SALAMANDRA **57**(1): 105–123
15 February 2021 | ISSN 0036–3375

SALAMANDRA
German Journal of Herpetology

Morphological differentiation of endemic water frogs (Ranidae: *Pelophylax*) from the southwestern Balkans

Petr Papežík[1], Maroš Kubala[2,3], Daniel Jablonski[1], Marie Doležálková-Kaštánková[4], Lukáš Choleva[4,5], Michal Benovics[6] & Peter Mikulíček[1]

[1)] Department of Zoology, Faculty of Natural Sciences, Comenius University in Bratislava, Ilkovičova 6, 842 15 Bratislava, Slovakia
[2)] Department of Ecology, Faculty of Natural Sciences, Comenius University in Bratislava, Ilkovičova 6, 842 15 Bratislava, Slovakia
[3)] Plant Science and Biodiversity Centre, Institute of Botany, Slovak Academy of Sciences, Dúbravská cesta 9, 845 23, Bratislava, Slovakia
[4)] Laboratory of Fish Genetics, Institute of Animal Physiology and Genetics, The Czech Academy of Sciences, 277 21 Liběchov, Czech Republic
[5)] Department of Biology and Ecology, Faculty of Science, University of Ostrava, Chittussiho 10, Ostrava 710 00, Czech Republic
[6)] Department of Botany and Zoology, Faculty of Science, Masaryk University, Kotlářská 2, 611 37 Brno, Czech Republic

Corresponding author: Petr Papežík, e-mail: petr.papezik.upol@gmail.com

Manuscript received: 23 September 2020
Accepted: 2 December 2020 by Stefan Lötters

Abstract. The southwestern Balkans is inhabited by three endemic water frog species, *Pelophylax shqipericus*, *P. epeiroticus* and *P. kurtmuelleri*. Although these species are genetically and bioacoustically distinct from each other, their morphological differentiation is less pronounced as is generally observed in the genus *Pelophylax*. In this study, we analyzed morphological data of 215 individuals of these Balkan endemics in order to evaluate their morphological variability and facilitate their identification in the field. Moreover, since *P. kurtmuelleri* is a disputable taxon, we compared it with closely related *P. ridibundus* from Central Europe. The most pronounced morphological differences expressed by the analysis of variance (ANOVA) and the discriminant analysis of the principal components (DAPC) were found between *P. kurtmuelleri* and the two other Balkan species. *Pelophylax kurtmuelleri* and *P. ridibundus*, as well as *P. shqipericus* and *P. epeiroticus* were only slightly differentiated by morphology, which demonstrates that the morphological characters measured do not reflect the genetic divergence of the species and are weak indicators of their taxonomic status. Morphological characters suitable for species identification include a combination of indices L/CINT, T/CINT, L/T, and DP/CINT (L – snout–vent length, CINT-length of the metatarsal tubercle, T – length of the tibia, DP – length of the first toe), and qualitative traits like the pattern and shape of the lateral spots, presence/absence of yellow pigment in the flanks and thighs, foot webbing colouration, size and shape of the metatarsal tubercle, and colouration of the male vocal sacs.

Key words. Amphibia, Anura, morphometry, endemism, hybridization, microsatellites, species identification.

Introduction

European water frogs (genus *Pelophylax*) are a widespread group of amphibians and have been intensively studied since Carl von Linné described the first representative, *Pelophylax esculentus* (Linnaeus, 1758). Modern herpetological research has since led to the discovery of up to 22 species and three hybrid asexual forms, which are distributed throughout the temperate and subtropical zones of the whole Palaearctic (Plötner 2005, Frost et al. 2020).

The southwestern Balkans is inhabited by three water frog taxa, all of them endemic to the peninsula. The Albanian water frog, *P. shqipericus* (Hotz, Uzzell, Günther, Tunner & Heppich, 1987), described from the Lake Skadar in Montenegro, is distributed throughout the lowlands of the Adriatic coast of southern Montenegro and western Albania (Günther 2004, Plötner 2005, Vucić et al. 2018). Mitochondrial phylogenetic analyses has shown that this species is closely related to *P. lessonae* (Camerano, 1882) and *P. bergeri* (Günther, 1986) (Ragghianti et al. 2004, Akin et al. 2010, Plötner et al. 2012, Dufresnes et al. 2017), forming the so-called *P. lessonae* group (cf. Plötner 2005). The Epirus water frog, *P. epeiroticus* (Schneider, Sofianidou & Kyriakopoulou-Sklavounou, 1984) was described from the Lake Ioannina (Greece) and is currently known from southern Albania and western Greece (Schneider et al. 1984, Sofianidou & Schneider 1989, Schneider & Haxhiu 1992, Oruçi 2010). Its phylogenetic position is not clearly resolved but the species is likely more related to *P. ridibundus* (Pallas, 1771) and other species from the so-called

P. ridibundus group (named according to Akin et al. 2010), than to the *P. lessonae* group (cf. Lymberakis et al. 2007, Akin et al. 2010). Another endemic taxon from the Balkans, *P. kurtmuelleri* (Gayda, 1940) was described as early as the 1940s, but until recently was not recognized as distinct from *P. ridibundus*. Research in the early 1990s, however, revealed that populations from the southwestern Balkans differed from other European *P. ridibundus* populations on the basis of bioacoustics (Schneider & Sinsch 1992, Schneider et al. 1993) and allozymes (Sinsch & Eblenkamp 1994, Sofianidou et al. 1994). Currently, the taxon is recognized as a separate species (Plötner et al. 2012, Frost et al. 2020) from, or a divergent evolutionary lineage of *P. ridibundus* (Lymberakis et al. 2007, Speybroeck et al. 2020).

Pelophylax kurtmuelleri lives sympatrically and often also syntopically, with *P. shqipericus* or *P. epeiroticus* and occasionally hybridizes with them (Hotz & Uzzell 1983, Schneider et al. 1984, Hotz et al. 1985, Sofianidou & Schneider 1989, Vucić et al. 2018). Ranges of the last two species do not overlap and are separated by a 60 km long mountain range in southern Albania inhabited only by *P. kurtmuelleri* (Plötner 2005, Speybroeck et al. 2016). However, hybridization between *P. kurtmuelleri* and its two endemic counterparts seems uncommon and hybrids are rare and likely do not exhibit hybridogenetic reproduction as opposed to other regions of Europe (Hotz & Uzzell 1983, Hotz et al. 1985, Sofianidou & Schneider 1989).

Water frogs have relatively uniform morphology, and, therefore, their systematics have often relied on genetic and bioacoustic characters. Morphology is, however, necessary for taxonomists who use morphological characters when describing new taxa, for evolutionary biologists studying adaptations, phenotypic plasticity, or life-history traits, and finally, for field biologists who need to identify species directly in the field. Morphological examination of taxa currently recognized as *P. shqipericus*, *P. epeiroticus*, and *P. kurtmuelleri* was carried out for the first time by Hotz & Uzzell (1982), later by Schneider et al. (1984, 1993) and Gavrilović et al. (1999). These authors found significant differences between the species in several morphometric indices and qualitative traits, such as colouration, head size, and size and shape of the metatarsal tubercle.

In this study, we aim to build on previous studies and revise the morphological characters and morphometric indices for three Balkan and one Central-European water frog species. Here we benefit from proper identification of each individual using genetic markers and specifically 1) examine external morphology of *P. shqipericus*, *P. epeiroticus* and *P. kurtmuelleri* and determine the quantitative and qualitative morphological traits suitable for their identification, and 2) compare morphological variation of *P. kurtmuelleri* with its closely related species, *P. ridibundus*.

Material and methods

Sampling and data collection

We collected 215 water frogs of the genus *Pelophylax* from 13 localities in Albania, Greece, and Montenegro between 2017 and 2019 (Tab. 1, Fig. 1). Selected localities covered the range of the species *P. epeiroticus*, *P. kurtmuelleri* and *P. shqipericus*. Since *P. kurtmuelleri* is closely related to Central-European *P. ridibundus* phylogenetically, we also included 89 individuals of this species for comparison. All *P. ridibundus* individuals were sampled during the years 1997 to 2009 in five localities in western Slovakia.

Table 1. A list of localities and water frog species (genus *Pelophylax*) included in the study.

Locality	Country	Coordinates		*P. epeiroticus*		*P. kurtmuelleri*		*P. shqipericus*		*P. ridibundus*	
		N	E	Males	Females	Males	Females	Males	Females	Males	Females
Berat	Albania	40.7043	19.9480	–	–	1	–	–	–	–	–
Dushnik	Albania	40.7594	19.9891	–	–	–	2	–	–	–	–
Qazim Pali	Albania	40.0497	19.8420	–	–	15	9	–	–	–	–
Ksamil	Albania	39.7454	20.0207	–	1	1	1	–	–	–	–
Mbrostar	Albania	40.7743	19.5897	–	–	2	3	–	–	–	–
Nishaj	Albania	41.6852	19.5877	–	–	–	–	27	19	–	–
Divjakë - Karavastë	Albania	40.9901	19.4975	–	–	10	11	7	4	–	–
Poçem	Albania	40.5051	19.7036	–	–	22	10	–	–	–	–
Xare	Albania	39.7330	20.0529	1	–	–	3	–	–	–	–
Zvërnec	Albania	40.5179	19.4007	–	–	1	–	3	–	–	–
Igoumenitsa	Greece	39.5366	20.2038	15	5	4	4	–	–	–	–
Ioannina	Greece	39.6886	20.8584	9	20	3	1	–	–	–	–
Virpazar	Montenegro	42.2450	19.0915	–	–	–	–	–	2	–	–
Brodské	Slovakia	48.6951	17.0089	–	–	–	–	–	–	3	1
Čunovo	Slovakia	48.0331	17.2006	–	–	–	–	–	–	3	1
Devín	Slovakia	48.1749	16.9766	–	–	–	–	–	–	15	9
Šúr	Slovakia	48.2268	17.2054	–	–	–	–	–	–	23	13
Šaštín-Stráže	Slovakia	48.6379	17.1400	–	–	–	–	–	–	13	8

Each individual was measured using a digital calliper for five morphometric characteristics: snout–vent length (L), length of the femur (F), length of the tibia (T), length of the first toe (DP), and length of the metatarsal tubercle (CINT). These characteristics vary between the taxa and are traditionally used in the morphometry of water frogs (Günther 1990, Plötner 2005). To minimize bias introduced by measurements, all frogs were measured by a single person (P. Mikulíček). The species status of every individual was determined by combining morphological characteristics and colour patterns in the field (Speybroeck et al. 2016). Additionally, the shape of the metatarsal tubercle was recorded, and individuals photographed. Only individuals with L ≥ 41.50 mm (*P. shqipericus*), 45.20 mm (*P. epeiroticus*), 51.50 mm (*P. ridibundus*) and 52.24 mm (*P. kurtmuelleri*) were involved in the study. Males with this body length had clearly visible vocal sacs and were distinguishable from females.

A tissue sample (a drop of blood or toe clip) from each individual was fixed in 96% ethanol for molecular analysis.

Genetic identification of Balkan species

Species identification of Balkan individuals was based on the size polymorphism and sequence variation in serum albumin intron-1 (SAI-1) (Plötner et al. 2009, Hauswaldt et al. 2012), and on microsatellite markers (see below). The size polymorphism of the species-specific SAI-1 fragments was examined on an agarose gel (Hauswaldt et al. 2012). Identification of the Central-European *P. ridibundus* frogs was based on the combined molecular markers in our previous works (Mikulíček et al. 2014, 2015) and is not subjected to this study.

Genomic DNA was extracted from blood or toe clips using NucleoSpin® Tissue kit (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol. For amplification of the SAI-1 fragment, the primers Pel-SA-F1 and Pel-SA-R1 were used (Hauswaldt et al. 2012). PCR was carried out in a total volume of 10 µl with 5 µl of VWR Red Taq 2x mix (VWR, Radnor, PA, USA), 0.3 µl of each primer (10 µM), 3.4 µl of ddH_2O, and 1 µl of DNA. PCR program was as follows: 2 min of initial denaturation at 94°C fol-

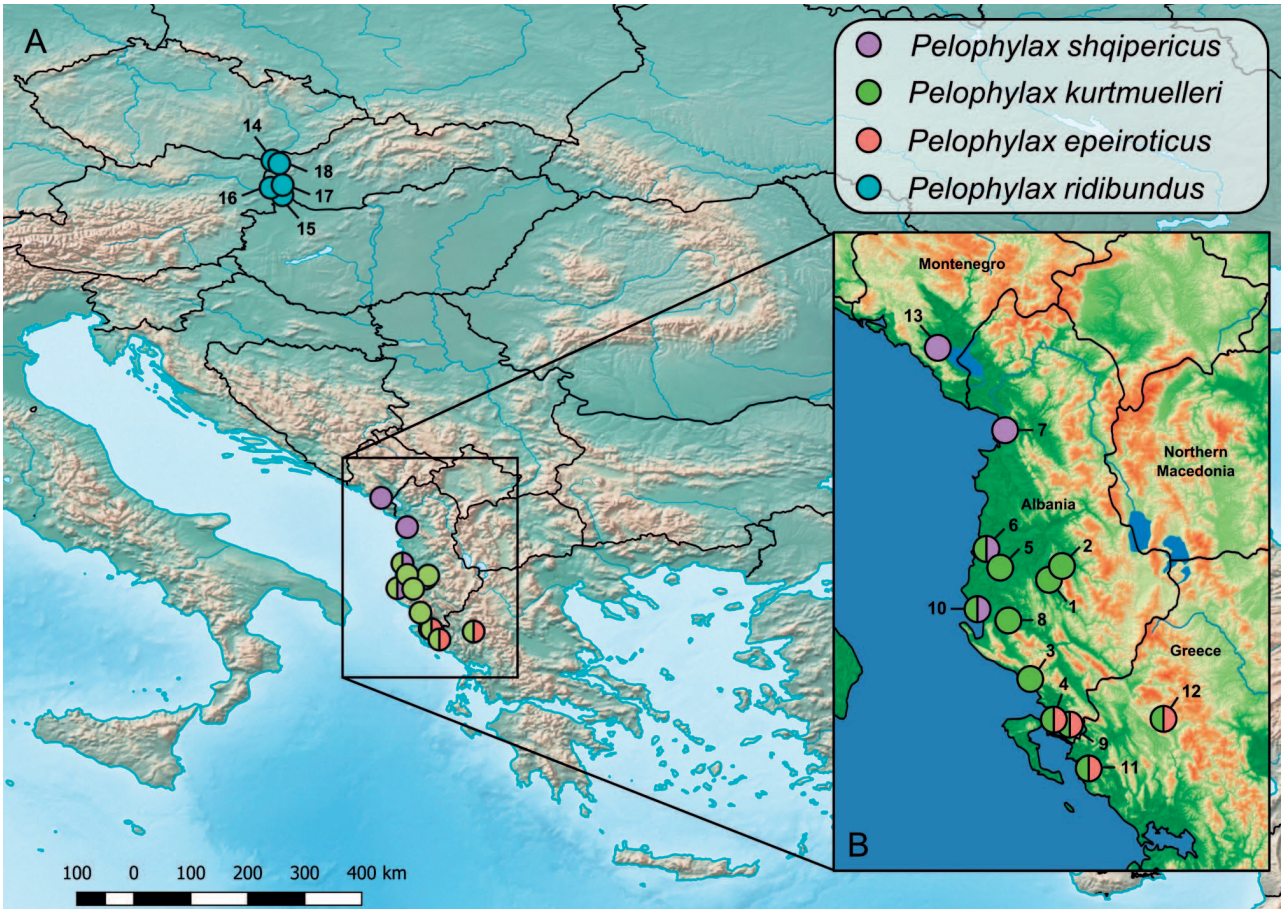

Figure 1. Localities of water frogs and species composition in the southwestern Balkans and Slovakia (A). The inset (B) shows sample sites from the southwestern Balkans in detail. 1 – Berat, 2 – Dushnik, 3 – Qazim Pali, 4 – Ksamil, 5 – Mbrostar, 6 – Divjakë – Karavastë, 7 – Nishaj, 8 – Poçem, 9 – Xare, 10 – Zvërnec, 11 – Igoumenitsa, 12 – Ioannina, 13 – Virpazar, 14 – Brodské, 15 – Čunovo, 16 – Devín, 17 – Šúr, 18 – Šaštín-Stráže.

lowed by 35 cycles of denaturation for 30 s at 94°C, primer annealing at 57°C for 30 s, and elongation at 72°C for 1 min, with a final elongation at 72°C for 10 min (modified from Hauswaldt et al. 2012). PCR products were run in agarose electrophoresis with a molecular-weight size marker. SAI-1 alleles of selected individuals were sequenced commercially in Macrogen Europe (Amsterdam, the Netherlands), and their similarity with published sequences was compared using the program BLAST (https://blast.ncbi.nlm.nih.gov/). Obtained sequences are deposited in the NCBI GenBank under the accession numbers MW296101–105 for *P. shqipericus*; MW287331–335 for *P. epeiroticus* and finally, MW296106–111 for *P. kurtmuelleri*. Relationships among SAI-1 alleles were reconstructed using a parsimony network algorithm of TCS (Clement et al. 2000) implemented in PopArt 1.7 (http:// http://popart.otago.ac.nz). All sequences were aligned and checked using Geneious Prime 2020 2.4 (Biomatters, Auckland, New Zealand). A final alignment involved 45 sequences of the size of 597 bp. *Pelophylax perezi* (López-Seoane, 1885) was used as an outgroup based on previous studies (Lymberakis et al. 2007).

Microsatellites were amplified in multiplex PCRs. Most of individuals were analyzed for five microsatellite loci: Ga1a19 (Christiansen 2009), RICA1b5 (Garner et al. 2000), Rrid013A (Hotz et al. 2001), RICA2a34 and Rrid-135A (Christiansen & Reyer 2009). PCR reactions were performed in a total volume of 10 µl and consist of 5 µl of Qiagen Microsatellite PCR Master mix (Qiagen, Hilden, Germany), 0.1 µl of each primer (10 µM), 3 µl of ddH_2O and 1 µl of DNA. In the case of 31 individuals of *P. shqipericus* and 40 individuals of *P. kurtmuelleri*, 13 microsatellite loci were used for species identification: Ga1a19 (Christiansen 2009), RICA1b5, RICA2a34F, RICA5 (Garner et al. 2000), RICA1b6, Re1Caga10, Re2Caga3 (Arioli et al. 2010), Res14, Res22 (Zeisset et al. 2000), Rrid013A, Rrid-082A (Hotz et al. 2001) and Rrid169A. PCR program was modified from Christiansen & Reyer (2009): 5 min of initial denaturation at 95°C followed by 30 cycles of denaturation for 30 s at 95°C, 60°C for 90 s and 72°C for 1 min, with a final extension at 60°C for 30 min. Microsatellite fragments were run on an automated ABI genetic analyser.

The Bayesian clustering implemented in the program Structure 2.3.4 (Pritchard et al. 2000) was used for microsatellite data analyses. In the first analysis, all three species were analysed together for five microsatellite loci. Subsequently, the frogs were analysed in two datasets involving sympatrically living species pairs *P. shqipericus*/*P. kurtmuelleri* (the first dataset based on thirteen loci), and *P. epeiroticus*/*P. kurtmuelleri* (the second dataset based on five loci), respectively. The admixture and non-correlated allele model was used to calculate the parameter Q, that is, the proportion of an individual's genome originating in one of the inferred clusters (K), corresponding to the species *P. shqipericus*, *P. epeiroticus* and *P. kurtmuelleri*. Fixed K = 3 was chosen when all three species were analysed together, fixed K = 2 when species pairs were analyzed. The analyses were based on runs of 10^6 iterations, following a burn-in period of 10^5 iterations. Five independent runs were carried out to test the accuracy of the results. Besides Bayesian analyses, the Discriminant Analysis of Principal Components (DAPC) based on five microsatellite loci was performed. The 'adegenet' package (Jombart et al. 2020) implemented in the R statistical environment (R Core Team 2019) was used for this analysis (for more details about the DAPC see below).

Morphometric analyses

Statistical analysis of morphometric characteristics was performed in the R statistical environment (R Core Team 2019). A suitable data format was created using 'tidyverse' (Wickham & RStudio 2019) and 'janitor' (Firke et al. 2019) packages. Seven morphometric indices (L/F, L/T, L/DP, L/CINT, F/T, T/CINT, DP/CINT) were calculated (Günther 1990, Plötner 2005). Before conducting analysis, correlation, outliers and the assumption of normality were checked via the 'PerformanceAnalytics' package (Peterson et al. 2020). Two-way ANOVA was performed to examine differences in calculated proportions among species and sexes. When ANOVA was significant, Tukey HSD post-hoc tests were used to perform multiple pairwise comparisons among the species. Both analyses were performed with respect to 'rstatix' package syntax (Kassambara 2020). Identification of clusters and distances in morphospace was performed via the Discriminant Analysis of Principal Components (DAPC) from 'adegenet' package (Jombart et al. 2020). This method finds the linear combinations of morphometric variables that have the largest between-group variance and the smallest within-group variance. DAPC uses the transformation of variables by the Principal Component Analysis (PCA) prior to performing the Discriminant Analysis (DA). This ensures that variables are uncorrelated, thus making it a suitable methodological approach for morphological data that usually demonstrate a high level of correlation. Based on the results of DAPC, group membership probabilities for each individual were computed. All visualizations were created using the 'ggplot2' (Wickham et al. 2019), 'ggpubr' (Kassambara 2019), 'adegenet' (Jombart et al. 2020) and 'gridExtra' (Auguie & Antonov 2017) packages.

Results

Genetic identification of Balkan species

Amplification of the SAI-1 revealed three PCR fragments of the length ~720 bp, ~840 bp, and ~1 200 bp, which corresponded to the species *P. kurtmuelleri*, *P. epeiroticus*, and *P. shqipericus*, respectively. The BLAST supported a correct species-specificity of sequenced PCR fragments. SAI-1 alleles formed species-specific clusters in a parsimony network (Supplementary Figure S1). Four individuals were heterozygous in SAI-1 (Supplementary Table S1). A female captured near Ksamil, Albania (ID: MIK3233), and a male

from the Ioannina Lake, Greece (MIK3309) carried one allele specific for *P. kurtmuelleri* and one for *P. epeiroticus*. A male from Divjakë – Karavastë, Albania (MIK3160) had one *P. kurtmuelleri* allele and one *P. shqipericus* allele. Finally, a female captured in Poçem, Albania (JAB5636), outside from *P. epeiroticus* distribution range, carried two fragments indicating it was a hybrid between *P. kurtmuelleri* and *P. epeiroticus*. In this case, the sequence of the fragment ~840 bp (MW296111) was not homologous with *P. epeiroticus* but matched *P. ridibundus* individuals from Kamchatka, Russia (99.50% identity, accession number KX503320.1), Aliartos, Greece (99.37% identity, accession number MF667644.1), and Atyrau, Kazakhstan (99.37% identity, accession number HE858213.1). This SAI-1 allele was assigned to the *P. ridibundus* cluster in a parsimony network (Supplementary Figure S1), and thus a female JAB5636 was not considered as a hybrid but as a *P. kurtmuelleri* individual with a *ridibundus*-specific allele

The program Structure assigned individuals to the clusters corresponding to the species *P. shqipericus*, *P. epeiroticus* and *P. kurtmuelleri* (Supplementary Table S2). The following four individuals revealed an admixed genome and were excluded from morphological analyses. A male from the Ioannina Lake (MIK3309) carried 57.5% of genome from *P. kurtmuelleri* and 42.5% from *P. epeiroticus*, and was considered an F1 hybrid. Two individuals from Ksamil (MIK3233) and Igoumenitsa (MIK3277) carried 75.0% and 77.5% of genome from *P. kurtmuelleri*, the remaining allelic variation was derived from *P. epeiroticus*, and both were considered backcrossed hybrids. However, MIK3277 was assigned to the *P. kurtmuelleri* cluster when all three species were analyzed together (with fixed K = 3; Supplementary Table S2). A male from Divjakë – Karavastë (MIK3160) possessed 30.1% of genome from *P. kurtmuelleri* and 69.9% from *P. shqipericus*, and was also evaluated as a backcrossed hybrid. A female from Poçem (JAB5636), which carried a SAI-1 allele specific for *P. ridibundus*, was clearly assigned to the *P. kurtmuelleri* cluster.

Microsatellite-based DAPC revealed three spatially separated clusters corresponding to particular species. All hybrid individuals but MIK3277 showed intermediate position in multivariate space (Fig. 3B).

Morphometric analyses

Morphometric characteristics and indices for both males and females of each species are summarized in Tables 2 and 3, and in Figure 2. The ANOVA revealed significant differences between *P. epeiroticus* and *P. kurtmuelleri* in six out of seven morphometric indices in males and in all seven in females (Table 4). *Pelophylax shqipericus* and *P. kurtmuelleri* differed significantly from each other at six indices for both sexes. *Pelophylax kurtmuelleri* and *P. ridibundus* differed significantly at six indices in males, whereas only four indices were significantly different in females. Interestingly, *P. epeiroticus* and *P. shqipericus* were the most similar species pair. No significantly different indices were found in males, and only two significantly different indices were in females. The significantly different indices across species were L/T, L/CINT, and DP/CINT (Tab. 4).

Four individuals identified as nuclear hybrids shared morphometric indices with both parental taxa (Supplementary Table S1). A specimen MIK3309 possessing 57.5% of genome from *P. kurtmuelleri* and 42.5% from *P. epeiroticus* had three and four indices similar to their parents, respectively. Specimens MIK3233 and MIK3277 (with about three-quarters of genome from *P. kurtmuelleri*) had the most indices similar to *P. kurtmuelleri*. Finally, a hybrid, MIK3160, composed of 69.9% from *P. shqipericus* genome and of 30.1% from *P. kurtmuelleri* had three and four indices similar to their parents, respectively.

Three principal components were retained for DAPC analysis. The first PC had an eigenvalue of 12.7, accounting for 95% of the variance (Supplementary Table S3). The other two retained components (PC_2, PC_3) had significantly lower eigenvalues (Supplementary Table S3). Overall, retained variance by all three principal components was 99%. The first principal component had noticeable loadings on L/CINT and T/CINT variables. The second principal component displayed strong loading on L/DP variable, and the third retained PC had strong loading on T/CINT variable (Supplementary Table S3). The examination of morphospace created via PCA (Supplementary Figure S2) showed a high overlap of all studied species. Similarly, the application of Discriminant Analysis (DA) introduced a considerable amount of overlap in all species (Fig. 3A). Both centroids of *P. epeiroticus* and *P. shqipericus* and *P. ridibundus, P. kurtmuelleri* appear to be closely situated in morphospace. Both *P. epeiroticus* and *P. shqipericus,* however, appear to be more distant in morphospace from *P. kurtmuelleri.* The hybrids either fell to the centroids of the parental species (MIK3160, MIK3277) or showed an intermediate position in multivariate morphospace (MIK3233 and MIK3309).

Colouration and qualitative morphological traits

The dorsal colouration of *P. shqipericus* (Fig. 4) varied from bright green to olive-brown or even dark brown. The dorsal pattern consisted of relatively large irregular dark green, olive, or dark grey spots. Almost uniformly coloured individuals with a strongly reduced spot pattern were rare (approx. 3% of individuals). A mid-dorsal yellow or light green stripe was present in the majority of individuals. Tympana can be green, olive, bronze, or dark brown with a lighter centre and darker surroundings or unicolour. Dorsolateral folds were visible but low, often distinguishable by a distinct colour. Male vocal sacs are light grey or olive green (Fig. 7A). Lateral sides are usually paler, marked with darker spots with diffused edges and yellow pigment. The upper side of the legs is coloured and spotted similar to the dorsum. Several darker transversal spots were present in the anterior part of the thighs, dark grey to black merged spots with yellow pigment were typical for a posterior part

Table 2. Summary statistics of morphometric characters measured in three water frog species (genus *Pelophylax*) from the southwestern Balkans and *P. ridibundus* from Central Europe. Min – minimal value, Max – maximal value, SD – standard deviation, SE – standard error.

Character	*P. epeiroticus*		*P. kurtmuelleri*		*P. ridibundus*		*P. shqipericus*	
	Males	Females	Males	Females	Males	Females	Males	Females
	N = 25	N = 26	N = 57	N = 43	N = 57	N = 32	N = 36	N = 24
L								
Mean	55.70	70.06	67.69	71.67	80.48	85.20	52.63	54.27
Min	45.20	46.87	52.24	54.69	51.50	50.00	41.50	42.00
Max	72.10	82.50	85.10	104.20	111.40	117.30	66.51	81.00
Median	53.90	71.35	67.50	71.80	86.40	89.40	51.45	52.45
SD	7.12	8.36	9.42	10.81	15.06	21.76	7.03	8.87
SE	1.42	1.64	1.25	1.65	1.99	3.85	1.17	1.81
F								
Mean	27.22	33.10	34.23	36.12	40.21	42.16	25.66	25.63
Min	22.00	22.15	26.59	28.33	24.95	22. 60	20.00	19.90
Max	37.27	40.20	44.30	50.50	53.10	59.65	32.70	39.30
Median	26.28	33.05	33.86	35.42	43.45	44.05	25.25	25.00
SD	3.70	4.16	4.78	4.93	7.50	11.16	3.55	4.19
SE	0.74	0.82	0.63	0.75	0.99	1.97	0.59	0.86
T								
Mean	28.49	34.23	37.51	39.95	42.90	45.23	27.04	27.07
Min	23.20	22.93	28.69	30.95	26.70	21.90	20.60	21.60
Max	36.31	39.20	48.00	56.00	56.35	62.50	34.30	41.00
Median	27.60	35.18	37.11	39.36	46.85	48.91	26.85	26.00
SD	3.25	3.92	4.91	5.46	8.19	12.50	3.75	4.33
SE	0.65	0.77	0.65	0.83	1.08	2.21	0.63	0.88
DP								
Mean	8.86	10.81	9.51	10.06	11.49	12.20	8.01	8.14
Min	6.44	7.31	6.92	7.73	6.20	5.30	6.20	6.00
Max	11.72	12.70	13.10	14.60	15.35	16.70	10.40	11.50
Median	8.75	10.88	9.46	10.00	12.25	13.80	8.20	8.05
SD	1.35	1.46	1.39	1.45	2.36	3.53	1.05	1.24
SE	0.27	0.29	0.18	0.22	0.31	0.62	0.18	0.25
CINT								
Mean	2.84	2.90	3.67	3.86	3.95	4.14	2.57	2.49
Min	2.10	1.90	2.40	2.74	2.30	2.05	1.80	1.90
Max	3.98	3.97	5.59	5.28	5.53	6.87	4.12	3.70
Median	2.72	2.90	3.50	3.81	3.85	4.35	2.50	2.40
SD	0.49	0.48	0.61	0.67	0.81	1.00	0.59	0.51
SE	0.10	0.09	0.08	0.10	0.11	0.18	0.10	0.10

(Fig. 8A). The venter was usually white or yellowish-white with diffused darker spots in some individuals and with a higher amount of yellow pigment. Similar colouration was also present on the ventral side of the thighs (Fig 9A). Rarely, there was brown spots in the anterior part of the venter. The metatarsal tubercle was either triangular or oblique, usually low, but sometimes higher and reached a maximum height in its distal part. The yellow or orange pigment was present in the foot webbing (Fig. 7A).

Dorsal colouration of *P. epeiroticus* (Fig. 5) formed a scale from yellowish green to dark green or olive green with relatively large, irregular, dark green to black spots. However, individuals without spots were also recorded. A yellow or light green mid-dorsal stripe was present in the majority of individuals. Dorsolateral glandular folds were low, but clearly visible and often olive or brown. Although, these folds can bear the same colour as the surrounding skin. The tympanum was either green, bronze, or brown, often with a darker margin and lighter centre. Vocal sacs of males were dark grey to almost black (Fig. 7B). Lateral sides were usually paler than, or similarly coloured to, a dorsal side with the yellow pigment.

Table 3. Summary statistics of morphometric indices in three water frog species (genus *Pelophylax*) from the southwestern Balkans and *P. ridibundus* from Central Europe. Min – minimal value, Max – maximal value, SD – standard deviation, SE – standard error.

Index	*P. epeiroticus*		*P. kurtmuelleri*		*P. ridibundus*		*P. shqipericus*	
	Males	Females	Males	Females	Males	Females	Males	Females
	N = 25	N = 26	N = 57	N = 43	N = 57	N = 32	N = 36	N = 24
L/F								
Mean	2.05	2.12	1.98	1.98	2.00	2.03	2.05	2.12
Min	1.91	1.99	1.80	1.82	1.88	1.86	1.89	1.94
Max	2.19	2.29	2.17	2.00	2.21	2.21	2.21	2.28
Median	2.05	2.11	1.97	2.00	2.00	2.02	2.04	2.12
SD	0.07	0.08	0.07	0.07	0.08	0.08	0.07	0.08
SE	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.02
L/T								
Mean	1.95	2.05	1.80	1.79	1.88	1.90	1.95	2.00
Min	1.86	1.91	1.68	1.67	1.71	1.75	1.85	1.84
Max	2.03	2.12	1.93	1.96	2.10	2.28	2.14	2.11
Median	1.96	2.05	1.81	1.80	1.87	1.88	1.94	1.99
SD	0.05	0.05	0.06	0.06	0.09	0.12	0.06	0.07
SE	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.01
L/DP								
Mean	6.32	6.50	7.14	7.13	7.07	7.15	6.	6.67
Min	5.57	6.04	6.01	5.78	5.89	5.99	5.77	5.89
Max	7.39	7.05	7.91	8.10	8.61	9.47	7.27	7.40
Median	6.33	6.43	7.16	7.20	7.02	7.10	6.52	6.64
SD	0.40	0.27	0.46	0.49	0.62	0.89	0.40	0.37
SE	0.08	0.05	0.06	0.07	0.08	0.16	0.07	0.08
L/CINT								
Mean	19.83	24.53	18.64	18.74	20.64	20.77	21.00	22.08
Min	15.82	19.32	14.76	14.98	15.14	14.14	15.36	17.71
Max	24.62	32.74	24.83	23.67	28.66	28.15	28.17	25.45
Median	19.64	24.42	18.28	18.71	20.21	21.48	21.31	22.34
SD	2.09	3.20	2.26	2.15	3.07	3.59	2.90	2.36
SE	0.42	0.63	0.30	0.33	0.41	0.64	0.48	0.48
F/T								
Mean	0.95	0.97	0.91	0.90	0.94	0.94	0.95	0.95
Min	0.89	0.92	0.85	0.86	0.86	0.86	0.89	0.90
Max	1.03	1.04	0.97	0.96	1.03	1.04	1.00	1.00
Median	0.95	0.96	0.91	0.90	0.94	0.93	0.95	0.94
SD	0.04	0.03	0.03	0.02	0.04	0.04	0.02	0.02
SE	0.01	0.01	0.00	0.00	0.01	0.01	0.00	0.00
T/CINT								
Mean	10.16	11.99	10.34	10.46	11.03	11.02	10.77	11.02
Min	8.12	9.35	7.82	8.15	7.63	6.81	8.19	8.78
Max	13.02	15.65	13.40	12.48	15.46	15.51	14.44	13.33
Median	10.12	11.97	10.33	10.36	10.94	11.20	10.78	11.14
SD	1.17	1.50	1.22	1.16	1.92	2.32	1.41	1.22
SE	0.23	0.29	0.16	0.18	0.25	0.41	0.24	0.25
DP/CINT								
Mean	3.15	3.78	2.63	2.64	2.96	2.98	3.21	3.32
Min	2.55	2.87	1.97	2.04	1.77	1.65	2.19	2.44
Max	4.03	5.13	3.53	3.48	4.62	4.43	4.56	4.24
Median	3.11	3.63	2.60	2.57	2.86	3.03	3.20	3.30
SD	0.39	0.49	0.38	0.39	0.62	0.76	0.50	0.41
SE	0.08	0.10	0.05	0.06	0.08	0.13	0.08	0.08

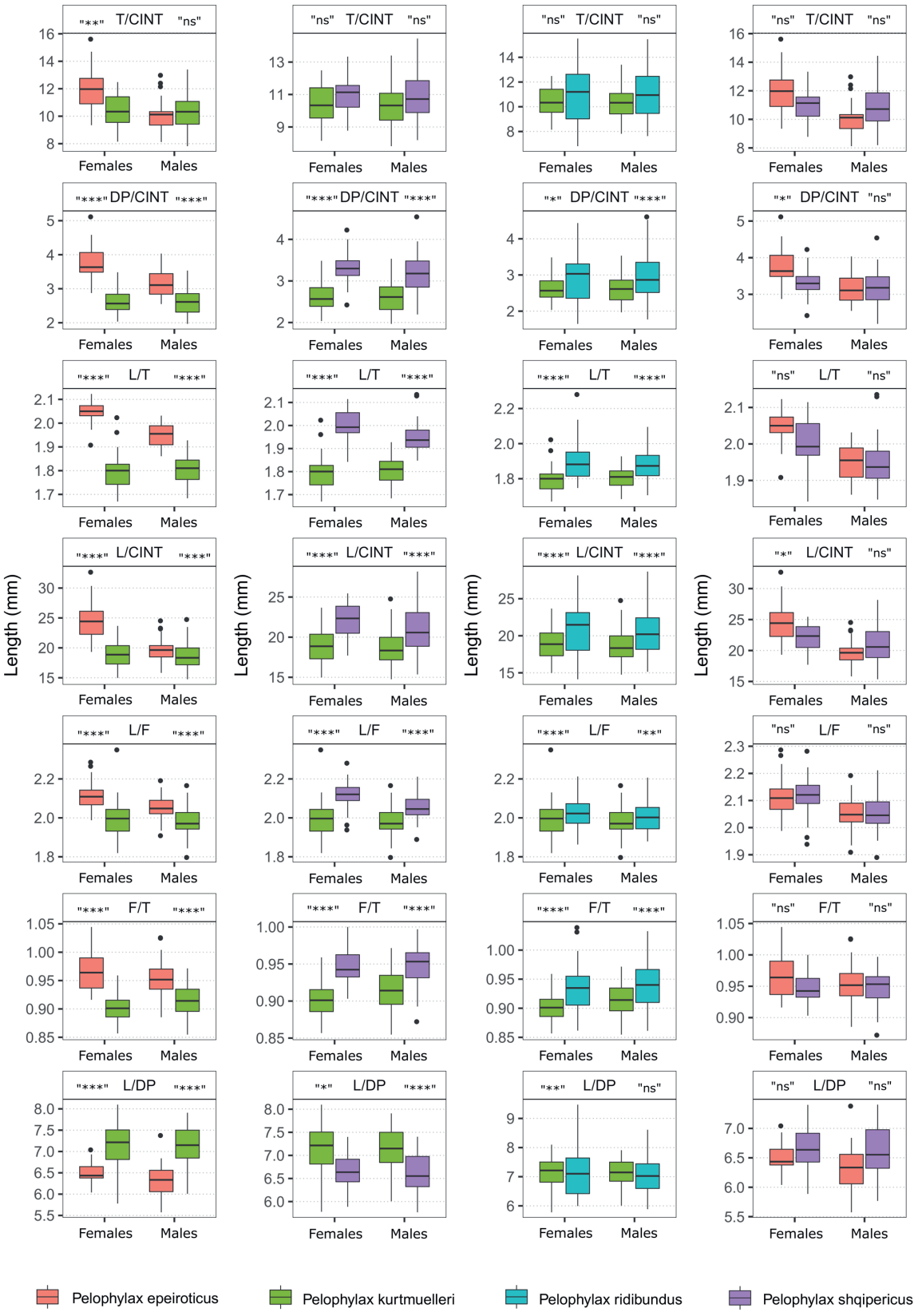

Figure 2. Comparison of morphometric indices among three Balkan water frog species (genus *Pelophylax*) and central-European *P. ridibundus*. * – $p < 0.05$, ** – $p < 0.005$, *** – $p < 0.0005$, ns – not significant.

Table 4. Differences between water frog species (genus *Pelophylax*) in morphometric indices revealed by ANOVA and Tukey HSD post-hoc tests with statistically significant values in bold. S – Significance, * – $p < 0.05$, ** – $p < 0.005$, *** – $p < 0.0005$, ns – not significant.

Species 1	Species 2	Sex	Variable	*p* value	S
P. epeiroticus	*P. shqipericus*	Males	L/F	1.00 e^{+0}	ns
P. epeiroticus	*P. shqipericus*	Males	L/T	2.99 e^{-1}	ns
P. epeiroticus	*P. shqipericus*	Males	L/DP	1.61 e^{-1}	ns
P. epeiroticus	*P. shqipericus*	Males	L/CINT	5.37 e^{-1}	ns
P. epeiroticus	*P. shqipericus*	Males	F/T	2.03 e^{-1}	ns
P. epeiroticus	*P. shqipericus*	Males	T/CINT	9.34 e^{-1}	ns
P. epeiroticus	*P. shqipericus*	Males	DP/CINT	1.79 e^{-1}	ns
P. epeiroticus	*P. kurtmuelleri*	Males	L/F	**1.36 e^{-12}**	***
P. epeiroticus	*P. kurtmuelleri*	Males	L/T	**6.68 e^{-13}**	***
P. epeiroticus	*P. kurtmuelleri*	Males	L/DP	**9.09 e^{-12}**	***
P. epeiroticus	*P. kurtmuelleri*	Males	L/CINT	**9.26 e^{-11}**	***
P. epeiroticus	*P. kurtmuelleri*	Males	F/T	**7.04 e^{-13}**	***
P. epeiroticus	*P. kurtmuelleri*	Males	T/CINT	6.92 e^{-2}	ns
P. epeiroticus	*P. kurtmuelleri*	Males	DP/CINT	**7.06 e^{-13}**	***
P. kurtmuelleri	*P. shqipericus*	Males	L/F	**7.89 e^{-13}**	***
P. kurtmuelleri	*P. shqipericus*	Males	L/T	**6.68 e^{-13}**	***
P. kurtmuelleri	*P. shqipericus*	Males	L/DP	**3.88 e^{-8}**	***
P. kurtmuelleri	*P. shqipericus*	Males	L/CINT	**4.26 e^{-8}**	***
P. kurtmuelleri	*P. shqipericus*	Males	F/T	**4.59 e^{-12}**	***
P. kurtmuelleri	*P. shqipericus*	Males	T/CINT	2.33 e^{-1}	ns
P. kurtmuelleri	*P. shqipericus*	Males	DP/CINT	**1.33 e^{-11}**	***
P. kurtmuelleri	*P. ridibundus*	Males	L/F	**9.15 e^{-3}**	**
P. kurtmuelleri	*P. ridibundus*	Males	L/T	**7.31 e^{-13}**	***
P. kurtmuelleri	*P. ridibundus*	Males	L/DP	9.59 e^{-1}	ns
P. kurtmuelleri	*P. ridibundus*	Males	L/CINT	**5.71 e^{-6}**	***
P. kurtmuelleri	*P. ridibundus*	Males	F/T	**5.87 e^{-9}**	***
P. kurtmuelleri	*P. ridibundus*	Males	T/CINT	**2.18 e^{-2}**	*
P. kurtmuelleri	*P. ridibundus*	Males	DP/CINT	**3.73 e^{-5}**	***
P. epeiroticus	*P. shqipericus*	Females	L/F	1.00 e^{+0}	ns
P. epeiroticus	*P. shqipericus*	Females	L/T	2.71 e^{-1}	ns
P. epeiroticus	*P. shqipericus*	Females	L/DP	7.00 e^{-1}	ns
P. epeiroticus	*P. shqipericus*	Females	L/CINT	**1.48 e^{-2}**	*
P. epeiroticus	*P. shqipericus*	Females	F/T	1.26 e^{-1}	ns
P. epeiroticus	*P. shqipericus*	Females	T/CINT	1.55 e^{-1}	ns
P. epeiroticus	*P. shqipericus*	Females	DP/CINT	**1.49 e^{-2}**	*
P. epeiroticus	*P. kurtmuelleri*	Females	L/F	**5.29 e^{-10}**	***
P. epeiroticus	*P. kurtmuelleri*	Females	L/T	**8.66 e^{-15}**	***
P. epeiroticus	*P. kurtmuelleri*	Females	L/DP	**1.05 e^{-4}**	***
P. epeiroticus	*P. kurtmuelleri*	Females	L/CINT	**1.91 e^{-12}**	***
P. epeiroticus	*P. kurtmuelleri*	Females	F/T	**7.50 e^{-12}**	***
P. epeiroticus	*P. kurtmuelleri*	Females	T/CINT	**1.25 e^{-3}**	**
P. epeiroticus	*P. kurtmuelleri*	Females	DP/CINT	**2.70 e^{-13}**	***
P. kurtmuelleri	*P. shqipericus*	Females	L/F	**1.64 e^{-9}**	***
P. kurtmuelleri	*P. shqipericus*	Females	L/T	**4.15 e^{-14}**	***
P. kurtmuelleri	*P. shqipericus*	Females	L/DP	**1.09 e^{-2}**	*
P. kurtmuelleri	*P. shqipericus*	Females	L/CINT	**6.23 e^{-5}**	***
P. kurtmuelleri	*P. shqipericus*	Females	F/T	**3.57 e^{-6}**	***
P. kurtmuelleri	*P. shqipericus*	Females	T/CINT	5.24 e^{-1}	ns
P. kurtmuelleri	*P. shqipericus*	Females	DP/CINT	**1.25 e^{-5}**	***
P. kurtmuelleri	*P. ridibundus*	Females	L/F	5.39 e^{-2}	ns
P. kurtmuelleri	*P. ridibundus*	Females	L/T	**2.07 e^{-7}**	***
P. kurtmuelleri	*P. ridibundus*	Females	L/DP	1.00 e^{+0}	ns
P. kurtmuelleri	*P. ridibundus*	Females	L/CINT	**1.49 e^{-2}**	*
P. kurtmuelleri	*P. ridibundus*	Females	F/T	**8.32 e^{-5}**	***
P. kurtmuelleri	*P. ridibundus*	Females	T/CINT	4.51 e^{-1}	ns
P. kurtmuelleri	*P. ridibundus*	Females	DP/CINT	**3.50 e^{-2}**	*

Lateral spots were usually present, dark grey or black, always with diffused edges. Yellow pigment is present on the margins of the ventral side. Legs were dorsally green or yellowish with olive, dark grey, or black irregular spots. These spots were transversal and merged or are separated (Fig. 8B). The ventral side was uniformly white to creamy, sometimes with grey or brown marbling. The ventral part of the thighs was uniformly white to creamy or covered by marbling (Fig. 9B). The metatarsal tubercle was predominantly very small and low and either the shape of a scalene triangle or oblique. Orange or yellow pigment was characteristically present in the foot webbing (Fig. 7B).

In *P. kurtmuelleri* (Fig. 6), the dorsal part of the body was green to olive green or brown. Green colouration was usually situated in the anterior part of the body; the posterior part was usually brown or grey. Brown, grey or black, mostly irregular spots were distributed over the dorsal part, uniformly coloured individuals were rare. A yellow or light green mid-dorsal stripe was present in the majority of individuals. Dorsolateral folds were clearly visible, often distinguishable from the surrounding skin by a distinct colour. Tympana were olive, bronze, brown, or rarely green, with a paler centre. Vocal sacs in males were olive, grey, or dark grey (Fig. 7C). Lateral sides have a lighter tint compared to the dorsum and usually many irregular solitaries or merged black spots with sharp (not diffused) edges. Hind legs were usually olive or brown with darker transversal spots in the anterior part and merged dots in the posterior part, forming a web-like pattern. Yellow pigment was absent (Fig. 8C). The ventral side was mainly white without any pattern, rarely with brown or dark grey spots situated between the forelegs, in the mandibular area, or across the whole belly. The ventral side of the legs was predominantly white and spotless (Fig. 9C). The metatarsal tubercle was low but higher than in *P. epeiroticus*, and either triangular, or oblique. The foot webbing lacks any yellow or orange pigmentation (Fig. 7C). Table 5 characterizes the Balkans species living in sympatry for their identification in the field.

Discussion

Proper taxon identification is a prerequisite of any biological study, with morphological and morphometric characters being the first to be examined. Univariate (ANOVA) and multivariate (DAPC) analyses of water frogs from the southwestern Balkans revealed substantial differences between the sympatric species pairs, *P. kurtmuelleri*/*P. shqipericus*, and *P. kurtmuelleri*/*P. epeiroticus*. The most useful characteristics for the species identification are morphometric indices L/CINT, T/CINT, L/T, and DP/CINT. Key morphological characters for identifying *P. epeiroticus* are the presence of yellow pigment in the flanks, venter, groins and thighs; lateral spots absent or with diffused edges and a small metatarsal tubercle. Similar characters also fit to *P. shqipericus*, with exception of the metatarsal tubercle, which is bigger and higher compared to *P. kurtmuelleri*. On the other hand, yellow pigment in *P. kurtmuelleri* is usually absent; lateral spots with sharp edges are present and finally, the metatarsal tubercle is bigger compared to *P. epeiroticus*, but smaller than in *P. shqipericus*. Such data extends the previous allozyme and immunological markers (Hotz & Uzzell 1982, Hotz et al. 1987), or bioacoustic parameters (species-specific differences in mating calls; Schneider et al. 1984, 1993) for species identification. The advantage of our morphological and morphometric indices is their applicability where no molecular analyses are available, allowing for non-invasive field identification. The availability of identification markers may significantly help in potential sympatric populations to revise species distribution ranges and aid in species conservation.

Validity of morphometric and morphological tools to differentiate water frog species

Our study on a number of species across a wide distribution range confirmed a general validity of morphometric indices obtained by Hotz & Uzzell (1982) and Hotz et al. (1987) from Montenegro and Schneider et al. (1984, 1993) from Greece. The only exceptions were indices, where CINT was involved. Our more extensive sampling shows that the values T/CINT and L/CINT in *P. shqipericus* and *P. kurtmuelleri*, and T/CINT and DP/CINT in *P. epeiroticus* were higher than the values presented in the aforementioned studies.

According to ANOVA, the most significantly different indices between the species pairs were L/T, L/CINT, and DP/CINT for *P. epeiroticus* and *P. kurtmuelleri*, as also proposed by Schneider et al. (1984), and L/T, L/F, F/T and DP/CINT for *P. shqipericus* and *P. kurtmuelleri*. ANOVA results are corroborated by multivariate analyses. In general, a multivariate approach revealed considerable overlap

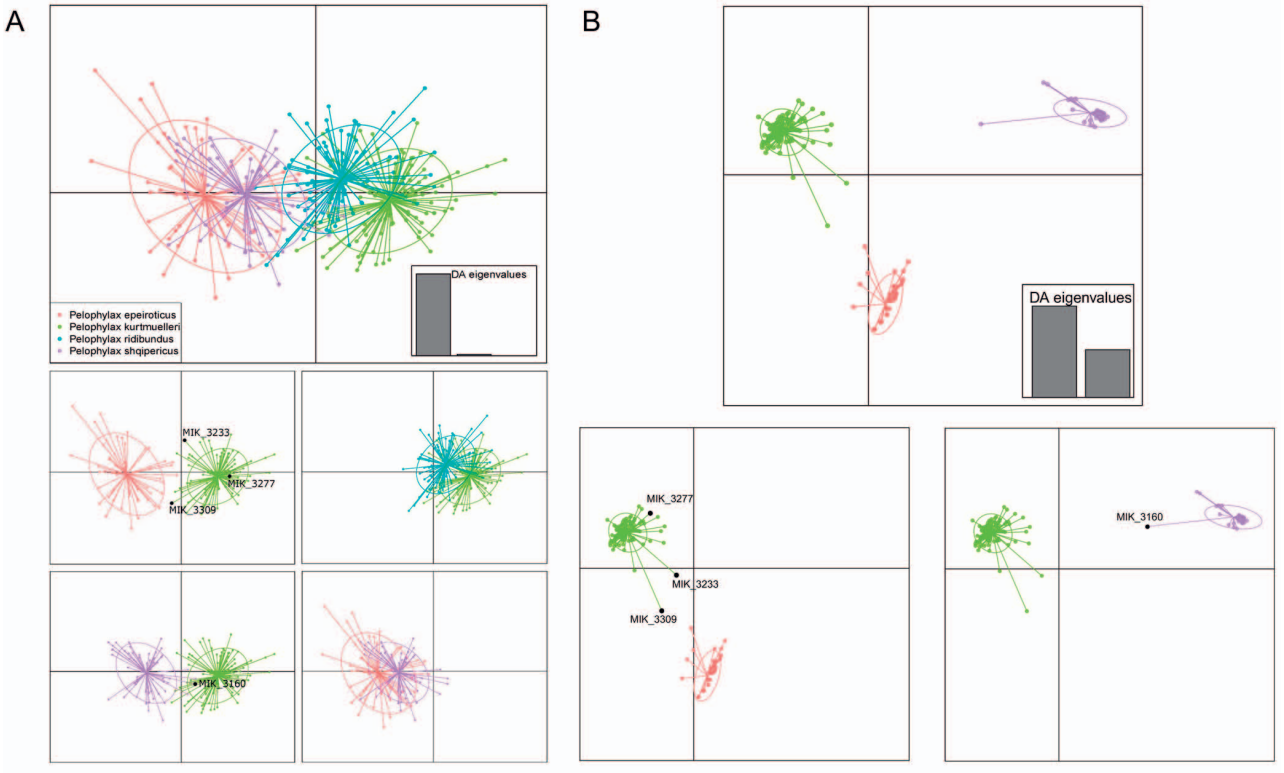

Figure 3. Discriminant analysis of principal components (DAPC) based on morphological (A) and microsatellite markers (B). Both analyses were carried out for all three (microsatellites) or four (morphological markers) species (upper panels) and subsequently for sympatrically living species pairs (lower panels). The position of hybrid individuals is displayed. A specimen MIK_3277 was identified as a hybrid only in a Bayesian analysis (see Results).

Figure 4. Variability of colour pattern in *Pelophylax shqipericus* from Nishaj, Albania (A, B, C); Velipojë, Albania (D, E, F,); Virpazar, Montenegro (G) and Divjakë, Albania (H).

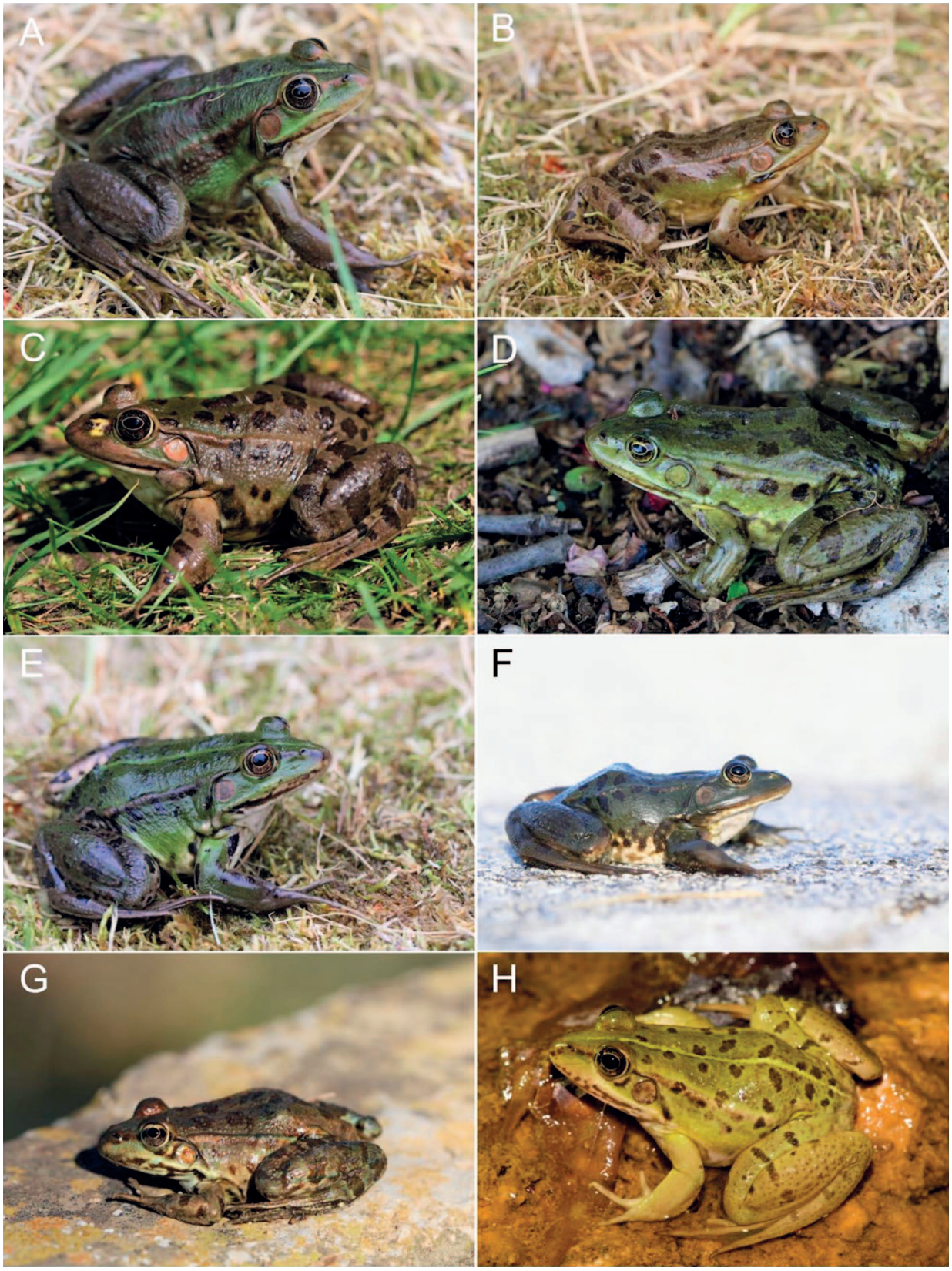

Figure 5. Variability of colour pattern in *Pelophylax epeiroticus* from Igoumenitsa, Greece (A, B); Ioannina, Greece (C, D, E); Corfu, Greece (F); Kalogria, Greece (G) and Stjar, Albania (H).

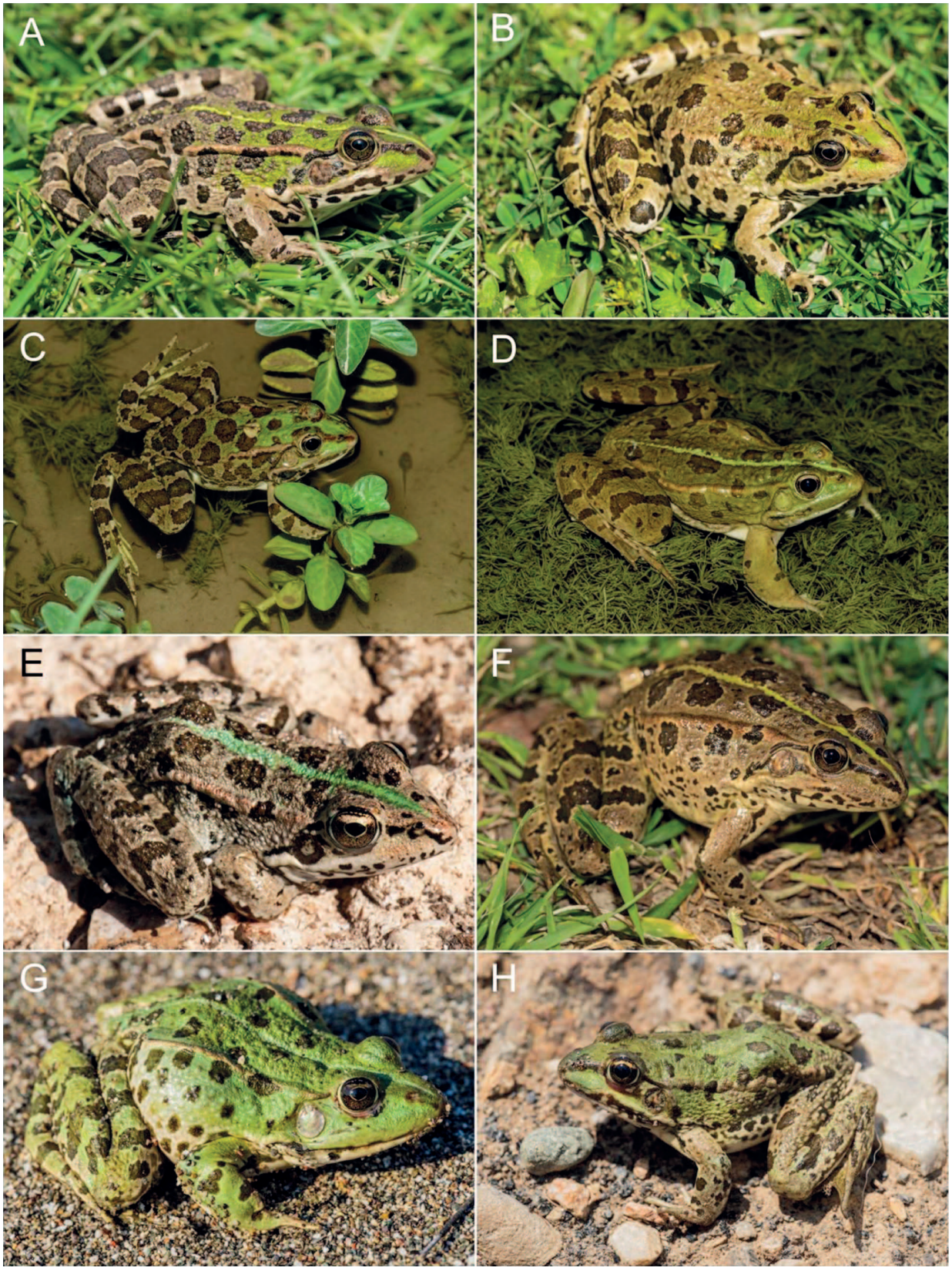

Figure 6. Variability of colour pattern in *Pelophylax kurtmuelleri* from Poçem, Albania (A, B); Qazim Pali, Albania (C, D); Shkalla, Albania (E); Mbrostar, Albania (F), Velipojë, Albania (G) and Perbreg, Albania (H).

among all species after the analysis of principal components (PCA). However, after the following DAPC, syntopically living *P. kurtmuelleri*/*P. shqipericus* or *P. epeiroticus* seem to be more separated in morphospace than other species pairs.

The morphological differentiation between the European water frog species is relatively narrow despite their deep phylogenetic divergence and high genetic differentiation as we also show from the studied regions. The valued discrimination characters are differences in the length of the body, hind legs, the first toe, and the metatarsal tubercle, as well as the head shape, the shape of the metatarsal tubercle and colouration (Günther 1990, Plötner 2005). Thus, the water frog species are rather challenging to correctly identify in the field, and this holds for the southwestern Balkans as well (e.g. Szabolcs et al. 2017). So, how to differentiate between sympatric *P. kurtmuelleri*/*P. epeiroticus*, and *P. kurtmuelleri*/*P. shqipericus* individuals correctly?

Species and hybrid identification in the field

According to our observations, *P. epeiroticus* differs from *P. kurtmuelleri* (Supplementary Figure S3) in the following traits: the presence of the yellow pigment in the groin, flanks, belly edges and upper as well as lower parts of the thighs, lateral spots are absent or, if present, with diffused edges, yellow or orange feet webbing, and a very small and oblique metatarsal tubercle. Moreover, vocal sacs of *P. epeiroticus* males are usually darker when compared to *P. kurtmuelleri*.

In morphometry, the best indices separating these two species are L/T (*P. epeiroticus* > 2, *P. kurtmuelleri* < 1.9), L/CINT (*P. epeiroticus* > 23.7, *P. kurtmuelleri* < 15.8), and DP/CINT (*P. epeiroticus* > 3.5, *P. kurtmuelleri* < 2.9). These findings agree with other authors (Hotz & Uzzell 1982, Schneider et al. 1984, Speybroeck et al. 2016). Additionally, *P. epeiroticus* should have a shorter tibia and femur (Hotz & Uzzell 1982), which is also evident in our data. On the other hand, we cannot confirm the unique green tympana colouration suggested by Schneider et al. (1984) as our observations also included *P. epeiroticus* individuals with bronze tympana, or even a combination of both.

Comparing another syntopically living species pair, *P. shqipericus* and *P. kurtmuelleri*, the best discriminative characteristics for *P. shqipericus* are yellow pigmentation in the groin, flanks, belly edges and on the thighs, lateral spots with diffused edges or absent, pronounced yellow or orange colouration of feet webbing, a relatively high and usually triangular metatarsal tubercle, and light greyish or greenish colouration of vocal sacs of males. The best indi-

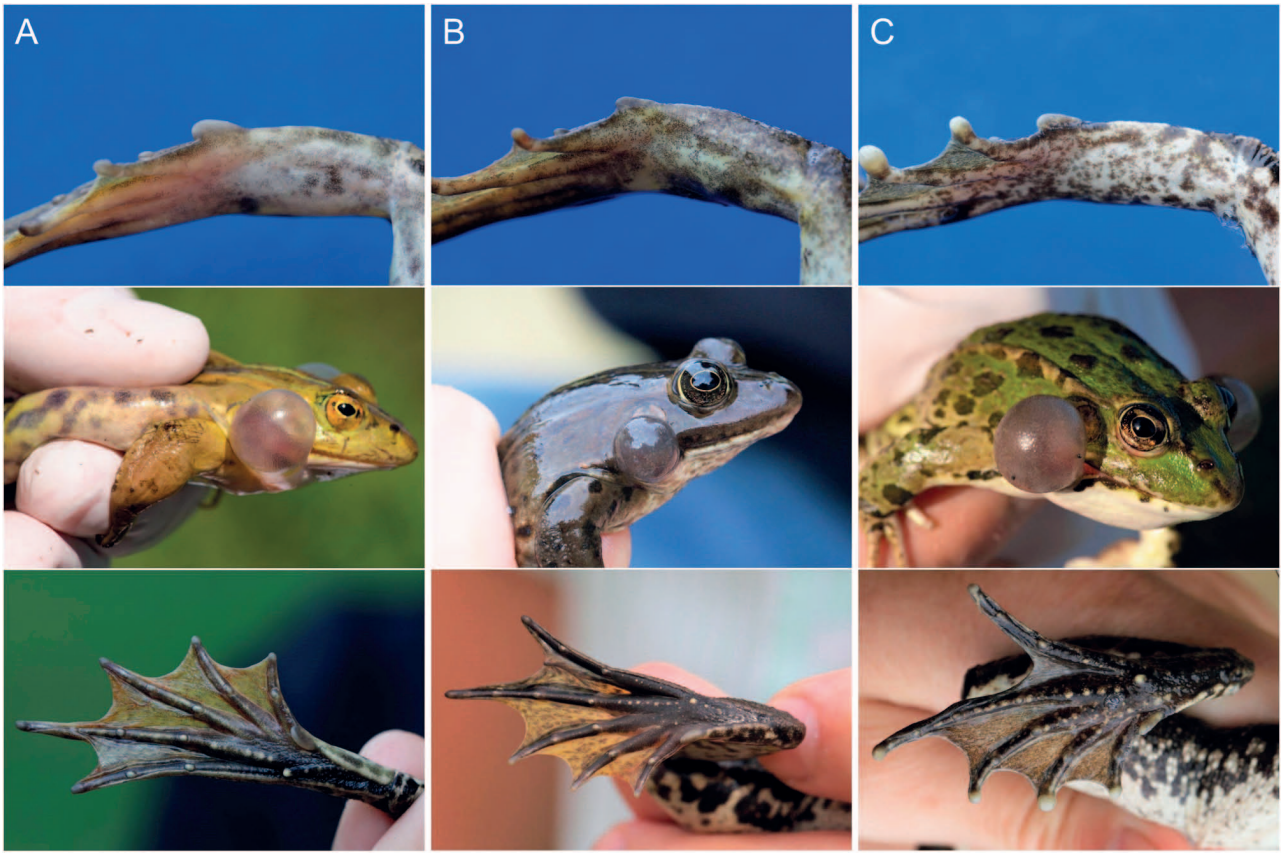

Figure 7. The shape of the metatarsal tubercle, vocal sacs colouration and the colouration of the foot webbing of *Pelophylax shqipericus* (A), *P. epeiroticus* (B) and *P. kurtmuelleri* (C).

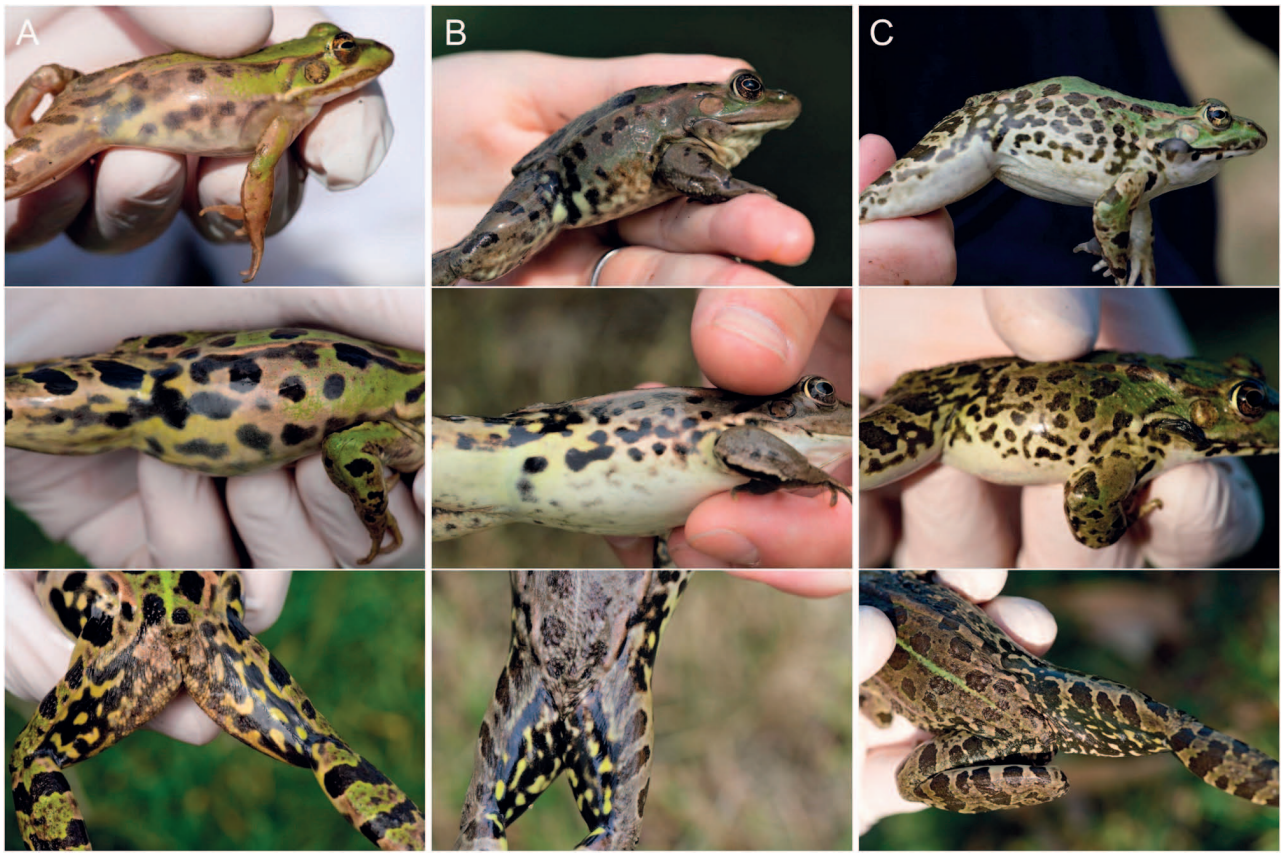

Figure 8. A pattern of lateral spots and yellow pigment in venter margins and upper thighs of *Pelophylax shqipericus* (A), *P. epeiroticus* (B) and *P. kurtmuelleri* (C).

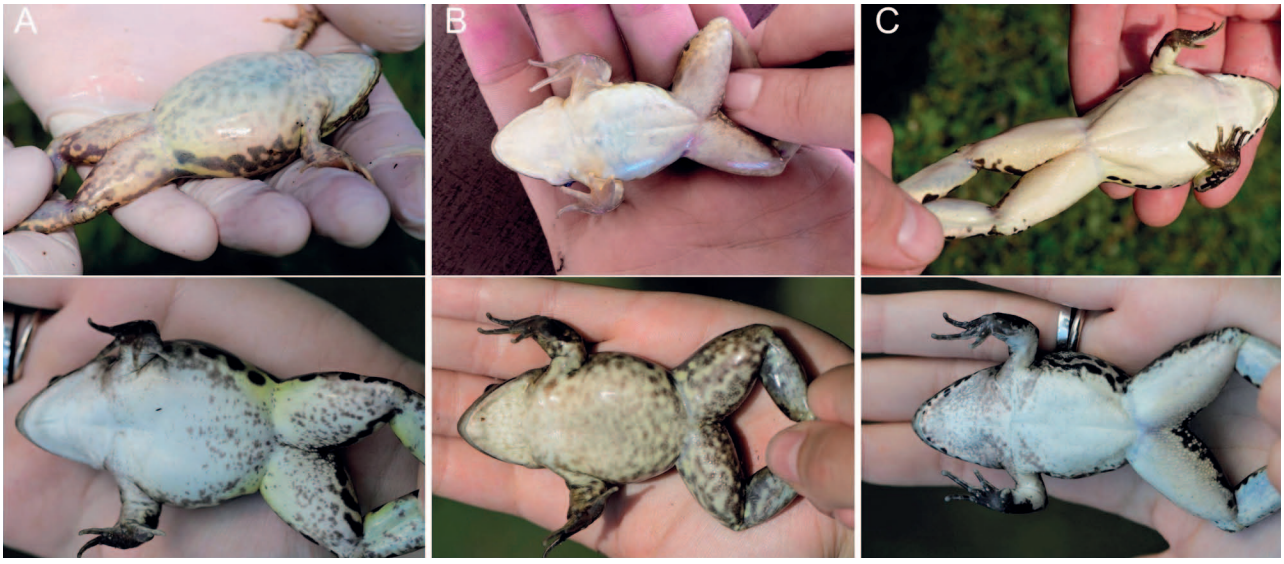

Figure 9. A ventral pattern and colouration of *Pelophylax shqipericus* (A), *P. epeiroticus* (B) and *P. kurtmuelleri* (C).

Table 5. Comparison of morphometric indices and qualitative morphological traits between sympatrically living species of water-frogs (genus *Pelophylax*) from the southwestern Balkans. [1] – external morphological characteristics according to Speybroeck et al. (2016).

Characteristics	*P. epeiroticus*	*P. kurtmuelleri*	*P. shqipericus*	*P. kurtmuelleri*
L/T	> 2.0	< 1.9	> 2.0	< 1.8
L/CINT	> 23.7	< 15.8	> 23.7	< 17.7
T/CINT	overlapped values	overlapped values	> 12.5	< 8.8
DP/CINT	> 3.5	< 2.9	> 3.5	< 2.4
Ventral colouration (Fig. 8)	Uniformly white or creamy or with dark marbling	Uniformly white or with dark spots, which can be situated over the belly or in the lower jaw and thighs	Usually white or yellowish-white, rarely with diffused dark spots	Uniformly white or with dark spots, which can be situated over the belly or in the lower jaw and thighs
Yellow pigment (Fig. 7)	Present in flanks, groins, venter, upper and lower thighs	Usually absent, rarely in upper thighs	Present in flanks, groins, venter, upper and lower thighs	Usually absent, rarely in upper thighs
Lateral spots (Fig. 7)	Absent or with diffused edges	Present, with sharp edges	Absent or with diffused edges	Present, with sharp edges
Shape and size of the metatarsal tubercle (Fig. 6)	The shape of scalene triangle or oblique; usually very low and small	Triangular or oblique; low and small, but higher and larger than in *P. epeiroticus*	Mostly triangular, rarely oblique; higher	Triangular or oblique; lower
Foot webbing colouration (Fig. 6)	Yellow to orange	Olive, brown or dark grey	Yellow to orange	Olive, brown or dark grey
Vocal sacs colour (Fig. 6)	Dark grey, dark olive, black Darker than in *P. kurtmuelleri*	Olive, grey, dark grey	Light grey or olive green Lighter than in *P. kurtmuelleri*	Olive, grey, dark grey
Head shape[1]	Medium length and blunt	Long with a sharp snout	Medium length and sharp	Long with sharp snout
Skin[1]	Smooth	Rough with warts and ridges	Smooth	Rough with warts and ridges
Heel joint when leg pulled forwards[1]	Reaches the eyes	Reaches beyond the eyes, usually beyond the snout	Reaches beyond the eyes, usually beyond the snout	Reaches beyond the eyes, usually beyond the snout
Thigh colour during breeding period[1]	Yellow-spotted	Grey or white-spotted, rarely yellow	Yellow-spotted	Grey or white-spotted, rarely yellow

ces to distinguish between the species are L/T (*P. shqipericus* > 2.0, *P. kurtmuelleri* < 1.8), L/CINT (*P. shqipericus* > 23.7, *P. kurtmuelleri* < 17.7), T/CINT (*P. shqipericus* > 12.5, *P. kurtmuelleri* < 8.8) and DP/CINT (*P. epeiroticus* > 3.5, *P. kurtmuelleri* < 2.4). Diffused lateral spots in *P. shqipericus* can continue to the ventral side and form dark marbling in contrast to *P. kurtmuelleri*, which has dark solid spots usually only on the jaw or thigh area, whereas the venter is uniformly white. Schneider et al. (1984), Hotz et al. (1987) and Speybroeck et al. (2016) reported other differences between the Balkan water frog species like an extension of the webbing to toe tips with an indentation between, the smoothness of the skin, shape of the head or the position of the heel joint when the leg is pulled forward, but none of these were measured in our study.

The morphological differentiation between *P. ridibundus* and *P. kurtmuelleri* were less pronounced. All seven measured morphometric indices to differentiate *P. kurtmuelleri* from the Central European *P. ridibundus* are cryptic. However, we cannot exclude that a comprehensive morphological analysis including more external, skeletal and other phenotypic characteristics would reveal a correlation between phylogenetic and morphological divergence and would shed light upon the taxonomy of the disputed species as was recently applicable, for instance, to the morphologically uniform bufonid genus *Bufotes* (Dufresnes et al. 2019).

Morphometric indices had a low-resolution power for identify hybrids among the Balkan water frog species. For instance, a hybrid MIK3160 possessing 69.9% of *P. shqipericus* genome and 30.1% *P. kurtmuelleri* clearly fell to the centroid of *P. kurtmuelleri* in a morphological DAPC analysis (Fig. 3A.). However, the same individual showed an intermediate position in a DAPC analysis of microsatellite markers, corroborating results of the Bayesian analysis (Fig. 3B). Similarly, a hybrid MIK3277 possessing two thirds of its genome from *P. kurtmuelleri* and the rest from *P. epeiroticus* was assigned to the *P. kurtmuelleri* centroid in both DAPC analyses. Two other *Pelophylax kurtmuelleri* × *P. epeiroticus* hybrids (MIK3233 and MIK3309) also showed an intermediate position in a multivariate space in DAPC analyses, however, only microsatellite markers sep-

arated them clearly from putative pure parental individuals. Therefore, a hybrid identification based solely on morphometry may lead to an inaccurate estimation of population structure, which is also evident in other amphibian taxa (e.g. Lehtinen et al. 2016, Arntzen et al. 2018). For detection of hybrids we advocate using nuclear molecular markers, including microsatellites and SAI-1. However, the length variability of the SAI-1 fragments examined on the agarose gel alone may not be sufficient, as shown by the example of the *P. kurtmuelleri* female from Poçem (Albania). This individual possessed SAI-1 fragments of ~720 bp and ~840 bp. The first fragment was specific for *P. kurtmuelleri* and the second one resembled the fragment specific for *P. epeiroticus*, indicating that this female is a hybrid between these two species. The sampling locality Poçem is, however, far northward from the *P. epeiroticus* range limit. Sequencing of the ~840 bp fragment and its comparison with other sequences using BLAST and a parsimony network showed that this fragment revealed the highest nucleotide similarity with SAI-1 sequences from *P. ridibundus*. The occurrence of the *P. ridibundus*-specific SAI-1 allele in the genome of *P. kurtmuelleri* might be a consequence of the ancestral polymorphism or historical hybridization between *P. kurtmuelleri* and *P. ridibundus*. Similarly, *P. kurtmuelleri*-specific alleles were found for instance in Poland, far away from the supposed range of *P. kurtmuelleri* (Kolenda et al. 2017).

Conclusion

The accurate identification of water frogs in the southwestern Balkans might be a challenging objective due to substantial morphological variability, interspecific hybridization, and sympatric occurrence. Despite these challenges, the most useful characteristics for the identification of the studied Balkan species are morphometric indices L/CINT, T/CINT, L/T, and DP/CINT, and qualitative traits like colouration and the size and shape of the metatarsal tubercle. Because some of these field markers may overlap between species when used for a taxon identification independently, both morphometric and morphological markers are necessary and recommended. Morphological markers are not suitable for the identification of hybrids where the application of genetic markers is necessary.

Acknowledgements

The authors would like to thank Adam Javorčík, Daniel Gruľa, Jana Christophoryová, Jana Poláková, Mária Hároníková, Michaela Mešková, Simona Papežíková and Žaneta Živčicová for their assistance during the fieldwork or in the laboratory. This study was supported by the Slovak Research and Development Agency under contracts APVV-19-0076 and APVV-15-0147, by the grant of the Scientific Grant Agency of the Slovak Republic VEGA 1/0286/19, by the grant of the Comenius University in Bratislava UK/7/2020, and by the grant of the Czech Science Foundation GA ČR 19-24559S, and by the grant of the Czech Academy of Sciences RVO: 67985904. The scientific permits were provided by the Directorate of Forest Management, Ministry for Environment and Energy of the Hellenic Republic (181012/807/28-3-2019), and the National Agency of Protected Areas, Ministry of Tourism and Environment of the Albanian Republic (No. 480/2019).

References

Akin, C., C. C. Bilgin, P. Beerli, R. Westaway, T. Ohst, S. N. Litvinchuk, T. Uzzell, M. Bilgin, H. Hotz, G.-D. Guex & J. Plötner (2010): Phylogeographic patterns of genetic diversity in eastern Mediterranean water frogs have been determined by geological processes and climate change in the Late Cenozoic. – Journal of Biogeography, **37**: 2111–2124.

Arioli, M., C. Jakob & H.-U. Reyer (2010): Genetic diversity in water frog hybrids (*Pelophylax esculentus*) varies with population structure and geographic location. – Molecular Ecology, **19**: 1814–1828.

Arntzen, J. W., N. Üzüm, M. D. Adjuković, A. Ivanović & B. Wielstra (2018): Absence of heterosis in hybrid crested newts. – PeerJ, **6**: e5317.

Auguie, B. & A. Antonov (2017): gridExtra: Miscellaneous Functions for "Grid" Graphics (version 2.3) [computer software]. – Available at: https://CRAN.R-project.org/package=gridExtra.

Clement, M., D. Posada & K. A. Crandall (2000): TCS: a computer program to estimate gene genealogies. – Molecular Ecology, **9**: 1657–1659.

Dufresnes, C., M. Denoël, L. di Santo & S. Dubey (2017): Multiple uprising invasions of *Pelophylax* water frogs, potentially inducing a new hybridogenetic complex. – Scientific Reports, **7**: 6506.

Dufresnes, C., G. Mazepa, D. Jablonski, R. C. Oliveira, T. Wenseleers, D. A. Shabanov, M. Auer, R. Ernst, C. Koch, H. E. Ramírez-Chaves, K. P. Mulder, E. Simonov, A. Tiutenko, D. Kryvokhyzha, P. L. Wennekes, O. I. Zinenko, O. V. Korshunov, A. M. Al-Johany, E. A. Peregontsev, R. Masroor, C. Betto-Colliard, M. Denoël, L. J. Borkin, D. V. Skorinov, R. A. Pasynkova, L. F. Mazanaeva, J. M. Rosanov, S. Dubey & S. N. Litvinchuk (2019): Fifteen shades of green: The evolution of *Bufotes* toads revised. – Molecular Phylogenetics and Evolution, **141**: 106615.

Firke, S., B. Denney, C. Haid, R. Knight & M. Grosser (2019): janitor: Simple tools for examining and cleaning dirty data (version 1.2.0) [computer software]. – Available at: https://CRAN.R-project.org/package=janitor.

Frost, D. R., T. Grant, I. Faivovich, R. H. Bain, A. Haas, C. F. B. Haddad, R. O. de Sá, A. Channing, M. Wilkinson, S. C. Donnellan, Ch. S. Raxworthy, J. A. Campbell, B. L. Blotto, P. Moler, R. C. Drewes, R. A. Nussbaum, J. D. Lynch, D. M. Green & W. C. Wheeler (2020): The Amphibian Tree of Life. – Bulletin of the American Museum of Natural History, **297**: 8–359.

Garner, T. W. J., B. Gautschi, S. Röthlisberger & H.-U. Reyer (2000): A set of CA repeat microsatellite markers derived from the pool frog, *Rana lessonae*. – Molecular Ecology, **9**: 2173–2174.

Gavrilović, V., D. D. Cvetković, G. Djukić & S. Petkovski (1999): Comparative morphological study of *Rana balcanica* and *Rana ridibunda*. – Contributions to the Zoogeography and Ecology of the Eastern Mediterranean Region, **1**: 205–210.

Günther, R. (1990): Die Wasserfrösche Europas. – Ziemsen Verglag, Leipzig.

Günther, R. (2004): *Rana shqiperica*. – pp. 156–157 in: Gasc, J.-P., A. Cabela, J. Crnobrnja-Isailovic, D. Dolmen, K. Grossenbacher, P. Haffner, J. Lescure, H. Martens, J. P. Martinez Rica, H. Maurin, M. E. Oliveira, T. S. Sofianidou, M. Veith & A. Zuiderwijk (eds): Atlas of amphibians and reptiles in Europe. – Muséum national d´Histoire naturelle, Paris.

Hauswaldt, J. S., M. Hoër, M. Ogielska, D. G. Christiansen, D. Dziewulska-Szwajkowska, E. Czernicka & M. Vences (2012): A simplified molecular method for distinguishing among species and ploidy levels in European water frogs (*Pelophylax*). – Molecular Ecology Resources, **12**: 797–805.

Hotz, H. & T. Uzzell (1982): Biochemically detected sympatry of two water frog species: two different cases in the Adriatic Balkans (Amphibia, Ranidae). – Proceedings of the Academy of Natural Sciences of Philadelphia, **134**: 50–79.

Hotz, H. & T. Uzzell (1983): Interspecific hybrids of *Rana ridibunda* without germ line exclusion of the parental genome. – Experentia, **39**: 538–540.

Hotz, H., G. Mancino, S. Bucci-Innocenti, M. Ragghianti, L. Berger & T. Uzzell (1985): *Rana ridibunda* varies geographically in inducing clonal gametogenesis in interspecific hybrids. – Journal of Experimental Zoology, **236**: 199–210.

Hotz, H., T. Uzzell, R. Günther, H. G. Tunner & S. Heppich (1987): *Rana shqiperica*, a new European water frog species from the Adriatic Balkans (Amphibia, Salientia, Ranidae). – Notulae Naturae of the Academy of Natural Sciences of Philadelphia, **468**: 1–3.

Hotz, H., T. Uzzell, G.-D. Guex, D. Alpers, R. D. Semlitsch & P. Beerli (2001): Microstellites: A tool for evolutionary genetic studies of western Palearctic water frogs. – Mitteilung aus dem Museum für Naturkunde Berlin, Zoologische Reihe, **77**: 43–50.

Christiansen, D. G. (2009): Gamete types, sex determination and stable equilibria of all-hybrid populations of diploid and triploid edible frogs (*Pelophylax esculentus*). – BMC Evolutionary Biology, **9**: 135.

Christiansen, D. & H.-U. Reyer (2009): From clonal to sexual hybrids: Genetic recombination via triploids in all-hybrid populations of water frogs. – Evolution, **63**: 1754–1768.

Jombart, T., Z. N. Kamvar, C. Collins, R. Lustrik, M.-P. Beugin, B. J. Knaus, P. Solymos, V. Mikryukov, K. Schliep, T. Maié, L. Morkovsky, I. Ahmed, A. Cori, F. Calboli, R. J. Ewing, F. Michaud & R. DeCamp (2020): adegenet: Exploratory analysis of genetic and genomic data (version 2.1.2) [computer software]. – Available at: https://CRAN.R-project.org/package=adegenet.

Kassambara, A. (2019): ggpubr: "ggplot2" based publication ready plots (version 0.2.4) [computer software]. – Available at: https://CRAN.R-project.org/package=ggpubr.

Kassambara, A. (2020): rstatix: Pipe-friendly framework for basic statistical tests (version 0.4.0) [computer software]. – Available at: https://CRAN.R-project.org/package=rstatix.

Kolenda, K., A. Pietras-Lebioda, S. Hofman, M. Ogielska & M. Pabijan (2017): Preliminary genetic data suggest the occurrence of the Balkan water frog, *Pelophylax kurtmuelleri*, in southwestern Poland. – Amphibia-Reptilia, **38**: 187–196.

Lehtinen, R. M., A. F. Steratore, M. M., Eyre, E. S. Cassagnol, M. L. Stern & H. A. Edgington (2016): Identification of widespread hybridization between two terrestrial salamanders using morphology, coloration, and molecular markers. – Copeia, **104**: 132–139.

Lymberakis, P., N. Poulakakis, G. Manthalou, C. S. Tsigenopoulos, A. Magoulas & M. Mylonas (2007): Mitochondrial phylogeography of *Rana* (*Pelophylax*) populations in the Eastern Mediterranean region. – Molecular Phylogenetics and Evolution, **44**: 115–125.

Mikulíček, P., M. Kautman, B. Demovič & K. Janko (2014): When a clonal genome finds its way back to a sexual species: evidence from ongoing but rare introgression in the hybridogenetic water frog complex. – Journal of Evolutionary Biology, **27**: 628–642.

Mikulíček, P., M. Kautman, J. Kautman & N. B. M. Pruvost (2015): Mode of hybridogenesis and habitat preferences influence population composition of water frogs (*Pelophylax esculentus* complex, Anura: Ranidae) in a region of sympatric occurrence (western Slovakia). – Journal of Zoological Systematics and Evolutionary Research, **53**: 124–132.

Oruçi S. (2010): Data on geographical distribution and habitats of the *Rana epeirotica* in Albania. – Natura Montenegrina, **7**: 419–423.

Peterson, B. G., P. Carl, K. Boudt, R. Bennett, J. Ulrich, E. Zivot, D. Cornilly, E. Hung, M. Lestel, K. Balkissoon, D. Wuertz, A. A. Christidis, R. D. Martin, Z. Zhou Zenith & J. M. Shea (2020): PerformanceAnalytics: Econometric tools for performance and risk analysis (version 2.0.4) [computer software]. – Available at: https://CRAN.R-project.org/package=PerformanceAnalytics.

Plötner, J. (2005): Die westpaläarktischen Wasserfrösche: von Märtyrern der Wissenschaft zur biologischen Sensation. – Laurenti Verlag, Bielefeld.

Plötner, J., F. Köhler, T. Uzzell, P. Beerli, R. Schreiber, G.-D. Guex & H. Hotz (2009): Evolution of serum albumin intron-1 is shaped by a 5´truncated non-long terminal repeat retrotransposon in western Palearctic water frogs (Neobatrachia). – Molecular Phylogenetics and Evolution, **53**: 784–791.

Plötner, J., F. Baier, C. Akin, G. Mazepa, R. Schreiber, P. Beerli, S. N. Litvinchuk, C. C. Bilgin, L. Borkin & T. Uzzell (2012): Genetic data reveal that water frogs of Cyprus (genus *Pelophylax*) are an endemic species of Messinian origin. – Zoosystematics and Evolution, **88**: 261–283.

Pritchard, J. K., M. Stephens & P. Donnelly (2000): Inference of population structure using multilocus genotype data. – Genetics, **155**: 945–959.

Ragghianti, M., S. Bucci, C. Casola, S. Marraci & G. Mancino (2004): Molecular investigations in western Palearctic water frogs. – Italian Journal of Zoology, **71**: 17–23.

R Core Team (2019): R: A language and environment for statistical computing. R Foundation for Statistical Computing. – Available at: https://www.R-project.org/.

Schneider, H., T. S. Sofianidou & P. Kyriakopoulou-Sklavounou (1984): Bioacoustic and morphometric studies in water frogs (genus *Rana*) of Lake Ioannina in Greece, and description of a new species (Anura, Amphibia). – Zeitschrift für Zoologische Systematik und Evolutionsforschung, **22**: 349–366.

Schneider, H. & I. Haxhiu (1992): The distribution of the Epirus frog (*Rana epeirotica*) in Albania. – Amphibia-Reptilia, **13**: 293–295.

Schneider, H. & U. Sinsch (1992): Mating call variation in lake frogs referred to as *Rana ridibunda* Pallas, 1771. Taxonomic implications. – Zeitschrift für Zoologische Systematik und Evolutionsforschung, **30**: 297–315.

Schneider, H., U. Sinsch & T. S. Sofianidou (1993): The water frogs of Greece. Bioacoustic evidence for a new species. – Zeitschrift für Zoologische Systematik und Evolutionsforschung, **31**: 47–63.

Sinsch, U. & B. Eblenkamp (1994): Allozyme variation among *Rana balcanica*, *R. levantina* and *R. ridibunda* (Amphibia: Anura): Genetic differentiation corroborates the bioacoustically detected species status. – Zeitschrift für Zoologische Systematik und Evolutionforschung, **32**: 35–43.

Sofianidou, T. S. & H. Schneider (1989): Distribution range of the Epirus frog *Rana epeirotica* (Amphibia: Anura) and the composition of the water frog populations in Western Greece. – Zoologischer Anzeiger, **223**: 13–25.

Sofianidou, T. S., H. Schneider & U. Sinsch (1994): Comparative electrophoretic investigation on *Rana balcanica* and *Rana ridibunda* from northern Greece. – Alytes, **12**: 93–108.

Speybroeck, J., W. Beukema, B. Bok, J. Van Der Voort & I. Velikov (2016): Field guide to the amphibians and reptiles of Britain and Europe. – Bloomsbury, London.

Speybroeck, J., W. Beukema, C. Dufresnes, U. Fritz, D. Jablonski, P. Lymberakis, I. Martínez-Solano, E. Razzeti, M. Vamberger, M. Vences, J. Vörös & P.-A. Crochet (2020): Species list of the European herpetofauna – 2020 update by the Taxonomic Committee of the Societas Europaea Herpetologica. – Amphibia-Reptilia, **41**: 139–189.

Szabolcs, M., E. Mizsei, D. Jablonski, B. Vági, B. Mester, Z. Végvári & S. Lengyel (2017): Distribution and diversity of amphibians in Albania: new data and foundations of a comprehensive database. – Amphibia-Reptilia, **38**: 435–448.

Vucić, M., D. Jelić, G. I. V. Klobučar, B. Prkljačić & M. Jelić (2018): Molecular identification of species and hybrids of water frogs (genus *Pelophylax*) from Lake Skadar, Southeast Adriatic drainages (Amphibia: Ranidae). – Salamandra, **54**: 147–157.

Wickham, H., W. Chang, L. Henry, T. L. Pedersen, K. Takahashi, C. Wilke, K. Woo, H. Yutani & RStudio (2019): ggplot2: Create Elegant Data Visualisations Using the Grammar of Graphics (version 3.2.1) [computer software]. – Available at: https://CRAN.R-project.org/package=ggplot2.

Wickham, H. & RStudio (2019): tidyverse: Easily install and load the "tidyverse" (version 1.3.0) [computer software]. – Available at: https://CRAN.R-project.org/package=tidyverse.

Zeisset, I., G, Rowe & T. J. C. Beebee (2000): Polymerase chain reaction primers for microstellite loci in the North European water frogs *Rana ridibunda* and *R. lessonae*. – Molecular Ecology, **9**: 1173–1174.

Supplementary data

The following data are available online:

Supplementary Table S1. Fragments of the serum albumin-intron 1 (SAI-1), the proportion of a species' genome based on microsatellites, and morphometric indices in water frog hybrids.

Supplementary Table S2. The proportion of an individual's genome originating in one of the inferred clusters, corresponding to the species *Pelophylax shqipericus, P. kurtmuelleri* and *P. epeiroticus*.

Supplementary Table S3. Summary statistics for PCA and PCA loadings for retained principal components of DAPC.

Supplementary Figure S1. Nuclear allele network of *Pelophylax* water frogs based on the 597 bp long fragment of SAI-1.

Supplementary Figure S2. PCA ordination plot displaying different clusters of water frog species and species pairs.

Supplementary Figure S3. Morphological comparison of the sympatric living species *Pelophylax epeiroticus* and *P. kurtmuelleri* from Ioannina Lake, Greece, in dorsal, lateral and ventral view.