

Article

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

Susanne Reier ^{1,2,*} , Nina Bogutskaya ¹ and Anja Palandačić ^{1,3}

¹ First Zoological Department, Vienna Museum of Natural History, Burgring 7, 1010 Vienna, Austria; nina.bogutskaya@nhm-wien.ac.at (N.B.); anja.palandacic@nhm-wien.ac.at (A.P.)

² Department of Evolutionary Biology, University of Vienna, Djerassiplatz 1, 1030 Vienna, Austria

³ Department of Biology, Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, 1000 Ljubljana, Slovenia

* Correspondence: susanne.reier@nhm-wien.ac.at

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 epigeal 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 epigeal 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 overflows 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 troglone (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 sympleiomorphies.

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. adsper-*

sus [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 Krbavsko 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.

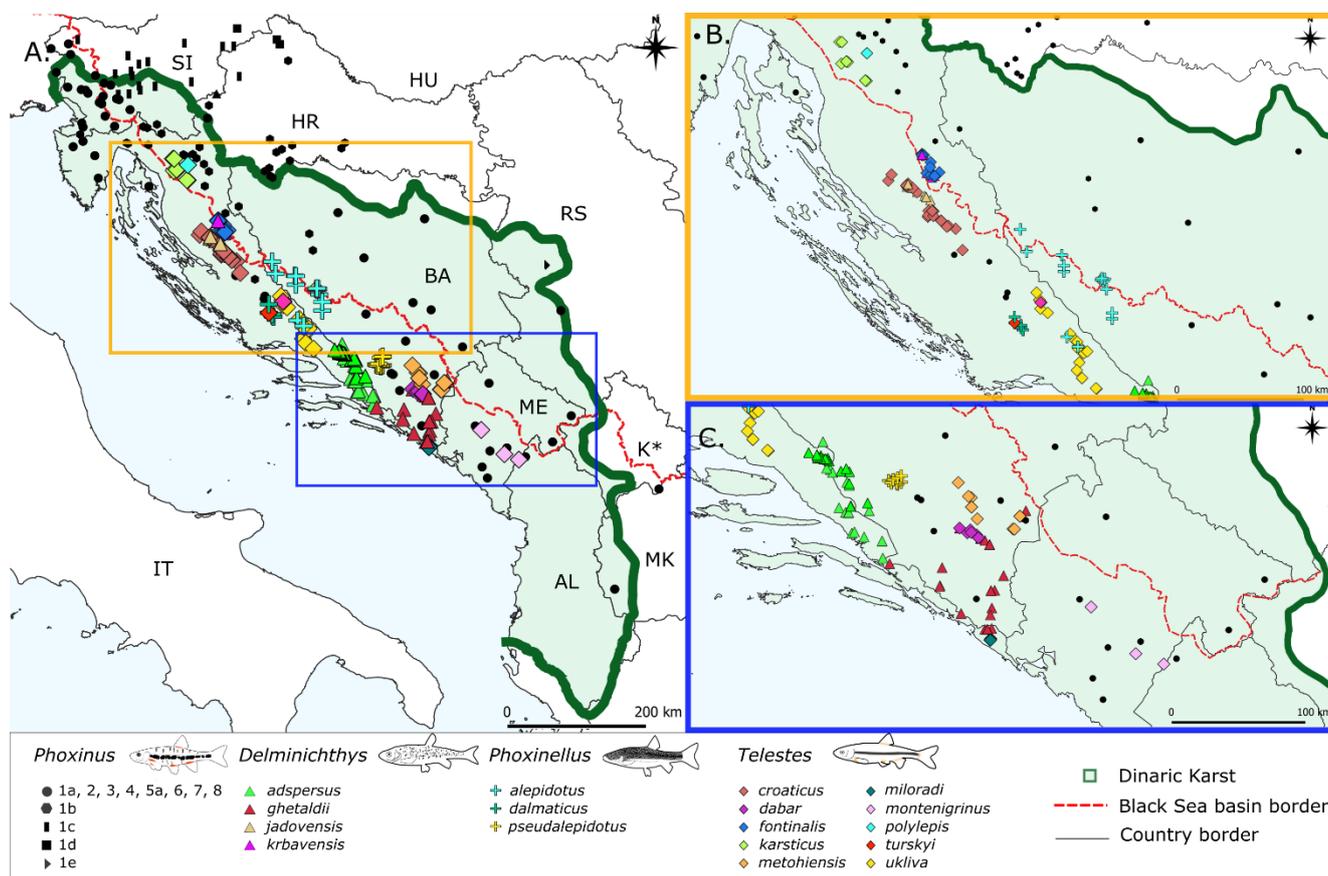


Figure 1. Distribution data of four fish genera *Phoxinus*, *Delminichthys*, *Phoxinellus* and *Telestes* in Dinaric Karst. *Phoxinus* is shown in black, while *Delminichthys*, *Phoxinellus* and *Telestes* are presented in color. (A) Overview of Dinaric Karst (B) Enlargement of the northern part of Dinaric 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 transverse 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.

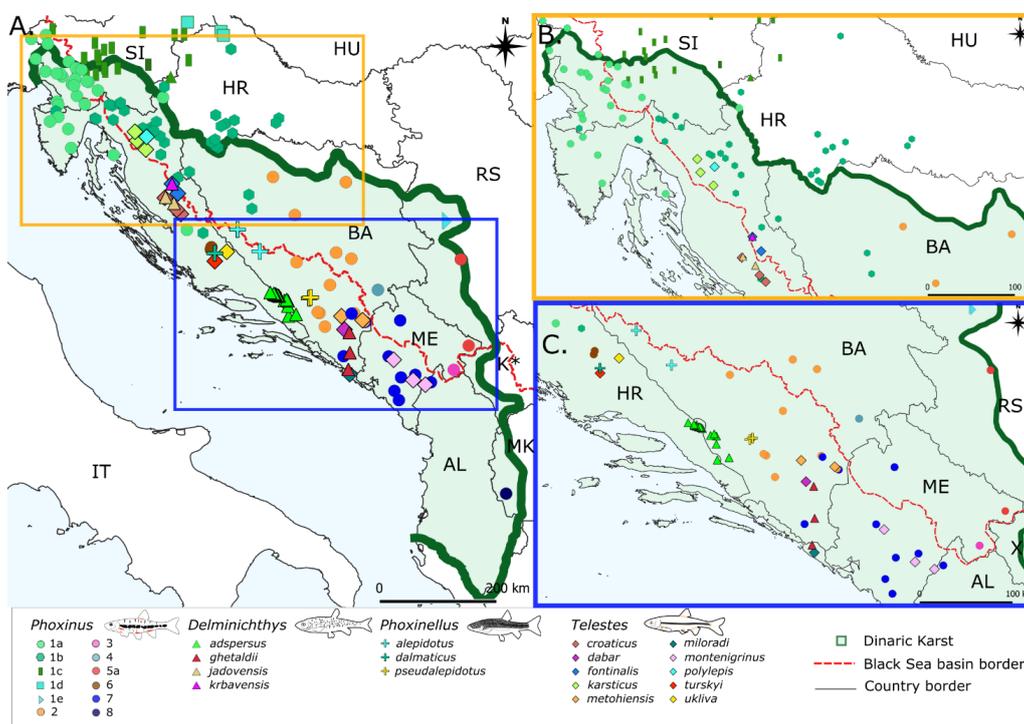


Figure 2. Distribution of mitochondrial (mt) lineages of the four fish genera *Phoxinus*, *Delminichthys*, *Phoxinellus*, *Telestes* in Dinaric Karst. In *Phoxinus*, not all lineages and sub-lineages represent valid species, and thus, they are encoded according to Palandačić et al. [37] (1a–1e, 2–4, 5a, 6–8). In the other three genera, *Deliminichthys*, *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.

For most analyses performed in this study, the species of four fish genera *Phoxinus*, *Delminichthys*, *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 *Delminichthys*, *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>Delminichthys</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, *Delminichthys*, *Phoxinellus* and *Telestes*, the mt lineages correspond to species, and thus, species names were used.

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 (Hd), 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 barbatus* (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) v4.2 × 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%.

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.

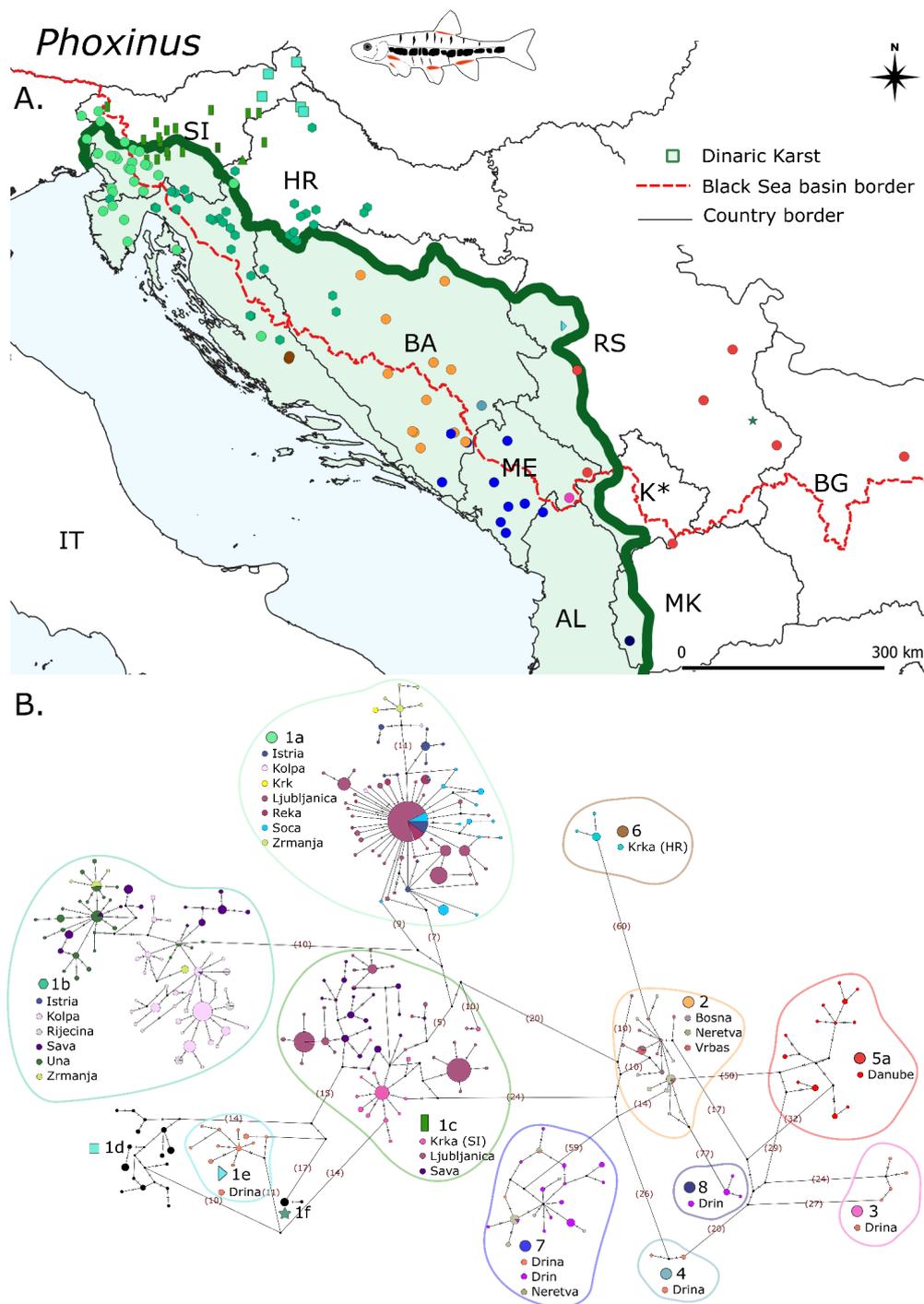


Figure 3. Distribution map and haplotype network (based on cytochrome *b* fragment) of *Phoxinus* mitochondrial (mt) lineages. The lineages are encoded following Palandačić et al. [37]. **(A)** Distribution of different mt (sub-)lineages of *Phoxinus* in Dinaric Karst and adjacent areas. Dinaric Karst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

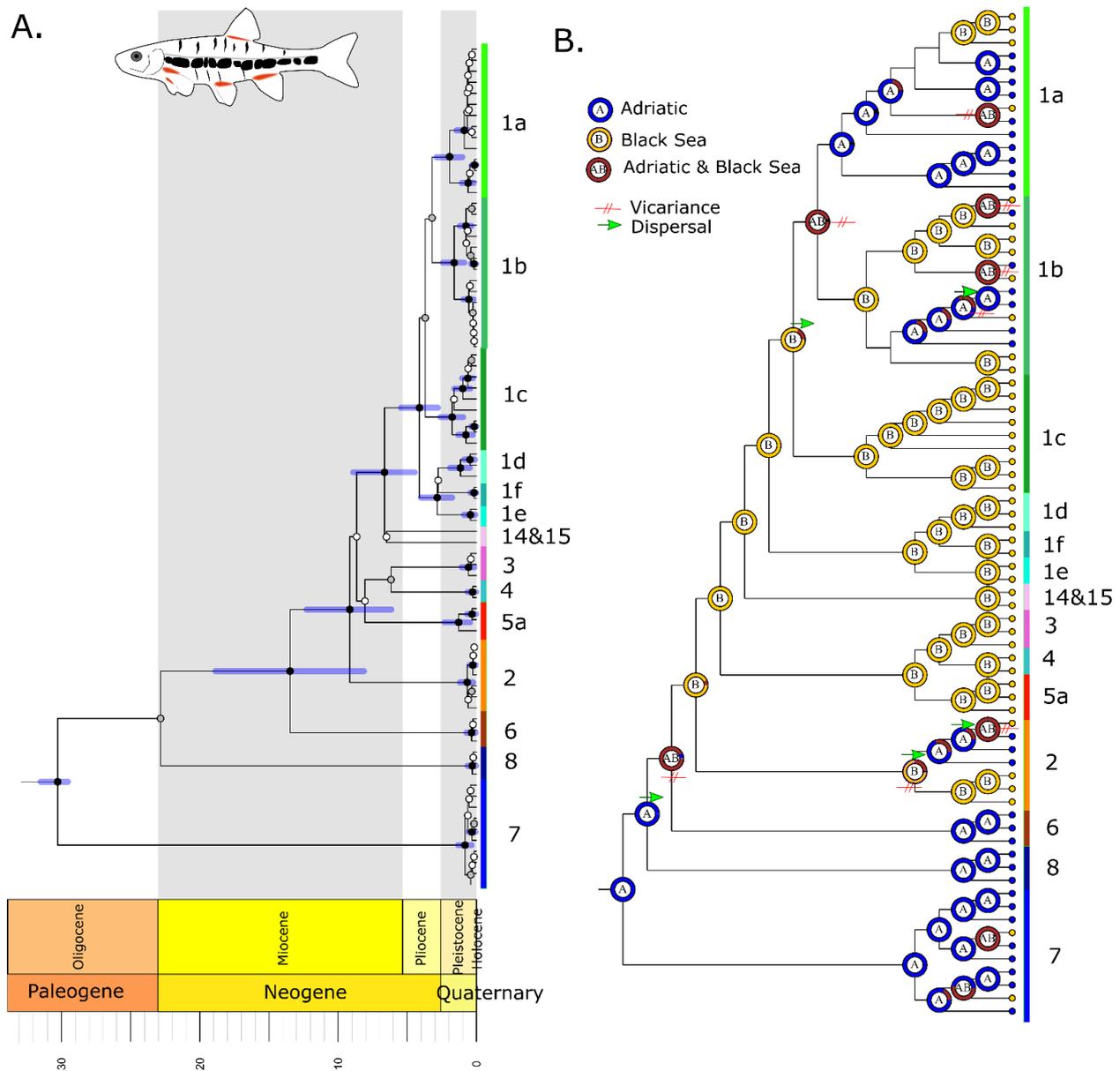


Figure 4. Divergence time estimates and reconstruction of ancestral areas (RASP) analyses of Balkan *Phoxinus* based on the cytochrome *b* fragment. **(A)** Divergence time estimates, calculated in BEAST [77], between mt lineages 1(a–f) and 8, 14 and 15, encoded following Palandačić et al. [37]. Time 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.

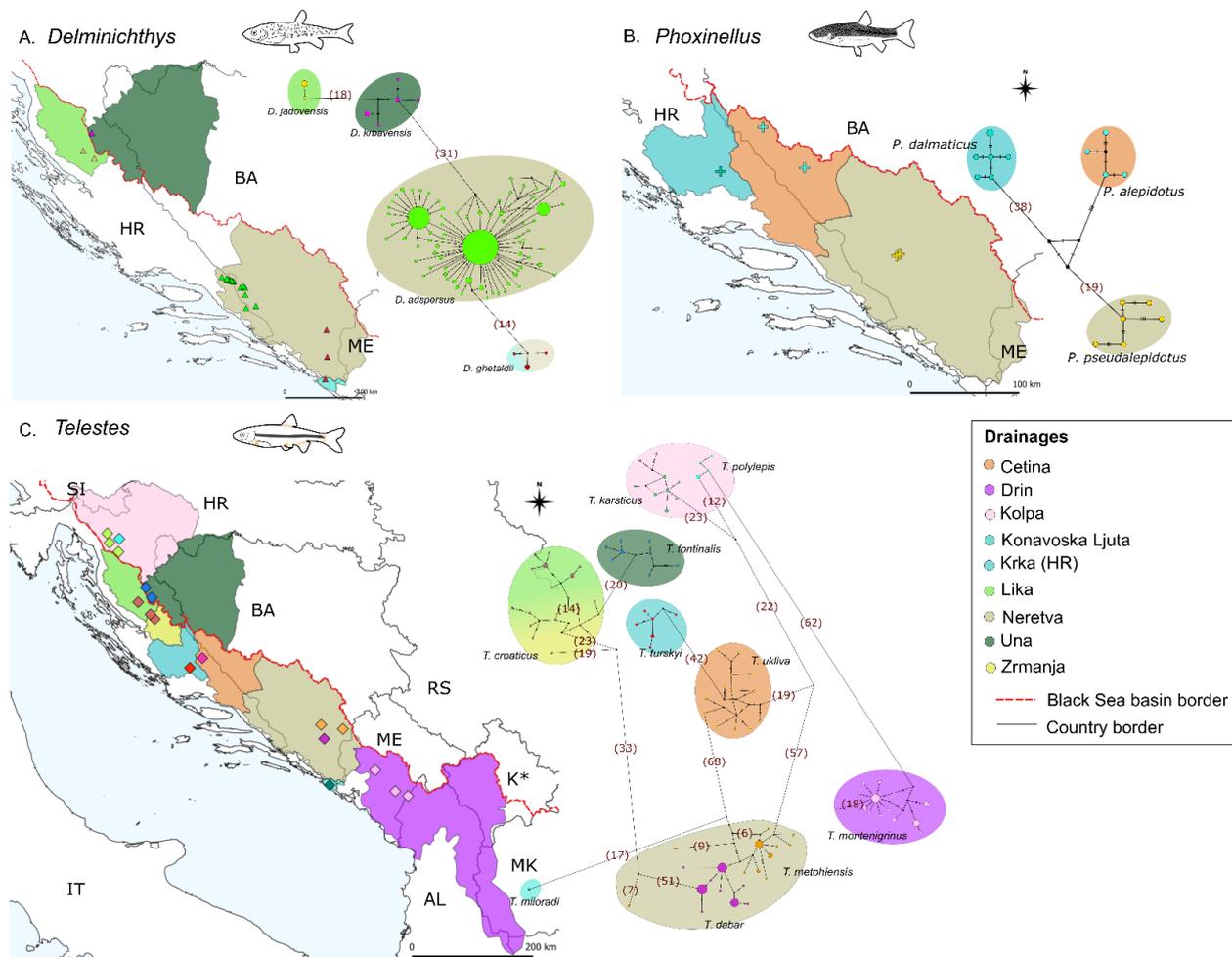


Figure 5. Distribution maps and haplotype networks (based on cytochrome *b* fragment) of mitochondrial lineages, corresponding to species, of the three fish genera *Delminichthys*, *Phoxinellus* and *Telestes* within Dinaric Karst. The river drainages are represented by different colors (see legend) 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*.

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%.

3.2.2. Divergence Times and Ancestral Biogeography

The divergence between the genera *Pelagius* 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 *Pelagius* 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 *Pelagius* and *Delminichthys* ancestor occupied the broader area of the Neretva and Drina 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.

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.

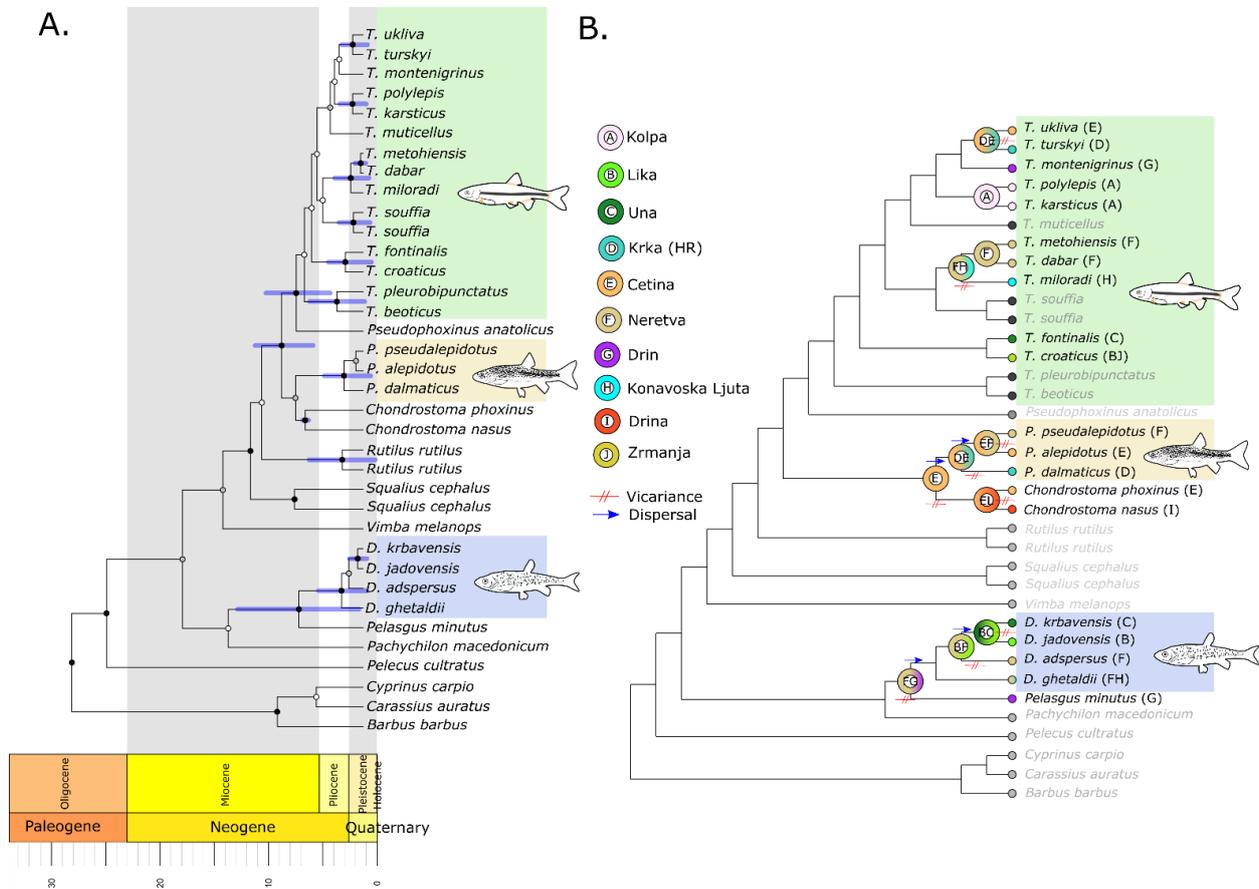


Figure 6. Divergence time estimates and reconstruction of ancestral areas (RASP) analyses of *Delminichthys*, *Phoxinellus* and *Telestes* (together with sister genera and outgroups; for details see Table S3) based on cytochrome *b* fragment. **(A)** Divergence time estimates for *Delminichthys* (highlighted 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.

3.4. *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, Zupančić, 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 Zalomka, 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 Kravsko 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. jadovensis*, 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 adaptation(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 *Congerina 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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