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Short Note

Cross-species testing of nuclear markers in *Pelophylax* water frogs in Greece and examination of their power to detect genetic admixture

Kostas Sagonas[1,2,*], Emmanouela Karameta[1,2], Panayiota Kotsakiozi[1,2], Nikos Poulakakis[1,2]

Abstract. The genus *Pelophylax* has been considered a model organism for understanding hybridization. Despite being the most diverse within the Western Palearctic group of *Pelophylax*, the *ridibundus/bedriagae* lineage that includes six species, remains largely understudied, revealing many knowledge gaps in regards to their evolution and conservation. Using genetic data from populations among species inhabiting contact zones could prove vital in filling these gaps. We tested 17 microsatellite markers for cross-species amplification in mainland *Pelophylax* species distributed in southern Balkans and evaluated their power to successfully detect population/species structure. Importantly, we examined their potential for identifying hybrids and backcrosses between known hybridized species. We detected 12 highly polymorphic loci that cross-amplified all species that showed no significant Linkage Disequilibrium and were able to discriminate among species and between parental and hybrids. We suggest their future use in genetic studies for the genus *Pelophylax* in Greece, including the identification of contact zones.

Keywords: amphibians, hybridization, microsatellites, primers, species differentiation.

Interspecific hybridization is long recognised to impact biological diversity. In the context of speciation, two highly opposing viewpoints have emerged. On one hand, introgressive hybridization has been suggested to foster adaptive radiation (Seehausen, 2004) and the generation of novel phenotypes able to occupy new niches (Rieseberg et al., 2003). On the other hand, hybridization may prevent local adaptation (Seehausen et al., 2008) and generate hybrid genotypes that tend to be less fit in the local environment than their parents. Hence, studying hybrid zones to understand the effects of hybridization and gene exchange on population dynamics is utterly important, both in the context of conservation and evolution.

The water frogs of the genus *Pelophylax* are probably one of the best candidate organisms for understanding speciation in the context of hybridization (Pagano et al., 2001; Plenet et al., 2005; Christiansen and Reyer, 2009; Christiansen et al., 2010; Hoffmann et al., 2015; Vucić et al., 2018), due to their outstanding reproductive mechanism of hybridogenesis, in which genetically distinct parental species produce viable and fertile hybrid forms (Ogielska, 2009). The Western Palearctic group of *Pelophylax* expands from the Iberian Peninsula and northern Africa to the eastern regions of Asia, and according to mitochondrial markers, encompasses three major phylogenetic lineages: the *perezi*, the *lessonae* and the *ridibundus/bedriagae* lineage (Plötner and Ohst, 2001; Lymberakis et al., 2007). The latter lineage is the most diverse and comprises six species that are distributed in southern Balkans and particularly in Greece, including *P. ridibundus*, *P. kurtmuelleri*, *P. epeiroticus*, *P. bedriagae*, *P. cretensis* (endemic to Crete island) and *P. cerigen-*

1 - Department of Biology, School of Sciences and Engineering, University of Crete, Vasilika Vouton, Heraklion, 70013, Greece

2 - Natural History Museum of Crete, School of Sciences and Engineering, University of Crete, 71409 Heraklion, Crete, Greece

*Corresponding author; e-mail: ksagonas@biol.uoa.gr

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sis (the most endangered frog in Europe and endemic to Karpathos island). Although cases of hybridization have been reported between *P. ridibundus* and *P.* cf. *bedriagae* (Holsbeek et al., 2008; Hotz et al., 2013), *P. ridibundus* (and likely *P. kurtmuelleri*) and *P. epeiroticus* (Sofianidou, 1996; Radojičić et al., 2015), as well as between *P. ridibundus* and *P. kurtmuelleri* (Hotz et al., 2013) in their overlapping geographic ranges in Greece, there is still an extensive lack of knowledge, from taxonomy and systematics to conservation and genome evolution.

The significant morphological variation and the overlapping ranges of morphological characters reported across *Pelophylax* species, and particularly between mainland *P. epeiroticus*, *P. ridibundus* and *P. kurtmuelleri* (Valakos et al., 2008) species, do not always allow a reliable morphological discrimination of the different species in their overlapping regions. Hence, to study hybridization in *Pelophylax* species and identify contact zones in mainland Greece, neutral molecular markers with high genetic variation, able to reliably discriminate the different species and hybrids, and acquire information on population structure are required. Among the different types of molecular markers, microsatellites can detect inter- and intra- population structure and differentiation. Microsatellite analyses are well established in the *Pelophylax esculentus* complex, the best characterized case of hybridization within the genus, and several markers are available. Here, we tested 17 markers (supplementary table S1) isolated in *P. ridibundus* (Zeisset et al., 2000; Hotz et al., 2001; Christiansen and Reyer, 2009), *P. lessonae* (Garner et al., 2000; Zeisset et al., 2000; Christiansen and Reyer, 2009), *P. perezi* (Sánchez-Montes et al., 2016) and the hybrid taxon *P. esculentus* (Christiansen, 2009; Arioli et al., 2010) for cross species amplification in *Pelophylax* water frogs distributed in mainland Greece. We selected these primers because they have proven ability to successfully amplify across a wide range of *Pelophylax* species and target loci with high number of alleles or high number of private alleles (Zeisset et al., 2000; Holsbeek et al., 2008; Radojičić et al., 2015; Dufresnes et al., 2017). To investigate their discriminatory power, we tested them in parental, sympatric populations and potentially hybrids in putative contact zones (supplementary fig. S1).

Total genomic DNA was extracted from toe clipping samples using the ammonium acetate method. The primers were tested in 189 specimens (56 specimens of *P. epeiroticus*, 54 of *P. kurtmuelleri*, 21 of *P.* cf. *bedriagae* (Akin et al., 2010) and 58 of *P. ridibundus*; supplementary fig. S1) for cross species amplification and for their ability to distinguish parental species from hybrids within the *ridibundus*/*bedriagae* lineage. When taxonomical assignment of specimens via morphological traits (e.g., development of tympanic membrane, the presence of dorsolateral skin folds, coloration etc.) (Valakos et al., 2008) was not possible, the mitochondrial DNA marker *cytochrome b* was sequenced given its power to identify the different species, following established protocols (Lymberakis et al., 2007). Noting, that the purely morphological identification of some samples in our study was not optimal. However, genetic characterization of all samples by mtDNA (cytb) was beyond the scope of this note. A gradient polymerase chain reaction (PCR) was conducted to optimize the annealing temperature across species and to find the optimal Mg^{2+} concentration for each primer pair. The PCR mix contained 0.2 mM of each primer, 1.5 or 3 mM $MgCl_2$, 0.2 μM dNTPs, 0.25 U Taq polymerase and 10-20 ng template of DNA in a 10 μl reaction volume. The conditions were set as follow: pre-heating at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, a gradient procedure with an annealing temperature ranging from 49 to 63°C for 1 min and 72°C for 1 min and a final extension step at 72°C for 10 min. The selected loci were amplified in three multiplex PCRs and genotyped on an ABI PRISM 3730 capillary sequencer (Applied Biosystems) with the GeneScan 500 LIZ size standard (Applied Biosystems). Fragments were scored using STRand software (Toonen and Hughes, 2001) and binned using FLEXIBIN 2 (Amos et al., 2007) in an attempt to minimize microsatellite allele miscalling.

On the basis of gradient PCR, specific conditions were selected for each locus (supplementary table S1) and new PCRs were conducted following the cycling program described above. For each locus for all species, we calculated measures of genetic diversity in the form of expected heterozygosity (*He*), observed heterozygosity (*Ho*), the number of alleles per locus and alleles size range using GENETIX (Belkhir et al., 2000). All loci were also analyzed for within-population deviations from Hardy-Weinberg equilibrium (HWE) and Linkage disequilibrium (LD) in Arlequin v.3.5 (Excoffier and Lischer, 2010). Both tests were run with 10^5 dememorizations, 10^4 batches and 10^6 iterations per batch. Bonferroni corrections were applied for multiple comparisons.

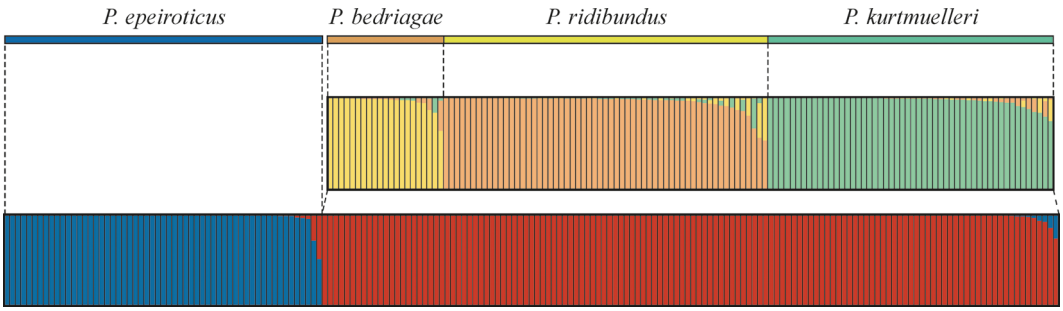

Figure 1. STRUCTURE bar plot for the entire dataset ($K = 11$) and the subset ($K = 7$) using the 12 selected microsatellite loci. Species names are reported on the top. Y axis reports the probability of each individual (Q-value) assigned to a genetic group identified by STRUCTURE. The genetic groups are represented by different colors. Each bar represents an individual. Individuals with 100% assignment to one group are identified by a single color. Individuals with mixed ancestry are represented by bars with different percentages of the two colors. The first dataset suggested the presence of two clusters (bottom: red and blue) while the analysis of the red sub-cluster indicated three genetic groups corresponding to three *Pelophylax* species.

We conducted Bayesian assignment test as implemented in STRUCTURE 2.3.4 (Pritchard et al., 2000) in two different datasets: the entire dataset and a sub-cluster obtained by the first run to evaluate the power of the targeted loci to detect genetic structure among species and populations. Both runs assumed an admixture model and correlated allele frequencies. We used 10^5 Markov chain Monte Carlo repeats and discarded the first 5×10^4 as burn-in. Calculations were repeated 10 times for each K. In the first dataset K was set from 1 to 11 (i.e., the total number of specimens and populations; supplementary fig. S1), while in the second dataset from 1 to 7 (limiting the analysis within the second STRUCTURE cluster; fig. 1). Evanno's test (Evanno et al., 2005) was applied to identify the most likely number of genetically homogeneous clusters in the two datasets. We used CLUMPP v1.1.2 to summarize the results from the different independent STRUCTURE runs, while DISTRUCT v.1.1 was used to plot the results. Additionally, we estimated the mean deviation from zero of the inbreeding coefficient (F_{IS}) and mean genetic differentiation (F_{ST}) across species and populations per locus using FSTAT (Goudet, 2001).

To assess the ability of the selected markers to differentiate parental species from hybrids a model-based Bayesian approach was used as implemented in NEWHYBRIDS v.1.1 (Anderson and Thompson, 2002). We focused on two cases of inter-specific hybridization, the *ridibundus-bedriagae* in the Northern regions of Greece and the *kurtmuelleri-epeiroticus* in the west (supplementary fig. S1) for which putative hybrid populations in contact zones were sampled or for which previous records of hybridization exist. For both cases we pursued the following approach. A threshold value (q) of 0.8 was used to assign specimens either to "pure" parental populations or to a certain hybrid category (i.e., F1 hybrids, F2 hybrids or backcrosses of F1 hybrids with either parental population). Based on STRUCTURE results we define "pure", non-hybridized individuals only those with q values > 0.80 and these were used to simulate pure parental and hybrid classes of each species in HYBRIDLAB (Nielsen et al., 2006). We simulated a dataset of 500 of each of the classes: i-ii) parental species P1 and P2, iii-iv) first- and second-generation hybrids (F1, F2, respectively), v-vi) backcrosses with either parental population (BC-P1 and BC-P2). We then re-run NEWHYBRIDS using the same settings as above to estimate the posterior probability of an individual falling into one of these six categories. The analysis was performed using default genotype frequency classes with 100 000 iterations as a burn-in phase and 500 000 iterations post burn-in. Two independent runs with different starting points were performed for both for "Jeffreys-like" prior and the Uniform prior.

From the 17 microsatellite markers tested, cross-species amplification was successful for 15 loci. Two primer pairs either did not amplify the targeted loci in the examined species (Rrid059A) or it was difficult to score mainly due to non-specific amplification (Res20). The remaining 15 markers were used for further characterization of *Pelophylax* populations in mainland Greece. The 15 loci, except Pper4.29 that was monomorphic in *P. epeiroticus*, proved to be polymorphic in all species and had a product size ranging between 112 and 516 bp. The number of alleles per locus varied, ranging from 2 to 15 for *P.* cf. *bedriagae*, 4 to 20 for *P. epeiroticus*, 2 to 19 for *P. kurtmuelleri* and 3 to 28 for *P. ridibundus*, with an average of 8.2 alleles per locus. The average observed and expected heterozygosity calculated using the 14 polymorphic microsatellite markers was

Table 1. Basic characteristics of the 15 cross-species amplified microsatellite loci for *Pelophylax epeiroticus*, *Pelophylax kurtmuelleri*, *Pelophylax ridibundus* and *Pelophylax* cf. *bedriagae*. The number of individuals genotyped for each species is given within the parentheses. A refers to the number of alleles and within the parenthesis the number of private alleles is shown, H_O and H_E refer to the observed and expected heterozygosity, respectively, while the allele size range for each locus is also given.

	P. epeiroticus (N = 56)				*P. kurtmuelleri* (N = 54)				*P. ridibundus* (N = 58)				*P.* cf. *bedriagae* (N = 21)			
Locus	A (P)	H_O	H_E	Range	A (P)	H_O	H_E	Range	A(P)	H_O	H_E	Range	A (P)	H_O	H_E	Range
Res5	7 (1)	0.46	0.64	142-150	4	0.20	0.51	140-156	3	0.43	0.58	142-154	5	0.30	0.73	142-146
Res14	5	0.11	0.62	132-144	6 (1)	0.08	0.59	138-146	6	0.44	0.65	136-146	5	0.25	0.76	132-146
Res16	7 (1)	0.34	0.58	114-128	3	0.28	0.36	112-124	8	0.63	0.75	116-120	8	0.71	0.68	114-128
Res17	4 (1)	0.02	0.17	152-170	2	0.28	0.24	150-160	5 (1)	0.49	0.44	156-160	6	0.56	0.73	154-176
Ga1a19	3 (1)	0.16	0.15	201-275	10 (1)	0.48	0.69	187-201	21 (3)	0.88	0.88	203-251	15 (2)	0.79	0.93	199-257
RlCA1b5	7	0.38	0.59	128-138	9	0.50	0.85	124-140	8	0.63	0.77	124-140	7	0.62	0.68	128-148
Rrid013A	8 (1)	0.54	0.70	285-291	6 (1)	0.45	0.67	273-297	5	0.65	0.67	282-300	4	0.33	0.38	282-297
Rrid064A	3	0.02	0.51	201-241	16 (2)	0.78	0.89	219-229	18 (5)	0.61	0.91	209-245	6	0.22	0.81	203-259
Rrid082A	12	0.64	0.78	193-195	19 (1)	0.85	0.91	149-197	11	0.78	0.82	153-205	2	0.00	0.67	163-185
Pper3.22	3 (1)	0.04	0.04	341-353	6	0.35	0.43	329-356	7 (1)	0.74	0.74	341-362	5 (1)	0.50	0.64	338-356
Pper3.24	4 (1)	0.06	0.09	263-275	4 (1)	0.27	0.42	236-263	4 (1)	0.47	0.58	251-263	4	0.10	0.22	257-266
Pper4.7	4	0.49	0.57	280-372	8	0.36	0.76	296-312	14	0.20	0.76	268-328	12 (2)	0.69	0.90	260-364
Pper4.23	20 (1)	0.77	0.93	440-496	14	0.39	0.89	364-500	17	0.76	0.93	436-512	14	0.71	0.93	448-516
Pper4.24	9 (3)	0.23	0.68	286-486	19 (1)	0.85	0.92	234-438	28 (2)	0.74	0.94	334-458	11 (1)	0.25	0.92	242-506
Pper4.29	1	0	0	313-329	2	0	0.11	317-317	3	0.16	0.27	317-321	4	0.05	0.56	313-321

0.43 ± 0.25 and 0.71 ± 0.20 for *P.* cf. *bedriagae*, 0.30 ± 0.24 and 0.50 ± 0.27 for *P. epeiroticus*, 0.44 ± 0.23 and 0.65 ± 0.22 for *P. kurtmuelleri* and 0.60 ± 0.17 and 0.74 ± 0.14 for *P. ridibundus* for the observed and expected heterozygosity respectively. The general characteristics and diversity parameters of all markers for each species are shown in table 1. We found no significant LD for any pair of loci for the examined populations, which satisfies the requirement of marker independence for population genetic studies. Even though all loci showed cases of deviation from HWE after Bonferroni correction, likely due to an excess of homozygotes, this pattern was not observed across all populations and therefore were not discarded for structure analyses. A more extensive sampling is required for future landscape genetic studies.

Pper4.23 and Pper4.24 although highly polymorphic (average population allele number: 12.5 ± 2.3 and 10.4 ± 4.7, respectively) showed a weak power for detecting genetic structure among mainland *Pelophylax* species (mean F_{ST}: 0.007 and 0.010, respectively; supplementary table S1). Conversely, preliminary STRUCTURE analyses suggested that the remaining 12 loci have the ability to detect genetic structure among species (mean F_{ST} values ranged from 0.051 to 0.214) and populations if differences exist (mean F_{ST} values ranged from 0.092 to 0.373), suggesting a high potential for their use in population genetics studies of *Pelophylax*.

In addition to population structure, NEWHYBRIDS analyses revealed a number of pure parental genotypes as well as hybrids between *P. kurtmuelleri* and *P. epeiroticus* (supplementary table S2) in sympatric regions for which no previous records exist (e.g., sites 6, 7, 9, 10; supplementary fig. S1), supporting the results of Radojičić et al. (2015) who found evidence of hybridization between *P. ridibundus* (likely *P. kurtmuelleri*) and *P. epeiroticus* in western Greece using haplotypic data of mitochondrial and microsatellite markers. However, no hybrids or admixed genotypes were detected in remote parental populations (e.g. Lake Doxa; supplementary fig. S1). Likewise, hybrids between *P. ridibundus* and *P.* cf. *bedriagae* were detected in Northern Greece (supplementary table S3), in regions that are close enough to be considered as contact zones (supplementary fig. S1) given also the overlapping distribution of these species as shown in the embedded map of supplementary fig. S1, resembling the study of Hotz et al. (2013).

Conclusively, our findings indicate that primers developed for studying particular *Pelophylax* complexes can be applicable to other species of the genus, providing valuable tools to address a variety of questions on population dynamics and demography, genetic structure, gene flow and hybridization. Most importantly, we assessed the power of the selected markers to successfully dissect pure parental from hybrid individuals for all known hybridized species pairs in Greece. This is of particular importance for future studies aiming to identify contact zones in Greece, understand the extent to which genomes are free to recombine and examine their unique mechanism of hybridization as an important source of genomic diversity in *Pelophylax* water frogs.

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References

Akin, C., Bilgin, C.C., Beerli, P., Westaway, R., Ohst, T., Litvinchuk, S.N., Uzzell, T., Bilgin, M., Hotz, H., Guex,

G.D., Plötner, J. (2010): Phylogeographic patterns of genetic diversity in eastern Mediterranean water frogs were determined by geological processes and climate change in the Late Cenozoic. Journal of Biogeography **37**: 2111-2124.

Amos, W., Hoffman, J.I., Frodsham, A., Zhang, L., Best, S., Hill, A.V.S. (2007): Automated binning of microsatellite alleles: problems and solutions. Mol. Ecol. Notes **7**: 10-14.

Anderson, E.C., Thompson, E.A. (2002): A model-based method for identifying species hybrids using multilocus genetic data. Genetics **160**: 1217-1229.

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

Belkhir, K., Borsa, P., Chikhi, L., Raufaste, N., Bonhomme, F. (2000): Genetix, a WindowsTM based software for population genetic analyses. Laboratoire génome, populations, interactions CNRS UMR 5000 Montpellier, France, Université de Montpellier II.

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

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

Christiansen, D.G., Jakob, C., Arioli, M., Roethlisberger, S., Reyer, H.-U. (2010): Coexistence of diploid and triploid hybrid water frogs: population differences persist in the apparent absence of differential survival. BMC Ecology **10**: 14.

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

Evanno, G., Regnaut, S., Goudet, J. (2005): Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol. Ecol. **14**: 2611-2620.

Excoffier, L., Lischer, H.E.L. (2010): Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol. Ecol. Resour. **10**: 564-567.

Garner, T.W.J., Gautschi, B., Röthlisberger, S., Reyer, H.U. (2000): A set of CA repeat microsatellite markers derived from the pool frog, *Rana lessonae*. Mol. Ecol. **9**: 2173-2175.

Goudet, J. (2001): FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). Available from http://www.unil.ch/izea/softwares/fstat.html.

Hoffmann, A., Plötner, J., Pruvost, N.B.M., Christiansen, D.G., Röthlisberger, S., Choleva, L., Mikulíček, P., Coglniceanu, D., Sas-Kovács, I., Shabanov, D., Morozov-Leonov, S., Reyer, H.U. (2015): Genetic diversity and distribution patterns of diploid and polyploid hybrid water frog populations (*Pelophylax esculentus* complex) across Europe. Mol. Ecol. **24**: 4371-4391.

Holsbeek, G., Mergeay, J., Hotz, H., Plotner, J., Volckaert, F.A.M., De Meester, L. (2008): A cryptic invasion within an invasion and widespread introgression in the European water frog complex: consequences of uncontrolled commercial trade and weak international legislation. Mol. Ecol. **17**: 5023-5035.

Hotz, H., Uzzell, T., Guex, G.D., Alpers, D., Semlitsch, R.D., Beerli, P. (2001): Microsatellites: a tool for evolutionary genetic studies of western Palearctic water frogs. Mitteilungen aus dem Zoologischen Museum in Berlin **77**: 43-50.

Hotz, H., Beerli, P., Uzzell, T., Guex, G.-D., Pruvost, N.B.M., Schreiber, R., Plötner, J. (2013): Balancing a cline by influx of migrants: a genetic transition in water frogs of eastern Greece. J. Hered. **104**: 57-71.

Lymberakis, P., Poulakakis, N., Manthalou, G., Tsigenopoulos, C.S., Magoulas, A., Mylonas, M. (2007): Mitochondrial phylogeography of *Rana* (*Pelophylax*) populations in the Eastern Mediterranean region. Mol. Phylogenet. Evol. **44**: 115-125.

Nielsen, E.E.G., Bach, L.A., Kotlicki, P. (2006): HYBRIDLAB (version 1.0): a program for generating simulated hybrids from population samples. Mol. Ecol. Notes **6**: 971-973.

Ogielska, M. (2009): Development and reproduction of amphibian species, hybrids, and polyploids. In: Reproduction of Amphibians. Ogielska, M., Ed., CRC Press, Boca Raton, Florida.

Pagano, A., Crochet, P.A., Graf, J.-D., Joly, P., Lodé, T. (2001): Distribution and habitat use of water frog hybrid complexes in France. Global Ecology and Biogeography **10**: 433-441.

Plenet, S., Joly, P., Hervant, F., Fromont, E., Grolet, O. (2005): Are hybridogenetic complexes structured by habitat in water frogs? J. Evol. Biol. **18**: 1575-1586.

Plötner, J., Ohst, T. (2001): New hypotheses on the systematics of the western Palearctic water frog complex (Anura, Ranidae). Zoosystematics and Evolution **77**: 5-21.

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

Radojičić, J.M., Krizmanić, I., Kasapidis, P., Zouros, E. (2015): Extensive mitochondrial heteroplasmy in hybrid water frog (*Pelophylax* spp.) populations from Southeast Europe. Ecology and Evolution **5**: 4529-4541.

Rieseberg, L.H., Raymond, O., Rosenthal, D.M., Lai, Z., Livingstone, K., Nakazato, T., Durphy, J.L., Schwarzbach, A.E., Donovan, L.A., Lexer, C. (2003): Major ecological transitions in wild sunflowers facilitated by hybridization. Science **301**: 1211-1216.

Sánchez-Montes, G., Recuero, E., Gutiérrez-Rodríguez, J., Gomez-Mestre, I., Martínez-Solano, I. (2016): Species assignment in the *Pelophylax ridibundus* × *P. perezi* hybridogenetic complex based on 16 newly characterized microsatellite markers. Herpetolog. J. **26**: 99-108.

Seehausen, O. (2004): Hybridization and adaptive radiation. Trends Ecol. Evol. **19**: 198-207.

Seehausen, O.L.E., Takimoto, G., Roy, D., Jokela, J. (2008): Speciation reversal and biodiversity dynamics with hybridization in changing environments. Mol. Ecol. **17**: 30-44.

Sofianidou, T.S. (1996): Electrophoretic studies of hybrids of water frogs (*Rana epeirotica*, *R. balcanica*) in the ionian zone of Greece. Isr. J. Zool. **42**: 149-157.

Toonen, R.J., Hughes, S. (2001): Increased throughput for fragment analysis on ABI Prism 377 automated sequencer using a membrane comb and STRand software. Biotechniques **31**: 1320-1324.

Valakos, E.D., Pafilis, P., Sotiropoulos, K., Lymberakis, P., Maragou, P., Foufopoulos, J. (2008): The Amphibians and Reptiles of Greece. Chimaira, Frankfurt am Main, Germany.

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

Zeisset, I., Rowe, G., Beebee, T.J.C. (2000): Polymerase chain reaction primers for microsatellite loci in the north European water frogs *Rana ridibunda* and *R. lessonae*. Mol. Ecol. **9**: 1173-1174.

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