

Molecular identification of species and hybrids of water frogs (genus *Pelophylax*) from Lake Skadar, Southeast Adriatic drainages (Amphibia: Ranidae)

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Abstract. Species composition of Eurasian water frogs, genus *Pelophylax*, in Lake Skadar (Montenegro) was analysed using mitochondrial (mtDNA) and nuclear (nuDNA) markers. Specimens were characterised at first using mtDNA sequences of NADH dehydrogenase subunit 3 (ND3) gene. Based on their mitochondrial genomes, 49 specimens were determined as *Pelophylax kurtmuelleri* while 39 specimens were identified as *Pelophylax shqipericus*. The systematic affiliation was evaluated further using serum albumin intron-1 (SAI-1) nuDNA. The results of SAI-1 analyses confirmed identification of both species but also their hybrids. The SAI-1 variant of *P. shqipericus* was ~600 nucleotide base pairs longer compared to *P. kurtmuelleri*. Five specimens contained both variants, indicating their hybrid origin. However, population allotment of hybrids was low, suggesting normal Mendelian inheritance in the interspecific mating of *P. kurtmuelleri* and *P. shqipericus* rather than a hybridogenetic mode of reproduction. Mito-nuclear discordance as a result of backcross hybridization was not observed in this study. However, the absence of mito-nuclear discordance is not surprising since backcross specimens were rarely detected in previous research of water frogs from Lake Skadar. This study showed that species composition in Lake Skadar is the same compared to 30 years ago, when a previous study analysed the water frogs using protein electrophoresis.

Key words. Hybridization, indigenous species, NADH dehydrogenase subunit 3, gene serum albumin intron-1, Ranidae.

Introduction

With a surface area of ~44,000 km², the Southeast Adriatic Drainages sensu ABELL et al. (2008) (hereinafter referred to as SEAD) is one of the smallest freshwater ecoregions in Europe (Fig. 1). The SEAD was delineated from its neighbouring freshwater ecoregions by endemic-rich fauna of fish (ABELL et al. 2008, SKOULIKIDIS et al. 2009), gastropods (ALBRECHT & WILKE 2009), crustaceans (WYSOCKA et al. 2014) and amphibians with particular reference to water frogs (DŽUKIĆ & KALEZIĆ 2004). The observed independent evolutionary histories of distinct biota in the SEAD were discussed in the light of complex geomorphologic events, climate history, and ecosystem diversity (STANKOVIĆ 1960, ALBRECHT & WILKE 2009).

In this study, we analysed species composition of water frogs in Lake Skadar, the largest freshwater body in the SEAD (surface area of ~370 km²), as well in all of entire Southeastern Europe. Lake Skadar was formed

due to complex tectonic folding and faulting within the northeast wing of Old Montenegro anticlinorium (High Karst Zone) during the Neogene or even Paleogene period (PEŠIĆ & GLÖER 2013). Together with Lake Ohrid and Lake Prespa, Lake Skadar is considered a hot-spot of endemic flora and fauna in Southeastern Europe (ALBRECHT & WILKE 2009, PEŠIĆ et al. 2009). Two Eurasian water frog species have been described from SEAD, the Albanian pool frog, *Pelophylax shqipericus* (HOTZ, UZZELL, GUNTHER, TUNNER & HEPPICH, 1987), and the Balkan pool frog, *Pelophylax kurtmuelleri* (GAYDA, 1940) (SCHNEIDER et al. 1993, UZZELL et al. 2009, AmphibiaWeb 2016). Both species are common in Lake Skadar (UZZELL et al. 2009) and their type localities are shown in Figure 1. The third species reported from the SEAD is the Epirus water frog, *Pelophylax epeiroticus* (SCHNEIDER, SOFIANIDOU & KYRIAKOPOULOU-SKLAVOUNOU, 1984). But it has never been observed in Lake Skadar (UZZELL et al. 2009, AmphibiaWeb 2016).

Pelophylax shqipericus was first biochemically recognised and described as an unnamed species from Lake Skadar by HOTZ & UZZELL (1982). Later SCHNEIDER & HAXHIU (1994) had difficulties in distinguishing the mating calls of *P. shqipericus* from that of the pool frog *P. lessonae* (CAMERANO, 1882). However, full-species status of *P. shqipericus* was supported by numerous studies on morphology, allozymes (HOTZ & UZZELL 1982, BEERLI et al. 1996), mtDNA (PLÖTNER 1998, AKIN et al. 2010a, 2010b, PLÖTNER et al. 2010) and nuDNA sequences (PYRON & WIENS 2011). Although the species status of *P. shqipericus* was confirmed, its hierarchical relationships among the Eurasian water frogs remained less clear; *P. shqipericus* was placed in a monophyletic group with *P. lessonae* and *P. bergeri* in mtDNA phylogenies (e.g. PLÖTNER et al. 2010), while its phylogenetic position based on nuDNA markers was uncertain (e.g. BEERLI et al. 1996, PYRON & WIENS 2011).

In Lake Skadar, *P. shqipericus* lives in sympatry with *P. kurtmuelleri* (SCHNEIDER et al. 1993, UZZELL et al. 2009). In comparison with *P. shqipericus*, the systematic status of *P. kurtmuelleri* is less clear and its full-species status has often been debated. CROCHET & DUBOIS (2004) and SPEYBROECK et al. (2010) argued that there is low genetic divergence between *P. kurtmuelleri* and *P. ridibundus*, implying that *P. kurtmuelleri* is conspecific with *P. ridibundus*. Also, the results of some allozyme analyses were controversial in

distinguishing *P. kurtmuelleri* from *P. ridibundus* (BEERLI et al. 1996, HOTZ et al. 2013). In earlier studies of water frogs from Lake Skadar, specimens distinct from *P. shqipericus* were assigned to the Adriatic populations of the Eurasian marsh frog *P. ridibundus* (PALLAS, 1771) (HOTZ & UZZELL 1982, 1983, HOTZ et al. 1985, GUERRINI et al. 1997, SPASIĆ-BOŠKOVIĆ et al. 1999). But within *P. ridibundus* in Greece, two taxa were discriminated based on their mating calls and morphometry (SCHNEIDER & SINSCH 1992, SCHNEIDER et al. 1993). The new taxon was named *Rana balcanica* SCHNEIDER & SINSCH, 1992. Later this taxon was recognised as a junior synonym of *P. kurtmuelleri* (DUBOIS & OHLER 1994). Further evidence for full-species status of *P. kurtmuelleri* was found in its non-hybridogenetic interactions with *P. ridibundus* and *P. lessonae* (HOTZ et al. 1985, BERGER et al. 1994), morphometrics (GAVRILOVIĆ et al. 1999), bioacoustics (SCHNEIDER & SINSCH 1992, SCHNEIDER et al. 1993, LUKANOV et al. 2015), electrophoretic investigation (SOFIANIDOU et al. 1994), phylogenetic inference based on DNA sequence data (LYMBERAKIS et al. 2007, PYRON & WIENS 2011, PLÖTNER et al. 2012, HOFMAN et al. 2015), and the results of research on cytogenetic differences and centromeric hybridization (MARRACCI et al. 2011). *Pelophylax kurtmuelleri* is distributed up to 1,000 m above sea level throughout much of Greece and Albania (UZZELL et al. 2009). Furthermore, it is considered a naturalised alien species in Italy from where it spread to Slovenia (BRESSI 2006). Also, *P. kurtmuelleri* is an introduced species in Denmark (LEVER 2003).

In areas where two species of water frog are in contact, their hybrids are extant (ARNOLD & OVENDEN 2002, MAYER et al. 2013). Among hybrid forms in Eurasian water frogs, there are three different fertile hybrids with reproductive mode termed hybridogenesis: the Edible frog *Pelophylax kl. esculentus* (LINNAEUS, 1758), the Graf's hybrid frog *Pelophylax kl. grafi* (CROCHET, DUBOIS, OHLER & TUNNER, 1995) and the Italian edible frog *Pelophylax kl. hispanicus* (BONAPARTE, 1839) (reviewed in GRAF & POLLS PELAZ 1989). Diploid hybridogenetic or hemiclinal hybrids exclude one of the parental genomes from the hybrid germline, pass the remaining one clonally to their gametes, and provide the excluded genome via backcross mating with the parental species (SCHULTZ 1969, SCHMELLER et al. 2001). The genome of *P. ridibundus* is considered to induce hybridogenetic reproduction in all three hybridogenetic complexes in Europe (GRAF & POLLS PELAZ 1989, HOLSBECK & JOORIS 2010). To date, the species of Eurasian water frogs and their hybrid forms were intensively studied as models for understanding speciation in the context of hybridization and polyploidization (PAGANO et al. 2001, PLENET et al. 2005, CHRISTIANSEN & REYER 2009, CHRISTIANSEN et al. 2010, JAKOB et al. 2010, HOFFMAN & REYER 2013, PRUVOST et al. 2013, HOFFMAN et al. 2015).

During the 1980s, water frogs from Lake Skadar were repeatedly studied using protein (allozyme) electrophoresis and chromosome analyses to investigate interspecific hybridization (HOTZ & UZZELL 1982, 1983, HOTZ et al. 1985, GUERRINI et al. 1997). The presence of *P. shqipericus* (at that time referred to as an unknown species), *P. kurtmuelleri*



Figure 1. Map presenting sampling sites of water frogs in Lake Skadar and type localities of species occurring in Southeast Adriatic drainages (SEAD) sensu ABELL et al. (2008). National boundaries are indicated by black lines while the border of SEAD is shown by a thick orange line.

leri (referred to as *P. ridibundus* from the Adriatic region) and their hybrids was reported for Lake Skadar (HOTZ & UZZELL 1982, 1983). The comparison of results obtained by enzyme electrophoresis from somatic tissues with those from individual primary oocytes indicated that *P. shqipericus* does not produce hybridogenetic hybrids in interspecific crosses with *P. kurtmuelleri* (referred to as “Adriatic *P. ridibundus*”) (HOTZ et al. 1985). The same was observed for hybrids of *P. kurtmuelleri* (referred to as “Balkan *Rana ridibunda*”) and *P. epeiroticus* (*Rana epeirotica*) (GUERRINI et al. 1997). Reported frequency of hybrids of *P. kurtmuelleri* and *P. shqipericus* from Lake Skadar was low (8.7%), unlike the abundant hemiclinal hybrid lineage *P. kl. esculentus* in Central and Western Europe (HOTZ & UZZELL 1982). It was indicated that the lack of hybridogenetic reproduction in these hybrids was caused by a failure of hybrids to “induce” the exclusion of one parental species genome during gametogenesis of hybrids, or a “resistance” of *P. shqipericus* genome to such exclusion, or both (GUERRINI et al. 1997). Nevertheless, it was acknowledged that the *P. kurtmuelleri* genome in hybrids from Lake Skadar does not contain putative inducing factors for the exclusion of *P. shqipericus* genome during gametogenesis (GUERRINI et al. 1997). However, a few backcross specimens were observed in Lake Skadar, indicating that non-hybridogenetic F₁ hybrids of both parental combinations were not completely sterile (HOTZ & UZZELL 1982, 1983).

In recent years, analyses of DNA sequence fragments have become common in water frog research. Mitochondrial DNA sequences such as cytochrome b, 16S rRNA (LYMBERAKIS et al. 2007), NADH dehydrogenase subunit 2 gene (ND2) and NADH dehydrogenase subunit 3 gene (ND3) (PLÖTNER et al. 2008, AKIN et al. 2010a, b, DOMENEGHETTI et al. 2013, HOTZ et al. 2013) were proven informative in phylogenetic inference, as well as nuDNA sequences, e.g. serum albumin intron-1 (SAI-1) (PLÖTNER et al. 2009, HAUSWALDT et al. 2012). Also, the difference in lengths of the SAI-1 region has become a widely used method for distinguishing among species and hybrids within *Pelophylax* species (HAUSWALDT et al. 2012, MAYER et al. 2013, HERCZEG et al. 2016). Therefore, one of the main focuses of this research was to obtain sequences of the SAI-1 nuclear region of *P. shqipericus* from its type locality. Further, our aim was also to apply nuclear (SAI-1) and mtDNA (ND3) markers to investigate, whether the species composition in Lake Skadar had changed compared to the study conducted 30 years ago. Also, we investigated occurrence of mtDNA introgression between *P. shqipericus* and *P. kurtmuelleri* via backcross mating of their hybrids.

Materials and methods

Sampling procedure

Eighty-eight water frogs were collected at four localities on Lake Skadar in Montenegro (Supplementary Table S1, Fig. 1): 27 specimens from Rijeka Crnojevića (42.35°N, 19.04°E); 29 from Mareza (42.45°N, 19.20°E); 26 from

Lake Skadar (42.33°N, 19.09°E); 6 from Morača (42.28°N, 19.13°E). The tip of a toe was clipped from each frog and preserved in 96% ethanol for further processing. Frogs were released after this procedure, no vouchers were maintained.

DNA extraction and PCR amplifications

Total genomic DNA was extracted from toe tissue using GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich) following the manufacturer's instructions. DNA quality and concentration was checked with a Nanodrop ND-100 spectrophotometer (Nanodrop Technologies). The primer pair used for ND3 was ND3L/ND3H (AKIN et al. 2010a) and the primer pair for SAI-1 was Pel-SA-F1/Pel-SA-R2 (HAUSWALDT et al. 2012). Each PCR was conducted using HotStarTaq Plus Master Mix (Qiagen) in a total volume of 10 µL containing 0.5 U of HotStarTaq Plus DNA Polymerase, 200 µM of each dNTP, 1.5 mM MgCl₂, 0.25 µM of each primer, and 40–120 ng of template DNA. Thermocycling profiles started with a 5 min activation step at 95°C, and were followed by 35 cycles of denaturation (30 s at 94°C), annealing (20 s at 62°C for ND3 or 40 s at 59°C for SAI-1) and extension (60 s at 72°C for ND3 or 100 s at 72°C for SAI-1), and a final extension step at 72°C for 10 min. PCR amplicons were purified using MinElute PCR Purification Kit (Qiagen).

Visualisation of PCR amplicons using gel electrophoresis, sequencing and cloning procedures

Three microliters of PCR amplicons were run on a 1.5% agarose gel. Eighty-eight purified amplicons of ND3 and sixteen randomly chosen SAI-1 amplicons (eight per species) were sequenced in both directions using the same primers used in PCR (Supplementary Table S1). Sequencing reactions were conducted on ABI 3730XL DNA Analyzer (Applied Biosystems) in Macrogen Europe sequencing service (Amsterdam/NL). Chromatographs were checked and edited using SEQUENCHER version 5.3 (Gene Codes Corp.). SAI-1 amplicons were cloned in case of heterozygous individuals. The PCR amplicons of heterozygous individuals were ligated into vectors and transformed into bacteria using the pGEM-T Vector System II (Promega). Plasmid DNA from nine positive clones per specimen was isolated and purified using the Wizard Plus SV Miniprep DNA Purification System (Promega), and inserts were sequenced with the primers used during PCR and new internal primers (INTSHQ-F1: 5'-GCACGGAGCATCGATAGTTT-3', and INTSHQ-R1: 5'-AGGCATAAGGTGCCCATCTA-3').

Phylogenetic inference using ND3 and SAI-1 sequences

Sequence traces were analysed using SEQUENCHER version 5.3 (Gene Codes Corp.). The SAI-1 sequences gen-

Table 1. List of unique sequences for nuDNA SAI-1 region in water frogs from Lake Skadar.

Sample ID	Locality	ID of SA-1 haplotypes	Taxonomic affiliation	NCBI GenBank Acc. No. of SAI-1
RC2	Rijeka Crnojevića	Seq1	<i>P. kurtmuelleri</i>	MH038010
RC3	Rijeka Crnojevića	Seq1	<i>P. kurtmuelleri</i>	MH038011
RC7	Rijeka Crnojevića	Seq1	<i>P. kurtmuelleri</i>	MH038012
RC18	Rijeka Crnojevića	Seq1	<i>P. kurtmuelleri</i>	MH038013
MA19	Mareza	Seq1	<i>P. kurtmuelleri</i>	MH038016
MA21	Mareza	Seq1	<i>P. kurtmuelleri</i>	MH038017
MA22	Mareza	Seq1	<i>P. kurtmuelleri</i>	MH038018
MA26	Mareza	Seq1	<i>P. kurtmuelleri</i>	MH038019
RC21	Rijeka Crnojevića	Seq2, Seq3	<i>P. shqipericus</i>	MH038014
RC23	Rijeka Crnojevića	Seq2, Seq4	<i>P. shqipericus</i>	MH038015

erated in this study were examined using Nucleotide Basic Local Alignment Search Tool (Nucleotide BLAST: <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to screen for the most similar sequences in the GenBank nucleotide database (National Center for Biotechnology Information, U.S. National Library of Medicine, USA). Sequence alignments were inferred using MAFFT version 7.187 (KATO et al. 2005) on the CIPRES Science Gateway version 3.1 OF MILLER et al. (2010) (<http://www.phylo.org>). Phylogenetic tree reconstructions were conducted using sequences obtained in this study and the sequences of ND3 and SAI-1 which were downloaded from the GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) and are those of SUMIDA et al. (2001), PLÖTNER et al. (2008, 2009, 2010), AKIN et al. (2010a, b), HOFMAN et al. (2012, 2015), DOMENEGHETTI et al. (2013), HOTZ et al. (2013), DUBEY et al. (2014), MIKULÍČEK et al. (2014); see Supplementary Table S1. The sequences obtained in this study were likewise deposited in GenBank; see Supplementary Table S1 and Table 1. Phylogenetic inference was performed using Maximum Likelihood (ML) and Bayesian Analysis (BA). The best-fit evolutionary model used in ML and BA was calculated using the Bayesian Information Criterion (BIC) in jModelTest2 version 2.1.6 (DARRIBA et al. 2012) as implemented on the CIPRES. Best-fit model of nucleotide substitution for ND3 gene was TrN (TAMURA & NEI 1993) while the model for SAI-1 sequences was HKY (HASEGAWA et al. 1985) with a gamma-distributed rate variation among sites and a significant proportion of invariable sites.

The ML analyses for ND3 and SAI-1 were run using MEGA version 6 (TAMURA et al. 2013) with optimised parameters. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining (NJ) method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach. The obtained phylogeny was tested using 1,000 nonparametric bootstrap replicates.

In the analyses of ND3, the BA was run in MrBayes 3.2 (RONQUIST et al. 2012) on CIPRES. Two independent runs with four MCMC chains were run for 50 million generations and sampled every 5,000 generations, with temperature parameter set to 0.2 and the first 12.5 million gen-

erations discarded as burn-in. In the analyses of SAI-1 sequences, the BA was conducted using BAlly-Phy version 2.3.8 to incorporate insertion/deletion information into phylogeny estimation (REDELINGS & SUCHARD 2005, 2007, SUCHARD & REDELINGS 2006). BA in BAlly-Phy was run using 100,000 iterations, the HKY model of nucleotide substitution, and RSo7 insertion/deletion model (REDELINGS & SUCHARD 2007). To reduce computational time in BAlly-Phy, SAI-1 sequence data set was reduced to one randomly chosen haplotype per species.

The convergence of runs in BA was screened using AWTY (NYLANDER et al. 2008) while effective sample sizes of parameters were checked using TRACER 1.5 (DRUMMOND & RAMBAUT 2007). Nodes in phylograms with bootstrap values $P \geq 70$ in ML and posterior probabilities (pp) ≥ 0.95 in BA were considered as support.

Results

Phylogenetic inference based on mtDNA (ND3)

ND3 sequences were generated for all individuals (Supplementary Table S1). Forty-nine sequences which corresponded to seven unique haplotypes were placed in the *P. kurtmuelleri* lineage. Thirty-nine sequences with eleven haplotypes were positioned in the *P. shqipericus* lineage in ML and BA phylograms (Fig. 2). Our phylogenetic inference based on ND3 placed *P. kurtmuelleri* haplotypes in the group “ridibundus-bedriagae” sensu PLÖTNER & OHST (2001), but they were genetically distinct from *P. ridibundus* (Fig. 2). Haplotypes of *P. shqipericus* were positioned within the major group “lessonae” sensu PLÖTNER & OHST (2001), and formed a distinct supported group in both ML and BA phylogenies using ND3 mtDNA.

Length variation of SAI-1 region in water frogs from Lake Skadar

The PCR amplification of SAI-1 fragments and subsequent visualisation on agarose gels were completed with

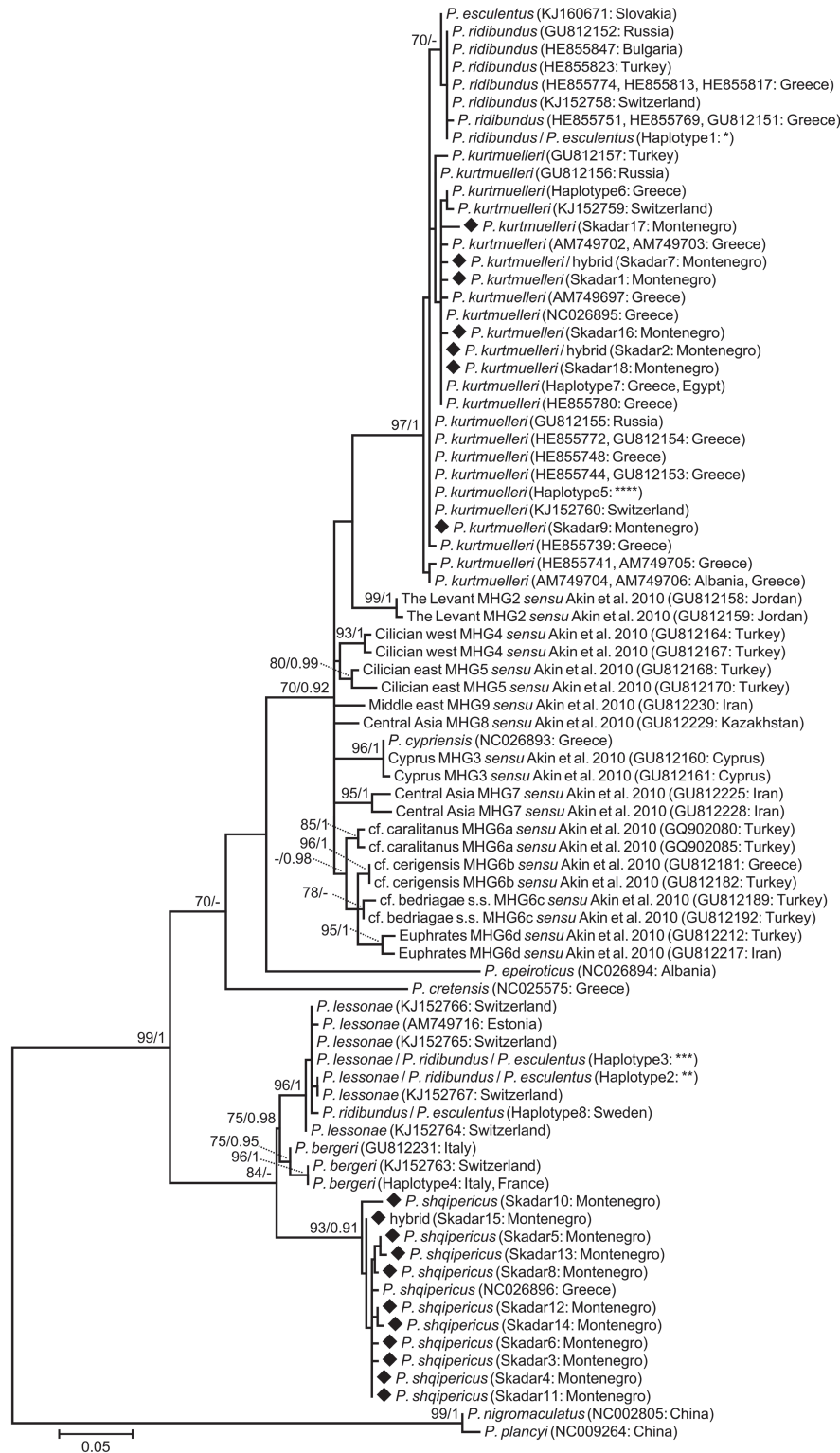


Figure 2. Phylogenetic tree inferred by ML analysis using ND3 sequences of water frogs. Newly obtained haplotypes in this study were marked by black rhombi. Node supports are given as bootstrap values (P) in ML analysis (showing values ≥ 70) and posterior probabilities (pp) in BA (showing values ≥ 0.9). Legend: * – Bulgaria, France, Greece, Hungary, Italy, Latvia, Poland, Romania, Russia, Serbia and Slovakia; ** – Germany, Poland, Romania and Slovakia; *** – Denmark, Estonia, Germany, Italy, Latvia, Lithuania, Poland, Romania, Slovakia and Sweden; **** – France (Acc. No. AM749707), Germany (Acc. No. AM900647), Latvia (Acc. No. AM900652), Lithuania (Acc. No. AM900648), Romania (Acc. No. AM900650), Ukraine (Acc. No. AM900651), Italy, Greece and Macedonia.

all samples (Supplementary Table S1, Fig. 3). Two amplicon lengths (~720 bp and ~1200 bp) were detected. Forty-six specimens were determined as *P. kurtmuelleri*, since they carried SAI-1 variants of ~720 bp length and *P. kurtmuelleri* mtDNA genomes. Thirty-seven specimens were identified as *P. shqipericus*, since they carried SAI-1 variants of ~1200 bp and *P. shqipericus* mtDNA genomes. Furthermore, a mixture of both SAI-1 variants was observed in five samples, indicating hybridization between *P. kurtmuelleri* and *P. shqipericus* in Lake Skadar. Three hybrid specimens had *P. kurtmuelleri* mtDNA genomes (haplotypes Skadar2 and Skadar7) while two hybrids had *P. shqipericus* mtDNA genomes (Skadar15). There were no cases of mito-nuclear discordance (mtDNA introgression) in *P. kurtmuelleri* or *P. shqipericus* from Lake Skadar.

Phylogenetic inference using nuDNA SAI-1 region

Sequencing of the SAI-1 region was done on 16 randomly chosen samples originating from *P. kurtmuelleri* and *P. shqipericus* while hybrid specimens were avoided. We managed to get 'good' SAI-1 sequences from eight specimens of *P. kurtmuelleri* and all sequences were identical (variant Seq1) (Table 1). This haplotype was identical to the GenBank sequence which originates from *P. kurtmuelleri* from Greece (FN432367).

However, we failed to obtain 'good' sequences for the eight samples of *P. shqipericus*. Therefore, we cloned amplicons of two individuals and sequenced nine clones per amplicon. Among the 18 sequences, we obtained three variants of SAI-1 (Table 1). After BLAST search, the variant Seq2 showed 99% similarity with the SAI-1 sequence of *P. shqipericus* obtained by DUBEY & DUFRESNES (2017).

Additionally, we reconstructed phylogenetic trees using the SAI-1 sequences of water frogs and new *P. shqipericus* sequences from this study (Figs. 4 and 5). The SAI-1 sequences of *P. shqipericus* form a monophyletic group

(Fig. 4). Hierarchical relationships of *P. shqipericus* towards other species are not resolved in ML and BA phylogenies (Figs. 4 and 5). However, a long branch of *P. shqipericus* group points to a deep evolutionary divergence between this species and its closest relatives.

Discussion

Phylogenetic inference of *P. shqipericus* and *P. kurtmuelleri* from Lake Skadar

In this study, we confirmed the presence of *P. shqipericus* and *P. kurtmuelleri* in Lake Skadar, using mitochondrial and nuclear genetic markers. Occurrence of both these taxa in Lake Skadar was first recognised by HOTZ et al. (1985). Our results suggest that species composition in Lake Skadar has remained unchanged since analysed by HOTZ et al. (1985) using protein electrophoresis.

The ND3 haplotypes of *P. shqipericus* were positioned within the group "lessonae" sensu PLÖTNER & OHST (2001), comprising three species, *P. lessonae*, *P. bergeri* and *P. shqipericus* (Fig. 1). However this group is designated as monophyletic only in the ML analysis and is not supported as monophyletic in the SAI-1 phylogenies (Figs. 4 and 5). Nevertheless, a tendency of *P. lessonae* and *P. shqipericus* as sister taxa can be seen in both ML and BA phylogenetic inference using SAI-1. Additionally, long branches in the SAI-1 phylograms indicate a deep divergence between these two species (Figs. 4 and 5). Similarly, ML phylogenetic inference based on protein electrophoresis data (BEERLI et al. 1996) revealed that *P. lessonae* and *P. shqipericus* shared the same ancestral node, and both were characterized by long branches.

Our phylogenetic inference on ND3 placed *P. kurtmuelleri* in the group "ridibundus-bedriagae" sensu PLÖTNER & OHST (2001), which is the most diverse group of water frogs comprising at least six species (Fig. 2, LYMBERAKIS et al. 2007, AKIN et al. 2010a, PLÖTNER et al. 2012). However, this group is supported only by a low bootstrap value in

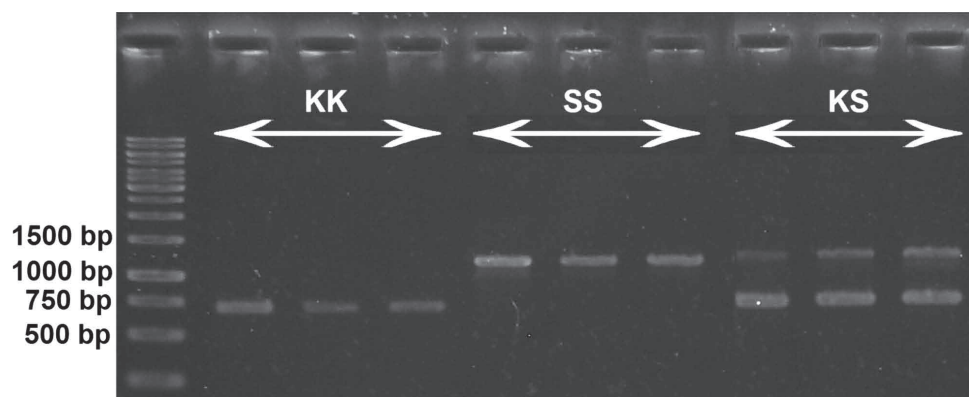


Figure 3. Image of an agarose gel showing the PCR products of SAI-1 originating from three specimens each of *Pelophylax kurtmuelleri* (KK), *Pelophylax shqipericus* (SS) and their hybrids (KS). In *P. kurtmuelleri* one band of ~720 bp and in *P. shqipericus* one of ~1200 bp were detected. Both bands were obvious in the hybrids.

ML analysis ($P = 70$) and it is not supported by BA (Fig. 2), thus, adding uncertainty to the monophyly of this group. In line with results of the mtDNA data, the group “*ridibundus-bedriagae*” sensu PLÖTNER & OHST (2001) is not supported by SAI-1 (Figs. 4 and 5).

Analyses of ND3 gene indicated low statistical support in ML ($P=70$) and no support in BA for *P. ridibundus* as a group distinct from *P. kurtmuelleri* (Fig. 2). Also, branch lengths are short indicating a shallow genetic divergence between *P. kurtmuelleri* and *P. ridibundus* (Fig. 2). However, sequences of *P. kurtmuelleri* form a supported group in SAI-1 phylogenies (both ML and BA) (Figs. 4 and 5).

It is also worth mentioning that the number of known ND3 haplotypes in *P. kurtmuelleri* ($h = 25$) is significantly larger than in *P. ridibundus* ($h = 8$) especially when considering the larger distribution area and more analysed specimens of the latter. High genetic variability in species distributed in southern European regions has been attributed to climate oscillations during the Pleistocene (HEWITT 2011). It can be assumed that *P. kurtmuelleri* had a better survival rate in glacial refugia across Southeast Europe (e.g. Lake Skadar) than *P. ridibundus* which probably survived the Last Glacial Maximum in a yet unknown southern refugium from where it has recolonized Central Europe.

Hybridization between *P. shqipericus* and *P. kurtmuelleri* in Lake Skadar

Analysis of SAI-1 of water frogs from Lake Skadar revealed two fragments with a large difference in length (~720 and ~1300 bp) (Supplementary Table S1). These fragments were associated to *P. kurtmuelleri* and *P. shqipericus*, respectively (Figs. 4 and 5). The shorter fragment was previously reported for *P. kurtmuelleri* (HAUSWALDT et al. 2012) while the long SAI-1 fragment of *P. shqipericus* was determined for the first time by DUBEY & DUFRESNES (2017).

In five cases, a mixture of both types of SAI-1 fragments was observed indicating hybridization between *P. kurtmuelleri* and *P. shqipericus* (Supplementary Table S1). In earlier studies, laboratory controlled hybridization experiments conducted on *P. shqipericus* showed its resistance to genome exclusion (HOTZ et al. 1985). Natural hybrids of *P. kurtmuelleri* and *P. shqipericus* were detected, but they were infrequent with 13 out of 150 individuals (8.7%) and only two recombinant backcrosses (1.3%) (HOTZ & UZZELL 1982). These previous results are comparable to our results which show 5.7% hybrids among the sampled populations at Lake Skadar (Supplementary Table S1).

In this research, mito-nuclear discordance, indicating recombinant backcrosses, was not observed in Lake Ska-

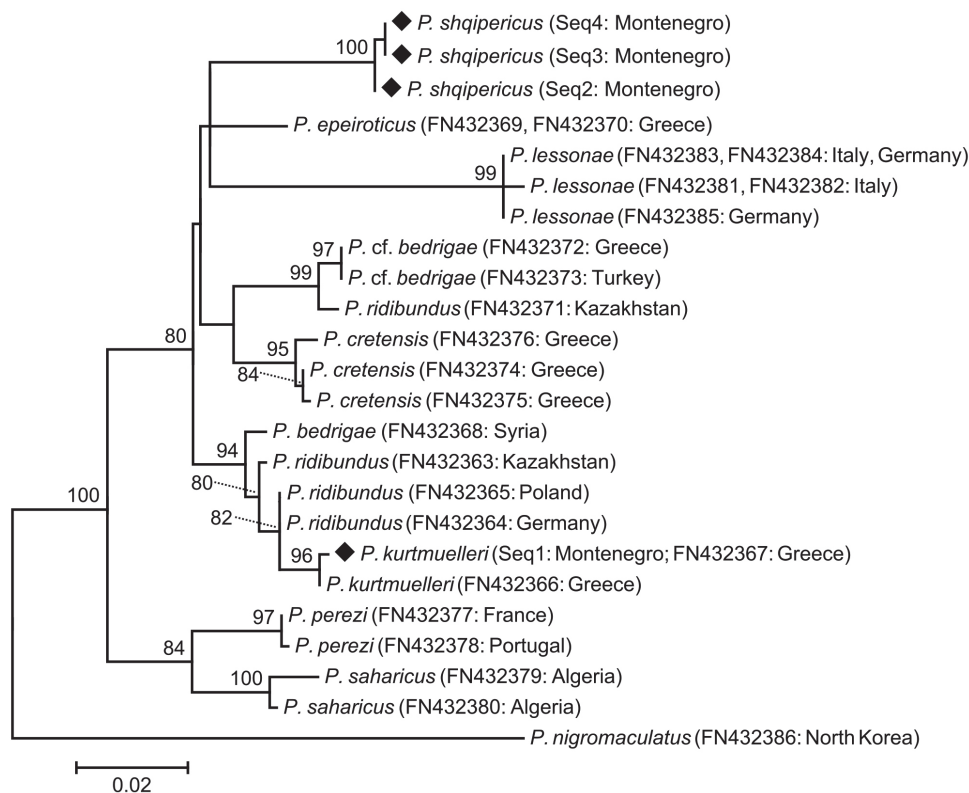
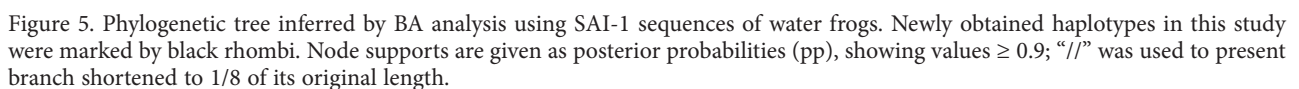


Figure 4. Phylogenetic tree inferred by ML analysis using SAI-1 sequences of water frogs. Newly obtained haplotypes in this study were marked by black rhombi. Node supports are given as bootstrap values (P), showing values ≥ 70 .

In their native range in Albania and Montenegro, *P. kurtmuelleri* and *P. shqipericus* are threatened by uncontrolled harvesting for commercial purposes (UZZELL et al. 2009). Moreover, this is a source for illegal introduction of non-native species in Europe, as for instance *P. kurtmuelleri* and *P. shqipericus* originating from Southeastern Europe were reported in central Italy (DOMENEGHETTI et al. 2013). To cope with the increasing problem of human-mediated translocations across Europe (HOLSBECK et al. 2010, DUBEY et al. 2014), we demonstrated the use of low-cost genetic determination of *P. kurtmuelleri*, *P. shqipericus* and their hybrids with mitochondrial and nuclear markers. The results of this research also provide a good basis for iden-

QUILODRÁN et al. (2015) discussed that *P. kurtmuelleri* (referred to as southern *P. ridibundus*) represents a serious threat to native water frogs in Western Europe in cases of human-mediated translocations. This species can disrupt the equilibrium in hybridogenetic systems of *P. kl. esculentus*, which can lead to the collapse of native frogs populations. The first action in the conservation of native water



tification of the geographic origin of *P. kurtmuelleri* and *P. shqipericus* genotypes in Europe in further studies.

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Supplementary material

Supplementary Table S1. List of analysed water frog specimens from Lake Skadar and their species/hybrid affiliations obtained by molecular markers.