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

Susanne Reier¹ ² | Luise Kruckenhauser² ³ | Aleš Snoj⁴ | Peter Trontelj⁵ | Anja Palandačić¹ ⁵

¹First Zoological Department, Natural History Museum Vienna, Vienna, Austria
²Department of Evolutionary Biology, University of Vienna, Vienna, Austria
³Central Research Laboratories, Natural History Museum Vienna, Vienna, Austria
⁴Department of Animal Science, Biotechnical Faculty, University of Ljubljana, Domžale, Slovenia
⁵Department of Biology, Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia

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 trogloxenes, 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

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

© 2022 The Authors. *Ecohydrology* published by John Wiley & Sons Ltd.

Ecohydrology. 2022;15:e2449.

https://doi.org/10.1002/eco.2449
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 (Petric 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; Zaksék 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 & Sbordon, 2001).

Nevertheless, it has been suggested that some fish can use underground passages to migrate between isolated, sinking streams (Palandačić, Matschner, et al., 2012; Snoj et al., 2008; Zupančić, 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 (Zaksék 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; Teržič et al., 2014). There are five major RS in Slovenian Dinaric Karst corresponding, respectively, to the main rivers Ljubljanica, 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 trogloxenes, that is, surface species that occasionally enter caves and groundwater, where they are able to survive but not reproduce (Howarth & Moldovan, 2018). Trogloxenes 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., Musteth et al., 2007; Miró & Ventura, 2015; Knebelsberger 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 sampling sites

In concordance with several abiotic properties (e.g., tectonic, lithological, relief, climatic and edaphic), Slovenia can be divided into five zoo-geographic 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) Ljubljanica 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 non-native 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 (Palandačić 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 (Palandačić et al., 2017). Sequencing was performed at Microsynth (Balbach, 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 (Vilienë, 2007). The best-fit models (K2P+G4 for COI; GTR+I+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 were Phoxinus was present; red dots, sampling sites that were not inhabited by Phoxinus.
## Table 1. Phoxinus lumaireul sampling sites from Slovenia

| ID | Sampling site           | Drainage | Clade–group | Number Present Study | Palandačić et al. (2015, 2017) | Total |
|----|-------------------------|----------|-------------|----------------------|-----------------------------|-------|
| Slovenian Dinaric Karst                      |          |            |                  |                                |       |
| Ljubljanica 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 |
| LJJU | Ljubljanica 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 | Tojnice | 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)                    |          |            |                  |                                |       |
| 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 |
| 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 |
| 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 |
| Subpannonian Region                          |          |            |                  |                                |       |
| PTU | Drava | B | 1d | 0 | 5 | 5 |
| RAT | Ratkovski | B | 1d | 0 | 4 | 4 |
| SCA | Ščavnica | B | 1d | 0 | 7 | 7 |

(Continues)
TABLE 1  (Continued)

| ID | Sampling site | Drainage | Clade–group | Number |
|----|---------------|----------|-------------|--------|
|    |               |          |             | Present study | Palandači 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 (Palandači 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 (Hd), 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 nucleoli 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 (FST), among sampling sites within groups (FSC) and among groups (FCT), 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 Schöhnhuth 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, Mödder 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, α, 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. lumaireul* 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 sub-clades. 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 KRKS (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 (Hd), nucleotide diversity (\(\pi\)), 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 \(Hd = 0.85\), 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 + VIP (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 + KR) 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 \(Hd = 0.876\), 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 (\(Hd = 0.84; \pi = 0.0061; S = 99\); 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 KRKS 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 KRKS 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 (\(Hd = 0.86; \pi = 0.00362\)) 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 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 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   | $H_d$ | $\pi$  | $S$  | $k$  | 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** |     |       |        |      |      |                  |
| **Ljubljanica river system (LRS)** |     |       |        |      |      |                  |
| 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              |
| **Reka river system (RRS)** |     |       |        |      |      |                  |
| MRZL             | 4   | 0.668 | 0.00047| 3    | 0.81053 | 29               |
| **Vipava river system (VRS)** |     |       |        |      |      |                  |
| PRED             | 6   | 0.617 | 0.00073| 6    | 1.27094 | 39               |
| VIPA             | 5   | 0.782 | 0.00106| 5    | 1.84615 | 12               |
| **Kolpa river system (KORS)** |     |       |        |      |      |                  |
| KOLP             | 17  | 0.933 | 0.00146| 20   | 2.54637 | 32               |
| MOKR             | 8   | 0.815 | 0.00112| 11   | 1.94474 | 34               |
| **Krka river system (KRRS)** |     |       |        |      |      |                  |
| 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: $\pi$, nucleotide diversity; $k$, mean number of pairwise differences; $H_d$, haplotype diversity; N, number of haplotypes; S, number of polymorphic sites.
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 MALL. SDK-C4 contained the north-eastern LRS BLOS, CERK, RASC, TOJN, IZIC and LJU. SDK-C5 included the two sampling sites of KRRS 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).
P ≤ 0.0001. Most (79.94%) of the variation was among clusters, with only 14.97% within sampling sites (P ≤ 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 krkæ*, Krka River in Croatia) split from the remaining clades 1–5, 14 and 15. The divergence of clade 5 (*Phoxinus csikî*) 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.
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   | $Hd$ | $\pi$  | $S$ | $k$   | 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** |      |      |        |     |       |                  |                    |
| Ljubljanica 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  | 22.83333| 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: $\pi$, 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 Ljubljanica 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-1d 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 minnows 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, Koša 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.
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 Ljubljanica, 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 (Pandalác, et al., 2015; Vučić et al., 2018), most frequently in karst areas, where Pandalác 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, HOTJ and the springs of Ljubljanica 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 (Bravnicar 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, Bravnicar et al., 2021; Hucho hucu, Snoj et al., 2022; and Thymallus thymallus [unpublished data]) and in invertebrate species (Kolpa: Trontelj et al., 2005; Klobočar 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 (Klobočar et al., 2013). Likewise, specific water conditions, where karst streams function as ‘freshwater islands’, are thus considered an isolating factor, proposed also in Previšič 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 (Marčič 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; Knebelberger 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 (Hd = 0.815) and also exhibits three unique haplotypes, findings that are incongruent with a recently introduced population. Similarly, anthropogenic influences might explain the heterogenous 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 Ljubljanica (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 populations in the spring area of Ljubljanica (TOJN) and that was followed by stocking. 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.

**ACKNOWLEDGEMENTS**

This study was funded by the Austrian Science Fund (FWF; project no. I 4131-B25). The work of PT was supported through the Slovenian Research Agency project no. N1-0096. We thank Sandra Kirchner for assistance during field trips, Alexandra Wanka for assistance in the laboratory and Jermom Bravničar for providing fish samples. We also thank Nina Bogutskaya and Elisabeth Haring for valuable discussions. Furthermore, we thank Iain Wilson for improving the manuscript. Freshly collected fish were acquired under permission no. 3420-30/2017/9 of the Slovenian Ministry of Agriculture, Forestry and Food.

**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.

**ORCID**

Susanne Reier [https://orcid.org/0000-0001-6839-7137](https://orcid.org/0000-0001-6839-7137)

Luise Kruckenhauser [https://orcid.org/0000-0001-8708-4347](https://orcid.org/0000-0001-8708-4347)

Ales Snoj [https://orcid.org/0000-0003-4708-0370](https://orcid.org/0000-0003-4708-0370)

Peter Trontelj [https://orcid.org/0000-0003-4057-6912](https://orcid.org/0000-0003-4057-6912)

Anja Palandačić [https://orcid.org/0000-0002-4555-5240](https://orcid.org/0000-0002-4555-5240)

**REFERENCES**

Bandelt, H. J., Forster, P., & Rohl, A. (1999). Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution*, 16(1), 37–48. [https://doi.org/10.1093/oxfordjournals.molevol.a026036](https://doi.org/10.1093/oxfordjournals.molevol.a026036)

Behrens-Chapuis, S., Herder, F., Esmaili, H. R., Freyhof, J., Hamidian, N. A., Özuluğ, M., Šanda, R., & Geiger, M. F. (2015). Adding nuclear rhodopsin data where mitochondrial COI indicates discrepancies—Can this marker help to explain conflicts in cyprinids? *DNA Barcodes*, 3(1), 187–199.

**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 Ljubljanica 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 (Habić, 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.
Halse (Eds.), Cave Ecology (pp. 41–67). Springer International Publishing. https://doi.org/10.1007/978-3-319-98852-8_4

Humphreys, W. F. (2009). Hydrogeology and groundwater ecology: Does each inform the other? Hydrogeology Journal, 17(1), 5–21. https://doi.org/10.1007/s10040-008-0349-3

Imoto, J. M., Saitoh, K., Sasaki, T., Yonezawa, T., Adachi, J., Kartavtsev, Y. P., Miyata, M., Nishida, M., & Hanzawa, N. (2013). Phylogeny and biogeography of highly diverged freshwater fish species (Leuciscinae, Cyprinidae, Teleostei) inferred from mitochondrial genome analysis. Gene, 514(2), 112–124. https://doi.org/10.1016/j.gene.2012.10.019

Ivković, M., & Plant, A. (2015). Aquatic insects in the Danarides: Identifying hotspots of endemism and species richness shaped by geological and hydrological history using Empididae (Diptera). Insect Conservation and Diversity, 8(4), 302–312. https://doi.org/10.1080/17550998.2015.1068647

Kalyaanamoorthy, S., Minh, B. Q., Wong, T. K. F., von Haeseler, A., & Warnow, T. (2015). FastTree2–approximately maximum-likelihood trees fast. Molecular Biology and Evolution, 32(1), 203–214. https://doi.org/10.1093/molbev/msv070

Katoh, K., & Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. Molecular Biology and Evolution, 30(4), 772–780. https://doi.org/10.1093/molbev/mss010

Kaufman, G., Mayaud, C., Kogovšek, B., & Gabrovšek, F. (2020). Understanding the temporal variation of flow direction in a complex karst system (Planinska Jama, Slovenia). Acta Carsoologica, 49(2–3), 213–228. https://doi.org/10.3986/ac.v49i2-3.7373

Ketmaier, V., Bianco, P. G., Coboli, M., Krivokapic, M., Caniglia, R., & De Matteis, A. (2004). Molecular phylogeny of two lineages of Leuciscinae cyprinids (Telestes and Scardinius) from the peri-Mediterranean area based on cytochrome b data. Molecular Phylogenetics and Evolution, 32(3), 1061–1071. https://doi.org/10.1016/j.ympev.2004.04.008

Klobučar, G. I. V., Podnar, M., Jelić, M., Franjević, D., Faller, M., Štambuk, A., … Maguire, I. (2013). Role of the Dinaric karst (western Balkans) in shaping the phylogeographic structure of the threatened crayfish Astrotapobamus torrentium. Freshwater Biology, 58(6), 1089–1105. https://doi.org/10.1111/twb.12110

Knebelberger, T., Dunz, A. R., Neumann, D., & Geiger, M. F. (2015). Molecular diversity of German freshwater fishes and lampreys assessed by DNA barcoding. Molecular Ecology Resources, 15(3), 562–572. https://doi.org/10.1111/1755-0998.12322

Konec, M., Delic, T., & Trontelj, P. (2016). DNA barcoding sheds light on hidden subterranean boundary between Adriatic and Danubian drainage basins: Subterranean drainage basins elucidated by DNA barcoding. Ecology Hydrology, 9(7), 1304–1312. https://doi.org/10.1002/eco.1727

Krystufek, B., Buzan, E. V., Hutchinson, W. F., & Hänfling, B. (2007). Phylogeography of the rare Balkan endemic Martinos vole, Dinaromys bogdanoi, reveals strong differentiation within the western Balkan Peninsula. Molecular Ecology, 16(1), 1221–1232. https://doi.org/10.1111/j.1365-294X.2007.03235.x

Kumar, S., Stecher, G., Li, M., Knys, C., & Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. Molecular Biology and Evolution, 35(6), 1547–1549. https://doi.org/10.1093/molbev/msy096

Leigh, J. W., & Bryant, D. (2015). POPART: Full-feature software for haplotype network construction, Methods in Ecology and Evolution, 6(9), 1110–1116. https://doi.org/10.1111/2041-210X.12410

Marčić, Z., Buj, I., Duplič, A., Čaleta, M., Mustafić, P., Zanella, D., Zupančič, P., & Mrakovčič, A. (2012). Morphological comparison of Delminichthys ghetalídi (Steindachner, 1882), D. adspersus (Heckel, 1843), D. adveniosis (Zupančič & Bogutskaya, 2002) and D. kravensisi (Zupančič & Bogutskaya, 2002), endemic species of the Dinaric Karst. Croatia. Journal of Applied Ichthyology, 33(2), 256–262. https://doi.org/10.1111/j.1365-294X.2007.03235.x

Mustafić, P., Buj, I., Opašić, M., Zanella, D., Marčić, Z., Čaleta, M., Štandar, R., Horvatić, S., & Mrakovčič, A. (2017). Morphological comparison of Delminichthys ghetalídi (Steindachner, 1882), D. adspersus (Heckel, 1843), D. adveniosis (Zupančič & Bogutskaya, 2002) and D. kravensisi (Zupančič & Bogutskaya, 2002), endemic species of the Dinaric Karst. Croatia. Journal of Applied Ichthyology, 33(2), 256–262. https://doi.org/10.1111/j.1365-294X.2007.03235.x

Palandačić, A., Bonacci, O., & Snaj, A. (2012). Molecular data as a possible tool for tracing groundwater flow in karst environment: Example of Delminichthys adspersus in Dinaric karst system: Fish as groundwater tracers in karst. Ecohydrology, 5(6), 791–797. https://doi.org/10.1002/eco.269

Palandačić, A., Bravničar, J., Zupančič, P., Štandar, R., & Snaj, A. (2015). Molecular data suggest a multispecies complex of Phoxinus (Cyprinidae) in the Western Balkan Peninsula. Molecular Phylogenetics and Evolution, 92, 118–123. https://doi.org/10.1016/j.mpev.2015.05.024

Palandačić, A., Knuckenhauser, L., Ahnelt, H., & Mikschi, E. (2020). European minnows through time: Museum collections aid genetic assessment of species introductions in freshwater fishes (Cyprinidae: Phoxinus species complex). Heredity, 124(3), 410–422. https://doi.org/10.1038/s41437-019-0292-1

Palandačić, A., Matschner, M., Zupančič, P., & Snaj, A. (2012). Fish migrate underground: The example of Delminichthys adspersus (Cyprinidae). Molecular Ecology, 21(7), 1658–1671. https://doi.org/10.1111/j.1365-294X.2012.05507.x

Palandačić, A., Naseka, A., Ramler, D., & Ahnelt, H. (2017). Contrasting morphology with molecular data: An approach to revision of species complexes based on the example of European Phoxinus (Cyprinidae). BMC Evolutionary Biology, 17(1), 1–17. https://doi.org/10.1186/s12862-017-1032-x

Perea, S., Bähme, M., Zupančič, P., Freyhof, J., Štandar, R., Özuluğ, M., Abdoli, A., & Doadrio, I. (2010). Phylogenetic relationships and biogeographical patterns in Circum-Mediterranean subfamily Leuciscinae (Teleostei, Cyprinidae) inferred from both mitochondrial and nuclear data. BMC Evolutionary Biology, 10(1), 1–27. https://doi.org/10.1186/1471-2148-10-265

Petrić, M., Ravbar, N., Gostiničar, P., Krsnik, P., & Gacin, M. (2020). GIS database of groundwater flow characteristics in carbonate aquifers: Tracer test inventory from Slovenian karst. Applied Geography, 118, 102191. https://doi.org/10.1016/j.apgeog.2020.102191

Pičanec, T., & Culver, D. C. (2007). Epikarst communities: biodiversity hotspots and potential water tracers. Environmental Geology, 53(2), 265–269. https://doi.org/10.1007/s00254-007-0640-y

Plević, M., Schnitzler, J., Kuzić, M., Graf, W., Ibrimihi, H., Kerovec, M., & Pauls, S. (2014). Microscale vicariance and diversification of Western Balkan caddisflies linked to karstification. Freshwater Science, 33(1), 250–262. https://doi.org/10.1086/674430

QGIS Development Team. (2009). QGIS Geographic Information System. Open Source Geospatial Foundation. Retrieved from http://qgis.org
Verovnik, R., Sket, B., & Trontelj, P. (2004). Phylogeography of subterranean and surface populations of water lice Asellus aquaticus (Crustacea: Isopoda): PHYLOGEOGRAPHY OF ASELLUS AQUATICUS. Molecular Ecology, 13(6), 1519–1532. https://doi.org/10.1111/j.1365-294X.2004.02171.x

Verovnik, R., Sket, B., & Trontelj, P. (2005). The colonization of Europe by the freshwater crustacean Asellus aquaticus (Crustacea: Isopoda) proceeded from ancient refugia and was directed by habitat connectivity: COLONIZATION OF EUROPE BY ASELLUS AQUATICUS. Molecular Ecology, 14(14), 4355–4369. https://doi.org/10.1111/j.1365-294X.2005.02745.x

Villesen, P. (2007). FaBox: An online toolbox for fasta sequences. Molecular Ecology Notes, 7(6), 965–968. https://doi.org/10.1111/j.1471-2229.2007.01821.x

Vučić, M., Jelić, D., Žutić, P., Grandjean, F., & Jelić, M. (2018). Distribution of Eurasian minnows (Phoxinus: Cypriniformes) in the Western Balkans. Knowledge & Management of Aquatic Ecosystems, (419), 11. https://doi.org/10.1051/kmae/2017051

Warnock, R. C. M., Yang, Z., & Donoghue, P. C. J. (2017). Testing the molecular clock using mechanistic models of fossil preservation and molecular evolution. Proceedings of the Royal Society B: Biological Sciences, 284(1857), 20170227. https://doi.org/10.1098/rspb.2017.0227

Wickham, H. (2016). ggplot2: Elegant Graphics for Data Analysis (2nd ed.). Springer International Publishing: Imprint: Springer. https://doi.org/10.1007/978-3-319-24277-4

Zakšek, V., Konc, M., & Trontelj, P. (2018). First microsatellite data on Proteus anguinus reveal weak genetic structure between the caves of Postojna and Planina. Aquatic Conservation: Marine and Freshwater Ecosystems, 28(1), 241–246. https://doi.org/10.1002/aqc.2822

Zakšek, V., Sket, B., Gottstein, S., Franjević, D., & Trontelj, P. (2009). The limits of cryptic diversity in groundwater: Phylogeography of the cave shrimp Troglocaris anophthalmus (Crustacea: Decapoda: Atyidae). Molecular Ecology, 18(5), 931–946. https://doi.org/10.1111/j.1365-294X.2008.04061.x

Žganec, K., Lunko, P., Stroj, A., Mamos, T., & Grabowski, M. (2016). Distribution, ecology and conservation status of two endemic amphipods, Echinogammarus acarinatus and Fontogammarus dalmitinus, from the Dinaric karst rivers, Balkan Peninsula. Annales de Limnologie - International Journal of Limnology, 52, 13–26. https://doi.org/10.1051/limn/2015036

Zogaris, S., Economou, A. N., & Dimopoulos, P. (2009). Ecoregions in the southern Balkans: Should their boundaries be revised? Environmental Management, 43(4), 682–697. https://doi.org/10.1007/s00267-008-9243-y

Zupančič, P. (2008). Rjetic in ugrozene slatkovodne ribe jadranskog slijeva Hrvatske, Slovenije i Bosne i Hercegovine (Rare and endangered freshwater fishes of Croatia, Slovenia and Bosnia and Hercegovina). AZV, Dolsko.

SUPPORTING INFORMATION

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

How to cite this article: Reier, S., Kruckenhauser, L., Snoj, A., Trontelj, P., & Palandachič, A. (2022). The minnow Phoxinus lumiaire (Leuciscidae) shifts the Adriatic–Black Sea basin divide in the north-western Dinaric Karst region. Ecology Hydrology, 15(6), e2449. https://doi.org/10.1002/eco.2449