lineage of *N. hebereri,* dispersed back into the central Dinarides during the Messinian salinity crisis and diversified in the coastal area (Delić et al., 2020). No other immigrant lineage is present in the central Dinarides, despite the several dispersal routes to Dinarides. One possible reason is a priority effect – an impact that a first arrival species can have on the development of a community. The Apulia is younger than the Dinarides and was colonised from the Dinarides. Therefore, Apulia is unlikely to be a donor of older lineages. Even if younger lineages dispersed into Dinarides, the competition with ecologically similar species prevented their speciation.

The phylogeographical history of the Apennine clade is less clear. The Apennine clade is nested within major *Niphargus* radiation, but its relation to other Dinaric clades is not resolved. The basal split of the clade divides it into smaller south Dinaric subclade and larger Apennine subclade. Given the paleogeographic data it seems plausible that ancestors of Apennine subclade migrated into Apennine Peninsula and massively diversified there, now occupying the entire peninsula. If the ancestor was indeed from the Dinarides, the question arise why south Dinaric subclade is much less speciose and narrowly distributed, compared to other equally old Dinaric clades. More molecular and distributional data is needed to answer these questions.

Dispersal from the Dinarides also occurred on the other side of the mountain ridge, into the Pannonian alluvial plains and toward the Carpathian foothills, as in the case of *N. mirocensis* from the West Balkan clade (Petkovič et al., 2015). We expect that comprehensive sampling could reveal more such cases of dispersal from the Dinarides towards the east, and possibly also in completely understudied south (Albania).

To sum up, the south-eastern Dinarides acted as a donor region from where species dispersed and occupied surrounding regions that later emerged from the sea. The existing Dinaric clades, which occupied all subterranean aquatic habitats, probably blocked the immigration of foreign lineages into this area. Consequently, the south-eastern Dinarides have relatively low phylogenetic diversity, despite their high species richness.

The evolutionary history of *Niphargus* was different in the north-western Dinarides, especially at the junction with the South-Eastern Alps and the Pannonian lowlands. Here, on the intersection of karst areas and alluvial plains, the *Niphargus* species richness peaks and representatives of most of *Niphargus* phylogenetic lineages occur. Unrelated clades that diverged from each other already in the Eocene spatially overlap (Borko et al., 2022). The area was occasionally submerged (Kováč et al., 2018). Aquatic connections between the Adriatic basin and the Paratethys Sea probably opened migration routes, resulting in congregation of species from several phylogenetically and geographically independent lineages. The established sympatries lead to high phylogenetic diversity and species richness, the highest in the region and overall.

This north-western hotspot is thus the result of a combination of dispersal from outside the Dinarides and diversification within the region. The north-western Dinarides acted as recipient area for various unrelated *Niphargus* lineages, that consequently diversified here, mostly within one habitat type. Such case are lineages from Pannonian and Pontic clades, that migrated in the north-western Dinarides. While Pannonian species only marginally penetrate into the Dinarides and are more common in the border regions of the South-Eastern Alps and the Pannonian lowlands, a subclade of 10 species from the Pontic clade has apparently diversified in the north-western Dinarides, in a specific habitat (fissure system) (Borko et al., 2022). We hypothesise that they prevented diversification of species from Dinaric clades in this particular habitat, where only few species occur (a case of competition driven constrain of adaptive radiation (Meier et al., 2019)). This would be an interesting study of colliding adaptive radiations and among clade competition.

As in the south-east, we noted some dispersal from the north-western Dinarides into neighbouring regions. A *N. rhenorhodanensis* lineage of six species in western Italy according to latest phylogenetic reconstructions belongs to the West Balkan clade. While

500 km away from closest relatives, they occur within relatively small area with radius around 50 km (Borko et al., 2022). More data is needed to confirm the lineage's phylogenetic position and to get insight into its phylogeography.

Given the contrasting differences between the patterns of species richness and phylogenetic diversity of *Niphargus* in the Dinarides and in general small distributional ranges (Bregović et al., 2019; Trontelj et al., 2009), speciation can be considered a universal generator of species richness patterns along the entire Dinarides (Borko et al., 2022). Nevertheless, dispersal played an important role in the formation of this hotspot. The dispersal acted mostly in the north-west, while in south-east the Dinarides were the donor area for surrounding regions (Borko et al., 2022). We acknowledge that the aforementioned inability to evaluate extinctions presents a potential source of misinterpretation of phylogenetic diversity patterns. However, the most recent driver of extinctions in Europe were Pleistocene glaciations, and the Balkan Peninsula was recognised as a southern refugium (Hewitt, 2000). Additionally, Alpine glaciers in the northwest of the study area prompted *Niphargus* speciation (Delić et al., 2022). Therefore, we consider dispersal as a more likely cause of high phylogenetic diversity in northwest.

3.2 CONCLUSIONS

We have comprehensively analysed the patterns of speciation and ecomorphological diversification of the genus *Niphargus* at the continental scale, within the subterranean hotspot, and within a community. This is the first in-depth analysis of *Niphargus* diversification and the first study of adaptive radiation in the subterranean realm.

The conclusions from our work can be summarised as follows:

1. Amphipods evolved in several diversification pulses that correspond to various ecological opportunities. A pattern of nested radiations is present within amphipods, *Niphargus* being one of them.

2. Tempo and mode of *Niphargus* speciation and ecomorphological diversification are consistent with the adaptive radiation hypothesis. Thus, adaptive radiation is indeed a universal phenomenon that also occurs in subterranean environment.

3. Several independent radiations unfolded within *Niphargus*. At least five *Niphargus* clades adaptively radiated, and one clade shows evidence of nonadaptive radiation. Although showing some degree of convergence, the clades adapted to distinct sets of adaptive optima.

4. The timing of increased diversification corroborate with the uplift of karst massifs in South-Eastern Europe and the subsequent formation of new subterranean habitats, representing a plausible ecological opportunity.

5. Although general patterns support the adaptive radiation hypothesis, explicit tests for ecological speciation are lacking. A community in a Greek cave is a possible case of ecological speciation of *Niphargus*, but more detailed analyses are needed.

6. The diversity of *Niphargus* within the Dinarides is 2 to 2.6 times higher than the number of nominal species indicates. Speciation and dispersal contributed differently in different parts of the Dinarides.

7. The diversity of the south-eastern Dinarides emerged mostly through local speciation. Southeastern Dinarides are a donor area, with repeated dispersal from the region, while dispersal in the region is limited.

8. In the north-western Dinarides, the high diversity is due to a combination of dispersal and local diversification.

9. More studies are needed for understanding the contribution of adaptive and nonadaptive speciation events within the Dinarides.

4 SUMMARY

4.1 SUMMARY

Understanding the origins of the various facies of biodiversity is a primary goal of evolutionary biology and macroecology. The current patterns of biodiversity have emerged through an interplay of speciation, dispersal, and extinction. Biodiversity hotspots form through high diversification, low extinction, high immigration, or a combination of these processes. It has been proposed that most of the extant diversity in many major clades around the globe emerged through adaptive radiations: a sudden burst of speciation accompanied by extensive ecological diversification.

In adaptive radiation, ecology-based divergent selection among populations leads to cessation of gene flow and, consequently, ecological speciation. The classic definition of adaptive radiation includes four criteria: common ancestry of species, phenotype-environment correlation, trait utility, and rapid speciation. An external prerequisite for adaptive radiation to unfold is an ecological opportunity, "a wealth of unexploited ecological resources," such as the extinction of a dominant group, the emergence of new resources, or the colonisation of a new area. Large adaptive radiations within discrete, replicated habitats, such as on islands or in lakes, often comprise of multiple parallel radiations that result in similar suites of ecomorphs. Famous examples of adaptive radiation include anole lizards in the Greater Antilles, Darwin's finches in Galapagos, or cichlids in African Great Lakes. Less attention has been paid to continental adaptive radiations, or radiations of invertebrates.

A counterpoint to adaptive radiation, nonadaptive radiation is a lineage diversification with minimal ecological differentiation that results in related and ecologically similar species. Most often, the speciation would be allopatric or parapatric, where gene flow reduces due to geographic isolation, sometimes fortified by intensified genetic drift in smaller populations. The process is affected by the similarity of environments of the diverging populations. Nonadaptive radiations often involve organisms with limited dispersal abilities or those living in highly subdivided environments with similar habitats and conditions on either side of barriers.

Due to the complete absence of light and thus photosynthetic production, subterranean ecosystems are among the most resource-limited environments on Earth. The subterranean fauna has long been considered an evolutionary dead end. Studies over the past two decades have shown that evolutionary processes continue after the colonisation of the subterranean environment, including speciation within it. A considerable number of subterranean species derived from subterranean ancestors. Some species retained similar morphology due to similar environmental conditions, and many species have further diversified morphologically and ecologically. In subterranean amphipod genus *Niphargus*, a highly diversified clades on one hand, and eco-morphologically similar or even cryptic clades on the other hand, suggest that this clade evolved through a combination of nonadaptive and adaptive processes. To date, no comprehensive analysis of speciation and ecomorphological diversification of the exclusively subterranean group has been made.

The aim of this dissertation was to comprehensively analyse the speciation and ecomorphological diversification of the genus *Niphargus* at the continental scale, within subterranean biodiversity hotspot, and within a community. The work was divided into five chapters. First, we analysed the diversification patterns of the order Amphipoda and provided the evidence for monophyly of *Niphargus*. Second, we explored whether the tempo and mode of *Niphargus* diversification patterns corroborate with adaptive radiation hypothesis. We tested whether shifts in speciation and diversification are spatially and temporally consistent with geological and paleogeographic events that may have grounded the ecological opportunity. Third, with aim to analyse a possible case of ecological speciation within *Niphargus*, we studied a community within a Melissotrypa Cave in Greece. Fourth, we aimed to investigate the extent to which speciation and dispersal from neighbouring regions contributed to the overall biodiversity of *Niphargus* in the Dinarides. Finally, we evaluated the Dinarides as a donor region of species, especially for the neighbouring Apennine Peninsula.

Amphipods are among the most speciose crustacean orders. Despite their global distribution and key role in aquatic environments, little is known about their phylogenetic relationships and diversification patterns. In the first chapter, we inferred a large-scale, time-calibrated multilocus phylogeny and reconstructed evolutionary patterns for major ecological traits. We showed that amphipods originate in the Late Palaeozoic, but their speciation and ecological diversification exploded only in the Late Mesozoic, after a protracted stasis in marine littoral habitats. Multiple independent post-Jurassic radiations occurred in deep-sea, freshwater, terrestrial, pelagic, and symbiotic environments. We identified deep-sea faunal extinctions, significant climatic cooling, tectonic reconfiguration, continental flooding, and increased oceanic oxygenation as potential ecological opportunities. *Niphargus* is a monophyletic lineage, sister to another subterranean clade comprised of *Pseudoniphargus* and *Microniphargus*.

Multiple potential adaptive radiations are nested within amphipods. One of these is the subterranean genus *Niphargus*, which accounts for 20-25 % of all amphipod species. In the second chapter, we inferred calibrated multilocus phylogeny of *Niphargus* and modelled the lineage diversification and the evolution of morphological and ecological traits. We have shown that the diversification of the genus *Niphargus* is a case of adaptive radiation, consisting of multiple subordinate adaptive radiations. Their spatio-temporal origin coincides with the uplift of carbonate massifs in south-eastern Europe 15 million years ago. The emerging subterranean environments likely provided an unoccupied, predator-free space that represented an ecological opportunity, a key trigger for adaptive radiation. Although dark and food-deprived, subterranean environment supported adaptive radiation and preserved it through major climatic perturbances on the surface. Our results support the hypothesis of the generality of adaptive radiations that can indeed unfold even in ecologically simple and resource-limited environments such as the subterranean. However, *Niphargus* is only one of many subterranean taxa and for now remains a rare exception of adaptive radiation in the subterranean realm. We suggest that further studies of subterranean radiations in various clades are needed to better understand the origins of subterranean biodiversity.

Although the macroevolutionary patterns suggest adaptive radiation in *Niphargus*, distinguishing between ecological and nonecological speciation can be challenging. In the third chapter, we characterized the amphipod community of Melissotrypa Cave (Greece) taxonomically, ecologically, and phylogenetically. We found three *Niphargus* species and one *Bogidiella* sp. The three *Niphargus* species form a well-supported monophylum, but differ in their ecology and morphology: *N. jovanovici* is a small and slender species inhabiting small freshwater voids, *N. lindbergi* is a large and stout species living in freshwater lakes, whereas *N. gammariformis* sp. nov. is a small and stout species found predominantly in a sulphidic lake. The first two are broadly distributed, although their occurrences are mostly not supported by molecular data. Third species is a single-site that shares convergent morphology with unrelated species from different sulphidic caves. Available evidence suggests that diversification was likely driven by ecological differentiation. However, more data is needed to rule out the alternative hypothesis that species evolved in allopatry and only later came into secondary contact. To answer this question, one would need reliable distributional and molecular data to infer detailed phylogeography and reconstruct past dispersal.

In the fourth chapter we focused on processes that shaped a subterranean biodiversity hotspot. We studied the biodiversity patterns of *Niphargus* in the Dinarides, using two biodiversity metrics, species richness and phylogenetic diversity, with a grid-based approach. To account for high cryptic diversity, we replaced nominal species with Molecular Operational Taxonomic Units (MOTUs) identified in unilocus delimitations. We built a time-calibrated multilocus phylogeny of 512 *Niphargus* MOTUs from within and outside the study area, and calculated Faith's phylogenetic diversity, standardized effect sizes of phylogenetic diversity, and residual of phylogenetic diversity regressed on species richness. Within the study area, we recognized 245 MOTUs belonging to different *Niphargus* clades. Species richness is highest in a north-western hotspot, although some species-rich cells were also detected in the southeast. High phylogenetic diversity coincides with high species richness in the northwest, while in the southeast it is lower than expected. This difference and the detailed analysis of clades distribution suggest that the north-west and the south-east hotspots emerged through different processes: by a combination of dispersal and speciation in the northwest, and by local radiation in the southeast, respectively. The south-eastern Dinarides can therefore be considered as a donor area of species for the surrounding regions, while the situation in the north-west, at the junction with the South-Eastern Alps and the

Pannonian lowlands, is more complex, with many immigrant species from various non-Dinaric clades, some of which diversified within specific habitats.

In the last chapter, we tested whether the distribution patterns support the hypothesis of repeated dispersal and vicariance between the Dinarides and the Apennine Peninsula caused by marine regression–transgression cycles. We collected data for most of *Niphargus* lineages, focusing on species living on both sides of the Adriatic Sea. We applied unilocus delimitations and calculated a time-calibrated multilocus phylogeny, which we used for modelling dispersal, vicariance, extinction, cladogenesis and ancestral ranges. We compared the time frame of events with the main regression–transgression events in the Miocene and Pleistocene. A clade containing South Dinaric and West Balkan clades is distributed on both sides of the Adriatic Sea. The reconstructions of the ancestral ranges suggest that the clade originated in the Dinarides, dispersed three times independently to the Apennine Peninsula and once back to the Dinarides. Adriatic Islands were colonized multiple times, mostly from the Dinarides. The dispersal–vicariance events correspond to historical regression–transgression cycles in the Miocene and Pleistocene, namely a land bridge between the Dinarides and the newly emerged Apulia, a regression during the Messinian salinity crisis, and most recent during the Pleistocene glaciations. The Dinarides indeed acted as a donor region for surrounding land masses that later emerged from the sea, while dispersal back into the area was limited, probably due to the priority effect.

We comprehensively analysed the patterns of speciation and ecomorphological diversification of the genus *Niphargus* at the continental scale, within the subterranean hotspot, and within a community. This is the first in-depth analysis of amphipod and *Niphargus* diversification and the first study of adaptive radiation in the subterranean realm overall. We were unable to distinguish between adaptive and nonadaptive radiations in the Dinarides due to unexpectedly high number of new MOTUs without reliable ecological and morphological data. Nevertheless, we provided a solid phylogenetic and methodological framework that will enable to further explore processes that shaped the evolutionary history of *Niphargus*, as well as other subterranean groups.

4.2 POVZETEK

Biotsko pestrost lahko merimo na več načinov: kot vrstno, fenotipsko ali filogenetsko pestrost (Devictor in sod., 2010). Razumevanje izvora biotske pestrosti je prvotni cilj evolucijske biologije in makroekologije. Današnji vzorci biotske pestrosti so nastali skozi preplet speciacije, disperzije in izumrtja (Davies in sod., 2007).

Glavni mehanizem nastanka biodiverzitete je speciacija, t. j. evolucija novih vrst iz skupnega prednika. Lahko je ekološka ali neekološka. Pri ekološki speciaciji se reproduktivna izolacija razvije s prilagoditvijo na različna okolja ali okoljske niše. Pri neekološki speciaciji pa se razvije skozi fiksacijo različnih koristnih mutacij v populacijah pod podobnim selekcijskim pritiskom (Rundell in Price, 2009; Schluter, 2009). Speciacijo običajno spremlja fenotipska diverzifikacija, ki je lahko naključna, ali vzročno povezana s speciacijo. Pri ekološki speciaciji je fenotipska diverzifikacija povezana z izkoriščanjem različnih ekoloških niš. Morfološka pestrost v tem primeru sledi ekološki pestrosti (Michaud in sod., 2018; Schluter, 2000).

Disperzija je proces premikanja s katerim vrste spreminjajo svoje areale. Vpliva na pretok genov, populacijsko dinamiko, kompeticijo in razširjenost vrste. Čeprav je pomembna, jo dostikrat slabo razumemo (Duarte in Mali, 2018). Razumevanje disperzije olajša filogenetska pestrost, mera pestrosti, ki vključuje sorodstvene odnose med vrstami (Faith, 1992). Vrstno pestra območja z nizko filogenetsko pestrostjo lahko nastanejo zaradi hitre nedavne speciacije in majhne disperzije. Nasprotno pa visoka disperzija ali dolga obdobja enakomerne speciacije vodijo v visoko vrstno in filogenetsko pestrost. Izumiranje povečuje filogenetsko pestrost, a ga je dostikrat težko rekonstruirati (Davies in sod., 2007).

Vroče točke biotske pestrosti nastanejo zaradi povečane speciacije, majhne stopnje izumiranja, obsežnega priseljevanja ali kombinacijo naštetega (Wiens in Donoghue, 2004). Večina današnje biotske pestrosti naj bi nastala v povečanih pulzih speciacije, t. i. evolucijskih radiacijah (Stroud in Losos, 2016). To tezo smo testirali v nepričakovanem okolju: ekološko preprostem in s hranili osiromašenem podzemlju.

Speciacija pri različnih skupinah poteka različno hitro. Lahko je počasna ali hitra, zvezna ali nenadna. Tempo speciacije se lahko skozi čas spreminja. Hitro vznikanje novih vrst iz skupnega prednika imenujemo evolucijska radiacija. Če pri tem pride do diferenciacije ekoloških niš, govorimo o adaptivni radiaciji. Pri neadaptivni radiaciji do te diferenciacije ne pride. Kladi lahko vsebujejo elemente obeh procesov, adaptivne in neadaptivne diferenciacije. Razmejitev je nejasna, ekološko diferenciacijo je tudi pogosto težko opredeliti (Czekanski-Moir in Rundell, 2019; Gittenberger, 1991; Rundell in Price, 2009; Schluter, 2009).

Adaptivna radiacija je hitro vznikanje novih ekološko diferenciranih vrst iz skupnega prednika (Rundell in Price, 2009; Schluter, 2000). Klasična definicija adaptivne radiacije vključuje štiri pogoje: skupnega prednika, povezavo med fenotipom in okoljem, funkcionalnost znaka in hitro speciacijo (Schluter, 2009). Model z ekoevolucijskimi procesi, ki delujejo na lokalni ravni (lokalna adaptacija, medvrstne interakcije), pojasnjuje makroevolucijske procese in globalne vzorce biotske pestrosti. Ekološko pogojena divergentna selekcija med populacijami vodi v zmanjšan genski pretok in posledično ekološko speciacijo. Hitrost ekološke divergence in speciacije je odvisna od moči divergentne selekcije. Včasih je težko določiti sosledje procesov, ko, na primer, pride do neekološke speciacije v alopatriji, ki ji sledi ekološka divergenca in sekundaren kontakt med novonastalima vrstama (Losos in Mahler, 2010; Schluter, 2009; Stroud in Losos, 2016).

Adaptivna radiacija je odvisna od notranjih in zunanjih dejavnikov. Notranji dejavniki klada so na primer spolna selekcija, hibridizacija in razvojna plastičnost. Zunanji dejavnik, oziroma predpogoj za zagon adaptivne radiacije pa je ekološka priložnost, t. j. neizkoriščen ekološki vir. Primeri ekoloških priložnosti so kolonizacija novega območja, pojav novega vira, izumrtje ekološko dominantne skupine, ali evolucija t. i. ključne inovacije (ang. key innovation), ki omogoča novo interakcijo z okoljem (Losos in Mahler, 2010; Schluter, 2009; Stroud in Losos, 2016).

Prve študije adaptivnih radiacij so se osredotočale na diskretne, ponavljajoče se habitate, kjer je primere relativno enostavno prostorsko in časovno vrednotiti. Klasični primeri adaptivne radiacije so ostrižniki v velikih afriških jezerih, ali kuščarji anoli in Darwinovi ščinkavci na otokih Velikih Antilov oziroma na Galapagosu (Grant in Grant, 2008; Losos, 2009; Seehausen, 2006). V takšnem okolju je omejeno priseljevanje in začetek diverzifikacije sovpada s poselitvijo. Prav tako je lažje definirati ekološke niše (Losos, 2009; Seehausen, 2006). Manj pozornosti je bilo namenjeno kontinentalnim radiacijam, ali radiacijam nevretenčarjev, med katerimi najdemo študije sulaveških jezerskih kozic, galapaških polžev ali havajskih pajkov (Blom in sod., 2016; Gillespie, 2004; Gillespie in sod., 2020; Von Rintelen in sod., 2010).

Velike adaptivne radiacije, ki so se odvile v diskretnih in ponavljajočih se ekosistemih pogosto sestojijo iz več vzporednih neodvisnih radiacij, skozi katere se oblikujejo podobni seti ekomorfov. Ekomorfi so ekološko in vedenjsko podobne vrste, ki živijo v podobnih habitatih, a niso nujno sorodne (Mahler in sod., 2013). Primeri vzporednih neodvisnih radiacij so že omenjeni anoli in afriški ostrižniki, pri katerih so se v več kladih neodvisno razvili podobni habitatni specialisti, ki so si morfološko podobni (Elmer in sod., 2014; Mahler in sod., 2013). Te vzporedne radiacije niso nujno identične replike. Radiacija anolov na primer sestoji iz fenotipsko konvergentnih vrst, pa tudi unikatnih vrst, s samosvojimi ekomorfološkimi značilnostmi (Mahler in sod., 2013).

Kadar hitro vznikanje vrst ne poteka sočasno z ekomorfološko diferenciacijo, govorimo o neadaptivni radiaciji. Speciacija poteka v ekološko podobnih okoljih. Večinoma je takšna speciacija alopatrična ali parapatrična, torej do omejenega genskega pretoka pride zaradi geografske izolacije (Rundell in Price, 2009). Učinek se še poveča v majhnih populacijah, kjer je prisoten genetski drs (Nürk in sod., 2020). Nastajajoče vrste so lahko tudi simparične, na primer v primeru spolne selekcije (Rundell in Price, 2009).

Neadaptivna radiacija je bolj pogosta pri organizmih z omejeno sposobnostjo razširjanja, ali pa v močno razčlenjenih okoljih, kjer so v posamičnih enotah podobni okoljski pogoji (Czekanski-Moir in Rundell, 2019). Na večji prostorski skali verjetno najhitreje speciirajo organizmi s srednjimi sposobnostmi razširjanja, saj še vedno lahko kolonizirajo nova območja, a nato ne zmorejo vzdrževati genskega pretoka (Agnarsson in sod., 2014). Drugi možni mehanizem neadaptivne radiacije je konservativizem filogenetskih niš, to je tendenca vrst, da ohranjajo predniške ekološke lastnosti. V nestabilnih okoljih bi tak mehanizem vodil v vikariantno izolacijo (Kozak in sod., 2006). Na splošno so definicije neadaptivnih radiacij še bolj ohlapne in raznolike od definicij adaptivnih radiacij, verjetno tudi zaradi težavnosti opredelitve (odsotnosti) ekološke diferenciacije.

Neadaptivna radiacija je bila leta 1872 opredeljena na primeru Havajskih kopenskih polžev (Gulick, 1872). Spolno selekcijo kot gonilo radiacije so potrdili pri več rodovih kačjih pastirjev in pri električnih ribah (Arnegard in sod., 2010; Wellenreuther in Sánchez-Guillén, 2016). Kriptična speciacija postranic v Bajkalskem jezeru, v kombinaciji z že omenjeno visoko fenotipsko pestrostjo nekaterih skupin, pa nakazuje na kombinacijo adaptivnih in neadaptivnih dogodkov (Schön in Martens, 2004). Podoben primer najdemo v podzemlju, kjer znotraj rodu *Niphargus* najdemo tako kriptične klade, kot močno morfološko raznolike klade (Delić in sod., 2017a; Delić in sod., 2017b; Trontelj in sod., 2012).

Do danes še nobena študija ni testirala adaptivne radiacije v podzemlju. Čeprav je ekološko zahtevno, je to okolje hkrati geografsko zelo razdrobljeno in ekološko pestro, torej dopušča razvoj adaptivnih in neadaptivnih radiacij. Naravnih virov hrane v podzemlju je malo, v veliki večini so zunanjega izvora. Le malo vrst je uspešno koloniziralo podzemlje, zato je dolgo veljalo, da so podzemne vrste evolucijska slepa veja, obsojene na izumrtje še preden uspejo diverzificirati (Barr in Holsinger, 1985; Culver in Pipan, 2019; Poulson in White, 1969). A študije zadnjih desetletij kažejo, da se evolucijski procesi nadaljujejo tudi v podzemlju. Mnogo vrst se je razvilo iz podzemeljskega prednika, prek neekološke in ekološke speciacije. Nekatere vrste so ohranile podobno morfologijo, lahko so celo morfološko nerazpoznavne (Esposito in sod., 2015; Faille in sod., 2013; Fišer in sod., 2015). Druge so ekomorfološko diverzificirale, v procesu specializacije na različne podzemne habitate in trofične niše. Tak primer so ditiscidni hrošči iz avstralskih vodonosnikov, kjer so se večkratno razvili dvojčki ali trojčki sestrskih, a morfološko zelo različnih vrst, domnevno prilagojenih različnim ekološkim nišam (Leijs in sod., 2012; Vergnon in sod., 2013). Do podobnega sklepa so prišli v študijah jamskih pajkov (Mammola in sod., 2018) in slepih postranic *Niphargus* (Delić in sod., 2016; Trontelj in sod., 2012).

Objavljeni podatki nakazujejo, da evolucijski procesi v podzemlju potekajo podobno kot na površju. Še več, podzemlje ustreza zgoraj naštetim lastnostim okolij, ki promovirajo radiacije. Nudi kompleksno strukturo raznolikih in zelo razdrobljenih habitatov, v katerih bi se linije lahko ekološko diferencirale in prostorsko ločevale. A celovita makro analiza speciacije in diverzifikacije v podzemlju še ni bila narejena. Namen pričujoče naloge je zapolniti to vrzel.

Kot modelni organizem za analizo adaptivne radiacije v podzemlju smo izbrali rod slepih postranic, *Niphargus*, največji rod sladkovodnih postranic na svetu (Väinölä in sod., 2008). Z več kot 420 opisanimi in številnimi še neopisanimi vrstami ima ta izjemno pestra skupina pomembno vlogo v vodnih podzemnih habitatih zahodne Palearktike in pomembno prispeva k njihovi biotski pestrosti (Fišer, 2019; Horton in sod., 2021; Zagmajster in sod., 2014). Slepe postranice so se najverjetneje razvile in podzemnega prednika in v celoti speciirale in ekomorfološko diverzificirale v podzemlju (Fišer in sod., 2008b). Dandanes jih najdemo v praktično vseh podzemnih vodnih habitatih, vse od površja do izjemnih globin. Morfološke značilnosti vrst lahko povežemo z lastnostmi habitatov v katerih živijo, kar kaže na morebitno adaptacijo znotraj podzemlja (Delić in sod., 2016; Trontelj in sod., 2012). *Niphargus* združuje vse ključne elemente adaptivnih radiacij: izvor iz skupnega prednika, veliko število vrst in ekomorfološko diferenciacijo. Kot tak je odličen model za testiranje hipoteze podzemne adaptivne radiacije.

Namen disertacije je bil celovito analizirati vzorce speciacije in ekomorfološke diverzifikacije rodu *Niphargus* na kontinentalnem nivoju, znotraj vroče točke biotske pestrosti in znotraj ene združbe. Disertacijo smo razdelili v pet sklopov. V prvem sklopu smo preučili filogenetsko zgodovino redu postranic in pokazali, da obdobja povečane diverzifikacije sovpadajo s potencialnimi ekološkimi priložnostmi (Copilaş-Ciocianu in sod., 2020). V drugem sklopu smo ovrednotili tempo in hitrost diverzifikacije rodu *Niphargus* in preverili, ali ustrezajo pričakovanjem modela adaptivne radiacije, ter če tudi te dogodke lahko pojasnimo z ekološko priložnostjo. Hipotetično ekološko priložnost bi lahko predstavljal dvig karbonatnih gorstev v jugovzhodni Evropi, ki je ustvaril množico prostih, še nenaseljenih podzemnih habitatov. Testirali smo dve hipotezi: evolucijsko zgodovino rodu sestavljajo adaptivni in neadaptivni dogodki; in da bodo večkratne neodvisne adaptivne radiacije na območjih dvigajočih se gorstev ugnezdene znotraj filogenije rodu (Borko in sod., 2021). V tretjem sklopu smo preučili potencialni primer ekološke speciacije znotraj rodu: združbo jame Melissotrypa, v kateri živijo tri vrste slepih postranic, ki zasedajo različne habitate (Borko in sod., 2019). V četrtem sklopu smo preučevali pestrost slepih postranic znotraj vroče točke podzemeljske biotske pestrosti – Dinaridov. Vrstno bogate regije lahko nastanejo z lokalno speciacijo ali z doseljevanjem iz okoliških regij (Rosindell in Phillimore, 2011). Namen tega sklopa je bil analizirati doprinos speciacije, bodisi adaptivne ali neadaptivne, in disperzije k pestrosti slepih postranic na Dinaridih. Izredno visoka pestrost in morfološka raznolikost vrst, ki živijo na Dinarskem krasu kaže na kombinacijo procesov in raznolik prispevek procesov znotraj regije (Borko in sod., 2022).

V petem sklopu smo analizirali disperzijo med Dinaridi in Apeninskim polotokom (Delić in sod., 2020).

Postranice so z več kot 10.000 vrstami, ki živijo v praktično vseh vodnih habitatih, med najbolj pestrimi redovi rakov (Arfianti in sod., 2018; Horton in sod., 2021). Kljub njihovi številčnosti, globalni razširjenosti in pomembni vlogi v vodnih ekosistemih, filogenetski odnosi med njimi do zdaj niso bili razrešeni. Temeljili so predvsem na morfologiji, hipoteze o časovnem poteku diverzifikacije pa so si močno nasprotovale (Lowry in Myers, 2017). Zato smo izračunali obsežno časovno kalibrirano molekularno filogenetsko drevo in rekonstruirali potek speciacije in ekološke diverzifikacije.

Zbrali smo molekularne podatke rodov postranic na način, da bi zajeli čim več filogenetske in ekološke pestrosti skupine. Kalibrirano filogenetsko drevo smo izračunali s štirimi informativnimi genskimi markerji: mitohondrijsko citokrom oksidazo (COI), delom gena 28S rRNK, delom 18S rRNK in histonom (H3). Filogenetske odnose smo izračunali z različnimi metodami: Bayesovim pristopom (Aberer in sod., 2014; Drummond in sod., 2012), metodo največjega verjetja (Stamatakis, 2014; Trifinopoulos in sod., 2016), in metodo največje varčnosti. Za kalibracijo smo uporabili pet kalibracijskih točk, ki temeljijo na fosilnih najdbah (Copilaş-Ciocianu in sod., 2019). Iz literature smo zbrali ekološke podatke o habitatu (morski/sladkovodni/kopenski, bentoški/pelaški, načinu življenja (simbiotski/prostoživeči), globini (5 diskretnih kategorij) in temperaturni preferenci (hladnoljubni/toploljubni) za vsak rod. Speciacijo skozi čas smo izračunali z metodo drsečega okna (Meredith in sod., 2011). Rekonstruirali smo predniška stanja za vseh pet ekoloških znakov z metodo največjega verjetja in Bayesovim pristopom (Revell, 2012) in rekonstruirali hitrost ekološke diverzifikacije.

Rekonstruirane filogenije so bile dobro podprte in so v veliki meri sovpadale. Čeprav skupina obstaja že od Perma, je do največje diverzifikacije prišlo kasneje in sicer v več pulzih: sredi jure, na prehodu v kredo, sredi krede in na prehodu v paleogen. Z izjemo prehoda na kopno so se vse spremembe habitata ponovile večkrat neodvisno in relativno pozno v evolucijski zgodovini redu (Copilaş-Ciocianu in sod., 2020).

Postranice so v glavnem hladnoljubne živali z nizko toleranco na hipoksijo. Prva ekološka priložnost bi lahko sledila permsko-triasnemu množičnemu izumrtju, po tem ko so oceani zopet postali oksigenirani. Iz teorije adaptivne radiacije lahko sklepamo, da je izumrtje izpraznilo prostor, ki je po paleoklimatskih spremembah postal prosto dostopno okolje za postranice. Drugi izbruh speciacije je najverjetneje sledil razpadu Pangeje, speciacija bi v bila tem primeru vikariantna. Zadnji veliki izbruh speciacije in ekološke diverzifikacije pa se je zgodil v pozni kredi, ko je zaradi povišane gladni morja nastala obilica plitvomorskih habitatov in so imela morja visok delež kisika. Čeprav smiselne, pa so našteto zgolj hipoteze, ki jih z dostopnimi podatki ni moč zanesljivo testirati. Rekonstrukcije tako oddaljenih dogodkov so zahtevne, še posebej za skupine z malo fosili, kot so postranice (Copilaş-Ciocianu in sod., 2020).

Postranice so najverjetneje doživele več ugnezdenih radiacij, še posebej se to kaže v starodavnih velikih jezerih, na primer v Bajkalskem jezeru (Naumenko in sod., 2017). Rod *Niphargus* je po številu vrst sodeč največja takšna ugnezdena radiacija, ki predstavlja kar 20 do 25 % vseh sladkovodnih postranic in 5 % vsega redu (Horton in sod., 2021). Zanimivo je, da se je ta izjemna radiacija zgodila v na prvi pogled precej drugačnem okolju kot so velika jezera: v podzemlju.

Za analizo vzorcev diverzifikacije rodu *Niphargus* smo zbrali vse dostopne molekularne, morfološke in ekološke podatke za opisane in še neopisane vrste slepih postranic. Da bi zajeli vso kriptično pestrost, smo kot osnovno enoto pestrosti namesto vrste uporabili MOTU (ang. Molecular Operative Taksonomic Unit, v prevodu molekularna operativna taksonomska enota). Za namene delimitacije smo upoštevali obstoječe večlokusne študije, če le-ta ni obstajala pa smo ubrali konservativni enolokusni pristop. Končni nabor podatkov je obsegal 377 MOTUjev. MOTUje smo razdelili v šest kategorij, glede na habitat v katerem se pojavljajo: sistem razpok v nezasičeni coni jam, intersticij, podzemna jezera, podzemni potoki, plitvo podzemlje in podzemna voda s specifičnimi kemijskimi lastnostmi. Podatke smo zbrali iz literature, terenskih zapiskov in relacijske baze SubBioDB. Morfološki podatki so vključevali 11 telesnih mer, ki odražajo lastnosti habitata, v katerem živi vrsta. Podatki so bili zbrani z merjenjem osebkov in iz literature.

Kalibrirano filogenetsko drevo smo izračunali na podlagi sedmih informativnih genskih markerjev: COI, dveh segmentov 28S rRNK, H3, dela 18S rRNK, del sekvence gena za fosfoenolpiruvat karboksinazo (PEPCK), gena za glutamil-prolil-tRNK sintetazo (EPRS), gen za beljakovino toplotnega šoka 70 (HSP70) in gena za arginin kinazo (ArgKin). Kalibrirano filogenijo smo izračunali z Bayesovim pristopom (Bouckaert in sod., 2014). Uporabili smo štiri notranje kalibracijske točke: 1) fosil slepe postranice, pri katerem se prvič pojavi trnast krempelj (Jazdzewski in Kupryjanowicz, 2010); 2) zadnjo zalitje povezave med Evrazijo in severno Ameriko kot zgornjo mejo starosti rodu (Brikiatis, 2016); 3) starost otoka Kreta, na katerem živijo monofiletske vrste z najbližjim sorodnikom na celinski Grčiji (Allegrucci in sod., 2011); 4) starost vodne povezave med Paratetido in Mediteranom, ki je ločila dinarske in bližnjevzhodne klade (Popov in sod., 2004).

V naslednjem koraku smo rekonstruirali potek speciacije, poseljevanja habitatov in morfološke diverzifikacije ter jih primerjali z modeli naključne diverzifikacije (ang. null model), kot bi jih pričakovali pri nevtralni evoluciji (Harmon in sod., 2003; Revell, 2012). Nato smo z metodo največjega verjetja testirali kateri model evolucije najbolje opiše naše podatke (Clavel in sod., 2015). Del analiz smo ponovili na šestih monofiletskih kladih z več kot 25 vrstami. Testirali smo konvergenco fenotipov različnih linij, da bi ugotovili, ali so se določeni fenotipi večkrat neodvisno razvili (Ingram in Mahler, 2013). Rekonstruirali smo predniške areale in analizirali potek poselitve (Pagel in sod., 2004).

Kalibrirana filogenija rodu *Niphargus* obsega vsaj dvakrat več vrst kot druga največja analizirana podzemeljska skupina (Morvan in sod., 2013). Pokazali smo, da rod slepih postranic izvira iz srednjega miocena (56 do 39 milijonov let nazaj). Analiza kopičenja filogenetskih linij skozi čas je pokazala pospešitev celokupne speciacije pred 15 milijoni let, z upočasnjevanjem zadnjih pet milijonov let. To je v skladu z predvidevanji adaptivno-radiacijskega modela, tako imenovano dinamiko zgodnjega izbruha (ang. early burst). Primerjava alternativnih modelov speciacije je pokazala, da proces speciacije rodu slepih postranic najbolje opiše model s spremembo hitrosti speciacije okoli 15 milijonov let nazaj. Sledi mu model s spremembo hitrosti speciacije in spremembo nosilnosti okolja (Borko in sod., 2021).

Rekonstruirali smo predniške habitate in pokazali, da so predniki danes živečih vrst prve 20 do 30 milijonov let živeli in se razširjali v intersticijskih obalnih ali aluvialnih habitatih. Nato so večkrat kolonizirali raznolike nove podzemne habitate. Pričetek kolonizacije novih habitatov sovpada s povišano speciacijo (Borko in sod., 2021).

Funkcionalna morfologija slepih postranic odraža pogoje, v katerih vrsta živi, na primer velikost podzemnih prostorov, hitrost toka, način premikanja ali prehranjevalne navade (Trontelj in sod., 2012). Povezava med morfologijo in habitati ni enoznačna, zato je smiselno uporabljati obe metriki komplementarno. Analizirali smo evolucijsko dinamiko morfološke raznolikosti skozi čas. Morfološka raznolikost se je prvič skokovito povišala približno 35 milijonov let nazaj. Sledilo je 15 milijonov let divezifikacije, kakršno bi pričakovali pri nevtralnem modelu evolucije. Pred približno 15 milijoni let je raznolikost spet poskočila, kar nakazuje na neodvisno diverzifikacijo morfologije znotraj več kladov. Faza visoke fenotipske diverzifikacije ustreza fazama visoke speciacije in ekološke diverzifikacije. Pri vseh 11 telesnih merah se je stopnja diverzifikacije sistematično višala skozi čas. Primerjava multivariatnih modelov evolucije morfologije je pokazala, da morfološko diverzifikacijo rodu najbolje opiše model s preskokom iz Brownovega gibanja v model zgodnjega izbruha okoli 15 milijonov let nazaj (Borko in sod., 2021).

V izjemnih pogojih so velike adaptivne radiacije lahko seštevek neodvisnih vzporednih radiacij znotraj klada. Na šestih geografsko opredeljenih monofiletskih kladih smo ponovili analize diverzifikacije. Speciacija znotraj kladov ustreza modelu zgodnjega izbruha, ki se je pričel pred 15 do pet milijoni let nazaj. Modeli spreminjanja ekološke pestrosti niso odstopali od pričakovanih. Analiza morfološke disparitete je pokazala adaptivno-radiacijski vzorec pri pontskem, panonskem, zahodnobalkanskem in severnodinarskem kladu, pri južnodinarskem kladu sprememba ni bila statistično značilna. Apeninski klad sestavljajo večinoma morfološko podobne vrste, za katere nismo imeli dovolj morfoloških podatkov, sklepamo pa, da bi lahko bil to primer neadaptivne radiacije. Preverili smo tudi, ali so se v neodvisnih vzporednih radiacijah razvili podobni morfotipi. Na podobno evolucijo morfotipov smo sklepali s pomočjo modeliranja t.i. adaptivnih vrhov morfoloških lastnosti. Adaptivni vrh v tem primeru predstavlja določeno vrednost morfološki lastnosti (ali skupka lastnosti); ena lastnost ima v različnih okoljih lahko različne vrednosti, ki na drevesu vznikenjo večrat neodvisno. Najverjetnejši modeli so predvideli 11 do 12 konvergentnih adaptivnih vrhov, od teh sta se dva razvila večkrat znotraj klada, devet pa se jih je razvilo v več kladih. Tri do štiri adaptivni vrhovi so se pojavili le enkrat. Čeprav kladi izkazujejo določen nivo konvergence so imel posamezne radiacije določene skupine adaptivnih vrhov (Borko in sod., 2021).

Rekonstrukcija predniških območij je pokazala, da slepe postranice izvirajo iz današnje zahodne Evrope. Preko intersticija so se razširjale proti jugovzhodni Evropi. Časovni in prostorski potek diverzifikacije analiziranih kladov sovpada z dvigom in zakrasevanjem kraških masivov jugovzhodnih Alp, Karpatov in Dinaridov, zato sklepamo, da novi habitati predstavljajo verjetno ekološko priložnost. Ob dvigu kraških masivov iz morja se je začelo zakrasevanje, to je formacija jam in množice drugih podzemnih habitatov. Proces je dosegel vrhunec v srednjem miocenu. Kraški masivi so takrat delovali kot otoki v Paratetidi, občasno povezani med seboj. Obsežna nova sladkovodna podzemna okolja so nudila popolnoma nov in še nenaseljen ekološki prostor, in ekološko priložnost za slepe postranice, ki so že bile prilagojene na podzemno okolje (Borko in sod., 2021).

Čeprav makroevolucijski vzorci kažejo na adaptivno radiacijo rodu *Niphargus*, je razlikovanje med ekološko in neekološko speciacijo na nivoju posamičnih speciacijskih dogodkov zapleteno. Kladi lahko vsebujejo elemente obeh procesov, včasih pa vzorci ustrezajo obema procesoma in jih je nemogoče razložiti (Rundell in Price, 2009). Taksonomsko, filogenetsko in ekološko smo ovrednotili združbo slepih postranic v grški jami Melissotrypa, ki bi se lahko razvila prek ekološke speciacije.

V jami živijo štiri vrste postranic, tri iz rodu *Niphargus* in ena iz rodu *Bogidiella*. Tri vrste slepih postranic živijo v različnih habitatih: v majhnih medzrnskih prostorih na dnu jezera, v freatski vodi sladkovodnega jezera in v freatski vodi jezera bogatega z žveplovodikom. Filogenetsko drevo 103 vrst slepih postranic smo izračunali s štirimi informativnimi genskimi markerji: COI, dvema odsekoma 28S rRNK in H3. Filogenetske odnose smo izračunali z Bayesovim pristopom (Ronquist in sod., 2012) in metodo največjega verjetja (Trifinopoulos in sod., 2016). Izvedli smo klastrsko analizo funkcionalnih morfoloških znakov za vrste z znano ekologijo.

V Melissotrypi smo našli vrste *N. lindbergi* S. Karaman, 1956, *N. jovanovici*, S. Karaman 1931, in še neopisano vrsto iz žveplenega jezera, ki smo jo opisali kot *N. gammariformis sp. nov.* Tri vrste so si ozko sorodne in tvorijo svojo evolucijsko linijo z nejasno pozicijo znotraj rodu. Vrste se med seboj razlikujejo v habitatu in morfologiji. Klastrska analiza je pokazala, da so morfološko podobne nesorodnim vrstam, ki živijo v podobnih habitatih (Borko in sod., 2019).

Morfološke prilagoditve na s strupenim žveplovodikom bogato okolje so se pri slepih postranicah neodvisno razvile vsaj štirikrat. Ekomorfološka raznolikost in filogenetsko združevanje nakazujeta ekološko speciacijo znotraj jame. A bi morali za nedvoumno potrditev ekološke speciacije pokazati, da se je reproduktivna izolacija zares razvila zaradi ekološke diferenciacije, za kar nimamo dovolj podatkov. Alternativna možnost bi bila, da so se vrste razvile v alopatriji, se prilagodile na različna okolja, nato pa prišle v sekundarni kontakt (Rundell in Price, 2009). Četudi je *N. gammariformis* znan samo iz študirane jame, pa sta drugi dve vrsti gelede na razpoložljive podatke razširjeni širše. Za razrešitev vprašanja bi potrebovali natančne podatke o razširjenosti in tudi dobro podprto molekularno filogenetsko drevo na nivoju populacij (Borko in sod., 2019).

V četrtem sklopu disertacije smo se osredotočili na vprašanje biotske pestrosti in procesov v prostoru. Analizirali smo filogenetsko in vrstno pestrost slepih postranic v območju vroče točke podzemeljske biotske pestrosti, Dinaridov (Borko in sod., 2022). Pregledali smo celotno zbirko slepih postranic Skupine za speleobiologijo, BF, UL. Za namene pokritja vseh obstoječih lokalitet znotraj Dinaridov in čim več morfološke variabilnosti smo odbrali preko 1000 osebkov za izolacijo DNK in pomnoževanje COI gena. Uspešno pomnožene sekvence smo analizirali skupaj z že obstoječimi COI sekvencami. Uporabili smo pet enolokusnih delimitacijskih metod (Puillandre in sod., 2012, 2021; Zhang in sod., 2013), jih primerjali z delimitacijami iz obstoječih večlokusnih študij in izbrali konsenzno določitev vsakega osebka.

Zaradi visokega deleža novih, prej nepoznanih MOTUjev, za katere še ne obstajajo zanesljivi ekološki in morfološki podatki, nismo mogli rekonstruirati evolucije morfologije in poseljevanja dinarskih kladov, zato smo ubrali drugačen pristop. Ponovili smo računanje kalibrirane filogenije z enakimi markerji in metodološkim pristopom kot v drugem sklopu (Borko in sod., 2021). Nato smo na analizirano območje položili mrežo kvadratov velikosti 20 km x 20 km in znotraj vsakega kvadrata računali vrstno in filogenetsko pestrost (Faith, 1992). Filogenetsko pestrost smo standardizirali glede na število vrst v celici (Kembel in sod., 2010). Nato smo primerjali vzorce obeh metrik v prostoru.

Glede na rezultate enolokusnih delimitacij na Dinaridih živi med 212 in 474 MOTUjev. Po preverjanju kakovosti in navzkrižnem preverjanju z že objavljenimi večlokusnimi študijami smo oblikovali končen nabor podatkov, ki je obsegal 598 lokalitet in 245 MOTUjev, med katerimi je 79% vseh nominalnih vrst na Dinaridih. Presenetljivo odkritje je bilo kar 148 novih MOTUjev, ki ne ustrezajo nominalnim vrstam in še niso bili obravnavani v preteklih študijah. Filogenetski odnosi so bili v večji meri podobni prvotnim rekonstrukcijam, a se je število MOTUjev znotraj dinarskih kladov (južnodinarski, severnodinarski in vzhodnobalkanski klad) močno povečalo (Borko in sod., 2022).

Vrstno najbolj bogate celice so na severozahodu Dinaridov. Vzorec filogenetske pestrosti le deloma sledi vzorcu vrstne pestrosti. Večina celic je imela nižjo filogenetsko pestrost kot bi pričakovali, če bi bile živali razvrščene v prostoru naključno. Celoten jugovzhodni del Dinaridov ima izjemno nizko filogenetsko pestrost. Severozahodni Dinaridi imajo razmeroma višjo filogenetsko pestrost. Dodatno vpogled v vzorce vrstne in filogenetske pestrosti na Dinaridih dobimo, če analiziramo razporeditev kladov v prostoru (Borko in sod., 2022).

Osrednje in jugovzhodne Dinaride poseljujejo predvsem dinarski kladi, to so zahodnobalkanski, južnodinarski in severnodinarski klad. Za njih smo pokazali, da so v celoti diverzificirali na območju Dinaridov. Egejski klad seže v Dinarski prostor le bežno, na skrajnem jugovzhodu. Na vzhodnih aluvialnih ravnicah pa so prisotni predstavniki široko razširjenih plitvo podzemeljskih vrst iz linije *N. sphagnicolus*. Vsi trije kladi so se iz Dinaridov tudi preseljevali, predvsem na Apeninski polotok, v obdobjih povezav med obema kopninama. Nasprotno pa je le ena linija prišla v Dinaride in se razširila po obmorskem delu (glej sklop 5). Kljub obstoju povezav z drugimi kraškimi masivi nobena druga linija ni uspešno naselila jugovzhodnih Dinaridov. Filogenetska pestrost na jugovzhodu je nizka in vzorec diverzifikacije vseh treh dinarskih kladov nakazuje, da je adaptivna radiacija dominirala nad disperzijo.

Drugače je v severozahodnih Dinaridih, še posebej na stičišču z jugovzhodnimi Alpami in Panonsko nižino. V območju, kjer se kraška območja stikajo z aluvialnimi ravnicami, sta tako vrstna kot filogenetska pestrost visoki, tu živijo predstavniki večine linij rodu slepih postranic. Dva dinarska klada segata do severozahoda. Zahodnobalkanski klad je razširjen le znotraj meja dinaridov, medtem ko je severnodinarski prisoten tudi v jugovzhodnih Alpah. Prisotne so vrste iz pontskega in panonskega klada. Panonske vrste le bežno segajo v ožje območje Dinaridov in so bolj prisotne v jugovzhodnih Alpah in Panonski nižini. Iz panonskega klada je v severozahodnih Dinaridih prisotna linija desetih MOTUjev, ki je najverjetneje diverzificirala v tem prostoru, znotraj specifičnega habitata (sistem razpok). Nadalje v severozahodnih Dinaridih najdemo še štiri nesorodne klade z vsaj petimi MOTUji na klad, ter 18 MOTUjev brez najbližjih sorodnikov znotraj tega prostora. Ta kompleksna struktura nesorodnih vrst, ki so se priselile v regijo in sorodnih vrst, ki so diverzificirale tu, se odraža v visoki biotski pestrosti (Borko in sod., 2022).

Glede na razlike v vzorcih vrstne in filogenetske pestrosti in na splošno majhne areale razširjenosti vrst slepih postranic lahko zaključimo, da je lokalna diverzifikacija najverjetneje glavno gonilo vrstne pestrosti v Dinaridih, disperzija pa je bila prisotna predvsem v severozahodnem delu regije (Borko in sod., 2022). Potencialna pomanjkljivost študije je nezmožnost oceniti delež izumiranja, ki lahko pomembno prispeva k visoki filogenetski pestrosti. A iz več razlogov sklepamo, da izumiranje ni imelo vloge pri nastanku današnjih vzorcev filogenetske pestrosti: podzemlje je v času katastrofičnih dogodkov predstavljalo pribežališče vrstam, ki na površju drugače ne bi preživele. V Evropi so bile poledenitve glavni vzrok izumiranja, Dinaridi pa so prepoznani kot južni refugij. Še več, pokazano je bilo da so alpski ledeniki na severozahodu spodbudili speciacijo slepih postranic in ne obratno (Delić in sod., 2022).

Z analizo vzorcev pestrosti smo pridobili pomemben vpogled v možne procese, ki so se odvijali v regiji. A zaradi višjega števila novih MOTUjev, kot smo jih pričakovali, in posledično pomanjkanja zanesljivih ekomorfoloških podatkov, nismo mogli oceniti doprinosa neadaptivnih in adaptivnih radiacij. Za podrobnejšo in predvsem zanesljivo analizo procesov na lokalni ravni bo potrebno še veliko terenskega in laboratorijskega dela (Borko in sod., 2022).

V zadnjem sklopu smo analizirali disperzijo rodu *Niphargus* med Dinaridi in širšim območje, konkretno Apeninskim polotokom (Delić in sod., 2020). Obdobja regresije in transgresije morja bi lahko povzročili izmenjavo disperzije in vikariance. Zbrali smo podatke za 494 osebkov slepih postranic, s poudarkom na vrstah ki živijo ob Jadranskem morju. Z enolokusnimi delimitacijami s COI markerjem (Puillandre in sod., 2012; Zhang in sod., 2013) smo prepoznali 169 MOTUjev, za katere smo izračunali filogenijo. Filogenijo smo izračunali s pomočjo sekvenc štirih markerjev: H3, COI in dvema fragmentoma 28S, z metodo največjega verjetja (Minh in sod., 2020) in z Bayesovim pristopom (Ronquist in sod., 2012). Kalibrirano filogenijo smo izračunali z Bayesovim pristopom (Bouckaert in sod., 2014) in enakimi kalibracijskimi točkami kot v sklopih 2 in 4. Nato smo na t. i. transjadranskem kladu, ki vključuje sestrska zahodnobalkanski in južnodinarski klad, z metodo zveznih markovskih procesov modelirali disperzijo, izumiranje, vikarianco, kladogenezo in predniške areale (Matzke, 2013).

Znotraj analiziranega klada so vgnezdeni trije dobro podprti podkladi z vrstami, prisotnimi na obeh straneh Jadranskega morja. Prav tako so nekatere sestrske vrste prisotne na Dinarskem krasu in na Jadranskih otokih. Dinaridi so bili, tako kot v študiji sklopa 2, prepoznani kot izvorno območje klada. Cepitve med vrstami na obeh straneh morja so se zgodile v treh časovnih oknih. Prva serija cepitev sega med 10 do 20 milijonov let nazaj, druga 4,4 do 8,9 milijonov let nazaj, tretja pa se je odvila v zadnjih dveh milijonih let. Vsi modeli so pokazali, da je bila disperzija mnogo večja od izumiranja. Najbolj podprt je bil model skokovite disperzije (ang. jump dispersal) (Delić in sod., 2020).

Prva serija cepitev sovpada s kopenskim mostom med Dinaridi in novo nastalo kopnino Apulija (Mazza in Rustioni, 2008). Potopitev mostu, ki je sledila, je razdelila populacije, kar je vodilo v obdobje, ko so potekle prve vikariantske speciacije. Sledila je regresija Jadranskega morja med mesinijsko krizo slanosti (Garcia-Castellanos in Villaseñor, 2011), ko je predniška linija *N. hebereri* najverjetneje migrirala nazaj iz Apeninskega polotoka na Jadranske otoke in obalo Dinaridov. Zadnje cepitve sovpadajo s pleistocensko poledenitvijo (Correggiaii in sod., 1996). Vsaj šest linij je takrat iz Dinaridov koloniziralo Jadranske otoke (Delić in sod., 2020).

Znotraj zahodnobalkanskega in južnodinarskega klada smo pokazali na večkratno disperzijo iz Dinaridov na Apeninski polotok. Disperzija iz Dinaridov se je najverjetneje zgodila še večkrat. Na vznožju karpatov je razširjena vrsta *N. mirocensis* z najbljižimi sorodniki na Dinaridih. Linija šestih vrst iz kompleksa *N. rhenorhodanensis* v zahodni Italiji je dispergirala iz severozahodnih Dinaridov. Dinaride zato lahko obravnavamo kot donorsko območje slepih postranic, ki so iz Dinaridov poseljevale kasneje vzdignjene kopnine. Nasprotno je bila disperzija v osrednje in jugovzhodne Dinaride od zunaj močno omejena, pokazali smo le en prehod nazaj iz Apeninskega polotoka. Najverjetneje so že prisotne dinarske vrste preprečevale uspešno priseljevanje.

V okviru doktorske disertacije smo celovito analizirali vzorce speciacije in ekomorfološke diverzifikacije znotraj rodu *Niphargus* na kontinentalnem nivoju, znotraj vroče točke podzemne biotske pestrosti in znotraj jamske združbe. Pokazali smo, da diverzifikacija slepih postranic ustreza adaptivno-radiacijskemu modelu, ter da se je znotraj rodu odvilo več neodvisnih radiacij na območjih dvigajočih se kraških gorstev. Pomembno smo razširili podatkovni nabor na območju Dinaridov in razkrili skrito pestrost slepih postranic znotraj vroče točke podzemeljske biotske pestrosti. Pokazali smo, da imajo slepe postranice znotraj Dinaridov heterogeno filogenetsko strukturo v prostoru, kar je posledica različnega doprinosa speciacije in disperzije k nastanku vrstne pestrosti tega območja. To je prva tovrstna študija adaptivne radiacije v podzemlju in kot taka predstavlja pomemben doprinos k razumevanju evolucije v podzemlju. Ne le, da odkriva potencial za nadaljnje raziskovanje radiacij v podzemlju, pomembno prispeva tudi k prepoznavanju Evrope kot kontinenta z visoko biotsko pestrostjo v okoljih, kjer je na prvi pogled ne bi pričakovali.

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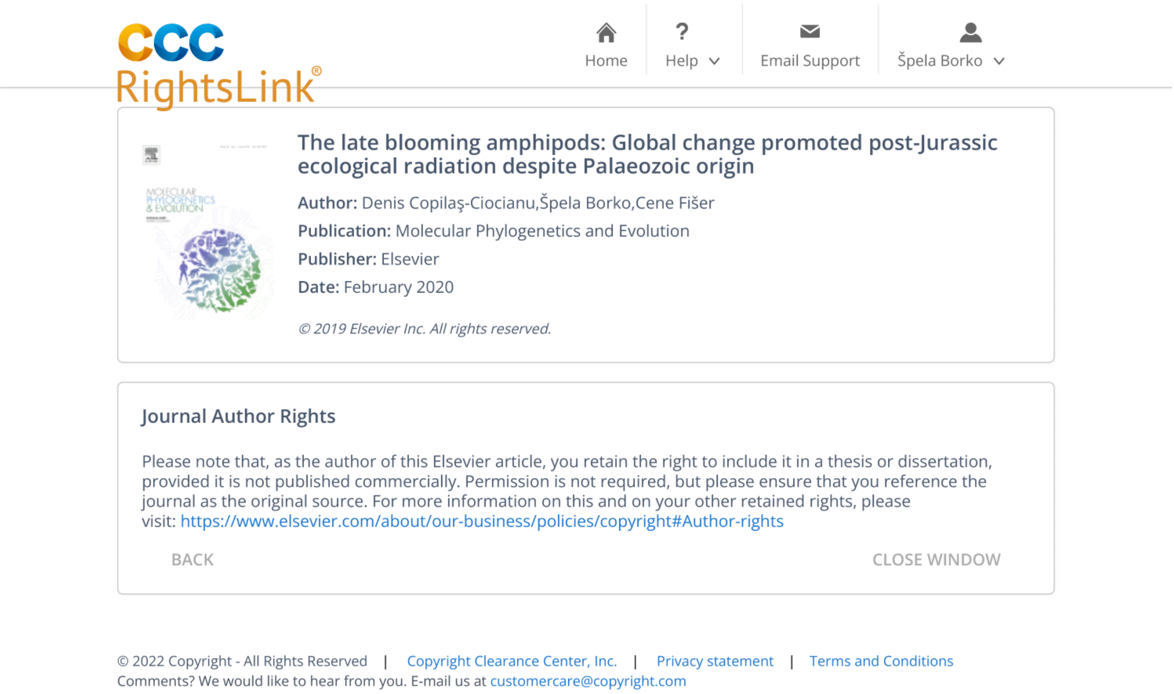
ANNEXES

ANEX A

Consent from publishers for the re-publication of article entitled *The late blooming amphipods: Global change promoted post-Jurassic ecological radiation despite Palaeozoic origin* in the print and electronic versions of the doctoral dissertation

Copilaş-Ciocianu D., Borko Š., Fišer C. 2020. The late blooming amphipods: Global change promoted post-Jurassic ecological radiation despite Palaeozoic origin. Molecular Phylogenetics and Evolution, 143: 106664, https://doi.org/10.1016/j.ympev.2019.106664: 12 p.



The late blooming amphipods: Global change promoted post-Jurassic ecological radiation despite Palaeozoic origin

Author: Denis Copilaş-Ciocianu,Špela Borko,Cene Fišer
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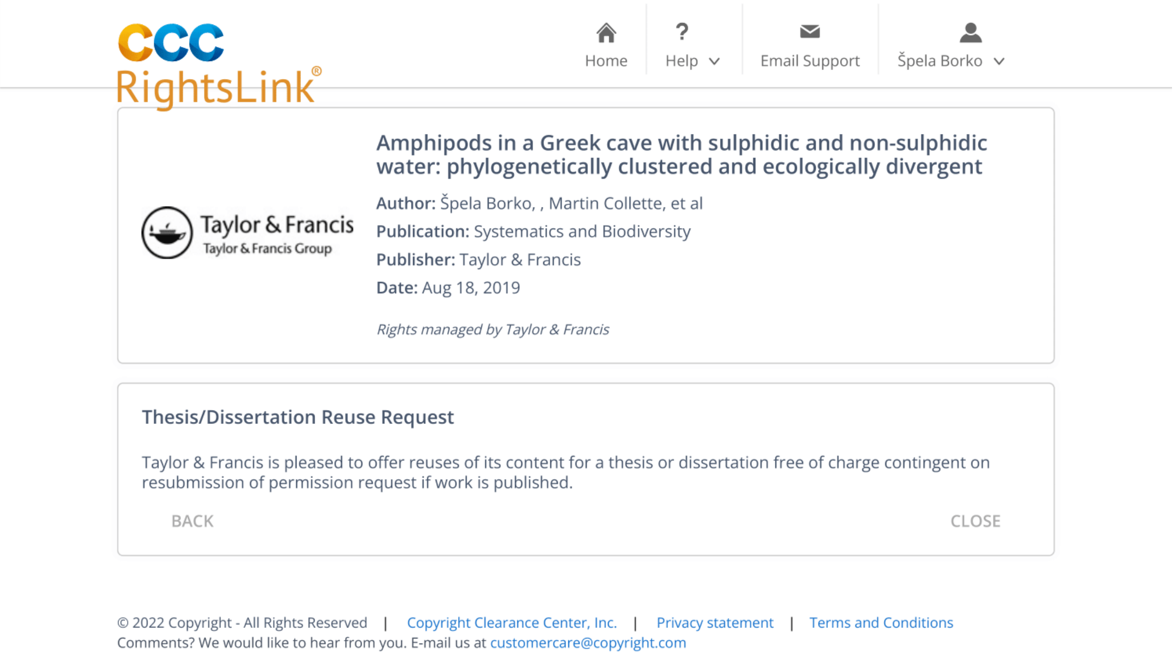
Borko Š., Collette M., Brad T., Zakšek V., Flot J.-F., Vaxevanopoulos M., Sarbu S. M., Fišer C. 2019. Amphipods in a Greek cave with sulphidic and non-sulphidic water: phylogenetically clustered and ecologically divergent. Systematics and Biodiversity, 17, 6: 558-572



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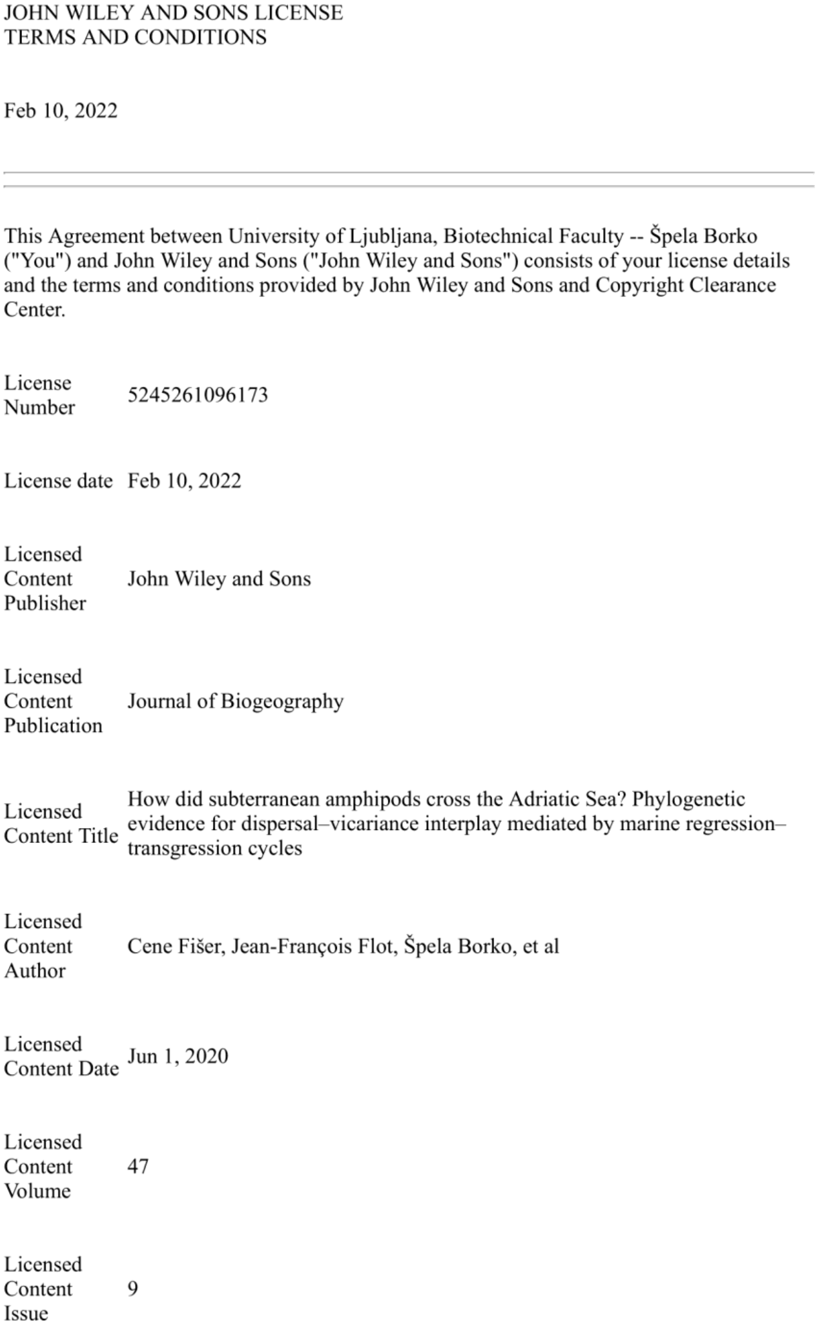
Delić T., Stoch F., Borko Š., Flot J.-F., Fišer C. 2020. How did subterranean amphipods cross the Adriatic Sea? Phylogenetic evidence for dispersal–vicariance interplay mediated by marine regression–transgression cycles. Journal of Biogeography 47: 1875–1887



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