

Article

Molecular Analysis of Two Endemic *Squalius* Species: Evidence for Intergeneric Introgression among Cyprinids and Conservation Issues

Damir Valić ^{1,*} , Matej Kristan Mirković ², Višnja Besendorfer ³ and Emin Teskeredžić ⁴

¹ Laboratory for Biological Effects of Metals, Division for Marine and Environmental Research, Ruđer Bošković Institute, Bijenička 54, 10000 Zagreb, Croatia

² Department of Biology, Faculty of Science, University of Zagreb, Ravnice 48, 10000 Zagreb, Croatia; matej.k.mirkovic@gmail.com

³ Division of Molecular Biology, Department of Biology, Faculty of Science, University of Zagreb, Horvatovac 102a, 10000 Zagreb, Croatia; visnja.besendorfer@biol.pmf.unizg.hr

⁴ Laboratory for Aquaculture and Pathology of Aquatic Organisms, Division of Molecular Biology, Ruđer Bošković Institute, Bijenička 54, 10000 Zagreb, Croatia

* Correspondence: dvalic@irb.hr; Tel.: +385-1-4561-076

Abstract: Conservation of indigenous species, especially endemic ones, is of the utmost importance. Morphological determination of species is usually not sufficient; therefore, molecular phylogenetic analyses of the Illyrian chub, *Squalius illyricus*, and the Zrmanja chub, *Squalius zrmanjae*, from the Krka River were performed. For the genetic characterization of the mitochondrial gene cytochrome *b* and the non-coding nuclear region *Cyfun P*, 15 specimens from each species were subjected to analysis. The obtained sequences were aligned with similar ones from GenBank to determine the taxonomic and phylogenetic position of these species. The obtained molecular results imply that *S. zrmanjae* from the Krka River has a nuclear region that resembles Dalmatian rudd, *Scardinius dergle*. This result implies an introgression event and the transfer of genetic information between the two genera. The investigated species are on the IUCN Red List of Threatened Species, their biological data are scarce, and further investigation and protection are needed.

Keywords: Leuciscidae; cyt *b*; endemic species; *Squalius*

Key Contribution: We investigated two fishes endemic to the Adriatic basin—the Illyrian chub, *Squalius illyricus* and the Zrmanja chub, *Squalius zrmanjae*—both on the IUCN Red List of Threatened Species. They are protected by national law but their biological and molecular data are scarce, and data for their nuclear markers do not exist. The results obtained from molecular markers imply an introgression event and the transfer of genetic information between the two genera, *Squalius* and *Scardinius*, which have to be taken into consideration in the future conservation of these species.



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1. Introduction

The cyprinid genus *Squalius* includes around 28 species in Europe [1]. They are small-to large-sized fishes adapted to diverse habitats. Specimens can be found in streams, slow-flowing rivers, and even lakes. According to Kottelat and Freyhof [1], seven species can be found in Croatia: Chub *Squalius cephalus* (L. 1758); Illyrian chub *S. illyricus*, Heckel & Kner 1858; Makal dace *S. microlepis*, Heckel 1843; Cavedano chub *S. squalus* (Bonaparte, 1837); Neretva chub *S. svallize*, Heckel & Kner 1858; Livno masnica *S. tenellus*, Heckel 1843; and Zrmanja chub *S. zrmanjae*, Karaman 1928).

Squalius illyricus (Figure 1a) is on the IUCN List of Near-Threatened species (NT) (<https://www.iucnredlist.org/species/61381/12469652>, accessed on 10 February 2023) and is internationally protected by the Bern Convention. Moreover, it is an indigenous Croatian species, as well as endemic to the Adriatic Basin [2]. It inhabits a very small area—the

Cetina and Krka river basins— and it can be found in clean and swift karstic rivers and lakes. Biological data on this species are very scarce. *S. zrmanjae* (Figure 1b) is a Croatian Near-Threatened (NT) species (<https://www.iucnredlist.org/species/60794/12399825>, accessed on 10 February 2023) that can be found only in the Zrmanja and Krka catchment areas [2–4]. It is also indigenous to Croatia and endemic to the Adriatic basin [2]. Karstic streams are a natural habitat for this species, but the biological data are not sufficient. These two species are morphologically very similar and scale pigmentation is used as a distinguishing criterion, as are eye diameter and snout appearance. Therefore, molecular analysis will provide additional information in determination of the species.



Figure 1. Investigated species, (a)—*Squalius illyricus* and (b)—*Squalius zrmanjae*.

Research in the fields of taxonomy and phylogeny of fishes has been both intensive and extensive over the last three decades, using a wide range of different techniques. Molecular methods such as the sequencing of a specific gene or the entire genome have generally been used for these purposes. The Leuciscidae is the most investigated fish family in Europe, particularly the genera *Leuciscus*, *Chondrostoma*, *Scardinius* and *Rutilus* [5–11].

DNA sequences of mitochondrial genes, especially of cytochrome *b* (*cyt b*), are widely used to establish phylogenetic relationships between different organisms [12]. *Cyt b* is a highly conserved protein-encoding region that evolves slowly, is suitable for monitoring evolutionary processes, and is convenient for detecting differences between closely related species [13,14]. Mitochondrial markers represent only the maternal lineage and provide very little information about hybridization unless combined with information obtained from nuclear markers or geographic data [15].

Cyprinid fishes are known for frequent intergeneric hybridization and numerous different intrageneric and intergeneric hybrids have been described [16–21]. In the subfamily Leuciscinae, the nuclear region *Cyfun P* (Cyprinid formerly unknown nuclear Polymorphism) displays large intergeneric length variations caused by various deletion or insertion events. This nuclear region is useful for the detection of intergeneric hybridization [22,23]. Therefore, nuclear DNA is suitable for resolving relationships among higher taxonomic levels and detecting hybridization due to segregation of species-specific alleles. Due to the

fact that nuclear genes have a low evolutionary rate and are unlikely to undergo mutational saturation, they provide important information complementary to mitochondrial genes.

As suggested by Vadas & Orth [24], the habitat use of fishes as well as their associations within the habitat can provide us with additional information about possible hybridization events between species occupying the same habitat. From this point of view, *S. illyricus* is a benthopelagic species that inhabits clean, fast karst rivers and lakes with a water temperature of 5–25 °C. *S. zrmanjæ* is a rheophilic species that mainly inhabits karst streams but can also be found in lake areas. During sampling, these two species have been found at the same locations, potentially giving them the opportunity for hybridization, although their spawn timings have not been studied. Seeing hybridization as a viable mode of speciation [25], these two species have numerous ways and opportunities to develop all the possible different forms of this process. Being a Near-Threatened species with a very small distributional area, this is an extremely important issue, which is essential to the future conservation of these two species. Looking at the history of potential hybrids in fishes [25–28], it is evident that hybridization as a process is constantly present in nature, potentially as a possible mode of speciation and evolution, but it is also true that hybrids in fishes are difficult to detect and to determine. For certain species of fish that were supposed to be hybrids, it was found not to be. To prove this, it was necessary to apply modern techniques and methods that have been developed for years. Sequencing is a convenient tool for identifying differences that is especially applicable to small populations of native species that face extinction. However, determining whether a certain species is a hybrid or a valid species requires numerous other data.

The purpose of this study was to perform molecular analysis of specimens of *S. illyricus* and *S. zrmanjæ* from the Krka River, due to their morphological resemblance, in order to genetically characterize these species. The aim was to obtain insight into the phylogenetic structure of the closely related species and to verify the taxonomic status of these two species, which are endemic to Croatia with a relatively small distribution area. Using mitochondrial and nuclear markers, we tried to assess the possibility of hybridization. The results obtained will provide additional information on these two endemic species and stimulate further research and the necessary measures to protect these Near-Threatened species.

2. Materials and Methods

The Krka River belongs to the Adriatic Sea basin and is 72.5 km long with a total slope of 224 m. The hydrological basin of the Krka River covers an area of approximately 2500 km². It is not considered polluted [29], and forms the basis of the Krka National Park (NP). About 20 species of fish inhabit the Krka River, more than half of which are endemic to the Croatian ichthyofauna [2,4,30].

Seven sampling sites (Figure 2, Table 1) were selected along the length of the Krka River. Sampling for the molecular analyses was carried out during the field trips from December 2006 to October 2008.

The fish were sampled with an electrofishing device (Hans Grassl, EL63 II GI, 5.0 KW, Honda GX270, 300/600 V max., 27/15A max.) according to the Croatian standard: HRN EN 14011:2005 Water quality—Sampling of fish with electricity. The captured fish were kept alive in a tank with aerated water until further processing. The fish were anesthetized in a separate tank with buffered MS-222 (Sigma-Aldrich, Taufkirchen, Germany). Vouchers (fixation with buffered 4% formaldehyde, deposited in 75% ethanol) were deposited at the Faculty of Science, University of Zagreb, Croatia. Identification of fishes was performed according to Kottelat and Freyhof [1]. First, biometric data were recorded, including total length, standard length and total mass, while the Fulton condition index was later calculated according to Rätz and Lloret [31]. The tip of the anal fin was cut off and preserved in 96% ethanol or stored at −80 °C until further DNA analyses in the laboratory.

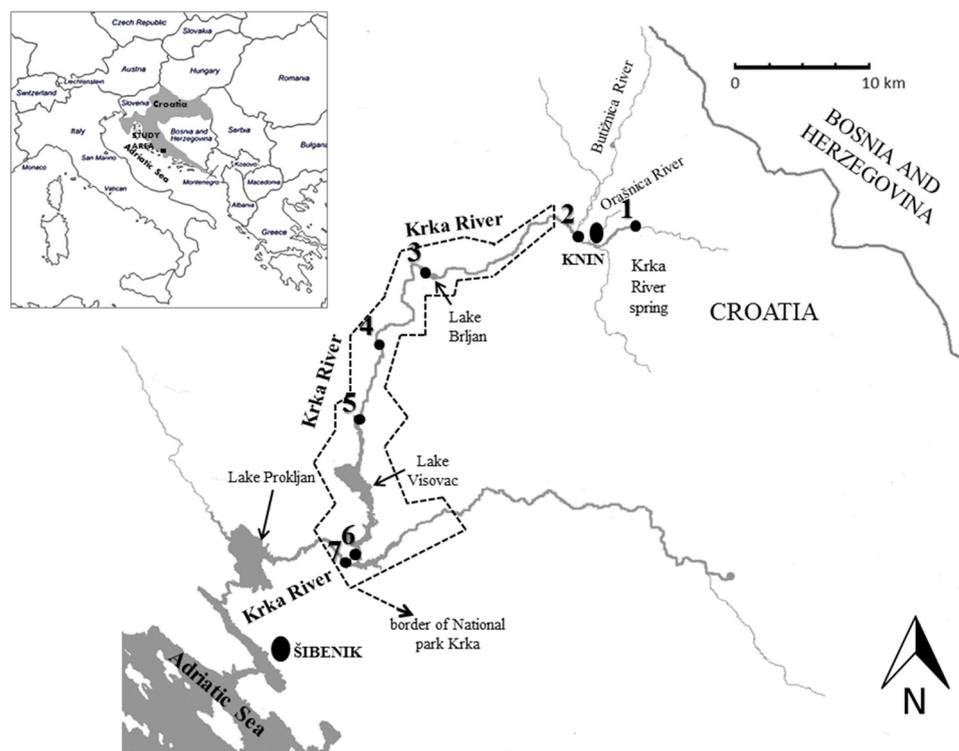


Figure 2. Study area marked with sampling sites along the length of the Krka River (1—Krka River spring; 2—Krka River upstream of the Butižnica River tributary; 3—Lake Brljan; 4—“Krka” monastery; 5—Roški Slap waterfall; 6—Lake Visovac; 7—Skradinski Buk waterfall).

Table 1. Sampling sites on the Krka River.

No.	Name of the Sampling Site	Coordinates
1.	Krka River spring	44°02.563' N, 16°14.412' E
2.	Krka River upstream of the Butižnica River tributary	44°02.295' N, 16°10.347' E
3.	Lake Brljan	44°00.343' N, 16°02.444' E
4.	“Krka” monastery	43°57.538' N, 15°59.833' E
5.	Roški Slap waterfall	43°54.140' N, 15°58.815' E
6.	Visovac Lake upstream from waterfall Skradinski Buk	43°48.349' N, 15°58.693' E
7.	Skradinski Buk waterfall	43°48.426' N, 15°58.110' E

A total of 237 specimens of *S. illyricus* and 267 of *S. zrmanjæ* were caught in the Krka River during sampling, representing 18.96% and 21.38% of the total catch, respectively (Table S1). Altogether, 30 specimens were subjected to DNA analyses, 15 from each species. The partial *cyt b* sequence (1140 bp) was used for phylogenetic analysis. Newly obtained *cyt b* sequences were uploaded into the GenBank database (GenBank ID: JQ663535.1, JQ663536.1 and OR791603-OR791610). The nucleotide composition of the *cyt b* was in accordance with previous findings on closely related genera [11,32–37].

Total genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the spin-column protocol. The partial *cyt b* gene (1140 bp) was amplified in two overlapping fragments using the following primers: L15267 (5'-AATGACTTGAAGAACCACCGT-3') and H16526 (5'-CTTTGGGAGYRRGGGTGRGA-3') [36]. The PCR reaction mixtures contained 4 U AmpliTaq DNA Polymerase (Applied Biosystems, Waltham, MA, USA), 1 × PCR buffer (without MgCl₂, Applied Biosystems, Waltham, MA, USA), 2.5 mM MgCl₂, 200 μM dNTPs (Sigma, Steinheim, Germany), 400 nM of each primer and approximately 5 μg/μL of DNA in a final volume of 100 μL. Reactions were subjected to the following thermocycling protocol: initial denaturation (94 °C: 3 min), 30 cycles (94 °C: 30 s; 55 °C: 30 s; 72 °C: 1 min) and final extension (72 °C: 7 min). PCR

products were examined on a 1.7% agarose gel using electrophoresis and subsequently purified (QIAquick Gel Extraction Kit, Qiagen, Hilden, Germany). Sequencing was performed using the ABI PRISM[®] 3100 Avant Genetic Analyzer (Ruđer Bošković Institute DNA service) or at Macrogen Inc. in Seoul, Republic of Korea.

To test for putative hybridization and to support the *cyt b* results, the highly variable noncoding nuclear region, *Cyfun P* [23], was sequenced. Primers for this nuclear region have already been used for several species of fish [22]: *Cyp_unFLP1F* (5'-AAGTGGTGC-ATCGTGTGTG-3') and *Cyp_unFLP1R* (5'-CAGCCTGAACAATCAAAACAG-3'). The PCR reaction mixtures were identical to those used for *cyt b*, but the cycling protocol was somewhat different: initial denaturation (94 °C: 3 min), 35 cycles (94 °C: 15 s; 55 °C: 20 s; 72 °C: 45 s) and final extension (72 °C: 7 min). Purification and further sequencing of the PCR products were performed as described for *cyt b*.

The BLAST network service (<http://www.ncbi.nlm.nih.gov>, accessed on 13 November 2023) was used for sequence homology search. Multiple alignments were performed with CLUSTALW Ver. 1.6 [38] using the default parameters. Ambiguously aligned regions were determined using the program Gblocks 0.91b under less stringent parameters [39] and excluded from further analyses. Aligned sequences were imported into MEGA version 6 [40], where the phylogenetic relations of sequences in the datasets were analyzed using maximum likelihood (ML) [41]. The tench *Tinca tinca* (L. 1758) (Tincidae) was selected as the outgroup. Support for the nodes in the trees was estimated via bootstrapping (1000 bootstrap replicates in ML). The model for the ML analysis was selected using Modeltest 3.7 [42]. The Akaike Information Criterion (AIC) indicated TrN + I (Tamura Nei with invariant sites) for the *cyt b* and HKY + G (Hasegawa–Kishino–Yano with gamma distributed sites) for the *Cyfun P*. Initially, a large maximum likelihood tree was constructed, containing almost 150 total sequences from various species. The phylogenetic tree was then reduced to the current number of sequences by eliminating the sequences. Pruning was performed by hand, leaving at least one specimen of each original species and taking into consideration only the most unusual and distant sequences. To ensure the correctness of the pruned tree, a new maximum parsimony (MP) [43] tree containing all original sequences was constructed and compared with the pruned tree to ensure that no major differences occurred.

3. Results

S. illyricus and *S. zrmanjae* showed differences in nucleotide composition; A:T:G:C were 26.1:28.4:16.8:28.6 and 27.0:28.6:15.9:28.5, respectively. The sequence identity matrix showed 0.918 similarity between these two species from the Krka River.

Phylogenetic relation between *S. illyricus* and *S. zrmanjae* from the Krka River was established in the phylogenetic analysis, which included 44 sequences of closely related species obtained from GenBank, the majority of which originated from the work of Perea et al. [44]. Previously known and published sequences for these two species (GenBank ID: AJ251092.1, AJ251093.1, AJ251094.1, HM560183.1, HM560184.1, HM560213.1, MN166108.1 and MN166113.1) were also included in the analysis (Figure 3). Our *S. illyricus* sequences (GenBank ID: JQ663535.1 and OR791603-OR791607) grouped together with an *S. illyricus* (GenBank ID: MN166108.1) from the Krka River and other *S. illyricus* (GenBank ID: AJ251094.1, HM560183.1 and HM560184.1) haplotypes from the Cetina River (Croatia) with high bootstrap values. *S. zrmanjae* (GenBank ID: JQ663536.1 and OR791608-OR791610) grouped together with another *S. zrmanjae* (GenBank ID: AJ251092.1) from the Krka River (Croatia). The positions of investigated *S. illyricus* and *S. zrmanjae* from the Krka River in the phylogenetic tree indicate two divergent species.

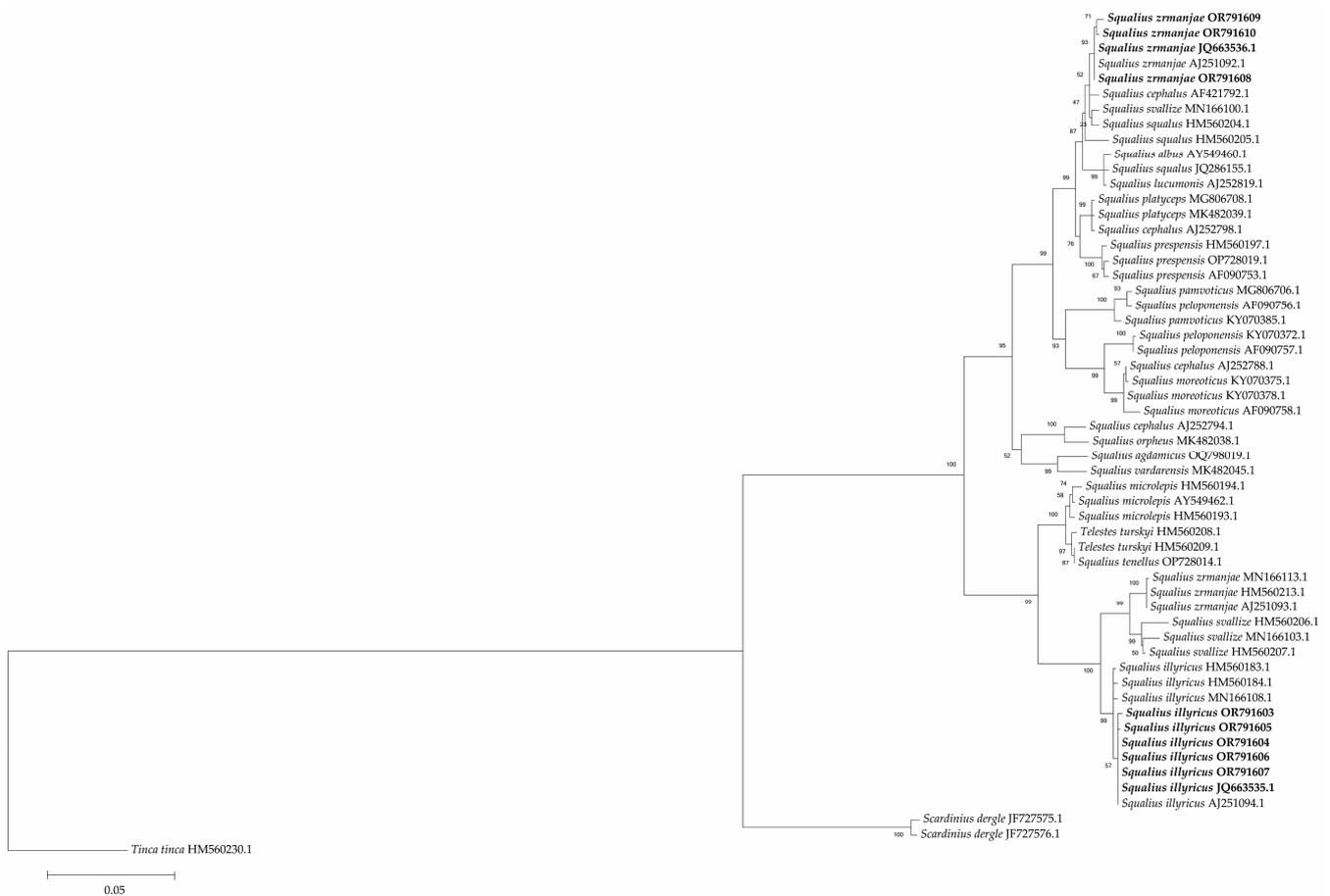


Figure 3. Phylogenetic relation of *Squalius illyricus* and *Squalius zrmanjae* (obtained sequences are shown in bold) based on mitochondrial cytochrome *b* (cyt *b*) nucleotide sequences. Bootstrap values (1000 replicates) are indicated by the line.

The same specimens subjected to cyt *b* analysis were used for characterization of the highly variable noncoding nuclear region, Cyfun P. The sequence identity matrix showed relatively low similarity between *S. illyricus* and *S. zrmanjae* from the Krka River (0.774), as base composition, A:T:G:C, was 39.8:28.4:16.2:15.5% and 35.0:31.2:16.9:16.9%, respectively. Further, the nucleotide sequence length of this marker was different in these two species, 394 and 343 bp, respectively. The Cyfun P sequences obtained were uploaded into the GenBank database (GenBank ID: JQ663537.1, JQ663538.1 and OR791611-OR791614).

Because hybridization of the species can be demonstrated with this marker, a phylogenetic analysis of the noncoding region was performed. The results show that the analyzed species were separated into two clusters supported by the high bootstrap values. *S. illyricus* was grouped with the *Squalius* species and *S. zrmanjae* with the *Scardinius* species (Figure 4). The sequence identity matrix showed a high similarity between *S. zrmanjae* and, the Dalmatian rudd, *Scardinius dergle* Heckel & Kner 1858 (GenBank ID: JF727578.1) from the Krka River (1.000), but also with the other sequences of *S. dergle* (GenBank ID: JF727577.1. and AY831428.1).

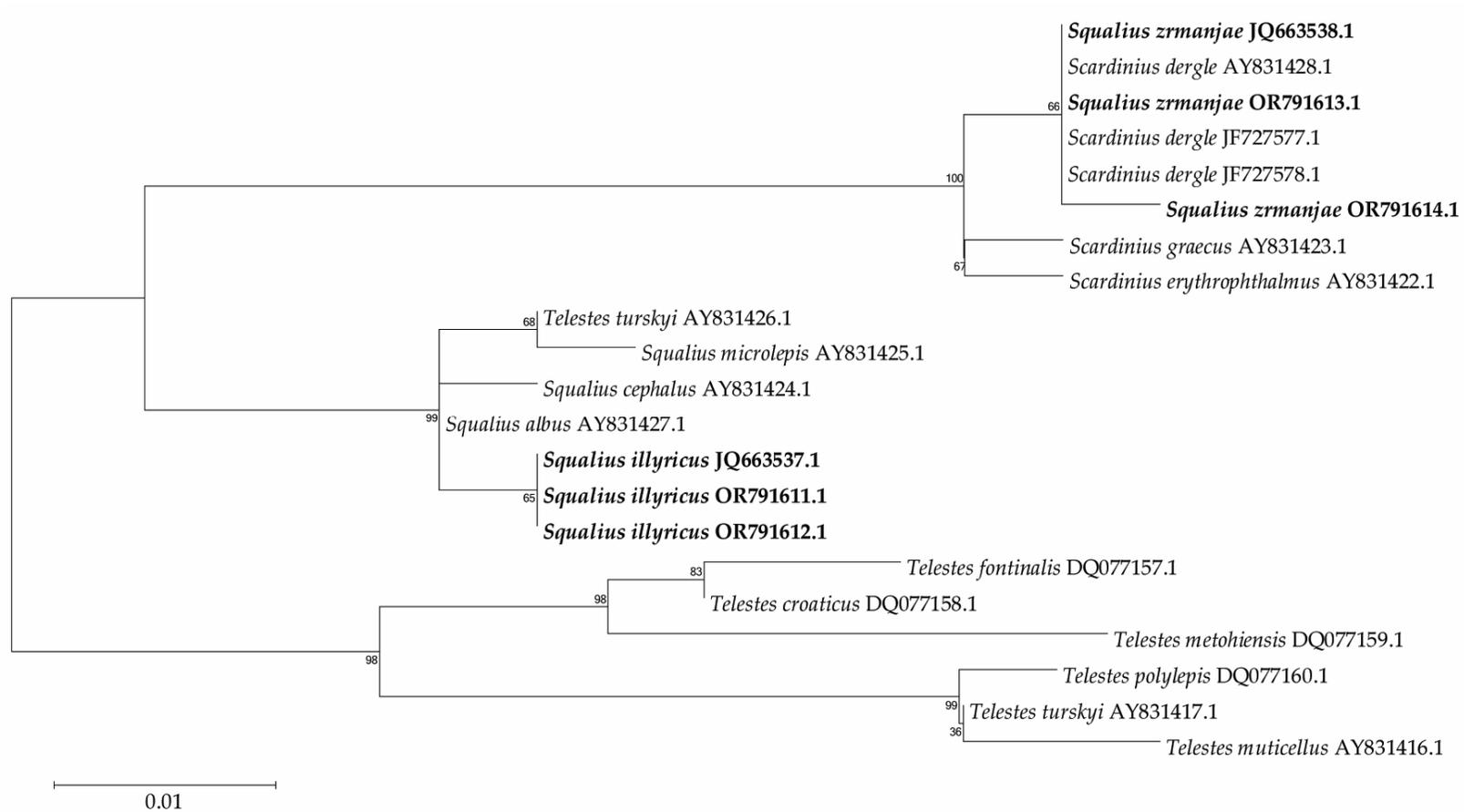


Figure 4. Phylogenetic position of *Squalius illyricus* and *Squalius zrmanjae* (obtained sequences are shown in bold) based on nuclear Cyfun P nucleotide sequences. Bootstrap values (1000 replicates) are indicated by the line.

4. Discussion

The analysis based on *cyt b* (Figure 3), showed that the *S. illyricus* haplotypes from the Krka River (GenBank ID: JQ663535.1 and OR791603-OR791607) were grouped in a cluster with the *S. illyricus* haplotypes (GenBank ID: AJ251094.1, HM560184.1, HM560183.1 and MN166108.1) from the Cetina River in Croatia. Because these two rivers are adjacent, this result was to be expected.

On the other hand, in the reconstructed phylogenetic tree there are two distinct, distant groups comprising the haplotypes of *S. zrmanjae*. In one group, which is closely related to the group of *S. illyricus*, there is one haplotype of *S. zrmanjae* from the Krka River (GenBank ID: MN166113.1) and two from the Zrmanja River, Croatia (GenBank ID: AJ251093.1 and HM560213), as well as three haplotypes of the closely related species *S. svallize* (GenBank ID: HM560206.1, HM560207.1 and MN166103.1) from Bosnia and Herzegovina. In the other group, which is distant from the previous one, our haplotypes of *S. zrmanjae* (GenBank ID: JQ663536.1 and OR791608-OR791610) are grouped together with another one from the Krka River (GenBank ID: AJ251092.1). The grouping of two haplotypes from the Krka River is to be expected, but it is curious that these two groups of *S. zrmanjae* are so far apart in the phylogenetic tree. The reason for this is probably that the haplotypes from the Krka and Zrmanja River do not belong to the same species. Although they are geographically close to each other (the Krka and Zrmanja river basins are connected through the karstic terrain), DNA sequences of fish from these two rivers comprise different species. It should be noted that the different grouping of haplotypes of *S. zrmanjae* probably presents two different and distinct species. This issue should be resolved with a larger number of samples of fishes from these two rivers.

The phylogenetic tree based on the highly variable noncoding nuclear region, Cyfun P, positions *S. illyricus* with other *Squalius* species and *S. zrmanjae* with *Scardinius* species (Figure 4). These results, together with the sequence identity matrix (high similarity between *S. zrmanjae* and *S. dergle* (GenBank ID: JF727578) from the Krka River—1.000), imply that our species, *S. zrmanjae*, in addition to obvious morphological characters of the genus *Squalius* (Table 2), had the nuclear region, Cyfun P, that resembles the genus *Scardinius*. “*S. zrmanjae*” could be a hybrid, and our results demonstrate an introgression event and transfer of genetic information between these two cyprinid genera. This would not be the first case of a hybrid specimen between *S. dergle* and a representative of the genus *Squalius*. In the work of Freyhof et al. [33], a hybrid specimen of “*S. dergle*” had a *cyt b* sequence similar to the sympatric species *S. tenellus*, although it did not show morphological characteristics of the genus *Squalius*. The authors demonstrated a transfer of genetic information between two related but distant cyprinid genera. The genera *Scardinius* and *Squalius* separated phylogenetically in the middle Miocene, approximately 10 Ma [45].

Table 2. Morphological characters of the genus *Squalius* and *Scardinius* [1,33].

Morphological Characters	<i>Scardinius</i>	<i>Squalius</i>
Body shape	Compressed	Cylindrical
Shape of the posterior anal-fin margin	Concave	Convex
Mouth position	Superior	Terminal
Pharyngeal teeth	Slightly serrated	Smooth
Branched anal rays	10	8–9½ *
Gill rakers	10–13	12–16 **
Lateral line scales	38–41	46–54 or 44–49 *

* *S. illyricus* and *S. zrmanjae*; ** *S. tenellus*.

Hybridization between species involves mating between unrelated organisms regardless of the taxonomic status and in some cases may lead to gene transfer, a complex evolutionary process occurring in freshwater fishes [34]. Introgression occurs when hybrids backcross with one or both parental species [46]. For freshwater fish, cyprinids are known to exhibit higher rates of hybridization than other groups of fish, which is especially

evident for Leuciscidae, for both interspecific introgressions [16,21,47,48] and intergeneric hybrids [33,49–52]. Of course, there are numerous cases of introgressive hybridization in other groups of fish [53–56], but at the beginning of the century, already, 62 different intra- and intergeneric hybrids were described for leuciscine species in the wild [19].

The geographic proximity of the investigated species explains the close phylogenetic relationship with the Italian species. The investigated species grouped together with the species from other countries in this region, which is in agreement with the hypothesis of peri-Mediterranean dispersal of freshwater fishes. This explanation concurs with our haplotypes of *S. zrmanjae* in the phylogenetic tree. The species related to our specimen occur in Albania, Greece, Italy, Slovenia and, of course, Croatia. Other haplotypes of *S. zrmanjae* from GenBank group together with our *S. illyricus* haplotypes. The majority of these haplotypes consist of native species from Croatia or the nearby countries of Bosnia and Herzegovina and Italy.

Research from Ketmaier et al. [57] supports the hypothesis of peri-Mediterranean dispersal of freshwater fishes during the “Lago Mare” phase of the Mediterranean Sea [58]. While this is accurate for the genus *Telestes*, the genus *Scardinius* had a different dispersal route. The intraspecific divergence of the genus *Scardinius* occurred between 1.35 ± 1 Ma [57]. Sea-level decline during the Messinian crisis probably increased the isolation of certain populations [59]. Future research on other groups of fish with a similar pattern of geographic dispersal can provide insight into the history and evolutionary relationships between species of fish, but also could clarify the events that led to the origin of new species and their dispersal in this region. The work of Sabolić et al. [60] on the morphological diversity and relationships of the populations of *S. dergle* and *S. plotizza* from Croatia and Bosnia and Herzegovina helps to clarify the taxonomic relationships and population status of one of the investigated genera in this work, but there is certainly a need for further molecular research on the phylogenetic relationships and taxonomy of these species.

Further work on the genus *Squalius* is needed, possibly with the introduction of additional markers (mitochondrial *COXI* and nuclear *RAG-1* and *S7* genes), as used by Buj et al. [35], and more samples from small tributaries should be included in this research. This is necessary because we found that the existing mitochondrial haplotypes of *S. zrmanjae* actually represent two different and distant species in the phylogenetic tree. Although further progress has been made on this topic [35], a similar finding has not been discovered. The authors were able to identify hybrid individuals among the *Squalius* species, confirming that hybridization is a widespread phenomenon in the Adriatic region. The haplotype from the Krka River is probably a hybrid whose mitochondrial genome originates from the species *S. dergle*, although morphological characteristics indicate a similarity with the genus *Squalius*. Buj et al. [35] found one sample from the Zrmanja River with *S. illyricus* mtDNA and the other from the Krka River with *S. zrmanjae* mtDNA. This shows that mtDNA intrageneric introgression and, in our case, intergeneric introgression are present.

Knowledge of the systematic relationships in the Croatian freshwater ichthyofauna is still incomplete, and further molecular analyses are required to solve taxonomic and systematic problems where morphology is not sufficient. There are 52 native species of fish in Croatian freshwaters [2] and intensive research is of great importance for their protection. If the probability of hybridization between endemic species of two different genera is relatively high, as we have found, then the importance of further molecular as well as morphological research of these species becomes even greater.

5. Conclusions

The aim of this study was to investigate both mitochondrial and nuclear markers of two native and Near-Threatened species. These species have very small distribution areas and phylogenetic analysis is of the utmost importance in addition to morphological analysis. Using the mitochondrial marker *cyt b*, we confirmed the previous phylogenetic position of the investigated species. However, the results of the nuclear marker indicated

that *S. zrmanjae* may be a hybrid and that there may have been a transfer of genetic information between two cyprinid genera, *Squalius* and *Scardinius*. For conservation measures, the occurrence of hybridization presents a potential problem that needs to be resolved. Following the guidelines proposed by Allendorf et al. [28], further genetic investigations with additional markers (e.g., COI, RAG1) are needed to identify which type of hybridization occurred, define hybrid zones and accordingly consider which protection measures would be most efficient.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/fishes9010004/s1>, The data in supplementary Table S1 are available from the corresponding author upon request.

Author Contributions: D.V., V.B. and E.T. developed the original research idea and designed the molecular analysis. D.V. and E.T. carried field sampling. D.V. performed molecular analysis, made analysis of aligned sequences and wrote the paper. M.K.M. performed analysis of aligned sequences. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The fish were returned to the wild after being held in an aerated water tank. No ethical review or approval was needed for this study.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data available in a publicly accessible repository (GenBank ID: JQ663535.1-JQ663538.1 and OR791603-OR791614).

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Conflicts of Interest: The authors declare no conflict of interest.

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