Charles University in Prague, Faculty of Science, Department of Ecology

Study programme: Ecology

Biogeography, phylogeny, ecology and systematics of epigean freshwater Amphipoda in the Carpathian Arc and beyond

Biogeografie, fylogeneze, ekologie a systematika blešivců povrchových vod Karpat a jejich okolí

Ph.D. Thesis
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Prague, 2017

I declare that this thesis has not been submitted for the purpose of obtaining of the same or another academic degree earlier or at another institution. My involvement in the research presented in this thesis is expressed through the authorship order of the included publications and manuscripts. All literature sources I used when writing this thesis have been properly cited.

Prague, July 2017

Denis Copilaş-Ciocianu

The research presented in this thesis was financially supported by the Charles University Grant Agency (project no. 1398214).

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Copilaş-Ciocianu D, Petrusek A (2015): The southwestern Carpathians as an ancient centre of diversity of freshwater gammarid amphipods: insights from the *Gammarus fossarum* species complex. *Molecular Ecology* 24, 3980–3992.

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Supporting information to all chapters is provided as an electronic supplement to the thesis.

PREFACE

Since as long as I can remember, I have been fascinated with biology, but also geography and geology. It probably all started when I was around six years old and I opened a spectacular dinosaur book that my older brother had. Soon after, I started exploring the nature around me, collecting animals and plants, observing and drawing them, taking notes. Aquatic life was the most fascinating because it was also the most secluded, mysterious and not easy to observe in the wild. Crustaceans were especially interesting because of their morphological complexity with many different legs doing different things. Later on, during my turbulent teenage years, my passion for biology dwindled in favour for music. However, my university studies had put me back on track, and my thirst for knowledge was greater than ever. It was during these years that I had to decide between a career in science or music and I am certain I made the right decision. I am very grateful to Adam Petrusek, my supervisor, for giving me the opportunity and freedom to choose a PhD topic. Working on phylogeography meant that I could marry my childhood passions: biology, geography and geology. I believe that during these four years my scientific thinking and knowledge has matured to the point that I can do science independently. I had the opportunity to travel around Europe and personally meet many great scientists that share my fascination with the amazing amphipods. I am grateful that we could collaborate and discuss new ideas. The end of my PhD studies means the beginning of a new chapter in my life and scientific career, to which I curiously look forward.

ACKNOWLEDGEMENTS

First of all, I express my gratitude to my supervisor, Adam Petrusek. He helped me significantly to improve my scientific writing and thinking, and most importantly, he taught me that I should be confident in my own work and not to underestimate myself. I appreciate his time and dedication, always replying to emails and being ready to help and advise. I thank my friend Marius G. Berchi for his great help and company during our many fieldwork adventures. Without his support this thesis wouldn't be where it is now. I am grateful to Cene Fišer for providing me with the opportunity for a research visit at the Subterranean Biology Lab in Ljubljana, Slovenia, during which I learned a lot about the fascinating subterranean amphipods. I also thank Cene for his insight and advice that helped to shape my PhD project proposal and his contributions to the chapters of the thesis. I greatly value the help and hard work of the students Tereza Rutová and Bianca Vanesa Boroş that resulted in fruitful collaborations from which two of the chapters of this thesis came to be. Many thanks go to Péter Borza, Petr Pařil, Lucian Pârvulescu, Michał Grabowski, Gergely Balázs and Dorottya Angyal for their contributions that significantly improved the quality of the chapters. I appreciate the training and advice I got from Jasna Vukić regarding the standard laboratory procedures. I thank all the colleagues at the department for the wonderful times we spent together at Cibulka, during bowling, hiking trips or nights out. I am grateful to my parents for their huge support and enthusiasm towards my work during all these years. Finally, I thank Eglė Šidagytė for her care, support, and very useful statistical advice.

ABSTRACT

Freshwater amphipods are poor dispersers due to the fragmentary nature of their habitat, benthic life-style and lack of resistant propagules. Consequently, most species are narrowly endemic, and widespread morphospecies consist of numerous divergent lineages. As such, these patterns are considered relict, mainly reflecting historical rather than contemporary factors. The Carpathian Mountains underwent a dramatic geomorphological evolution during the last 20 million years, rising from the Paratethys Sea through a series of islands that eventually coalesced into one of the longest mountain chains in Europe. Moreover, these mountains and surrounding basins are inhabited by a ubiquitous and diverse amphipod fauna, providing an ideal setting for testing historical biogeographical hypotheses. Our results have shown that in the southern half of the Carpathians, montane *Gammarus* taxa display fragmented and allopatric distribution patterns while lowland species belonging to the genera *Niphargus* and *Synurella* are mostly sympatric and widespread over large distances. Subsequent molecular investigations revealed that the patchy distribution of *G. fossarum* in the western and southwestern Carpathians has likely resulted from the breakup of a formerly contiguous landmass 16 million years ago, suggesting that geological subsidence can play an important role in shaping biogeographical patterns. Moreover, the Western Carpathian lineages in eastern Czechia and Slovakia are narrowly endemic and date back to the Miocene, implying *in situ* survival of the Pleistocene climatic fluctuations closer to the northern ice-sheets than previously thought. Similarly, the phylogeography and lineage distribution of the *G. balcanicus* species complex reflect several chronologically distinct uplift episodes of the islands that once formed the Carpathian archipelago 10 to 15 million years ago, indicating that the genetic legacy of long-gone archipelagos can persist for millions of years in contemporaneous continental biotas. In contrast to montane gammarids, the two lowland species *Niphargus hrabei* and *N. valachicus* exhibit a much shallower genetic differentiation despite their substantially wider ranges. Although *Niphargus* is a groundwater genus, our analyses reveal that the focal species have independently invaded surface waters from subterranean ancestors and rapidly dispersed throughout the Danube lowlands during the second half of the Pleistocene. Although these two species are not closely related, they are remarkably similar in habitat preferences, functional morphology and zoogeographical patterns. Coupled with very rare cases of syntopy, we conclude that they are weakly differentiated ecologically. However, they do coexist with other less related amphipod taxa, as demonstrated on a case study of *N. valachicus* co-occurring with the unrelated *G. dacicus* and *Synurella ambulans*. These form an assemblage that exhibits a suite of contrasting life-history strategies which might reflect their divergent morphologies or distant evolutionary relationships.

ABSTRAKT (IN CZECH)

Sladkovodní různonožci mají poměrně špatnou schopnost šíření, mimo jiné v důsledku izolovanosti obývaných stanovišť a absence odolných propagulí v životním cyklu. Většina jejich druhů je proto úzce endemická a rozšířené morfologicky definované druhy („morfospecies“) se skládají z četných geneticky odlišných evolučních linií. Takové rozšíření a genetická struktura jsou považovány za relikty historických procesů. Oblast Karpatského oblouku prodělala během posledních 20 milionů let dramatický geomorfologický vývoj: Karpaty byly postupně vyzdviženy z moře Paratethys a v miocénu tvořily souostroví, které se nakonec spojilo do jednoho z nejdelších horských pásem v Evropě. Tyto hory a okolní nížiny jsou navíc obývané různorodou faunou blešivců, což poskytuje ideální prostředí pro testování historických biogeografických hypotéz. Výsledky prezentované v této disertační práci ukázaly, že horské taxony rodu *Gammarus* v jižní části Karpat mají roztříštěné a alopatrické areály, zatímco druhy rodů *Niphargus* a *Synurella* se většinou vyskytují sympatricky a na rozsáhlých územích. Následné molekulární studie doložily, že roztříštěná distribuce druhového komplexu *Gammarus fossarum* v jihozápadních Karpatech je pravděpodobně důsledkem rozpadu dříve souvislé pevniny před cca 16 miliony let. I lokální endemické západokarpatské linie na území Moravy a Slovenska jsou miocenního stáří, což naznačuje, že tito blešivci přežili pleistocenní klimatické výkyvy na místě, podstatně blíže severskému kontinentálnímu ledovci, než se dříve předpokládalo. Fylogeografie a rozšíření druhového komplexu *Gammarus balcanicus* odráží několik chronologicky odlišných epizod vzniku ostrovů, které před 10 až 15 miliony let tvořily karpatské souostroví. To naznačuje, že odkaz dávných souostroví může přetrvávat v genomech současné kontinentální fauny i po mnoho milionů let. Na rozdíl od horských blešivců vykazují dva nížinné druhy *Niphargus hrabei* a *N. valachicus* mnohem mělčí genetickou diferenciaci, a to navzdory rozsáhlým areálům. *Niphargus* je typický taxon podzemních vod a naše analýzy odhalují, že studované druhy se oddělily od stygobiotických předků nezávisle na sobě, obsadily povrchové vody a rychle se během druhé poloviny pleistocénu rozšířily po celé oblasti podunajských nížin. Ačkoli oba druhy nejsou blízce příbuzné, mají pozoruhodně podobné habitatové preference, funkční morfologii i zoogeografii, přičemž syntopicky se vyskytují jen velmi vzácně. To naznačuje, že ekologicky jsou jen slabě diferencované a kompetičně se vytěsňují. Společně se ale mohou vyskytovat se zástupci jiných rodů různonožců. To demonstruje i studie kontrastních životních strategií koexistujících druhů *N. valachicus, Gammarus dacicus* a *Synurella ambulans*. Ty zřejmě reflektují jak relativně vzdálené evoluční vztahy dotyčných druhů, tak jejich odlišnou morfologii.

INTRODUCTION

Freshwater Amphipoda

The order Amphipoda represents one of the largest and diverse orders of the crustacean class Malacostraca, comprising almost 10000 species of which more than 1900 inhabit freshwater environments (Väinölä *et al.* 2008; Lowry & Myers 2013, 2017). Freshwater amphipods are polyphyletic, as they belong to various taxonomical groupings that are not closely related (Barnard & Barnard 1983; Lowry & Myers 2013). As such, multiple continental invasions have taken place at different times and places around the world (Väinölä *et al.* 2008). By far, the most diverse region in freshwater taxa is the Palaearctic, followed by the Nearctic, Neotropic and the Australasian regions. The parvorders Gammaridira and Crangonyctidira have extensively radiated in freshwater and form the bulk of this diversity (Fig. 1).

Amphipods inhabit virtually all kinds of freshwater environments, from cave lakes located at more than 1.5 km under the surface, to springs, brooks and lakes at high altitudes and almost everything in between (Fišer *et al.* 2008; Väinölä *et al.* 2008; Sidorov *et al.* 2015). However, they are particularly diverse in groundwater and cool running waters, and explosive radiations have occurred in some of the world's ancient lakes such as Baikal, Ohrid, Titicaca, Fuxian Hu and the brackish Ponto-Caspian basin (Barnard & Barnard 1983; Martens 1997; Väinölä & Kamaltynov 1999; Cristescu & Hebert 2005; Macdonald *et al.* 2005; Sket & Fišer 2009; Wysocka *et al.* 2013, 2014; Zagmajster *et al.* 2014; Hou & Sket 2016; Naumenko *et al.* 2017). The latitudinal diversity patterns of freshwater amphipods are unusual; the highest diversity is reached at temperate latitudes while epigean freshwater taxa are almost absent in the tropics with the exception of a few species that belong to one genus in the Amazon basin (Barnard & Barnard 1982, 1983). Throughout Eurasia, the most common inhabitants of surface waters are members of the genus *Gammarus* with more than 200 described species (Hou & Sket 2016) while the subterranean realm is dominated by *Niphargus* which comprises more than 350 described species (Esmaeili-Rineh *et al.* 2015).

Amphipods, especially freshwater species, are generally considered as poor dispersers. This is due to external factors such as the fragmented nature of freshwater habitats but also due to their biology and ecology. Epigean freshwater amphipods are benthic animals, living on the bottom of water bodies under stones and leaves (Cărăuşu *et al.* 1955). As such, their dispersal is constrained in comparison to pelagic or freely swimming species (e.g. Cristescu *et al.* 2003). They do not possess dormant stages or resistant propagules that can be passively dispersed or withstand harsh environmental periods like desiccation or anoxia (Barnard & Barnard 1983). Furthermore, the development of amphipods is direct, lacking highly mobile larval stages commonly observed in marine crustaceans, and juveniles hatch from eggs which have been brooded by the female in a special brood pouch, called marsupium (Schram 1986; Väinölä *et al.* 2008). These developmental characteristics coupled with the benthic life-style and the poorly connected nature of freshwater environments makes amphipods especially susceptible to population isolation and genetic differentiation.

The limited dispersal of amphipods is often reflected in their biogeographical patterns. Both surface and subterranean species are characterized by narrow geographical ranges and mosaic-like distribution patterns thought to reflect historical geological or climatic events (Notenboom 1991; Holsinger 1994; Hou *et al.* 2007). Moreover, most of the species that were presumed as widespread are actually diverse species complexes with narrowly distributed, endemic lineages. This pattern has been extensively observed in distantly related taxa belonging to independent freshwater radiations across Europe, Asia, North America, Australia and New Zealand (Müller 2000; Witt & Hebert 2000; Witt *et al.* 2006; Hogg *et al.* 2006; Lefébure *et al.* 2006, 2007; Cooper *et al.* 2007; Murphy *et al.* 2009; Seidel *et al.* 2009; Trontelj *et al.* 2009; Westram *et al.* 2011; Hou *et al.* 2011; Ethridge *et al.* 2013; Mamos *et al.* 2014, 2016; Copilaş-Ciocianu & Petrusek 2015; Katouzian *et al.* 2016; Copilaș-Ciocianu & Petrusek 2017; Copilaș-Ciocianu *et al.* 2017b). Out of nearly 200 freshwater species of *Gammarus*, only one, *G. lacustris*, is indeed widespread, having a Holarctic distribution with modest genetic differentiation between distant populations (Vainio & Väinölä 2003; Usjak 2009; Alther *et al.* 2016). A similar pattern seems to hold for the subterranean genus *Niphargus* where apparently only two epigean species have ranges greater than 1500 km across the longest diagonal (Copilaș-Ciocianu *et al.* 2017a).

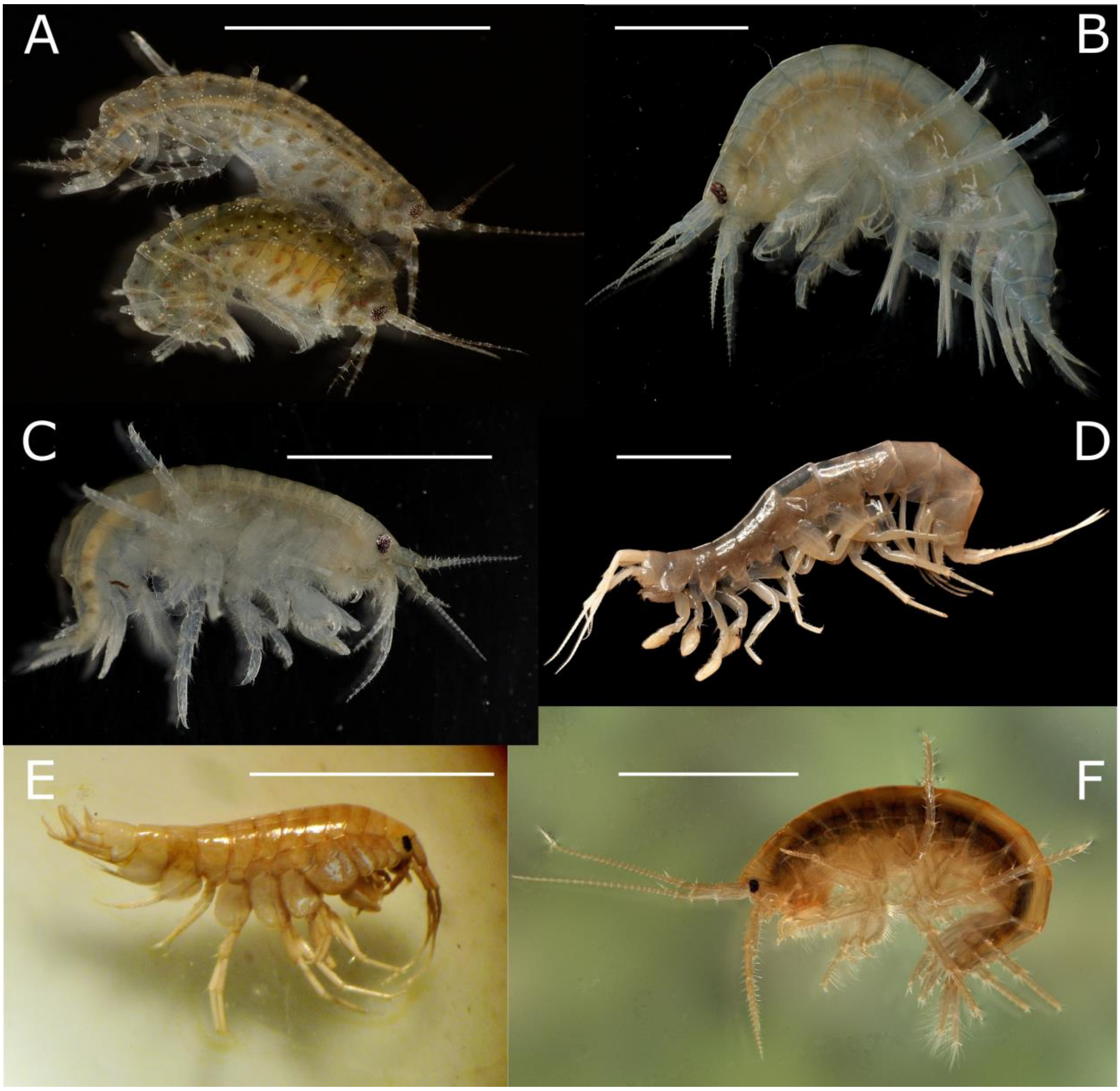

FIGURE 1. Various representatives of Palaearctic freshwater amphipods. A) *Chaetogammarus warpachowskyi* Sars, 1894, B) *Pontogammarus robustoides* Sars, 1894, C) *Obesogammarus crassus* Sars, 1894, D) *Niphargus pannonicus* Karaman 1950, E) *Synurella ambulans* Müller, 1846, and F) *Gammarus fossarum* Koch in Panzer, 1836. Scale bars = 0.5 cm. Photographs: Denis Copilaş-Ciocianu.

The limited dispersal of freshwater amphipods coupled with their geographically structured genetic variation makes them appropriate model organisms for the study of historical biogeography. Indeed, studies linking contemporary distribution patterns of subterranean species with water-level fluctuations of the ancient Tethys Sea have appeared decades ago, before the widespread use of molecular markers (Notenboom 1988, 1991; Boutin 1994). Even a few decades earlier, some authors already suspected a relict, pre-Pleistocene origin for the Ponto-Caspian endemic gammarid radiation or for the subterranean genus *Niphargus* (Ruffo 1953; Cărăuşu *et al.* 1955; Mordukhai-Boltovskoi 1964).

Recent research, employing modern molecular techniques, mostly supports these early claims and has uncovered a multitude of additional patterns. Deep phylogenetic splits between species of the genus *Metacrangonyx* inhabiting subterranean freshwaters in the Caribbean, peri-Mediterranean, Middle East and interspersed oceanic islands are concordant with Late Cretaceous plate tectonics vicariance (Bauzà-Ribot *et al.* 2012) and sea-level fluctuations during the Messinian Salinity Crisis (Bauzà-Ribot *et al.* 2011). Similarly, diversification in *Gammarus* was linked to the major regressive phases of the Parathetys from Eurasia which increased the availability of freshwaters during the second half of the Cenozoic (Hou *et al.* 2011, 2014b; Mamos *et al.* 2016).

Mountain uplift is considered a major driver of biodiversity in many organismal groups (Hoorn *et al.* 2010). This pattern has also been reported in freshwater *Gammarus* species whose diversification time coincides with the uplift of the Carpathians, Dinaric Alps, Balkans, Pontic, Alborz, Zagros mountains and the Tibetan Plateau (Hou *et al.* 2007, 2014a; Copilaș-Ciocianu 2013; Wysocka *et al.* 2014; Katouzian *et al.* 2016; Mamos *et al.* 2016; Copilaș-Ciocianu & Petrusek 2017). Conversely, the opposite phenomenon, geological subsidence, can cause fragmentation of landmasses and also lead to diversification through vicariance. This has been reported only in the *Gammarus fossarum* species complex inhabiting the western and southwestern Carpathians (Copilaş-Ciocianu & Petrusek 2015). In certain cases, the present-day distribution of groundwater genera in arid landscapes can also reflect ancient drainage patterns (Finston *et al.* 2007).

The dynamic past of the Ponto-Caspian region with drastic fluctuations in salinity and water-level, and numerous episodes of isolation and connection between basins have left their traces on the diverse radiation of Ponto-Caspian endemics, creating a euryhaline and phenotypically plastic fauna that gave rise to numerous invasive species currently spreading throughout Europe and North America (Bij de Vaate *et al.* 2002; Reid & Orlova 2002; Cristescu *et al.* 2003, 2004; Cristescu & Hebert 2005; Nahavandi *et al.* 2013; Rewicz *et al.* 2015; Šidagytė *et al.* 2017). Apart from geological events, climatic changes such as the Pleistocene glacial cycles or increased aridification have left their signatures on the genetic structure of various taxa, both in the northern and southern hemispheres (Witt & Hebert 2000; Väinölä *et al.* 2001; Vainio & Väinölä 2003; Cooper *et al.* 2007; Seidel *et al.* 2009; Copilaș-Ciocianu *et al.* 2017b; chapter 7). However, there are several noteworthy cases where very old and endemic species or even families have endured tens of millions of years of climate change in groundwater in the British Isles and Iceland, apparently unaffected by the presence of glaciers (Kristjánsson & Svavarsson 2007; Kornobis *et al.* 2010, 2011; McInerney *et al.* 2014).

The Carpathian Mountains

Geological history

The Carpathian Mountains are the second most extensive mountain system in Europe, forming an arc that spans ca. 1500 km. Their geomorphological evolution has been shaped by the Alpine orogeny (Late Mesozoic, Cenozoic), an event that triggered the uplift of many Palaearctic mountain systems, from the Atlas to the Himalayas, forming the Alpide belt (Moores & Fairbridge 1998). The Alpine orogeny was caused by the collision of the African, Arabian and Indian tectonic plates with the Eurasian plate. The uplift of the Carpathians started during the Late Cretaceous from the Tethys Sea, however most of the mountain chain formed during the Miocene and geological activity continues even in the present day in the youngest, southeastern parts (Maţenco 2017). The uplift of the oldest parts of the Carpathians occurred between 65 to 40 Ma. These regions are represented nowadays by the central part of the Western Carpathians, the Apuseni Mountains and western part of the Southern Carpathians (Banat Mountains) (Rögl 1998; Popov *et al.* 2004; Golonka *et al.* 2006; Kováč *et al.* 2016)(Fig. 2A,B). During this period the marine Carpathian foreland basin covered most of the Western and Eastern Carpathian landmasses and the Transylvanian Plateau. Approximately 16 Ma, the Eastern Carpathians began their uplift from the sea. This event caused the disintegration of Tisza Land, a continuous landmass that incorporated the Apuseni and Banat mountains as well as the present-day Pannonian region. Consequently, the Pannonian Basin transformed into a marine embayment of the Parathethys, and the Apuseni and Banat mountains became isolated islands or peninsulas (Meulenkamp & Sissingh 2003; Popov *et al.* 2004; Golonka *et al.* 2006; Maţenco 2017)(Fig. 2C,D). Furthermore, the Eastern Carpathians themselves formed an island chain as well (Kováč *et al.* 2007). During this time frame, the Middle Miocene climatic cooling started and the tropical vegetation of Central Europe was progressively substituted with temperate species (Jiménez-Moreno 2006; Kvaček *et al.* 2006). The youngest region, the eastern part of the Southern Carpathians started its uplift ca. 12 Ma, and in the same period, the marine conditions temporarily became brackish (Palcu *et al.* 2015)(Fig. 2E). Beginning with 9 Ma, marine regression coupled with sedimentation caused progressive drying of the region and the isolated Carpathian islands began to merge (Popov *et al.* 2004)(Fig. 2F). Marine or brackish conditions still persisted in the Pannonian Lake (Magyar *et al.* 1999). Due to progressive climatic cooling, planetary sea level drop and continued sedimentation, the marine or lacustrine areas gradually dried up towards the Pliocene (Haq *et al.* 1987; Zachos *et al.* 2001; Matoshko *et al.* 2016)(Fig. 2G-H). The Carpathians continued their uplift during this time, especially their southern portions (Popov *et al.* 2004). The Pannonian Basin became a freshwater lake that eventually dried up at the end of the Pliocene and the Dacic Basin (between the Southern Carpathians and the Balkans) turned brackish and progressively became filled with sediment until the Pleistocene (Kázmér 1990; Magyar *et al.* 1999; Popov *et al.* 2006). By the end of the Pliocene, volcanic activity and uplift came to an end, with the exception of the eastern part of the Southern Carpathians. The Pleistocene glaciations left their mark only in the highest portions of the mountain range (Zasadni & Kłapyta 2014; Popescu *et al.* 2017).

Biodiversity hotspot and glacial refugium

The Carpathians are regarded as one of the most important biodiversity hotspots in Europe, yet also one of the least studied (Bálint *et al.* 2011). This diversity stems from the cumulative effects of geographical position and isolation, large surface, landscape and geomorphological heterogeneity, relatively well-preserved habitats and the apparently low impact of the Pleistocene glaciations (Mráz & Ronikier 2016). Due to their southeastern European position, the biota of the Carpathians is further enriched since it lies at the intersection of Alpine, Mediterranean and Asiatic regions (Finnie *et al.* 2007). Aside from Scandinavia and Russia, the Carpathians harbour one of the largest continuous forests in Europe, including some of the last remains of primeval forest (Oszlányi *et al.* 2004). As such, these areas are inhabited by one of the largest populations of large mammals (Chapron *et al.* 2014). The region is also characterized by high endemism, alpha and beta diversity, especially in taxa with low dispersal abilities such as high altitude plants, or subterranean arthropods (Hurdu *et al.* 2016; Iepure *et al.* 2016; Mráz & Ronikier 2016). However, endemic mammals, amphibians and especially fish are also known (Antal *et al.* 2016; Mráz & Ronikier 2016).

Due to the low ice coverage during the Pleistocene glaciations, the region acted as a major glacial refugium. The Southern Carpathians, given their location below the continuous permafrost line (Vandenberghe *et al.* 2012; Tzedakis *et al.* 2013), are prime candidates for a refugium region. However, direct evidence for microrefugia has been provided also for more northerly located Western Carpathians, based on the presence of fossil remains in plants (Willis & Van Andel 2004), mammals (Neumann *et al.* 2005; Sommer & Nadachowski 2006), molluscs (Harl *et al.* 2014a; Juřičková *et al.* 2014) and pollen deposits (Mitka *et al.* 2014). These studies revealed that patches of deciduous forests persisted in various locations across the Carpathians, enabling the survival of various species. The largest body of evidence for the role of Carpathians as a glacial refugium, albeit indirect, comes from molecular data and climatic modelling that corroborates the patterns found in the fossil data. Numerous taxa, including plants, mammals, fish, amphibians, reptiles, crustaceans, insects and molluscs bear the signatures of glacial survival in microrefugia throughout the Carpathians (Kotlík & Berrebi 2002; Kotlik *et al.* 2006; Pârvulescu *et al.* 2013; Theissinger *et al.* 2013; Fehér *et al.* 2013; Homburg *et al.* 2013; Schrimpf *et al.* 2014; Copilaş-Ciocianu & Petrusek 2015; Wielstra *et al.* 2015; Dénes *et al.* 2016; Drees *et al.* 2016; Jablonski *et al.* 2016; Copilaș-Ciocianu & Petrusek 2017; Copilaș-Ciocianu *et al.* 2017b). Estimated divergences in these taxa usually range from Late Pleistocene to Middle Miocene (0.2 to 17 Ma), suggesting that some have survived only the Last Glacial Maximum in the Carpathians while others well predate the onset of the Pleistocene glaciations (reviewed in Mráz & Ronikier 2016). In general, the divergence in plants seems to be younger than in animals with most of the splits dating back to the second half of the Pleistocene; in animals, the splits range from Holocene to the Middle Miocene (Mráz & Ronikier 2016). Among the taxa analysed so far, the oldest seem to be the freshwater amphipods belonging to the genus *Gammarus* (Copilaş-Ciocianu & Petrusek 2015; Mamos *et al.* 2016; Copilaș-Ciocianu & Petrusek 2017; Copilaș-Ciocianu *et al.* 2017b). Divergences that date back to the Miocene have also been reported in Diptera and land snails (Harl *et al.* 2014b; Dénes *et al.* 2016). Although most of the old splits were identified in the southern half of the Carpathians, it is surprising that divergences that date back to the Middle Miocene were uncovered in lineages of *G. fossarum* inhabiting the Western Carpathians, which were located much closer to the Pleistocene ice-sheets (Copilaș-Ciocianu *et al.* 2017b).

OUTLINE OF PUBLICATIONS AND MANUSCRIPT

This thesis comprises seven chapters which are structured in two main parts. Part I is the core of the thesis and consists of five chapters that are focused on the biogeography and phylogeny of epigean amphipods in the Carpathians and surrounding areas. Part II tackles some ecological and systematic aspects of these amphipods in two chapters. I am a first-author on all of the seven chapters, six of which have been published in international peer-reviewed journals while one is a submitted manuscript.

Chapter 1 represents the foundation on which this thesis is built upon. It focuses on the distribution of inland epigean freshwater amphipods in Romania, which comprises a substantial part of the Carpathian region. Although the amphipod fauna of this country is quite diverse, information on the distribution of most taxa was sketchy with small number of records from disparate regions (Cărăuşu *et al.* 1955; Petrescu 1996, 1997; Copilaș-Ciocianu & Pârvulescu 2012). Consequently, biogeographical interpretations were very limited. Based on years of field sampling and reliable literature records, we aimed at providing high resolution distribution maps that would serve as a background upon which we could form biogeographical hypotheses that would be subsequently tested using molecular tools. We have uncovered conspicuous distributional patterns among the three genera that inhabit inland waters of Romania. *Gammarus* species exhibit high altitudinal variability, patchy, allopatric distribution patterns and inhabit well oxygenated waters such as springs and streams. On the contrary, the genera *Niphargus* and *Synurella* are restricted to the lowlands and display largely sympatric distributions and high environmental tolerance.

Chapter 2 focuses on the phylogeography and phylogeny of the *G. fossarum* species complex in the southwestern Carpathians. This taxon is one of the most common of amphipods in Europe, being encountered throughout the central and southeastern parts of the continent (Barnard & Barnard 1983; Živić & Marković 2007; Copilaș-Ciocianu *et al.* 2014) and previous studies have indicated the presence of highly divergent cryptic lineages (Müller 2000; Hou *et al.* 2011; Westram *et al.* 2011; Weiss *et al.* 2014). In the southwestern Carpathians *G. fossarum* occurs in two small regions that are separated from its main European distribution area as well as from each other (Copilaș-Ciocianu *et al.* 2014). We therefore hypothesized that this distribution pattern is of relictary nature and predicted that the southwestern Carpathian lineages should be divergent from the ones found throughout Europe. Our fine-scale multilocus phylogenetic analyses indicated that the study area harbours a remarkable diversity that consists of 16 to 23 microendemic lineages (depending on species delineation method applied) exhibiting an intricate, mosaic-like distribution pattern. Their divergence times apparently date back to ca. 16 Ma, a period when a formerly contiguous landmass called Tisza Land (encompassing the present day Pannonian Basin and southwestern Carpathians) had disintegrated due to the uplift of the Eastern Carpathians and was largely replaced by the marine Lake Pannon (Kázmér 1990; Magyar *et al.* 1999; Popov *et al.* 2004). Consequently, the regions presently inhabited by *G. fossarum* in the southwestern Carpathians (i.e. Apuseni and Banat Mountains) became isolated islands or peninsulas throughout the Middle to Late Miocene. Thus, we propose that this taxon was widespread throughout the Tisza Land during the Middle Miocene and that its insular distribution in the southwestern Carpathians resulted from being stranded after the breakup of this landmass.

Chapter 3 investigates the phylogeographical and phylogenetic patterns of the most widely distributed amphipod in the Carpathians, the *G. balcanicus* species complex. This taxon is represented in the Carpathian region by two morphotypes that have a complementary distribution pattern. *G.* cf. *balcanicus*, which is morphologically similar but genetically distinct from *G. balcanicus* in a strict sense, is mostly distributed over the regions that were uplifted by 15 Ma, while *G. dacicus* inhabits newer landmasses that started their uplift ca. 12 Ma (Popov *et al.* 2004; Golonka *et al.* 2006; Mațenco 2017). Given that previous research has indicated that *G. balcanicus* is a diverse species complex with lineages that date back to the Miocene (Hou *et al.* 2011; Mamos *et al.* 2016), we hypothesized that the complementary distribution patterns of the Carpathian morphotypes represents two distinct orogenic episodes that occurred during the Middle to Late Miocene. Thus we presumed that *G.* cf. *balcanicus* would comprise older and more numerous lineages than *G. dacicus* as it is distributed over older landmasses. Our molecular analyses were consistent with this scenario and indicated that *G.* cf. *balcanicus* represents an older and more diverse radiation than *G. dacicus*, consisting of up to 20 lineages in three main clades that diverged throughout the Miocene and originated on the old palaeo-islands. Conversely, *G. dacicus* is comprised of only two lineages that diversified on the younger landmasses at the onset of the Late Miocene. These results support our hypothesis of different timings of landmass uplift and indicate that the genetic legacy of dynamic archipelagos can persist in continental biotas millions of years after their disappearance.

Chapter 4 compares the diversity of the *G. fossarum* complex between the Bohemian Massif and the Western Carpathians. The border between these geomorphologically distinct regions is considered an important biogeographical boundary for invertebrates as well as plants (e.g., Meusel *et al.* 1965; Illies 1978), and our preliminary analyses indicated that some cryptic diversity within *G. fossarum* exists at the border area between Czechia and Slovakia. Our aim was to test if the observed diversity patterns are more likely to stem from recent factors such as drainage divides and environmental conditions, or historical circumstances such as survival in glacial refugia. The results indicated that the two regions exhibit contrasting patterns regarding the diversity of lineages and also their demographic histories. Two lineages which exhibit local signatures of recent population expansion inhabit both regions. However, the Western Carpathians harbour six additional lineages that display a relict pattern. These lineages are phylogenetically diverse and apparently diverged throughout the Miocene, and two of them could not be attributed to any of the three previously known major clades of *G. fossarum*. Seven out of eight lineages were found in adjacent drainages and the four most widespread ones had overlapping altitudinal ranges, suggesting that the drainage divides and local environmental conditions do not play a major role in shaping their distributions. Therefore, due to its old age and relictual pattern, the observed diversity is better explained by the differential survival of lineages during the Pleistocene glaciations across the studied region. The results indicate that the conditions in the Western Carpathians, probably local hydrogeology, promoted the long-term survival of ancient lineages through millions of years of climatic fluctuations closer to the northern ice sheets than previously thought possible.

Chapters 5 and **7** examine the phylogenetic positions, phylogeography, ecology, functional morphology and taxonomy of two epigean *Niphargus* species, *N. hrabei* and *N. valachicus*. **Chapter 7** compared their distribution patterns, ecologies and functional morphologies, and tested the taxonomic validity of two previously described similar taxa occurring in the Danube basin. The results indicated that *N. hrabei* and *N. valachicus* are weakly differentiated ecologically, exhibiting little ecological and functional morphological differentiation. Due to its larger size *N. valachicus* might be a stronger competitor while *N. hrabei* could be a better disperser due to its wider environmental tolerance. Despite large-scale sympatry, the frequency of co-occurrence was very low, indicating competitive exclusion and further evidence for weak ecological differentiation. We also synonymized the previously described species *N. thermalis* and *N. mediodanubialis* with *N. hrabei* and *N. valachicus*, respectively, due to high morphological and genetic similarities.

Although chapter 7 investigated the distribution patterns, ecology and taxonomy of *N. hrabei* and *N. valachicus*, in **chapter 5** we focused on their evolutionary ecology and phylogeography. These species are atypical because they inhabit surface waters and have very large geographical ranges, whereas most of their congeners are narrowly endemic species that inhabit groundwater, although some of them also have populations that inhabit the surface/subsurface boundary (Fišer *et al.* 2014; Copilaș-Ciocianu *et al.* 2017a). This suggests that *N. hrabei* and *N. valachicus* colonized surface waters from subterranean ancestors and that this ecological shift could have promoted their large-scale dispersal. Phylogenetic and ancestral state analyses indicated that these two species are not closely related and have independently invaded surface waters from groundwater ancestors. Their morphologies are more similar to one another than to other ecotonal species, suggesting a convergent evolution due to similar ecology. Their phylogeographies reveal dispersal episodes across the Danube lowlands throughout the second half of the Pleistocene. We concluded that the ecological barrier between the surface and subterranean environment might not be as impenetrable as previously thought. Therefore, adaptation to groundwater is not a one way evolutionary path and subterranean species can occasionally recolonize and widely disperse in surface waters.

Chapter 6 analyses the life-history strategies of three species which occasionally co-occur throughout the Danube lowlands, *G. dacicus*, *N. valachicus* and *Synurella ambulans*. Due to their distant phylogenetic relationships (each belonging to a different family) and conspicuous morphological differences, we predicted that they should exhibit divergent life-history strategies, a common feature of species in ecological communities (Bonsall *et al.* 2004). Monthly sampling during a one-year period indicated significant differences among species regarding fecundity, size at maturity, number of generations and the duration and timing of the reproductive period. The most fecund species was *S. ambulans*, *G. dacicus* had the highest reproductive potential, while *N. valachicus* was the least fecund although females matured at a relatively smaller body size than those of the other two species. Despite overlap in some life-history traits, each species possessed a unique combination. Although the factors that are responsible for these differences are not yet clear, this phylogenetically diverse assemblage might represent a suitable model system for further work focusing on the ecology of poorly studied native freshwater amphipod communities.

CONCLUSIONS AND FUTURE PERSPECTIVES

The results presented in this thesis constitute a significant step forward in understanding the biogeography of Carpathian amphipods and permanent aquatic fauna in general. Very little was known about the diversity of Carpathian amphipods before this work was started. Even the distribution patterns of morphospecies were unclear and incomplete. At the end of my studies I can confidently say that the Carpathians are one of the most thoroughly studied and best known regions regarding freshwater amphipod biogeography. The complex geomorphological history of these mountains coupled with the limited dispersal, old age and ubiquity of their freshwater amphipod fauna provided a particularly suitable model system for testing explicit historical biogeographical hypotheses at fine spatial scales. The results of this thesis reveal that the evolution of Carpathian amphipods is profoundly connected with that of the mountains they inhabit. The turbulent geological and climatic history of the region has left deep genetic signatures on contemporaneous diversity patterns, promoting ancient diversification of lineages as well as recent ecological shifts. Therefore, biology can inform geology and *vice versa*. As such, freshwater amphipods can be regarded as powerful indicators of deep biogeographic history and can improve our insight on the connection between patterns and processes in biogeography.

Below I outline what I think could be relevant avenues for future research.

Carpathian biogeography

Further phylogeographic research should be focused on different organismal groups in order to test the generality of the patterns outlined in this thesis. This could be aimed at groups with limited dispersal abilities, high intra- and interspecific genetic differentiation and old age such as subterranean fauna, Opiliones, the spring-habitat specialist turbellarian *Crenobia alpina*, and terrestrial or aquatic gastropods. So far, some similarity between patterns of endemism seem to exist in the Southern Carpathians between plants and *Gammarus* amphipods (Hurdu *et al.* 2016; Copilaș-Ciocianu & Petrusek 2017). Future integrative studies are also needed to shed light on the generality of these patterns.

On more local scales, the role of deep circulation mineral springs in the Western Carpathians as stable habitats throughout the Pleistocene glacial cycles (as hypothesized in Chapter 4) could be tested by comparing levels of genetic diversity within local communities of permanent aquatic fauna with comparable regions without such springs, or in other areas with similar hydrogeology outside the Carpathians.

Factors promoting microscale divergence in *Gammarus*

The results of this thesis provide a general biogeographical framework but do not give many direct clues as to how lineage splitting has occurred in Carpathian *Gammarus* or even if these morphologically cryptic entities are distinct biological species. Although the deep splits can be associated with ancient historical factors, the data available so far indicates that there is a high diversity of relatively young lineages (of Late Pliocene and Pleistocene age) at the microgeographic scale. For instance, almost every sampling locality contained endemic haplotypes or lineages separated by only a few kilometres in some cases. There are also several cases of syntopy, so far without molecular evidence for hybridization. The factors that promote isolation at such local scales are unknown. Physical barriers seem to be unlikely in this case. Future research should thoroughly investigate these patterns across local scales and along environmental gradients to identify if adaptive or non-adaptive processes or even their interplay are behind these patterns of microendemicity and morphological stasis. Data on *G. fossarum* in human-impacted landscapes indicate strong genetic isolation of populations on the local scale which is further strengthened by anthropogenic impacts (Weiss & Leese 2016). Further lines of evidence apart from genetic data should be investigated in order to understand if these cryptic lineages do indeed represent distinct species. These could come in the form of mate choice experiments, comparative functional morphology, ecological niche, behaviour, comparative parasitology, or cytogenetics. Several recent examples suggest that cryptic lineages of *G. fossarum* in Western Europe are most likely distinct species as they can differ with respect to habitat requirements, dispersal, ecological niche, levels of parasite infections, and probably also exhibit premating isolation barriers (Westram *et al.* 2013; Lagrue *et al.* 2014; Galipaud *et al.* 2015, 2017; Eisenring *et al.* 2016). Cryptic taxa of the subterranean genus *Niphargus* seem to play similar ecological roles at regional but not at local scales, suggesting that ecological equivalence relates to the scale of the study (Fišer *et al.* 2015; Delić *et al.* 2017).

Amphipods play a key role in nutrient cycling throughout aquatic ecosystems and their restricted dispersal can quickly lead to local adaptation and genetic isolation. Therefore, they are likely to be used as target taxa for studies on adaptation, speciation, adaptive radiation, evolutionary ecology and eco-evolutionary dynamics also in the coming years.

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