



Phylogenetic relationships among Southern Balkan *Rutilus* species inferred from cytochrome *b* sequence analysis: Micro-geographic resolution and taxonomic implications



Miranta Tsoumani^a, Andreas Georgiadis^b, Ioannis A. Giantsis^b,
Ioannis Leonardos^a, Apostolos P. Apostolidis^{b,*}

^aLaboratory of Zoology, Biological Applications and Technology Department, University of Ioannina, Ioannina, Greece

^bDepartment of Animal Production, School of Agriculture, Faculty of Agriculture, Forestry and Natural Environment, Aristotle University of Thessaloniki, Thessaloniki, Greece

ARTICLE INFO

Article history:

Received 17 November 2013

Accepted 1 February 2014

Available online 22 February 2014

Keywords:

Rutilus species
Southern Balkan lakes
Phylogeny
Sequence analysis
Cytochrome *b*
Taxonomy

ABSTRACT

The phylogenetic relationships and geographic distribution of Greek roaches (*Rutilus* spp.) were investigated by analyzing the complete mitochondrial cytochrome *b* gene sequence of 84 specimens collected from 15 southern Balkan lakes. Phylogenetic analysis revealed the existence of five highly divergent haplotype groups with mean pairwise sequence divergence between them ranging from 4.1 to 9%, namely at the level of values reported for different species. These five groups correspond to four species namely *Rutilus rutilus*, *Rutilus prespensis*, *Rutilus panosi* and *Rutilus ylikiensis*. On the other hand, the existence of two highly divergent haplotype groups, which are currently attributed to *R. rutilus*, suggests the re-examination of the current taxonomic status of this species.

© 2014 Elsevier Ltd. All rights reserved.

1. Introduction

The clarification of the taxonomic status of individual species aids greatly in the proper managing of their populations, in establishing regulations regarding their exploitation, in evaluating the impact of human interventions and in recognizing and protecting those at risk (Lillywhite and Lee, 2011; Paquin and Hedin, 2004). Fish species taxonomy was traditionally based on morphological features; however, the advent of molecular genetic techniques gave us the opportunity to re-examine the existing classification and answer many unresolved problems, especially in cases of closely related species with overlapping habitats (Apostolidis et al., 2011).

This is particularly true in the case of southern Balkan which is regarded as the most diverse ichthyofauna in Europe (Economidis and Banarescu, 1991).

The genus *Rutilus* (Rafinesque, 1820), which is placed in the Cyprinidae family, is one of the most common and widely distributed in the Central and Western Palearctic region. It includes at least 16 species (<http://www.fishbase.org>), which show a clear preference for riverine and lacustrine warm waters (Tsoumani et al., 2013). *Rutilus* species are among the most common cyprinids of the Balkan Peninsula, an area harbors a large number of endemic taxa. Thus, while three *Rutilus* species

* Corresponding author. Tel./fax: +30 2310998683.

E-mail address: apaposto@agro.auth.gr (A.P. Apostolidis).

have been recognized throughout Central Europe (Ketmaier et al., 2008), at least four have been reported in the lakes of inland Greece (*Rutilus prespensis*, *Rutilus panosi*, *Rutilus ylikiensis* and *Rutilus rutilus*) (Tsoumani et al., 2013).

A number of morphological studies on Greek roaches have been carried out to clarify their distribution (Economidis, 1991; Economidis and Banareescu, 1991; Economou et al., 2007; Kottelat and Freyhof, 2007; Stephanidis, 1950), and to investigate their phylogenetic relationships (Bogutskaya and Iliadou, 2006; Tsoumani et al., 2013). However, molecular approaches have been confined to scattered individuals embodied in three broad-scale genetic studies, of which the first concerned the phylogeny of the European cyprinids (Zardoya and Doadrio, 1999), the second studied *Rutilus* specimens from Central and Eastern Europe (Ketmaier et al., 2008) and the third focused on the species *R. rutilus* (Larmuseau et al., 2009). In addition, in a recent study, Milosevic et al. (2011) explored *Rutilus* specimens from the Lake Prespa, which is shared by Albania, Greece and FYROM. Despite the significant contribution of these studies in *Rutilus* taxonomy, the phylogeny and distribution of the numerous *Rutilus* populations in Greece remains under debate (Tsoumani et al., 2013); therefore a more assiduous genetic study is clearly needed in order to clarify these issues.

In this study we report on DNA sequence variation of the full mitochondrial (mt) cytochrome *b* (*cytb*) gene. The new sequences were analyzed together with previously published *cytb* sequences in order to examine the validity of the current taxonomy and to compare the phylogenetic relationships among Greek populations of *Rutilus* sp. inferred from molecular data with those based on morphological characters.

2. Materials and methods

During the autumn of 2007 and 2008, 84 *Rutilus* specimens were collected from 15 southern Balkan lakes (Fig. 1, Table 1). Sampling was followed by genomic DNA isolation from muscle tissue, according to a standard protocol of C-TAB/Phenol/Chloroform (Hillis et al., 1996). Thereafter, a pair of primers designed by Kotlik et al. (2008) was used to amplify the complete mitochondrial *cytb* gene under the following thermal-cycling conditions: initial denaturation at 95 °C for 3 min, 30 cycles of 95 °C (30 s) – 61 °C (20 s) – 72 °C (30 s), and a final extension at 72 °C for 5 min. Each amplification reaction was performed in 60 µl volume containing the following reagents: 2.4 units of *Taq* DNA polymerase, 1 × reaction buffer (HyTest Ltd., Finland), 2 mM MgCl₂, 0.2 mM of each dNTP and 24 pmol of each primer. The amplified products were purified using the NucleoSpin Clean-up Kit (Macherey–Nagel, Germany) and double strand sequenced with the same pair of primers used for PCR amplification, by VBC-Biotech (Vienna, Austria). Sequences were aligned and edited using the BioEdit 7.1.9 software (Hall, 1999).

The resulted sequences were analyzed together with twelve GenBank-obtained *Rutilus* records: nine Greek *Rutilus* haplotypes (Table 1), two haplotypes of *Rutilus frisii* (EU285042, EU285048) and one of *Rutilus ohridanus* (FJ025085). Also, two *cytb* records of *Leuciscus cephalus* (AY509827) and *Telestes souffia* (AY509859) were used as outgroups (Fig. 2). The software DNASP 5.0 (Librado

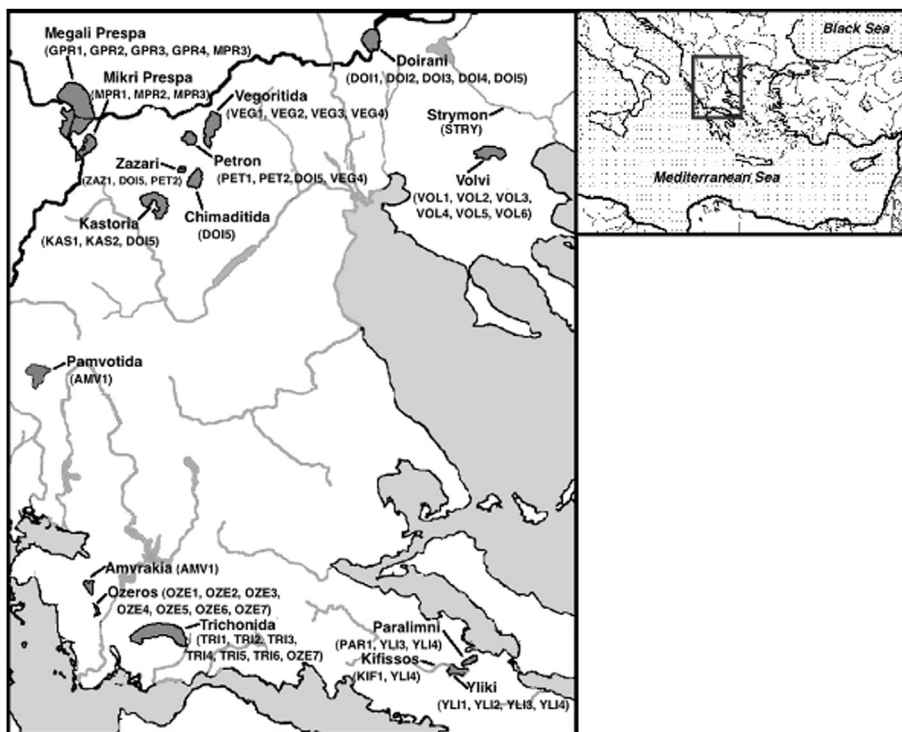


Fig. 1. Map of mainland Greece that shows the locations of the 15 lakes and 2 rivers from which *Rutilus* specimens were collected. The haplotypes found in each lake, as coded in Table 1, are shown in brackets.

Table 1

Sampling locations, sample sizes (*N*), taxa, number of haplotypes scored (*H*), Haplotype codes and GenBank Accession Numbers of the Greek *Rutilus* specimens from the 15 lakes and the 2 rivers included in the present study.

| Location | <i>N</i> | Taxon | <i>H</i> | Haplotype code (Number encountered) | Accession No./Reference |
|--------------------|----------|----------------------|----------|----------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------|
| Lake Doirani | 5 | <i>R. rutilus</i> | 5 | DOI1 (1) DOI2 (1) DOI3 (1) DOI4 (1) DOI5 (1) | KF784819 KF784820 KF784821 KF784822 KF784808 |
| Lake Volvi | 8 | <i>R. rutilus</i> | 6 | VOL1 (2) VOL2 (1) VOL3 (1) VOL4 (1) VOL5 (1) VOL6 (3) | KF784810 KF784811 KF784812 KF784839 KF784838 FJ025074 ^a |
| Lake Vegoritida | 6 | <i>R. rutilus</i> | 4 | VEG1 (1) VEG2 (1) VEG3 (1) VEG4 (3) | KF784815 KF784814 KF784813 FJ025073 ^a |
| Lake Petron | 5 | <i>R. rutilus</i> | 4 | PET1 (1) PET2 (1) DOI5 (1) VEG4 (2) | KF784831 KF784832 KF784808 FJ025073 ^a |
| Lake Zazari | 5 | <i>R. rutilus</i> | 3 | ZAZ1 (1) DOI5 (3) PET2 (1) | KF784841 KF784808 KF784832 |
| River Strymon | – | <i>R. rutilus</i> | 1 | STRY | AF090772 ^b |
| Lake Chimaditida | 5 | <i>R. rutilus</i> | 1 | DOI5 (5) | KF784808 |
| Lake Kastoria | 5 | <i>R. rutilus</i> | 3 | KAS1 (1) KAS2 (1) DOI5 (3) | KF784833 KF784840 KF784808 |
| Lake Mikri Prespa | 7 | <i>R. prespensis</i> | 3 | MPR1 (4) MPR2 (1) MPR3 (2) | KF784842 KF784845 FJ025062 ^a |
| Lake Megali Prespa | 7 | <i>R. prespensis</i> | 5 | GPR1 (1) GPR2 (1) GPR3 (1) GPR4 (1) MPR3 (3) | KF784843 KF784844 KF784846 AF090771 ^b FJ025062 ^a |
| Lake Amvrakia | 5 | <i>R. panosi</i> | 1 | AMV1 (5) | KF784809 |
| Lake Pamvotida | 5 | <i>R. panosi</i> | 1 | AMV1 (5) | KF784809 |
| Lake Ozeros | 8 | <i>R. panosi</i> | 7 | OZE1 (1) OZE2 (1) OZE3 (2) OZE4 (1) OZE5 (1) OZE6 (1) OZE7 (1) | KF784823 KF784824 KF784825 KF784826 KF784836 KF784837 KF784816 |
| Lake Trichonida | 9 | <i>R. panosi</i> | 7 | TRI1 (1) TRI2 (1) TRI3 (1) TRI4 (1) TRI5 (2) TRI6 (1) OZE7 (2) | KF784817 KF784818 KF784834 KF784835 FJ025071 ^a AF090774 ^b KF784816 |
| Lake Yliki | 6 | <i>R. ylikiensis</i> | 5 | YLI1 (1) YLI2 (2) YLI3 (1) YLI4 (1) | KF784827 KF784829 KF784828 FJ025070 ^a |
| Lake Paralimni | 4 | <i>R. ylikiensis</i> | 3 | PAR1 (1) YLI3 (2) YLI4 (1) | KF784830 KF784828 FJ025070 ^a |
| Kifissos River | – | <i>R. ylikiensis</i> | 2 | KIF1 YLI4 | AF090773 ^b FJ025070 ^a |

^a Ketmaier et al. (2008).^b Zardoya and Doadrio (1999).

and Rozas, 2009) was used to calculate the average pairwise sequence divergence across taxa. The phylogenetic analysis was performed following the neighbor-joining, maximum parsimony and maximum likelihood approaches with 1000 bootstrap replicates, using the software MEGA 5.05 (Tamura et al., 2011). Additionally, the Bayesian analysis was performed using the software BEAST 1.7.5 (Drummond et al., 2012). All analyses were performed using the default settings of the programs.

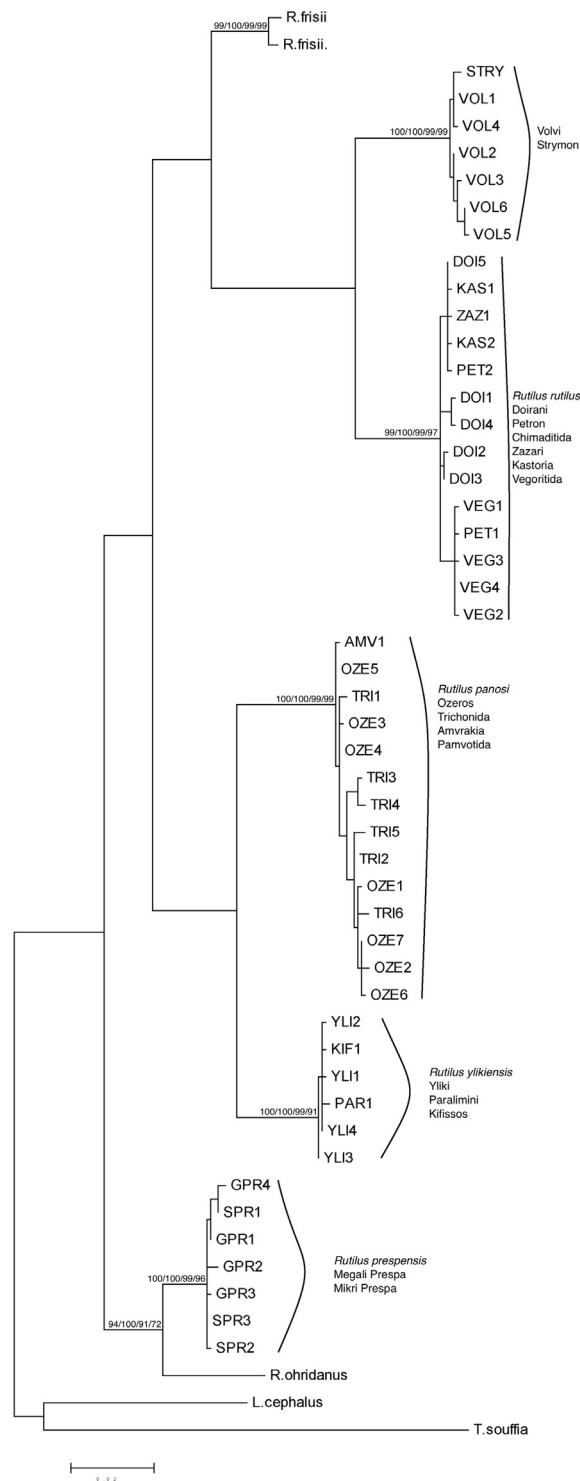


Fig. 2. Consensus dendrogram of the four phylogenetic methods among the different *Rutilus* haplotypes found based on the sequence analysis of the *cyt b* gene. Bootstrap values and posterior probabilities greater than 70% are shown for the main clades as follows: ML/NJ/MP/Bayesian.

3. Results

The sequencing of the complete *cytb* gene (1141 bp) from 84 *Rutilus* specimens revealed a total of 186 variable sites and 44 different haplotypes, 39 of which were new and deposited in GenBank (accession numbers KF784808–KF784846, Table 1).

The number of haplotypes found in each population range from one (Chimaditida, Pamvotida and Amvrakia) to seven (Ozeros and Trichonida), whereas most of the haplotypes found were unique i.e. except from eight haplotypes that were observed in more than one populations (DOI5, VEG4, PET2, MPR3, AMV1, OZE7, YL13, YL14), all others were observed in only one population (Table 1 and Fig. 1).

The phylogenetic relationships within *Rutilus* sp were inferred using four different methods (ML, NJ, MP and Bayesian) which all concluded in highly congruent tree topologies (Fig. 2). In particular, the *Rutilus* haplotypes found in southern Balkan populations were clustered into five strongly supported phylogenetic groups (clades) separated by mean sequence divergence estimates ranging from 4.1 to 9% (Table 2). The first group consist of haplotypes found in populations of Yliki, Paralimni and Kifissos river; the second comprises haplotypes found in lakes Ozeros, Trichonida, Amvrakia and Pamvotida; the third group consist of haplotypes found in Mikri and Megali Prespa; the fourth comprises of haplotypes found in the lakes Doirani, Vegoritida, Chimaditida, Petron, Zazari and Kastoria; and the fifth group consist of the 6 haplotypes found in lake Volvi and of the haplotype found in one individual from the Strymon river (Table 1, Fig. 2).

4. Discussion

The distribution of *Rutilus* species in Southern Balkan Peninsula appears to follow the biogeographical pattern of other European cyprinids, whereas they are considered of Plio-Pleistocene origin (Tsoumani et al., 2013 and references therein). In the last decades, there have been several revisions in the taxonomy and nomenclature of Greek roaches (Bogutskaya and Iliadou, 2006; Economidis and Banarescu, 1991; Economou et al., 2007; Kottelat and Freyhof, 2007), indicating the difficulty in reconstructing their phylogeny and classification. However, with the advent of molecular genetic techniques we have now an opportunity to improve our knowledge and resolution in *Rutilus* taxonomy. The results of this study suggest the clustering of the Southern Balkan (Greek) populations of *Rutilus* into five strongly supported clades (Fig. 2), with levels of mean pairwise sequence divergence (Table 2) comparable with those reported for different cyprinid species (Larmuseau et al., 2009 and references therein). Moreover, our results are generally in accordance with the results derived from a recent morphological study of roaches coming from the same locations (Tsoumani et al., 2013), although it should be noted that the significant differentiation of the population from Lake Volvi is more profound in the present study (Fig. 2). Nevertheless, to verify the results of mtDNA sequence analysis, particularly those regarding the classification and taxonomy of the *Rutilus* species, further genetic studies using nuclear DNA markers are required.

4.1. *R. ylikiensis* and *R. panosi*

All roaches from central continental Greece (Sterea Ellada) were initially classified as *R. ylikiensis*. However, Bogutskaya and Iliadou (2006) proposed that the *Rutilus* populations of Central-Western Greece belong to a new species named *R. panosi*, and that the *R. ylikiensis* is restricted only in the central-eastern Greece. Our data fully support the existence of two distinct *Rutilus* species as proposed by Bogutskaya and Iliadou (2006), as the group of haplotypes found in Yliki, Paralimni, and Kifissos River (*R. ylikiensis*, Central-Eastern Greece) is highly divergent from the group of those found in Ozeros, Trichonida, Amvrakia and Pamvotida (*R. panosi*, Central-Western Greece) (Fig. 2). The differentiation between the central-eastern and the central-western *Rutilus* populations can be attributed to Pindos mountain range, which influenced greatly the demography and distribution of fish species (Economou et al., 2007). The fact that the populations of lake Pamvotida and lake Amvrakia exhibit the same fixed haplotype (Table 1), could be attributed to previous stocking activities. Actually, there are some evidence of fish stock introduction in Lake Pamvotida at mid-50s (Leonardos et al., 2008), and is likely that this stock was originated from the lake Amvrakia. Finally, it should be noted that the species *R. ylikiensis* faces serious survival threats, as Yliki lake and Kifissos River are used for water supplying and irrigation purposes, respectively (Freyhof and Kottelat, 2008).

4.2. *R. prespensis*

The *Rutilus* populations that inhabit the neighbor lakes Megali and Mikri Prespa were previously classified as *R. ohridanus prespensis* (Economidis, 1991), and were considered endemic to these lakes (Bogutskaya and Iliadou, 2006; Crivelli et al., 1997). However, Kottelat and Freyhof (2007) classified these populations as a new species, *R. prespensis*, which, according

Table 2
Values of the average pairwise sequence divergence.

| | <i>R. rutilus</i> | Volvi/Str | <i>R. prespensis</i> | <i>R. panosi</i> | <i>R. ylikiensis</i> | <i>R. frisii</i> | <i>R. ohridanus</i> |
|----------------------|-------------------|-----------|----------------------|------------------|----------------------|------------------|---------------------|
| <i>R. rutilus</i> | – | | | | | | |
| Volvi/Strymon | 0.04098 | – | | | | | |
| <i>R. prespensis</i> | 0.07890 | 0.08050 | – | | | | |
| <i>R. panosi</i> | 0.08865 | 0.08606 | 0.06299 | – | | | |
| <i>R. ylikiensis</i> | 0.08790 | 0.08980 | 0.05774 | 0.04380 | – | | |
| <i>R. frisii</i> | 0.06017 | 0.06278 | 0.05248 | 0.06382 | 0.05960 | – | |
| <i>R. ohridanus</i> | 0.08116 | 0.08596 | 0.04808 | 0.09502 | 0.08924 | 0.07943 | – |

to Ketmaier et al. (2008) and Milosevic et al. (2011), inhabits also the Lake Skadar in sympatry with *Rutilus albus*. Our findings support further this classification, as the haplotypes found in Megali and Micri Prespa were clustered in a group significantly divergent from the other Greek *Rutilus* haplotypes and also from the *R. ohridanus* (Fig. 2). The high genetic divergence between *R. prespensis* and *R. rutilus*, despite their habitat proximity (Fig. 1), could be attributed to the mountainous terrain of the area between the two regions, which probably acted as a physical barrier promoting genetic divergence.

4.3. *R. rutilus*

In the present study, the species *R. rutilus* is represented by specimens from seven lakes of Northern Greece; Doirani, Vegoritida, Kastoria, Zazari, Chimaditida, Petron and Volvi. However, the mtDNA haplotypes found in Volvi were highly divergent and clustered, together with the haplotype found in Strymon River, in a separate, highly supported, clade (Fig. 2). Notably, significant differentiation between the *Rutilus* specimens of Lake Volvi and those of Vegoritida and Doirani was previously reported by Ketmaier et al. (2008) and Triantafyllidis et al. (2011), based on mtDNA *cytb* and *COI* sequences, respectively. According to Larmuseau et al. (2009), the populations of *R. rutilus* across Europe are divided in two divergent clades: (i) the West-European clade (WE clade) and (ii) the Ponto-Caspian clade (PC clade), whereas Northern Greece is considered the home of both *R. rutilus* clades. Therefore, in order to examine if the *Rutilus* population of Lake Volvi belongs to the Ponto-Caspian clade of *Rutilus rutilus*, a GenBank sequence of *R. rutilus caspicus* (AF095610), was initially included in the analysis. Similarly to the study of Ketmaier et al. (2008), who also used the same haplotype as a reference sequence, the *R. rutilus caspicus* haplotype was clustered together with those of *R. frisii*. The low genetic divergence between the *R. rutilus caspicus* and *R. frisii* puzzled also Ketmaier et al. (2008), as the two species are clearly distinguished by several morphological features (Kottelat and Freyhof, 2007). A possible explanation for this issue is that the specimen identified as *R. rutilus caspicus* could result from introgressive hybridization of *R. frisii* mitochondria into *R. rutilus*. Introgressive hybridization is a common phenomenon in the Cyprinidae family, for example, Larmuseau et al. (2009) described a case of *R. frisii* mtDNA introgression in individuals identified morphologically as *R. rutilus*. For this reason the *R. rutilus caspicus* haplotype (AF095610) was excluded from our analysis. Then, on further searching in Genbank for similar sequences, we found two sets of partial *cytb*-like sequences of *R. rutilus caspicus*, (JN244029–JN244030 and KF056853–KF056855) which, although were listed as unverified, were highly similar to the *R. rutilus* haplotypes found in Lake Volvi (data not shown). Therefore, it is likely that the *R. rutilus* populations inhabiting the North-Eastern Aegean drainage, which encompasses the Lake Volvi and the basin of the river Strymon, belong to the Ponto-Caspian clade of *R. rutilus*, as was previously noted (Larmuseau et al., 2009). Furthermore, as the average pairwise sequence divergence between the haplotypes found in Lake Volvi and those of the WE-clade (Doirani, Vegoritida, Kastoria, Zazari, Chimaditida and Petron) is in the range of values reported between different cyprinid species, it seems reasonable to adopt the suggestion of Larmuseau et al. (2009) that the two *R. rutilus* clades should be recognized as different taxa.

In conclusion, since all taxa examined in this study are listed in the 'IUCN Red list of threatened species' (Crivelli, 2006; Freyhof and Kottelat, 2008), any findings regarding their phylogeny and distribution should be considered very important. The results of the present study support the existence of at least four *Rutilus* species in Greece (*R. prespensis*, *R. panosi*, *R. ylikiensis* and *R. rutilus*). However, based on the significant divergence of the populations of Volvi and Strymon River from all the other southern Balkan *R. rutilus* populations studied, we cannot exclude their classification, together with other populations from the Ponto-Caspian zone, into a new *Rutilus* species. Nevertheless, additional mitochondrial and nuclear DNA analyses of *Rutilus* specimens from various locations, particularly from the north-eastern Balkan and Turkey, will be necessary to address the phylogenetic relationships within the *R. rutilus* group.

References

- Apostolidis, A.P., Stouboudi, M.T., Kalogianni, E., Cote, G., Bernatchez, L., 2011. Genetic divergence among native trout *Salmo trutta* populations from southern Balkans based on mitochondrial DNA and microsatellite variation. *J. Fish Biol.* 79, 1950–1960.
- Bogutskaya, N.G., Iliadou, K., 2006. *Rutilus panosi*, a new roach from Western Greece (Teleostei: Cyprinidae). *Zoosyst. Ross.* 14, 293–298.
- Crivelli, A.J., Catsadorakis, G., Malakou, M., Roscchi, E., 1997. Fish and fisheries of the Prespa lakes. *Hydrobiologia* 351, 107–125.
- Crivelli, A.J., 2006. *Rutilus prespensis*. In: IUCN 2013. IUCN Red List of Threatened Species. Version 2013.1. <www.iucnredlist.org>. Downloaded on 01 October 2013.
- Drummond, A.J., Suchard, M.A., Xie, D., Rambaut, A., 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Mol. Biol. Evol.* 29, 1969–1973.
- Economidis, P.S., 1991. Check List of Freshwater Fishes of Greece. Recent Status of Threats and Protection. Hellenic Society for the Protection of Nature, Athens.
- Economidis, P.S., Banarescu, P.M., 1991. The distribution and origins of freshwater fishes in the Balkan Peninsula, especially in Greece. *Int. Rev. Ges. Hydrobiol.* 76, 257–283.
- Economou, A.N., Giakoumi, S., Vardakas, L., Barbieri, R., Stouboudi, M., Zogaris, S., 2007. The freshwater ichthyofauna of Greece – an update based on a hydrographic basin survey. *Medit. Mar. Sci.* 8/1, 91–166.
- Freyhof, J., Kottelat, M., 2008. *Rutilus ylikiensis*, *Rutilus panosi*, *Rutilus rutilus*. In: IUCN 2013. IUCN Red List of Threatened Species. Version 2013.1. <www.iucnredlist.org>. Downloaded on 01 October 2013.
- Hall, T.A., 1999. Bioedit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids Symp. Ser.* 41, 95–98.
- Hillis, D.M., Moritz, C., Mable, B.K., 1996. *Molecular Systematics*, second ed. Sinauer Associates Inc., Sunderland.
- Ketmaier, V., Bianco, P.G., Durand, J.D., 2008. Molecular systematics, phylogeny and biogeography of roaches (*Rutilus*, Teleostei, Cyprinidae). *Mol. Phylogenet. Evol.* 49, 362–367.
- Kotlik, P., Markova, S., Choleva, L., Bogutskaya, N.G., Ekmekci, F.G., Ivanova, P.P., 2008. Divergence with gene flow between Ponto-Caspian refugia in an anadromous cyprinid *Rutilus frisii* revealed by multiple gene phylogeography. *Mol. Ecol.* 17, 1076–1088.
- Kottelat, M., Freyhof, J., 2007. *Handbook of European Freshwater Fishes*. Kottelat, Cornol, Switzerland and Freyhof, Berlin, Germany.

- Larmuseau, M.H., Freyhof, D.J., Volckaert, F.A.M., Van Houdt, J.K.J., 2009. Matrilinear phylogeography and demographical patterns of *Rutilus rutilus*: implications for taxonomy and conservation. *J. Fish. Biol.* 75, 332–353.
- Leonardos, I.D., Kagalou, I., Tsoumani, M., Economidis, P.S., 2008. Fish fauna in a protected Greek lake: biodiversity, introduced fish species over a 80-year period and their impacts on the ecosystem. *Ecol. Freshw. Fish.* 7, 239–250.
- Librado, P., Rozas, J., 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25 (11), 1451–1452.
- Lillywhite, K., Lee, D., 2011. Automated fish taxonomy using evolution-constructed features. In: Bebis, G., Boyle, R., Parvin, B., Koracin, D., Fowlkes, C., Wang, S., Choi, M.-H., Mantler, S., Schulze, J., Acevedo, D., Mueller, K., Papka, M. (Eds.), *Advances in Visual Computing, Lecture Notes in Computer Science*, vol. 6938. Springer, Berlin/Heidelberg, pp. 541–550.
- Milosevic, D., Winkler, K.A., Maric, D., Weiss, S., 2011. Genotypic and phenotypic evaluation of *Rutilus* spp. from Skadar, Ohrid and Prespa Lakes supports revision of endemic as well as taxonomic status of several taxa. *J. Fish. Biol.* 79, 1094–1110.
- Paquin, P., Hedin, M., 2004. The power and perils of 'molecular taxonomy': a case study of eyeless and endangered *Cicurina* (Araneae: Dictynidae) from Texas caves. *Mol. Ecol.* 13, 3239–3255.
- Stephanidis, A., 1950. Contribution to the study of freshwater fish of Greece. *Prak. Acad. Athens* 18, 200–210.
- Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28, 2731–2739.
- Triantafyllidis, A., Bobori, D., Koliimitra, C., Gbandi, E., Mpanti, M., Petriki, O., Karaiskou, N., 2011. DNA barcoding analysis of fish species diversity in four north Greek lakes. *Mitochondr. DNA* 22 (S1), 37–42.
- Tsoumani, M., Apostolidis, A.P., Leonardos, I.D., 2013. Biogeography of *Rutilus* species of the southern Balkan Peninsula as inferred by multivariate analysis of morphological data. *J. Zool.* 289, 204–212.
- Zardoya, R., Doadrio, I., 1999. Molecular evidence on the evolutionary and biogeographical patterns of European cyprinids. *J. Mol. Evol.* 49, 227–237.