

Article

Tackling the Phylogeny of Lampreys—Insight from the Croatia's Danube Basin

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Abstract: This research addresses the pressing issue of protecting endangered lamprey species in Croatia, a crucial element in preserving biodiversity, particularly in the face of increasing human-induced impacts on natural ecosystems due to global warming. Lampreys, a group of vertebrates with an ancient lineage, are not fully understood taxonomically, posing a challenge to conservation efforts. In the Danube and Adriatic basins of Croatia, where lampreys are found, the lack of modern molecular methods and analyses has hindered an accurate determination of species numbers. This study aimed to bridge this knowledge gap by assessing the genetic diversity and structure of identified lamprey species and lineages in Croatia using the gene for cytochrome *b*. The research revealed four distinct lineages within the species *Eudontomyzon vladykovi* Oliva and Zanandrea, 1959 and confirmed the presence of the species *Eudontomyzon danfordi* Regan, 1911 in Croatia. Genetic diversity and differentiation tests, coupled with molecular diagnostic analyses, indicated moderate to high levels of genetic diversity within and between the identified species and lineages, emphasizing the deep structuring within *Eudontomyzon vladykovi* species. These results highlight the significance of understanding lamprey taxonomy and genetic diversity for effective conservation. The study provides important insights into the intricate relationships and conservation needs of lampreys, and provides a basis for future discussions involving additional genetic markers. By gaining a comprehensive understanding of the taxonomy, ecology, and genetic diversity of lampreys, we can ensure their conservation and that of associated ecosystems.

Keywords: lamprey diversity; phylogenetic insights; taxonomy; evolution; freshwater conservation; *Eudontomyzon* genus



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1. Introduction

The Croatian Danube basin is home to a remarkable diversity of fish species, a result of the interplay of historical events and geological processes. The Danube River and its tributaries, which cover a large part of the country, are home to a wide variety of species as a result of biogeographical separation and evolutionary processes. Geological forces, including the uplift of the Dinarides, tectonic activity, and the formation of the Central Paratethys Basin, have significantly influenced the area. These events, coupled with the effects of glacial and interglacial periods, have facilitated the creation of harbors for fish populations during glacial periods and their subsequent dispersal to rivers in central and northern Europe after the last glaciation [1]. With its distinctive geomorphological and hydrological features, the Danube basin and its tributaries are emerging as a hotspot of European ichthyofaunal diversity, housing an impressive 81 species of freshwater fish species (64 native and 17 invasive species) [2–5].

Among this great diversity of fish in the Croatian Danube basin, a fascinating group of animals, the lampreys, can be found, either buried in the substrate or swimming freely in the water. Lampreys are elongated and scaleless organisms widely distributed in temperate waters, with the exception of tropical and polar regions [6]. Their fascinating life cycle has attracted the interest of scientists [7–12] and varies among species and is influenced by

diet and ecological conditions. During the larval stage, known as ammocoetes, lampreys burrow in the sand for several years before metamorphosing and growing, making them ecosystem engineers [13]. Some species migrate to the sea to feed or reproduce, while others remain in freshwater. During the trophic phase, lampreys feed as parasites, and in some cases, this phase is absent. These peculiar life cycles and behaviors explain why they are rarely caught in their natural habitats and why they are so little studied. Lampreys belong to the Agnatha class, which means that they lack jaws and have a permanently open mouth [6,14,15]. Despite belonging to a distinct superclass (Agnatha) that has not undergone significant anatomical change in the past 360 million years [16], lampreys are often lumped together with fishes (superclass: Gnathostomata) in the literature.

The reported number of lamprey species in Croatia varies in different publications from the last decade and earlier [3,4,17]. Globally, the Petromyzontidae family comprises 10 genera and 40 described species [18], and 13 species are found in Europe, with one species now extinct [17]. Earlier field studies in Croatia identified five species, including *Lethenteron zanandreaei* (Vladykov, 1955), *Petromyzon marinus* Linnaeus, 1758, *Eudontomyzon danfordi* Regan, 1911, *Eudontomyzon mariae* (Berg, 1931), and *Lampetra planeri* (Bloch, 1784) [4]. However, a recent list of Croatian freshwater ichthyofauna only includes three species: *Eudontomyzon vladykovi* Oliva and Zanandrea, 1959, *Lampetra soljani* Tutman, Freyhof, Duli, Glamuzina, and Geiger, 2017, and *Petromyzon marinus* Linnaeus, 1758 [3]. The difference in the number of species is explained by incorrect determination, the frequent use of synonyms in the literature, and the lack of molecular genetic analysis [3]. The identification of lamprey is usually based on morphological observations, such as the position of the dorsal fins, the relative position of the cloaca and dorsal fins, the position of the eyes, the structure of the oral funnel, the teeth and laminae, and the number of myomeres [17–19]. As morphological determinations are not always accurate and lampreys lack countable structures such as fin rays, shells, or ossified structures [18,20], it is necessary to use molecular and genetic methods in order to resolve their taxonomy. To solve the problem, modern genetic techniques, molecular analyses, and phylogenetic reconstructions are necessary but have not yet been carried out for lampreys in Croatia.

The purpose of this research is to provide important information on the genetic diversity and distribution of lamprey species in the Danube basin in Croatia, which will help in their conservation and management. Furthermore, the results of this study will contribute to a better understanding of the evolutionary history and biogeography of lampreys in Europe and the Black Sea basin. Owing to the combination of methods applied to the Petromyzontidae family, the results of the analyses conducted will finally reveal some of the secrets hidden in the genetic material of these secretive organisms and provide accurate data on the phylogeny of lampreys in the study area.

2. Materials and Methods

2.1. Sampling

Individuals of adult lampreys and ammocoetes were sampled from 20 streams (a total of 39 different localities along 13 different river catchments): Bednja, Mura, Drava, Voćinska river, Krapina, Sutla, Mrežnica, Dobra, Kupa, Kupčina, Korana, Radonja, Una, Glina, Ilova, Česma, Sava, Danube, Orjava, and Bosna (Figure 1). A total of 103 individuals were caught using the electrofishing method or randomly by hand (Table 1). A small part of the dorsal fin was cut off with scissors, and the individuals were released. The collected fin samples were stored in tubes filled with 96% ethanol, together with information on the location of the catch. The samples were stored in a freezer at $-20\text{ }^{\circ}\text{C}$ (to prevent degradation of the DNA sample) until further analysis.

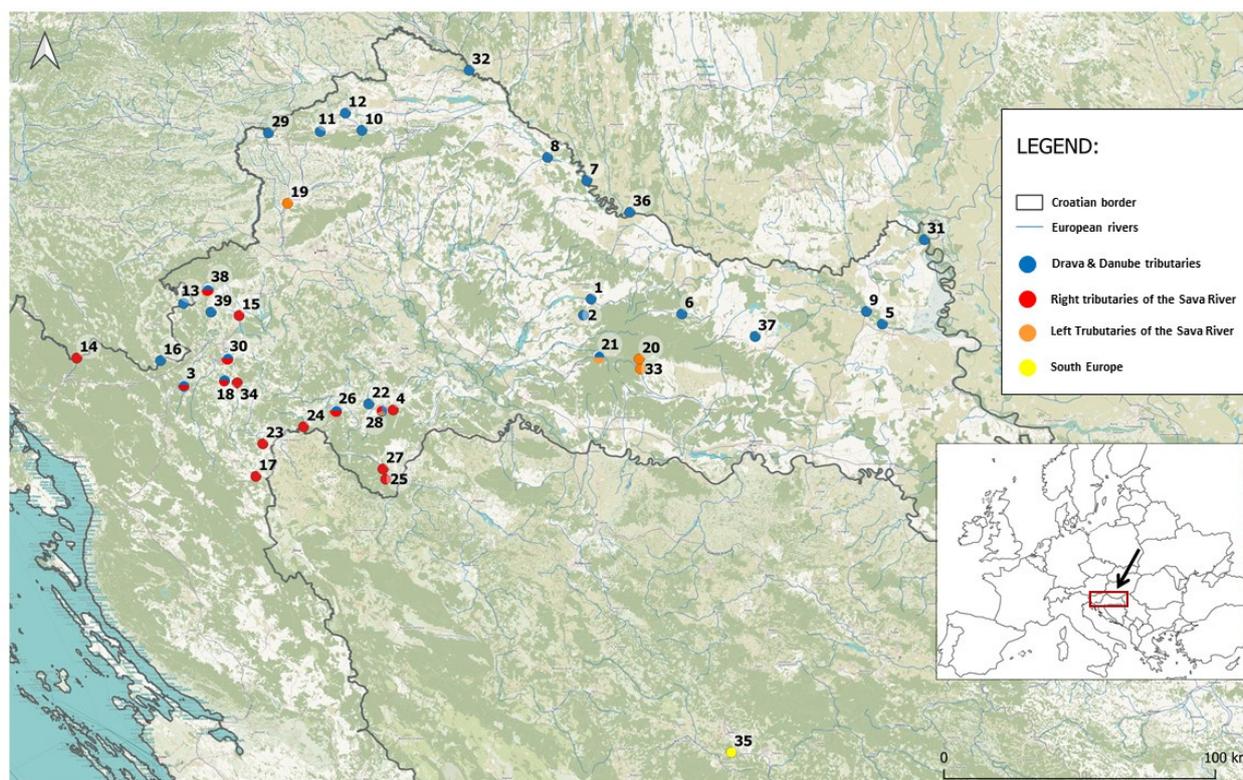


Figure 1. Map of the research area with sampling localities marked with numbers from 1 to 39. Location numbers correspond with numbers in Table 1, where more information on localities is presented.

Table 1. Distribution (localities) and number of samples of cytochrome *b* haplotypes of lampreys in the rivers of the Danube basin.

Locality (Name & Number)	Drainage System	Number of Samples (<i>n</i>)	cyt <i>b</i> Haplotype	GenBank Accession Numbers
Maslenjača (1)	Ilova	1	DUN2	PP661384
Toplica, Daruvar (2)	Ilova	2	DUN2	PP661384
Lešće (3)	Dobra	7	DOB1, DUN2	PP661393, PP661384
Sunja (4)	Sava	8	KUP6, SAV5	PP661399, PP661390
Osijek (5)	Drava	1	DRA1	PP661378
Voćin (6)	Drava	7	DRA2, DRA3, DRA5	PP661379, PP661380, PP661382
Štorgač (7)	Drava	1	DUN1	PP661383
Repaški most (8)	Drava	1	DRA4	PP661381
Petrijevci (9)	Drava	1	DRA1	PP661378
Ivanečka Željeznica (10)	Bednja	1	BED1	PP661376
Bednja (11)	Bednja	2	BED1	PP661376
Voća (12)	Bednja	4	BED1, BED2	PP661376, PP661377
Bubnjarci (13)	Kupa	1	DUN 2	PP661384

Table 1. Cont.

Locality (Name & Number)	Drainage System	Number of Samples (<i>n</i>)	cyt <i>b</i> Haplotype	GenBank Accession Numbers
Brod na Kupi (14)	Kupa	2	KUP1, KUP2	PP661394, PP661395
Lazina (15)	Kupa	1	KUP3	PP661396
Pribanjci (16)	Kupa	1	DUN2	PP661384
Furjašnica (17)	Korana	2	KUP4	PP661397
Brezova Glava (18)	Korana	2	KUP5, DUN2	PP661398, PP661384
Stubička Slatina (19)	Sava	1	KRA1	PP661385
Brzaja (20)	Sava	5	SAV4	PP661389
Pakra (21)	Sava	7	PAK1, PAK2, PAK3, DUN2	PP661402, PP661403, PP661404, PP661384
Bručina (22)	Kupa	3	DUN2	PP661384
Ruševnica (23)	Kupa	1	KUP6	PP661399
Glinica (24)	Kupa	1	KUP6	PP661399
Čemernica (25)	Kupa	1	UNA1	PP661400
Buzeta, Šibine (26)	Kupa	4	KUP6, DUN2	PP661399, PP661384
Žirovnica (27)	Una	2	UNA1, UNA2	PP661400, PP661401
Petrinjšica (28)	Kupa	5	SAV3, SAV4, DUN2	PP661388, PP661389, PP661384
Lupinjak (29)	Sava (Sutla)	3	SAV1, SAV2	PP661386 PP661387
Mostanje (30)	Mrežnica	2	MRE1, DUN2	PP661392, PP661384
Batina (31)	Dunav	1	DUN1	PP661383
Goričan (32)	Mura	1	DUN1	PP661383
Orljava (33)	Orljava	4	SAV4, SAV6	PP661389, PP661391
Živković kosa (34)	Korana	1	RAD1	PP661407
Ušće Lašve (35)	Bosna	2	BOS1, BOS2	PP661405, PP661406
Terezino polje (36)	Drava	5	DRA3	PP661380
Bukvik (37)	Drava	5	DRA3	PP661380
Jaševnica (38)	Kupa	2	KUP3, DUN2	PP661396, PP661384
Ozalj (39)	Kupa	2	DUN2	PP661384

Note(s): GenBank accession numbers will be provided in the proof, however, alignments can be sent to the editor, if needed.

2.2. Laboratory Protocols

Laboratory procedures included DNA isolation, gene amplification by polymerase chain reaction, and verification of amplification by agarose gel electrophoresis. DNA

isolation was conducted using a DNeasy Blood & Tissue chemical kit according to the protocol of the kit manufacturer, QIAGEN (Amsterdam, Netherlands). The protocol for gene amplification by polymerase chain reaction was optimized to obtain the best products, and PCR conditions and primers are listed in Table 2. PCR products obtained and verified by electrophoresis were sent to the Macrogen Service Centre for sequencing using the same primers that were used for PCR amplification.

Table 2. Protocol for PCR reaction and used primers.

Genetic Marker	<i>cyt b</i>
PCR conditions	35 cycles of 45 s at 92 °C, following 90 s at 48 °C, following 105 s at 72 °C
PCR primers	ProK (5'TTATTTAATGTTAAGATRCTAGCTTTGG3') Pak-Glu F (5'CACCGTTGTAGAATTCAA CTATAAG3')

The molecular marker chosen for analysis in this study was the cytochrome *b* (*cyt b*) gene. As part of mitochondrial DNA and also a coding gene, its mutation rate is usually lower than other gene markers, making it suitable for phylogenetic analyses at species and supraspecific levels. It was chosen because it has shown the best performance and yielded the most reliable results in previous studies of phylogenetic structure, relationships, and taxonomic research [2,21], particularly in recovering phylogenetic relationships among closely related freshwater fish taxa [22,23].

2.3. Data Analyses

2.3.1. Sequence Alignment

Sequences were aligned using the BioEdit Sequence Alignment Editor version 7.2.5 [24] and compared to previously published sequences. Visual checks were performed on chromatograms and alignments to ensure accuracy.

2.3.2. Neutrality Test and Analyses of Population Genetic Diversity and Polymorphism

Genetic diversity within the identified lineages and species was assessed by estimating measures of DNA sequence polymorphisms using the DnaSP 5.10 program [25]. To gain an understanding of gene flow and genetic differentiation of lineages, estimates of genetic differentiation (χ^2 , Hst, Kst, Kst*, Z, Z*, and Snn) of the lineages and their support were computed in addition to specific measurements of DNA polymorphisms. Sequences obtained from the captured individuals were used as input data for genetic statistical tests of diversity. Neutrality tests Tajima's D, Fu and Li's D, and F tests were performed and showed that the data set is in mutation-drift equilibrium (not statistically significant, $p < 0.05$) also using the DnaSP 5.10 program [25].

2.3.3. Phylogeographic and Evolutionary Analyses

Phylogenetic reconstruction was performed to confirm the phylogenetic position of lampreys from the Croatian Danube basin within the phylogenetic tree of the genus *Eudontomyzon*, to reveal relationships among them, and to test whether the phylogenetic position of each population is consistent with reported taxonomic hypotheses. Two different phylogenetic reconstruction methods were employed: maximum parsimony (MP) and maximum likelihood (ML), implemented in PAUP v.4.0a [26]. For MP analysis, a heuristic search mode with 100 replicates was used, with a randomized input order of taxa and tree bisection-reconnection (TBR) branch swapping, giving equal weight to all codon sites and nucleotide substitution types weighted equally. ML analysis was performed under the heuristic search option using the TBR branch-swapping algorithm. Branch support (BS) was assessed by nonparametric bootstrapping (1000 pseudoreplicates, ten additional

sequence replicates). The length of the MP tree was 163, with a consistency index of 0.6994, a homoplasy index of 0.3006, and a retention index of 0.7461. With haplotypes as input sequences for phylogenetic tree and network construction, sequences from the GenBank were also used (Table 3). Sequences of several *Eudontomyzon* species from the GenBank were included in the phylogenetic analyses to provide a clearer insight into the phylogenetic position of the lampreys from the study area in relation to their European relatives, while the sequence of *Lampetra fluviatilis* (accession number: GQ206175.1; Table 3) was used as an outgroup. The median-joining approach (MJ) was employed as an additional method using the computer program Network 4.5.1.6 [27]. In the resulting phylogenetic network, horizontal gene transfers were observed, which can be useful in reconstructing phylogenetic relationships among closely related taxa.

Table 3. Lamprey *cyt b* sequences retrieved from the GenBank and included in the phylogenetic and evolutionary history reconstruction.

Species	bp (Number of Base Pairs)	Locality	GenBank Accession Number	Reference
<i>Eudontomyzon danfordi</i> Regan, 1911	1133	Zdychava River (SVK)	GQ206158.1	[28]
<i>Eudontomyzon mariae</i> (Berg, 1931)	1133	Ivianka River (UKR)	GQ206162.1	[28]
<i>Eudontomyzon stankokaramani</i> (Karaman, 1974)	1133	Zeta River (MNE)	GQ206189.1	[28]
<i>Eudontomyzon vladkyovi</i> Oliva and Zanandrea, 1959	1133	Studenc Creek (SVK)	GQ206161.1	[28]
<i>Lampetra fluviatilis</i> (Linnaeus, 1758)	16,159	Garonne Estuary (FRA)	NC001131.1	[29]
<i>Eudontomyzon lanceolata</i> (Kux and Steiner, 1972)	1133	Chakhtsutsyr River (RUS)	GQ206176.1	[28]
<i>Lampetra planeri</i> (Bloch, 1784)	1133	Kalte Moldau River (DEU)	GQ206149.1	[28]
<i>Lethenteron zanandreaei</i> (Vladykov, 1955)	1133	Vipava River (SLO)	GQ206184.1	[28]
<i>Eudontomyzon</i> sp. Dnieper	1164	Ugra River (RUS)	KP135487.1	[30]
<i>Eudontomyzon</i> sp. Dnieper	1164	Rudyanka River (RUS)	KP135483.1	[30]
<i>Eudontomyzon</i> sp. Dnieper	1164	Sigosa River (RUS)	KP135485.1	[30]
<i>Eudontomyzon</i> sp. Dnieper	1164	Vyazma River (RUS)	KP135482.1	[30]
<i>Eudontomyzon hellenicus</i> (Vladykov, Renaud, Kott and Economidis, 1982)	1133	Strymon River (GRC)	GQ206160.1	[28]
<i>Lampetra fluviatilis</i> (Linnaeus, 1758)	1133	Luga River (RUS)	GQ206175.1	[28]
<i>Eudontomyzon stankokaramani</i> (Karaman, 1974)	1191	unknown river (SLO)	KX787432.1	[31]
<i>Eudontomyzon lanceolata</i> (Kux and Steiner, 1972)	1191	unknown river (TUR)	KX787431.1	[31]

Divergence times between phylogenetic lineages were estimated by a Bayesian MCMC coalescent method using BEAST 2.4.7 software [32]. The analysis was conducted on the *cyt b* data set. Rate homogeneity across phylogenetic lineages was assessed by the log-likelihood ratio test (LRT), comparing the likelihood of phylogenetic trees (reconstructed using the maximum likelihood approach) with and without molecular clock enforcement in PAUP software version 4.0. As the likelihood scores were not the same in both cases for the *cyt b* data set, we applied a relaxed molecular clock. Molecular clock calibration was based on the divergence rate of the *cyt b* gene in Petromyzontidae of 0.12% per lineage per million years, as reported by [33]. The divergence rates were drawn from an uncorrelated lognormal distribution and a Yule speciation prior to a random starting tree. The substitution model

used was HKY with a gamma-site heterogeneity model. We used default prior distributions for kappa, frequencies, and alpha, while substitution rate parameters were unlinked across codon positions. The number of MCMC steps (the length of the chain) was ten million.

The ancestral distribution ranges of lineages were reconstructed using Statistical Dispersal-Vicariance Analysis implemented in the S-DIVA software version 1.9 [34]. This method reconstructs the ancestral distribution in a phylogeny by optimizing a three-dimensional cost matrix, in which extinctions and dispersals 'cost' more than vicariance and determines the statistical support for the ancestral range [34]. Reconstruction of ancestral geographic ranges was conducted using the *cyt b* data set. An input set of trees was generated using Bayesian analysis (BAY). A total of six recent geographic ranges for *Eudontomyzon* were denoted (4 of them are marked in Figure 1): A—right tributaries of the Sava River, C—North Europe, D—tributaries of the Drava and Danube Rivers, E—Central Europe, F—South Europe.

3. Results

3.1. Phylogenetic Relationships and Distribution of Lampreys in Continental Croatia

The phylogenetic reconstruction of the Petromyzontidae family in the area of continental Croatia (the Danube basin) is based on the sequences of the gene for cytochrome *b* (*cyt b*) of sampled lampreys and sequences retrieved from the GenBank (Tables 2 and 3). The *cyt b* gene sequences were 1191 base pairs long, with 68 variable and 46 parsimony significant sites (sites with at least two nucleotides occurring at least twice).

A total of 103 newly obtained sequences were combined with sequences retrieved from GenBank (Table 3) and included in the analyses. With the Croatian sample set, 32 haplotypes were identified, and five phylogenetic lineages were recovered in the phylogenetic tree (Figure 2). Four of these lineages, including samples from both the Sava and Drava River basins in Croatia, belong to *E. vladykovi*, according to current taxonomy. However, several samples from the Drava R. basin, particularly from the easternmost part of this river basin in Croatia and from the Bednja River (the largest left tributary of the Drava River), clustered together with sequences of *E. danfordi* from Slovakia in a separate phylogenetic lineage. As this lineage corresponds to the species *E. danfordi*, this study confirms for the first time that the Croatian Drava and Bednja rivers are part of the distribution range of this species.

Another unexpected result is the high amount of cryptic diversity within *E. vladykovi*, with at least four clearly separated phylogenetic units whose geographic distributions are also largely separated. *Eudontomyzon vladykovi* lineage I is so far, and based on our results, only reported from Croatian waters, where it mainly inhabits the northwest part (area of Hrvatsko zagorje), but also the Mrežnica and Sunja rivers, so that it was found in both the Sava and Drava river basins. The second lineage, although found in a small number of samples, occupies a wide but fragmented distribution area. It was also found in both river basins in continental Croatia. The third lineage within *E. vladykovi* comprises the largest number of specimens from Croatia (it is present only in the Sava River basin, but it was found in a large number of localities), but also from Bosnia and Herzegovina. Lineage IV is also restricted to the Sava River basin, but is much rarer.

The MJ phylogenetic network (Figure 3), based on the *cyt b* gene sequences obtained in this study, corroborates the results visible in the phylogenetic trees. A clear separation of two species—*Eudontomyzon danfordi* and four lineages within the species *Eudontomyzon vladykovi*—is evident. Once again, there is a particularly high level of diversity within the *E. vladykovi* III lineage that needs to be recognized.

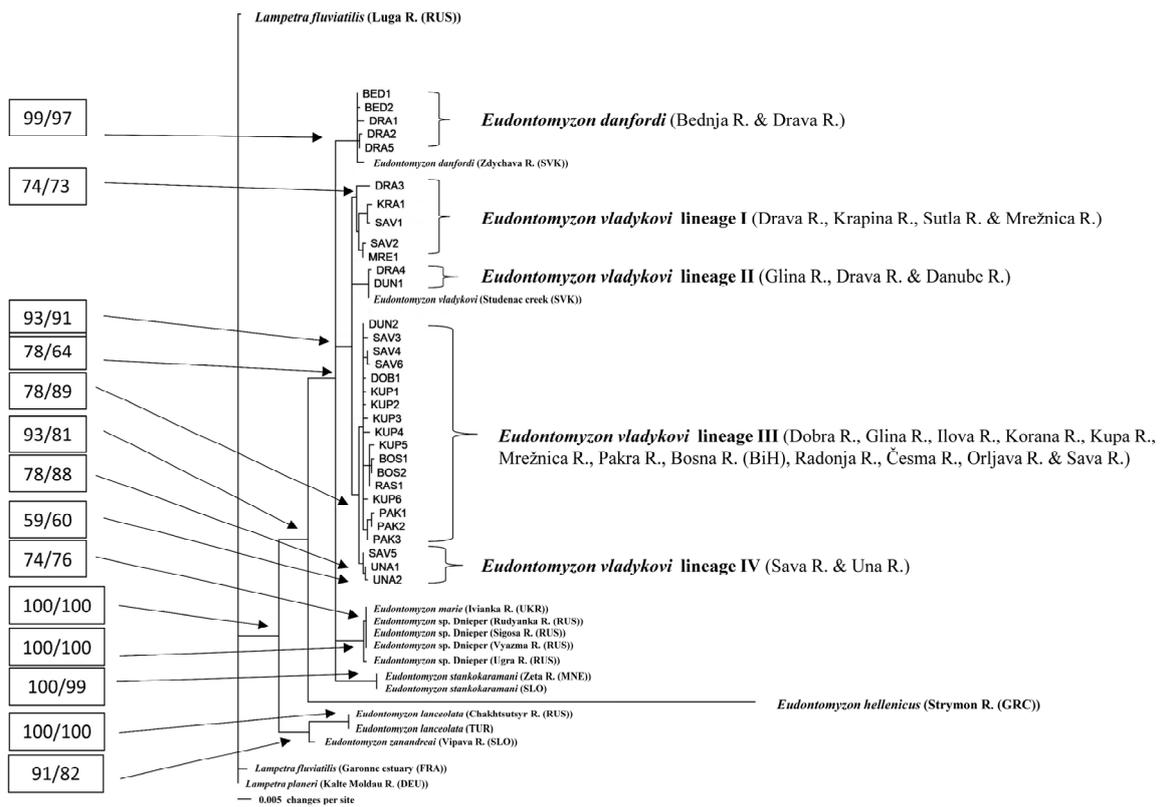


Figure 2. ML phylogram showing the position of *Eudontomyzon* species in the Danube basin, together with sequences retrieved from the GenBank based on the *cyt b* gene. Numbers at nodes represent ML and MP bootstrap values.

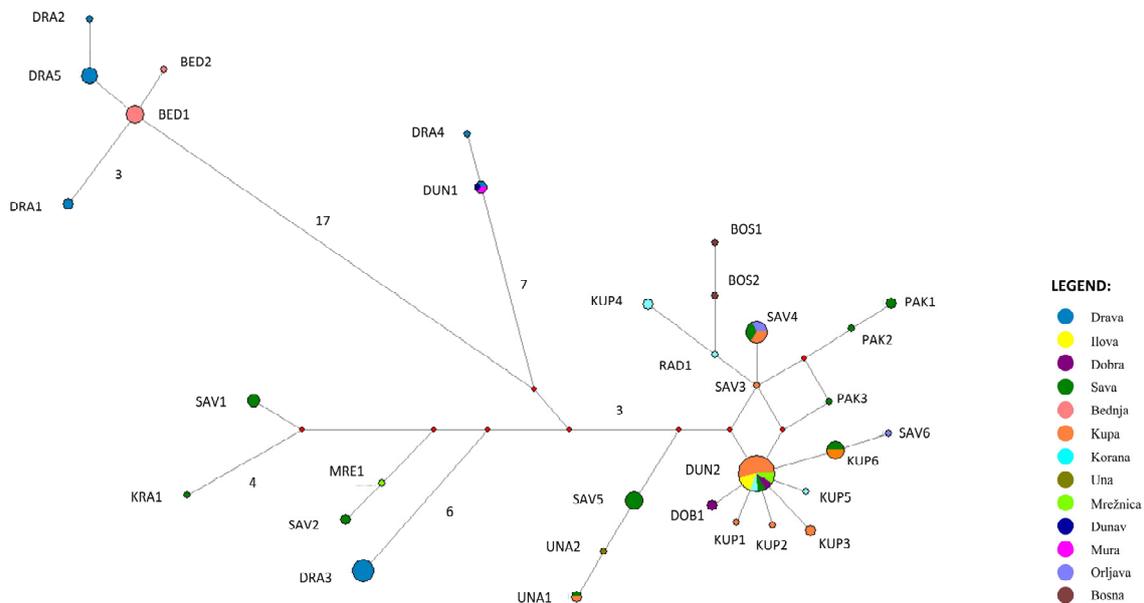


Figure 3. Median-joining network of *cyt b* haplotypes. Red circles represent median vectors. The number of mutations is indicated by branches when there are more than two mutations.

3.2. Inter- and Intraspecific Genetic Diversity and Differentiation

The genetic diversity of both species distributed in continental Croatia, as well as within each of the four *E. vladkovi* lineages, is moderate to high. A large number of

haplotypes were determined, as was their high diversity. Furthermore, the average number of nucleotide differences for the *Eudontomyzon vladykovi* species and the vladykovi I lineage is extremely high, indicating a deeper structure and the possibility of the presence of cryptic diversity (Table 4). It is noteworthy that the haplotype diversity of *E. danfordi* is only 15% lower than the haplotype diversity of *E. vladykovi*, although its distribution area in Croatia is much smaller and it is present in a much lower number of individuals (15 vs. 88) caught in this study. However, the average number of nucleotide differences is almost five times higher in *E. vladykovi*, indicating that deep structuring is present, in contrast to the uniform genetic structure of *E. danfordi*. Moreover, the number of shared polymorphisms is very low, and they exist only between the *E. vladykovi* lineage III and all other units, including *E. danfordi* (Table 5).

Table 4. Measures of genetic polymorphism for the *Eudontomyzon vladykovi* lineages and for the *Eudontomyzon danfordi* species. N—number of sequences, h—number of haplotypes, S—number of polymorphic sites, Hd—haplotype diversity, k—average number of nucleotide differences, π —nucleotide diversity, η —total number of mutations.

Species/Lineage	N	h	S	Hd	k	π	η
<i>E. danfordi</i>	15	5	6	0.752	1.524	0.00128	6
<i>E. vladykovi</i>	88	30	53	0.895	7.158	0.00601	54
vladykovi I	16	5	19	0.667	6.983	0.00586	19
vladykovi II	4	2	1	0.5	0.5	0.00042	1
vladykovi III	59	20	23	0.8	2.414	0.00203	23
vladykovi IV	9	3	2	0.556	0.722	0.00061	2

Table 5. Shared polymorphisms on the *cyt b* gene between lineages.

Species/Lineage	Vladykovi I	Vladykovi II	Vladykovi III	Vladykovi IV
<i>E. danfordi</i>	0	0	1	0
vladykovi I	-	0	3	0
vladykovi II	-	-	1	0
vladykovi III	-	-	-	0

The pairwise distances (p-distances) (Table 6) between species and lineages are always higher than the p-distance within *E. danfordi* and each of the *E. vladykovi* lineages. *Eudontomyzon vladykovi* lineages III and IV are the most closely related lineages, which is reflected in the lowest p-distance between them. On the other hand, as expected, p-distances between *E. danfordi* and all *E. vladykovi* lineages are the highest.

Table 6. p-values between and inside lineages (expressed as a percentage).

Species/Lineage	<i>E. danfordi</i>	Vladykovi I	Vladykovi II	Vladykovi III	Intraspecific/Intralineage
<i>E. danfordi</i>					0.08–0.41 (0.20)
vladykovi I	1.84–2.68 (2.26)				0.08–1.25 (0.70)
vladykovi II	2.01–2.35 (2.18)	1.09–1.51 (1.30)			/ (0.10)
vladykovi III	1.93–2.35 (2.14)	0.83–1.59 (1.21)	1.09–1.42 (1.255)		0.80–0.58 (0.30)
vladykovi IV	1.84–2.09 (1.97)	0.83–1.34 (1.09)	0.92–1.09 (1.01)	0.33–0.75 (0.54)	0.08–0.16 (0.10)

The distinction not only of species but also of lineages of *E. vladykovi* lineages is corroborated by the results of the genetic differentiation tests (Table 7), since there was no gene flow between species and lineages, indicating that there is no reproduction of individuals belonging to different lineages.

Table 7. Estimation of the number of migrants per generation between the lamprey lineages of the Danube basin in Croatia according to [35–37].

Species/Lineage	Nm [35]	Nm [36]	Nm [37]
<i>E. danfordi</i> : <i>E. vladykovi</i>	0.61	0.1	0.11
vladykoviI:vladykoviII	0.67	0.15	0.15
vladykoviI:vladykoviIII	0.53	0.26	0.26
vladykoviI:vladykoviIV	0.51	0.21	0.21
vladykoviII:vladykoviIII	0.74	0.6	0.6
vladykoviII:vladykoviIV	0.06	0.03	0.03
vladykoviIII:vladykoviIV	1.19	0.2	0.2

3.3. Molecular Identification of Species and Lineages

Molecular diagnostic analyses identified sites of fixed differences based on the *cyt b* gene. More fixed differences (Table 8) show that the species/lineages diverged earlier and indicate a large genetic difference between the species/lineages. As expected, the highest number of fixed differences was observed between *Eudontomyzon danfordi* and the phylogenetic lineages of *Eudontomyzon vladykovi*. However, there are also fixed differences present between all phylogenetic lineages of the *Eudontomyzon vladykovi* species. The table of diagnostic sites (Table 9) shows exactly which sites are involved. It is noteworthy that each of the *E. vladykovi* lineages can be unambiguously recognized on the basis of diagnostic sites in *cyt b* gene.

Table 8. Fixed differences based on the *cyt b* gene between lineages.

Species/Lineage	Vladykovi I	Vladykovi II	Vladykovi III	Vladykovi IV
<i>E. danfordi</i>	19	24	21	21
vladykovi I	-	9	6	6
vladykovi II	-	-	12	10
vladykovi III	-	-	-	3

Table 9. Fixed differences on the *cyt b* gene with specified nucleotide sites of fixed differences and bases present in each lineage indicated. Diagnostic sites for a particular lineage are represented by nucleotides in colored boxes.

Nucleotide Spot	Species/Lineage				
	<i>E. danfordi</i>	Vladykovi I	Vladykovi II	Vladykovi III	Vladykovi IV
36	T	C	C	C	C
57	C	T	T	T	T
72	C	T	T	T	T
156	G	A	A	A	A
157	C	T	T	T	T
162	T	C	C	C	C
312	A	G	A	A	A
378	A	A	G	A	A
390	T	T	C	T	T
480	A	G	G	G	G

Table 9. Cont.

Nucleotide Spot	Species/Lineage				
	<i>E. danfordi</i>	Vladykovi I	Vladykovi II	Vladykovi III	Vladykovi IV
483	A	G	G	G	G
531	C	C	C	C	T
588	A	A	G	A	A
630	T	T	C	T	T
639	G	A	A	A	A
675	T	C	C	C	C
726	C	T	T	T	T
771	A	C	C	C	C
807	G	A	G	G	G
813	C	C	C	T	C
909	G	A	A	A	A
1155	G	G	A	G	G

3.4. Evolutionary History of the Family Petromyzontidae in Europe

Evolutionary history analyses based on the *cyt b* gene (Figure 4) revealed an ancient origin and long-term evolutionary history of both *E. vladykovi* and *E. danfordi*. Separation of the clade comprising all *E. vladykovi* lineages from its sister clade comprising *E. danfordi*, *E. stankokaramani* and *E. sp. Dnieper* occurred in the early Miocene, about 22 mya (million years ago), whereas these two clades started to diversify around 17 mya. The origin of the different species and *E. vladykovi* lineages occurred mostly during the Middle Miocene, 11.6–8.5 mya.

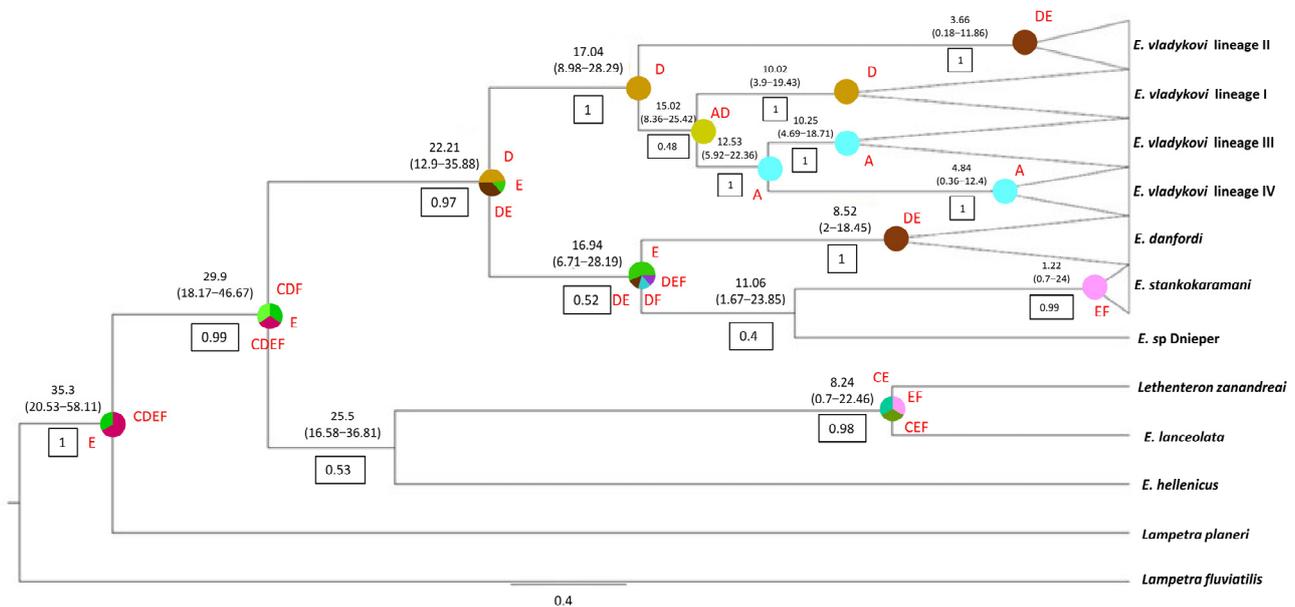


Figure 4. Estimates of divergence time based on *cyt b* sequences of lamprey species. The timing of divergence events is presented as a mean and a 95% credible interval (in millions of years ago). Lower and upper bounds of the highest posterior density (HPD) interval; the HPD is the shortest interval containing 95% of the sampled values. Numbers in squares are bootstrap values. Colored circles represent the highest probability of the ancestral range (A—right tributaries of the Sava River, C—North Europe, D—tributaries of the Drava and Danube Rivers, E—Central Europe, F—South Europe).

4. Discussion

4.1. Diversity of the Petromyzontidae Family in the Danube Basin in Croatia

As this study was the first to investigate the structure of lamprey populations in continental Croatia at the molecular genetic level, it provided new and very important insights. Our results show significantly higher diversity on all levels—species, intraspecific, and genetic diversity. In addition to *E. vladykovi*, which was thought to be the only lamprey species in continental Croatia [3], this study proved that *E. danfordi* is also present and that its distribution range includes localities in the Drava River basin. Since its current distribution range in Croatia comprises the Bednja River (located in the western part of the Drava River basin) and the easternmost localities (Osijek and Petrijevci), with no reports of its presence in the Drava River in between, it is possible that the current localities are remnants of the once wider distribution range of this species, which has been reduced as a consequence of anthropogenic activities, especially habitat modifications. Regardless of its small and fragmented distribution range in Croatia, the genetic diversity of *E. danfordi* is moderately high, which is consistent with its long-term diversification in the Miocene.

Eudontomyzon vladykovi has a wider distribution in Croatia, inhabiting localities in both the Drava and Sava river basins. Its genetic diversity is high, and its intraspecific structure is also very pronounced. Four *E. vladykovi* lineages can be observed in the phylogenetic structure of this species, with no observed gene flow between them and mostly distinct distribution ranges. The origin of *E. vladykovi* can be dated to the early Miocene (22 mya). The intraspecific divergences of this species are also of very ancient origin and started already in the Miocene, 17 mya.

It is noteworthy that although *E. vladykovi* now has a larger distribution area in the Sava River basin, our results suggest that its ancestral distribution area was located in the Drava and Danube River basins, similar to *E. danfordi*, whose ancestral area was in the Drava and Danube River basins in Croatia or even further north, in Central Europe. The time frame of the origin of these two species (early and middle Miocene) corresponds to the development and evolution of the Danube River basin, which is concordant with the results of their ancestral ranges. Namely, the Proto-Danube was formed in the North Alpine foreland basin around 19–18 mya and started its southward migration [38,39]. Interestingly, the period between 17 and 18 mya, which was recovered in this study as the time of divergence of the *E. vladykovi* lineages and the origin and evolution of the species *E. danfordi*, *E. stankokaramani* and *E. sp. Dnieper*, has already been observed as an important period in the evolutionary history of the fish species distributed in the Danube River basin. The first major diversification event within the genus *Barbus* was also associated with this period [5]. Since the Proto-Danube progressed slowly, reaching the Vienna basin at 14–13 mya and the Pannonian basin at 10–5 mya [39], it is possible that the evolutionary history of both *E. danfordi* and *E. vladykovi* was largely shaped by the evolution of the Danube River basin, especially since 10–5 mya is the exact time frame when the origin of *E. vladykovi* lineages and the origin of diversification of the Croatian *E. danfordi* population occurred. Moreover, the ancestral range of the Croatian *E. danfordi* and *E. vladykovi* lineages was reconstructed in the Drava and Danube River basins. On the other hand, the ancestral ranges of *E. vladykovi* lineages I and II are associated with the right tributaries of the Sava River, indicating the colonization of the Sava River basin at the same time and the subsequent evolution and diversification of *E. vladykovi* there. Another important observation is that some *E. vladykovi* lineages inhabit the same rivers and river basins, with no obvious barriers between their localities, that would prevent migration and gene flow. This implies the presence of reproductive isolation between lineages, but it is also consistent with their ecology and behavior, particularly their connection with small patches of suitable habitats. Discovering various aspects of life cycles and diversities in the ecology and behavior of different *E. vladykovi* lineages and populations should be the focus of further investigation.

Given the long-term evolutionary history of both, *E. vladykovi* and *E. danfordi*, their high and moderately high genetic diversity is not surprising and is positive from a conservational

perspective. Moreover, the absence of population genetic patterns that would imply bottleneck events indicates that both species have survived glaciations in the waters of continental Croatia and that glacial events did not have a significant effect on them. Namely, there is no abrupt change in their evolutionary history; the intraspecific structure of *E. vladykovi*, which originated in the Miocene, is well preserved until now, and intraspecific divergences of *E. danfordi* also started in the Miocene and continued until recent times. The waters of the Danube River basin in Croatia have been reported to serve as glacial refugia for other freshwater fish species, e.g., *Cobitis elongatoides* Bacescu & Mayer, 1969 [40], and it is likely that they also served as glacial refugia for lampreys. Nevertheless, lamprey populations with high genetic diversity have also been recognized as candidates for protection at several sites in Italy, Ireland, and Spain [7].

4.2. Taxonomic Implications

Besides revealing a high amount of cryptic diversity within lampreys in the Danube basin in Croatia, the results of all analyses conducted in this investigation indicate the necessity of a taxonomic revision of *E. vladykovi* (also observed by [33] at the European level) and the possibility that a species complex exists under this name, which, on the other hand, also has strong consequences for the practical conservation of lampreys in Croatia. The deep structuring within *E. vladykovi* observed by phylogenetic reconstruction and evolutionary history analyses (at least four phylogenetically distant and evolutionarily independent lineages were observed in this study) is comparable to the structuring within its sister clade, which comprises *E. danfordi*, *E. stankokaramani*, and *E. sp. Dnieper*. The origins of both clades occurred in a similar time frame, and the emergence of lineages included within both clades occurred mostly in the Miocene (11.6–10.25 mya), so it is surprising that lineages within one clade are all considered to belong to a single species (*E. vladykovi*), whereas lineages within the second clade each represent a separate species. Importantly, no gene flow was detected between the different *E. vladykovi* lineages, each of which can be recognized by molecular diagnostic sites in the *cyt b* gene. Although the above results are strong indications that each of the recovered *E. vladykovi* lineages may represent a separate species, a detailed comparison of morphological characteristics is necessary to find morphological diagnostic characters. Furthermore, although *cyt b* recovered cryptic diversity within *E. vladykovi*, it is necessary to investigate more genetic markers, including nuclear genes, in order to reliably resolve the taxonomy of this species or species complex.

4.3. Conservation Recommendations

The channeling of watercourses has been highlighted as the primary cause of endangerment at the sites where individuals were captured [41,42]. To preserve these populations, which harbor such a high level of genetic diversity and contribute to the biodiversity and ecosystem stability of Croatia, it is necessary to conserve, possibly protect, and prevent further endangerment of these sites. There is no reason why protective measures should not be implemented even before taxonomic issues are resolved. Among the sites highlighted in this study as unique and worthy of protection because of their natural richness are the areas inhabited by populations of the *E. danfordi* species, namely the Bednja River area, and the sites where the Vladykovi II lineage is found, specifically the Mura River basin and the confluence of the Mura River and Drava rivers. In addition to their genetic diversity, these sites collectively yielded the fewest individuals captured, suggesting the possible presence of smaller populations that, due to their size (currently indeterminable), may be more vulnerable to threats than some populations found in the tributaries of the Sava River. Furthermore, individuals of the Vladykovi II lineage indicate that populations in these areas may be crucial for unraveling the evolutionary history of the species *E. vladykovi* in Croatia.

Continued conservation efforts are essential to protecting lamprey populations and their habitats. Habitat restoration initiatives should be prioritized to improve the quality and connectivity of aquatic environments, particularly in regions where lamprey popula-

tions are threatened by habitat degradation [8]. Furthermore, the establishment of protected areas or reserves in critical lamprey habitats is imperative to ensure their long-term survival and to mitigate human disturbance. Monitoring programs must be developed and used to track population trends, habitat changes, and human impacts over time, providing essential data for adaptive management strategies [43]. In addition, outreach and education initiatives are essential to raise awareness of the ecological significance of lampreys and to build support for conservation efforts among local communities and among stakeholders. Cross-border collaboration is also necessary, working with other countries and international organizations to coordinate conservation efforts across transboundary river basins, recognizing the migratory nature of lamprey species and the interconnectedness of their habitats [44].

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