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Elucidating phylogeographic patterns of minnows in complex aquifers of
the Dinaric Karst

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ABSTRACT

Karst landscapes, covering approximately 14% of the Earth's land surface, harbor complex ecosystems shaped by geological processes such as the dissolution of soluble rocks. These landscapes feature complex underground water systems connected to surface water, underscoring the importance of understanding underground pathways within karst aquifers for both karst hydrology and biodiversity studies. The Dinaric Karst boasts rich biodiversity, notably characterized by its endemic freshwater fish fauna.

Chapter 1 presents a comprehensive analysis of distribution patterns and biology based on the mitochondrial genetic lineages of four genera of Dinaric Karst minnows: *Delminichthys*, *Phoxinellus*, *Phoxinus*, and *Telestes*. In this chapter, the influence of vicariance and dispersal in shaping species ranges was assessed. While *Delminichthys*, *Phoxinellus*, and Dinaric *Telestes* share a similar lifestyle adapted to sinking streams in karst poljes and thus have a restricted distribution, *Phoxinus* is widespread with an unconventional distribution pattern, unaffected by current hydrological barriers. *Phoxinus* distribution ranges often cross drainage and basin boundaries, and suggest the ability for underground dispersal. Conversely, the other three genera are restricted by their specialization of dwelling in sinking streams within karst poljes and cannot disperse along main river courses.

Chapters 2 and 3 shift focus to the widespread, surface-dwelling species *Phoxinus lumaireul*, on the influence of karst hydrology on its population genetic structure, as well as its dispersal ability through the karst landscape using underground connections. Chapter 2, based on mitochondrial DNA and one nuclear gene, identified significant genetic diversity and highlighted the influence of ongoing gene flow through underground connections and remnants of past hydrological networks on population structure. Building upon the results of Chapter 2, Chapter 3 further explored the impact of karstification on the genetic diversity and adaptation of *P. lumaireul* by comparing genetic structure between populations connected through underground water links with those connected through surface water network. Genome-wide data derived from ddRAD (double digest restriction-site associated DNA) sequencing revealed complex population structures influenced by historical climatic fluctuations rather than karst hydrology. Despite widespread distribution, localized adaptations and limited gene flow between river systems render *P. lumaireul* populations vulnerable to extinction events, particularly due to anthropogenic disturbances and habitat degradation.

In conclusion, this thesis advances our understanding of the intricate dynamics of the Dinaric Karst ecosystem by integrating genetic approaches with hydrological and geological knowledge. The findings underscore the importance of holistic management strategies for the conservation of the unique biodiversity in karst regions.

ZUSAMMENFASSUNG

Karstlandschaften, die etwa 14 % der Landfläche der Erde bedecken, beinhalten komplexe Ökosysteme, die durch geologische Prozesse wie die Lösungsverwitterung entstanden sind. Diese Landschaften weisen komplexe unterirdische Wassersysteme auf, die mit Oberflächengewässern verbunden sind. Die Kenntnis und das Verständnis dieser unterirdischen Verbindungen innerhalb der Karstaquifere, ist nicht nur für die Karsthydrologie von Bedeutung, sondern auch für die Biodiversitätsforschung. Der Dinarische Karst zeichnet sich durch seine hohe Biodiversität aus, die unter anderem eine beachtliche Anzahl an endemischen Fischarten aufweist.

Kapitel 1 enthält eine umfassende Analyse der phylogeographischen Verbreitungsmuster von vier Gattungen kleiner Süßwasserfische im Dinarischen Karst: *Delminichthys*, *Phoxinellus*, *Phoxinus* und *Telestes*, auf der Grundlage der mitochondrialen genetischen Linien. In diesem Kapitel wurde der Einfluss von Vikarianz und Ausbreitung auf die Verbreitungsgebiete der Arten untersucht, wobei unterschiedliche Verbreitungsmuster festgestellt wurden. Während *Delminichthys*, *Phoxinellus* und dinarische *Telestes* eine ähnliche Lebensweise, die an Bäche in Karstpoljes angepasst ist, aufweisen, zeichnet sich *Phoxinus* durch ein ausgedehntes Verbreitungsmuster aus, das wenig durch aktuelle hydrologische Barrieren beeinflusst wird und häufig Fluss- und Einzugsgebietsgrenzen überschreitet und damit auf eine unterirdische Ausbreitungsfähigkeit hinweist. Die anderen drei Gattungen sind durch ihre Spezialisierung auf das Leben in Karstflüssen innerhalb von Karstpoljes begrenzt, was ihre Ausbreitung entlang der Hauptflüsse behindert.

Kapitel 2 und 3 befassen sich mit der weit verbreiteten, oberirdisch lebenden Art *Phoxinus lumaireul*, dem Einfluss der Karsthydrologie auf die genetische Struktur ihrer Populationen und ihrer Fähigkeit, sich über unterirdische Verbindungen in der Karstlandschaft auszubreiten. In Kapitel 2 wurde auf der Basis der Daten der mitochondrialen DANN und einem Kerngen eine beträchtliche genetische Diversität festgestellt und der Einfluss des kontinuierlichen Genflusses über unterirdische Verbindungen sowie historischer hydrologischer Netzwerke auf die Populationsstruktur hervorgehoben. Aufbauend auf den Ergebnissen von Kapitel 2 wurden in Kapitel 3 die Auswirkungen der Verkarstung auf die genetische Diversität und Adaptation von *P. lumaireul* vergleichend untersucht. Genomweite ddRAD Sequenzdaten (double digest restriction-site associated DNA) zeigen komplexe Populationsstrukturen, die eher durch historische Klimaschwankungen als durch die Karsthydrologie beeinflusst wurden. Trotz der weiten Verbreitung sind die Populationen von *P. lumaireul* aufgrund lokaler Anpassungen und

des begrenzten Genflusses zwischen den Flusssystemen anfällig für Aussterbeereignisse, insbesondere durch anthropogene Störungen und Beeinträchtigungen des Habitats.

Zusammenfassend erweitert diese Arbeit das Verständnis der komplexen Dynamik des dinarischen Karst-Ökosystems durch die Integration genetischer Ansätze mit hydrologischem und geologischem Wissen. Die Ergebnisse unterstreichen die Bedeutung umfassenden Managementstrategien zur Erhaltung der einzigartigen Biodiversität in Karstgebieten.

GENERAL INTRODUCTION

Understanding how different habitats influence biodiversity is crucial for ecological research and conservation genetics. Karst landscapes, with their complex networks of caves, sinkholes, and underground rivers are vital yet vulnerable ecosystems. Climate change, pollution, and invasive species jeopardize these environments and the diverse flora and fauna they support (e.g., Fišer et al., 2022; Kačaroğlu, 1999; Šilc et al., 2020; Sket, 1999). Therefore, understanding the factors which create conditions for rich biodiversity in karst landscapes is crucial for conservation of these unique ecological niches.

The primary objective of this thesis is to investigate the (underground) dispersal abilities and their influence on phylogeographic patterns of epigean and subtroglophile fish species within karst landscapes. Part of this study thus represents, to the best of my knowledge, the first comprehensive population genetic and phylogeographic analysis of small epigean fish species inhabiting complex karst systems. Given that underground migration is seldom considered in biogeographical studies, despite karst landscapes representing approximately 14% of the world's surface (e.g., Williams, 2008), insights into subterranean dispersal could significantly influence our understanding of biodiversity patterns. Additionally, the methodology of integrating genetic with hydrological data will influence karst hydrological studies, and offering additional insights into paleohydrological dynamics in karst systems. Furthermore, the findings of this thesis hold relevance beyond ichthyological research, with potential implications for all aquatic organisms dwelling in karst regions.

Geological evolution of the Balkan Peninsula: Shaping freshwater ichthyofauna diversity

The Central Peri-Mediterranean region covers the Balkan and the Italian Peninsula, and is distinguishable among the seven biogeographical regions of Europe for its remarkable species diversity and high level of endemism (Bănărescu, 1989, 1992; Bianco, 1990; Reyjol et al., 2006). This diversity is attributed to significant geological transformations over the past 30 million years, resulting in a complex landscape (Grabowski et al., 2017). In the Early Oligocene period (approximately 30 million years ago) the Turgai Strait, which had previously separated Europe from Asia, dried out (Rögl & Steininger, 1981). When Europe and Asia were finally connected, the initial migration of Cypriniform fish (Actinopterygii: Cypriniformes) from Asia to Europe started (e.g., Bănărescu, 1989; Tang et al., 2008; Zardoya & Doadrio, 1999). During

the Miocene epoch (from the end of the Burdigalian through the Langhian stage, approximately 15.9-13.8 million years ago), central and southern Balkan regions became increasingly separated from the European landmass (Rögl, 1999). For European freshwater fishes, geomorphological events fostering speciation and shaping population structure (e.g., Dubut et al., 2012) are usually associated with three stages in geological history. (i) First is the Messinian Salinity Crisis, approximately 5.96 MYA by the end of the Late Miocene, (ii) followed by the oligosaline phase of the Paratethys, known as the "Lago Mare" phase (around 5.5–5.33 MYA) (Hsü et al., 1977; Krijgsman et al., 1999). Final is (iii) the Quaternary period, marked by cyclic glacial events from 2.4 million years ago to the Last Glacial Maximum (LGM) around 24,000 to 18,000 years ago (Heiri et al., 2014). This geological and climatic changes influenced the formation of hydrological networks, creating a complex system with large lake systems, fragmented drainages, and river captures (Grabowski et al., 2017). They played a significant role in shaping the diverse freshwater fish fauna found on the Balkan Peninsula, as indicated by several studies (e.g., Bănărescu, 1992; Bianco, 1990; Ketmaier et al., 2004; Perea et al., 2010; Schönhuth et al., 2018).

Study area - The Dinaric Karst

The movement of the African tectonic plate towards the north, leading to its collision with the Eurasian plate approximately 20 million years ago during the Neogene period, resulted in the Tethys regression and the uplift of the Hellenic and Dinaric Mountains (Zupan Hajna, 2019). In Quaternary, intense neotectonics movements of the Dinaric Mountains exposed the underlying carbonate rock and enabled the development of karst landscape on the outer belt of the mountain chain – the Dinaric Karst (Zupan Hajna, 2019). This complex geological landscape situated in the western region of the Balkan Peninsula and spanning the Adriatic littoral belt, is characterized by a massive system of limestone and dolomite formations exposed to continuous karstification: a process by which water dissolves soluble rock (Bosak, 2008). Karstification was particularly intense during the Pliocene and early Pleistocene (Mihevc, 2001; Trontelj et al., 2007; Zupan Hajna et al., 2011). With progressing karstification, surface streams moved underground, causing complex hydrographical changes (Ford & Williams, 2007). Influenced by lithology, tectonic structures, climate, and geomorphic processes, the hydrology of the Dinaric Karst drains water towards the Adriatic and Black Sea (Zupan Hajna, 2019). Despite abundant precipitation, surface water flows are scarce (Mihevc et al., 2016). While large rivers only occasionally cross the carbonate belts of the Dinaric Karst, sinking rivers are common in karst aquifers. Karstic depressions, known as poljes, are connected to each other

through underground water connections (e.g., Bonacci et al., 2013; Bonacci & Jelin, 1988; Mihevc et al., 2010; see Fig. 1). Due to the dynamic nature of hydrological processes and interconnectivity among different water bodies, karst aquifers are particularly susceptible to contamination (Petrič et al., 2020).

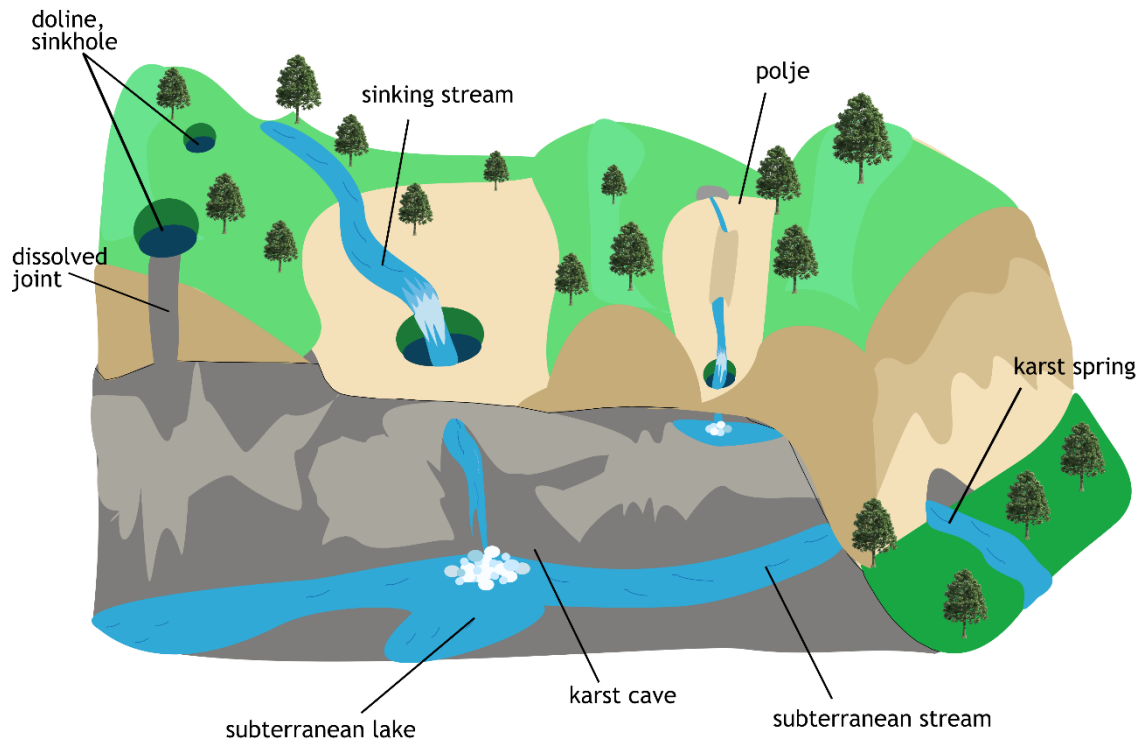


Fig. 1 Schematic overview of karst aquifer, showing characteristic karst features.

Thus, the knowledge and understanding of the underground pathways within a karst aquifer connecting karst poljes is of importance not only for karst hydrology, but also for biodiversity studies. The Dinaric Karst hosts a diverse array of species, that have evolved to thrive within its complex ecosystem, making it a hotspot of European biodiversity (Sket, 1999). The Dinaric Karst is characterized by a noticeably high endemism of freshwater ichthyofauna (Jelić & Jelić, 2015; Lukač et al., 2023).

Focus on the north-western Dinaric Karst

While Chapter 1 incorporates the main river systems of the Dinaric Karst, Chapters 2 and 3 primarily focused on the north-western part of the Dinaric Karst, mainly spanning the territory of Slovenia. Despite the modest size of Slovenia compared to other European countries, it exhibits a considerably high biodiversity per unit area, ranking second highest in biodiversity index across Europe (e.g., Mršić et al., 1997; Šilc et al., 2020). Slovenia is situated at the

convergence of four distinct biogeographical units – the Mediterranean, Pannonian, Alpine, and Dinaric regions (Mršić et al., 1997). The karst of Slovenia stands as *locus typicus* for karst landscapes globally, with research dating back to the late 19th century (Mihevc et al., 2016). As a result, Slovenia's karst areas are labelled the "Classical Karst," and many Slovene terms have been integrated into international karst terminology (e.g., Williams, 2008). Due to the extensive research conducted over centuries in this area of the Dinaric Karst, the hydrological system, including underground connections, has been comprehensively studied through more than 200 tracing tests. The results of these studies have been recently digitized (Petrič et al., 2020). Tracing tests are the most common and effective method utilizing fluorescent dyes or soluble salts to track the movement of water through underground connections, providing valuable insights into the hydrological connectivity and flow pathways within karst landscapes (Field, 1999). Thus, the well-studied aquifer of the north-western Dinaric Karst stands out as an exceptional model for further studies that integrate phylogeographic and population genetic data with hydrological information.

Study organisms – Minnows inhabiting the Dinaric Karst

My thesis focuses on four genera of small minnows occurring in the Dinaric Karst region: *Delminichthys*, *Phoxinellus*, *Phoxinus*, and *Telestes*, all belonging to the family Leuciscidae. While *Phoxinus* belongs to the subfamily Phoxininae, the other three belong to the subfamily Leuciscinae. The Leuciscidae family comprises six subfamilies with distribution ranges in Europe, Asia, and North America. Formerly, all six subfamilies were combined under the subfamily Leuciscinae within the family Cyprinidae, until the elevation to family status as Leuciscidae (Schönhuth et al., 2018; Stout et al., 2016).

The taxonomic relationships among palearctic Leuciscinae were debated, initially grouping various small Mediterranean leuciscins under the genus *Phoxinellus*, which was later revised (summarized in Freyhof et al. (2006)). In 2003, the genus *Phoxinellus* was restricted to ten species, all endemic to Dalmatia (Bogutskaya & Zupancic, 2003). However, in 2006, three of these species were reclassified under the genus *Telestes*, and the genus *Delminichthys* was introduced with four endemic species in the Dinaric Karst (Freyhof et al., 2006). Further cryptic species were later identified within the genus *Telestes* (Bogutskaya et al., 2012). While *Telestes* includes both widespread and endemic species, *Delminichthys* and *Phoxinellus* are limited to river systems in the Dinaric Karst, making both genera endemic to this region.

The genus *Phoxinus*, including many widespread species, harbors surprising species diversity. Initially, numerous species were identified in Europe, but they were later merged into the "common" minnow *P. phoxinus*. However, in 2004 and 2007, based on morphology, new species were described or old ones re-validated in Spain and France (Bogutskaya & Naseka, 2004; Kottelat, 2007). The true complexity of *Phoxinus* species was revealed from 2014 onwards with genetic research, highlighting the challenge of morphological differentiation and leading to the recognition of 14 species out of 23 evolutionary lineages in Europe (e.g., Bogutskaya et al., 2023; Corral-Lou et al., 2019; Denys et al., 2020; Geiger et al., 2014, Palandačić et al., 2015, 2017, 2020; Vučić et al., 2018). *Phoxinus* species do not strictly adhere to river catchment areas like other leuciscids, complicating identification and leading to hybrid formation when different genetic lineages come into contact (e.g., Palandačić et al., 2022). They have been introduced into various habitats, becoming invasive species in some regions (García-Raventós et al., 2020; Miró & Ventura, 2015; Museth et al., 2007). Therefore, native *Phoxinus* minnows face threats from both human activities and environmental changes, such as pollution and rising temperatures. The diversity of *Phoxinus* species is a good example, that hidden diversity can exist even in well-studied regions.

In Chapters 2 and 3 of my thesis, I primarily focus on the species *Phoxinus lumaireul*, which inhabits rivers draining the southern slopes of the Alps and the Dinaric Karst region in central and southern Europe. Within the Adriatic Sea basin, its range extends eastward from the Po River in Italy to the Zrmanja River in Croatia, including Istria and the island of Krk (e.g., Palandačić et al., 2015, 2017, 2020; Vučić et al., 2018). Isolated subpopulations are found in the Esino and Potenza rivers in central Italy, both of which were connected to the Po during the last glacial maximum (Caputo Barucchi et al., 2022). Additionally, *P. lumaireul* is found in the Danube river system, with most confirmed records occurring in tributaries of the upper Drava River downstream to the Mur River, and the upper Sava River downstream to the Una River (Palandačić et al., 2015, 2017, 2020; Vučić et al., 2018). However, the precise limits of its range in the Danube catchment remain unclear, and additional isolated subpopulations inhabit the Drina, Kolubara, and Svrliški Timok rivers in Serbia (Palandačić et al., 2020). To date, six distinct mitochondrial sub-lineages have been identified, with most restricted to the Western Balkans region (Palandačić et al., 2017). This somewhat patchy distribution is attributed to a complex evolutionary history (e.g., Palandačić et al., 2015, 2020; Vučić et al., 2018). Due to its genetic diversity and distribution in the north-western Dinaric Karst, *P. lumaireul* is a suitable model species to study population dynamics in a complex karst aquifer.



Fig. 2. *Phoxinus phoxinus*

Research aims and chapter overview

In my thesis I aim to assess the impact of karstification, geological and hydrological history, and the current hydrology of the Dinaric Karst region on the distribution ranges, dispersal abilities and population structures of small freshwater fishes belonging to the family Leuciscidae. With karst terrain covering approximately 14% of the Earth's land surface (Williams, 2008), understanding organismal dispersal in such environments holds significance for phylogenetic, population genetic, and biodiversity studies. While past research on biogeographic patterns in the Dinaric Karst has primarily focused on cave-dwelling species (e.g., Delić et al., 2022; Recknagel et al., 2023; Trontelj et al., 2007; Zakšek et al., 2009), it is equally crucial to investigate surface-dwelling organisms to comprehensively understand regional biodiversity and ecological dynamics.

Chapter 1: The distribution of many Dinaric Karst fish species, especially those inhabiting underground hydrological networks for several months (so-called sub-troglophiles according to (Sket, 2008)), remains poorly documented. This lack of clarity is compounded by the complex ecology of these species and limited data availability for their characterization. Therefore, the aim of Chapter 1 is to provide a comprehensive overview of the phylogeographic and distribution patterns of four genera of the family Leuciscidae (*Delminichthys*, *Phoxinellus*, *Phoxinus*, and *Telestes*) based on their mitochondrial genetic lineages. Subtroglophiles, as well as surface-dwellers, widespread as well as endemic species are included in this comparative phylogeographic approach. Through molecular dating, the research aims to estimate the divergence time of species within these genera and discern whether the observed distribution patterns were shaped by vicariance or dispersal events. The study also offers potential

explanations for the differences in species ranges within and between the genera, by conducting an extensive literature review on the biology of these species.

Chapter 2: The objective of this chapter is to investigate the genetic patterns of *Phoxinus lumaireul* in the north-western part of the Dinaric Karst in Slovenia. The high genetic diversity exhibited by *P. lumaireul*, along with its presence in both non-karstic surface streams and interconnected subterranean karstic streams within the well-explored Slovenian Dinaric Karst system, presents a unique chance to elucidate how a surface-dwelling fish species has evolved in response to karstic environments. Hence, the study explores three scenarios to account for the observed genetic patterns, utilizing data from two mitochondrial genes and one nuclear gene: (1) ongoing gene flow through underground connections, (2) remnants of historical hydrological networks, and (3) human-induced translocations.

Chapter 3: The main objective of this chapter is to further explore the impact of karstification on the genetic diversity and adaptation of *P. lumaireul* in karst environments, building upon the findings of Chapter 2. The study expands the sampling sites to include additional specimens, particularly from the surrounding regions of the Dinaric Karst. By employing genome-wide single nucleotide polymorphism (SNP) data obtained through ddRAD (double digest restriction-site associated DNA) sequencing, this chapter aims to assess the impact of karst hydrology by comparing the population genetic structure of *P. lumaireul* populations both within and outside karst areas. Furthermore, the chapter investigates the potential for underground dispersal of *P. lumaireul* in the karst landscape with the aim to compare it as a genetic tracer with the findings of the traditional tracing tests, reported in the literature (Petrič et al., 2020).

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CHAPTER 1

Comparative Phylogeography of *Phoxinus*, *Delminichthys*, *Phoxinellus* and *Telestes* in Dinaric Karst: Which Factors Have Influenced Their Current Distributions?

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Article

Comparative Phylogeography of *Phoxinus*, *Delminichthys*, *Phoxinellus* and *Telestes* in Dinaric Karst: Which Factors Have Influenced Their Current Distributions?

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Abstract: The waters of the Dinaric Karst drain into both the Adriatic and the Black Sea basins. Precipitation is high, yet surface waters are scarce, with the exception of sinking streams. Dinaric Karst is a biodiversity hotspot, with diverse speleo- and epigean freshwater fauna. The distribution patterns of taxa in Dinaric Karst have arisen from a combination of vicariance and dispersal and their alteration over time. Within fishes, there are genera that include both species that are widespread and species with restricted ranges (e.g., *Phoxinus*, *Telestes*), and genera with only restricted distributions (e.g., *Delminichthys*, *Phoxinellus*). Some (*Delminichthys*, *Phoxinellus* and Dinaric *Telestes*) have a similar lifestyle, specialized for dwelling in sinking streams in karst poljes. The present study compares the distribution ranges of *Phoxinus*, *Delminichthys*, *Phoxinellus* and *Telestes* in Dinaric Karst based upon their mitochondrial genetic lineages, including dating of divergence times and reconstruction of ancestral geographic ranges. The biology of *Phoxinus* has played a major role in its wider distribution than the other three genera, which exhibit some limited ability to migrate underground, but which cannot disperse along main river courses due to their specific adaptation for dwelling in the sinking streams of karst poljes.

Keywords: Dinaric Karst; subterranean; stygophily; freshwater fishes; molecular clock; molecular phylogeny; vicariance; dispersal; endemism; Phoxininae; Leuciscinae

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1. Introduction

Karst landscapes originate from water dissolving soluble base rocks (e.g., limestone and dolomite) and are characterized by an array of surface and underground geomorphological features, as well as a complex water system [1]. One of the best researched karst areas is Dinaric Karst, located in the western Balkans and extending from Italy to Albania along the Adriatic coast. The hydrology of this mostly carbonate region is well studied, with water draining toward both the Adriatic and the Black Sea basins [2]. Despite the high levels of precipitation, surface water courses are scarce and mostly take the form of sinking streams flowing on karst poljes (see study area for the definition) that are often interconnected underground [2]. Dinaric Karst is a biodiversity hotspot, and while it is especially known for its speleofauna [3], there is also a high species richness of epigean organisms [4–6]. Furthermore, the region is known for its diverse freshwater fauna [7,8], including fishes that are characterized by numerous endemics [9–11].

As in other areas, the distribution patterns of organisms or species in Dinaric Karst are allocated to two factors involved in speciation processes, namely, vicariance and dispersal. Among the macro events are the orogenesis of the Dinarids and the Alps, e.g., [12–

16], as well as climatic changes with reoccurring glacial periods, during which the Balkan Peninsula has repeatedly served as a faunal refugia [17], with most modern phylogeographic studies indicating the presence of multiple microrefugia forming a ‘refugia within refugia’ pattern [6,7,18]. Another important factor contributing to vicariant events were regional paleohydrological processes such as pre-Pleistocene changes in catchments and isolation of drainages [19,20]. On a smaller scale, there was the process of karstification, for example the formation of karst poljes, which caused fragmentation and further speciation of the resident biota [21,22]. Meanwhile, dispersal took place following glacial periods, when the taxa spread out of their refugia [23]. In Dinaric Karst, dispersal on a smaller scale could be assigned to extreme oscillations in the groundwater level, causing over-spills and floods. An additional, obvious means of dispersal would be through underground water connections that link surface streams or underground water bodies, or both, e.g., [24,25]. However, a combination of vicariance and dispersal, and their alteration throughout history, probably produced the distribution patterns observed today [26,27]. Finally, in fish, but also in other freshwater taxa, the impact of human translocations on current species ranges need to be considered [28,29].

The distribution patterns of fishes in Dinaric Karst can be divided roughly into two categories. On the one hand, there are some genera that include both widespread species, distributed among several river drainages and even outside of the region, and species with restricted ranges (e.g., only upper reaches of a river system or a few sinking streams) within Dinaric Karst, such as *Chondrostoma* and *Squalius* [30–32]. On the other hand, there are genera endemic to Dinaric Karst (e.g., *Aulopyge*, [31,33]). The first category includes the ubiquitous genus *Phoxinus* with numerous species common all over Europe but with an unusual distribution pattern. In contrast to other members of Leuciscidae [34–36], *Phoxinus* ranges do not seem to be affected by current hydrological barriers, often traversing drainage and basin boundaries [37]. Within Dinaric Karst, there are some *Phoxinus* species (hereinafter, Dinaric *Phoxinus*) with a distribution range restricted to a single river drainage, such as the endemic *P. krkae* Bogutskaya, Jelić, Vucić, Jelić, Diripasko, Stefanov and Klobučar, 2019, inhabiting the upper Krka drainage in Croatia [38]. In contrast, there are widespread species, for example, *P. csikii* Hankó, 1922, which is distributed in the Danube drainage of the Balkan Peninsula, as well as Central Europe, and *P. lumaireul* (Schinz, 1840), which inhabits both Adriatic and Black Sea drainages [37,39,40]. The genus *Telestes* also falls within the first category, though there are only a few species in more than one river drainage (for details see [41,42]), while nine of the ten species occurring in Dinaric Karst are restricted to only one polje, or a system of a few karst poljes (hereinafter, Dinaric *Telestes*, excluding *T. montenigrinus* (Vukovic, 1963) in the Lake Skadar, or Lake Shkodër, basin). In the second category, there are *Delminichthys* and *Phoxinellus*, which are endemic to Dinaric Karst. However, species in these genera, along with Dinaric *Telestes*, share a peculiar lifestyle specialized for dwelling in sinking streams in karst poljes.

Species of *Delminichthys*, *Phoxinellus* and Dinaric *Telestes* are subtroglophiles (definition according to [43]), or stygophiles (definition according to [44]), and while they spend up to several months underground, they require surface habitats for spawning [45–48]. These species lack obvious cave adaptations such as reduced eyes and pigmentation, though they exhibit other characteristics that might be connected to their partially subterranean habitat, such as some thickening of the skin, reduced cephalic sensory canals and increased number of poorly ossified, deeply embedded scales [49]. Nevertheless, reduced scalation has also been observed in *Phoxinus* [38], which can be classified as troglaxene (an accidental occupant of subterranean streams [43]). In addition, sharing a scalation pattern, the members of all four genera are cold-adapted species, with similar dietary preferences [50–53]. While similar in these aspects, the genus *Phoxinus* and the three other genera (*Delminichthys*, *Phoxinellus* and Dinaric *Telestes*) are phylogenetically distant, belonging to subfamilies Phoxininae and Leuciscinae, respectively [54]. Thus, their common features are either homoplastic specializations or leuciscid symplesiomorphies.

Several recent studies of *Phoxinus*, *Delminichthys*, *Phoxinellus* and *Telestes* in Dinaric Karst deal with their phylogeny [39,55–57], population structure [24], morphology [58] and taxonomy [49,59], yet these are mostly studies of selected single species (e.g., *D. adspersus* [24]), based on low number of genetic markers (e.g., [56]) and lack overarching findings. Together with small distribution ranges in some of the species (e.g., *Telestes fontinalis* (Karaman, 1972) on Kravsko polje, some 67 km² in size; [29]) and anthropogenic pressure in the form of pollution and introduction of non-native fish species, the Dinaric Karst endemics are seriously endangered [44]. Comparing the distributional data of the four genera with the available literature on the biology of these species might suggest some reasons for the difference in species ranges within and between *Phoxinus*, *Delminichthys*, *Phoxinellus* and *Telestes*. Thus, the goal of the present study was to compare the phylogeography of the four fish genera in Dinaric Karst based on their mitochondrial (mt) genetic lineages. Molecular dating was performed to estimate the time of divergence of the species within the genera and statistical estimates of whether they were a consequence of vicariance or dispersal. Some explanations are offered to explain the observed differences between the distribution patterns, and some suggestions given on whether Dinaric karstification could be a factor in promoting either vicariance or dispersal.

2. Materials and Methods

2.1. Study Area

The study area—Dinaric Karst—is presented in Figure 1.

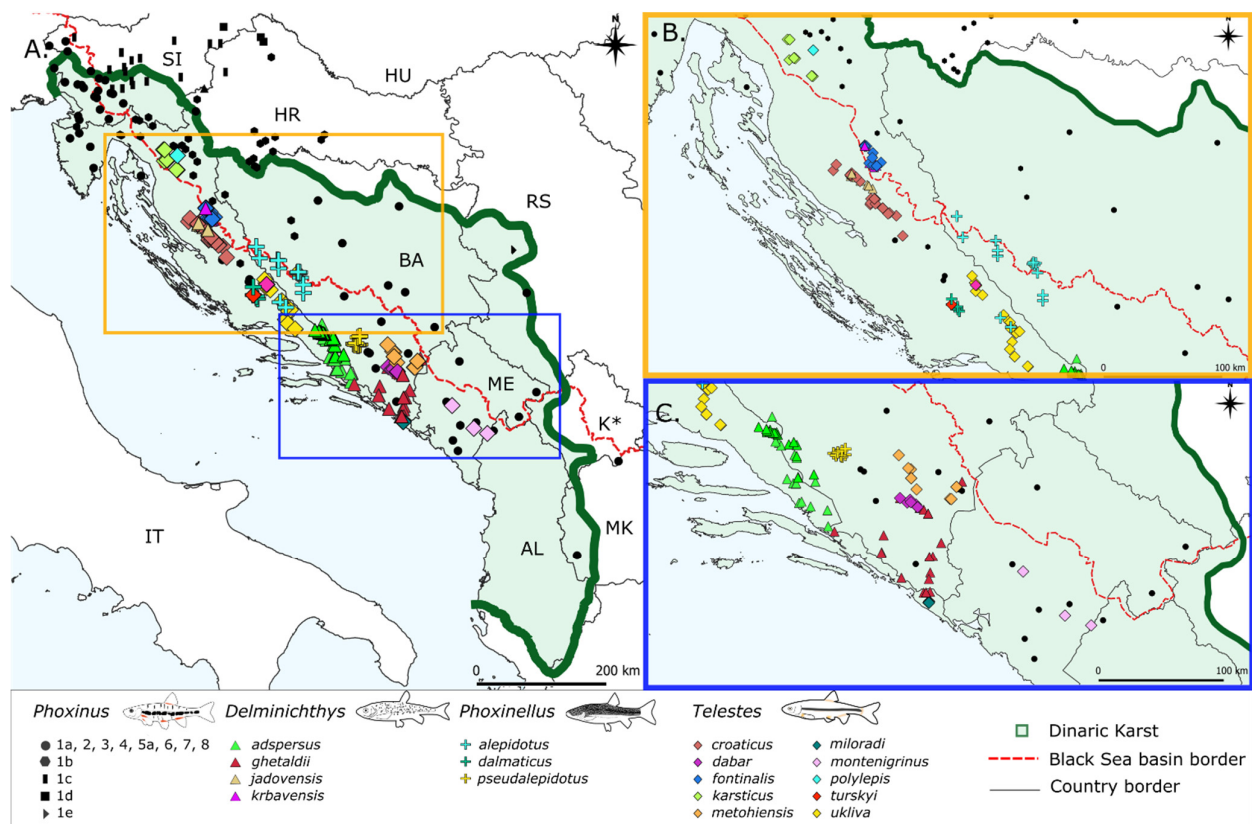


Figure 1. Distribution data of four fish genera *Phoxinus*, *Delminichthys*, *Phoxinellus* and *Telestes* in Dinaric Karst. *Phoxinus* is shown in black, while *Delminichthys*, *Phoxinellus* and *Telestes* are presented in color. (A) Overview of Dinaric Karst (B) Enlargement of the northern part of Dinaric Karst (framed in orange) (C) Enlargement of the southern part of Dinaric Karst (framed in blue). *Phoxinus* sub-lineages 1a–1e are represented with symbols shown in the key.

While the line separating the Adriatic and the Black Sea basins (hereinafter, the Adriatic–Black Sea basin divide) is delineated, there are numerous underground connections throughout Dinaric Karst, which transverses this boundary [60,61]. Due to the complex hydrology and hydrography, it is difficult to define hydrological entities. However, some analysis employed in the present study requires the definition of biogeographic areas, or in the case of aquatic organisms, hydrological areas (e.g., S-DIVA [62]). Thus, three levels of hydrological units were specified for the purpose of this study (hereinafter also used throughout the text): (1) sea basins (Adriatic and the Black Sea); (2) river drainages, including surface hydrological network of a river with its confluences, as well as all the sinking streams, which mostly drain into this river underground (Figure S1); (3) the smallest unit, karst polje, a closed depression with a flat floor draining mostly underground, across which there may be a sinking stream and which may be liable to flooding and becoming a lake [63] (but see [64] on variability of definitions). Some karst rivers have also developed valleys that could be treated as poljes [53,64]. There are about 130 [65,66] to 200 [67] poljes in the Dinaric Karst, 50 of which are large, with a total surface area of about 1500 km², approximately 2.5% of the area covered by karst in the region.

River drainages were obtained from [68], while missing river drainages were drawn manually. The boundaries of the Dinaric Karst were obtained from http://geonode.iwlearn.org/layers/geonode:diktas_dinaric_karst_boundary_hg (accessed on 8 December 2021) and boundaries of the Black Sea basin were downloaded from [69]. All shapefile layers were plotted in QGIS v3.4.9-Madeira [70].

2.2. Samples

In Figure 1, the distribution of all samples with known localities are shown. These data were collected from the literature, personal observations and personal communications. In Figure 2 only samples used in the molecular analyses are presented. For the combined dataset see Table S2.

For most analyses performed in this study, the species of four fish genera *Phoxinus*, *Deliminichthys*, *Phoxinellus* and *Telestes*, which are distributed in Dinaric Karst, were used, except when specified otherwise (Table S1). Most of the specimens (sequences) originated from previous studies, while a few new ones were added (Table 1). The samples of *Deliminichthys*, *Phoxinellus* and *Telestes* originated from the Fish collection, Natural History Museum Vienna, all the specimens were preserved in alcohol. The two additional *Phoxinus* samples were collected under permission no. 3420-30/2017/9 of the Slovenian Ministry of Agriculture, Forestry and Food. For detailed information and GenBank numbers see Table S2.

Table 1. An overview of sequences and alignment length of mitochondrial cytochrome *b* sequences used. Details are found in Table S1.

Genus	Newly Sequenced	Previous Studies	Alignment Length (bp)
<i>Deliminichthys</i>	15	344	1006
<i>Phoxinus</i>	2	1006	1092
<i>Phoxinellus</i>	9	8	1121
<i>Telestes</i>	7	190	1043

In *Phoxinus*, of the eight mt lineages dispersed in Dinaric Karst, four correspond to species (1, *P. lumaireul*; 5, *P. csikii*; 6, *P. krkae*; 7, *P. karsticus*), while the status of four (2, 3, 4, 8) has not yet been resolved. In addition, there are six sub-lineages within lineage 1 (1a–1f), and two within 5, of which 1d, 1f and 5b are not found in Dinaric Karst. Thus, throughout the text, lineage numbers as defined in [37] were used. In the other three genera, *Deliminichthys*, *Phoxinellus* and *Telestes*, the mt lineages correspond to species, and thus, species names were used.

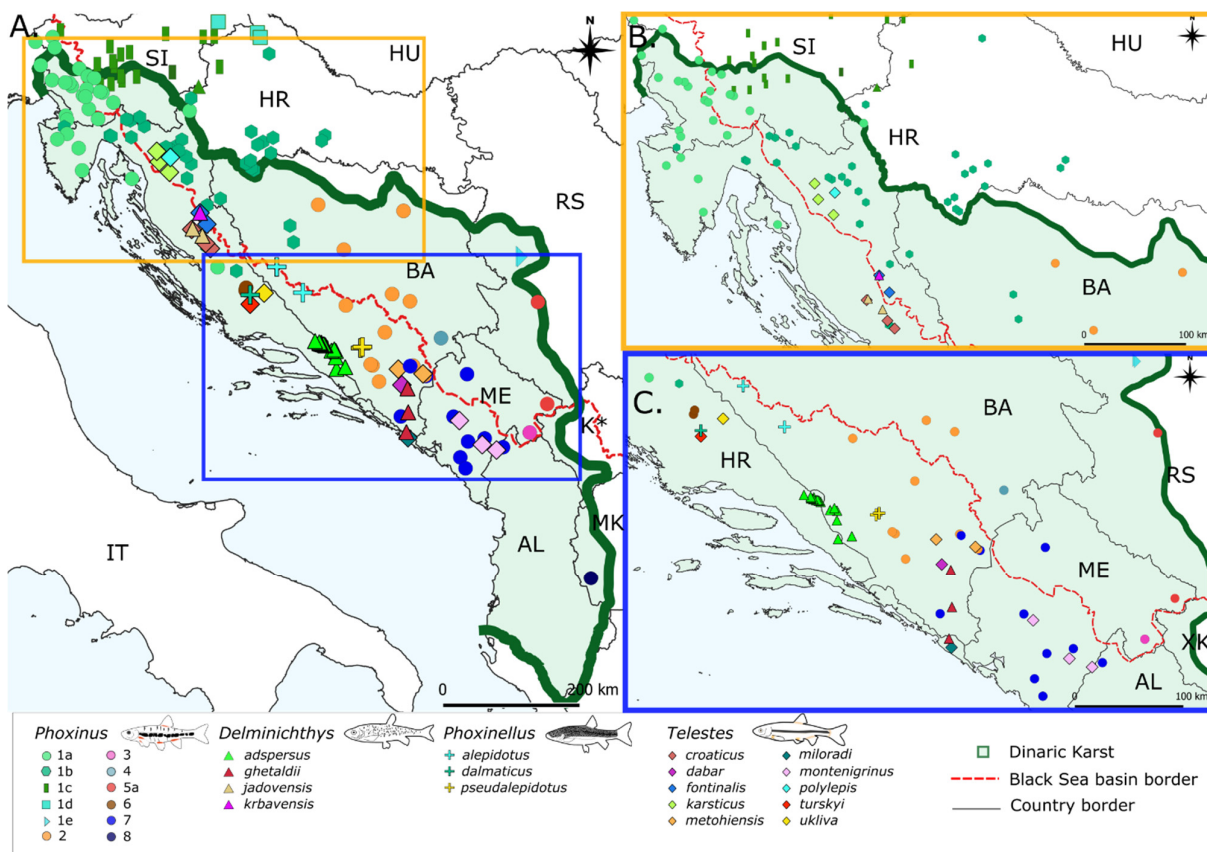


Figure 2. Distribution of mitochondrial (mt) lineages of the four fish genera *Phoxinus*, *Delminichthys*, *Phoxinellus*, *Telestes* in Dinaric Karst. In *Phoxinus*, not all lineages and sub-lineages represent valid species, and thus, they are encoded according to Palandačić et al. [37] (1a–1e, 2–4, 5a, 6–8). In the other three genera, *Delminichthys*, *Phoxinellus* and *Telestes*, the mt lineages correspond to species, and thus, species names were used. (A) Overview of Dinaric Karst (B) Enlargement of the northern part of Dinaric Karst (framed in orange) (C) Enlargement of the southern part of Dinaric Karst (framed in blue). *Phoxinus* sub-lineages 1a–1e are represented with symbols shown in the key.

2.3. DNA Extraction, Amplification and Sequencing

All the analyses were based on a sequence of the mitochondrial (mt) cytochrome *b* (cytb) gene fragment. For the newly sequenced partial cytb, genomic DNA was extracted from fin clips in a clean room using QIAmp DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) and DNeasy Mini spin columns. The extraction and amplification protocols are described in detail in [71], either the one for fresh material or when unsuccessful the one for museum specimens. The sequencing was performed at Microsynth (Balgach, Switzerland) in both directions using the same primers. The sequences were aligned and shortened to correspond to the lengths of the sequences downloaded from GenBank (see Table 1).

2.4. Haplotype Networks, Diversity Indices and Pairwise Distances

Templeton, Crandall and Sing's (TCS) parsimonious haplotype networks were calculated for each genus, using only the species distributed in the Dinaric Karst (Table S5) and the software PopART v1.7 [72]. Haplotype diversity (H_d), nucleotide diversity (π), number of polymorphic sites (S) and mean number of pairwise differences (k) were calculated using the software DnaSP v6 [73]. Uncorrected pairwise distances (p -distances) were calculated using MEGA v10.0.5 [74].

2.5. Phylogenetic Tree Reconstruction and Divergence Time Dating

For the phylogenetic tree reconstruction and divergence time dating, several preliminary analyses with different datasets/outgroups were conducted (see Supplementary Material for details). By observing the best marginal likelihood values as well as ESS values, the reconstruction was performed on two datasets.

The first dataset included *Phoxinus* (sub-)lineages distributed in the Dinaric Karst, as well as three additional (sub-)lineages from the Balkan Peninsula, helping to stabilize the timing tree (sub-lineages 1d, 1f, 14 and 15). *Alburnus alburnus* (L.), *Rutilus rutilus* (L.) and *Squalius cephalus* (L.), and the cyprinid *Cyprinus carpio* (L.) were chosen as outgroups [54]. The tree was calibrated with fossil data of Leuciscidae (23.5–24.5 Mya, according to [75]) and fossil data basal to *Phoxinus* (29.2–30.2 Mya [76]).

The second dataset of *Delminichthys*, *Phoxinellus* and *Telestes* included cytb sequences of the three genera, including the *Telestes* species, which are distributed outside of Dinaric Karst. In addition, *Pelagus minutus* (Karaman, 1924) as a sister group to *Delminichthys*, *Chondrostoma nasus* (L.) and *C. phoxinus* Heckel, 1843 as a sister group to *Phoxinellus*, and *Pseudophoxinus anaticus* (Hankó, 1925) as a sister group to *Telestes* were added. Further groups used within the leuciscins were *Pelecus cultratus* (L.), *R. rutilus*, *S. cephalus*, *Vimba melanops* (Heckel, 1837) and *Pachychilon macedonicum* (Steindachner, 1892) [54]. As in *Phoxinus*, the additional groups were added to stabilize the phylogenetic tree and improve the ESS values. As outgroups, the cyprinid *Carassius auratus* (L.), *Barbus barbus* (L.) and *C. carpio* were chosen [54]. The applied fossil calibration points were for Leuciscidae (23.5–24.5 Mya, according to [75]), as well as for fossil data basal to *Chondrostoma* (5.21 Mya [75]) and *Squalius* (6.56 Mya [75]). All the sequences used for both datasets are reported in Tables S3 and S4.

Phylogenetic trees were calculated using BEAST v2.6.3 [77] under the best fitting evolutionary site model chosen for both datasets as the GTR + F + G4, selected under the BIC criterion using ModelFinder [78]. Further settings were chosen by comparing different options (strict relaxed lognormal clock models, different tree priors, e.g., coalescent Bayesian skyline and Yule model) with the nested sampling analyses in BEAST v2.6.3 [77]. An uncorrelated relaxed lognormal clock corresponding to an evolutionary rate of 0.53% divergence per lineage per million years was applied as an additional calibration point, a rate cited for Leuciscidae [79] according to [80]. For fossil calibration points (as described below) a lognormal distribution was applied starting with an offset of the minimum age and an adjusted standard deviation, so that the divergence time span of the fossils was covered by 95% of the sampled divergence date priors [81]. A birth-death prior was used to estimate node times. Three separate runs were conducted for both sets, with each run for more than 50,000,000 generations and sampled every 1000 generations. The log-files were inspected in Tracer v1.7.1 (available at <http://tree.bio.ed.ac.uk/software/tracer/>, accessed on 13 April 2022) and revealed high ESS values (>300). The three runs were combined using LogCombiner v2.6.6 [82] and the maximum clade credibility (MCC) was estimated using TreeAnnotator v2.6.3 [82] with a burn-in of 25% and a 50% majority rule. The final trees were plotted against a geological time scale using the R package strap v1.4 [83] in R v3.6.3 [84].

2.6. Ancestral Biogeography

To reconstruct the ancestral geographic ranges of the mt lineages of the four genera, the Statistical Dispersal-Vicariance analysis (S-DIVA; [62]) as implemented in RASP (Reconstruct Ancestral State in Phylogenies) v.4.2 × 64 [85] was conducted. S-DIVA is commonly applied to reconstruct ancestral biogeographic areas, as it enables the use of the entire posterior distribution of phylogenetic trees to account for phylogenetic uncertainty, as well as uncertainty regarding ancestral states [85]. The annotated BEAST2 time tree and the collection of time trees (BEAST2 output) for the two datasets were used as input and 1000 random trees were employed for S-DIVA analysis.

The S-DIVA analysis requires the user to set biogeographic areas according to the hypothesis they want to test, and the software allows the defining of up to 15 different areas. Thus, in this study, first the sea basins were designated as biogeographic areas, i.e., the Adriatic and the Black Sea basins. This analysis was named S-DIVA basins. For a more detailed analysis, the river drainages as specified under “Study area” were used, because the karst poljes are too numerous. The defining of the river drainages as biogeographic areas was supported also with the timing analysis, which suggested that most of the splits on the phylogenetic tree predated the start of karstification (including the formation of the karst poljes). The defined river drainages are shown in Figure S1 and listed in Table S4. The second analysis was named S-DIVA drainages. To assign terminal leaves (=mt lineage) to one of the preselected regions, their current distributions ranges were considered. Only ancestral ranges with a probability of >0.5 were accepted as sufficiently supported.

3. Results

3.1. *Phoxinus*

3.1.1. Haplotype Networks and Distribution Patterns, Diversity Indices and p -Distances

As shown in previous studies, there are eight different mt lineages of *Phoxinus* distributed in Dinaric Karst (1–8, Figure 3), including sub-lineages, 1a–c, 1e, and 5a. Lineages and sub-lineages 1a, 1b, 2 and 7 are spread across both the Adriatic and the Black Sea basins, while 1c, 1e, 3, 4 and 5a are found only in the Black Sea basin. Lineages 6 and 8 are distributed in the Adriatic basin.

Along with sub-lineages 1a and 1b, lineage 2 is the most widely distributed. On the Adriatic side, it includes the Neretva river drainage (including some sinking streams), while on the Black Sea side, it is distributed in the Bosna and Vrbas river drainages, both connected to the Danube through the Sava. Lineage 7 is mostly distributed in the Adriatic basin; however, comprises two relatively distant haplotypes (six mutational steps) originating from the same sampling point (Tara River) in the Black Sea basin. These sequences originate from a previous study (GenBank Nos. MG681489–90) and could also be a mistake in the sampling site data. For more details on the distribution of the different lineages and sub-lineages in the river drainages, see Figure 2 and Table S2.

The diversity indices for *Phoxinus* are reported in Table S5. Altogether, there were 1038 sequences resulting in 297 unique haplotypes. In ten of 14 lineages and sub-lineages, a very high haplotype diversity ($H_d = >0.8$) was calculated while in lineages 4, 6 and 8, and sub-lineage 1f, the haplotype diversity ranged from 0.25 to 0.66. Comparatively, all mt lineages and sub-lineages showed rather low levels of nucleotide diversity, between 0.0118 (sub-lineage 1d, lineage 2) and 0.0006 (lineages 6 and 8).

Pairwise distances are reported in Table S6. The largest p -distances were found between lineages 7 and 8 (7.3–9%) and the rest of the lineages. The shortest p -distances were found between lineages 7 and 8 (0.8%). On average, p -distances of 5% were found between mt lineages 1, 2, 3, 4 and 5a, while those among sub-lineages 1a–f were between 2% and 3%.

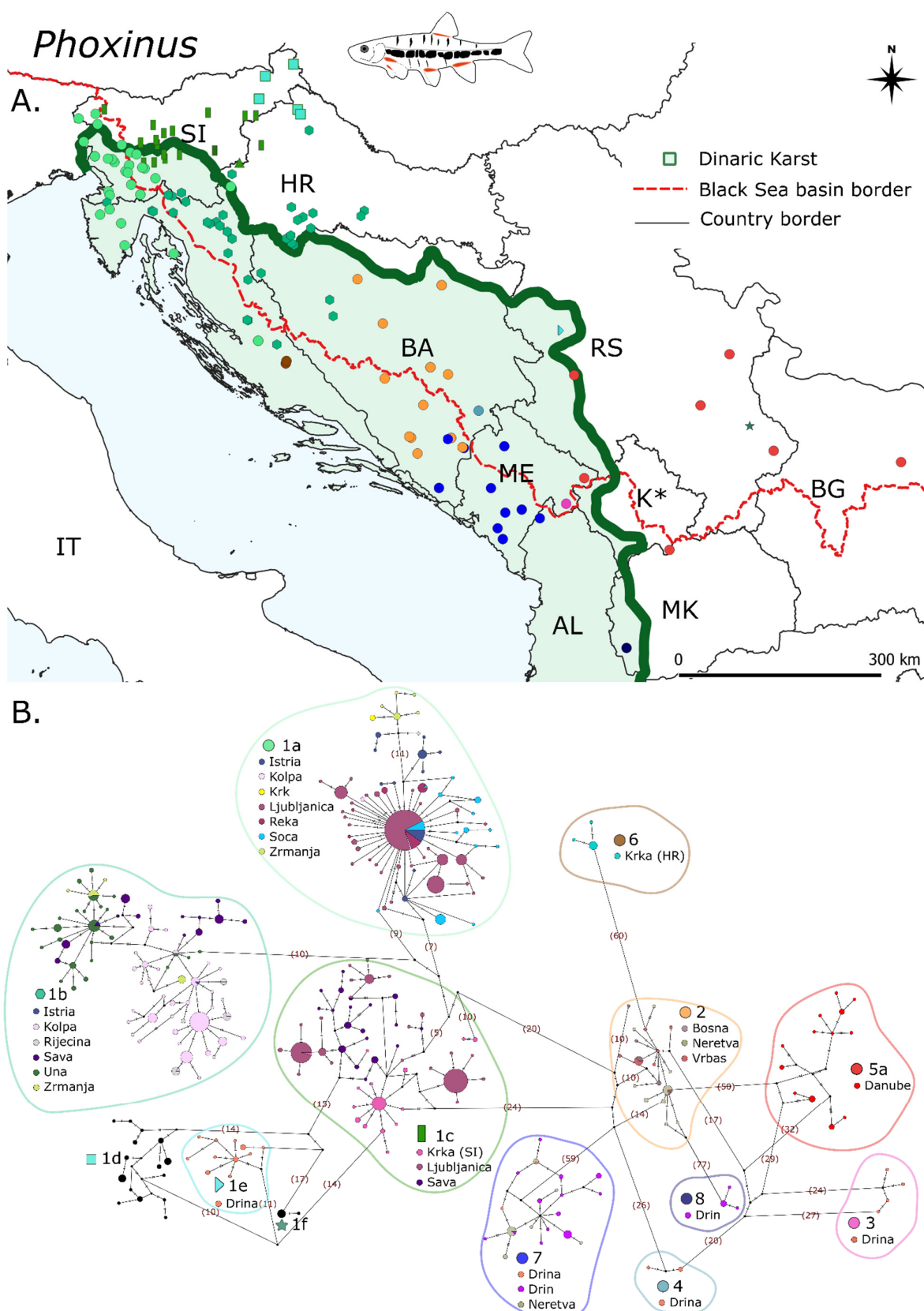


Figure 3. Distribution map and haplotype network (based on cytochrome *b* fragment) of *Phoxinus* mitochondrial (mt) lineages. The lineages are encoded following Palandačić et al. [37]. (A)

Distribution of different mt (sub-)lineages of *Phoxinus* in Dinaric Karst and adjacent areas. Dinaric Karst is highlighted in green; the Adriatic–Black Sea basin divide is marked with a red dashed line. **(B)** Haplotype network of the cytochrome *b* (cytb) marker. (Sub-)Lineages according to Palandačić et al. [37] are encircled. Haplotypes are colored according to the river drainages in which they occur. River drainages are also written next to each mt (sub-)lineage. Mutational steps < 5 are given as dashes, mutational steps ≥ 5 are written in parentheses. *Phoxinus* mt sub-lineages 1d and 1f are represented in black, as they do not occur in Dinaric Karst (see **A**)).

3.1.2. Divergence Times and Ancestral Biogeography

The timing tree based on the *Phoxinus* dataset showed poor statistical support for some of the phylogenetic nodes, as well as large confidence intervals (Figure 4A). In addition, the order of the divergence proved to be unstable and phylogenetic relationships, especially at the level of the deeper nodes of the tree, were unclear (i.e., not statistically supported). The branching of the individual lineages was unstable though the nodes were stable. Nevertheless, some of the divergences were well-supported, starting with the split of lineage 2 from lineages 1, 3, 4, 5a, 14 and 15 in the Miocene. Second, the divergence of the two non-Dinaric Karst lineages 14 and 15 from lineage 1 was well supported and also took place in the Miocene. Sub-lineages 1a–f diverged from each other in the mid-Pliocene, while the divergence within the sub-lineages took place in the Pleistocene, with a similar diversification shown for lineages 2 and 5a.

The S-DIVA basin analysis based on two biogeographic areas—the Adriatic and the Black Sea basins—is shown in Figure 4B. It suggests that the whole group of the “Balkan *Phoxinus*”, including 14 and 15 (not distributed in Dinaric Karst) originated in the Adriatic Sea basin, while 6, 7 and 8 remained and spread within this basin, sequentially diverging from the others in a series of vicariant events. Further events for lineages 1–5a, but including 14 and 15, are unsupported statistically, but the results suggest that the lineages spread to the Black Sea basin. Finally, the analysis pointed to sub-lineages 1a and 1b dispersing back into the Adriatic Sea basin. Dispersal from the Black Sea to the Adriatic Sea basin was suggested also within lineage 2. The S-DIVA drainages analysis was even less supported.

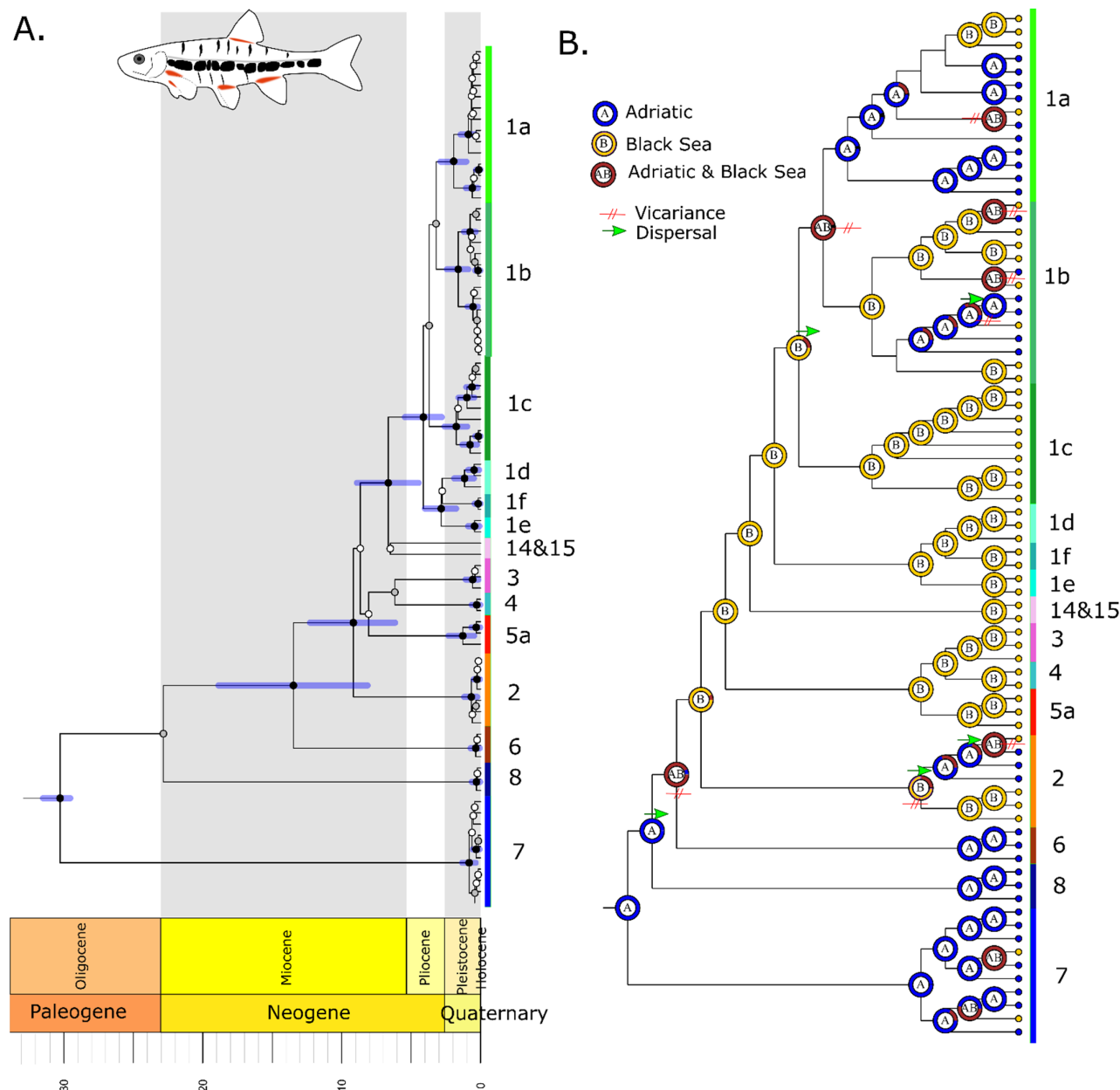


Figure 4. Divergence time estimates and reconstruction of ancestral areas (RASP) analyses of Balkan *Phoxinus* based on the cytochrome *b* fragment. **(A)** Divergence time estimates, calculated in BEAST [77], between mt lineages 1(a–f) and 8, 14 and 15, encoded following Palandačić et al. [37]. Time of splitting is represented by horizontal bars corresponding to a 95% HPD interval. Posterior probabilities (pp) = ≥ 0.95 are indicated as black dots, pp = < 0.95 and ≥ 0.75 are grey and pp = < 0.75 are white. The scale represents millions of years. **(B)** Results from reconstruction of ancestral areas based on two defined biogeographic areas: Adriatic and the Black Sea basins. The analysis was performed with S-DIVA [62] and implemented in RASP [85] based on the phylogenetic trees calculated in 4A. Pie charts at nodes represent the most likely ancestral distributions and letters denote the regions (Adriatic, Black Sea or both) encoded by the respective color. Colors of tip labels indicate the current distribution of each tip. Only ancestral areas with a probability of > 0.5 are shown. Vicariance or dispersal events, or both, are given next to the pie chart. Only the topology is shown. For better visualization, outgroups are not shown.

3.2. *Delminichthys*

3.2.1. Haplotype Networks and Distribution Patterns, Diversity Indices and *p*-Distances

There are four mt lineages in the genus *Delminichthys*, corresponding to the four species (Figure 5A): *D. ghetaldii* (Steindachner, 1882) is distributed in the karst poljes of the left bank of the Neretva river drainage and in the Konavosko poljes (Konavska Ljuta river), both in the Adriatic Sea basin; *D. adspersus* (Heckel, 1843) is distributed in the karst poljes of the right-bank Neretva river drainage; *D. jadovensis* (Zupančič and Bogutskaya, 2002) inhabits karst poljes of the Lika–Jadova river drainage, in the Adriatic Sea basin; while *D. krbavensis* (Zupančič and Bogutskaya, 2002) can only be found in Krbavsko polje in the Una river drainage of the Black Sea basin. Distribution of the species in the karst poljes is reported in Table S2.

The diversity indices for *Delminichthys* are reported in Table S5. Altogether, there were 359 sequences resulting in 84 unique haplotypes. The haplotype diversity of three species—*D. adspersus*, *D. ghetaldii* and *D. krbavensis*—were considerably high, ranging from 0.7 to 0.86, while the nucleotide diversities were low, from 0.0012 to 0.0032. Only *D. jadovensis* exhibited little haplotype diversity (0.33) and nucleotide diversity (0.0003).

The *p*-distances are reported in Table S5. Those between two pairs of species, *D. adspersus* and *D. ghetaldii*, and *D. jadovensis* and *D. krbavensis*, were considerably small: 1.8% and 2.1%, respectively. In contrast, the distance between the two pairs was 4%.

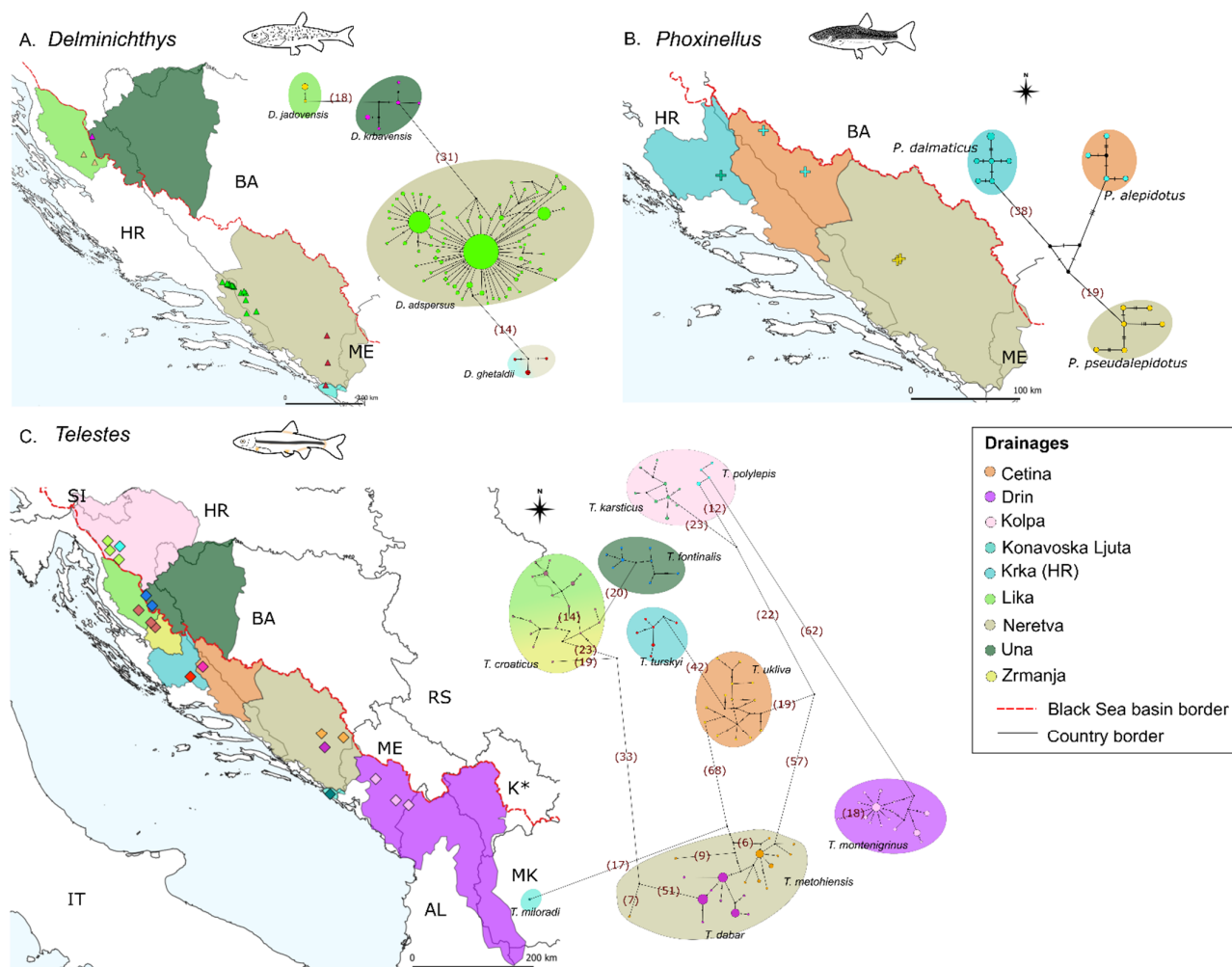


Figure 5. Distribution maps and haplotype networks (based on cytochrome *b* fragment) of mitochondrial lineages, corresponding to species, of the three fish genera *Delminichthys*, *Phoxinellus* and

Telestes within Dinaric Karst. The river drainages are represented by different colors (see legend) and the Adriatic–Black Sea basin divide is marked with a red dashed line. Mutational steps < 5 are given as dashes, mutational steps ≥ 5 are written in parentheses. Colors of haplotypes denote species. The ovals correspond to the drainage basin where each species occurs. (A) *Delminichthys*; (B) *Phoxinellus*; (C) *Telestes*.

3.2.2. Divergence Times and Ancestral Biogeography

The divergence between the genera *Pelagus* and *Delminichthys* was supported statistically and dates to the end of the Miocene, though with a broad confidence interval (1.65–12.84 Myr). The first split within *Delminichthys* was also well supported, with the branching of *D. ghetaldii* dating to the end of the Pliocene (0.5–4.9 Myr). The branching of *D. adspersus* was unsupported, while *D. jadovensis* and *D. krbavensis* split in the middle of the Pleistocene, around 1.5 million years ago (0.05–1.7 Myr).

According to the S-DIVA basins analysis (Figure S2), the ancestor of *Pelagus* and *Delminichthys* was distributed in both basins, while the first splits within *Delminichthys* occurred within the Adriatic Sea basin through vicariance and later dispersal. Finally, the ancestor of *D. krbavensis* and *D. jadovensis* dispersed back into the Black Sea basin, with the two species splitting by a vicariance event.

According to the S-DIVA drainages analysis (Figure 6B), the *Pelagus* and *Delminichthys* ancestor occupied the broader area of the Neretva and Drin river drainages in the Adriatic Sea basin. The two genera split in a vicariance event, separating the two genera into Drina and Neretva river drainages. Divergence of *Delminichthys* in a vicariance event into the left-bank *D. ghetaldii* and the rest of *Delminichthys* (right-bank group) within the Neretva drainage followed, with *D. ghetaldii* spreading further south to the Ljuta drainage. This divergence was followed by a divergence of *D. adspersus* from *D. jadovensis* and *D. krbavensis* in a vicariance event. The ancestor of the two species dispersed into the Una drainage and finally separated in a vicariance event into, respectively, Ličko polje and Kravsko polje, which they inhabit today.

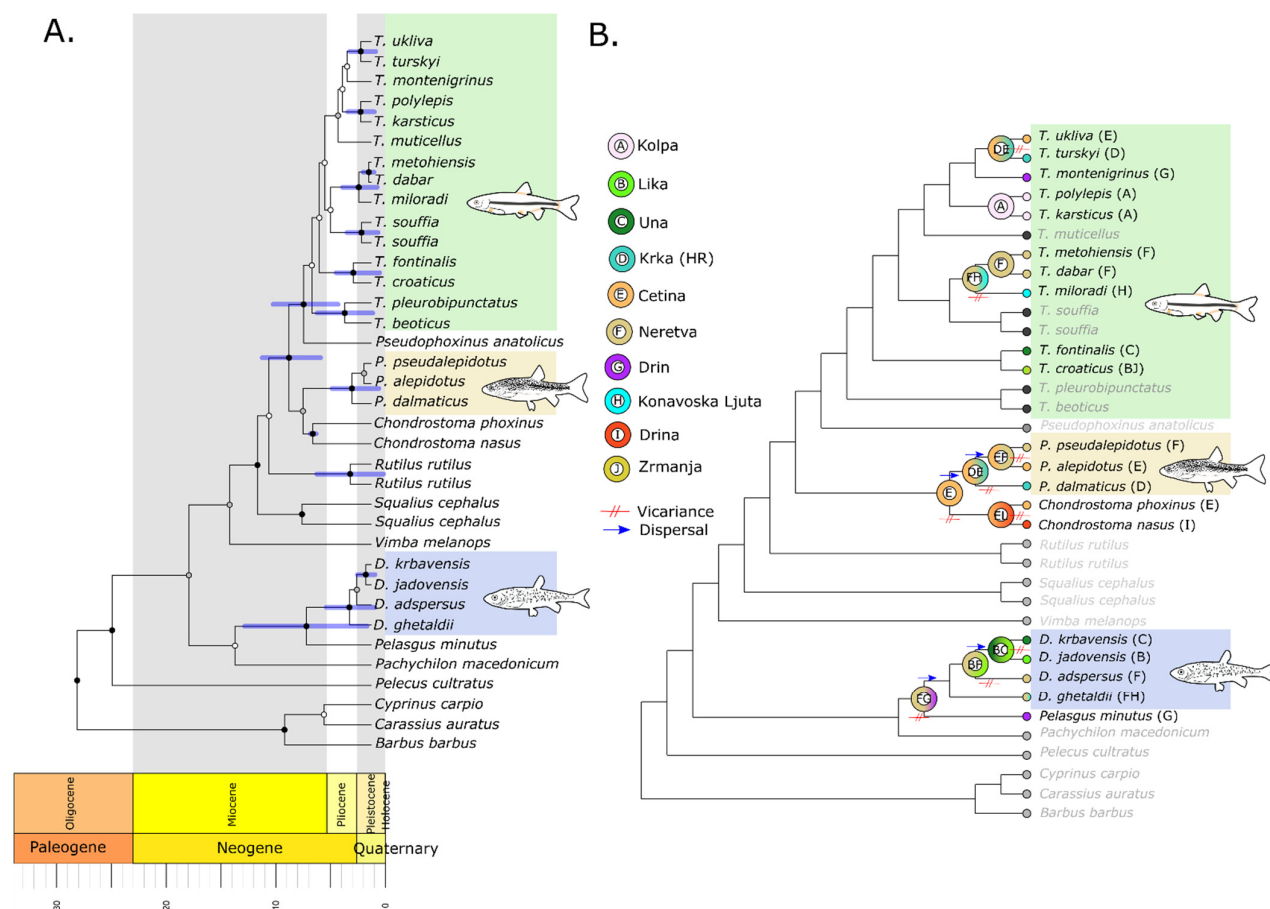


Figure 6. Divergence time estimates and reconstruction of ancestral areas (RASPs) analyses of *Delminichthys*, *Phoxinellus* and *Telestes* (together with sister genera and outgroups; for details see Table S3) based on cytochrome *b* fragment. **(A)** Divergence time estimates for *Delminichthys* (highlighted in blue), *Phoxinellus* (orange) and *Telestes* (green) with sister genera, calculated in BEAST [77]. Time of splitting is represented by horizontal bars corresponding to a 95% HPD interval. Posterior probabilities (pp) = ≥ 0.95 are indicated as black dots, pp = < 0.95 and ≥ 0.75 are grey and pp = < 0.75 are white. The scale represents millions of years. **(B)** Results from reconstruction of ancestral areas based on 10 biogeographic areas (see legend). The analysis was performed with S-DIVA [62] and implemented in RASP [85] based on the phylogenetic trees calculated in **(A)**. Pie charts at nodes represent the most likely ancestral distributions and letters denote the regions encoded by the respective color (legend next to tree). Letters following tip labels indicate the recent distribution of each tip. Only ancestral areas with a probability of > 0.5 are shown. Vicariance or dispersal events, or both, are given next to the pie chart. Only the topology is shown.

3.3. *Phoxinellus*

3.3.1. Haplotype Networks and Distribution Patterns, Diversity Indices and *p*-Distances

The *Phoxinellus* genus includes three mt lineages (Figure 5B), corresponding to three different species, which are distributed in the karst poljes of three neighboring river drainages, namely, Krka (*P. dalmaticus* Zupančič and Bogutskaya, 2000), Cetina (*P. alepidotus* Heckel, 1843) and Neretva (*P. pseudalepidotus* Bogutskaya and Zupančič, 2003). The occurrence of each species is restricted to the respective river system poljes.

The diversity indices for *Phoxinellus* are reported in Table S5. Altogether, there were 17 sequences resulting in 16 unique haplotypes. Haplotype diversities of individual species are high (0.95–1), while the nucleotide diversities are low (0.002–0.0035).

Pairwise distances are reported in Table S5. The largest distance is between *P. dalmaticus* and *P. pseudalepidotus* (5.4%), and the smallest between *P. alepidotus* and *P. pseudalepidotus* (2.4%).

3.3.2. Divergence Times and Ancestral Biogeography

According to the divergence time analysis (Figure 6A), the split between *Phoxinellus* and *Chondrostoma* was unsupported, while the first split within the *Phoxinellus* group was well supported and dates to the late Pliocene (0.3–4.6 Myr).

According to the S-DIVA basin analysis (Figure S2), the ancestor of *Chondrostoma* and *Phoxinellus* occupied both the Adriatic Sea and Black Sea basins and, subsequently, the two genera split in a vicariance event, with *Phoxinellus* distributed in the Adriatic Sea basin only. The analysis produced no indication of whether the splits within *Phoxinellus* occurred through vicariance or dispersal.

The S-DIVA drainages analysis (Figure 6B) suggested that the ancestor of *Chondrostoma* and *Phoxinellus* resided in the Cetina river drainage, in the Adriatic Sea basin. The two genera split through a vicariance event and *Phoxinellus* kept dispersing in the Cetina and as well as further in Krka drainage. *Phoxinellus dalmaticus* was the first to branch off through vicariance and the ancestor of the other two species dispersed to the Neretva drainage. Finally, *P. alepidotus* and *P. pseudalepidotus* split in a vicariance event.

3.4. Telestes

3.4.1. Haplotype Networks and Distribution Patterns, Diversity Indices and *p*-Distances

There are thirteen mt lineages in the genus *Telestes*, corresponding to thirteen different species. However, only *Telestes* distributed in the area of the Dinaric Karst are presented in the haplotype network, which comprises ten mt lineages or species (Figure 5C). The difference between *T. metohiensis* (Steindachner, 1901) and *T. dabar* Bogutskaya, Zupančič, Bogut and Naseka, 2012 is only five mutational steps. In this part of the network, there are at least two individual haplotypes, which are up to 16 mutational steps away from the main haplogroup. *Telestes* species are distributed in river drainages belonging to either the Adriatic or Black Sea basin. In the Kolpa river drainage two species—*T. karsticus* Marčić and Mrakovčić, 2011 and *T. polylepis* Steindachner, 1866—occur, while *T. dabar* and *T. metohiensis* are found in the Neretva river drainage.

The diversity indices for *Telestes* are reported in Table S5. Altogether, there were 197 sequences analyzed, resulting in 96 unique haplotypes. The haplotype diversities were high (0.74–1), while nucleotide diversities were low (0.0017 in *T. polylepis* and 0.0147 in *T. croaticus* (Steindachner, 1866)). The pairwise distances are reported in Table S5, with the largest *p*-distances (up to 11%) reported for *T. dabar* and *T. fontinalis*, and *T. fontinalis* and *T. miloradi* Bogutskaya, Zupančič, Bogut and Naseka, 2012. The smallest *p*-distances (just less than 3%) were calculated for *T. fontinalis* and *T. croaticus*, *T. dabar* and *T. miloradi*, and *T. miloradi* and *T. metohiensis*, with that between *T. metohiensis* and *T. dabar* only 0.9%.

3.4.2. Divergence Times and Ancestral Biogeography

According to the divergence time analysis (Figure 6A), the split between *Pseudophoxinus* and *Telestes* was well supported, and dates to the end of the Miocene (3.6–9.5 Myr). Whereas the deeper nodes within *Telestes* were unsupported, the splits between sister pairs comprised mostly *T. ukliva* (Heckel, 1843) and *T. turskyi* (Heckel, 1843), *T. polylepis* and *T. karsticus*, and *T. dabar* and *T. miloradi*, all dating to the end of the Pliocene or the middle of the Pleistocene.

The S-DIVA basins analysis (Figure S2) produced few supported nodes within the genus *Telestes*, and neither did the S-DIVA drainages analysis (Figure 6B). The supported nodes include the splitting of *T. turskyi* and *T. ukliva* into the Krka and Cetina river drainages in the Adriatic Sea basin, and *T. karsticus* and *T. polylepis* within the Kolpa drainage, in the Black Sea basin. The ancestor of *T. miloradi*, *T. metohiensis* and *T. dabar* was

distributed in the Neretva and Ljuta river drainage, and later on *T. miloradi* split in a vicariance event to reside in the Ljuta river drainage.

4. Discussion

The distribution areas of four sub-lineages (1a–1c, 5a) and two lineages (2, 7) of the *Phoxinus* genus in Dinaric Karst span several river drainages, while the ranges of (sub-)lineages 1a–c and 5a also include drainages outside of the region, together with three additional lineages distributed in the Balkan Peninsula and eleven distributed elsewhere in Europe [37,86]. Four of the (sub-)lineages (1a, 1b, 2 and 7) distributed in Dinaric Karst transverse the Adriatic–Black Sea basin divide, while (sub-)lineages 1a–1c, 2 and 7 encompass several karst poljes (Table S2). The range of *Telestes* species also includes some outside of Dinaric Karst, yet the Dinaric *Telestes* are all endemic to the region and have distribution ranges restricted to one karst polje, or a few interconnected poljes. The only exception is *T. montenigrinus*, which inhabits mountain rivers in the south of the Dinaric Karst and has, according to the reviewed literature, never been recorded in subterranean habitats [87,88]. All species of *Delminichthys* and *Phoxinellus* are endemic to Dinaric Karst and, as with Dinaric *Telestes*, inhabit one or a few interconnected karst poljes, with a minimum size of only one square kilometer (Konavosko polje, Ljuta sinking stream, inhabited by *T. miloradi* and *D. ghetaldii*) and a maximum of 200 square kilometers (sinking streams of Imotsko polje, Rastocko and Vrgoracko polje; *D. adspersus*). Three of the lineages of Dinaric *Phoxinus* are restricted to one locality (3, 4, 8), while one inhabits the upper part of the Krka river drainage (6), indicating their species ranges are restricted (further sampling might reveal more populations within these lineages). Nevertheless, in sum, the lineages or species ranges of Dinaric *Phoxinus* are generally broad and include river drainages and sinking streams on karst poljes, while those of *Delminichthys*, *Phoxinellus* and Dinaric *Telestes* are narrow, restricted to sinking streams on karst poljes (karst river valleys, see “Study area”). Finally, several species of all four genera occur sympatrically (see Figures 1 and 2) and inhabit the same sinking streams, e.g., *T. croaticus* and *D. jadovensis*, *T. fontinalis* and *D. krbavensis*, *Phoxinus karsticus* and *D. gethaldii*, and *Phoxinus* sp. (mt lineage 2 according to [37]) and *T. metohiensis*.

The common homoplastic specializations of the four non-related genera suggest some similarities in their ecology. However, as summarized above, *Phoxinus* species have wider distribution ranges than the species of *Delminichthys*, *Phoxinellus* and Dinaric *Telestes*, suggesting a factor that is influencing their potential to migrate. *Phoxinus* can endure cold climatic conditions, as well as severe winters under ice [52,89]. At the same time, some species can tolerate high water temperatures, providing that the concentration of the oxygen is high [90,91]. In Europe, the genus inhabits a variety of habitats across a broad geographical range throughout its native distributional area, in brackish water, as well as in various types of freshwater: streams, rivers, ponds and large lakes, ranging from coastal areas to high mountains [92]. In rivers, it is found in mountain streams inhabited by trout, which it shares with only a few other rheophilic species (e.g., *Cottus* sp. and *Salmo* sp.), and downstream to the barbel zone, as well as some lowland rivers and lakes [93–96]. Thus, *Phoxinus* is resilient to predation and plastic with regard to dietary preferences. When introduced to non-native rivers and lakes, *Phoxinus* species quickly spread and are considered invasive in Scandinavia and Spain [52,79,97], attesting again to the adaptability of the genus to new environments [92]. It is clear that *Phoxinus* species are generalists with a high migratory potential, confirmed also in previous studies [37,39,98], which detected several occasions of the crossing of the Adriatic–Black Sea divide in Dinaric Karst. Especially in lineages 1a and 1b, the genetic structure points to a recurrent, ongoing contact between surface-isolated water systems that are connected underground, keeping the populations in Hardy–Weinberg equilibrium. In congruence with this finding are also data on observations of *Phoxinus* in caves (personal observation NB), classifying it as a trogluxene (according to [43]). In lineage 2, the genetic structure was not studied in great detail, but there are two sub-clusters in the tree, corresponding to Adriatic and Black Sea

divergence. These two sub-clusters separated late in the Pleistocene, well into the process of karstification, which, according to Trontelj et al. [19] and confirmed by Zupan Hajna et al. [99], started 2–5 million years ago. Thus, if not a recurrent migration, it is possible that *Phoxinus* at one time used the underground connections to spread across the Adriatic–Black Sea divide and reach its present distribution. In congruence with this suggestion, the S-DIVA basins analysis supported dispersal within this lineage, namely, dispersal from the Adriatic to the Black Sea drainage. In addition, along the left bank of the Neretva drainage, lineage 2 is distributed in Bunica and Bregava rivers, while lineage 7 spreads to Trebisnjica. Meanwhile, in the two streams Musnica and Zalotka, hybrids of the two lineages were detected (Figure 2A, and also based on nuclear DNA; see [39]), yet again pointing to migration between subterranean connected streams [100]. Even though this pattern could possibly be a consequence of human translocations (see comments in “Results” on lineage 7), which are frequent for *Phoxinus* (e.g., [101] and the references within), a recent study [37] suggested that anthropogenic influence on the distribution of *Phoxinus* lineages is less than expected. Unfortunately, dispersal and vicariance patterns suggested by the S-DIVA analysis conducted on *Phoxinus* were mostly unsupported, and thus, there are no meaningful results for other lineages.

In Dinaric *Phoxinus* species, a set of characteristics—such as reduced scalation and cephalic sensory canals, also observed in cave-dwelling fishes (numerous literature sources, summarized in [102])—might have facilitated the migration of *Phoxinus* through an underground karst system. These characteristics were also observed in *Delminichthys*, *Phoxinellus* and Dinaric *Telestes*, yet they exhibit much more restricted distribution ranges. According to [44], which compared four stygophile species—*T. croaticus* and *T. fontinalis*, and *D. jadovenis* and *D. krbavensis* (whereby species pairs *T. croaticus* and *D. jadovenis*, and *T. fontinalis* and *D. krbavensis* are sympatric)—there are different levels of cave adaptation among these two genera: simple stygophiles (*T. fontinalis* and *T. croaticus*) and advanced stygophiles (*D. jadovenis* and *D. krbavensis*). Simple stygophiles retreat underground during dry periods in winter and summer but commonly stay in surface waters of karst poljes with vegetation and rich mud habitats. In contrast, the two advanced stygophiles mostly stay underground and only emerge for spawning. In general, adaptations to cave dwelling are considered a limiting factor for their dispersal potential, making highly specialized cave species unable to survive outside of specific environments to which they are adapted [103,104]. However, recurrent underground migration was suggested for *D. adspersus* in the poljes of the right bank of the Neretva river drainage [24,25]. Furthermore, while *T. fontinalis* is restricted to Krbavsko polje (Una river drainage in the Black Sea basin), *T. croaticus* is distributed in two river drainages: Lika and Zrmanja (Adriatic Sea basin), which are connected underground [44,105]. For *Phoxinellus alepidotus*, Bogutskaya and Zupancic [49] observed no morphological differences between individuals from Livanjsko and Sinjsko polje that are separated by Dinara Mountain but linked underground, indicating a single population. Therefore, there is some evidence indicating, as for *Phoxinus*, *Delminichthys*, *Phoxinellus* and *Telestes*, some limited ability to migrate underground. On the other hand, *T. karsticus* and *T. polylepis* seem to inhabit the same system of karst poljes belonging to Mreznica river drainage, while *D. jadovenis*, inhabiting the same stream as *T. croaticus* in Lika, has not managed to move into the Zrmanja river drainage. The divergence time dating and S-DIVA analyses (Figure 6) suggested that most of the sister species of *Delminichthys*, *Phoxinellus* and Dinaric *Telestes* diverged through vicariance at the end of the Pliocene towards the middle of the Pleistocene. While the three genera are not closely related (Figure 6A, but see also [36,54]), their adaption(s) to the sinking streams on karst poljes with periodical (seasonal) alterations of drought and flood developed in parallel and over a relative short period (some 3 million years). Thus, the convergent evolution of *Delminichthys*, *Phoxinellus* and Dinaric *Telestes*, resulting in their adaptation to partial underground lifestyle, ran parallel to the karstification process [19,99], locking the species into their respective karst polje systems. In turn, Dinaric *Phoxinus*, whose sub-lineages also diverged at the end of the Pliocene to the middle of the Pleistocene, were able

to disperse through the karst landscape. Therefore, regardless of the level of stygophily and some common adaptations that they share with *Phoxinus*, *Delminichthys*, *Phoxinellus* and *Telestes* have less migratory potential, suggesting there must be additional differences in species biology preventing them from migrating through the main river courses and reaching wider areas of distribution. For example, in comparison to Dinaric *Phoxinus*, *Phoxinellus*, *Delminichthys* and Dinaric *Telestes* species also seem to be more sensitive to water eutrophication and to the introduction of non-native predatory species [44]. In Trebisnjica (Popovo polje), where *Phoxinus karsticus* and *Delminichthys gethaldii* occur in sympatry, in the past few years, the population of *Phoxinus* has been increasing, while *Delminichthys* is declining (NB, personal observation and communication with Dusan Jelic). In contrast, the pair of sympatric species *Telestes* and *Delminichthys* (*T. croaticus* + *D. jadovensis* and *T. fontinalis* + *D. krbavensis*; as well as other species from the three genera; see Figures 1 and 2) co-inhabit without evident competition.

As specified in the Introduction, the genus *Phoxinus* and the other three genera belong to two different subfamilies, and it is important to emphasize that these subfamilies have different evolutionary backgrounds that have contributed to differences in their distributions. While Leuciscinae in Europe are well represented (>320 species), the closest relatives of *Phoxinus* are distributed in the Pacific basin [54,106,107]. Morphologically, *Phoxinus* is, in general, characterized by a low level of phenotypic diversity, in contrast to its high level of genetic diversity [38,98,108], whereas Leuciscinae (including *Delminichthys*, *Phoxinellus* and *Telestes*) are highly phenotypically divergent and taxonomically structured in a complex system that also corresponds to their genetic patterns. Nevertheless, when comparing the distribution ranges with other unrelated taxa spread in the Dinaric Karst, some common patterns emerge. For example, several studies of surface and subterranean taxa in vertebrates and invertebrates have observed distribution areas that traverse the Adriatic–Black Sea basin divide (e.g., [9,109,110]. Especially in the north Dinaric Karst, where there are numerous underground connections between the two basins, *Phoxinus* has a distribution in common with the obligate subterranean cave salamander *Proteus anguinus* Laurenti, 1768, as well as the freshwater crustacean *Asellus aquaticus* [111,112]. Notably, there is a similarity in the distributions of *Phoxinus* (sub-)lineages and the mt lineages of the cold-adapted stone crayfish *Austropotamobius torrentium* (von Paula Schrank, 1803) (summarized in Figure S3), which is spread both in and outside of the Dinaric Karst. Especially, lineage CSE (according to [109,113,114]) has a distribution comparable to *Phoxinus* lineage 1, while two additional lineages of *A. torrentium* are distributed across the Adriatic–Black Sea basin divide. Nevertheless, the proposed times of divergence within these two lineages differ considerably: while divergence of *Phoxinus* lineage 1 was dated to the middle of the Pliocene, the *A. torrentium* CSE lineages either split at the end of the Miocene or much later, in the middle of the Pleistocene, depending on the calibration method employed [113]. While the latter timing, as in *Phoxinus*, would suggest dispersal upon or despite the karstification, the differences in the two estimates point to problems with the divergence time analysis methods, whose results should always be treated with extreme caution [115,116]. Without reliable timing analysis, it is difficult to evaluate how much vicariance induced by formation of geomorphological forms in karst influenced speciation. Verovnik et al. [18] pointed out that other geological events predating karstification need to be considered when studying the distribution ranges of Dinaric taxa. Indeed, in the present study, most of the divergence time dating suggests that the initial diversifications within all four genera predate the onset of karstification. Nevertheless, the final splits on the phylogenetic tree at the end of the Pliocene to the middle of the Pleistocene produced ten Dinaric *Telestes* species (but see also comments on *T. metohiensis* and *T. dabar* in “Results”), increasing the species richness of fish in Dinaric Karst. Similarly, previous studies [36,58,59,117] suggested karstification functioned as an isolating factor promoting speciation, confirmed also for other taxa such as in speciation of *Drusus* flies [118] and the cave bivalve *Congeria kusceri* Bole, 1962 [119]. Thus, further studies, based on more reliable genetic analysis, including of nuclear genes, as well as well-dated

and well-supported phylogenetic trees are needed to fully understand the distribution patterns of taxa in Dinaric Karst. In conclusion, while comprising an analysis of the known data and literature, the present study highlights the importance of comparative phylogeographic studies in Dinaric Karst.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/d14070526/s1>, Table S1. Mitochondrial lineages of Phoxinus used for the various types of analysis. Table S2. Details for sampling localities and source of data. Table S3. All samples with GenBank accession numbers and references included in the divergence timing analysis of Phoxinus mt lineages. Table S4. All samples with GenBank accession numbers and references included in the divergence timing analysis of Delminichthys, Phoxinellus and Telestes and sister/outgroups. Table S5. Genetic diversity parameters of the mitochondrial cytochrome b marker of the four fish genera. Abbreviations: h, number of haplotypes; Hd, haplotype diversity; π , nucleotide diversity; S, number of polymorphic sites; k, mean number of pairwise differences; N, number of sequences. Table S6. Pairwise uncorrected p-distances between species/lineages of each genera. Species names of Delminichthys, Phoxinellus and Telestes are abbreviated in the horizontal line. Figure S1. Major river drainages in the Dinaric karst with the distribution of the four fish genera Phoxinus, Delminichthys, Phoxinellus and Telestes). Each Symbols correspond to species (see legend). Figure S2. Reconstruction of ancestral areas (RASP) analyses of Delminichthys, Phoxinellus and Telestes (together with sister genera and outgroups, for details see Table S4) using two biogeographic areas: Adriatic and Black Sea basin (see legend) and based on cytochrome b fragment. Figure S3. Distribution map of the mitochondrial lineages of stone crayfish Astopotamobius torrentium.

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Chapter 1: Supplementary material

1. List of tables

Table S1. Mitochondrial lineages of *Phoxinus* used for the various types of analysis.

Mt (sub-)lineage/species	Distributed in Dinaric karst	Haplotype network	Diversity indices	p-distances	Divergence time dating	Ancestral biogeography
1a – <i>P. lumiareul</i>	x	x	x	x	x	x
1b – <i>P. lumiareul</i>	x	x	x	x	x	x
1c – <i>P. lumiareul</i>	x	x	x	x	x	x
1d – <i>P. lumiareul</i>		x		x	x	x
1e – <i>P. lumiareul</i>	x	x	x	x	x	x
1f – <i>P. lumiareul</i>		x		x	x	x
2	x	x	x	x	x	x
3	x	x	x	x	x	x
4	x	x	x	x	x	x
5a – <i>P. csikii</i>	x	x	x	x	x	x
6 – <i>P. krkae</i>	x	x	x	x	x	x
7 – <i>P. karsticus</i>	x	x	x	x	x	x
8	x	x	x	x	x	x

Table S2. Presented as a separate Excel file with four sheets for each genus. Details for sampling localities and source of data are given. For samples colored in black genetic data are available, while those marked in red correspond to distribution data without genetic information, collected from the literature, personal observations and personal communications. [available at <https://www.mdpi.com/article/10.3390/d14070526/s1>]

Add Material & Methods section 2.5

1. For the phylogenetic tree reconstruction for and divergence time dating, several preliminary analyses with different datasets/outgroups were conducted. The datasets/outgroups were chosen based on [10], [15] and [19]. The datasets tested: *Phoxinus* from Balkan with only one haplotype per lineage (*Phoxinus phoxinus* from Mongolia as outgroup)
2. *Phoxinus* with one haplotype per lineage with all occurring lineages in Europe
3. *Phoxinellus* only (*Phoxinus* as outgroup)
4. *Telestes* only (*Phoxinus* as outgroup)
5. *Delminichthys* only (*Phoxinus* as outgroup)
6. *Phoxinellus* and *Telestes* (with their sistergroups *Chondrostoma* and *Pseudophoxinus*)
7. *Phoxinellus*, *Delminichthys* and *Telestes* (with their sistergroups (*Chondrostoma*, *Pseudophoxinus* and *Pelagius*), *Squalius cephalus*, *Rutilus rutilus*, *Alburnus alburnus*, *Leuciscus leuciscus*, *Vimba vimba*; outgroups: *Phoxinus*, *Cyprinus carpio* and *Carassius auratus*)

The two datasets described in the Material & methods section of the manuscript revealed the overall highest ESS values in the trace files, as well as the highest marginal likelihood values.

Table S3. All samples with GenBank accession numbers and references included in the divergence timing analysis of *Phoxinus* mt lineages.

Species	Clade	GenBank	Reference
<i>Alburnus alburnus</i>		AB239593	Saitoh et al. [2]
<i>Cyprinus carpio</i>		NC_001606	Chang, Huang, and Lo [3]
<i>Phoxinus lumaireul</i>	1a	KT166665	Palandačić et al. [4]
<i>Phoxinus lumaireul</i>	1a	KT166757	Palandačić et al. [4]
<i>Phoxinus lumaireul</i>	1a	ON494605	Reier et al. [5]
<i>Phoxinus lumaireul</i>	1a	KT166668	Palandačić et al. [4]
<i>Phoxinus lumaireul</i>	1a	ON494698	Reier et al. [5]
<i>Phoxinus lumaireul</i>	1a	ON494839	Reier et al. [5]
<i>Phoxinus lumaireul</i>	1a	ON494712	Reier et al. [5]
<i>Phoxinus lumaireul</i>	1a	KT166604	Palandačić et al. [4]
<i>Phoxinus lumaireul</i>	1a	KT166722	Palandačić et al. [4]
<i>Phoxinus lumaireul</i>	1a	KT166647	Palandačić et al. [4]
<i>Phoxinus lumaireul</i>	1a	KT166734	Palandačić et al. [4]
<i>Phoxinus lumaireul</i>	1a	MG681503	Vučić et al. [6]
<i>Phoxinus lumaireul</i>	1a	KT166828	Palandačić et al. [4]
<i>Phoxinus lumaireul</i>	1a	KT166832	Palandačić et al. [4]
<i>Phoxinus lumaireul</i>	1a	ON494890	Reier et al. [5]
<i>Phoxinus lumaireul</i>	1b	ON494754	Reier et al. [5]
<i>Phoxinus lumaireul</i>	1b	ON494856	Reier et al. [5]
<i>Phoxinus lumaireul</i>	1b	KT166653	Palandačić et al. [4]
<i>Phoxinus lumaireul</i>	1b	KT166742	Palandačić et al. [4]
<i>Phoxinus lumaireul</i>	1b	KT166759	Palandačić et al. [4]
<i>Phoxinus lumaireul</i>	1b	KT166743	Palandačić et al. [4]
<i>Phoxinus lumaireul</i>	1b	KT166784	Palandačić et al. [4]
<i>Phoxinus lumaireul</i>	1b	KT166785	Palandačić et al. [4]
<i>Phoxinus lumaireul</i>	1b	KT166729	Palandačić et al. [4]
<i>Phoxinus lumaireul</i>	1b	KT166631	Palandačić et al. [4]
<i>Phoxinus lumaireul</i>	1b	MG681431	Vučić et al. [6]
<i>Phoxinus lumaireul</i>	1b	MG681432	Vučić et al. [6]
<i>Phoxinus lumaireul</i>	1b	KT166824	Palandačić et al. [4]
<i>Phoxinus lumaireul</i>	1b	KT166829	Palandačić et al. [4]
<i>Phoxinus lumaireul</i>	1c	ON494640	Reier et al. [5]
<i>Phoxinus lumaireul</i>	1c	ON494611	Reier et al. [5]
<i>Phoxinus lumaireul</i>	1c	KT166627	Palandačić et al. [4]
<i>Phoxinus lumaireul</i>	1c	ON495044	Reier et al. [5]
<i>Phoxinus lumaireul</i>	1c	ON494783	Reier et al. [5]
<i>Phoxinus lumaireul</i>	1c	ON494807	Reier et al. [5]
<i>Phoxinus lumaireul</i>	1c	KT166716	Palandačić et al. [4]
<i>Phoxinus lumaireul</i>	1c	ON738222	this study
<i>Phoxinus lumaireul</i>	1c	KT166588	Palandačić et al. [4]
<i>Phoxinus lumaireul</i>	1d	KT166725	Palandačić et al. [4]
<i>Phoxinus lumaireul</i>	1d	KT166708	Palandačić et al. [4]
<i>Phoxinus lumaireul</i>	1d	KT166584	Palandačić et al. [4]
<i>Phoxinus lumaireul</i>	1e	KT166790	Palandačić et al. [4]

<i>Phoxinus lumaireul</i>	1f	KT166762	Palandačić et al. [4]
<i>Phoxinus lumaireul</i>	1f	KT166769	Palandačić et al. [4]
<i>Phoxinus csikii</i>	5a	KT166638	Palandačić et al. [4]
<i>Phoxinus csikii</i>	5a	KT166753	Palandačić et al. [4]
<i>Phoxinus csikii</i>	5a	KT166553	Palandačić et al. [4]
<i>Phoxinus sp.</i>	2	KT166780	Palandačić et al. [4]
<i>Phoxinus sp.</i>	2	KT166557	Palandačić et al. [4]
<i>Phoxinus sp.</i>	2	KT166558	Palandačić et al. [4]
<i>Phoxinus sp.</i>	2	KT166813	Palandačić et al. [4]
<i>Phoxinus sp.</i>	2	KT166589	Palandačić et al. [4]
<i>Phoxinus sp.</i>	2	KT166620	Palandačić et al. [4]
<i>Phoxinus sp.</i>	2	KT166645	Palandačić et al. [4]
<i>Phoxinus sp.</i>	3	KT166691	Palandačić et al. [4]
<i>Phoxinus sp.</i>	3	KT166692	Palandačić et al. [4]
<i>Phoxinus sp.</i>	3	KT166693	Palandačić et al. [4]
<i>Phoxinus sp.</i>	4	KT166574	Palandačić et al. [4]
<i>Phoxinus sp.</i>	4	KT166575	Palandačić et al. [4]
<i>Phoxinus csikii</i>	5a	KT166638	Palandačić et al. [4]
<i>Phoxinus krkae</i>	6	KT166546	Palandačić et al. [4]
<i>Phoxinus krkae</i>	6	KT166548	Palandačić et al. [4]
<i>Phoxinus krkae</i>	6	KT166551	Palandačić et al. [4]
<i>Phoxinus karsticus</i>	7	KT166776	Palandačić et al. [4]
<i>Phoxinus karsticus</i>	7	KT166778	Palandačić et al. [4]
<i>Phoxinus karsticus</i>	7	MG681299	Vučić et al. [6]
<i>Phoxinus karsticus</i>	7	MG681489	Vučić et al. [6]
<i>Phoxinus karsticus</i>	7	MG681490	Vučić et al. [6]
<i>Phoxinus karsticus</i>	7	KT166806	Palandačić et al. [4]
<i>Phoxinus karsticus</i>	7	KT166820	Palandačić et al. [4]
<i>Phoxinus karsticus</i>	7	KT166649	Palandačić et al. [4]
<i>Phoxinus sp.</i>	7	KT166815	Palandačić et al. [4]
<i>Phoxinus sp.</i>	7	KT166817	Palandačić et al. [4]
<i>Phoxinus sp.</i>	8	KT166678	Palandačić et al. [4]
<i>Phoxinus sp.</i>	8	KT166679	Palandačić et al. [4]
<i>Phoxinus sp.</i>	8	KT166683	Palandačić et al. [4]
<i>Rutilus rutilus</i>		AP010775	Saitoh et al. [2]
<i>Squalius cephalus</i>		NC031540	Iwasaki et al. [7]

Table S4. All samples with GenBank accession numbers and references included in the divergence timing analysis of *Delminichthys*, *Phoxinellus* and *Telestes* and sister/outgroups.

Species	GenBank	Reference
<i>Barbus barbus</i>	NC_008654	Saitoh et al. [8]
<i>Carassius auratus</i>	KJ874430	Cheng, Liang, and Sun [9]
<i>Chondrostoma nasus</i>	MG806657	Schönhuth et al. [10]
<i>Chondrostoma phoxinus</i>	MG806658	Schönhuth et al. [10]
<i>Cyprinus carpio</i>	NC_001606	Chang, Huang, and Lo (1994)
<i>Delminichthys adspersus</i>	JQ315974	Palandačić et al. [11]
<i>Delminichthys ghetaldii</i>	MG806663	Schönhuth et al. [10]
<i>Delminichthys jadovensis</i>	ON738200	this study
<i>Delminichthys krbavensis</i>	ON738195	this study
<i>Pachychilon macedonicum</i>	MG806671	Schönhuth et al. [10]
<i>Pelagus minutus</i>	MZ230788	Viñuela Rodríguez et al. [12]
<i>Pelecus cultratus</i>	AY838938	Freyhof et al. [13]
<i>Phoxinellus alepidotus</i>	ON738208	this study
<i>Phoxinellus dalmaticus</i>	ON738213	this study
<i>Phoxinellus pseudoalepidotus</i>	ON738206	this study
<i>Pseudophoxinus anatolicus</i>	AY494754	Hrbek et al. [14]
<i>Rutilus rutilus</i>	AP010775	Saitoh et al. (2011)
<i>Rutilus rutilus</i>	MG806695	Schönhuth et al. [10]
<i>Squalius cephalus</i>	MG806701	Schönhuth et al. [10]
<i>Squalius cephalus</i>	NC031540	Iwasaki et al. [7]
<i>Telestes beoticus</i>	MG372658	Buj et al. [15]
<i>Telestes croaticus</i>	AY509828	Ketmaier et al. [16]
<i>Telestes dabar</i>	MG372497	Buj et al. [15]
<i>Telestes fontinalis</i>	MG372533	Buj et al. [15]
<i>Telestes karsticus</i>	JN188371	Marčić et al. [17]
<i>Telestes metohiensis</i>	MK482048	Benovics et al. [18]
<i>Telestes miloradi</i>	MG372657	Buj et al. [15]
<i>Telestes muticellus</i>	MG372593	Buj et al. [15]
<i>Telestes montenegrinus</i>	AY509849	Ketmaier et al. [16]
<i>Telestes pleurobipunctatus</i>	MG372650	Buj et al. [15]
<i>Telestes polylepis</i>	HM560222	Perea et al. [19]
<i>Telestes souffia</i>	AY509861.1	Ketmaier et al. [16]
<i>Telestes souffia</i>	MG372582	Buj et al. [15]
<i>Telestes turskyi</i>	MG372555	Buj et al. [15]
<i>Telestes ukliva</i>	MG372543	Buj et al. [15]
<i>Vimba melanops</i>	MG806725	Schönhuth et al. [10]

Table S5. Genetic diversity parameters of the mitochondrial cytochrome b marker of the four fish genera. Abbreviations: *h*, number of haplotypes; *Hd*, haplotype diversity; π , nucleotide diversity; *S*, number of polymorphic sites; *k*, mean number of pairwise differences; *N*, number of sequences.

ID	<i>h</i>	<i>Hd</i>	π	<i>S</i>	<i>k</i>	<i>N</i>
<i>Phoxinus</i>						
all	297					1,008
1	225	0.95	0.0183	243	19.95	886
1a	88	0.81	0.0072	149	7.81	393
1b	84	0.96	0.0085	124	9.23	267
1c	40	0.83	0.0077	81	8.43	186
1e	9	0.94	0.0026	13	2.82	12
2	22	0.91	0.0111	80	12.10	40
3	4	1.00	0.0029	6	3.17	4
4	2	0.67	0.0012	2	1.33	3
5a	13	0.94	0.0074	31	8.10	21
6	3	0.42	0.0006	3	0.67	9
7	19	0.92	0.0032	26	3.53	41
8	3	0.60	0.0006	2	0.67	6
<i>Delminichthys</i>						
all	84					359
<i>adspersus</i>	74	0.74	0.0012	78	1.23	340
<i>ghetaldii</i>	3	0.70	0.0022	5	2.2	5
<i>jadovens</i>	2	0.33	0.0003	1	0.33	6
<i>krbavensis</i>	5	0.86	0.0032	9	3.18	8
<i>Phoxinellus</i>						
all	16					17
<i>alepidotus</i>	4	1.00	0.0024	5	2.67	4
<i>dalmaticus</i>	6	0.95	0.0021	6	2.29	7
<i>pseudoaleptidotus</i>	6	1.00	0.0035	10	3.93	6
<i>Telestes</i>						
all	96					197
<i>croaticus</i>	20	0.97	0.0147	75	15.30	25
<i>dabar</i>	9	0.74	0.0023	11	2.40	55
<i>fontinalis</i>	8	0.97	0.0042	16	4.39	9
<i>karsticus</i>	9	0.95	0.0036	17	3.72	13
<i>miloradi</i>	1					1
<i>methionensis</i>	11	0.78	0.0072	85	7.49	26
<i>montenegrinus</i>	15	0.86	0.0047	38	4.94	42
<i>polylepis</i>	4	0.80	0.0017	4	1.80	6
<i>turskyi</i>	6	0.95	0.0036	11	3.71	7
<i>ukliva</i>	12	1.00	0.0057	26	5.97	12

Table S6. Pairwise uncorrected p-distances between species/lineages of each genera. Species names of *Delminichthys*, *Phoxinellus* and *Telestes* are abbreviated in the horizontal line.

Delminichthys, Phoxinellus and Telestes are abbreviated in the horizontal line.										
<i>Phoxinus</i>	1a	1b	1c	1e	5	2	3	4	6	7
1a										
1b	0.02									
1c	0.02	0.02								
1e	0.03	0.03	0.03							
5	0.06	0.06	0.06	0.06						
2	0.04	0.05	0.04	0.05	0.05					
3	0.05	0.06	0.05	0.06	0.05	0.04				
4	0.05	0.06	0.05	0.06	0.05	0.04	0.05			
6	0.07	0.07	0.07	0.07	0.08	0.06	0.08	0.07		
7	0.08	0.09	0.08	0.08	0.09	0.07	0.08	0.08	0.08	
8	0.08	0.09	0.09	0.09	0.09	0.07	0.08	0.09	0.09	0.08
<i>Delminichthys</i>	<i>ads</i>	<i>ghe</i>	<i>jad</i>							
<i>adspersus</i>										
<i>ghetaldii</i>	0.02									
<i>jadovens</i>	0.04	0.04								
<i>krbavensis</i>	0.04	0.04	0.02							
<i>Phoxinellus</i>	<i>ale</i>	<i>pse</i>	<i>dal</i>							
<i>alepidotus</i>										
<i>pseudoalepidotus</i>	0.02									
<i>dalmaticus</i>	0.04	0.05								
<i>Telestes</i>	<i>cro</i>	<i>dab</i>	<i>fon</i>	<i>kar</i>	<i>met</i>	<i>mil</i>	<i>mon</i>	<i>pol</i>	<i>tur</i>	
<i>croaticus</i>										
<i>dabar</i>	0.11									
<i>fontinalis</i>	0.03	0.11								
<i>karsticus</i>	0.10	0.10	0.09							
<i>metohiensis</i>	0.11	0.01	0.11	0.09						
<i>miloradi</i>	0.11	0.03	0.11	0.10	0.03					
<i>montenegrinus</i>	0.09	0.10	0.10	0.07	0.10	0.10				
<i>polylepis</i>	0.09	0.09	0.08	0.04	0.09	0.09	0.07			
<i>turskyi</i>	0.10	0.10	0.10	0.07	0.10	0.10	0.08	0.07		
<i>ukliya</i>	0.10	0.08	0.09	0.07	0.08	0.08	0.07	0.06	0.05	

2. List of figures



Figure S1. Major river drainages in the Dinaric karst with the distribution of the four fish genera *Phoxinus*, *Delminichthys*, *Phoxinellus* and *Telestes*. Each Symbols correspond to species (see legend).

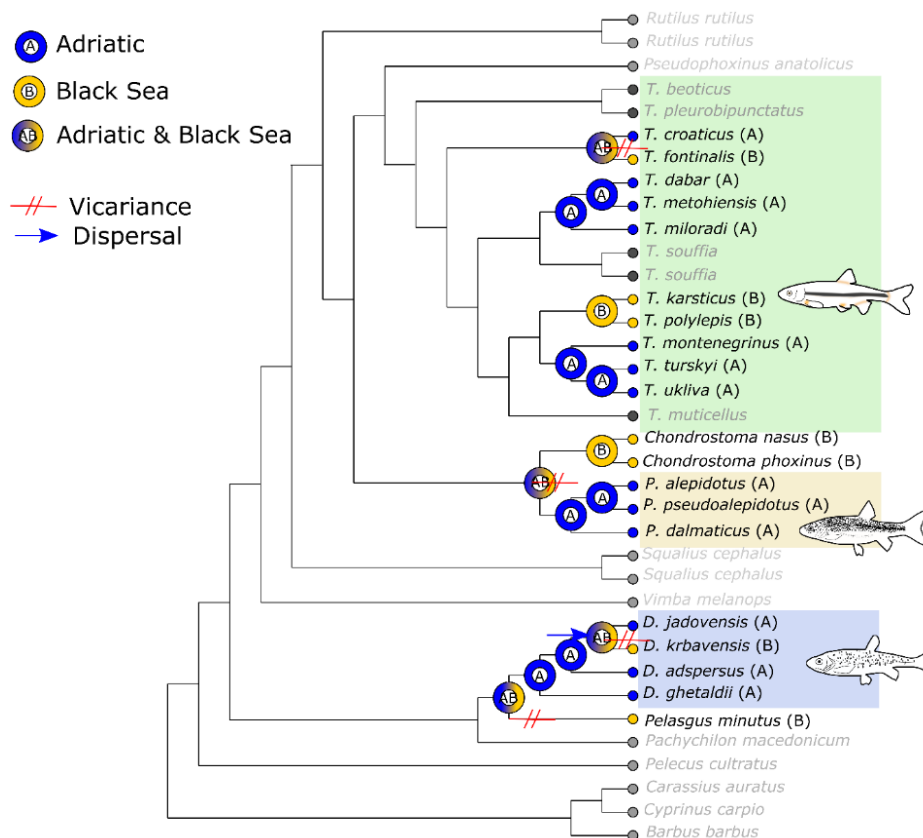


Figure S2. Reconstruction of ancestral areas (RASP) analyses of *Delminichthys*, *Phoxinellus* and *Telestes* (together with sister genera and outgroups, for details see Table S4) using two biogeographic areas: Adriatic and Black Sea basin (see legend) and based on cytochrome b fragment. The analysis was performed with S-DIVA [20] and implemented in RASP [21] based on the phylogenetic trees calculated in the divergence timing analysis (BEAST [1] see Materials and Methods for details). Pie chart at node represent the most likely ancestral distributions and letters denote which regions (Adriatic, Black Sea, or both) are coded by each color. Letters following tip labels indicate the recent distribution of each tip. Only ancestral areas with a probability >0.5 are shown. Vicariance and/or dispersal events are given next to the pie chart.

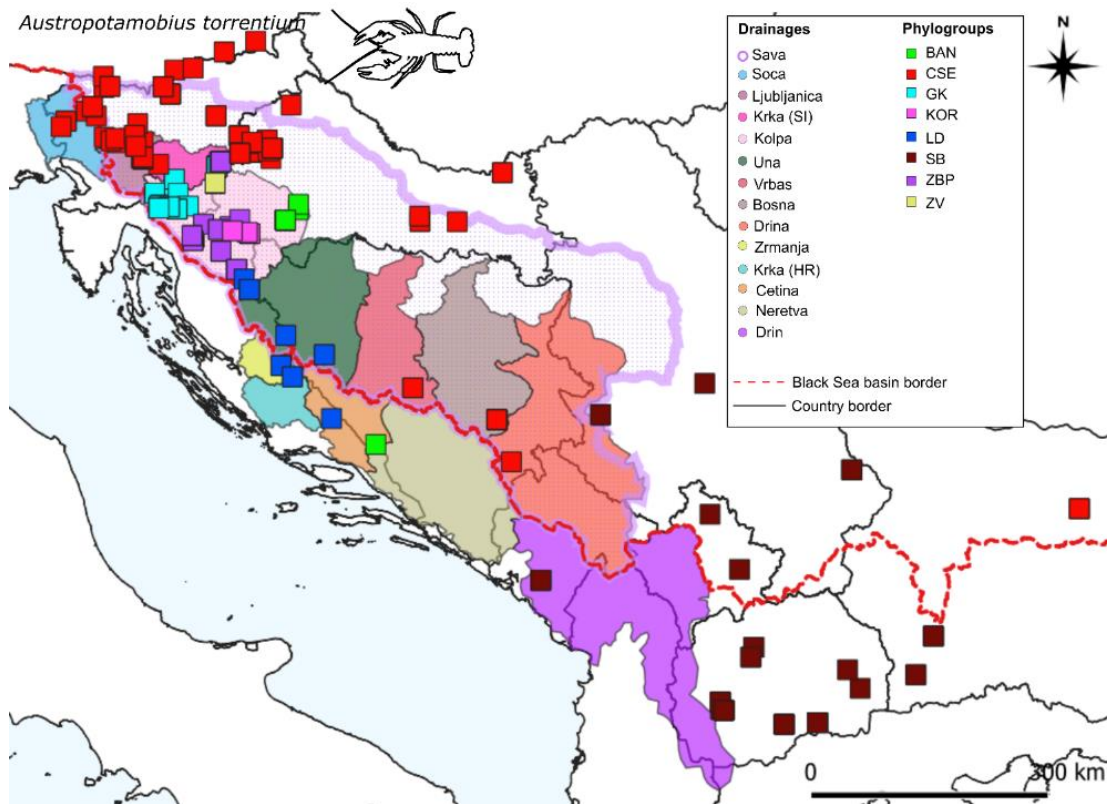


Figure S3. Distribution map of the mitochondrial lineages of stone crayfish *Astopotamobius torrentium* summarized according to [22, 23, 24]; based on cytochrome oxidase I. The lineages are coded in Klobucar et al. [22] and were thereafter used with these names throughout the literature. CSE – central and south-eastern Europa, SB – southern Balkans, GK – Gorski Kotar, BAN - Banovina, ZV – Zeleni vir, KOR - Kolpa, LD – Lika and Dalmacija, ZPB – Zumberak, Plitvice and Bjelolasica. The red dashed line represents the Adriatic-Black Sea basin divide.

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CHAPTER 2

The minnow *Phoxinus lumaireul* (Leuciscidae) shifts the Adriatic–Black Sea basin divide in the north-western Dinaric Karst region

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RESEARCH ARTICLE

The minnow *Phoxinus lumaireul* (Leuciscidae) shifts the Adriatic–Black Sea basin divide in the north-western Dinaric Karst region

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Abstract

Karst landscapes are characterized by intermittent and sinking streams. The most common method used to study underground hydrological connections in karst is tracing tests. However, a more biologically oriented approach has been suggested: analysis of the genetic structure of aquatic organisms. Biological tracers can be sought among troglomenes, that is, surface species that occasionally enter caves and groundwater. One such example is the fish genus *Phoxinus*, which exhibits high genetic diversity and complex phylogeography in the Balkan Peninsula. In the north-western Dinaric Karst, the complex hydrological network was digitalized in 2020. Contemporaneously, *Phoxinus lumaireul* populations in the Slovenian Dinaric Karst were intensively sampled and analysed for fragments of two mitochondrial genes and one nuclear gene. The derived phylogeographic structure and data on hydrological connections were compared to evaluate support for three alternative scenarios: The genetic structure (1) is a consequence of the ongoing geneflow through underground connections, (2) reflects a previous hydrological network or (3) is an outcome of anthropogenic translocations. The results suggest that the first two scenarios seem to have played a major role, while the third has not had profound effects on the genetic composition. Comparison between the genetic structure of Slovenian Dinaric Karst sampling sites and that of hydrologically isolated reference sampling sites indicated a greater genetic connectivity in the former. Moreover, the range of Adriatic (1a) and Black Sea (1c) haplotypes does not correspond to the Adriatic–Black Sea basin divide but is shifted northwards.

KEYWORDS

Dinaric Karst, paleohydrology, *Phoxinus*, underground connections, underground migration

1 | INTRODUCTION

Karst landscapes are formed by soluble carbonate rocks, commonly limestone or dolomite, which, besides possessing specific features (e.g., sinkholes and caves), exhibit a lack of contiguous surface river systems (RS) and are characterized instead by intermittent and sinking streams. However, karst landscape patchy surface waters may be interconnected via complex underground drainage networks, with extreme spatial and temporal oscillations in groundwater levels (Bonacci, 1987; Bonacci & Živaljević, 1993). Several scientific disciplines, for example, hydrology, geology and biology, attempt to understand the intricate structure of karst aquifers in order to support water management, control pollution and foster conservation of endangered species (Ford & Williams, 2007). The most commonly used method for studying underground hydrological connections in karst is the tracing test (Field, 1999) for which tracers are substances (physical, chemical or isotopic) carried (as, e.g., a dye or salt) by water and that provide information on flow direction, rate and velocity (Petrič et al., 2020). Complementary to classical water tracing methods, a more recent, biologically oriented approach has been suggested: analysis of the genetic structure of aquatic organisms inhabiting underlying karstic aquifers (Pipan & Culver, 2007; Verovnik et al., 2003). In addition to revealing links between water bodies, biological connectivity reflects the ecology of the studied organisms and can convey information on pore size, water quality and, by combining active and passive swimmers, alternative flow directions (Humphreys, 2009; Konec et al., 2016; Verovnik et al., 2005).

The Dinaric Karst of the Western Balkan Peninsula is particularly suited for studies comparing genetic and hydrological connectivity, as it comprises among the best-investigated karst areas in terms of hydrogeology (Bonacci, 1987) and population structure of aquatic fauna associated with subterranean waters (e.g., Bilandžija et al., 2013; Palandačić, Bonacci, & Snoj, 2012; Zakšek et al., 2009). One of the characteristics of Dinaric Karst is the presence of poljes, flat alluvial depressions encircled by higher ground composed of permeable rocks that can be traversed by sinking rivers. Superficial sections of these rivers, which flow partly underground, are the only sizable surface wetlands in karst landscapes in which, as a result, they act as habitat islands.

Thus, in fish, karst has often been considered an isolating factor in organismal dispersal (e.g., Buj et al., 2014; Mustafić et al., 2017; Perea et al., 2010), promoting speciation and consequently increasing the species richness of the area. Similarly, the dissected and patchy karst landscape is considered to have promoted vicariance also in other epigean organisms, for example, various mammals, crayfish and insects (e.g., Klobučar et al., 2013; Krystufek et al., 2007; Previšić et al., 2014) as well as in cave fauna (e.g., Bilandžija et al., 2013; Caccone & Sbordón, 2001).

Nevertheless, it has been suggested that some fish can use underground passages to migrate between isolated, sinking streams (Palandačić, Matschiner, et al., 2012; Snoj et al., 2008; Zupančič, 2008) and some underground migration has also been shown in the epigean isopod *Asellus* (Konec et al., 2016) and

amphipods *Echinogammarus* and *Fontogammarus* (Žganec et al., 2016), as well as in subterranean *Proteus* (Zakšek et al., 2018).

Recently, the complex hydrological network of the north-western Dinaric Karst in Slovenia (hereafter referred to as Slovenian Dinaric Karst) was digitalized by Petrič et al. (2020), who employed more than 200 tracing tests. As a result, the hydrology of the area is fairly well understood: Whereas the western part of the Slovenian Dinaric Karst drains towards the Adriatic Sea, the central and southern parts flow to the Black Sea. Meanwhile, because the water flow fluctuates spatially and temporally (Bonacci, 1987, 2013; Konec et al., 2016), the karstic subterranean watershed separating the basins is difficult to determine (Habič, 1989; Terzić et al., 2014). There are five major RS in Slovenian Dinaric Karst corresponding, respectively, to the main rivers Ljubljana, Vipava, Reka, Krka and Kolpa (Gams, 1993). The watersheds of all five rivers include both karstic and non-karstic areas, as well as several underground connections among them.

Biological tracers revealing subterranean flows can be sought among obligate cave species or among troglonexes, that is, surface species that occasionally enter caves and groundwater, where they are able to survive but not reproduce (Howarth & Moldovan, 2018). Troglonexes seem to be especially suitable as indicator species, because they connect different isolated surface wetlands such as karst poljes, as well as karstic and non-karstic areas. One such example is the group of ubiquitous small minnows of the genus *Phoxinus* Rafinesque, 1820 (formerly in the family Cyprinidae, now in Leuciscidae; Schönhuth et al., 2018) that inhabit diverse habitats, from mountain streams to lowland rivers and lakes (Frost, 1943; Tack, 1940). Recent genetic studies of *Phoxinus* spp. in Europe have revealed a high level of genetic diversity and complex phylogeography in the Balkan Peninsula (Palandačić et al., 2015; Palandačić et al., 2017; Vučić et al., 2018) that do not follow zoogeographic patterns defined by drainage boundaries (e.g., Konec et al., 2016; Palandačić et al., 2017; Palandačić et al., 2020; Zogaris et al., 2009). This distribution of *Phoxinus* genetic lineages and the admixture detected among them was ascribed to human-mediated actions (see, e.g., Museth et al., 2007; Miró & Ventura, 2015; Kneibelsberger et al., 2015). However, it has also been suggested that, besides human introductions (Vučić et al., 2018), the admixture of lineages in the Western Balkans can also be attributed to natural causes, occurring as a consequence of historical and recent migration patterns of minnows (Palandačić et al., 2015, 2020). These effects also apply to the species *Phoxinus lumaireul* (Schinz, 1840) (clade 1 sensu Palandačić et al., 2017), which inhabits the Adriatic and the Black Sea basins from Italy via Slovenia, Croatia, Austria and Bosnia-Herzegovina to Serbia and which harbours notable genetic diversity with six distinct genetic lineages (subclades 1a–f sensu Palandačić et al., 2017).

The combination of an organism, such as *P. lumaireul*, with high levels of genetic diversity inhabiting a hydrological system and a complex but well studied system of subterranean water connections (Slovenian Dinaric Karst) provides an excellent research opportunity for deciphering the factors that might shape phylogeographic patterns and biological diversity of surface-dwelling species in karstic areas. Thus, *P. lumaireul* populations in Slovenian Dinaric Karst were

intensively sampled and analysed for partial fragments of two mitochondrial genes (*cytochrome b* and *cytochrome c oxidase subunit 1*) and one nuclear gene (*ribosomal protein S7*). The derived phylogeographic structure, along with data on past and present hydrological connections, was compared in order to assess support for three alternative scenarios: that the genetic structure (1) is a consequence of the ongoing geneflow through underground connections, (2) reflects the past hydrological network or (3) is an outcome of anthropogenic translocations.

2 | MATERIAL AND METHODS

2.1 | Study area and grouping of samples

In concordance with several abiotic properties (e.g., tectonic, lithological, relief, climatic and edaphic), Slovenia can be divided into five zoogeographic regions (Figure 1a): Slovenian Dinaric Karst (SDK), Submediterranean Region (SMR), Subpannonian Region (SPR) and Alpine and Prealpine Region (herein merged into one group, APR) (Mršić et al., 1997). The main part of the karst area of Slovenia comprises the Dinaric and Alpine regions but also partly the Prealpine and SMRs (Mihevc et al., 2016).

The main study area was SDK, which included 20 sampling sites. Additionally, there were five sampling sites in APR, four in SMR and three in SPR, adopted from previous studies and included as reference sampling sites.

The sampling sites in SDK were sub-divided into five groups corresponding to five RS: (1) Ljubljana RS (LRS), (2) Vipava RS (VRS), (3) Reka RS (RRS), (4) Krka RS (KRRS) and (5) Kolpa RS (KORS) (Figure 1b). LRS includes 13 sampling sites (MALI, CERK, RAKO, NANO, RAKU, HOTE, LOGA, TOJN, IZIC, BLOS, RASK, CERJ, LJU), VRS two sites (PRED, VIPA), RRS one site (MRZL), KRRS two sites (KRKA, RAD) and KORS two sites (KOLP, MOKR); see Table 1 and Figure 1b.

In the APR, there were five sampling sites (BOH, KRN, LOZN, NAD, SOC), in the SMR four sites (BELS, KORO, OSP, RIZA) and in the SPR, three sampling sites (PTU, RAT, SCA).

In comparison to the reference sampling sites, those of SDK exhibit high hydrological connectivity (Petrič et al., 2020). As the hydrological system is very complex and the local names of the rivers might be challenging for nonnative speakers, a detailed description of the sampled streams and their mutual underground connections is presented in the Supporting Information, while a simplified presentation of the main interconnections is depicted in Figure 1b. None of the reference sampling sites is connected underground to the SDK sampling sites (Figures 1b and S1).

2.2 | Sampling design

The data from Petrič et al. (2020) are deposited at the website of the Slovenian Environment Agency (<http://gis.arso.gov.si/>) and were

downloaded and plotted in QGIS 3.12.2 (QGIS Development Team, 2009). The division of the sampling sites as described above was drawn manually in QGIS (depicted in Figure 1) and plotted together with the underground connections in Figure S1. A simplified depiction of the underground connections is shown in Figure 1b.

In total, 477 specimens of *P. lumaireul* were collected between the years 2016 and 2020 from 22 sampling sites (Figure 1, Table 1) using electrofishing, fishing nets and traps. Besides the freshly collected samples, the dataset also includes 101 previously published sequences (Palandačič et al., 2015, 2017) from 10 additional sampling sites. The combined dataset of new and GenBank sequences comprises 578 sequences (for respective GenBank accession numbers see Table S1).

2.3 | Mitochondrial DNA

2.3.1 | DNA extraction, amplification, sequencing and alignment

Total DNA was extracted from 477 fin clips in a clean room using QIAmp DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) and following the manufacturer's protocol. Partial fragments of the mitochondrial (mt) *cytochrome c oxidase subunit 1* (COI) gene (the so-called barcoding region) and the mt *cytochrome b* (*cytb*) gene were amplified using the primer pairs FishF1/FishR1 (COI; Behrens-Chapuis et al., 2015) and GluF/ThrR (*cytb*; Bergsten et al., 2014) with the polymerase chain reaction (PCR) protocol described in (Palandačič et al., 2017). Sequencing was performed at Microsynth (Balgach, Switzerland) in both directions using the PCR primers.

Sequences were manually checked, trimmed and unambiguously aligned (no missing data) in Geneious v2.10.3 (<https://www.geneious.com>). COI and *cytb* sequences were subsequently concatenated using Geneious, and the concatenated alignment, including available sequences from GenBank, was used for all further genetic analysis (hereinafter referred to as combined COI–*cytb*).

2.3.2 | Phylogenetic tree reconstruction

Sequences of COI–*cytb* were collapsed to unique haplotypes using FaBox v1.5 (Villesen, 2007). The best-fit models (K2P+G4 for COI; GTR+F+G4 for *cytb*) were selected using ModelFinder (Kalyaanamoorthy et al., 2017) applying the BIC criterion. Phylogenetic relationships were inferred using maximum likelihood (ML) and Bayesian inference (BI) performed in RAXML-HPC v8 (Stamatakis, 2014) and Mr. Bayes v3.2 (Ronquist et al., 2012), respectively. We applied the GTR+G model for ML tree search with 10,000 bootstrap replicates to assess nodal support. BI was performed with three runs, each with four chains, and run for 50,000,000 generations. Trees and parameters were sampled every 1000 generations. After inspecting effective sample size (ESS) parameters that exceeded 300 in Tracer v1.7.1 (available at <http://tree.bio.ed.ac.uk/software/>)

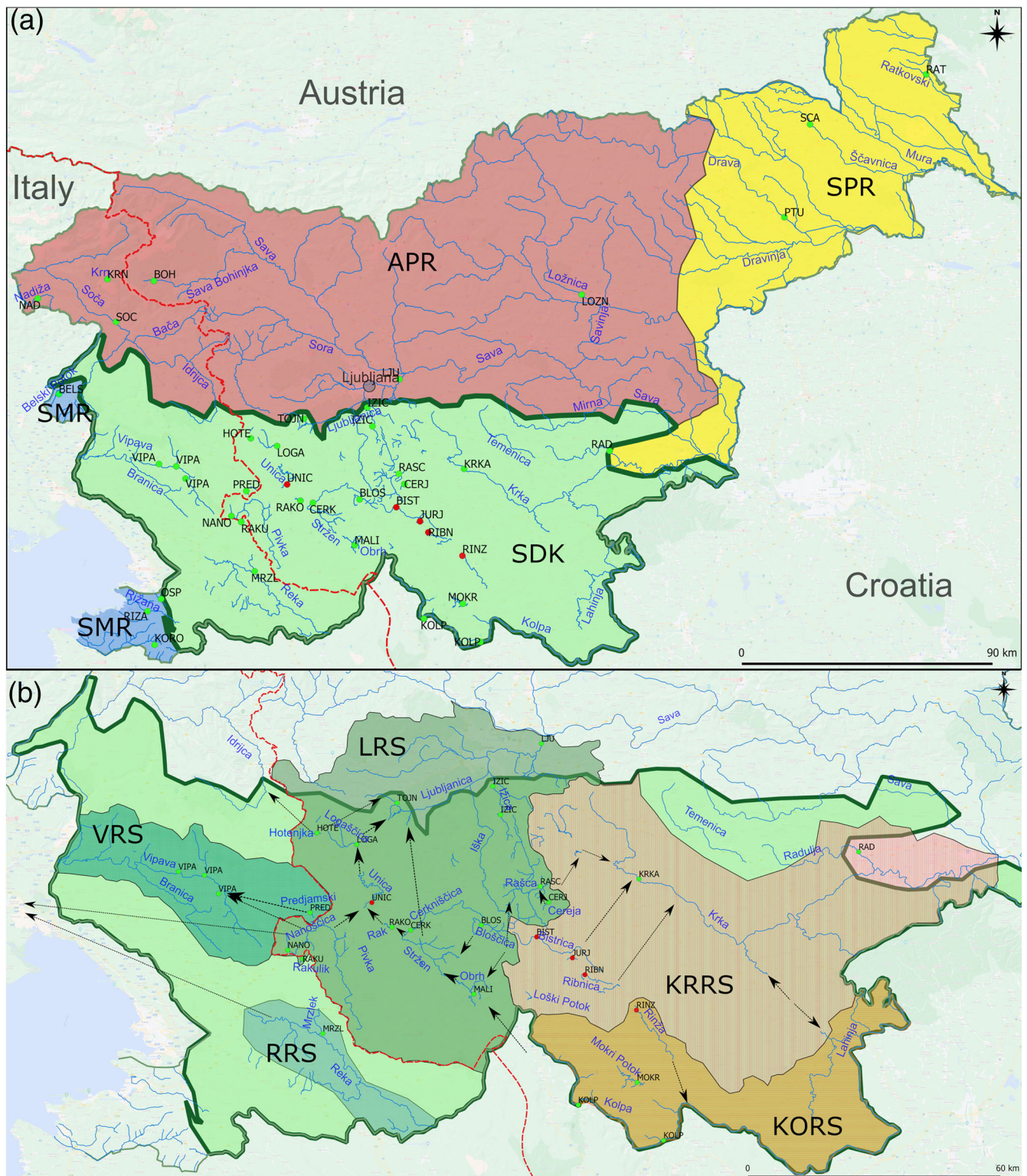


FIGURE 1 North-western Dinaric Karst and adjacent regions. (a) Zoogeographic regions of Slovenia including sampling sites. Names of main rivers of Slovenia are given. Abbreviations: APR, Alpine and Prealpine Regions; SDK, Slovenian Dinaric karst; SMR, Submediterranean region; SPR, Subpannonian region. (b) Major River systems in SDK. Arrows represent underground connections between rivers. Red dashed line represents divide between Adriatic and Black Sea basins. Abbreviations: VRS, Vipava river system; RRS, Reka river system; LRS, Ljubljanica river system; KRRS, Krka river system; KORS, Kolpa river system. Green dots represent sites where *Phoxinus* was present; red dots, sampling sites that were not inhabited by *Phoxinus*.

TABLE 1 *Phoxinus lumaireul* sampling sites from Slovenia

				Number		
ID	Sampling site	Drainage	Clade-group	Present study	Palandačić et al. (2015, 2017)	Total
Slovenian Dinaric Karst						
Ljubljana river system (LRS)						
BLOS	Bloščica	B	1c	29	4	33
CERJ	Cereja	B	1c	36	0	36
CERK	Cerkniščica	A, B	1a/1c	28	8	36
HOTE	Hotenjka	B	1a	19	0	19
IZIC	Izica	B	1c (1a)	25	0	25
LJU	Ljubljana River	B	1c	0	2	2
LOGA	Logaščica	B	1a	20	0	20
MALI	Mali Obrh	A, Danubian	1a (1c)	19	6	25
NANO	Nanoščica	A, B	1a	42	6	48
RAKO	Rak	B	1a (1c)	19	0	19
RAKU	Rakulik	B	1a	26	0	26
RASC	Raska	B	1c (1a)	17	0	17
TOJN	Tojnica	B	1c (1a)	18	0	18
Reka river system (RRS)						
MRZL	Stream Mrzlek	A	1a	25	4	29
Vipava river system (VRS)						
PRED	Lovka	A	1a	33	6	39
VIPA	Vipava and tributaries	A	1a	10	2	12
Kolpa river system (KORS)						
						0
KOLP	Kolpa	B	1b	26	6	32
MOKR	Mokri Potok	B	1b	34	0	34
Krka river system (KRRS)						
KRKA	Krka	B	1c	33	0	33
RAD	Radulja	B	1c	0	10	10
						513
Alpine & Prealpine Regions						
BOH	Bohinj Lake	B	1c	0	5	5
KRN	Lake Krn	A	1a	0	4	4
LOZN	Ložnica	B	1c	3	3	6
NAD	Nadiža	A	1a	0	2	2
SOC	Soča	A	1a	0	1	1
						18
Submediterranean Region						
BELS	Belski Potok	A	1a	6	5	11
KORO	Malinska	A	1a	3	4	7
OSP	Osapska	A	1a	0	2	2
RIZA	Rižana	A	1a	6	5	11
						31
Subpannonian Region						
PTU	Drava	B	1d	0	5	5
RAT	Ratkovski	B	1d	0	4	4
SCA	Ščavnica	B	1d	0	7	7
						16

(Continues)

TABLE 1 (Continued)

ID	Sampling site	Drainage	Clade-group	Number		
				Present study	Palandačić et al. (2015, 2017)	Total
Summary				477	101	578

Note: Sampling sites, belonging to the Adriatic (A) or Black Sea (B) basins (drainages), clade-group affiliation, number of examined specimens per location (and subdivision of number of specimens from this study and from previous studies (Palandačić et al., 2015, 2017) per location) and total number are reported. Clade-group is reported in parentheses when only 1 or 2 individuals from the sampling site belong to this clade-group

tracer/), the first 25% of trees were discarded as burn-in, and a 50% majority rule consensus tree was built from the remaining trees.

2.3.3 | Median-joining network, population diversity indices

The median-joining haplotype network (Bandelt et al., 1999) was calculated for *COI-cytb* using the software PopART v1.7 (Leigh & Bryant, 2015). Haplotype diversity (H_d), nucleotide diversity (π), number of polymorphic sites (S) and mean number of pairwise differences (k) were calculated using DnaSP v6 (Rozas et al., 2017).

2.3.4 | Pairwise distances

Uncorrected pairwise (p-)distances were calculated using MEGA 10.0.5 (Kumar et al., 2018) and plotted using ggplot2 v3.3.5 (Wickham, 2016) in R v3.6.3 (R Core Team, 2020).

2.3.5 | Spatial population clustering and analysis of molecular variance (AMOVA)

In order to gather information on the spatial distribution of populations, cluster analysis was performed with GENELAND v4.9.2 (Guillot et al., 2005; Guillot et al., 2008) in R v3.6.3 (R Core Team, 2020) using the *COI-cytb* dataset. GENELAND enables the detection and location of genetic discontinuities between populations and the correlation of these discontinuities with landscape and environmental features (Guillot et al., 2005). The number of populations (K) was allowed to vary between 1 and 20. Twenty independent runs were conducted, each with 1,000,000 Markov Chain Monte Carlo (MCMC) iterations and with sampling at every 100 steps. The maximum number of nuclei in the Poisson-Voronoi tessellation was fixed to 1665 (full GENELAND, see below) and 1500 (sub-set GENELAND), and 1000 iterations were discarded as burn-in.

The GENELAND analysis was run on two levels: 1, full GENELAND, including all sampling sites from SDK, APR, SMR and SPR; and 2, sub-set GENELAND, including SDK sampling sites only in order to gain a better insight into the fine-scale population structure within this area.

To test whether the distribution of genetic variance corresponds with the clustering of populations suggested by GENELAND, AMOVA

(Excoffier et al., 1992) was conducted using Arlequin v3.5.2.2 (Excoffier & Lischer, 2010) for both full and sub-set GENELAND analysis. The proportion of genetic variation found among sampling sites (F_{ST}), among sampling sites within groups (F_{SC}) and among groups (F_{CT}), was estimated. Significance associated with the fixation indices was evaluated through random permutation procedures (10,000 permutations).

2.3.6 | Divergence time dating

In order to estimate the timing of the splits between and within the mt-clades of the genus *Phoxinus*, divergence time dating was performed based upon the mt-*cytb* phylogenetic tree. While divergence time estimation inferred from phylogenetic trees is frequently applied (Hedges et al., 2015), the results are often questioned (Bromham & Woolfit, 2004) and exhibit large confidence intervals (Warnock et al., 2017). To ensure reliable divergence time estimates, *cytb* was chosen because the evolutionary rate has already been estimated for Plagopterinae (a subfamily of Leuciscidae), which contains the true minnows including *Phoxinus* (Dowling et al., 2002). Second, outgroups were selected on the basis of the phylogenies of the complete mt genome published in Imoto et al. (2013) and a recently published study of Schönhuth et al. (2018). All sequences used are reported in Table S2 along with the corresponding Genbank numbers. Finally, fossil data were used as additional calibrating points to increase the accuracy of the evolutionary rate estimation, and the chosen model was tested by nested sampling (Russel et al., 2019) to check the reliability of the results (Ritchie et al., 2016).

The trees were constructed using BEAST2 v2.5 (Bouckaert et al., 2019). The chosen site model was GTR+F+G4, selected using ModelFinder (Kalyaanamoorthy et al., 2017) under the Bayesian information criterion (BIC). The tree was time-calibrated based on the estimated divergence times of Cyprininae (68–97.1 Mya, Saitoh et al., 2011), Leucisinae (23.5–24.5 Mya, Böhme & Ilg, 2003), *Chondrostoma* (5.21 Mya, Böhme & Ilg, 2003), *Squalius* (6.56 Mya, Böhme & Ilg, 2003) and *Phoxinus* (29.2–30.2 Mya, Mödden et al., 2000). A lognormal distribution with an offset of the minimum age was used, and the standard deviation adjusted so that the above-stated divergence time span was covered by 95% of the sampled divergence date priors (Drummond et al., 2006; Ho, 2007). As an additional calibration point, an uncorrelated, relaxed log-normal clock, corresponding to an evolutionary rate of 0.0053 mutations per site per million years, was applied, a rate generally used for the

Leuciscidae family (Dowling et al., 2002). Node times were estimated under a birth-death tree prior, which is often used when extinction rates are considered negligible and which has been shown to produce stable results (Ritchie et al., 2016). The analysis was run for 50,000,000 generations and sampled every 1000 generations. For further support of the model, nested sampling analyses (Russel et al., 2019) were applied in BEAST2 to compare the marginal likelihood of the above-described model and a strict clock model and different tree priors (coalescent Bayesian skyline and Yule model, detailed information can be found in Table S3). Forty-five particles for all models were used to compute the marginal likelihood and its standard deviation. Then, the marginal likelihoods of the models were compared and the log Bayes factor calculated.

The log-file of the final model was inspected in Tracer v1.7.1 and showed large values (>300) for the ESS. A maximum clade credibility (MCC) tree was estimated using Tree Annotator (Drummond & Rambaut, 2007). The final tree was plotted against the geological timescale using the R package strap v1.4 (Bell & Lloyd, 2014) in R v3.6.3 (R Core Team, 2020).

2.4 | Nuclear DNA

2.4.1 | DNA amplification, cloning, sequencing and alignment

The usefulness of the nuclear (nc) markers *recombination activating gene 1* (RAG1) and *internal transcribed spacer 1* (ITS1) for the detection of hybrids within the genus *Phoxinus* has been questioned previously (Palandačić et al., 2020). Thus, another nuclear partial fragment, the first intron of the single-copy *nuclear ribosomal protein S7* (RPS7) gene (used in other phylogenetic studies, e.g., Perea et al., 2010) was chosen for analysis to test whether admixture within *P. lumaireul* can be recognized. For amplification, primers S7RPEX1F/S7RPEX2R (RPS7; Chow & Hazama, 1998) were used. The PCR mixture had a final volume of 50 µl containing 27.75 µl nuclease-free water, 1× PCR buffer, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.2 µM of each primer and 1.25 U/µl AmpliTaq Gold® DNA Polymerase. PCR conditions were as follows: 95°C for 4 min, 35 cycles (94°C for 30 s, 55°C for 30 s, 72°C for 50 s) and final elongation at 72°C for 7 min. Sequencing was performed at Microsynth (Balgach, Switzerland) in both directions using the PCR primers.

After sequencing, heterozygous individuals were detected. However, due to length polymorphism, their sequences could not be read. Therefore, PCRs of 10 random individuals per sampling site (where available) were repeated using the proofreading Phusion® High-Fidelity Polymerase (New England Biolabs, Ipswich, UK) chosen due to its low error rate (Hestand et al., 2016). The PCR conditions were as follows: 98°C for 30 s, 35 cycles (98°C for 10 s, 64°C for 30 s, 72°C for 30 s) and final elongation for 10 min at 72°C. PCR fragments were purified by adding 2 µl ExoSAP (ExoSAP-IT; Amersham Biosciences, Arlington Heights, IL) into 5 µl of the PCR product to remove excess primers and dNTPs and cloned with the TOPO-TA© cloning

kit (Invitrogen, Carlsbad, CA, USA). Six clones per individual were sequenced in both directions using M13 universal primers.

Subsequent to cloning, multiple, repeated haplotypes of RPS7 within an individual were excluded from further analysis. The sequences of RPS7 exhibited different lengths, and therefore, an alignment including gaps was produced using the MAFFT algorithm (Katoh & Standley, 2013; fasta file available in the Supporting Information). Simple indel coding was applied in FastGap (Borchsenius, 2009) following the approach of Palandačić et al. (2020).

2.4.2 | Phylogenetic tree reconstruction

A phylogenetic tree for RPS7 was constructed using both BI and ML as described for the mtDNA analysis. ModelFinder (Kalyaanamoorthy et al., 2017) was used to select the best-fit model (F81+F+I+G4) using the BIC criterion.

2.4.3 | Median-joining network, population diversity indices

The median-joining allele network for RPS7 and population diversity indices (Hd, π , S, k) were calculated as described for mtDNA analysis.

2.4.4 | Pairwise distances

Uncorrected p-distances were calculated as described for mtDNA analysis.

3 | RESULTS

3.1 | Mitochondrial DNA

3.1.1 | DNA extraction, amplification, sequencing and alignment

A total of 651 base pairs (bp) of the COI and 1091 bp of the cytb gene were resolved with sequence analysis of 458 individuals. Together with the sequences from previous studies, the combined COI-cytb dataset final alignment included 555 sequences that were 1742 bp in length.

3.1.2 | Phylogenetic tree reconstruction

The complete COI-cytb dataset was collapsed to 133 unique haplotypes. Both the ML tree and BI tree resulted in a similar topology, though the BI tree had higher statistical support (Figure S2A,B).

In previous research, four genetic lineages were recognized for *P. lumaireui* in Slovenia: 1a, 1b, 1c and 1d (see Introduction); this numbering was also adopted in the present study. However, while the calculated tree supported the clustering of the newly sampled haplotypes to one of the three clades 1a, 1b and 1d, the rest of the haplotypes were not monophyletic but rather formed several subclades. The mt-clades are named mt-1a, mt-1b and mt-1d, while mt-1c is referred to as a group, to emphasize its possibly polytomous origin.

Clade mt-1a included samples from the APR (KRN, NAD, SOC) and SMR (OSP, RIZA, KORO, BELS).

Of the SDK samples, RRS (MRZL) VRS (VIPA and PRED) and south-western LRS (NANO, RAKU, HOTE, LOGA, MALI, RAKO and CERK, which are hydrologically allocated to the Black Sea basin) clustered to this clade. Of 13 LRS sampling sites, seven clustered to clade mt-1a.

Clade mt-1b included only two sampling sites belonging to the KORS (KOLP and MOKR) in the SDK.

Group mt-1c consisted of sampling sites from the APR (BOH, LOZN) and the SDK samples from KRRS (KRKA, RAD), as well as north-eastern LRS (BLOS, CERK, CERJ, IZIC, LJU, RASC and TOJN).

Sampling site CERK (LRS) exhibited haplotypes from clade mt-1a and group mt-1c; 30 samples bore mt-1a and six, mt-1c haplotypes. In IZIC, TOJN and RASC, only one specimen at each sampling site exhibited the mt-1a haplotype, while the others bore mt-1c haplotypes. Thus, for the discussion, CERK was grouped with the south-western LRS sampling sites and the other three to the north-eastern LRS sampling sites (for details see Median-joining network).

mt-1d was the most distinctive clade and included samples from the SPR (PTU, RAT, SCA).

3.1.3 | Median-joining network, population diversity indices

The haplotype network reflected the structure of the phylogenetic tree with clearly separated clades mt-1a, mt-1b and mt-1d. The haplotypes denoted as the group mt-1c were located in the centre of the network, forming several haplogroups and including single haplotypes (Figure 2). Numbers for haplotype diversity (H_d), nucleotide diversity (π), number of polymorphic sites (S) and mean number of pairwise differences (k) are reported in Table 2.

Clade mt-1a consisted of 59 haplotypes and showed a radial structure. It exhibited a relatively high level of H_d ($H_d = 0.85$), though the nucleotide diversity ($\pi = 0.00152$) was low (Table 2).

Clade mt-1a included the SDK sampling sites, RRS (MRZL), VRS (PRED, VIPA) and the south-western LRS (NANO, RAKU, HOTE, LOGA, MALI, RAKO). Besides, 30 specimens from CERK and one single specimen from IZIC, RASC and TOJN (north-western LRS) grouped in mt-1a. Sampling sites from APR (KRN, NAD, SOC), as well as from SMR (BELS, OSP, RIZA, KORO), clustered in this clade.

The dominant haplotype, representing the centre of the major haplogroup, was shared by 107 individuals (Figure 2) and included specimens from only certain parts of the SDK: the south-western LRS

(CERK, HOTE, MALI, NANO, RAKO, LOGA, RAKU, RASC), RRS (MRZL) and VRS (VIPA, PRED). Five additional haplogroups with more than 10 individuals from different SDK sampling sites were detected: (i) LOGA + NANO + PRED (LRS and VRS), (ii) CERK + MALI + RAKO (only LRS), (iii) a haplogroup formed by CERK + RAKO + MALI, where also the single specimens from TOJN and IZIC cluster (LRS) and (iv) PRED + RAKU + VIPA (LRS and VRS). There was one mixed haplogroup, which, besides the LRS sampling sites NANO + RAKO + RAKU, included also individuals from the SMR (KORO) and APR (NAD).

Besides KORO and NAD, which clustered with some of the SDK sampling sites, other sampling sites from the APR (SOC + KRN) and SMR (BELS, OSP, RIZA) formed their own, exclusive haplogroups. Of the LRS, sampling site HOTE formed its own haplogroup.

Clade mt-1b consisted of 21 haplotypes and exhibited a high level of H_d ($H_d = 0.876$), whereas the nucleotide diversity was low ($\pi = 0.00128$). This clade included sampling sites KOLP and MOKR of the SDK KORS. They shared five most abundant haplotypes, while they also exhibited unique haplotypes (MOKR, 3; KOLP, 13; Figure 2).

Group mt-1c consisted of 48 haplotypes with separated haplogroups and included several very distant haplotypes represented by only one individual. The group showed high levels of haplotype and nucleotide diversity but also a larger number of polymorphic sites compared to the clades ($H_d = 0.84$; $\pi = 0.0061$; $S = 99$; but see Table 2).

The two biggest haplogroups were formed by individuals from SDK: The first haplogroup consisted of north-eastern LRS sampling sites BLOS, CERK, CERJ, RASC and TOJN, and the second, north-eastern LRS sampling sites CERJ, IZIC, RASC and TOJN but including also KRRS sampling site KRKA. These two groups possessed 21 mutational steps between them. Some LRS individuals from IZIC and TOJN also formed two distinct haplogroups, in different parts of the mt-1c network: one including eight and the other five individuals. Another big haplogroup (with surrounding haplotypes) was formed by samples from the KRRS KRKA + RAD. Samples BOH and LOZN from the APR formed their own haplogroups (with a few haplotypes each).

Clade mt-1d consisted of seven haplotypes, had high levels of haplotype and nucleotide diversity ($H_d = 0.86$; $\pi = 0.00362$) and was formed exclusively of samples from the SPR.

3.1.4 | Pairwise distance matrix (COI-cytb)

Among the three clades and the mt-1c group, clade mt-1d of the SPR was most distant (2–3%).

Within the clade mt-1a, the genetic distances between the SDK and other sampling sites were up to 2% percent (Figure 3a), while the SDK sampling sites (within their respective clade) exhibited genetic distances of up to 0.2%. The only exception was CERK (0.2–0.5%), which bore haplotypes from mt-1a and mt-1c.

Within the group mt-1c, the genetic distances between the SDK sampling sites were up to 1.5%.

The two sampling sites of the KORS exhibited the largest divergence compared to all other SDK sampling sites (1.5–2%).

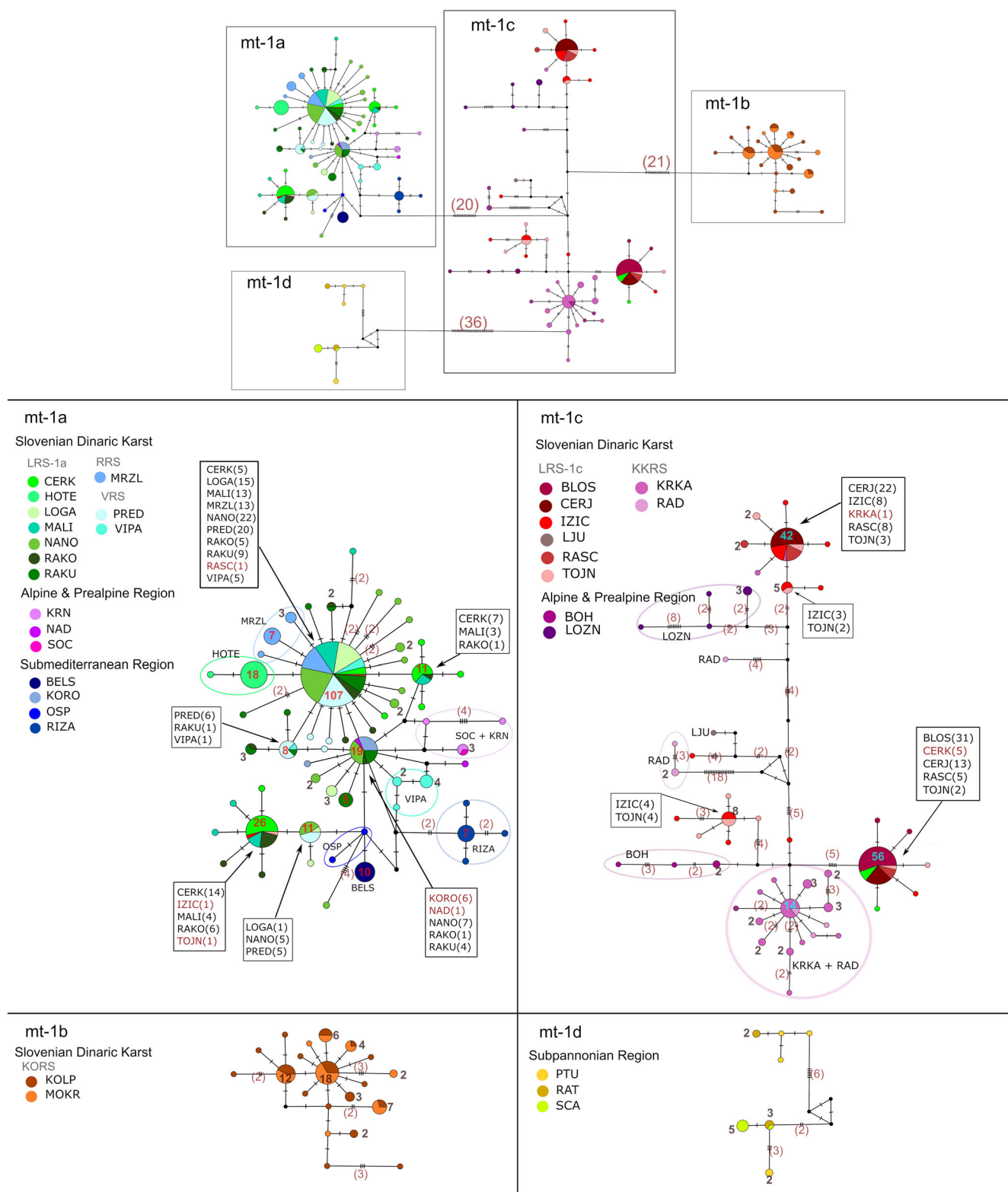


FIGURE 2 Overview of mitochondrial (*COI-cytb*) median-joining haplotype network of *Phoxinus phoxinus* in Slovenia. The dataset includes sequences generated in this study and sequences obtained from previous studies (Palandačić et al., 2015, 2017). A total of 134 unique haplotypes are present. Single clades are presented with a high magnification. Mutation steps are indicated with vertical lines. Number of individuals (when $N \geq 1$) contributing to each haplotype are given next to the haplotype. Black dots represent haplotypes missing in the sampling study. Abbreviations of localities are explained in Table 1.

TABLE 2 Genetic diversity parameters of the concatenated mitochondrial dataset

ID	N	Hd	π	S	k	No. of sequences
Alpine & Prealpine Regions						
BOH	3	0.833	0.00163	5	2.83333	4
KRN	3	0.833	0.00191	6	3.33333	4
LOZN	4	0.8	0.00329	14	5.73333	6
Slovenian Dinaric Karst						
<i>Ljubljana river system (LRS)</i>						
BLOS	3	0.154	0.00007	2	0.12121	33
CERJ	2	0.481	0.00414	15	7.21008	36
CERK	10	0.797	0.00613	38	10.67937	27
HOTE	2	0.00846	0.00006	1	0.10526	19
IZIC	12	0.866	0.00725	59	12.63043	24
LOGA	4	0.432	0.00066	5	1.14211	20
MALI	6	0.656	0.00131	10	2.28458	23
NANO	12	0.744	0.00095	17	1.65217	47
RAKO	10	0.854	0.00167	12	2.91228	19
RAKU	9	0.823	0.00095	9	1.64667	26
RASC	4	0.675	0.00602	40	10.48333	17
TOJN	10	0.922	0.00804	50	14.01307	18
<i>Reka river system (RRS)</i>						
MRZL	4	0.668	0.00047	3	0.81053	29
<i>Vipava river system (VRS)</i>						
PRED	6	0.617	0.00073	6	1.27094	39
VIPA	5	0.782	0.00106	5	1.84615	12
<i>Kolpa river system (KORS)</i>						
KOLP	17	0.933	0.00146	20	2.54637	32
MOKR	8	0.815	0.00112	11	1.94474	34
<i>Krka river system (KRRS)</i>						
KRKA	14	0.856	0.00214	33	3.72727	33
RAD	7	0.944	0.00907	39	15.80556	10
Submediterranean Region						
BELS	3	0.4725	0.00084	4	1.46154	10
KORO	2	0.286	0.00016	1	0.28571	7
RIZA	4	0.533	0.00046	4	0.8	6
Subpannonian Region						
PTU	4	0.9	0.00471	14	8.2	5
RAT	2	0.667	0.00421	11	7.33333	4
SCA	2	0.333	0.00019	1	0.33333	8
mt-1a	59	0.846	0.00152	89	2.64734	295
mt-1b	20	0.876	0.00128	24	2.23776	66
mt-1c	48	0.84	0.00606	99	10.55646	179
mt-1d	7	0.857	0.00362	16	6.30476	15
Summary	133	0.939	0.01253	215	21.8324	555

Abbreviations: π , nucleotide diversity; k, mean number of pairwise differences; Hd, haplotype diversity; N, number of haplotypes; S, number of polymorphic sites.

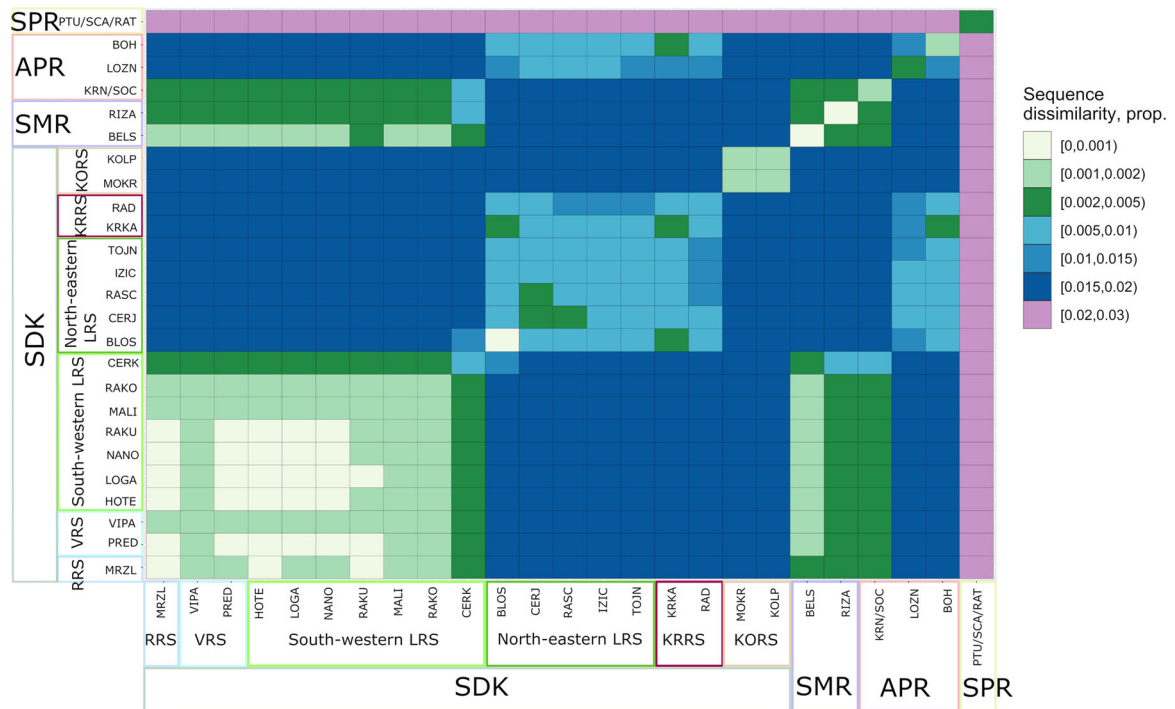
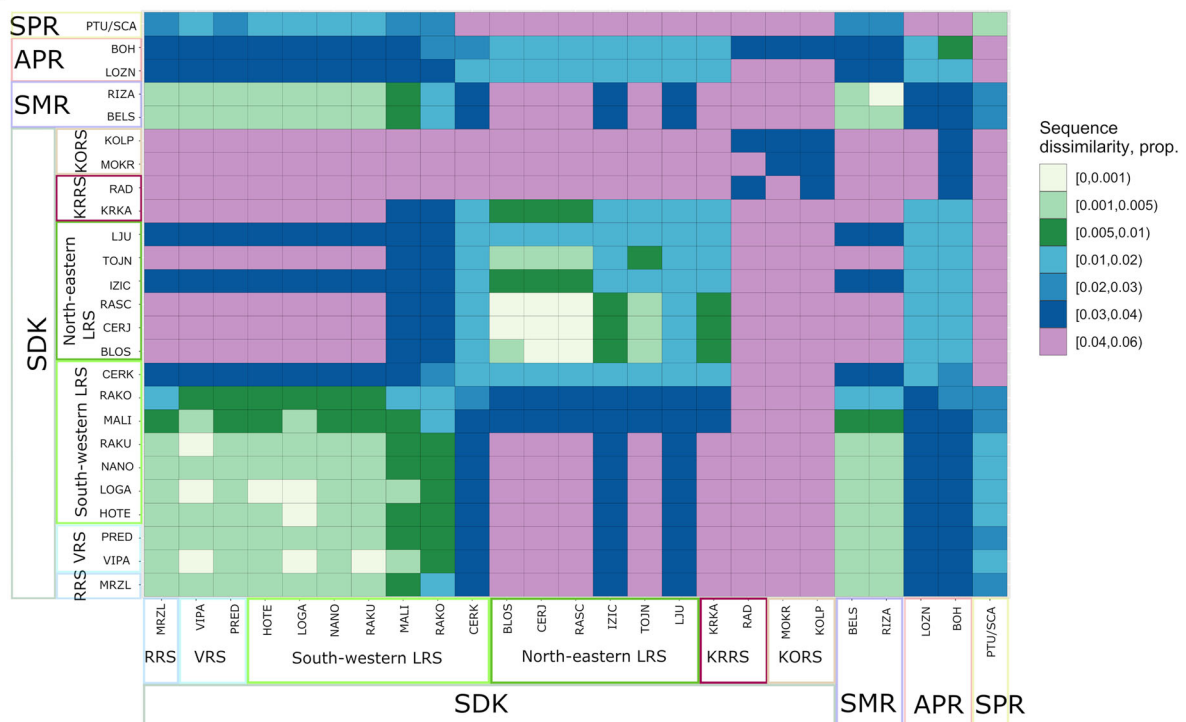
(a) *COI-cytb*(b) *RPS7*

FIGURE 3 Matrix of uncorrected pairwise p-distances between sampling sites of *Phoxinus lumaireul* in Slovenia. Distances are pooled to intervals. (a) Mt DNA (*COI-cytb*). (b) Nc DNA (*RPS7*)

3.1.5 | Spatial population clustering and AMOVA

The genetic groups detected in GENELAND analysis were denoted as 'clusters', abbreviated to C for the full GENELAND and SDK-C for sub-set GENELAND.

Full GENELAND

All 20 runs conducted revealed the same eight clusters. However, in four of 20 runs, single sampling sites from the APR either formed its own cluster (BOH) or clustered to C7 (LOZN) or both. Figure 4 shows the output of the model with the highest log posterior probability.

The eight clusters (C1–C8) are presented in Figure 4a, while posterior probabilities for each sampling site belonging to a given cluster are depicted in Figure 4b.

The sampling sites from the APR and SMR clustered together (C2), as did those from the SPR (C7), while LOZN, located in the APR, formed a distinct cluster (C8). The clustering of the SDK sampling sites was stable in all runs and revealed the following clusters: C1 comprised the sampling site HOTE (south-western LRS), C3 included the sampling sites from VRS, RRS and south-western LRS, C4 the KRRS, C5 the north-eastern LRS and C6, the KORS.

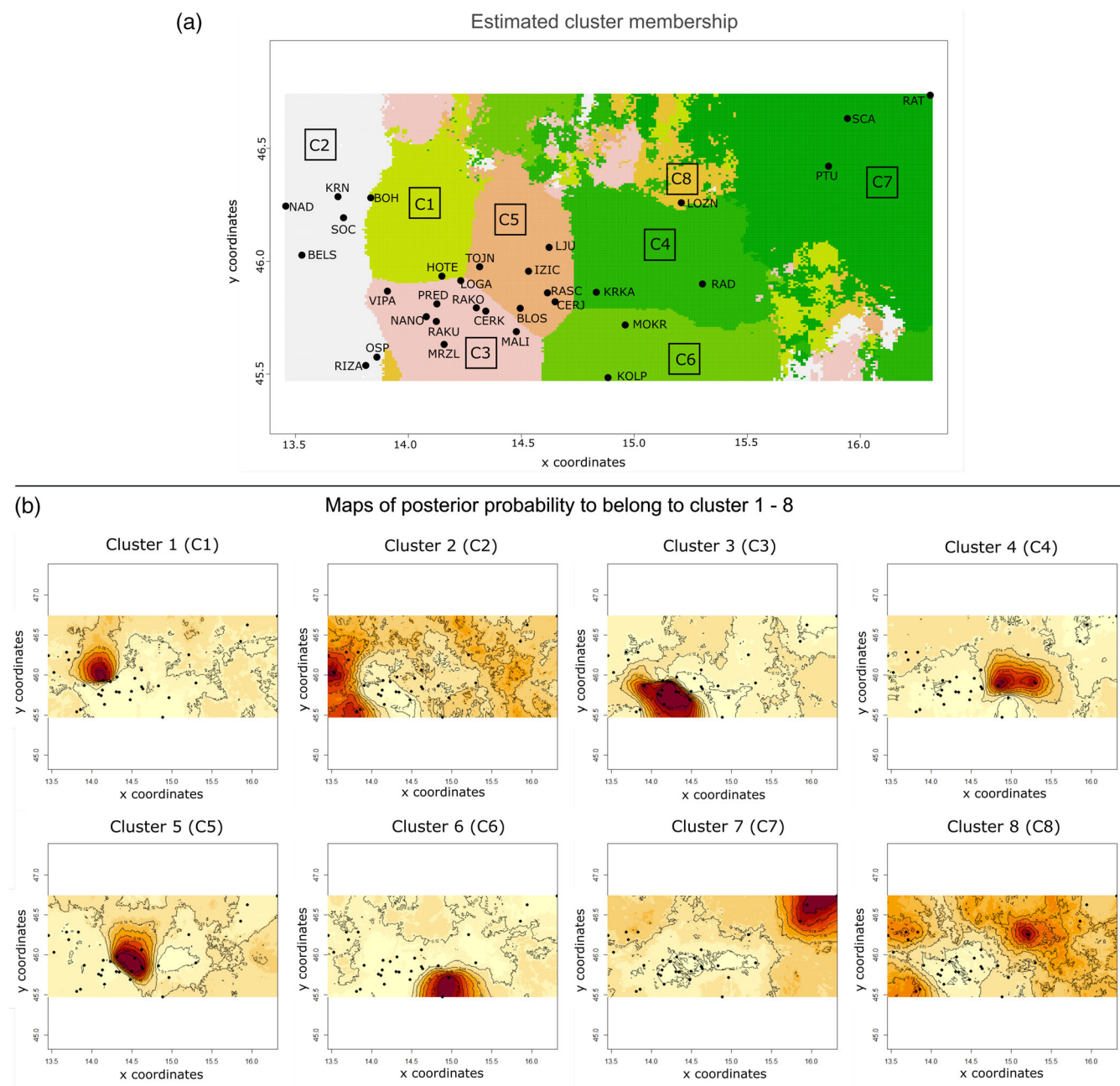


FIGURE 4 Population Bayesian cluster analysis (GENELAND). Maps showing geographic distribution of sampling sites (black points). (a) Map of cluster membership for each sampling site ($K = 8$). (b) Relative posterior probability of belonging to each of the eight inferred groups. The darker colour reflects a higher posterior probability.

Overall, genetic differentiation among the clusters revealed by GENELAND was significant (AMOVA $F_{ST} = 0.853$; $P \leq 0.0001$; Table 4). The results indicated that 78.17% of the variation was among the clusters and 14.74% within sampling sites ($P \leq 0.0001$, Table 3).

Sub-set GENELAND

GENELAND analysis of the SDK sampling sites revealed six clusters (SDK-C1–C6; Figure 5), which were reproduced stably in all 20 runs. Posterior probabilities for each sampling site to belong to a given

group are depicted in Figure S3. The clusters comprised SDK-C1, which includes HOTE (south-western LRS); SDK-C2, which contains the VRS and RRS and LOGA, NANO and RAKU from the south-western LRS. SDK-C3 included the remaining sites from south-western LRS RAKO, CERK and MALI. SDK-C4 contained the north-eastern LRS BLOS, CERK, RASC, TOJN, IZIC and LJU. SDK-C5 included the two sampling sites of KRKS KRKA and RAD, while the sixth cluster (SDK-C6) comprised the sampling sites of KORS KOLP and MOKR.

Results of the AMOVA using the revealed GENELAND clusters in the SDK showed also high genetic differentiation ($F_{ST} = 0.85$;

TABLE 3 Analysis of molecular variance (AMOVA) and degrees of freedom (d.f.) for COI-cytb sequences in all sampling sites of the present study (full GENELAND) and in the samples located in Slovenian Dinaric karst (sub-set GENELAND)

Source of variation	d.f.	Sum of squares	Variance components	Percentage variation	Fixation indices	P values
Full GENELAND						
Among clusters	7	4531.898	10.80656	78.17	$F_{CT} = 0.78171$	<0.0001
Among sampling sites within clusters	22	434.713	0.97991	7.09	$F_{SC} = 0.32471$	<0.0001
Within sampling sites	524	1067.837	2.03786	14.74	$F_{ST} = 0.85259$	<0.0001
Total	553	6034.448	13.82432			
Sub-set GENELAND						
Among clusters	4	3804.980	11.461	79.94	$F_{CT} = 0.79937$	<0.0001
Among sampling sites within clusters	15	294.373	0.731	5.1	$F_{SC} = 0.25399$	<0.0001
Within sampling sites	470	1008.621	2.146	14.97	$F_{ST} = 0.85033$	<0.0001
Total	489	5107.973	14.33781			

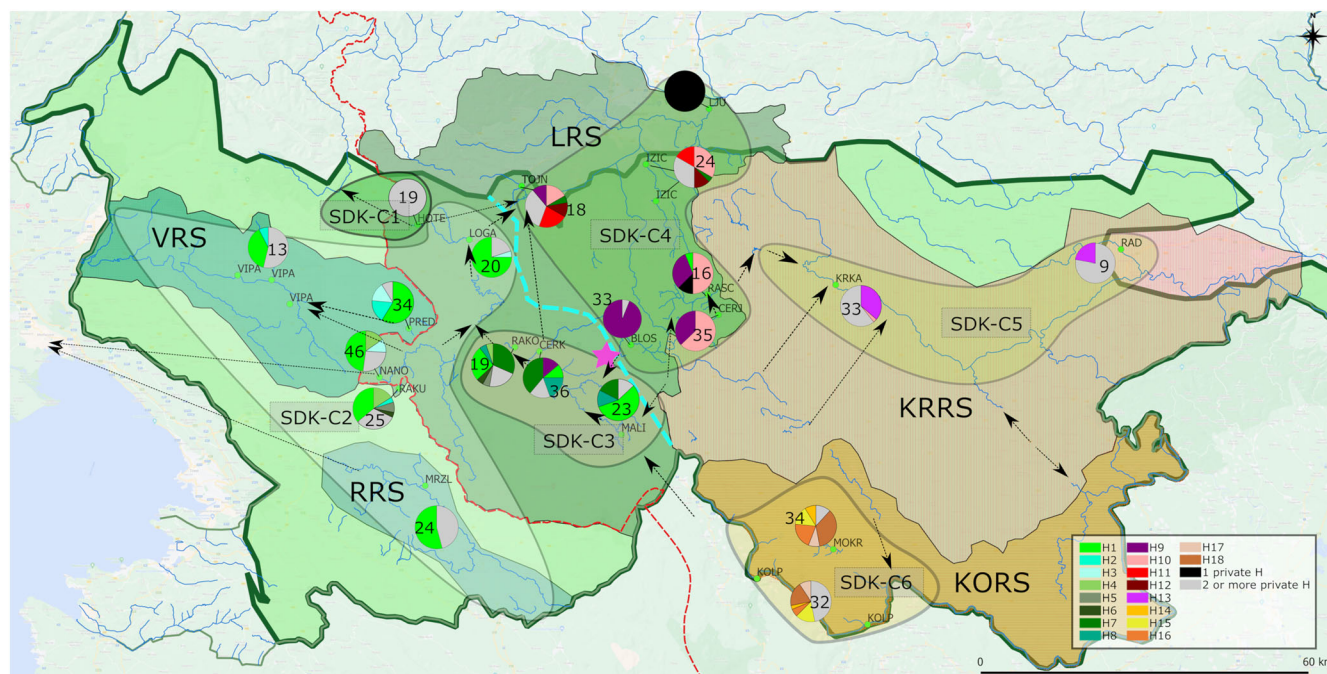


FIGURE 5 Map showing the sampling sites of *Phoxinus lumaireul* within examined river system in Slovenian Dinaric karst (SDK) and the simplified hydrological underground connections (black arrows) between them. Haplotype frequencies of the *mt* dataset for each sampling site are reported as a pie chart with the number of individuals reported within or next to the chart. One private haplotype is coloured black, while two or more private haplotypes within one sampling site are coloured grey. Population clusters revealed by GENELAND are indicated and named SDK-C1–SDK-C8. Dashed red line represents the divide between the Adriatic and Black Sea basins; turquoise dashed line represents the divide within Ljubljana river system (LRS) between clade 1a and 1c. Pink star shows possible zone of admixture between clades 1a and 1c.

$P \leq 0.0001$). Most (79.94%) of the variation was among clusters, with only 14.97% within sampling sites ($P \leq 0.0001$; Table 3).

3.1.6 | Divergence timing

A log Bayes factor of >150 was a result that favours our chosen model (log-normal clock model and birth-death tree prior) and indicated lack of evidence of the strict clock model and other tree priors (Table S4).

According to the divergence timing (measured in millions of years), diversification of the genus *Phoxinus* began in the middle Miocene (95% Highest Posterior Density, HPD: 11.25–18.52; Figure 6). Meanwhile, diversification of the 'Balkan minnows' (clades 1–6, 14 and 15 sensu Palandačić et al., 2017) was dated to the middle–late Miocene (95% HPD: 7.05–11.43), when the ancestor of clade 6 (*Phoxinus krkae*, Krka River in Croatia) split from the remaining clades 1–5, 14 and 15. The divergence of clade 5 (*Phoxinus csikii*) from the remaining clades occurred in the late Miocene (95% HPD: 5.51–8.62) and further split into two clades (clades 5a and 5b) in the late Pliocene (95% HPD: 1.61–4.11). The node separating clades 3 and 4 from the

remaining clades was not supported, while the split between clades 3 and 4 was dated to the late Miocene–early Pliocene (95% HPD: 3.11–6.82).

Separation of clades mt-1a-c and 1d-f was well supported and placed in the early Pliocene. There was no support for the split within mt-1a-c, which supposedly diverged at the end of the Pliocene to Pleistocene. Clade 1a split into an Italian and a Slovenian lineage in the late Pleistocene (node well supported with 95% HPD: 0.78–2.32).

3.2 | Nuclear DNA

3.2.1 | DNA amplification, cloning, sequencing and alignment

The amplification and cloning of 208 individuals resulted in 262 *RPS7* sequences, with a length of 940 bp and where 90 different alleles were detected. The *RPS7* alignment displayed 164 polymorphic sites, indicating the usefulness of the marker for studies of closely related populations and species.

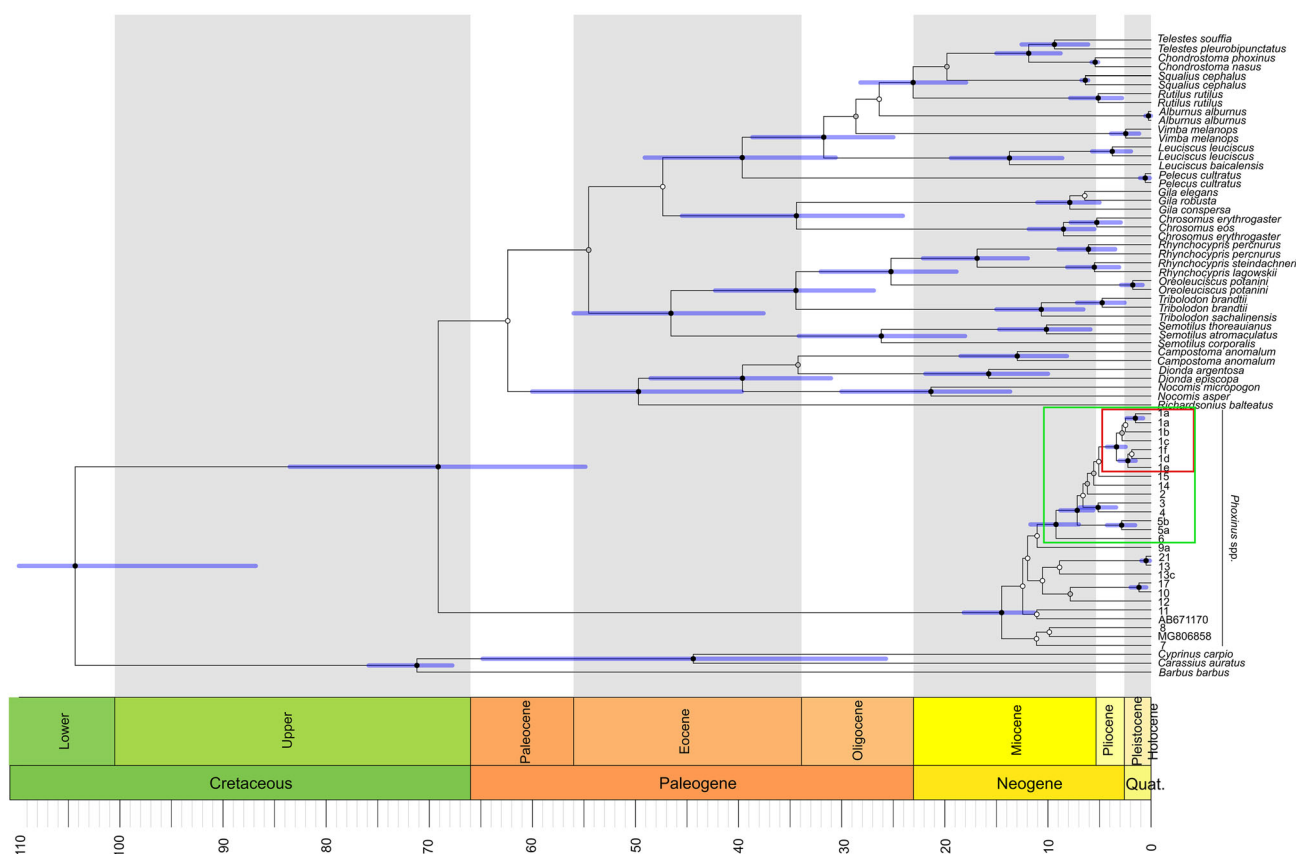


FIGURE 6 Divergence time estimates within the genus *Phoxinus* and related genera (details are reported in Table S2) based on the mitochondrial marker cytb. *Phoxinus* clades 1–6, 14 and 15 sensu Palandačić et al. (2017) are highlighted in green and designated as Balkan minnows. Clades 1a–c, lineages of *Phoxinus lumaireul* occurring in Dinaric karst of Slovenia, are highlighted in red. Time of splitting is presented by horizontal bars corresponding to a 95% HPD interval. Posterior probabilities (pp) ≥ 0.95 are indicated as black dots, pp ≤ 0.95 and ≥ 0.75 are grey and pp ≤ 0.75 are white.

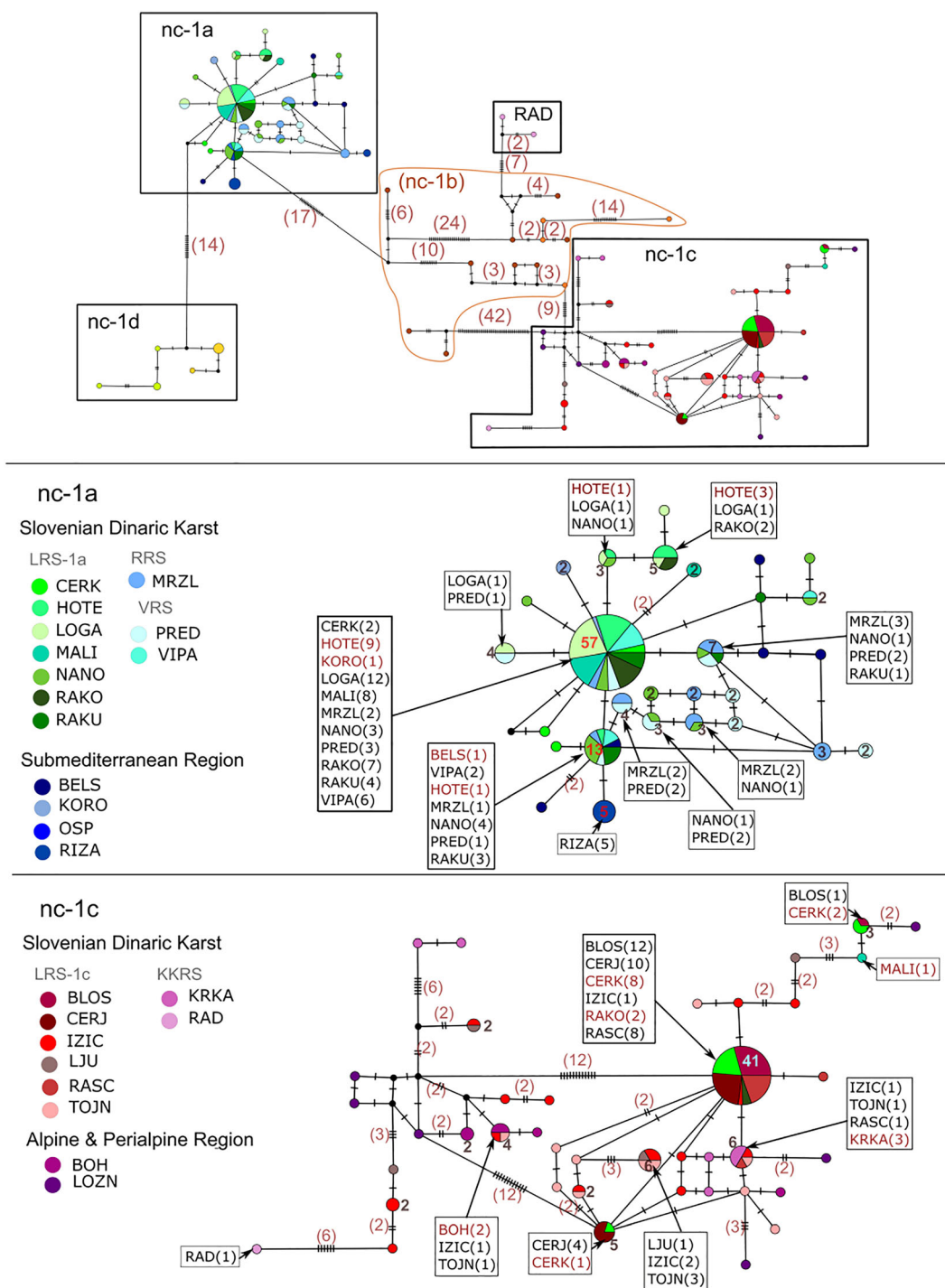


FIGURE 7 Overview of nuclear (*RPS7*) median-joining haplotype network of *Phoxinus lumaireul* in Slovenia. The dataset includes sequences generated in this study and sequences obtained from previous studies (Palandačić et al., 2015, 2017). A total of 90 alleles are present. Clades nc-1a and nc-1c are presented in a higher magnification. Number of individuals (when $N \geq 1$) contributing to each allele are reported next to the haplotype. Mutation steps are indicated with vertical lines. Black dots represent haplotypes missing in the sampling study. Abbreviations of localities are explained in Table 1.

3.2.2 | Phylogenetic tree reconstruction

The constructed phylogenetic tree lacked support for the clades (data not shown), and thus, only the allele network (below) is presented, where the structure is denoted with nc-groups.

3.2.3 | Median-joining network, population diversity indices

In the calculated allele network, specimens that clustered with clades mt-1a and mt-1d were clearly separated, while those that clustered

with mt-1b were ungrouped though scattered around the centre of the network (Figure 7). Individuals from the non-monophyletic group mt-1c formed a separate part of the RPS7 network.

Group nc-1a comprised 29 different alleles ($Hd = 0.803$) and a considerably low level of nucleotide diversity ($\pi = 0.0019$; Table 4). The dominant allele group included individuals from SDK sampling

TABLE 4 Genetic diversity parameters of the RPS7 dataset

ID	N	Hd	π	S	k	No. of sequences	No. of individuals
Alpine & Prealpine Regions							
BOH	4	0.867	0.0067	17	2.88	6	5
LOZN	6	1	0.01305	24	12.06667	6	5
Slovenian Dinaric Karst							
Ljubljana river system (LRS)							
BLOS	2	0.154	0.00116	7	1.07692	13	12
CERJ	2	0.44	0.0005	1	0.43956	14	10
CERK	6	0.714	0.01977	47	18.26667	15	11
HOTE	4	0.571	0.00102	3	0.94336	14	11
IZIC	14	0.983	0.01297	32	12	16	11
LOGA	5	0.507	0.00094	4	0.86765	17	16
LJU						4	2
MALI	3	0.473	0.00877	43	8.10909	11	9
NANO	10	0.917	0.00246	8	2.41092	16	12
RAKO	3	0.582	0.01451	41	13.41818	12	11
RAKU	4	0.75	0.00102	3	0.94444	9	8
RASC	3	0.378	0.00043	2	0.4	10	10
TOJN	10	0.955	0.00662	24	6.12121	12	11
Reka river system (RRS)							
MRZL	7	0.905	0.0021	4	1.94286	15	7
Vipava river system (VRS)							
PRED	9	0.935	0.00249	6	2.30719	18	12
VIPA	3	0.556	0.0009	3	0.8333	9	7
Kolpa river system (KORS)							
KOLP	9	1	0.03905	86	36.08333	9	7
MOKR	4	1	0.02468	43	22.8333	4	4
Krka river system (KRRS)							
KRKA	5	0.857	0.01196	7	11.04762	7	6
RAD	3	1	0.02013	26	17.333333	3	2
Submediterranean Region							
BELS	5	1	0.00324	6	2.88	5	4
KORO	2	0.667	0.00072	1	0.66667	3	3
RIZA	1	0	0	0	0	5	5
Subpannonian Region							
PTU	2	0.4	0.00086	2	0.8	5	4
SCA	3	0.833	0.0027	5	2.5	4	3
nc-1a	29	0.803	0.0019	19	1.809	134	
nc-1b	16	1	0.0364	101	33.58	16	
nc-1c	40	0.834	0.00816	46	7.5443	103	
nc-1d	4	0.81	0.0034	7	3.143	7	
Summary	90	0.923	0.02561	164	23.58676	262	208

Abbreviations: π , nucleotide diversity; Hd, haplotype diversity; k, mean number of pairwise differences; N, number of haplotypes; S, number of polymorphic sites.

sites of the south-western LRS (CERK, MALI, NANO, LOGA, RAKU and HOTE), RRS (MRZL) and VRS (VIPA and PRED), as well as from the SMR sampling site KORO. Another allele group was formed with the SDK sampling sites from the south-western LRS (RAKU, NANO and HOTE) and VRS (VIPA and PRED) and also included individuals from the SMR sample BELS. There were several additional allele groups sharing individuals from the LRS, RRS and VRS sampling sites (Figure 3). Of the non-SDK samples, only RIZA formed an exclusive allele group. Notably, HOTE clustered with nc-1a but did not form its own allele group. Only three CERK individuals (with four different alleles; two homozygotes and one heterozygote individual) were found in nc-1a, while the others clustered with nc-1c.

Allele frequencies in the SDK are shown in Figure S4. The most common allele in nc-1a (H-n1) is highlighted in light green and was present in all sampling sites of the SDK.

Group nc-1b. As reported above, individuals that clustered to the clade mt-1b were not clearly separated in the nuclear network. The group nc-1b consisted of single alleles that were separated by up to 42 mutational steps (Figure 3; population parameters $Hd = 1$, $\pi = 0.0364$), while two alleles belonging to the sampling site RAD (based on mt-DNA belonged to mt-1c) also clustered to this part of the network (highlighted in Figure 3).

Group nc-1c. In total, 40 different alleles with 46 polymorphic sites were distinguished within the part of the network that represented group nc-1c (Table 4). This group exhibited large Hd ($Hd = 0.834$) and large nucleotide diversity ($\pi = 0.00816$). The dominant group was formed by alleles from individuals from the north-eastern LRS (BLOS, CERK, RAKO, IZIC, CERJ and RASC). An allele group was shared between CERJ + CERK, while other mixed allele groups were formed by LJU + IZIC + TOJN, IZIC + KRKA + RASC + TOJN and BOH + IZIC + TOJN. None of the Alpine and Prealpine sampling sites, which clustered to group mt-1c and formed exclusive haplogroups BOH and LOZN, formed exclusive allele groups.

Three individuals exhibiting admixture between nc-1a and nc-1c were detected and where one allele clustered to nc-1a and the other to nc-1c. These individuals came from sampling sites in the south-western LRS: CERK, RAKO and MALI. There was a high frequency of allele H-n11 (among sampling sites BLOS, CERJ and RASC) in group nc-1c, and the most common allele among north-eastern Ljubljana sampling sites (IZIC, LJU, TOJN) was allele H-n17 (Figure S4). KRKA and RAD both possessed a large number of private alleles.

Group nc-1d comprised five alleles only ($Hd = 0.81$; $\pi = 0.0364$) and was separated by 14 mutational steps from nc-1a. DNA amplification and sequencing failed for sampling site RAT.

3.2.4 | Pairwise distance matrix

The p-distances between groups detected by nc-DNA analysis were considerably larger than by mt-DNA analysis (Figure 3a,b). Within nc-1a, the distances were quite small (0–0.05%), with the exceptions of sampling sites MALI, RAKO, CERK, which exhibited greater distances to the remaining sites from nc-1a (mostly 0.05–1%, though with CERK up

to 3–4%). The distances within nc-1c ranged from 0% to 2% (Figure 3b). KOLP and MOKR differed strongly from the remaining sites, with differences of up to 6% (with mean distances 3–4%). Group nc-1d showed very small distances (1–3%) from group nc-1a.

4 | DISCUSSION

In the present study, three possible scenarios explaining the complex genetic structure of *Phoxinus phoxinus* in SDK were considered: (1) ongoing geneflow through underground connections, (2) legacies of the past hydrological network and (3) anthropogenic translocations. The results suggest that the first two scenarios have played a major role, while the third has not had a profound effect on the genetic composition of minnows in karst, though this scenario cannot be excluded. Support for the first scenario is found in the mitochondrial genetic structure of the samples from SDK, which is characterized by numerous mutual underground water connections, and that exhibit greater genetic connectivity in comparison to hydrologically isolated reference sampling sites (Alpine and Prealpine, Submediterranean, SPRs) (Figures 2 and 5). Further, within SDK clade mt-1a sampling sites, among Reka, Vipava and south-western LRS, extensive mt haplotype sharing was observed (Figure 5, SDK-C2 and C3, H1 haplotype, denoted in bright green). Finally, the range of Adriatic haplotypes (1a) compared with Black Sea haplotypes (1c) did not correspond to the Adriatic–Black Sea basin divide but was shifted northwards in this area (Figure 5, red dashed line and bright blue dashed line, respectively). Evidence against the first scenario is provided by the genetic barrier found between the south-western and north-eastern LRS sampling sites, which share several direct subterranean connections. Regardless, they did not cluster to the same clade (phylogenetic tree, haplotype network) or cluster (full or sub-set GENELAND) and showed only very limited haplotype sharing (e.g., CERK). The genetic isolation of the remaining two major SDK RS, Kolpa and Krka, support the second scenario. Both RS formed their own genetic clusters (SDK-C5, SDK-C6). The third scenario—human introductions—might be reflected in the genetic composition of the north-eastern LRS, which consists of several haplogroups rather than a monophyletic clade. When considering the nuclear marker (Figure 7), the genetic make-up was less structured and included allele sharing between SDK and other sampling sites. Together with the conducted timing analysis, this could point to a relatively recent divergence among populations, consistent with the beginning of karstification in this area (Figure 6). While further sampling and more in-depth analysis of additional genetic markers is required, the present study offers a valuable insight into the factors that have shaped the genetic composition of populations of minnows dwelling in a complex karst aquifer.

4.1 | Genetic structure as a consequence of ongoing geneflow through underground connections

The SDK and reference sampling sites within the mt-1a clade were typically well separated genetically, a finding supported by all the mt-DNA

analyses that were conducted (haplotype network, Figure 2; phylogenetic tree, Figure S2; pairwise distance, Figure 3; GENELAND, Figure 4). Moreover, while reference sampling sites each clustered to their own mt-haplogroups, the SDK samples often shared haplotypes and formed one coherent population, with AMOVA indicating low genetic variation among the sampling sites within the designated clusters (7%). This finding is supported also by the considerably high levels of Hd and rather low levels of nucleotide diversity present in small streams (e.g., PRED, NANO, RAKU, RAKO), pointing to the existence of a single cohesive hydrological network in which the high levels of genetic diversity are maintained through recurring dispersal and population interchange. Thus, the detected genetic structure partially reflects underlying hydrological connections, with the most prominent example being the area within the LRS (sampling sites NANO, RAKU), which is characterized by hydrological bifurcation, where water is flowing to two different water basins (Pivka valley, Habič, 1989). Indeed, here, extensive haplotype sharing and population connectivity were detected between the south-western Ljubljana, Vipava and RRS sampling sites, and instead of matching the Adriatic–Black Sea basin divide running through this area, the genetic delimitation between mt-1a and mt-1c was shifted to the north-east (blue dotted line in Figure 5). The discrepancy between the distribution of *Phoxinus* genetic lineages and river-sea drainages has been observed previously (Palandačič et al., 2015; Vučić et al., 2018), most frequently in karst areas, where Palandačič et al. (2020) suggested the difference had a natural origin. However, even though shifted, the genetic divide between south-western (mt-1a, full GENELAND C3) and north-eastern (mt-1c, full GENELAND C5; Figure 5) LRS sampling sites still exists, despite numerous underground water connections between them (Figures 1b and 6). Further expansion of clade 1a to the north-east into the Black Sea basin was possibly obstructed by hydrogeological barriers between LOGA, HOTE and the springs of Ljubljana River (TOJN; Grabovšek & Turk, 2010; Blatnik, 2020), as well as the barrier to flow between the Idrija Fault Zone (sampling sites NANO, RAKU, MRZL, PRED, CERK, RAKO, MALI) and Ljubljana Basin (TOJN; Kaufman et al., 2020). There are few other studies on fishes with a dense sampling set in this area, though in the case of bullheads (Bravničar et al., 2021), the genetic structure follows the Adriatic–Black Sea basin divide, with one species populating the Adriatic and other the Black Sea basin. When comparing the two genera, *Phoxinus* seems to be much more adaptable to different environmental conditions, while *Cottus* is stenotopic and sedentary. Thus, the latter might have reduced dispersal abilities, and the genetic structure reflects hydrological conditions in the area before the beginning of karstification. A similar genetic pattern (albeit with reduced sampling) consistent with the Adriatic–Black Sea basin divide has been observed in chub (*Squalius* sp.; Bogutskaya & Zupančič, 2010) and *Telestes* (Ketmaier et al., 2004). In *Phoxinus*, there is possibly some interchange between the north-east and south-west LRS sampling sites, as haplotype sharing has been identified between ‘border’ sampling sites CERK, MALI, RAKO and IZIC + TOJN. However, the discordance between the mt and nc analysis of the MALI and RAKO sampling sites, which points to incomplete lineage sorting, suggests a recent isolation of populations

with no subsequent gene flow, with the populations slowly drifting apart and reflecting the past hydrological connections.

4.2 | Genetic structure as a legacy of a past hydrological network

The two major RS Krka and Kolpa form their own mt-clusters (C4 and C6, Figure 4; SDK-C5 and SDK-C6, Figure 5) and in the case of KORS even its own clade (mt-1b), suggesting vicariance induced by hydrological fragmentation rather than ongoing migration through underground links. While the difference might relate to different hydrological conditions (e.g., greater flow velocities and water volumes in rivers with fewer underground connections compared with small streams in the LRS with numerous subterranean water connections), the genetic isolation of the Krka and KORS has also been noticed in other fish (e.g., *Cottus* sp. in Kolpa, Bravničar et al., 2021; *Hucho hucho*, Snoj et al., 2022; and *Thymallus thymallus* [unpublished data]) and in invertebrate species (Kolpa: Trontelj et al., 2005; Klobučar et al., 2013; Ivković & Plant, 2015; and Krka: Verovnik et al., 2004). The timing analysis suggests a split between mt-1a–c and mt-1d–f within *P. lumaireul* to the middle or late Pliocene, with the start of genetic isolation coinciding with the beginning of the karstification process (Trontelj et al., 2007). While the dating of the further splits (mt-1a to mt-1c) is unreliable due to low levels of support at the nodes, it is possible that it took place following the split of mt-1d at the beginning of, or later during, the Pleistocene. Meanwhile, the polytomous relationship (Figure S2) supports this suggestion, with diversification most probably occurring within a relatively short period (3–5 MYA), consistent also with the genetic structure being influenced by the onset of karstification proposed for stone crayfish in the northern-central Dinarides (Klobučar et al., 2013). Likewise, specific water conditions, where karst streams function as ‘freshwater islands’, are thus considered an isolating factor, proposed also in Previšić et al. (2014) and Bilandžija et al. (2013). In fish, extreme genetic structuring was found in *Salmo marmoratus* in small streams of SDK, explained by impassable barriers preventing immigration into the main RS (Fumagalli et al., 2002). Furthermore, hydrologic isolation was used to explain the genetic structure of karst-dwelling fishes such as *Telestes croaticus* (Marčić et al., 2011) and *Cobitis* sp. (Buj et al., 2014).

4.3 | Genetic structure as a consequence of anthropogenic translocations

Despite numerous studies reporting the impact of human introduction on the dispersal patterns of *Phoxinus* species (Corral-Lou et al., 2019; Garcia-Raventós et al., 2020; Kneibelsberger et al., 2015; Miró & Ventura, 2015; Museth et al., 2007), its influence on the dispersal of *P. lumaireul* in SDK seems to be minimal. Nevertheless, there are some cases that point to anthropogenic translocations. For example, MOKR, a small sinking stream within KORS (mt-1b), forms a single population

with KOLP (GENELAND C6 and SDK-C6) despite the lack of underground connections. It is possible, therefore, that minnows have been introduced here, even though MOKR appears genetically very heterogeneous ($H_d = 0.815$) and also exhibits three unique haplotypes, findings that are incongruent with a recently introduced population. Similarly, anthropogenic influences might explain the heterogeneous genetic composition of *Phoxinus* at the sampling sites IZIC and TOJN (Figure 2, mt-1c), where there are two main haplogroups more than 25 mutational steps apart, several additional haplogroups shared by both and two specimens (one each) bearing mt-1a haplotypes. One possible explanation for this pattern is the fire at chemical waste processing plant Kemis in Vrhnika in the year 2017 that killed the fish populations in the spring area of Ljubljana (TOJN) and that was followed by stocking. Additionally, one mt-1a specimen bore the most prominent mt-1a H1 haplotype in RASC, with nc analysis of all three individuals showing no sign of admixture (Figure 3), pointing to a recent introduction of new genetic lineages.

4.4 | *Phoxinus lumaireul* as a biological tracer

The species *P. lumaireul* is eurytopic and inhabits both karstic and non-karstic streams in Slovenia and thus seems to be a perfect candidate for assessment of the usefulness of population composition for deciphering a complex underlying aquifer. Certainly, the genetic structure seems to reflect a combination of past and ongoing hydrological connections. Within mt-1a, haplotype sharing (H1, bright green in Figure 5) between the (i) southwestern LRS (PRED, NANO, RAKU, RAKO, CERK, MALI and LOGA), VRS (VIPA) and RRS (MRZL) sampling sites (GENELAND C3, Figure 4) was observed, as well as between all these listed sampling sites, and (ii) RAKO, CERK and MALI. According to full GENELAND analysis, all sampling sites were also designated to C3, though finer-scale analysis showed the first group clustered to SDK-C2 while the second formed SDK-C3 (Figure 5). Hydrological analysis indicates that the SDK-C2 Ljubljana and VRS sampling sites are connected by permanent water connections (Figures 1 and S1, Petrič et al., 2020), while RRS at present seems to be only occasionally or indirectly connected, or both, to LRS (Figure S1), though they were linked several times during the Pleistocene (Habič, 1989). According to Hartl and Clark (2007), occasional gene flow, with a few migrants per generation, is enough to maintain a population in genetic equilibrium, suggested also for karst-dwelling fish species *Delminichthys adspersus* by Palandačić, Matschiner, et al. (2012). In contrast, SDK-C3 seems to be permanently connected to farther distant LOGA (SDK-C2) sampling site through the Planina cave and Unica River but exhibits partial genetic isolation. The reason for this situation might be the temporary flow diversion along the Rak (RAKO) branch detected by Kaufman et al. (2020) that maintains some, though incomplete, population connectivity. Meanwhile, within the northeast LRS (RASC, CERJ, BLOS, IZIC and TOJN, SDK-C4), the genetic structure is in line with the underground connections between RASC and CERJ. However, there are no known subterranean water connections

between the remaining sampling sites. Together with the considerably large genetic distances among these sampling sites (up to 1.5%), the results, which still indicate the sampling sites as supporting a single population, possibly point to the past hydrological situation rather than recent gene flow. Similarly, it seems that the population at the sampling site HOTE, which exhibits incomplete lineage sorting when comparing the results of mt and nc analysis, was previously connected to the LOGA population, while at the present, it is isolated, despite the underground connections between these sites. Possibly, these water connections no longer allow the passage of *Phoxinus*, but the result might also point to a minimal effect of the underground links upon their population structure. Thus, while the genetic composition of *P. lumaireul* has helped to shed some light on past and previous hydrology of the karst area, a more in-depth analysis with further reference sampling sites and nuclear markers, such as microsatellites, is needed to assess the usefulness of this species as a biological tracer. Furthermore, a detailed comparison with other fishes and other organisms is required to draw conclusions on how the biology of a species influences its dispersal potential in karst aquifers.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the supplementary material of this article. Sequence data (sequences generated in the course of the present study as well as previously published once) are available online in the NCBI Database.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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Chapter 2: Supplementary material

1. Detailed description of the study area

1.1. River systems in Slovenian Dinaric Karst

1.1.1. Ljubljana river system

Ljubljana RS includes several karst poljes developed along the Idrija Fault connected by eight sinking streams under different names.

1. Trbuhovica first appears in the field of Prezidsko polje in Croatia and disappears in Babno polje. No *Phoxinus* are recorded according to BIOSWEB (Biological database of the Fisheries Research Institute of Slovenia, <http://www.biosweb.org/index.php?task=map&tid=4026>, accessed 30.03.2021)
2. Obrh flows on Loško polje, disappears and reoccurs on the Cerknica polje. *Phoxinus* were sampled in Mali Obrh (MALI).
3. Stržen is located in Cerknica polje, where the Cerkniščica (*Phoxinus* sampled there at CERK) runs from the north (Blatnik 2020). It is also connected underground to the Bloke Plateau (Kogovšek, Prelovšek, and Petrič 2008), where river Bloščica (BLOS) was sampled.
4. Rak drains through Rakov Škocjan polje (*Phoxinus* sampled at RAKO) and disappears in Planinska Jama Cave, where it meets River Pivka (Perko et al. 2020).
5. Pivka flows through Pivka basin and Postojna Cave. In Pivka no *Phoxinus* were recorded; thus fish were collected in its tributary Nanoščica (NANO) and a sinking stream flowing in the vicinity: Rakulik (RAKU).
6. Unica is formed where Pivka and Rak join in Planinska Jama Cave; it flows along Planinsko polje (Blatnik 2020). According to BIOSWEB, *Phoxinus* were recorded in Unica only once, in the year 1984, while during the collecting for this study, regardless of extensive efforts, none were observed.
7. Logaščica partly involves the sinking system of Rovte, Hotenjsko podolje (Hotenka (HOTE) was sampled) and Logaško polje (Blatnik 2020). *Phoxinus* were sampled in Logaščica stream (LOGA).
8. Ljubljana springs near Vrhnika (confluence Tojnica (TOJN) was sampled), runs through the Ljubljana marshes (confluence Ižica (IZIC) was sampled), and flows into the Sava at Podgrad (sampling site LJU, located outside of the SDK territory).

On the edge of the Ljubljana RS, near the Krka river system, rivers Rašica (RASC) and Cereja (CERJ) were sampled. While they are connected underground, tracing tests show that Rašica is also indirectly linked to Mali Obrh (MALI) through its tributary Kozmanjka (Figure 1B & Figure S1 for more details).

Although the geographic position of the Ljubljana RS is in the Black Sea basin, it represents a bifurcation of the Adriatic–Black Sea basins: some streams of Pivka basin (NANO) flow underground to the rivers Timavo and Vipava, while Hotenka (HOTE) is connected underground with Idrijca (Blatnik 2020); (Perko et al. 2020).

1.1.2. Vipava river system

The main water body of the Vipava RS mostly flows on flysch. *Phoxinus* were sampled in Vipava and some of its tributaries (all pooled: VIPA). Vipava springs in the karst and from there, 14 km to the northwest, it is connected underground to River Lovka, sinking into Predjama Cave (Šebela and Turk 2014). *Phoxinus* were sampled there (PRED). The Vipava RS is located in the Adriatic basin.

1.1.3. Reka river system

Reka is the longest sinking stream in Slovenia, which flows about 50 km on the surface on impermeable flysch rocks and another 7 km on limestone. At the end of its surface flow it reaches Dinaric Karst and sinks underground in the Škocjanske jame (Škocjan caves) (Gabrovšek and Perić 2006). Its underground course to Timavo (Italy) is around 33 km (Timavo is not inhabited by *Phoxinus*). Reka is represented by only one locality, by its tributary Mrzlek (MRZL). The Reka RS is located on the Adriatic basin.

1.1.4. Krka river system

The sampling included rivers Krka (KRKA) and Radulja (RAD). In this area, additional sinking streams exist (Bistrica (BIST), Ribnica (RIBN), small stream near Jurjevica (JURJ)), which are connected underground to the collected sampling sites. However, according to BIOSWEB and confirmed at the site, they are not populated with *Phoxinus* (red dots in Figure 1B). RAD is a tributary of Krka and belongs to the non-karstic area of the Krka RS (and zoogeographically to the Subpannonian Region). The Krka RS is located in the Black Sea basin.

1.1.5. Kolpa river system

Two sampling points (Kolpa, KOLP; Mokri Potok, MOKR) belong to the Kolpa RS, which extends from Slovenia to Croatia, and also includes a small part of Bosnia and Herzegovina. MOKR is a small sinking stream and no subterranean links to surrounding water are known. The sinking stream Rinža (RINZ in Figure 1B), which is connected underground to Kolpa is not inhabited by *Phoxinus* according to BIOSWEB and confirmed at the site (Figure 1B). The Kolpa RS is located in the Black Sea basin.

1.2. Reference sampling sites

1.2.1. Alpine & Prealpine Regions

There are five sampling sites in Alpine & Prealpine Regions: Krn Lake (KRN, introduced), Nadiža (NAD), and Soča (SOC) in the Adriatic basin and Bohinj Lake (BOH) and Ložnica (LOZN) in the Black Sea basin.

1.2.2. Submediterranean Region

The Submediterranean Region includes three coastal rivers (Malinska (KORO), Osapska (OSP), Rižana rivers (RIZA)), and the stream Belski Potok (BELS) in the western part of Slovenia near the Italian border.

1.2.3. Subpannonian Region

Subpannonian Region sampling sites include rivers located in the Drava river system (Drava (PTU)) and the Mura river system (Ratkovski (RAT), Ščavnica stream (SCA)).

2. Tables

Table S1. All examined specimens with GenBank accession numbers from this study and previous studies (Palandačić et al. 2015; Palandačić et al. 2017). Sampling sites with coordinates, drainage basins, clades, and sampling dates are given.

Table S1 is available as separate in Excel sheet at <https://onlinelibrary.wiley.com/doi/full/10.1002/eco.2449>

Table S2. All samples with GenBank accession numbers and references included in the divergence timing analysis.

Species	Clade	GenBank	Reference
<i>Phoxinus lumaireul</i>	1a	KT166578	Palandačić et al. (2015)
<i>Phoxinus lumaireul</i>	1a	ON495039	this study
<i>Phoxinus lumaireul</i>	1c	ON494620	this study
<i>Phoxinus lumaireul</i>	1b	KT166653	Palandačić et al. (2015)
<i>Phoxinus lumaireul</i>	1d	MF408226	Palandačić et al. (2017)
<i>Phoxinus lumaireul</i>	1e	KT166790	Palandačić et al. (2015)
<i>Phoxinus lumaireul</i>	1f	KT166762	Palandačić et al. (2015)
<i>Phoxinus sp.</i>	2	KT166621	Palandačić et al. (2015)
<i>Phoxinus sp.</i>	3	KT166691	Palandačić et al. (2015)
<i>Phoxinus sp.</i>	4	KT166574	Palandačić et al. (2015)
<i>Phoxinus sp.</i>	5a	KT166612	Palandačić et al. (2015)
<i>Phoxinus sp.</i>	5b	KX673469	Ramler et al. (2017)
<i>Phoxinus sp.</i>	6	KT166548	Palandačić et al. (2015)
<i>Phoxinus sp.</i>	7	MN820807	Palandačić et al. (2017)
<i>Phoxinus sp.</i>	8	KT166678	Palandačić et al. (2015)
<i>Phoxinus sp.</i>	9a	MF408194	Palandačić et al. (2017)

<i>Phoxinus phoxinus</i>	10	MF408222	Palandačić et al. (2017)
<i>Phoxinus sp.</i>	11	MG806683	Schönhuth et al. (2018)
<i>Phoxinus sp.</i>	12	MK787687	Corral-Lou et al. (2019)
<i>Phoxinus bigerri</i>	13	HM560122	Perea et al. (2010)
<i>Phoxinus sp.</i>	13c	MG681510	Vučić et al. (2018)
<i>Phoxinus sp.</i>	14	MG806685	Schönhuth et al. (2018)
<i>Phoxinus sp.</i>	15	MG806686	Schönhuth et al. (2018)
<i>Phoxinus sp.</i>	17	EU352213	Strange and Mayden (2009)
<i>Phoxinus sp.</i>	21	MK787690	Corral-Lou et al. (2019)
<i>Phoxinus sp.</i>	Mongolia	AB671170	Imoto et al. (2013)
<i>Phoxinus sp.</i>	Bolshoy	MG806682	Schönhuth et al. (2018)
<i>Oreoleuciscus potanini</i>		KJ754935	Chen et al. (2016)
<i>Oreoleuciscus potanini</i>		AB626851	Imoto et al. (2013)
<i>Tribolodon sachalinensis</i>		AB626856	Imoto et al. (2013)
<i>Tribolodon brandtii</i>		AB626853	Imoto et al. (2013)
<i>Tribolodon brandtii</i>		AB626854	Imoto et al. (2013)
<i>Rhynchocypris percunurus</i>		AP009149	Imoto et al. (2013)
<i>Rhynchocypris percunurus</i>		NC_008684	Saitoh et al. (2006)
<i>Rhynchocypris lagowskii</i>		AP009148	Imoto et al. (2013)
<i>Rhynchocypris lagowskii</i>		AP009147	Imoto et al. (2013)
<i>Pelecus cultratus</i>		AB239597	Saitoh et al. (2006)
<i>Pelecus cultratus</i>		AY838938	Freyhof et al. (2006)
<i>Richardsonius balteatus</i>		AP012106	Iwasaki et al. (2013)
<i>Campostoma anomalum</i>		DQ536421	Broughton and Reneau (2006)
<i>Campostoma anomalum</i>		KP013113	Renshaw et al. (unpublished)
<i>Nocomis micropogon</i>		MH324421	Schroeter et al. (2020)
<i>Nocomis asper</i>		AP012096	Iwasaki et al. (2013)
<i>Dionda episcopa</i>		AP012077	Iwasaki et al. (2013)
<i>Dionda argentosa</i>		JN812352	Schönhuth et al. (2012)
<i>Chondrostoma nasus</i>		MG806657	Schönhuth et al. (2018)
<i>Chondrostoma phoxinus</i>		MG806658	Schönhuth et al. (2018)
<i>Telestes pleurobipunctatus</i>		MG806720	Schönhuth et al. (2018)
<i>Telestes souffia</i>		MG806721	Schönhuth et al. (2018)
<i>Rutilus rutilus</i>		AP010775	Saitoh et al. (2011)
<i>Rutilus rutilus</i>		MG806695	Schönhuth et al. (2018)
<i>Squalius cephalus</i>		NC031540	Iwasaki et al. (2013)
<i>Squalius cephalus</i>		MG806701	Schönhuth et al. (2018)
<i>Alburnus alburnus</i>		AB239593	Saitoh et al. (2006)
<i>Alburnus alburnus</i>		MG806649	Schönhuth et al. (2018)
<i>Vimba melanops</i>		MG806725	Schönhuth et al. (2018)
<i>Vimba melanops</i>		AP011212	Iwasaki et al. (2013)
<i>Leuciscus leuciscus</i>		MG806667	Schönhuth et al. (2018)
<i>Leuciscus leuciscus</i>		DQ664302	Costedoat et al. (2006)
<i>Chrosomus erythrogaster</i>		AP011276	Imoto et al. (2013)
<i>Chrosomus erythrogaster</i>		AY281055	Mayden (2002)
<i>Chrosomus eos</i>		AP009151	Imoto et al. (2013)
<i>Gila elegans</i>		MT364326	Osborne et al. (2020)

<i>Gila robusta</i>	DQ536424	Broughton and Reneau (2006)
<i>Gila conspersa</i>	AP009315	Saitoh et al. (2006)
<i>Semotilus atromaculatus</i>	MG570419	Schroeter et al. (2019)
<i>Semotilus corporalis</i>	MG806644	Schönhuth et al. (2018)
<i>Semotilus thoreauianus</i>	MG806645	Schönhuth et al. (2018)
<i>Cyprinus carpio</i>	NC_001606	Chang, Huang, and Lo (1994)
<i>Barbus barbus</i>	NC_008654	Saitoh et al. (2006)
<i>Carassius auratus</i>	KJ874430	Cheng, Liang, and Sun (2012)
<i>Leuciscus baicalensis</i>	KF673863	Hu et al. (2015)

Table S3. Marginal likelihoods with standard deviation of four models tested with nested sampling for divergence time analysis (further information in Material & methods section). M1 is the model used for further analysis. Abbreviations: uclc, uncorrelated relaxed log-normal clock; strict, strict clock; BD, Birth-death model; Yule, Yule model; BS, Coalescent Bayesian skyline.

	M1	M2	M3	M4
molecular clock	uclc	strict	uclc	uclc
tree prior	BD	BD	Yule	BS
marginal likelihood	-16062.639	-47816.488	-18347.818	-18344.599
standard deviation	3.582	1.116	4.546	4.299

3. List of figures

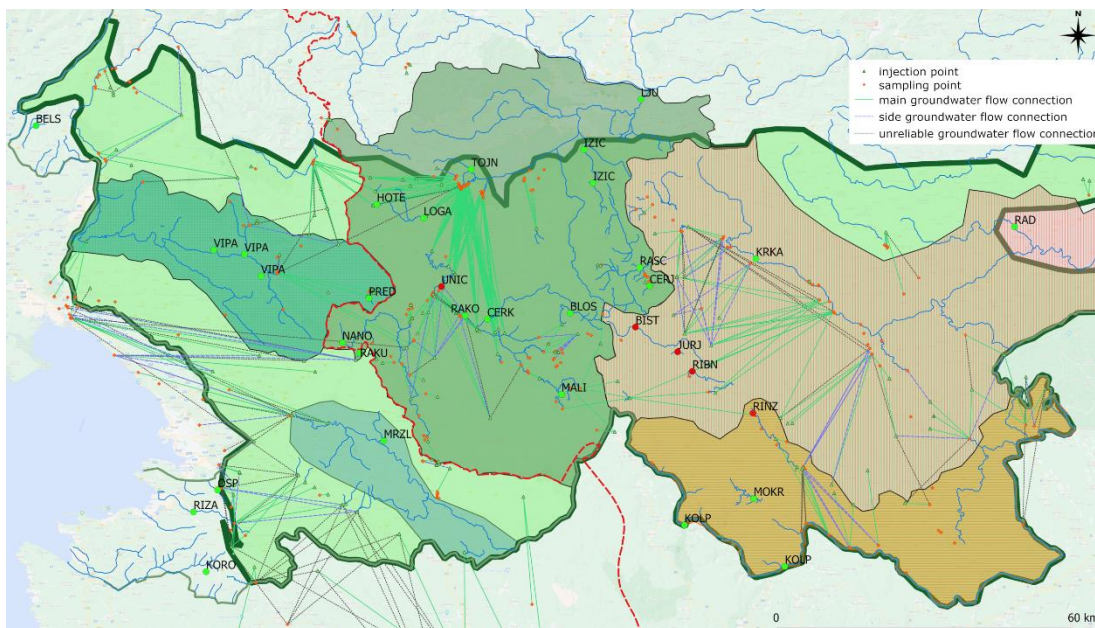


Figure S1. Map showing all main, side and unreliable groundwater flow connections in Slovenian Dinaric Karst (SDK), evaluated by tracer tests and digitalized by Petrič et al. (2020). All sampling points are included, red line indicates the border between Adriatic and Black Sea basins. Main river systems in SDK are depicted. Green bold line indicates the border of SDK.

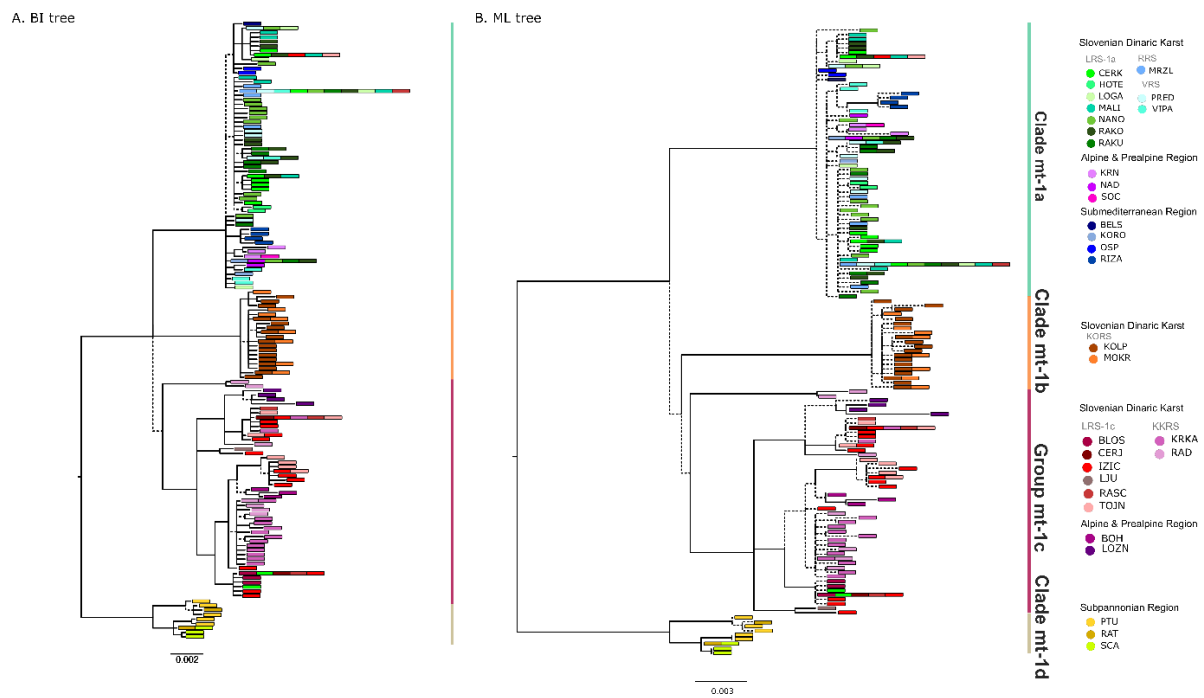


Figure S2. Trees based on a collapsed alignment of concatenated COI and cytb sequences of *Phoxinus lumaireuli*. A total of 134 unique haplotypes from samples of this study and previous studies (Palandačić et al., 2015; Palandačić et al., 2017; Table S1 for details) are distinguishable in three clades (mt-1a, -1b, -1d) and one group (mt-1c). Sample localities are given with abbreviations (explained in Table 1) **A.** Bayesian Inference (BI) tree. Branches with a Bayesian probability ≤ 0.95 are dashed. **B.** Maximum likelihood tree. Branches with a bootstrap support ≤ 0.9 are dashed.

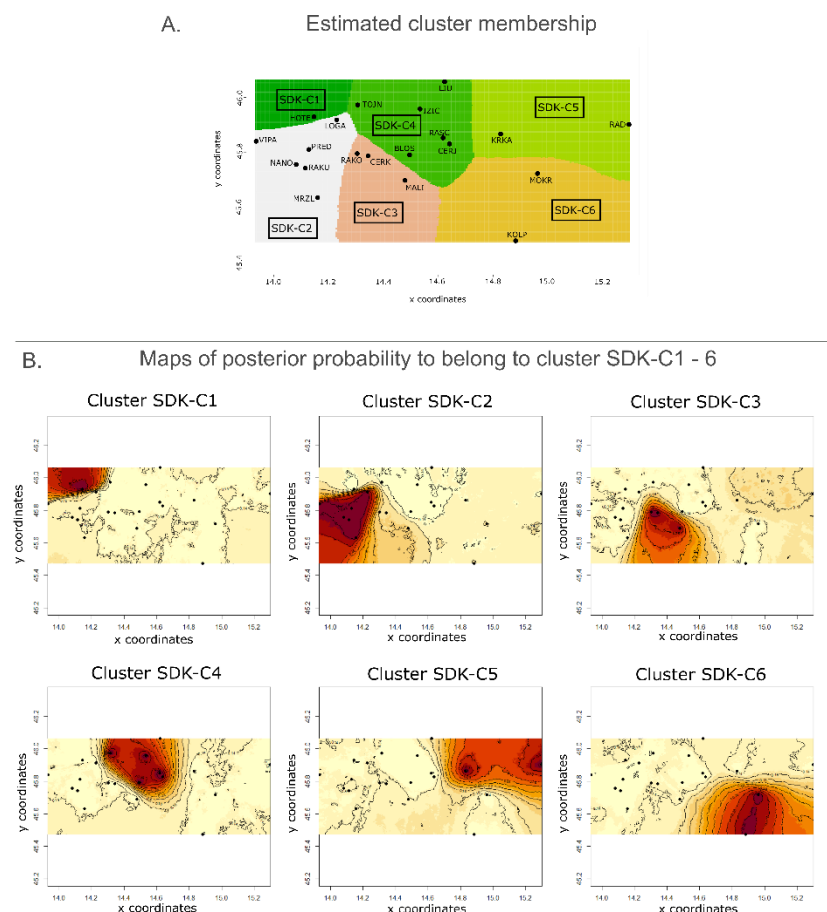


Figure S3. Population Bayesian cluster analysis (GENELAND). Only sampling sites of Slovenian Dinaric Karst (SDK) were analysed (sub-set GENELAND). Maps show the geographic distribution of samples (black points) **A.** Map of cluster membership for each sampling site ($K = 6$). **B.** Relative posterior probability of belonging to each of the six inferred clusters. Darker colour reflects higher posterior probability.

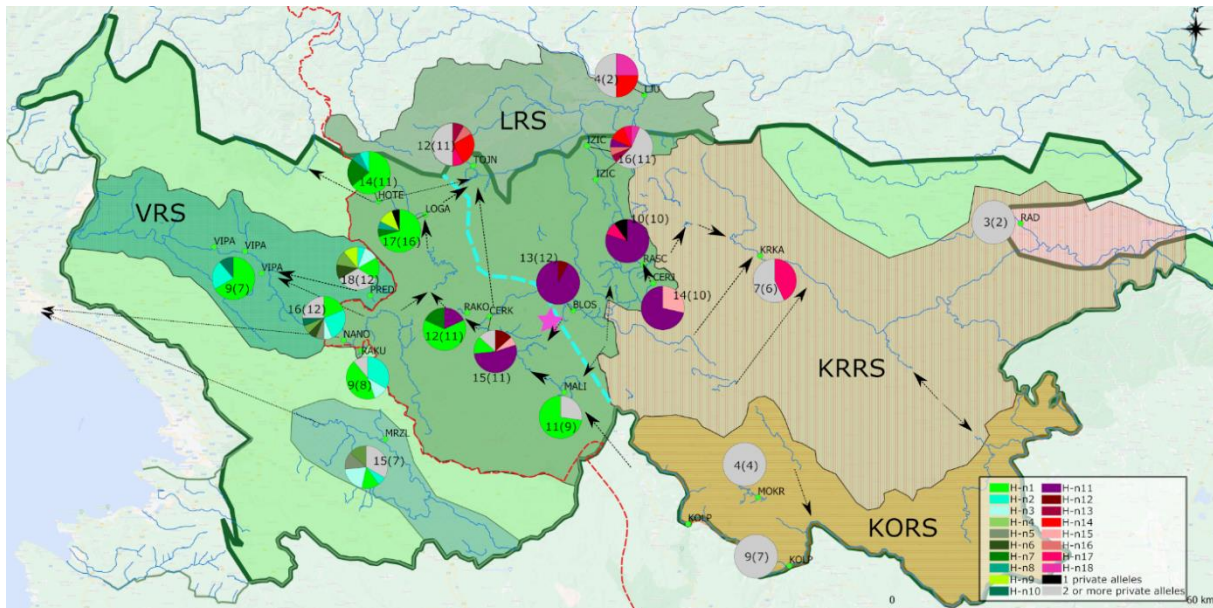


Figure S4. Haplotype frequencies of the nc dataset for each sampling site. Haplotype frequencies are given as a pie chart and the number of alleles and number of individuals in brackets is reported within or next to the pie chart. One private haplotype is coloured black, while two or more private haplotypes within one sampling site are coloured grey. Dashed red line represents the divide between the Adriatic and Black Sea basins; turquoise dashed line represents the divide within the Ljubljana river system (LRS) between clades 1a and 1c. Pink star shows possible zone of admixture between clades 1a and 1c.

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CHAPTER 3

Factors beyond karstification have shaped the population structure of a surface-dwelling minnow (*Phoxinus lumaireul*, Leuciscidae) able to disperse underground

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Factors beyond karstification have shaped the population structure of a surface-dwelling minnow (*Phoxinus lumaireul*) able to disperse underground

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Abstract

The Dinaric Karst, renowned for its rich biodiversity, is characterized by a network of interlinked surface and subterranean water bodies. Traditionally, tracing tests have been utilized to explore underground hydrological connections in this complex system. However, interest in a biological-centered approach that focuses on the population structure of resident aquatic organisms as genetic tracers is growing. The present study aimed to elucidate how the process of karstification has influenced the past and present population structure of the widespread, surface-dwelling fish species *Phoxinus lumaireul*. Populations of this minnow inhabiting surface streams and rivers in non-karst areas were compared genetically with those in karst areas that are connected exclusively underground. Additionally, the study assessed the potential for underground dispersal of *P. lumaireul*, comparing the identified population structure with the results of traditional tracing tests using dyes. Conducted in the densely sampled north-western Dinaric Karst, the study analyzed mitochondrial DNA and genome-wide single nucleotide polymorphism (SNP) data. The analysis revealed significant genetic divergence among three major groups of *P. lumaireul*, shaped by historical climatic fluctuations, with karst hydrology playing only a minor role. Indeed, consistent patterns emerged across river systems irrespective of whether the areas were karst or non-karst. Moreover, within the karst region, gene flow was restricted to specific areas, with populations exhibiting a robust structure and indicating local adaptations of *P. lumaireul* to its respective river systems. Further, this study highlighted the value of genetic tracers alongside traditional tracing methods.

Keywords: genetic water tracing, Dinaric Karst, freshwater species, ddRAD sequencing, dispersal, Leuciscidae

1. Introduction

Understanding how different habitats influence biodiversity is crucial in ecological research and conservation genetics. Complex habitats provide diverse niches, reducing their overlap and enhancing overall diversity (e.g., Smith et al., 2014; Stein et al., 2014; Tokeshi & Arakaki, 2012). Examples of a highly structured habitat include karst systems, where geological formations interact with hydrological processes to create a mosaic of unique ecosystems (Ford & Williams, 2007). One such system is Dinaric Karst, a well-developed karst region in the Western Balkan Peninsula characterized by limestone and dolomite formations (Zupan Hajna, 2019). From five to two million years ago (Mihevc, 2001; Trontelj et al., 2007) Dinaric Karst underwent intense karstification, in which water dissolves soluble rock (Bosak, 2008), resulting in a complex landscape of sinking streams, sinkholes, and caves (Ford & Williams, 2007) with hydrological connections between surface and subterranean water bodies (Bonacci, 1987; Bonacci & Živaljević, 1993; Legrand & Stringfield, 1973). Identifying these underground connections, which facilitate water movement, nutrient transfer, but also pollutant dispersal, is a central challenge in karst hydrology research. The use of tracing tests using dyes or salts is the classical, and regarded as the most effective, method for this purpose (e.g., Field, 1999; Petrič et al., 2020). However, a promising approach involves assessing genetic subdivisions of populations on either side of hypothesized hydrological connections, providing insights into their historical development and suitability for aquatic taxa dispersal (Konec et al., 2016; Palandačić, Bonacci et al., 2012; Reier, Kruckenhauser et al., 2022).

Dinaric Karst is well known for its high levels of biodiversity and endemism of freshwater and subterranean fauna (e.g., Francuski et al., 2019; Freyhof & Brooks, 2011; Gibert & Culver, 2009; Marčić et al., 2021; Recknagel et al., 2023; Žganec et al., 2016), the consequence of numerous species evolving under unique conditions in restricted habitats, e.g., subterranean aquatic caves (e.g., Amin et al., 2019; Delić et al., 2020; Lukić et al., 2023; Sket, 2012; Trontelj et al., 2007), or in surface water bodies located in karstic depressions (*polje*) (e.g., Bogutskaya & Zupancic, 2003; Freyhof et al., 2006; Sket, 1999). Vicariance and dispersal are emerging as key factors shaping genetic structure in karst regions (Delić et al., 2020; Trontelj, 2019), with their relative importance varying from region to region (Culver et al., 2009). While karstification can create landscape barriers fostering small-scale vicariance (Klobučar et al., 2013; Previšić et al., 2014), underground water connections may facilitate gene flow and connectivity among populations (Palandačić, Matschiner et al., 2012; Zakšek et al., 2009). Fluctuations in groundwater levels further contribute to dispersal in karst areas (Katz et al.,

2018; Reier, Bogutskaya et al., 2022; Zakšek et al., 2009). Meanwhile, some genetic patterns may be remnants of older geological events predating karstification (Sket, 2002; Verovnik et al., 2004; Žganec et al., 2016). The limited distributions of freshwater species in Dinaric Karst pose a threat to their survival, exacerbated by anthropogenic impacts such as water regulation (Fišer et al., 2022), pollution (Kačaroğlu, 1999), and invasive species introduction (Žganec et al., 2020). Therefore, understanding population connectivity through underground connections and water movement in karst aquifers is essential for biodiversity conservation efforts in the region.

The combination of older geological rearrangements and subsequent alternation of vicariance and dispersal, or both, during the formation of Dinaric Karst, seem also to have shaped the present genetic (sub)structures in the genus *Phoxinus* inhabiting the area (Palandačić et al., 2015; Reier, Bogutskaya et al., 2022; Reier, Kruckenhauser et al., 2022; Vučić et al., 2018). These small minnows belong to the family Leuciscidae and inhabit a variety of oxygen-rich waters throughout Europe and Asia (Frost, 1943; Tack, 1940). Several species of *Phoxinus* inhabit Dinaric Karst, with some being limited to a specific river basin (e.g., endemic *P. krkae*; Bogutskaya et al., 2020) and others widespread, inhabiting Adriatic and Black Sea basins in the Karst and beyond, such as *P. lumaireul* (Palandačić et al., 2015; Reier, Bogutskaya et al., 2022; Reier, Kruckenhauser et al., 2022; Vučić et al., 2018). *Phoxinus lumaireul* exhibits a eurytopic characteristic that enables the species to thrive under varying conditions, including both karstic and non-karstic streams (Reier, Kruckenhauser et al., 2022).

Whereas anthropogenic impacts on the distribution of *Phoxinus* species have been reported previously (e.g., Denys et al., 2020; Miró & Ventura, 2015; Museth et al., 2007), studies focusing on *P. lumaireul* have reported a minor role of human translocations in shaping its current genetic structure (Palandačić et al., 2015, 2020). Meanwhile, the study of Reier, Kruckenhauser et al. (2022) provides evidence for ongoing underground dispersal and historical water connections shaping the genetic structure of *P. lumaireul* in the NW Dinaric Karst, with higher genetic similarity among populations within the karst area compared to those outside. That study identified three mitochondrial (mtDNA) lineages distributed irrespective of the Adriatic–Black Sea basin divide, with Adriatic haplotypes found in the Black Sea basin. Nevertheless, limited genetic markers and representation of non-karst sampling sites have hindered a comprehensive understanding of demographic processes in the NW Dinaric Karst. Thus, a large-scale genomic approach with more sampling sites from non-karstic areas was required. The first objective of the present study was to investigate the influence of karst hydrology on the population structure and diversity of *P. lumaireul* by comparing populations

delineated by surface streams and rivers (in non-karst areas) with those where surface rivers are connected underground (karst areas). The second objective was to assess the potential for underground dispersal of the surface-dwelling species *P. lumaireul* inhabiting karstic surface streams through underground water connections, and compare the results with those obtained from the traditional technique of tracing tests (literature summarized in Petrič et al., 2020).

2. Material & Methods

2.1 Study organism and sampling

Finclips from 357 specimens of *P. lumaireul* from across eight localities were collected in August 2021 and stored in 96% EtOH prior to further analysis. Additionally, DNA samples from two earlier studies (Palandačić et al., 2015; Reier, Kruckenhauser et al., 2022) (see Table S1 for details) were included. In total, DNA of 841 specimens from 32 sampling sites was analyzed (Table S1). All freshly collected fish specimens were acquired in accordance with national regulations.

2.2 Study area

The study area (Fig. 1a, pink-bordered rectangle) is situated in the north-western part of the Dinaric Karst, with 20 sampling sites within the territory of Slovenia and one in Croatia (Table 1). Recently, the underground connections of this karst area were digitized by (Petrič et al., 2020) with information from more than 200 tracing tests dating back more than 100 years. The hydrology of the area is now well understood, with the western part draining towards the Adriatic Sea, and the center and south flowing to the Black Sea. A simplified scheme of connections is depicted in Fig. 1b. The divide between the two drainages is marked by a red dashed line (Fig. 1a&b). These include five major river systems (RSs): Vipava and Reka in the Adriatic Sea basin, and Ljubljana, Krka, and Kolpa in the Black Sea basin (Gams, 1993), with all RSs also encompassing non-karstic areas. Among the RSs, Ljubljana is the most complex, comprising several sinking and underground karst rivers, as well as many surface and underground karst features (Gabrovšek & Turk, 2011).

To assess the influence of the karst and its processes (karstification) on the genetic structure of *P. lumaireul*, ten sampling sites (Table 1) of the neighboring (non-Dinaric Karst) area were also collected and included in the study (Fig. 1a). These samples were collected from the Soča RS in the Adriatic Sea basin and the coastal streams flowing directly into the Adriatic (designated

herein as Coast). In addition, populations from tributaries of the Sava River in the Black Sea basin were also sampled.

Table 1. *Phoxinus phoxinus* sampling sites, by location on the Adriatic or Black Sea drainage basin. The site and the corresponding river system are included as an abbreviation, including genetic clusters from the Ljubljana river system (Fig. 1b). Whether the site is from a karst area or not, and the type of hydrological connectivity (surface connection or exclusively underground) is indicated, along with the genetic group (1a–c).

Adriatic Sea	ID Sampling site	Dinaric Karst	Connection type	Group
Coast	BADA OSPO RIZA	no	surface and underground	1a
Soča	BELS IDRI NADI SOCA	no	surface	1a
Reka	MRZL	yes	underground	1a
Vipava	BRAN VIPA	yes	surface and underground	1a
Black Sea				
Ljubljana - admix	CERK MALI RAKO	yes	underground	1a, 1c
Ljubljana - akarst	HOTE LOGA PRED NANO RAKU	yes	underground	1a
Ljubljana - ckarst	BLOS CERJ RASC	yes	underground	1c
Ljubljana - lju	TOJN IZIC	no	surface and underground	1c
Krka	CRMO KRKA RADU	yes	surface and underground	1c
Sava	BOHI KOKR SORA	no	surface	1c
Kolpa	KOLP KUPI MOKR	yes	surface	1b

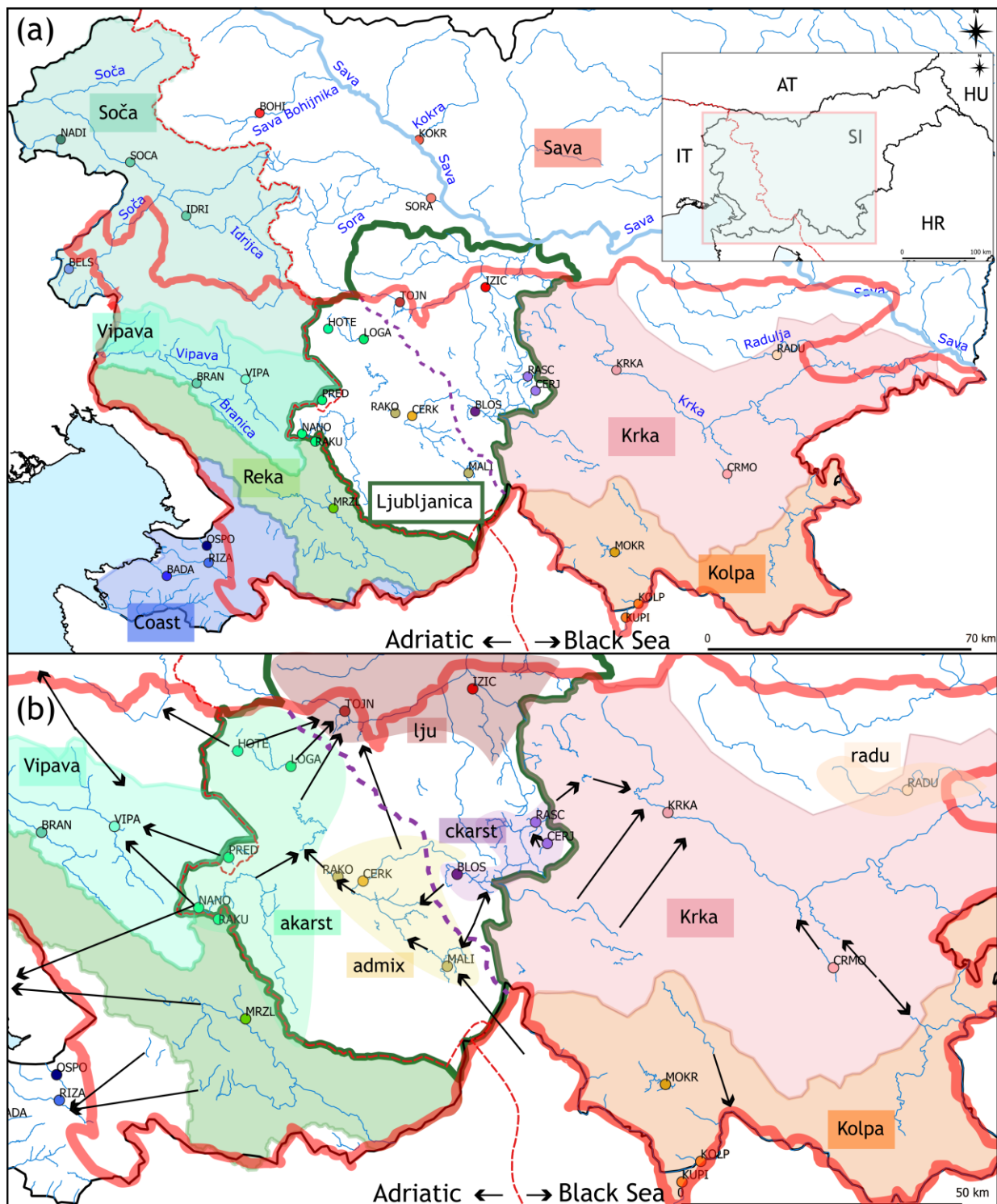


Fig. 1 Study area in the north-western Dinaric Karst and adjacent regions, showcasing the distribution of sampling localities. **(a)** View of the 8 main river systems. **(b)** Closer detail highlighting the karstic river systems. Arrows depict underground connections between surface rivers pointing in the direction of flow. Red dashed line indicates the border of the Adriatic–Danubian drainage basins, while the purple dashed line represents the border shift. The pink line indicates the border of Slovenian Dinaric Karst.

2.3 DNA extraction and analysis of mitochondrial DNA (mtDNA)

Genomic DNA was extracted from fin clips using QIAmp DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol.

The barcoding region of the mitochondrial COI gene was amplified using the primer pairs FishF1/FishR1 (Behrens-Chapuis et al., 2015) and the polymerase chain reaction (PCR) protocol described in (Palandačić et al., 2017). Sanger sequencing was conducted at Microsynth (Balgach, Switzerland) using the FishF1 forward primer. Sequences underwent manual verification, trimming, and alignment without ambiguity (no missing data) in Geneious v2.10.3 (<https://www.geneious.com>). Subsequently, a median-joining (MJ) haplotype network (Bandelt et al., 1999) was computed using PopART v1.7 software (Leigh & Bryant, 2015).

2.4 Double-digest RAD sequencing data sets

2.4.1 Library preparation and sequencing

Double-digest restriction site associated (ddRAD) sequencing libraries were prepared following the protocols outlined in Peterson et al. (2012) and Paulus et al. (2022), with a few custom modifications. The amount of input DNA ranged from 100 to 200 ng and, based on the DNA concentration, samples were organized into batches of 16. The protocol began with restriction digestion, conducted at 37°C for 2 hours, using a mastermix comprising 3 µl of rCutSmart™ buffer from New England BioLabs (NEB), 0.3 µl *MspI* (NEB), 0.3 µl *EcoRI* (NEB), 1.4 µl nuclease-free water (NFW), and 25 µl DNA. Subsequently, the samples were cleaned with 1.2x magnetic beads (AmpliClean™ Cleanup kit, Nimagen), followed by rinsing twice with 80% EtOH, and a final elution in 21 µl NFW. To attach adaptors for the specific restriction enzymes, we ligated 31.6 µM *MspI* and 2 µM *EcoRI* adaptors to 19 µl digested DNA. This ligation step comprised 3 µl of each adaptor, 3 µl 10x T4 ligase buffer, 0.75 µl T4 Ligase (NEB), and 1.25 µl NFW. Adaptors were tagged with unique barcodes to ensure a distinct barcode combination for each sample within every batch of 16 samples. The ligation was conducted at 22°C for 1 hour and heat-terminated at 65°C for 10 minutes. Subsequently, the 16 samples from each batch were pooled and cleaned up using 1.2x magnetic beads, followed by two washes with 80% EtOH, and final elution in 30 µl TE buffer. A crucial size selection step was implemented to narrow the fragment range to between 250 and 450 bp. This range had been previously determined in-silico using ddRADseqTools (v0.42, Mora-Márquez et al., 2017). Size selection was undertaken on a BluePippin instrument (Sage Science) utilizing a 2% agarose gel cassette and V1 marker. Subsequently, four PCR replicates were carried out for each pool to minimize

potential PCR amplification bias. PCR reactions were performed with 5 µl of NEBNext Q5 Hot Start HiFi PCR Master Mix, 0.5 µl of each primer, and 4 µl of the library with 10 cycles. The forward and reverse primers included eight distinct 8-bp long indices, which were combined to assign a unique combination to each pool. The four replicates of each pool were then combined and cleaned using 1× volume of magnetic beads, with elution in 16 µl NFW. DNA concentration was determined using a Qubit fluorometer (Thermo Fisher Scientific), while the mean fragment size was assessed using a TapeStation (Agilent). Finally, within each library, 16 pools were combined at equal molarity, resulting in a total of five libraries, which were sent to MacroGen Europe for sequencing on five Illumina HiSeqX lanes, generating 100 bp paired-end reads.

2.4.2 ddRAD data processing

Raw reads were first pre-demultiplexed by MacroGen and subsequently trimmed with trim-galore v0.6 (Krueger et al., 2023) using a quality threshold of 30 and minimum length filter of 40 bp. STACKS v2.59 (Rochette et al., 2019) was used to remove potential PCR duplicates (function *clone_filter*) and to demultiplex the individuals by barcodes (*process_radtags*). Each demultiplexed individual was aligned to a reference genome of *P. phoxinus* (PRJNA1030283; Oriowo et al., 2023) using bwa-mem v0.7.13 (Li, 2013) and default parameters. The output bam files were further sorted by reference position with SAMtools v1.12 (Danecek et al., 2021). A catalog file of loci was built with the *gstacks* program implemented in STACKS v2.59. Since the underlying model in some of the analyses assumes unlinked loci, two different single nucleotide polymorphism (SNP) sets based on distinct filtering criteria were applied using the program *populations* implemented in STACKS v2.59. A first run was performed to retain all SNPs matching the required filtering criteria, using the parameters: ‘-r 0.5’; ‘-p 9’; ‘-min-maf 0.03’. This step aimed to create the diversity dataset. A second run was conducted for unlinked SNPs, employing the same parameters as the first filtering, but including an additional ‘-write-random-snp’ to address linkage disequilibrium to generate the popgen dataset.

Individuals with a significant amount of missing data (>50%, assessed with ‘--missing-indv’) were removed from both datasets using vcftools v0.1.13 (Danecek et al., 2011). In four sampling sites, fewer than ten individuals remained after filtering, leading to the exclusion of the site from subsequent analysis. The final datasets were further filtered using only the remaining individuals to eliminate SNPs with missing data exceeding a threshold of 5% per site (command ‘--max-missing 0.95’). Missing data per individual per sampling site are represented in Fig. S1.

2.4.3 Inferring population structure

Using the popgen dataset, two distance-based methods were employed to visualize divergence between putative genetic lineages: (1) a principal component analysis (PCA) conducted using the R package SNPRelate (Zheng et al., 2012), and (2) an unrooted phylogenetic network calculated using SplitsTree4 v4.19.2 (Huson & Bryant, 2006) with the Neighbor-Net algorithm and uncorrected p -distances. To examine substructure within different genetic lineages detected by previous methods and to confirm robustness of the results of population structure and ancestry, four complementary clustering methods were applied using the popgen dataset.

ADMIXTURE v1.3.0 (Alexander et al., 2009), which utilizes a likelihood-based approach, was run for K values ranging from 1 to 18, with 10 runs conducted for each K . Membership coefficients for each K were visualized as bar charts using BITEV2 (Milanesi et al., 2017) in R. Additionally, the model fit for each K was evaluated using evalAdmix (Garcia-Erill & Albrechtsen, 2020). When individuals deviate from the model, those with comparable demographic backgrounds or relatedness, typically from the same population, will exhibit a positive correlation. Conversely, individuals with distinct histories are represented as having common ancestral populations as sources of admixture will display a negative correlation (Garcia-Erill & Albrechtsen, 2020).

Sparse Non-Negative Matrix Factorization (sNMF) (Frichot et al., 2014) implemented in the R package LEA (Frichot & François, 2015) was performed with K values ranging from 1 to 18, and 10 runs conducted for each K . The sNMF algorithm is a prior-free clustering algorithm used to compute regularized least-squares estimates of individual ancestry coefficients (Frichot & François, 2015). The optimal K value was determined through a 10-fold cross-validation (CV), and the run with the lowest CV value for each K was plotted as a pie chart on a map using the R package ggplot2 (Wickham, 2016) and rworldmap (South, 2011). Spatial information based on geographically constrained non-negative matrix factorization was integrated using the TESS3 algorithm as implemented in the R package tess3r v1.1.0 (Caye et al., 2016). TESS3 results were visualized using ggplot2 (Wickham, 2016) and rworldmap (South, 2011).

A fourth method—Discriminant Analysis of Principal Components (DAPC; Jombart et al., 2010)—was performed using the R package adegenet v.2.1.1 (Jombart, 2008). In contrast to the model-based methods, DAPC does not assume an evolutionary model. It utilizes k-means clustering and the Bayesian information criterion (BIC) to identify the most likely number of genetic clusters in the dataset by applying the function find.clusters. The number of principal components to be retained in the DAPC analysis was determined using the xvalDAPC function of adegenet v.2.1.1.

The diversity dataset was used to further quantify co-ancestry and detect subtle population substructures through conducting RADpainter and fineRADstructure analysis (Malinsky, Trucchi et al., 2018), a modification of fineSTRUCTURE and ChromoPainter (Lawson et al., 2012), tailored for RAD data and non-model organisms. The analysis used default parameters recommended by the developers (Malinsky, Trucchi et al., 2018) and the results plotted using the provided Rscript FinestructureLibrary.R (<https://github.com/millanek/fineRADstructure/blob/master/FinestructureLibrary.R>, accessed 29 December 2023).

Using the popgen dataset, estimates of genetic differentiation (pairwise F_{ST} values) between sampling sites and river systems and clusters (see Results section 3.2.1) were calculated using Arlequin v3.5 (Excoffier & Lischer, 2010) with 10,000 permutations. Additionally, genetic divergence between sampling sites was quantified through the calculation of D_{XY} values, using the program populations in STACKS v2.59 (Rochette et al., 2019). A pairwise matrix containing F_{ST} and D_{XY} values among all sampling sites was plotted as heatmap using the R package circlize v0.4.15 (Gu et al., 2014) and ComplexHeatmap v2.18 (Gu, 2022; Gu et al., 2016).

Analysis of molecular variance (AMOVA) was conducted to examine the genetic variation among i) the three major groups (see Results section 3.2.1), ii) river systems and clusters (based on $K=12$, see Results section 3.2.1), and iii) rivers with surface connections and rivers with underground connections (exclusively karst, see Table 1). The four distinct sampling site structures utilized for the different AMOVAs are reported in Table S2. AMOVA calculations were carried out with the software Arlequin v3.5 (Excoffier & Lischer, 2010) using the popgen dataset. Significance of fixation indices was evaluated through 10,000 random permutation procedures.

2.4.4 Calculating genetic diversity

Using the diversity dataset, a set of descriptive population genetics statistics, including mean observed (H_O) and expected (H_E) heterozygosity, and their corresponding standard errors (SE), were calculated with the program populations of STACKS v2.59 for each separate sampling site. Furthermore, standardized multi-locus heterozygosity and number of segregating sites for each site were evaluated using the R package inbreedR v0.3.3 (Stoffel et al., 2016) implemented in the SambaR pipeline (De Jong et al., 2021). All R packages used in the present study were run in R v4.3.2 (R Core Team, 2023).

2.4.5 Testing contemporary gene flow

The popgen dataset was used to assess recent migration rates between the sampling sites using BA3-SNPs v3.0.4 (Mussmann et al., 2019), an adaptation of BayesAss (Wilson & Rannala, 2003), designed to handle extensive SNP datasets. Ten repetitions in BA3-SNP-autotune (Mussmann et al., 2019) were conducted to determine optimal mixing parameters for migration rate ($\delta M=0.05$), allele frequencies ($\delta=0.1$), and inbreeding coefficients ($\delta F=0.025$). Subsequently, BA3-SNPs was executed with the specified mixing parameters for ten million generations, sampling every 100th generation. The initial million generations were discarded as burn-in, and chain convergence evaluated using Tracer v1.7.1 (Rambaut et al., 2018). Migration rates were determined to be statistically significant if the 95% credible intervals (mean migration rate $\pm 1.96 \times$ mean standard deviation) did not include zero. Hereby, only migration rates exceeding 0.01 were considered. The R package circlize v0.4.15 (Gu et al., 2014) was utilized to plot the results.

2.4.6 Testing historical gene flow and introgression

Since the results of PCA, ADMIXTURE, and sNMF may be hard to interpret historically (Malinsky et al., 2021), the software package Dsuite v.0.4 (Malinsky et al., 2021) was used to calculate Patterson's D, the f_4 -ratio (Patterson et al., 2012), and f -branch (f_b) statistics (Malinsky, Svandal et al., 2018) for all possible population trios to assess for potential historical introgression. Population clustering for the Dsuite analysis was determined based on previous analyses (clusters determined in ADMIXTURE, sNMF, fineRADstructure), and described further under Results. Clusters showing signs of admixture in the ADMIXTURE and sNMF analyses were excluded from the dataset, as they would bias the estimation by introducing signals of recent introgression (Secci-Petretto et al., 2023). Two individuals of *P. phoxinus* (mitochondrial clade 10 *sensu* Palandačić et al. (2017)) were used as outgroup (Table S1). The dataset was generated employing identical parameters to those used for the popgen dataset through the program populations (STACKS), with the inclusion of the two outgroup individuals. The analysis used block sizes of 399 variants to obtain 20 jackknife blocks. Assignment of signals of introgression to specific branches of a given population tree was undertaken with the use of the f_b metric, based on the outcomes of the f_4 -ratio analysis (Malinsky et al., 2021). The input population topology used as the guide tree for the f_b estimation was calculated by executing 1000 bootstrapping runs in TreeMix v1.13 (Pickrell & Pritchard, 2012), with the resulting trees summarized in a maximum likelihood (ML) consensus tree with the

program consens in PHYLIP v3.698 (<https://evolution.genetics.washington.edu>, accessed on 20 October 2023).

TreeMix v1.13 (Pickrell & Pritchard, 2012) was used to infer historical relationships among sampling sites by generating an ML tree for the clusters identified by the population structure analyses described above and considering historical gene flow events using the popgen dataset. The same grouping as for the Dsuite analysis was used (see also Results section), though this analysis allows for admixed groups (identified by ADMIXTURE and sNMF) to also be included. The analysis was conducted allowing for 0–10 migration events per 100 runs. Calculation of SEs for migration rates was done using the ‘-se’ option, and, subsequent to the addition of all populations, a round of global rearrangements was executed using the ‘-global’ option. No root was specified for the tree. Furthermore, the R package OptM v0.1.6 (Fitak, 2021) was used to evaluate the best number of migration events, while TreeMix v1.13 was run again with 1000 replications, sampling each tree to construct an ML consensus tree with consens (PHYLIP v3.698; <https://evolution.genetics.washington.edu/>, accessed on 20 October 2023). Once obtained the ML consensus tree was used as input for an additional TreeMix run with the evaluated optimal number of migration events. The R package BITE v2 (Milanesi et al., 2017) was used to plot the final tree with migration events, with heatmaps of the residuals showing deviations from the model fit to the data and genetic drift between populations.

2.4.7 Inference of effective population size history

Demographic changes in effective population size (N_e) over time for the three major geographic and genetic groups (1a–c; see Results) were estimated using Stairway plot v2.1 (Liu & Fu, 2020) using the diversity dataset. The Folded Site Frequency Spectrum (SFS) for each population was generated with the python script easySFS (<https://github.com/isaacovercast/easySFS/>; accessed on 29 October 2023). A preview of the data was initially generated to identify values for projecting down each group to account for missing data, but at the same time with the goal of maximizing the number of segregating sites (Gutenkunst et al., 2009). After running the script for projection, the SFS values obtained were imported into a blueprint file for Stairway Plot v2.1. The parameter settings for Stairway Plot were configured with 67% of SFSs used for training with four random breakpoints set at $(n-2)/4$, $(n-2)/2$, $(n-2)*(3/4)$, and $n-2$ as suggested by (Liu & Fu, 2020), whereby n corresponds to the number of SFSs. Additionally, a generation time of 2+ years was applied, considering that the age of the individuals typically does not exceed 3+ years (Altinişik & Yağlıoğlu, 2022; Frost, 1943; Mills, 1987; Nunn et al., 2007; Saç & Özuluğ, 2020). No mutation rate of *Phoxinus* is available, and,

therefore, a rate of 3.51×10^{-9} per site per generation was used (Tian et al., 2022), as for *Gymnocypris przewalskii*, to the authors' knowledge the closest relative of *Phoxinus* for which a mutation rate is known.

3. Results

3.1 Mitochondrial population structure and phylogeographic patterns

Three mtDNA clades (1a–c) were identified in the NW Dinaric Karst and neighboring areas (Fig. 2a) using the COI MJ haplotype network. Groups 1a and 1c span Dinaric Karst and neighboring sampling sites, while 1b is restricted to the karst area (Fig. 2a&b). Groups 1a and 1b are monophyletic, while 1c is not. Thus, instead of using the term mtDNA clade, the term mtDNA group will be used hereafter to emphasize 1c's paraphyletic origin. Group 1a comprised two main haplotypes (Fig. 2b). The first encompassed 184 individuals from all Adriatic Basin RSs (karst and non-karst) and included some collected in the Black Sea basin (Ljubljana RS). The second, comprising 163 individuals, included only sampling sites in the Dinaric Karst area, from both Adriatic and Black Sea basin RSs (including Vipava and Ljubljana). Group 1b included sampling sites from the Black Sea basin (Kolpa RS), all within the karst area. Sampling site RADU from Krka RS forms its own (haplo)group, not clustering with any of the three major groups (Fig. 2b). In group 1c, most individuals also clustered with two major haplotypes: one of 115 individuals from Black Sea basin RSs Ljubljana, Sava and Krka, and another of 56 individuals from Black Sea basin RSs Ljubljana and Krka. These major haplogroups include individuals from sampling sites both inside and outside of the karst area (Fig. 2b). CERK sampling site (Ljubljana RS), which lies at the border between the distributions of groups 1a and 1c, predominantly exhibited 1a haplotypes, with a lower frequency of 1c haplotypes.

3.2 ddRAD dataset

For the ddRAD dataset, a total of 841 individuals from 32 sites were sequenced. Four sites, BADA (Coast RS), BELS (Soca RS), CRMO (Krka RS) and MRZL (Reka RS), comprising a total of 46 specimens, did not meet quality standards and were excluded from further analysis (Table S1). MRZL was the only representative of the Reka RS. A further 79 individuals from other sites were excluded due to excessive missing, or poor quality, data, leaving 722 individuals for analysis. Nevertheless, the number of remaining specimens per sampling site was large enough to allow for meaningful analysis (Table S1). After filtering, the final datasets

comprised 8,085 unlinked SNPs (popgen dataset) and 28,764 linked SNPs (diversity dataset) with a missingness per site of less than 5%. Plots illustrating missingness per population are provided in Fig. S1.

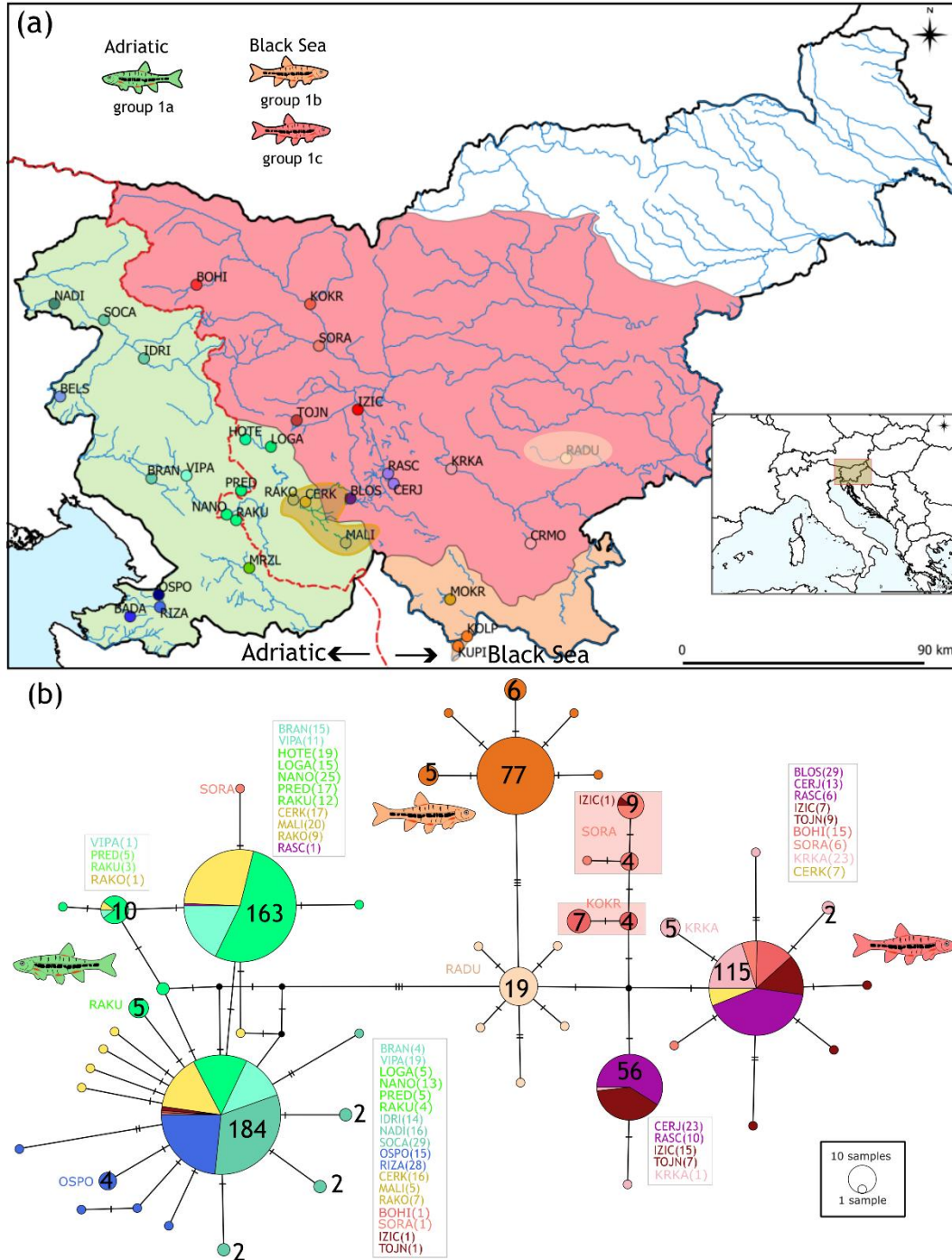


Fig. 2 (a) Distribution patterns of the three major mitochondrial groups according to the findings of Reier, Kruckenhauser et al. (2022): group 1a (pale green) spans the Adriatic drainage basin, crossing the border into the Danubian drainage basin; groups 1b (pale orange) and 1c (pale red) are situated on the Danube drainage basin. Circled localities indicate sampling points without clear grouping. Genetic group is visually represented by colored fish: green, group 1a; orange, group 1b; red, group 1c. (b) COI MJ network supporting the grouping into three major clusters. Group 1c lacks a distinct separate cluster in the COI MJ network.

3.2.1 Population structure

The PCA analysis unveiled three major groups corresponding to the three major mtDNA haplogroups (1a–c, Fig. 3a) with additional structures (Fig. 3b), resulting in seven distinct clusters: group 1a (including sampling sites from RSs Coast, Soca, Vipava, and some sites from Ljubljana), group 1b (Kolpa RS) and RADU confirmed as separate clusters (Fig. 3b), two clusters separated in Ljubljana RS, comprising individuals from sampling sites CERK and RAKO, and group 1c, split into two clusters with the first formed from individuals from Ljubljana and Sava RSs (SORA+TOJN+IZIC) and the second from the remaining Sava RS sites, some Ljubljana RS sites (BLOS+RASC+CERJ) and Krka RS (Fig. 3b). The PCA analysis revealed a subdivision of the Ljubljana RS (Fig. 1b, Fig. 3b) into (i) akarst in the west (mt group 1a, and including sampling sites NANO, RAKU, PRED, HOTE, LOGA), (ii) ckarst in the east (mt group 1c, and including sampling sites BLOS, CERJ, RASC), (iii) an admix between mtDNA groups 1a and 1c (including sites RAKO, and CERK, and MALI, though this last site is relatively close to those in the 1a cluster), and (iv) lju in the north (mt group 1c, and including non-karst sampling sites TOJN and IZIC). The above pattern is also evident in the SplitsTree network, which displayed (the same) seven clusters. However, sampling site SORA (Sava RS) was also divided into two clusters, with one of them forming an additional, eighth, cluster. Within lju additional sub-structuring was detected (see Fig. 3c).

No distinct lowest CV value was evident in ADMIXTURE, sNMF, or TESS3 analysis, though a plateau emerged from K=9 onwards, indicating the presence of at least nine ancestral groups with fine-scale population structuring (Fig. S2). Further evaluation with evalADMIX revealed the best model fit for most populations at K=18 (Fig. S4), though K-values should be interpreted carefully to avoid over-interpretation (Caye et al., 2016), as genetic groups identified by ancestry estimation programs may not always align with the number of biologically meaningful populations in the sample (François & Durand, 2010). Since population structure in our dataset is shown to be hierarchical, the focus of the results rests on K=12, corresponding to the main RSs, with K=5, 9, 15, and 18 shown in Fig. S4. When including spatial information with the TESS3 algorithm using tess3r, the clusters corresponded to the Coast, Soca RS, Vipava RS, Kolpa RS, Krka RS, sampling site RADU (Krka RS), Sava RS, and sampling site SORA (Sava RS) (Fig. 4a). Ljubljana RS was divided into four clusters: akarst, admix, ckarst, and lju. Sampling site MALI clustered with akarst.

In the ADMIXTURE analysis (Fig. 4b) the same clusters identified in tess3r were found, with the following exceptions: SORA (a sampling site of Sava RS) exhibited admixed ancestries

with lju, and to a lesser extent with group 1a, while Ljubljana RS was now divided into five groups. Admixed ancestries were found between HOTE and akarst (sampling site LOGA), admix and ckarst (sampling sites RAKO and CERK), and Vipava (sampling site VIPA) and akarst.

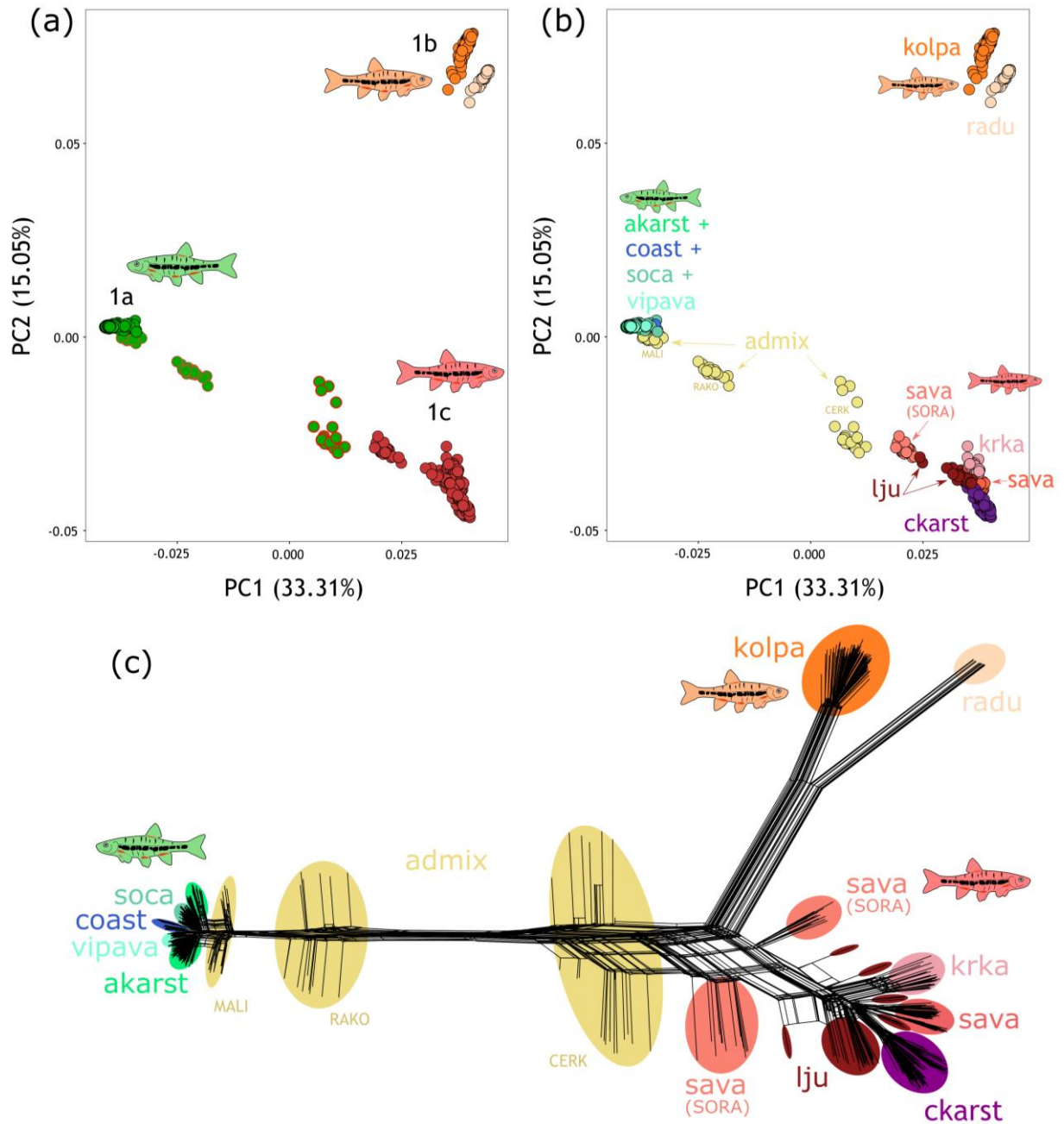


Fig. 3 PCA, showing PC1 and PC2 with each dot representing an individual, with colors representing (a) the major groups 1a–1c (admix sampling sites are colored green and circled in red) and RADU, and (b) the river systems and clusters, including sub-divisions of the Ljubljana river system. (c) Unrooted phylogenetic network with SplitsTree analysis. The groups are visually represented by the colored fish.

sNMF analysis (Fig. 4c) revealed the following clusters: Coast, Soca RS, Krka RS, RADU, Sava RS, SORA (sampling site of Sava RS) and Kolpa RS. Within the Ljubljana RS five ancestral groups were again revealed: akarst, admix, lju and two groups within ckarst. Admixture was revealed between admix and akarst (MALI and RAKO), as well as admix and ckarst (CERK). Within ckarst two ancestral clusters were revealed. Vipava RS had no distinct ancestral group and was admixed between Soca RS, Coast and akarst.

DAPC analysis (Fig. 4d) identified the lowest BIC value at K=15 (Fig. S3). The first axis of DAPC identified highly diverse clustering. Kolpa RS, Krka RS, RADU, and ckarst (Ljubljana RS) were separated into distinct clusters. Meanwhile, four clusters, although very slightly separated on axis 1, were found within akarst: sampling sites PRED and HOTE clustered separately, sampling sites NANO and LOGA were split into two clusters, one with RAKU and one with VIPA (Vipava RS), while the second Vipava sampling site BRAN was a separate cluster. The admix cluster was split into two: MALI and RAKO, and CERK.

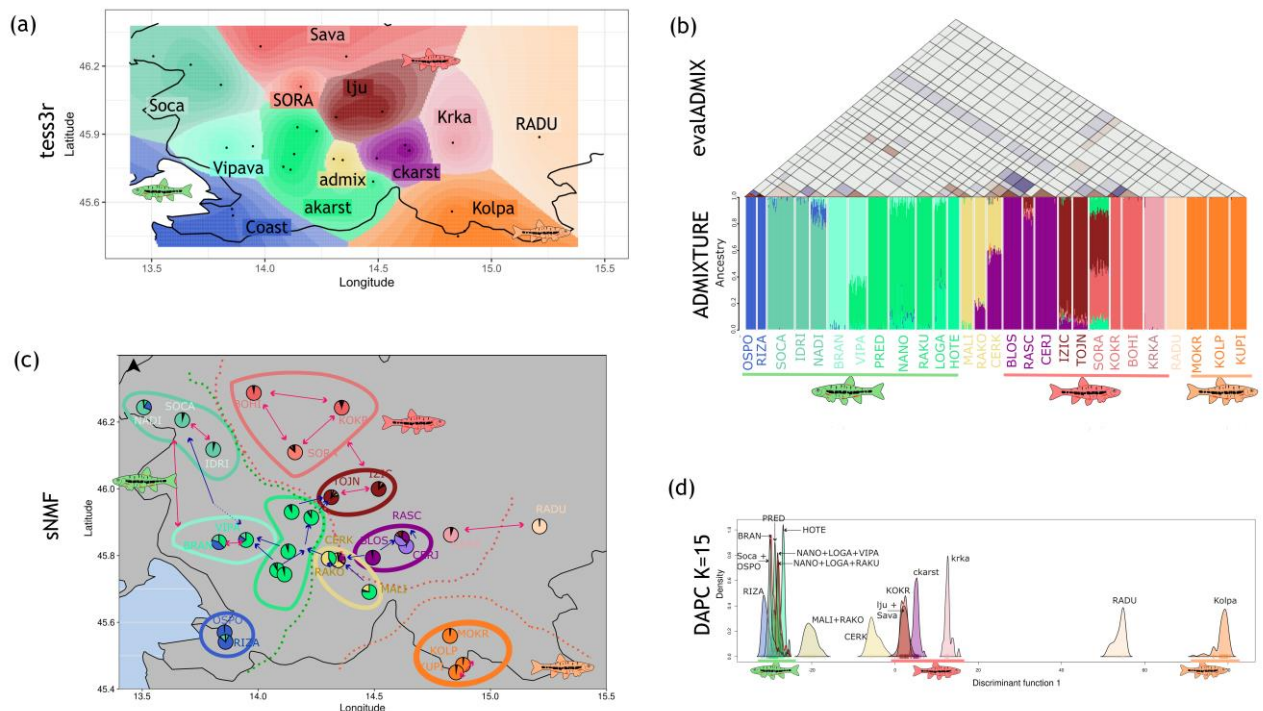


Fig. 4 presents a comprehensive view of the genetic structure using four different analyses based on genome-wide unlinked SNP data. In (a–c) ancestry proportions are inferred at K=12 using (a) tess3r, (b) ADMIXTURE, and (c) sNMF. The colored polygons in (a), the colored bars in (b), and the colored pie charts in (c) represent estimated admixture proportions, with the number of ancestries equal to K. In (b), colored lines and fish icons below population labels indicate group designations. Pairwise correlations of residuals, as estimated by evalAdmix, are shown above and below the respective ADMIXTURE barplots, ranging from –0.1 to 0.1 on the color scale. Dashed lines in (c) indicate the geographic separation of the three major groups identified in this study, further marked by colored fish icons. Respective river systems and sub-divisions within river systems are circled, and underground connections are denoted by blue arrows (with blue dashed lines indicating side underground connections) pointing in the flow direction. Pink arrows signify surface connections between

sampling sites. **(d)** Illustrates the first axes of a DAPC analysis based on K=15. The red outlines of peaks of BRAN and NANO+LOGA+RAKU are included for visual clarity, and the three major groups are indicated as colored lines and fish icons below the clustered peaks.

The fineRADstructure co-ancestry analysis highlighted co-ancestries within the major groups 1a, 1b and 1c, emphasizing genetic cohesion within each group but revealing the substructure based on RSs (Fig. 5). Admixed co-ancestry patterns were evident for specific sampling sites, such as CERK in the admix cluster, showing significant co-ancestry with 1c and, to a lesser extent, 1a. MALI and RAKO from admix showed predominantly ancestry of group 1a, though co-ancestry with 1c was also indicated. Two clusters, SORA and lju, while belonging to group 1c, also showed co-ancestry with 1a. Notably, group 1b and RADU exhibited the highest co-ancestry patterns within their respective groupings when compared to other sites (Fig. 5).

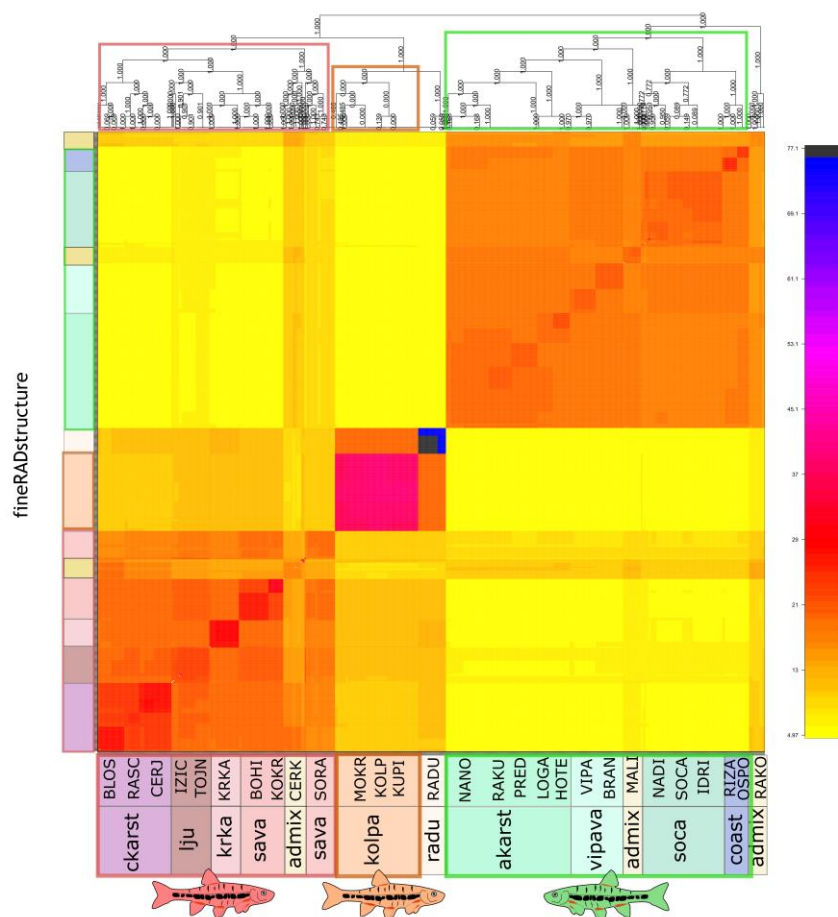


Fig. 5 Clustered fineRADstructure coancestry matrix. Colors correspond to the level of co-ancestry: low, yellow; high, purple–blue–black. Colors of sampling site names correspond to river systems and clusters, including sub-divisions of the Ljubljana river system. The groups are visually represented by colored fish.

Pairwise comparisons of F_{ST} and of D_{XY} values highlighted distinct genetic structures among the three major groups (1a, 1b, 1c), underscoring their significant genetic differentiation ($F_{ST} > 0.2$; Fig. 6a&b). The pairwise F_{ST} estimates among sampling sites varied widely (0.02–0.7 and higher), with differing values within river systems and clusters. Low F_{ST} values ($F_{ST} \leq 0.04$) were observed between all sampling sites within the Soca RS and cluster lju, linked through surface connections, while cluster akarst, characterized by exclusive underground connections, also displayed mostly low F_{ST} values (≤ 0.04). Low mean pairwise F_{ST} values were also found between three Adriatic RSs and clusters (Soca, Vipava, akarst; $F_{ST} \leq 0.04$). Meanwhile, across other clusters and river systems within the major groups, pairwise F_{ST} values ranged mostly from 0.04 to 0.09 (even higher), irrespective of type of connectivity (Fig. 6b). Among pairwise D_{XY} values, the lowest values within group 1a were observed for SOCA (Soca RS), and MALI and RAKO (both Ljubljana RS), while within group 1c, the lowest values were found for TOJN (cluster lju; Fig. 3). Conversely, the highest pairwise D_{XY} values were consistently observed for RADU across all sampling sites.

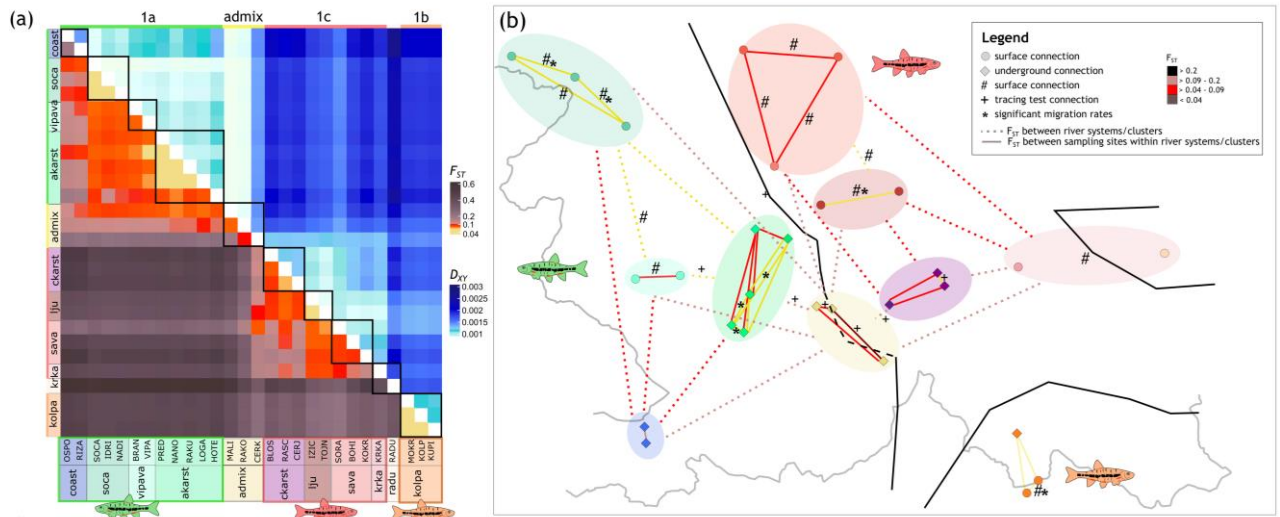


Fig. 6 (a) Pairwise F_{ST} (below diagonal) and D_{XY} (above diagonal) values using genome-wide unlinked SNP data for *P. lumaireul*. Each river system or cluster is indicated by a black-edged square. **(b)** Map of study area with representation of pairwise F_{ST} values and significant migration rates (*) between sampling sites within each river system or cluster, and mean pairwise F_{ST} values between the river systems or clusters as lines (see legend). The different groups are represented by colored fish, with the color corresponding to river system or cluster in which the sampling sites are located.

AMOVA found the largest source of variation to be among the three major groups (65.7%) and the RSs and clusters (56%), both highly significant (Table 2). However, the variation between

rivers connected on the surface (mostly non-karst) and underground (karst) was low and not statistically significant ($F_{CT}=-0.042$, $p=0.634$; Table 2).

Table 2. Analysis of molecular variance (AMOVA) and degrees of freedom (df) for ddRADseq data among the three major groups identified in the present study (Fig. 2a), the 12 clusters from the population genetic analyses, corresponding to the river systems, including the clusters of the Ljubljana river system (Fig. 1b), and the type of hydrological connectivity (underground or surface) corresponding to karst or non-karst rivers (see Table 1 and Table S2 for details).

Source of variation	d.f.	Sum of squares	Variance component	Percentage variation	Fixation index	P-value
among major groups (1a-c)	2	26168.62	33.85	65.72	$F_{CT}=0.558$	<0.0001
among sampling sites within major groups	21	2334.71	1.83	3.56	$F_{SC}=0.140$	<0.0001
within sampling sites	1242	19659.481	15.83	30.73	$F_{ST}=0.693$	<0.0001
Total	1265	48162.81	51.52			
among river systems/clusters	11	31230.968	22.87	55.78	$F_{CT}=0.657$	<0.0001
among sampling sites within river systems/clusters	16	2262.205	2.54	6.20	$F_{SC}=0.104$	<0.0001
within sampling sites	1416	22076.891	15.59	38.02	$F_{ST}=0.62$	<0.0001
Total	1443	55570.064	41.0			
between karst and non-karst rivers	1	335.11	-1.41	-4.24	$F_{CT}=-0.042$	0.634+-0.005
among sampling sites within karst and non-karst rivers	19	19020.1	19.31	58.23	$F_{SC}=0.558$	<0.0001
within sampling sites	1061	16187.48	15.26	46.01	$F_{ST}=0.539$	<0.0001
Total	1081	35542.690	33.158			

3.2.2 Genetic diversity

Within-population genetic variation measures (H_E , H_O) exhibited a consistent pattern across all sampling sites, as reported in Table 3. Generally, sampling sites within groups 1b and 1c displayed higher values of within-population genetic variation than did sites from group 1a, with the exception of RADU in Krka RS, which, along with sampling sites from Coast OSPO and RIZA, exhibited the least genetic variation in the dataset. In contrast, CERK and SORA had the highest values, followed by RAKO, indicating admixture. This pattern was supported by plots depicting the proportion of segregating sites among all sites (Fig. S3a), heterozygosity vs. nucleotide diversity (Fig. S3b), and standardized multi-locus heterozygosity (sMLH; Fig. S3c).

Table 3. Genetic diversity parameters and their corresponding standard error (SE) of genome-wide SNP data derived from ddRAD sequencing (H_O , observed heterozygosity; H_E , expected heterozygosity)

PopID	RS	H_o	SE	H_E	SE
OSPO	coast	0.04175	0.00075	0.04126	0.00071
RIZA	coast	0.04208	0.00077	0.04085	0.00071
IDRI	Soca	0.06848	0.00087	0.06833	0.00083
NADI	Soca	0.06605	0.00084	0.06649	0.00082
SOCA	Soca	0.06764	0.00083	0.06929	0.00083
BRAN	Vipava	0.06549	0.00085	0.06579	0.00083
VIPA	Vipava	0.06508	0.00084	0.06625	0.00083
HOTE	akarst	0.05790	0.00086	0.05694	0.00081
LOGA	akarst	0.06795	0.00087	0.06813	0.00084
PRED	akarst	0.06585	0.00085	0.06605	0.00083
NANO	akarst	0.06915	0.00084	0.07042	0.00084
RAKU	akarst	0.06670	0.00087	0.06671	0.00084
MALI	admix	0.08845	0.00088	0.08912	0.00085
RAKO	admix	0.17124	0.00110	0.16959	0.00103
CERK	admix	0.21812	0.00126	0.21706	0.00118
BLOS	ckarst	0.10435	0.00105	0.10517	0.00102
RASC	ckarst	0.11830	0.00111	0.11903	0.00105
CERJ	ckarst	0.11091	0.00110	0.10864	0.00104
IZIC	lju	0.13642	0.00104	0.14331	0.00104
TOJN	lju	0.14977	0.00104	0.15483	0.00102
BOHI	Sava	0.11554	0.00105	0.11902	0.00104
KOKR	Sava	0.09924	0.00105	0.10019	0.00101
SORA	Sava	0.19326	0.00109	0.19938	0.00108
KRKA	Krka	0.11696	0.00103	0.12267	0.00104
RADU	Radu	0.03662	0.00068	0.03729	0.00066
KUPI	Kolpa	0.11850	0.00105	0.13094	0.00109
KOLP	Kolpa	0.11953	0.00104	0.13194	0.00109
MOKR	Kolpa	0.11807	0.00104	0.12998	0.00109

3.2.3 Contemporary gene flow

The three independent BA3-SNP runs conducted to quantify the degree of migration rates agreed upon the patterns of gene flow. Significant migration rates were revealed primarily among sampling sites within the akarst cluster from NANO to the close sampling sites of LOGA ($m=0.14$), RAKU ($m=0.15$), and PRED ($m=0.15$) (Fig. 6b & 7a). Further, significant migration rates were found between sampling sites connected through the surface: from IZIC to TOJN ($m=0.14$; cluster lju, non-karst), from SOCA to IDRI ($m=0.15$) and NADI ($m=0.13$; all Soca RS, non-karst), and from KOLP to KUPI (Kolpa RS, karst). Other migration rates within akarst and between akarst and Vipava RS, ckarst, and Kolpa RS were not significant.

3.2.4 Historical gene flow and introgression

The f -branch method to detect all possible population trios using *P. phoxinus* as an outgroup identified four introgression events between a given population (Fig. 7b, x-axis) and branch b (y-axis). The strongest signal of introgression ($f_b=0.51$) was observed for Kolpa RS (group 1b) and RADU, while a strong signal was also detected between lineages of group 1c (encompassing ckarst, Sava RS, and Krka RS) and both Kolpa RS ($f_b=0.5$) and RADU ($f_b=0.37$), indicating shared ancestry. Within group 1c, the signals suggested gene flow between ckarst and KRKA ($f_b=0.22$).

Assessment of the optimal number of migration edges (m) using OptM revealed two migration events ($m=2$) optimal for TreeMix analysis as they were sufficient to explain more than 99.8% of the variance in the data (Fig. S3c). These two gene flow events indicated genetic exchange from ckarst to admix and from group 1a to SORA (Sava RS) (Fig. 7c). However, the TreeMix residuals showed that the model fit for $m=2$ may not fully capture certain aspects of the observed genetic data (see Fig. 7d), indicating the possibility of additional migration events, particularly between RADU and ckarst, Sava RS, and Krka RS, all belonging to group 1c. Analysis of genetic drift among populations showed high values for RADU (Fig. 7e).

3.2.5 Effective population size history

Stairway plot analysis revealed a reduction in N_e across all three groups, with distinct temporal patterns. Group 1a experienced a decline from 4,000 years ago, followed by rapid expansion from about 1,000 years ago, with stability maintained for the last 800 years (Fig. 8a). Group 1b showed a sharp decrease in N_e some 35,000 years ago, followed by a stable period, and then, 15,000 years ago, a subsequent rapid decline to the lowest level some 8,000–6,000 years ago, and a rapid recovery but to a comparatively lower level than prior to 35,000 years ago (Fig. 8c). In contrast Group 1c exhibited a more gradual, though still marked, decline in N_e from 20,000 years ago, reaching a low some 6,000–5,000 years ago, followed by a rapid recovery, though an ongoing decline began within the last 100 years (Fig. 8d).

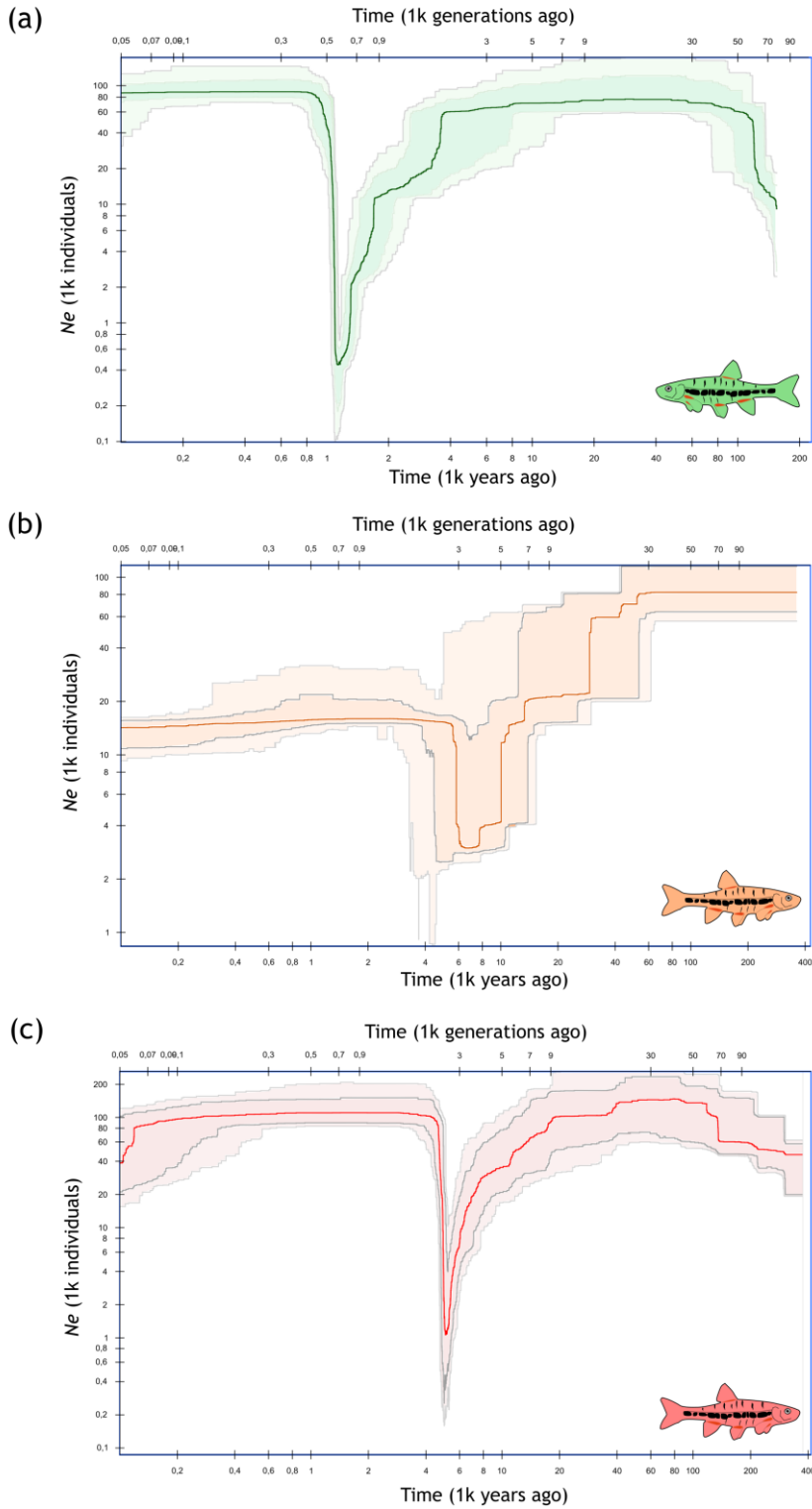


Fig. 8 Stairway plots showing the changes in effective population size (N_e). **(a)** Group 1a. **(b)** Group 1b. **(c)** Group 1c. Y-axis indicates N_e per 1,000 individuals. Lower x-axis shows the time in thousands of years before the present; upper x-axis shows the time in thousands of generations before the present.

4. Discussion

The present study aimed to understand how karstification has affected the population dynamics and diversity of the surface-dwelling minnow *P. lumaireul* over time, by comparing the past and present genetic connectivity within and outside karst regions (objective 1). Based on the mtDNA dataset, significant genetic divergence was found among three major groups (1a–c) across the NW Dinaric Karst and neighboring areas, consistent with previous mtDNA-based research (Palandačić et al., 2015, 2017, 2020; Reier, Kruckenhauser et al., 2022), and confirmed this structure with genome-wide SNP data. Two of the groups (1a and 1c) spanned both karst and non-karst river systems, with the third (1b) confined to the Dinaric Karst Kolpa RS. Significant genetic differentiation was found both among and within these three groups, with significant differences among the RSs. The robust structuring that exists among and within river systems was revealed by all analysis undertaken of the ddRAD datasets. Nevertheless, no notable differences were found among rivers connected through surface (non-karst areas) or subterranean (karst areas) connections (AMOVA, Table 2), revealing consistent structuring across sampling sites, irrespective of their linkage through either surface or subterranean connections. This finding suggests that factors beyond karst hydrology have played a crucial role in shaping the population structure and distribution patterns of *P. lumaireul*.

Ongoing gene flow within the karst region (subject of objective 2) is limited to a specific area (akarst) within the Ljubljana RS, with an historic admixture event detected between two genetically separate karst clusters (Adriatic akarst and Black Sea ckarst). These results indicate that the population structure of *P. lumaireul* does not completely reflect the hydrological network identified by classical tracing test methods, underpinning the potential of population genomic studies for provision of supplementary information on the type of hydrological connections in karst aquifers (e.g., Tobin et al., 2024).

Reier, Kruckenhauser et al. (2022) dated the divergence times of the six recognized mtDNA lineages of *P. lumaireul* (1a–f, *sensu* Palandačić et al., 2017) to the mid-Pliocene, though with the splits among 1a–c having low support (see also Reier, Bogutskaya et al., 2022). Thus, the Stairway plot analysis performed in the present study offers a more detailed picture of the population histories of the three major groups, and suggests that their evolutionary origins are relatively recent, all within the Pleistocene epoch (Fig. 8). Adriatic Sea group 1a emerged as the youngest (~200,000 years), while Black Sea groups 1b and 1c appear to have longer evolutionary histories (~400,000 years). In addition, the Black Sea groups exhibited a gradual decline in effective population size over tens of thousands of years, coinciding with the last cold period, the Würm glaciation (Heiri et al., 2014). During the Pleistocene–Holocene

transition, hydrological fluctuations, aligning with broader climatic changes, including cold-wet climates around 7,500–7,200 years and 5,800–5,200 years ago, as well as a dry phase approximately 6,000 years ago (Andrič et al., 2008; Heiri et al., 2014), seem to have further impacted the decline of N_e in the Black Sea groups. These findings align with previous studies suggesting the presence of multiple glacial and Pleistocene microrefugia in the Dinaric Karst region that have shaped the genetic structure of various resident freshwater taxa (e.g., Klobučar et al., 2013; Pârvulescu et al., 2013; Previšić et al., 2009). The Upper Sava River with its pre-Pleistocene origin (Bavec & Verbič, 2011), and corresponding to the range of group 1c, likely hosted several such microrefugia within its upper part, as observed for the sculpin *Cottus* spp. (Bravničar et al., 2021). This suggestion is in congruence with the patchy mtDNA haplotype network of group 1c observed in this study (Fig. 2b), supported further by the fine population structure within the (Upper) Sava RS (Fig. 4). Similarly, Pleistocene refugia may explain the presence of endemic invertebrate lineages in the Kolpa RS (e.g., Ivković & Plant, 2015; Klobučar et al., 2013; Trontelj et al., 2005), in accordance with a separate group identified in this RS also in the present study (1b). In contrast, the star-shaped haplotype network and haplotype sharing in group 1a (Fig. 2b) suggest a single mutual refuge, likely in the Adriatic region, with subsequent rapid dispersal towards the NW Dinaric Karst. This suggestion is supported by a recent bottleneck approximately 1,000 years ago (Fig. 8a), exclusively affecting group 1a and indicating a climatic event in the Adriatic region followed by rapid recovery or re-colonization. The impact of Pleistocene glaciations on the genetic structure of several other freshwater fish species has been observed in other karst areas (e.g., Day et al., 2014; Perea & Doadrio, 2015), where karstification likely influenced the genetic structure on a local scale. Regional studies at smaller scales complement broader genetic analyses, revealing distinctive local population structures influenced by independent histories of river systems within the same drainage basin (e.g., Chiba et al., 2015; Wu et al., 2016). As observed in several fish species, gene flow between river systems is limited (e.g., Hughes et al., 2012; Perea & Doadrio, 2015; Zbinden et al., 2023). In congruence, AMOVA showed that the majority of genetic variation detected in the present study is attributed to differences among river systems regardless of whether *P. lumaireul* inhabits karst or non-karst rivers. The hydrological network's specific characteristics define the population structure of the resident *P. lumaireul*. The observed genetic patterns indicate reduced connectivity between populations with fewer underground connections. For instance, there is a more pronounced fine-scale structure in Black Sea ckarst (with fewer underground connections) compared to greater connectivity in Adriatic akarst (with more underground connections). Simultaneously, a fine-scale structure similar to that found in

karst systems with few underground connections was identified along the tributaries of the Sava, suggesting that major rivers like the Sava could potentially act as barriers to the dispersal of *P. lumaireul*. Here, also the habitat preferences of *P. lumaireul* play a role, as it favors areas with slow-flowing water, such as near the shore (Frost, 1943; Ramler et al., 2017; Tack, 1940). Recent gene flow, as detected by BA3-SNPs (Mussmann et al., 2019), within the karst region is confined to a restricted area of the cluster akarst, supported further by all admixture analyses, which reveal genetic similarity and shared ancestry. Despite the absence of direct underground connections among the sampling sites within that area, fluctuations in water levels and extreme floods may have facilitated dispersal under specific circumstances (e.g., Zakšek et al., 2009), as observed previously in lowland rivers (e.g., Hughes, 2007). Gene flow identified in its sampling sites was in all cases unidirectional, originating from sampling site NANO in sinking river Pivka. This sinking river drains underground into both the Adriatic and Black Sea drainage basins and might have enabled *P. lumaireul* to traverse the basins, producing a distribution not observed in other freshwater fish species of the area (e.g., Bogutskaya & Zupančič, 2010; Bravničar et al., 2021; Ketmaier et al., 2004). Thus, as suggested previously (Palandačić et al., 2015), *P. lumaireul* may utilize underground connections for dispersal while other surface-dwelling fish species may not. The crossing of the Adriatic–Black Sea divide in the NW Dinaric Karst region has also been observed for subterranean fauna (e.g., Delić et al., 2022; Konec et al., 2016; Recknagel et al., 2023; Zakšek et al., 2009), indicating a correspondence between them and the distribution of the Adriatic *P. lumaireul* lineage, rather than epigeal fish fauna, and supporting the hypothesis of its subterranean dispersal ability. Further evidence for underground dispersal of *P. lumaireul* is seen in observations of this species in caves (Reier, Bogutskaya et al., 2022; personal observation PT) and while being surface-dwelling, *Phoxinus* exhibits morphological features shared with subtroglophile fish (Reier, Bogutskaya et al., 2022). In congruence, at least sporadic connectivity among sampling sites connected according to tracing tests (e.g., the underground connection from akarst to Vipava RS; underground connection within ckarst) is suggested by relatively low F_{ST} and D_{XY} values (Fig. 6), and signs of admixed ancestry (Fig. 4, 5). This suggestion is corroborated by extensive sharing of mtDNA haplotypes among karst populations even across river systems, demonstrated by this and previous research (Reier, Kruckenhauser et al., 2022). However, some of the possible underground pathways show no genetic link, potentially due to the quality of these water connections, which are passable for water but not for fish. On the other hand, while the genetic structure of *P. lumaireul* does not always align with classical tracing tests documented in Petrič et al. (2020), genetic tracing possibly uncovered additional connections not evident with

traditional methods, as in the akarst cluster. Such discordance between genetic and classical tracers has been also noted in other studies (Tobin et al., 2024).

The present study shows potential for tracing historical connections which no longer exist. Thus, historical introgression between group 1b (Kolpa) and the ancestor of group 1c, as well as between Ljubljana RS (specifically ckarst cluster) and Krka RS (Fig. 7b), hints at past river system connections, as suggested by Sket (2002) based on the distribution patterns of some subterranean fauna. A major admixture event between the karst groups akarst and ckarst indicates past gene flow, supported by TreeMix (Fig. 7a), with shared ancestry detected among sampling sites (Fig. 3, 4, 5). This population mixing might possibly have its origins in the redirection of the river Cerknjšica (CERK) in the late Pleistocene (Šušteršič et al., 2002). Despite no direct hydrological connections, historical gene flow between Adriatic RSs (group 1a) and SORA (Sava RS, 1c) is implied (Fig. 7b). Thus, a recent connection might have existed, further supported by these two systems sharing the same species (Trontelj et al., 2005).

In conclusion, genetic tracing offers valuable insights into the complex dynamics of karst hydrological systems and adds value to the traditional tracing tests performed by dye or salt. In particular, studies with extensive sampling and a genome-wide approach, possibly including several taxa, and a multidisciplinary perspective with a combination of geological and genetic data, are needed to better understand the demographic processes experienced by freshwater fauna in different karst landscapes. About 14% of the world's land surface is covered by karst, which occurs in different climatic zones and other varying conditions (Williams, 2008), preventing the generalization of the results of specific karst areas. Nevertheless, vulnerability is common to all of such areas, and thus deciphering population patterns, factors influencing them and challenges they face is crucial for effective conservation and management strategies in these environments. Thus, the minnow *P. lumaireul* seems to be a suitable organism for use as a genetic tracer in karst systems. Moreover, it exhibits a robust population structure across the NW Dinaric Karst and neighboring regions, indicating significant local adaptations to river systems. One of the groups—1c—is distributed only in the limited area of the Upper Sava (including Krka RS and part of Ljubljana RS) and has experienced a reduction in N_e over the last century. Thus, despite it being known as a widely distributed species, individual populations of *P. lumaireul* seem to be adapted to specific ecological conditions, with some factors (e.g., large rivers) preventing dispersal and making them susceptible to local extinction. The present study is, to the authors' knowledge, the first comprehensive investigation of the population structure and dispersal capabilities of a widely distributed surface-dwelling fish species within

a karstic hydrological network, and emphasizes the importance of conserving freshwater habitats within Dinaric Karst to protect its remarkable biodiversity.

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Author contributions

AP and PT conceptualized the study. AP, LK, MK and SR designed the research methodology. Laboratory work was performed by AW and SR. Data analysis was performed by SR with support from MK. The manuscript was initially drafted by SR, with help from AP. All authors participated in reviewing and editing the manuscript. SR did the visualization of data. AP, LK, MK and PT provided supervision throughout the project. Funding acquisition was led by AP and PT. All authors have read and agreed to the latest version of the manuscript.

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Chapter 3: Supplementary material

1. List of Tables

Table S1. All examined specimens with SRA (ddRAD) and GenBank (COI) accession numbers from this study and previous studies (Palandačić et al. 2015; Palandačić et al. 2017; Reier, Kruckenhauser et al., 2022). Sampling sites with river names, coordinates, and river systems are given. Table S1 is provided as Excel file.

Table S2. Sampling sites structured by major group, river system or cluster, and whether on karst or not, used for hierarchical AMOVA, with results reported in Table 2.

Major group		River system or cluster		Underground or surface river	
group 1a	HOTE	coast	OSPO	Underground (karst)	HOTE
	NANO		RIZA		LOGA
	PRED	soca	NADI		NANO
	RAKU		IDRI		PRED
	LOGA		SOCA		RAKU
	OSPO	vipava	BRAN		CERK
	RIZA		VIPA		RAKO
	NADI	akarst	HOTE		MALI
	IDRI		NANO		BLOS
	SOCA		PRED		CERJ
	BRAN		RAKU		RASC
	VIPA		LOGA		
group 1b	MOKR	admix	CERK	Surface (non-karst)	NADI
	KOLP		RAKO		KOKR
	KUPI		MALI		IZIC
group 1c	KOKR	ckarst	RASC		IDRI
	BOHI		CERJ		SOCA
	IZIC		BLOS		SORA
	SORA	lju	IZIC		TOJN
	TOJN		TOJN		BRAN
	KRKA	sava	KOKR		BOHI
	RASC		BOHI		VIPA
	CERJ		SORA		
	BLOS	krka	KRKA		
		radu	RADU		
		kolpa	MOKR		
			KOLP		
			KUPI		

2. List of Figures

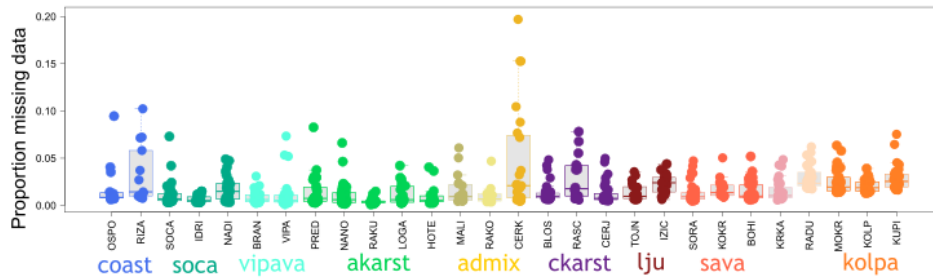


Fig. S1 Overview of proportion of missing data per individual for each population.

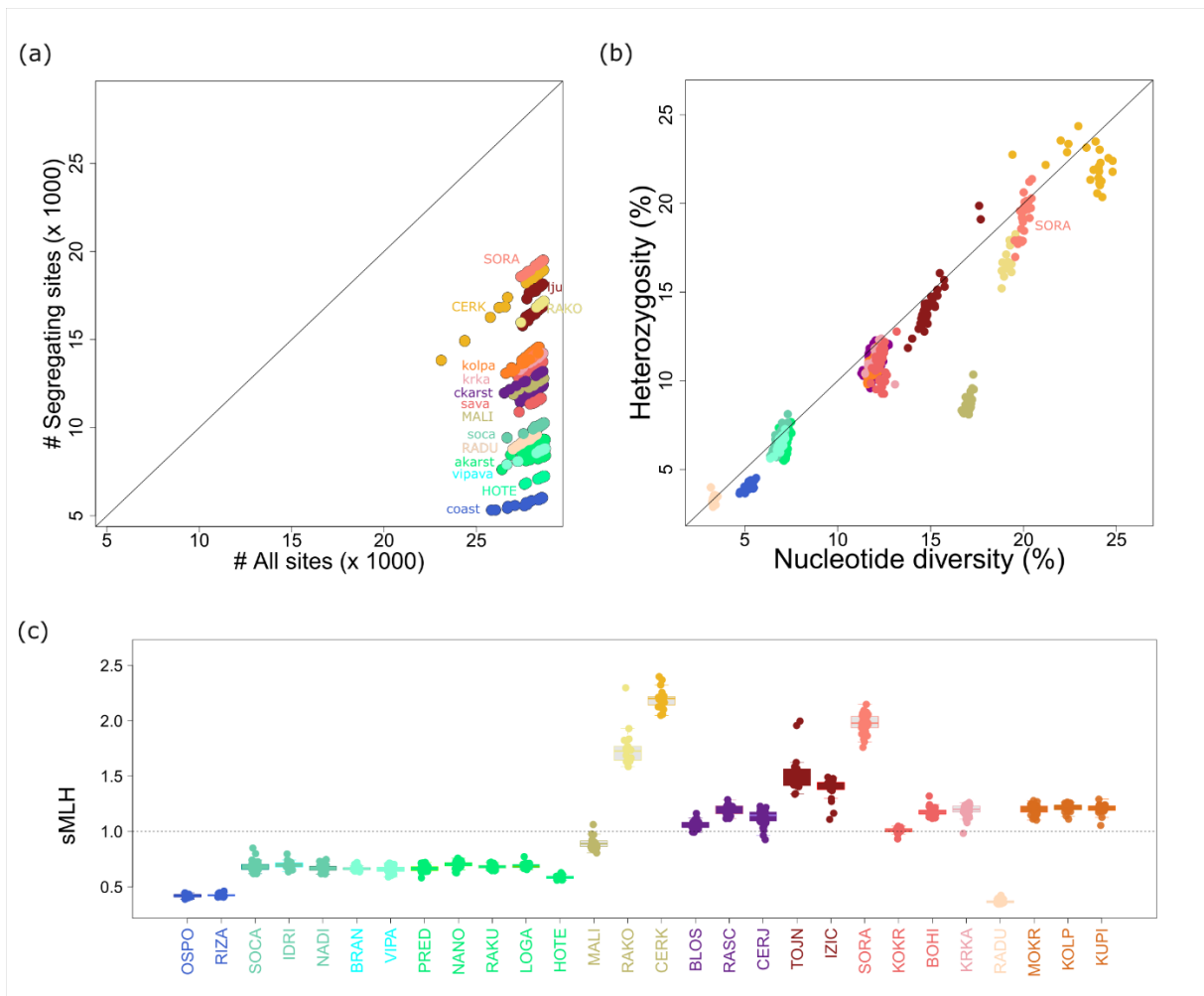


Fig. S2 Results of the various diversity analyses using the diversity dataset (28,764 linked SNPs). **(a)** Number of segregating sites vs. total number of sites, revealing that admix populations CERK and RAKO, along with group C populations SORA and lju, exhibit the largest proportion of segregating sites, while coast populations and HOTE display the smallest. **(b)** Percentage of heterozygosity vs. % nucleotide diversity. Again RADU has the smallest values, and CERK the largest. Samples in group A cluster together (excluding coast populations), indicating overall smaller values than groups B and C, which also cluster together. Admix populations RAKO, CERK, SORA, and lju have larger values, with MALI standing out because of its high level of nucleotide diversity compared to heterozygosity. **(c)** Standardized multi-locus heterozygosity (sMLH) barplots, where groups B and C exhibit larger values than group A. The smallest value is observed in RADU, while admix populations (especially CERK and RAKO) and SORA exhibit the largest values.

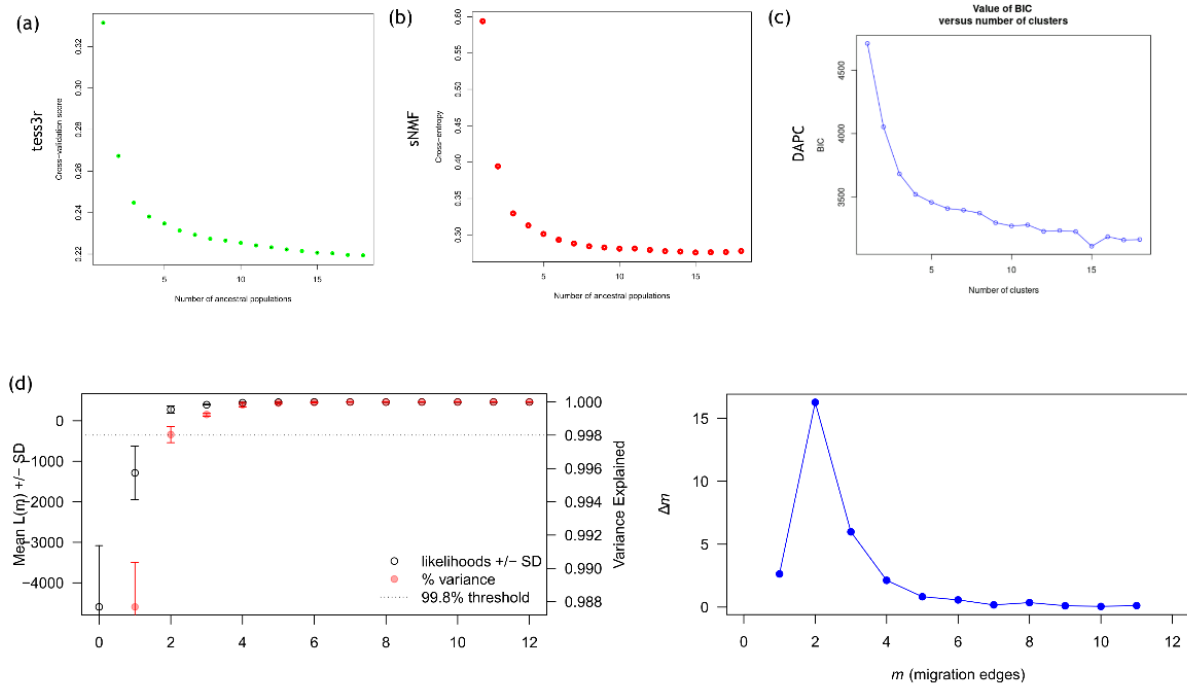


Fig. S3 Plot of cross-validation (CV) error from K=1 to K=18 for **(a)** tess3r and **(b)** sNMF. **(c)** Plot of BIC values of DAPC analysis from K=1 to K=18, revealing the lowest BIC value for K=15. **(d)** OptM output for TreeMix analysis. The plot on the left depicts the mean and standard deviation (SD) across 100 iterations for the composite likelihood $L(m)$ (left axis, black circles) and proportion of variance explained (right axis, red circles). The 99.8% threshold (horizontal dotted line) is recommended by Pickrell & Pritchard (2012). The plot on the right shows the second-order rate of change (Δm) across values of m , with $m=2$ being the chosen migration edge for the TreeMix analysis.

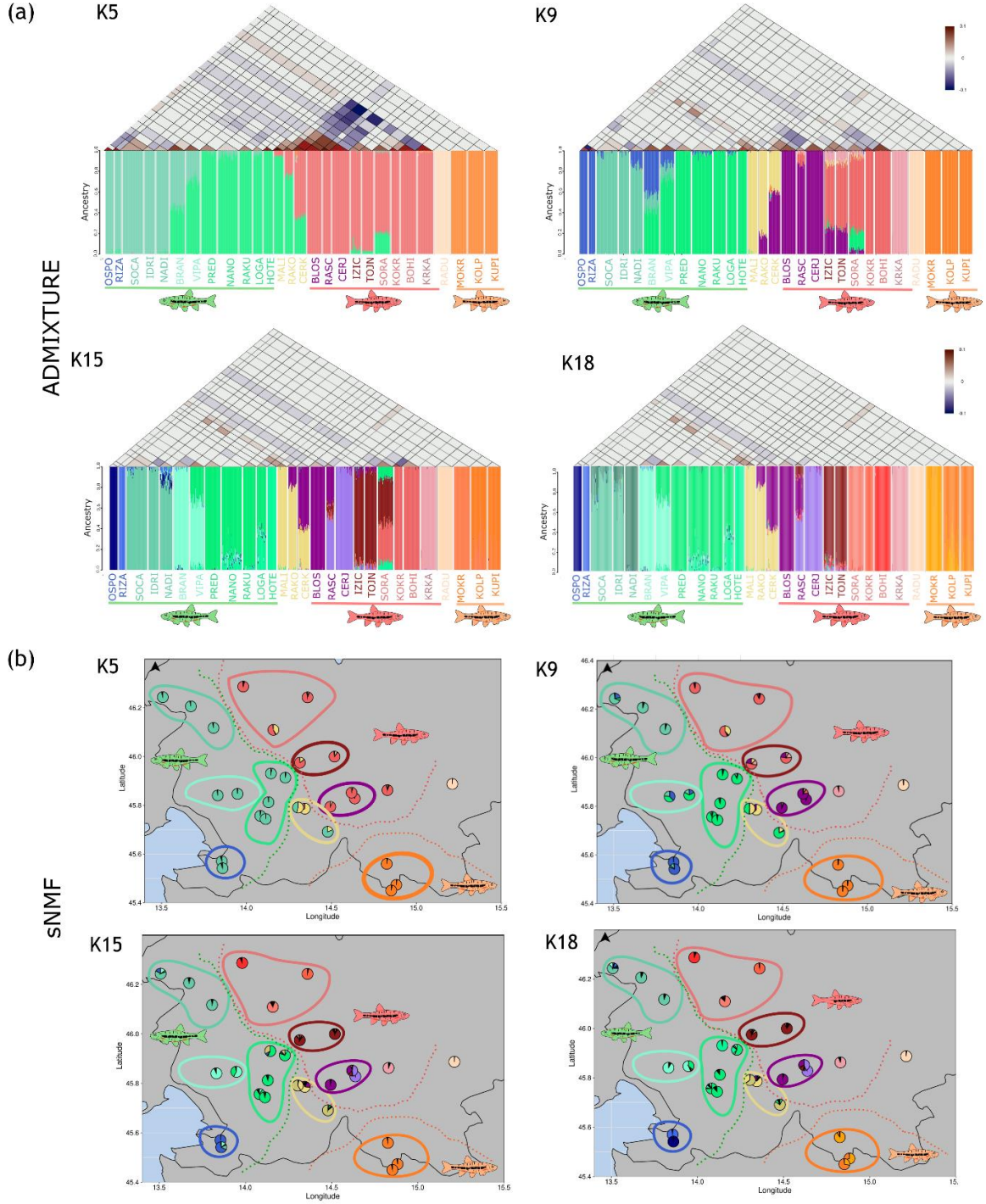


Fig. S4. Population genetic structure analysis using (a) ADMIXTURE and (b) sNMF. The colored bars in (a) and colored pie maps in (b) represent estimated admixture proportions, with K=5, K=9, K=15, and K=18 equivalent to the number of ancestries. (a) Pairwise correlations of residuals, as estimated by evalAdmix, are shown above and below the respective ADMIXTURE barplots, ranging from -0.1 to 0.1 on the color scale. (b) Dashed lines indicate the geographic separation of the three major groups identified in this study, further marked by colored fish icons. Respective river systems and clusters within river systems are circled.

KEY FINDINGS AND CONCLUDING REMARKS

The scientific work in this thesis represents a comprehensive study examining the phylogeographic patterns and dispersal abilities of epigean and subtroglophile freshwater fishes in a complex karst aquifer, covered across three chapters. Therefore, the findings presented here contribute significantly to our understanding of how organisms disperse in karst regions. The following outlines the key results of each chapter and their significance:

Chapter 1 provided a comprehensive examination of distribution patterns and mitochondrial genetic lineages among four genera of minnows dwelling in Dinaric Karst, emphasizing the crucial role of vicariance and dispersal in shaping species ranges. The study highlighted the varied distribution patterns, with *Phoxinus* species displaying broad ranges across multiple river drainages and interconnected poljes, while other genera showed more restricted distributions. This comparative analysis underscored the importance of protecting karst systems, particularly given the limited distribution of certain species to specific karst poljes and their restricted ability to occupy any other kind of water type, which could lead to their extinction in case of pollution of these environments. Additionally, the study revealed parallels between species of *Delminichthys*, *Phoxinellus* and Dinaric *Telestes* regarding their adaptation to karst poljes, which act as so-called freshwater islands within the karst landscape. Despite the divergence of species within all four genera coinciding with the onset of karstification, *Phoxinus* exhibited superior dispersal abilities compared to the other three genera, probably attributed to its biology, which allows it to disperse across river systems.

In the future, the comparative phylogeographic approach could be extended, with future comparative studies incorporating thorough morphological analysis within and across genera of subtroglophile and surface-dwelling fish species to identify physical adaptations related to their respective habitats. Further, by applying genomic approaches, researchers could identify genes associated with local adaptation (e.g., Bernatchez, 2016) to different habitats within karst environments. Such an integrative approach would offer a powerful tool to delve into the underlying mechanisms driving adaptations in fish species inhabiting karst environments.

In **Chapter 2**, the focus shifted to the species *Phoxinus lumaireul* and the study area of the north-western part of the Dinaric Karst in Slovenia. The genetic structure observed within the north-western Dinaric Karst and reference sampling sites suggests a cohesive hydrological network facilitating gene flow and population interchange based on mtDNA and one nuclear

gene. However, genetic barriers between certain sampling sites within the karst region indicate that hydrological connections alone cannot explain the observed patterns, suggesting a combination of historical factors and ongoing processes. Overall, this chapter provided first insights into the interplay of different intrinsic (biology of the species) and extrinsic (karst hydrology) factors in shaping population structure of a surface-dwelling species, however, further analysis should not rely mostly on mitochondrial sequences, but should also include wider nuclear data.

Thus, building on the results of **Chapter 2**, a more detailed approach was taken in **Chapter 3**, which included several additional sampling sites outside the karst area and genome-wide SNP data produced using ddRAD sequencing. Despite the assumption that karst hydrology would influence genetic patterns, as indicated in **Chapter 2**, the research revealed no significant genetic divergence among populations within and outside karst regions. This suggests that factors beyond hydrological karst features, such as habitat preferences and historical climate fluctuations, played essential roles in shaping population dynamics. Population history reconstructions indicated the Pleistocene epoch as a critical period shaping genetic diversity, with fluctuations in effective population size coinciding with climatic oscillations. These findings corroborate previous studies highlighting the presence of Pleistocene refugia and microrefugia in the Dinaric Karst region (e.g., Bravničar et al., 2021; Klobučar et al., 2013; Pârvulescu et al., 2013; Previšić et al., 2009). Further, despite being widely distributed, based on a fine-scale population structure, *P. lumaireul* exhibits distinct population clusters restricted to river systems, with limited gene flow between these river systems and clusters. This localized adaptation implies vulnerability to local extinction events, particularly in the face of anthropogenic disturbances and habitat degradation. This becomes particularly apparent when one considers the decline in the effective population size of one of the Black Sea groups (group 1c *sensu* Palandačić et al. (2017)) over the last 100 years. Previous studies that have looked at the distribution of genetic mitochondrial lineages of *P. lumaireul* have found no evidence of *P. lumaireul* group 1c outside the Upper Sava River system and north-western Dinaric Karst (e.g., Palandačić et al., 2015, 2017, 2020; Vučić et al., 2018). This means that if the decline of this genetic lineage continues, it could be lost.

In both, **Chapter 2** and **3**, *P. lumaireul* shows potential to add additional information as genetic tracer for studying karst aquifers due to its adaptability to both karstic and non-karstic environments. The genetic structure of *P. lumaireul* reflects a combination of past and ongoing hydrological connections, highlighting the utility of the population genetic structure of this

species in deciphering complex aquifer dynamics through time. Also, the absence of a recorded connection via classical tracing methods (e.g., dyes, salts) does not negate the possibility of animal dispersal. For instance, classical tracing tests cannot be conducted during floods, yet floods can facilitate animal dispersal (e.g., Zakšek et al., 2009). Further, positive water tracing results do not necessarily mean that dispersal is possible for animals (perhaps pores are too small or several other reasons). Thus, classical tracing methods and genetic tracers can complement each other to gain a more comprehensive understanding of a karst system. However, due to the high biodiversity in the Dinaric Karst, especially among cave-dwelling taxa, further taxa should definitely be included in such a study design in the future.

Nonetheless, this thesis underscored the exceptional nature of *Phoxinus*, exhibiting dispersal mechanisms not observed in other fish species, both epigean in previous studies (e.g., Bogutskaya & Zupančič, 2010; Bravničar et al., 2021; Ketmaier et al., 2004) and subtroglophiles in **Chapter 1**, within the Dinaric Karst landscape. Through a comprehensive analysis spanning three chapters, the dispersal ability of *P. lumaireul* was elucidated, showcasing its remarkable adaptability to the diverse environments found within the Dinaric Karst and beyond. **Chapter 1** highlighted the wide-ranging distribution of *Phoxinus* species and evolutionary lineages across multiple river drainages and interconnected poljes, indicative of its robust dispersal capability within the karst landscape. Furthermore, population genetic investigations of the species *P. lumaireul* in **Chapters 2** and **3** unveiled intricate structures of major genetic groups shaped by climatic oscillations, historical hydrological networks and ongoing processes. However, despite its widespread distribution, localized adaptations and limited gene flow between river systems render *P. lumaireul* populations susceptible to local extinction events. Compared to other genera under study, *Phoxinus* stands out for its ability to thrive in diverse habitats within the karst landscapes, highlighting the necessity of ongoing research and conservation endeavors to preserve its genetic diversity and population resilience.

Collectively, the three chapters of my thesis contribute to a comprehensive understanding of the complex dynamics of the Dinaric Karst ecosystem. By integrating genetic approaches with hydrological and geological knowledge from other disciplines, the thesis advances our knowledge of species distribution, population dynamics, and evolutionary processes in karst environments. The findings have implications for conservation efforts and underscore the need for holistic management strategies to preserve the unique biodiversity of karst regions amidst ongoing environmental changes.

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APPENDIX

Conference contributions and advanced training

During my PhD journey, I gave six talks at various conferences to share my ongoing research progress.

My PhD started towards the end of 2019, and due to the pandemic, my first opportunity to present my work occurred virtually at the **15th Network of Biological Systematics (NOBIS) meeting** on November 26, 2021. During this event, I discussed some preliminary findings from Chapter 2 of my thesis.

Following the submission of the paper for Chapter 2, I presented the outcomes at the **18. Tagung der Gesellschaft für Ichthyologie (GfI)** from November 17 to 20, 2022, hosted at the Natural History Museum of Vienna.

Subsequently, I presented the results of Chapter 1 at the **16th NOBIS meeting** held in Graz from November 25 to 26, 2022. Additionally, I participated in the **"Phylogeny and Diversification Rates" workshop**.

From May 26 to June 5, 2023, I attended the **Workshop on Molecular Evolution** at the Marine Biological Laboratory (MBL) in Woods Hole, MA, USA, where I received intensive training in bioinformatic tools from renowned experts, which helped me a lot to carry out the analyses and research for Chapter 3.

At the **XVII European Congress of Ichthyology** in Prague from September 4 to 8, 2023, I shared the findings from Chapter 2 and provided an overview of preliminary results from Chapter 3.

A detailed discussion on the preliminary findings of Chapter 3 took place during my presentation at the **Biodiversitätstage 2023** held at BOKU in Vienna from November 8 to 10, 2023. I won the "Biodiversitätspreis" for the best PhD talk.

Finally, I presented the final results of Chapter 3 on February 16, 2024, at the **Symposium of the Vienna Doctoral School of Ecology and Evolution (VDSEE)**. I won the prize for the best oral presentation in the category "Ecology".

Underground connections and paleohydrology rather than human translocations are causing the genetic complexity of *Phoxinus lumaireul* (Schinz, 1840) in the Slovenian Dinaric Karst

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The Dinaric karst on the Balkan Peninsula is known as a hot spot of European biodiversity. Karst landscapes exhibit specific underground drainage systems and complex connections to the surface water. The most common method to study underground hydrological connections in karst are tracing tests, whereby tracers are substances carried by water. In this study a biologically orientated approach of water tracing was applied, namely the analysis of the genetic structure of animal populations inhabiting a hydrological network with known underground connections. Thus, populations of the fish species *Phoxinus lumaireul* (Schinz, 1840) in the Slovenian Dinaric karst were densely sampled and analyzed for different genetic markers (*COI*, *cytb*, *RPS7*). Subsequently, the genetic structure was compared to the database of tracing tests and paleohydrology of the area in order to present support for three different scenarios: the genetic structure (1) is a consequence of the ongoing geneflow through underground connections, (2) reflects a past hydrological network, or (3) is an outcome of human translocations. Although none of the three possible scenarios can be unambiguously confirmed as main driver for shaping the current genetic composition, the population structure rather indicates a natural distribution, influenced by a mixture of current and past gene flow, than to human-mediated translocations.



***Phoxinus lumaireul* (Schinz, 1840) shifts the Adriatic-Black Sea drainage basin in the Dinaric Karst**

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The Dinaric Karst on the Balkan Peninsula is a hotspot of European biodiversity. Karst landscapes have underground water systems with complex connections to surface water. The most common method for investigating these underground connections in the karst are so-called "tracing tests" with tracers. Tracers are substances carried by water (e.g., dyes, salts) that provide information on flow direction, velocity and quantity. Another biologically oriented approach is the analysis of the genetic relationship of aquatic organisms inhabiting the investigated underground karst system. Here, the genetic population structure not only provides information about the water connections, but also reflects the ecology of the studied organisms. Thus, populations of the fish species *Phoxinus lumaireul* (Schinz, 1840) in the Slovenian Dinaric karst were densely sampled and analyzed for different genetic markers (*COI*, *cytb*, *RPS7*). Subsequently, the genetic structure was compared to known underground connections and the palaeohydrology of the area. Support for three different scenarios were evaluated: the genetic structure (1) is a consequence of the ongoing geneflow through underground connections, (2) reflects a past hydrological network, or (3) is an outcome of human translocations. Although none of the three possible scenarios can be unambiguously confirmed as main driver for shaping the current genetic composition, the population structure rather indicates a natural distribution, influenced by a mixture of current and past gene flow, than to human-mediated translocations. Moreover, the range of Adriatic and Black Sea haplotypes does not correspond to the Adriatic–Black Sea basin divide but is shifted northwards.

Near vs. wide: Comparative phylogeography of four fish genera in the Dinaric Karst

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The Dinaric Karst is a biodiversity hotspot, especially known for its speleofauna. However, there is also a high species richness of epigean organisms and the region is especially known for a diverse freshwater fish fauna, which is characterized by numerous endemics. The distribution patterns of fishes in the Dinaric karst can roughly be divided in two categories: (1) Genera, which include both widespread species, distributed in several river drainages in- and outside of the region, as well as species with ranges restricted to the Dinaric karst e.g., *Phoxinus*, *Telestes*. (2) Genera endemic to Dinaric karst (e.g. *Phoxinellus*, *Delminichthys*). The present study compares the distribution ranges and the biology of the two categories, including genera *Phoxinus*, *Delminichthys*, *Phoxinellus* and *Telestes*, based upon their mitochondrial genetic lineages. While *Delminichthys*, *Phoxinellus* and Dinaric *Telestes* have a similar lifestyle, specialized for dwelling in sinking streams in karst poljes, the genus *Phoxinus* is ubiquitous with numerous species common all over Europe. Yet, *Phoxinus* have an unusual distribution pattern, which, in contrast to other members of Leuciscidae, do not seem to be affected by current hydrological barriers, often traversing drainage and basin boundaries. One possible reason for that is the biology of *Phoxinus* that includes high migratory potential. In other three genera some limited ability to migrate underground is exhibited, but they cannot disperse along main river courses due to their specific adaptation for dwelling in the sinking streams of karst poljes.



Do surface-dwelling fish migrate underground? The case of the minnow *Phoxinus lumaireul* (Schinz, 1840) in the Dinaric Karst

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The Dinaric karst on the Balkan peninsula is a hotspot of European biodiversity. Karst landscapes are characterized by underground water systems with complex connections to surface water. The most common method for studying these underground connections in karst are so-called "tracer tests" with tracers (e.g., dyes, salts). Another more biologically oriented approach is the analysis of the genetic relationship of aquatic organisms inhabiting the investigated karst system. In this context, genetic population structure not only provides information on aquatic relationships, but also reflects the ecology of the studied organism. In a preliminary study of our working group, populations of the minnow *Phoxinus lumaireul* (Schinz, 1840) were densely sampled in the Slovenian Dinaric karst and analyzed for various genetic markers (COI, cytb, RPS7). This study indicated that the population structure of *P. lumaireul* suggests a natural distribution influenced by a mixture of current and past gene flow rather than human-mediated translocations. To test whether recent gene flow is due to subsurface migration through underground water connections in karst, in the present study we compare double digest restriction-site associated DNA (ddRAD-seq) data from populations that are connected underground. We aim to evaluate whether minnows use the underground water connections, or the karst landscape represents a barrier to (recent/ongoing) gene flow. The second objective is to evaluate the influence of the paleohydrology of the area, which changed rapidly and continuously due to karstification, on the present genetic structure. Finally, we discuss the divergence of the different genetic lineages of *P. lumaireul* in the Dinaric karst.



Subterranean Voyagers: Investigating Genetic Population Structure and Underground Migration of *Phoxinus lumaireul* (Schinz, 1840)

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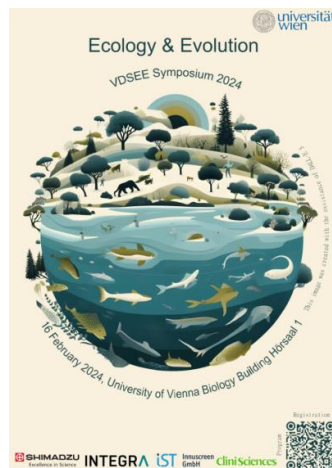
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The Dinaric karst on the Balkan peninsula harbors a remarkable variety of European biodiversity. Karst landscapes, known for intricate underground water systems interconnected with surface water, are prevalent in this region. A biologically-oriented approach to study these underground connections involves analyzing the genetic relationships among aquatic organisms residing in the investigated karst system. In this context, examining genetic population structure not only reveals aquatic relationships but also sheds light on the ecology of the studied organism.

In our preliminary study, we sampled populations of the minnow species *Phoxinus lumaireul* in the Slovenian Dinaric karst. Genetic markers (COI, cytb, RPS7) indicated a natural population structure influenced by historical and current gene flow, not human translocations. Our ongoing research employs genome-wide ddRAD-seq data to compare populations connected through underground pathways. We aim to determine if minnows use underground water connections or if karst acts as a gene flow barrier. Additionally, we assess how paleohydrology affected present genetic structure. The rapid hydrological changes in the Dinaric karst impact species distribution and adaptation.

Our findings shed light on the divergence of different genetic lineages of *P. lumaireul* in the Dinaric karst, providing valuable insights into the complex dynamics of biodiversity in this unique region. Understanding genetics and historical adaptations emphasizes karst landscape preservation, crucial due to susceptibility to human impact, highlighting the need for protective measures.



Exploring the Impact of Paleohydrology and Underground Migration on the Population Genetic Structure of the minnow *Phoxinus phoxinus* (Schinz, 1840)

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The Dinaric karst on the Balkan peninsula harbors a remarkable variety of European biodiversity. Karst landscapes, known for intricate underground water systems interconnected with surface water, are prevalent in this region. A biologically-oriented exploration of these subterranean connections involves analyzing the genetic relationships among aquatic organisms inhabiting the studied karst system. By examining genetic population structure, insights not only into aquatic relationships but also into the ecology of the organism under investigation can be gained.

In our preliminary study, we sampled several populations of the minnow species *Phoxinus phoxinus* in the Slovenian Dinaric karst. Genetic markers (*COI*, *cytb*, *RPS7*) revealed a natural population structure influenced by historical and current gene flow, rather than human translocations. Our ongoing research utilizes genome-wide ddRAD-seq data to compare populations linked through underground connections. Our goal is to determine whether minnows utilize underground water connections or if the karst serves as a barrier to gene flow. Furthermore, we examine how paleohydrology has shaped the current genetic structure. The rapid hydrological changes in the Dinaric karst impact species distribution and adaptation.

Our results shed light on the divergence of different genetic lineages of *P. phoxinus* in the Dinaric karst, providing valuable insights into the intricate dynamics of biodiversity in this unique region. Understanding genetics and historical adaptations underscores the importance of preserving karst landscapes, particularly due to their vulnerability to human impact, emphasizing the necessity for protective measures.