Panacea or nemesis? Re-assessing the reliability of serum-albumin intron 1 for genetic analyses in Western Palearctic water frogs

Dufresnes, C.; Dubey, S.; Wielstra, B.M.; Jablonski, D.; Denoël, M.

Alytes, 2024, **41** (1–4): 5–17.

Panacea or nemesis? Re-assessing the reliability of *serum albumin* intron 1 for genotyping Western Palearctic water frogs

Christophe DUFRESNES[1,*], Sylvain DUBEY[2,3], Ben WIELSTRA[4,5], Daniel JABLONSKI[6] & Mathieu DENOËL[7]

[1] Institut de Systématique, Evolution, Biodiversité (ISYEB), Muséum national d'Histoire naturelle, CNRS, Sorbonne Université, EPHE, Université des Antilles, Paris, France.
<christophe.dufresnes@hotmail.fr>, https://orcid.org/0000-0002-8497-8908

[2] Department of Ecology and Evolution, University of Lausanne, Lausanne, Switzerland;
[3] HW Romandie SA, Montreux.
<dubey@hw-romandie.ch>, https://orcid.org/0000-0003-4342-9432

[4] Institute of Biology, Leiden University, Leiden, The Netherlands;
[5] Naturalis Biodiversity Center, Leiden, The Netherlands.
<ben.wielstra@naturalis.nl>, https://orcid.org/0000-0002-7112-5965

[6] Department of Zoology, Comenius University in Bratislava, Bratislava, Slovakia.
<daniel.jablonski@uniba.sk>, https://orcid.org/0000-0002-5394-0114

[7] Laboratory of Ecology and Conservation of Amphibians (LECA), FOCUS, University of Liège, Belgium.
<mathieu.denoel@uliege.be>, https://orcid.org/0000-0002-3586-8323

[*]Corresponding author

Water frogs (genus *Pelophylax*) are one of the most widespread and diverse, but also most invasive amphibians of the Western Palearctic region. As such, *Pelophylax* studies face the challenge of identifying similar taxa that hybridize in sympatry. For this purpose, the nuclear marker *serum albumin* intron 1 (*SAI-1*) has been used for over a decade in *Pelophylax*. Initially praised for its diagnosticity, notably to discriminate common species such as the pool frog (*P. lessonae*), the marsh frog (*P. ridibundus*) and their hybridogenetic hybrid the edible frog (*P. esculentus*) without sequencing (by amplicon length polymorphism), *SAI-1* was later questioned due to misidentifications and doubtful patterns of genetic divergence. In this study, we incorporate an up-to-date multilocus phylogeographic framework spanning the entire *Pelophylax* diversification, to re-assess the performance of *SAI-1* for lineage identification and discovery. We show that *SAI-1* sequences discriminate all Palearctic water frog species and most of their phylogeographic lineages, enabling us to map their distributions and identify the genomes of hybridogenetic hybrids. However, the phylogeny of *SAI-1* is aberrant and unrepresentative of the evolution

of the genus. In particular, differentiated *P. l. lessonae* alleles segregating in the Alpine region mimic a species-level divergence that is not recovered by any other marker. Moreover, the indel polymorphism that supposedly distinguishes *P. lessonae* from *P. ridibundus*, as well as the main *P. ridibundus* lineages from the Balkans (*P. r. ridibundus vs kurtmuelleri*), are not diagnostic across the entire range of these taxa. Hence, *SAI-1* is neither the panacea for nor the nemesis of *Pelophylax* genotyping. Sequencing *SAI-1* shall continue to offer a reliable and informative preliminary approach of single-gene barcoding identification of lineages, but analyses without sequencing, and other applications such as phylogenetic and taxonomic inferences, should be avoided.

urn:lsid:zoobank.org:pub:06563AFB-403E-4FBF-9B94-072790044C0D

INTRODUCTION

Molecular analyses are decisive in distinguishing closely related taxa when traditional field criteria cannot, and DNA-based identification methods have become common practice in amphibian studies on taxonomy, biogeography, distribution, conservation and biological invasion. With their high cost-effectiveness, mitochondrial DNA (mtDNA) barcodes, such as 16S rRNA and COI, have been preferentially sequenced, despite their known limitations (Rubinoff *et al.* 2006). This is particularly true of amphibians (Dufresnes & Jablonski 2022), where recurrent hybridization between diverging lineages has created convoluted situations involving widespread introgression, mitochondrial replacement and hybrid taxa, which cannot be deciphered from clonal, maternally-inherited, mitochondrial sequences (e.g., Zieliński *et al.* 2013; Wielstra *et al.* 2017; Dufresnes *et al.* 2019). In such situations, genetic identification must then rely on nuclear DNA, but such analyses have to cope with the technical challenges of genotyping diploid markers and finding taxon-diagnostic polymorphisms in the generally more conserved nuclear genes.

Nuclear DNA-based identification is particularly relevant for Palearctic water frogs of the genus *Pelophylax* Fitzinger, 1843. Widespread across Europe, North Africa, Central and Far-East Asia, this group has diversified in more than 40 lineages, delimited in 15 species (Dufresnes *et al.* 2024), for which phenotypic resemblance renders field identification problematic. One major additional complication is the propensity of water frogs to hybridize, both between phylogeographic lineages meeting in secondary contact zones and between deeply-diverged species co-occurring in sympatry. In particular, the genus is notorious for hybridogenesis, a peculiar reproductive mode that allows the maintenance of F1-like hybrids, sometimes known as “kleptons” (reviewed by Dufresnes & Mazepa 2020). For instance, the edible frog *Pelophylax esculentus* (Linnaeus, 1758) results from ancestral hybridization between the pool frog *Pelophylax lessonae* (Camerano, 1882) and the marsh frog *Pelophylax ridibundus* (Pallas, 1771). Unlike regular sexual vertebrates, *P. esculentus* discards the genome of one parental species during the production of gametes, and it is perpetuated by back-crossing with that parental species. Combined with a dynamic biogeographic history, the propensity to hybridize in *Pelophylax* has resulted in complex patterns of genetic diversity such as far-ranging introgression, especially of mtDNA (Plötner *et al.* 2008). Nowadays, these patterns have become even more intricate due to uncontrolled introductions of

numerous species outside their native range, linked to the frog-leg commerce (Dubois 1983), trade for ornamental ponds (Puky *et al.* 2005) and experimental use and academic teaching (Neveu 1989), which has resulted in biological invasions (Dufresnes & Dubey 2020; Dufresnes *et al.* 2024).

The high interest in the genus has motivated many studies that implemented multilocus analyses of nuclear markers to identify water frog taxa and their hybrids, such as the genotyping of allozymes (e.g., Hotz *et al.* 2013) and microsatellites (e.g., Dufresnes *et al.* 2017; Sagonas *et al.* 2020; Papežík *et al.* 2021), or more recently the sequencing of Restriction-Associated-DNA (RAD-seq, e.g., Dubey *et al.* 2019; Dufresnes & Dubey 2020; Doniol-Valcroze *et al.* 2021). However, these approaches are expensive, often require cumbersome laboratory implementation and optimization, and independently-obtained datasets usually cannot be compared for technical reasons. A more efficient and universal method is to target specific genes with species-diagnostic polymorphism, especially if this polymorphism can be genotyped without sequencing, i.e., by screening size polymorphism on agarose gels, with (e.g., Patrelle *et al.* 2011; Cuevas *et al.* 2022) or without enzyme restriction beforehand (e.g., Hauswaldt *et al.* 2012; Ermakov *et al.* 2019).

In this respect, perhaps the most informative nuclear gene to identify *Pelophylax* taxa is the hyper-variable s*erum albumin* intron 1 (*SAI-1*). Initially developed for phylogenetic studies, this marker contains a ~ 500 base pairs (bp) retrotransposon (Chicken Repeat 1, CR1) in some species, but not in others (Plötner *et al.* 2009), putatively enabling their distinction by comparing amplicon sizes. A cost-effective standardized protocol of *SAI-1* genotyping on agarose gels was thus designed (Hauswaldt *et al.* 2012), in which marsh frogs carry much larger alleles (~ 800 bp) than pool frogs (~ 300 bp), with their hybridogenetic hybrids featuring both. Moreover, preliminary data showed that additional taxa could theoretically be distinguishable by indels elsewhere in the sequence, notably *Pelophylax shqipericus* (Hotz, Uzzell, Günther, Tunner, & Heppich, 1987) (~ 1,200 bp) and *Pelophylax ridibundus kurtmuelleri* (Gayda, 1940) (~ 720 bp) (Hauswaldt *et al.* 2012).

Despite its widespread use in phylogeographic surveys (e.g., Plötner *et al.* 2012), for characterizing the genomes involved in hybridogenetic systems (e.g., Miura *et al.* 2021), to screen for native (e.g., Herczeg *et al.* 2017; Vucić *et al.* 2018) and introduced taxa (e.g., Plötner *et al.* 2015; Kolenda *et al.* 2017; Litvinchuk *et al.* 2020; Jelić *et al.* 2022), and even for a taxonomic description (Plötner *et al.* 2012), the diagnosticity and reliability of *SAI-1* have repeatedly been questioned (Mayer *et al.* 2013; Dubey *et al.* 2019; Cuevas *et al.* 2022). For instance, amplicon length profiles failed to distinguish the water frogs found in hybridogenetic populations from southern Germany (Mayer *et al.* 2013). In Italy and southern Switzerland, the sequencing of *SAI-1* revealed new lineages that were not recovered as such by multilocus phylogenies (Dubey & Dufresnes 2017; Dubey *et al.* 2019; Dufresnes *et al.* 2024). These discrepancies call for a re-assessment of how the *SAI-1* polymorphism, especially its allele length variation, associates with the phylogeography and taxonomy of populations, in order to verify its diagnosticity.

In a recent study, we revisited the evolution, diversity and distribution of *Pelophylax* across the entire range, combining available mitochondrial and nuclear sequences spanning more than 1,700 localities (Dufresnes *et al.* 2024). This

phylogeographic framework can now serve as a baseline to re-assess patterns of allele divergence and length polymorphism at *SAI-1* in the Western Palearctic species, notably to draw lessons for nuclear DNA-based taxon identification and discovery with this particular marker.

METHODS

We re-used the *SAI-1* alignment and metadata recently compiled by Dufresnes *et al.* (2024) and reposited online (Dufresnes 2024). This dataset contains 106 haplotypes obtained from 906 aligned sequences (1,410 bp) representing 453 diploid individuals. All individuals originate from the Western Palearctic (217 localities) except one that belongs to *Pelophylax nigromaculatus* (Hallowell, 1861), an Eastern Palearctic species. Heterozygous individuals, notably the hybridogenetic hybrids *P. e. esculentus* and *Pelophylax esculentus hispanicus* (Bonaparte, 1839), were phased, unique haplotypes were identified, and lineages were defined and labelled in the original study (Dufresnes *et al.* 2024; see methods therein).

The haplotype dataset was visualized in two ways. First, a phylogenetic network was built with SplitsTree 4.18.3 (Huson & Bryant 2006) using default settings. Second, a phylogeny was inferred by maximum-likelihood with the web version of IQtree 1.6 (Trifinopoulos *et al.* 2016), using model finder to find and apply the best model of sequence evolution, and 1,000 ultrafast bootstraps to assess node support. The *P. nigromaculatus* haplotype was used as an outgroup. The *SAI-1* phylogeny was then compared against the mitochondrial tree and the nuclear species tree obtained by Dufresnes *et al.* (2024) based on mitogenomes (~ 16.8 kb) and five introns (~ 4.9 kb), respectively.

To assess the reliability of scoring amplicon length at *SAI-1* for taxon identification following the approach of Hauswaldt *et al.* (2012), we examined sequence length variation between and within lineages. To this end, we trimmed the *SAI-1* haplotype alignment to the portion amplified by the primers Pel-SA-F1 and Pel-SA-R2 from Hauswaldt *et al.* (2012) and determined the number of base-pairs of each haplotype in each taxon/lineage. Two haplotypes that were not sequenced across the entire portion of interest were discarded.

RESULTS

As of August 2023 (when the dataset of Dufresnes *et al.* 2024 was compiled), 105 haplotypes were reported for *SAI-1* in Western Palearctic *Pelophylax*. These are arranged in 11 major haplogroups (Fig. 1) that correspond to:

(1) *Pelophylax perezi* (López-Seoane, 1885).

(2) *Pelophylax saharicus* (Boulenger *in* Hartert, 1913).

(3) Most populations of *P. lessonae lessonae* (Camerano, 1882) (thereafter "regular" *P. l. lessonae*) and *Pelophylax lessonae bergeri* (Günther *in* Engelmann, Fritzsche, Günther & Obst, 1986).

(4) *Pelophylax l. lessonae* from the Alpine region (thereafter "Alpine" *P. l. lessonae*).

(5) *Pelophylax epeiroticus* (Schneider, Sofianidou & Kyriakopoulou-Sklavounou, 1984).

(6) *Pelophylax shqipericus* (Hotz, Uzzell, Günther, Tunner & Heppich, 1987).

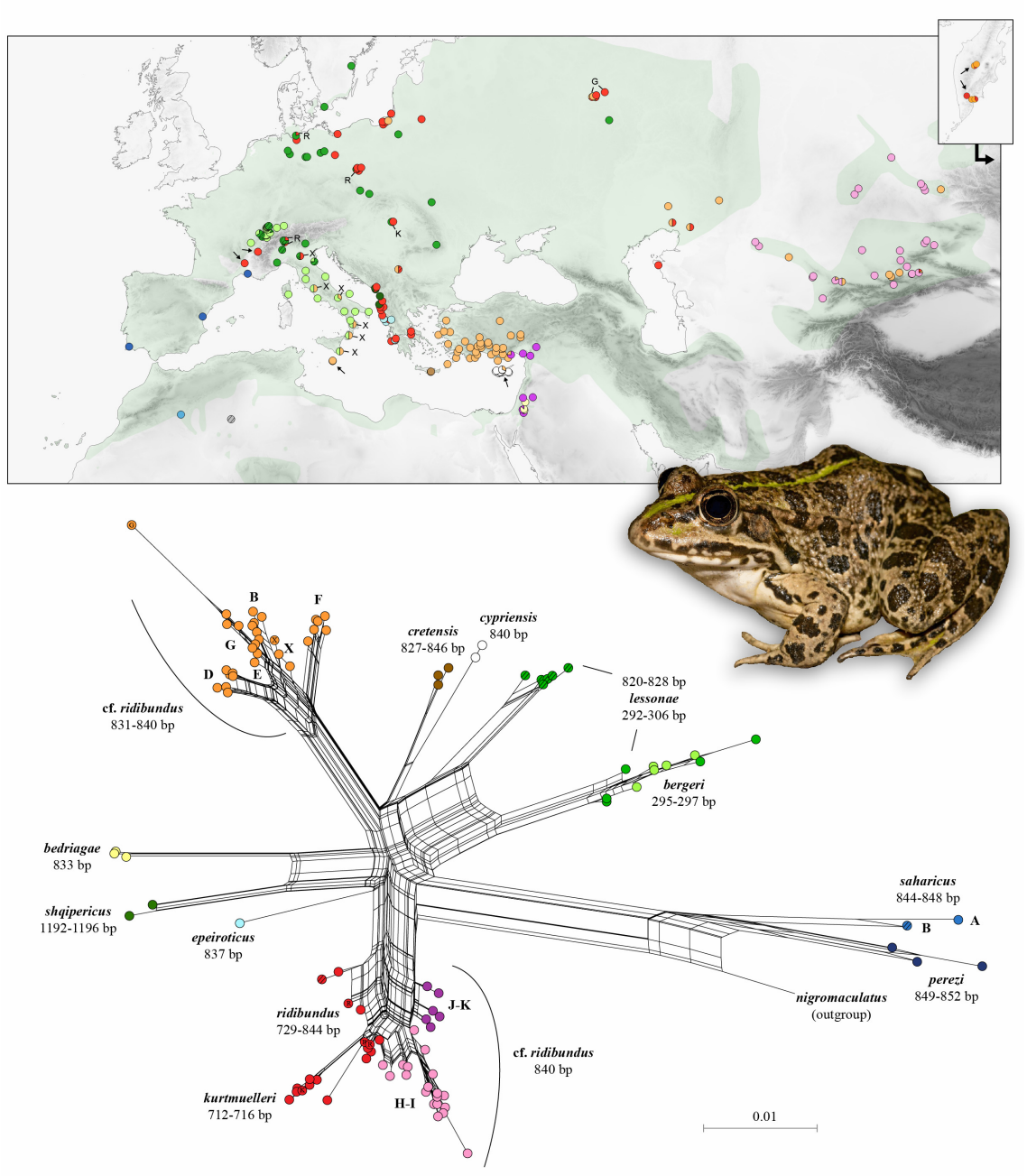

Figure 1. Phylogenetic network of *SAI-1* haplotypes and distributions of the main haplogroups/lineages. Haplotypes found in the *ridibundus* genomes of hybridogenetic hybrids are identified with letters (R: *P. r. ridibundus*; K: *P. r. kurtmuelleri;* G: *P. r.* cf. *ridibundus* G; X: *P. r.* cf. *ridibundus* X). Haplotypes with unusual sequence lengths are emphasized with dash lines (see Fig. 4). The distribution of the *P. ridibundus* lineages are further detailed in Fig. 2. On the map, green shading shows *Pelophylax* presumably native occurrence, grey shading shows relief, and arrows point to non-native populations (see Dufresnes *et al.* 2024). The inset map shows the Kamchatka Peninsula. Photo: DJ.

(7) *Pelophylax cretensis* (Beerli, Hotz, Tunner, Heppich & Uzzell, 1994).

(8) *Pelophylax ridibundus* from Cyprus: *Pelophylax ridibundus cypriensis* Plötner, Baier, Akin, Mazepa, Schreiber, Beerli, Litvinchuk, Bilgin, Borkin & Uzzell, 2012.

(9) *Pelophylax ridibundus* from Europe: *P. ridibundus ridibundus* and *P. r. kurtmuelleri*; Central Asia: *Pelophylax ridibundus* cf. *ridibundus* H–I; and southern Anatolia: *P. r.* cf. *ridibundus* J–K.

(10) *Pelophylax ridibundus* from the Levant: *Pelophylax ridibundus bedriagae* (Camerano, 1882).

(11) *Pelophylax ridibundus* from the Near and Middle East: *P. r.* cf. *ridibundus* B, D–F, X, which includes *Pelophylax ridibundus caralitanus* (Arikan, 1988) and *Pelophylax ridibundus cerigensis* (Beerli, Hotz, Tunner, Heppich & Uzzell, 1994).

Several taxa falling within the same haplogroups are identifiable by their private haplotypes, notably in *P. ridibundus* (Fig. 2). In total, up to 20 *Pelophylax* genetic forms are distinguished and mapped, including hybridogenetic hybrids (Fig. 1–2).

Only two pairs of closely related lineages are not distinguishable by *SAI-1* due to allele sharing: *P. r.* cf. *ridibundus* H and I from Central Asia; *P. l. bergeri* A and B from Italy. A third pair from Cilicia (*P. r.* cf. *ridibundus* J/K) might also lack differentiation, but it is unclear whether both lineages were actually sampled as only a few individuals were sequenced. Four lineages otherwise documented in Dufresnes *et al.* (2024) are absent from the *SAI-1* dataset: *P. r.* cf. *ridibundus* A [*P. r. persicus* (Schneider, 1799)], C and M from the Middle East; and *P.* cf. *saharicus* C from Tunisia/E-Algeria.

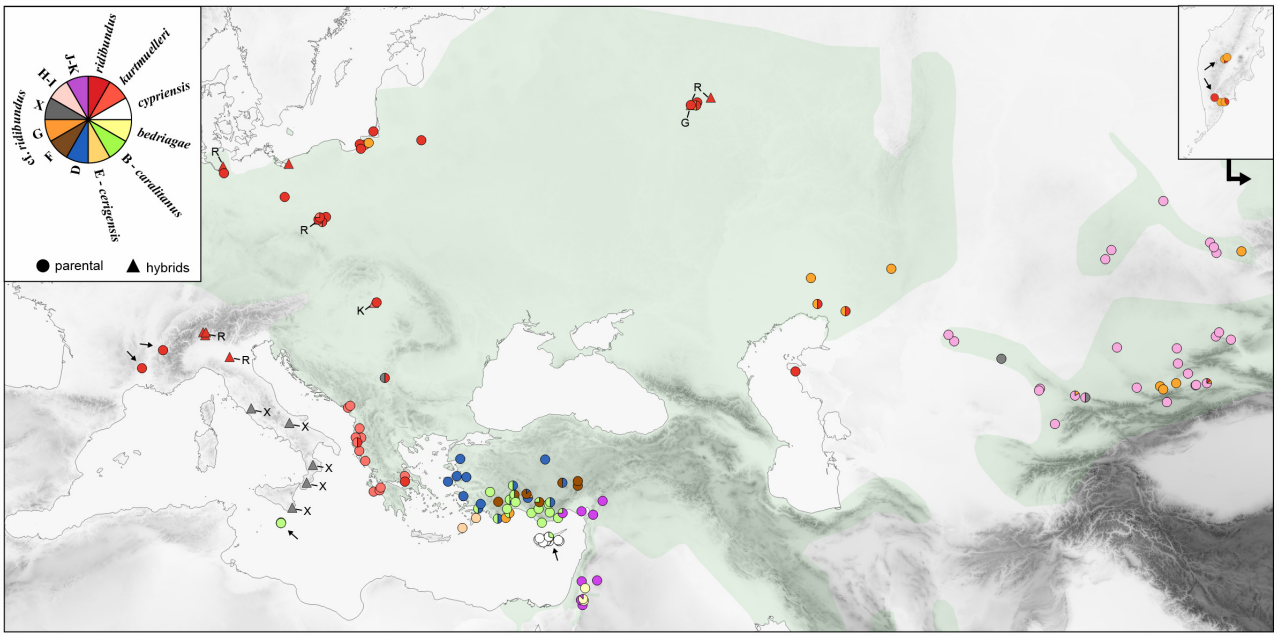

Figure 2. Distribution of the *P. ridibundus* lineages identified by *SAI-1*. Triangles show lineages found in hybridogenetic hybrids. On the map, green shading shows *P. ridibundus* presumably native occurrence, grey shading shows relief, and arrows point to non-native populations (see Dufresnes *et al.* 2024). The inset map shows the Kamchatka Peninsula.

The geographic distributions of the *SAI-1* lineages (Fig. 1–2) generally follow the mitochondrial phylogeography (Dufresnes *et al.* 2024) and suggest instances of introgressive hybridization in the Levant (between *P. r.* cf. *ridibundus* J/K and *P. r. bedriagae*), in the Balkans (between *P. r. ridibundus* and *P. r. kurtmuelleri*), in Anatolia (between *P. r.* cf. *ridibundus* B, D, F, G and J–K) and in Central Asia (between *P. r. ridibundus* and *P. r.* cf. *ridibundus* H–I and G).

The phylogenetic analysis retrieved the major *SAI-1* haplogroups and most of their lineages within as distinct monophyla (Fig. 3). The few exceptions are caused by the basal positions of haplotypes from *P. r. ridibundus* and "regular" *P. l. lessonae* in their respective phylum (as also visible on the network, Fig. 1). The topology of the *SAI-1* tree is, however, largely inconsistent with the mitochondrial and nuclear species trees (Fig. 1). In contrast with both, the *SAI-1* tree groups *P. shqipericus* with *P. r. bedriagae* in a basal phylum, groups *P. cretensis*/*P. r. cypriensis* with *P. lessonae*, and separates the remaining *P. ridibundus* lineages into two distantly-related groups (Fig. 1). It also recovers the two *SAI-1* haplogroups of *P. lessonae* as deeply-diverged lineages, a phylogenetic divergence that was not retrieved by other nuclear genes (Fig. 3).

The *SAI-1* haplotypes of most *Pelophylax* species/lineages include a CR1 retrotransposon and their amplicons measure ~ 800–850 bp (Fig. 1, 4), except for three lineages: (1) *P. shqipericus*, which has much longer haplotypes (~ 1200 bp); and (2) "regular" *lessonae*/*bergeri*, which lack CR1 and have much shorter haplotypes (~ 300 bp); (3) *P. r. kurtmuelleri*, which have moderately shorter haplotypes (~ 710–720 bp) than other *P. ridibundus* lineages. The last two polymorphisms, are, however, not diagnostic for the identification of their respective lineages. First, haplotypes from the "Alpine" *P. l. lessonae* haplogroup measure ~ 820–830 bp, thus overlapping with most other *Pelophylax* lineages. Second, one *P. r. ridibundus* allele sampled in Serbia was shorter than usual (~ 730 bp) and broadly measures the same length as typical *P. r. kurtmuelleri* haplotypes.

DISCUSSION

Building on our recent multilocus phylogeographic framework for Palearctic water frogs (Dufresnes *et al.* 2024), the present study aims to revisit patterns of divergence, diversity and diagnosticity at the widely used *SAI-1* nuclear marker to assess its reliability for evolutionary, ecological, taxonomic and conservation studies on *Pelophylax*. Our findings emphasize both the pros and cons that must be acknowledged when considering this marker in future surveys.

On the one hand, the variability of the *SAI-1* sequences is sufficient to identify all known Western Palearctic species and > 90 % of their phylogeographic lineages. With the availability of a comprehensive reference dataset (Dufresnes 2024), *SAI-1* is thus adequate to infer the nuclear identity of populations for phylogeographic (e.g., Plötner *et al.* 2012) or biological invasion surveys (e.g., Papežík *et al.* 2024). *SAI-1* sequencing might be particularly relevant to confirm the identity of the many introduced populations so far only screened with mtDNA (e.g., Dufresnes *et al.* 2024) before implementing management actions. In principle, the marker is also informative to identify the genomes involved in hybridogenesis (e.g., Miura *et al.* 2021), noting that the direct sequencing (e.g., by Sanger technology) of hybrids might be compromised by indel polymorphisms, thus requiring other approaches instead (e.g., cloning or amplicon sequencing).

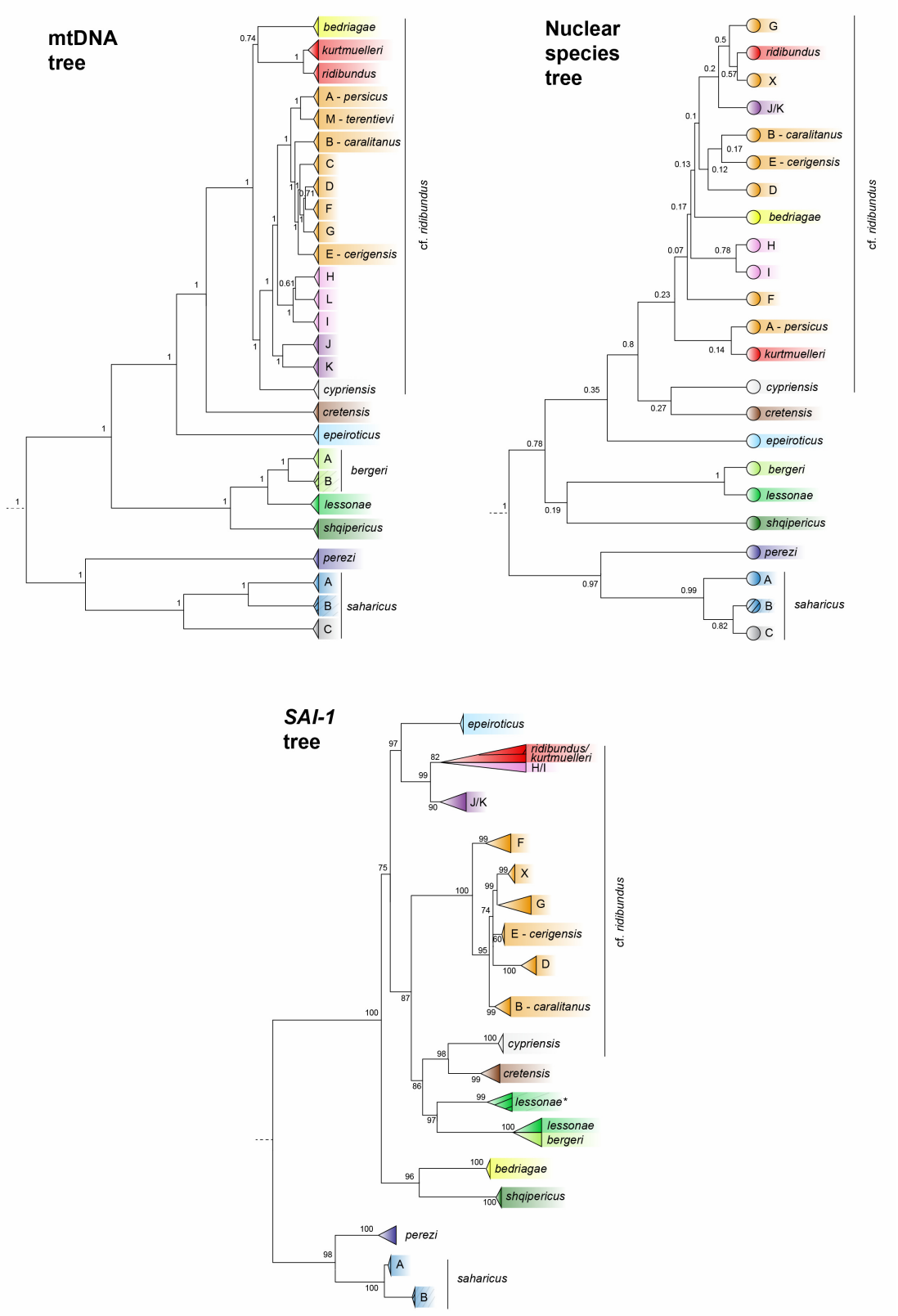

Figure 3. Mitochondrial, nuclear, and *SAI-1* trees in Western Palearctic *Pelophylax*. The first two, built from mitogenomes (~ 17 kb) and five nuclear genes (~ 4.9 kb), are adapted from Dufresnes *et al.* (2024). Values indicate node support (posterior probabilities or % of bootstrap replicates). Dash lines emphasize the phylogenetic placement of *SAI-1* haplotypes with unusual sequence lengths (see Fig. 4).

On the other hand, the analyses revealed discrepancies between divergence patterns, lineage identity, and sequence length, which hinders the use of *SAI-1* sequences for phylogenetic analyses and species identification by amplicon scoring. The issues are best emphasized by the populations found in Italy and southern Switzerland, where patterns of *SAI-1* divergence were initially mistaken for putatively new cryptic species (Dubey & Dufresnes 2017), namely the Alpine populations of *P. l. lessonae* and the marsh frog ancestor of *P. e. hispanicus* – both of which were later refuted by the analysis of additional markers and samples (Dubey *et al.* 2019; Dufresnes *et al.* 2024). The doubts surrounding the *SAI-1* phylogeny have also led to question the species status of *P. cypriensis* (Speybroeck *et al.* 2020) since the evidence for nuclear differentiation of this taxon only comes from *SAI-1*.

In addition, the Alpine *lessonae SAI-1* haplotypes, which include the CR1 retrotransposon, are undistinguishable from *P. ridibundus* by size, making the approach of Hauswaldt *et al.* (2012) unreliable in areas where these haplotypes co-occur. So far, the Alpine *lessonae SAI-1* haplotypes were only detected in northern Italy and in Switzerland, but they could potentially extend across western Europe and the Pannonian Basin, which remains largely unsampled for *SAI-1* (Fig. 1). For instance, the *SAI-1* misidentification reported in southern Germany (Mayer *et al.* 2013) could be due to the incursion of long *lessonae SAI-1* variants in the region. A similar situation may happen in Belgium (K. Cox pers. comm.). In the Balkans, the variability of the *SAI-1* allele size in *P. r. ridibundus* may also prevent proper discrimination from *P. r. kurtmuelleri* without sequencing. Assumptions regarding the diagnosticity of *SAI-1* size-polymorphism generally rely on few samples, unrepresentative of the whole range of lineages – the method by Hauswald *et al.* (2012) was optimized based on samples that mostly originate from the Baltic region. Any *SAI-1*-based water frog identification without sequencing should thus be treated with caution. The warning extends to *P. shqipericus*, an Adriatic endemic species of conservation concern, which has been monitored based on its uniquely long *SAI-1* alleles, both to document its natural distribution (e.g., Zimić *et al.* 2020) and introductions in allochthonous ranges (e.g., Jelić *et al.* 2022).

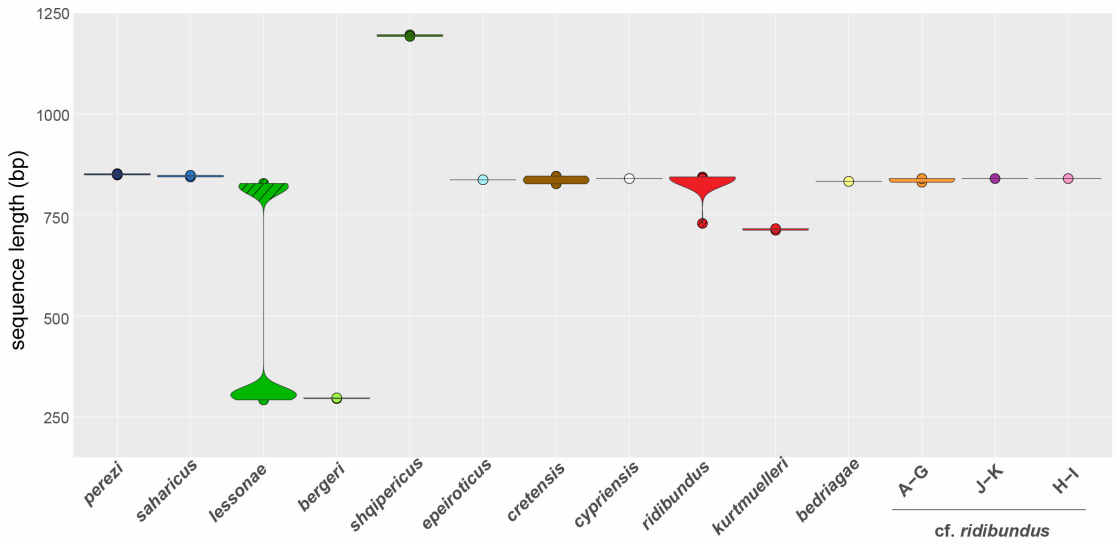

Figure 4. Variation of *SAI-1* sequence length across *Pelophylax* taxa/lineages, as amplified with the primers of Hauswaldt *et al.* (2012). Dash lines emphasize the unusually long alleles from the Alpine *lessonae* haplogroup (similar in size as marsh frog alleles), and the unusually short allele found in one *P. r. ridibundus* sample from Serbia (similar in size as *P. r. kurtmuelleri* alleles).

In conclusion, *SAI-1* appears to be a useful and reliable nuclear marker for *Pelophylax* identification if it is sequenced. However, any deviation from its known diversity (e.g., new lineages) should not be interpreted in a phylogeographic or taxonomic context without further evidence. Likewise, identification without sequencing should be avoided unless the diagnosticity of size-polymorphism can be ascertained in the study area, notably by sequencing representative sets of samples from all the potentially co-occurring lineages. Genotyping *SAI-1* with lineage-specific primers may be an alternative for populations inhabited by a few lineages, and providing that these lineages have been well characterized beforehand (Ermakov *et al.* 2019). Although more costly and demanding in terms of implementation, multilocus nuclear approaches pre-optimized for given sets of *Pelophylax* species, such as microsatellite panels (Christiansen 2005) and PCR-RFLP-like protocols (Patrelle *et al.* 2011; Cuevas *et al.* 2022) may be more suited to reliably identify and monitor local water frog communities.

ACKNOWLEDGMENTS

We thank two anonymous reviewers for their useful comments. MD is a Research Director of Fonds de la Recherche Scientifique – FNRS. This study was partly funded by a PDR F.R.S. – FNRS grant (T.0070.19 to MD).

REFERENCES

Arikan, H. (1988) On a new form of *Rana ridibunda* (Anura, Ranidae) from Turkey. *İstanbul Üniversitesi fen Fakültesi Biyoloji Dergisi*, **53**: 81–87.

Beerli, P., Hotz, H., Tunner, H. G., Heppich, S. & Uzzell, T. (1994) Two new water frog species from the Aegean Islands Crete and Karpathos (Amphibia, Salientia, Ranidae). *Notulae Naturae of the Academy of natural Sciences of Philadelphia*, **470**: 1–9.

Bonaparte, C. L. J. L. (1839) *Iconografia della Fauna Italica per le quattro classi degli Animali Vertebrati*. Tomo II. Amphibi. Fascicolo 24. Rome (Salviucci): 1–560, 54 pl.

Camerano, L. (1882) Recherches sur les variations de la *Rana esculenta* et du *Bufo viridis* dans le Bassin de la Méditerranée. *Comptes rendus de l'Association française pour l'Avancement des Sciences. Paris*, **10**: 680–690.

Christiansen, D. G. (2005) A microsatellite-based method for genotyping diploid and triploid water frogs of the *Rana esculenta* hybrid complex. *Molecular Ecology Notes*, **5** (1): 190–193.

Cuevas, A., Patrelle, C., Ciavatti, F., Gendre, T., Sourrouille, P., Geniez, P., Doniol-Valcroze, P. & Crochet, P.-A. (2022) A new PCR-RFLP method for the identification of parental and hybridogenetic western European water frogs, including the *Pelophylax perezi-grafi* system. *Salamandra*, **58** (3): 218–230.

Doniol-Valcroze, P., Mazepa, G., Grimal, F., Sourrouille, P., Perrin, N., Litvinchuk, S. N. & Crochet, P.-A. (2021) Discovery of a *Pelophylax saharicus* (Anura, Ranidae) population in southern France: a new potentially invasive species of water frogs in Europe. *Amphibia-Reptilia*, **42** (4): 427–442.

Dubey, S. & Dufresnes, C. (2017) An extinct vertebrate preserved by its living hybridogenetic descendant. *Scientific Reports*, **7** [12768]: 1–6.

Dubey, S., Maddalena, T., Bonny, L., Jeffries, D. L. & Dufresnes, C. (2019) Population genomics of an exceptional hybridogenetic system of *Pelophylax* water frogs. *BMC evolutionary Biology*, **19** [164]: 1–13.

Dubois, A. (1983) A propos de cuisses de grenouilles. *Alytes*, **2** (3): 69–111.

Dufresnes, C. (2024) *Data from: Piecing the barcoding puzzle of Palearctic water frogs (*Pelophylax*) sheds light on amphibian biogeography and global invasions*. *Zenodo*. <www.doi.org/10.5281/zenodo.10423702>.

Dufresnes, C., Denoël, M., Di Santo, L. & Dubey, S. (2017) Multiple uprising invasions of *Pelophylax* water frog, potentially inducing a new hybridogenetic complex. *Scientific Reports*, **7** [6506]: 1–10.

Dufresnes, C. & Dubey, S. (2020) Invasion genomics supports an old hybrid swarm of pool frogs in western Europe. *Biological Invasions*, **22** (2): 205–210.

Dufresnes, C. & Jablonski, D. (2022) A genomics revolution in amphibian taxonomy. *Science*, **377** (6612): 1272.

Dufresnes, C. & Mazepa, G. (2020) Hybridogenesis in water frogs. *eLS*, **1** (4): 718–726.

Dufresnes, C., Mazepa, G., Jablonski, D., Caliari Oliveira, R., Wenseleers, T., Shabanov, D. A., Auer, M., Ernst, R., Ramírez-Chaves, H. E., Mulder, K. P., Simonov, E., Tiutenko, A., Kryvokhyzha, D., Wennekes, P. L., Zinenko, O. I., Korshunov, O. V., Al-Johany, A. M., Peregontsev, E. A., Betto-Colliard, C., Denoël, M., Borkin, L. J., Skorinov, D. V., Pasynkova, R. A., Mazanaeva, L. F., Rosanov, J. M., Dubey, S. & Litvinchuk, S. N. (2019) Fifteen shades of green: the evolution of *Bufotes* toads revisited. *Molecular Phylogenetics and Evolution*, **141** [106615]: 1–25.

Dufresnes, C., Monod-Broca, M., Bellati, A., Canestrelli, D., Ambu, J., Wielstra, B., Dubey, S., Crochet, P.-A., Denoël, M. & Jablonski, D. (2024) Piecing the barcoding puzzle of Palearctic water frogs (*Pelophylax*) sheds light on amphibian biogeography and global invasions. *Global Change Biology*, **30** (3) [e17180]: 1–30.

Engelmann, W.-E., Fritzsche J., Günther, R. & Obst, F. J. (1986) *Lurche und Kriechtiere Europas*. Stuttgart (Ferdinand Enke): 1–420.

Ermakov, O., Ivanov, A., Titov, S., Svinin, A. & Litvinchuk, S. N. (2019) New multiplex PCR method for identification of East European green frog species and their hybrids. *Russian Journal of Herpetology*, **26** (6): 367–370.

Fitzinger, L. J. F. J. (1843). *Systema Reptilium. Fasciculus Primus*. Wien (Braumüller et Seidel): 1–106.

Gayda, H. S. (1940). Su alcuni anfibi e rettili dell'Albania esistenti nel Museo zoologico di Berlino. *Atti della Societa italiana di Scienze naturali e del Museo civico di Storia naturale di Milano*, **79** (4): 263–272.

Hallowell, E. (1861) Report upon the Reptilia of the North Pacific Exploring Expedition, under command of Capt. John Rogers, U.S. N. *Proceedings of the Academy of natural Sciences of Philadelphia*, **1860**: 480–510.

Hartert, E. (1913) Expedition to the central western Sahara. V. Reptiles and Batrachians. *Novitates zoologicae*, **20**: 76–84.

Hauswaldt, J. S., Höer, M., Ogielska, M., Christiansen, D. G., Dziewulska-Szwajkowska, D., Czernicka, E. & Vences, M. (2012) A simplified molecular method for distinguishing among species and ploidy levels in European water frogs (*Pelophylax*). *Molecular Ecology Resources*, **12** (5): 797–805.

Herczeg, D., Vörös, J., Christiansen, D. G., Benovics, M. & Mikulíček, P. (2017) Taxonomic composition and ploidy level among European water frogs (Anura: Ranidae: *Pelophylax*) in eastern Hungary. *Journal of zoological Systematics and evolutionary Research*, **55** (2): 129–137.

Hotz, H., Uzzell, T., Günther, R., Tunner, H. G. & Heppich, S. (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., 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. *Journal of Heredity*, **104** (1): 57–71.

Huson, D. H. & Bryant, D. (2006) Application of phylogenetic networks in evolutionary studies. *Molecular Biology and Evolution*, **23** (2): 254–267.

Jelić, M., Franjević, D., Đikić, D., Korlević, P., Vucić, M., Jelić, D., Becking, T., Grandjean, F. & Klobučar, G. (2022) Hybrid swarm as a result of hybridization between two alien and two native water frog species (genus *Pelophylax*) in central Croatia: Crna Mlaka fishpond acting as a species melting pot? *Biological Invasions*, **24** (10): 3291–3304.

Kolenda, K., Pietras-Lebioda, A., Hofman, S., Ogielska, M. & Pabijan, M. (2017) Preliminary genetic data suggest the occurrence of the Balkan water frog, *Pelophylax kurtmuelleri*, in southwestern Poland. *Amphibia-Reptilia*, **38** (2): 187–196.

Linnaeus, C. (1758) *Systema Naturae per regna tria naturae, secundum classes, ordines, genera, species, cum characteribus, differentiis, synonymis, locis.* Editio decima, reformata. Tomus I. Holmiae (Laurentii Salvii): [i–iv] + 1–824.

Litvinchuk, S. N., Ivanov, A. Y., Lukonina, S. A. & Ermakov, O. A. (2020) A record of alien *Pelophylax* species and widespread mitochondrial DNA transfer in Kaliningradskaya Oblast' (the Baltic coast, Russia). *BioInvasions Records,* **9** (3): 599–617.

López-Seoane, V. (1885) On two new forms of *Rana* from N.W. Spain. *The Zoologist: a monthly Journal of natural History*, (3), **9** (101): 169–172.

Mayer, M., Hawlitscheck, O., Zahn, A. & Glaw, F. (2013) Composition of twenty green frog populations (*Pelophylax*) across Bavaria, Germany. *Salamandra*, **49** (1): 31–44.

Miura, I., Vershinin, V., Vershinina, S., Lebedinskii, A., Trofimov, A., Sitnikov, I. & Ito, M. (2021) Hybridogenesis in the water frogs from western Russian territory: intrapopulation variation in genome elimination. *Genes*, **12** (2): 244.

Neveu, A. (1989) *Rana ridibunda*. *In*: J. Castanet & R. Guyétant (ed.), *Atlas de répartition des amphibiens et reptiles de France,* Paris (Société Herpétologique de France): 90–91.

Pallas, P. S. (1771) *Reise durch verschiedene Provinzen des Russischen Reichs*. Theil 1. St. Pétersbourg (Kayserlichen Academie der Wissenschaften): 1–504.

Papežík, P., Kubala, M., Jablonski, D., Doležálková-Kaštánková, M., Choleva, L., Benovics, M. & Mikulíček, P. (2021) Morphological differentiation of endemic water frogs (Ranidae: *Pelophylax*) from the southwestern Balkans. *Salamandra*, **57** (1): 105–123.

Papežík, P., Sciberras, A., Benovics, M., Sciberras, J., Deidun, A. & Mikulíček, P. (2024) Far from home: tracing the origin of non-native water frogs (genus *Pelophylax*) in Malta by molecular markers. *Biological Invasions*, **26** (4): 1045–1059.

Patrelle, C. Ohst, T., Picard, D., Pagano, A., Sourice, S., Dallay, M.-G. & Plötner, J. (2011) A new PCR-RFLP-based method for an easier systematic affiliation of European water frogs. *Molecular Ecology Resources,* **11** (1): 200–205.

Plötner, J., Akın Pekşen, Ç., Baier, F., Uzzell, T. & Can Bilgin, C. (2015) Genetic evidence for human-mediated introduction of Anatolian water frogs (*Pelophylax* cf. *bedriagae*) to Cyprus (Amphibia: Ranidae). *Zoology in the Middle East*, **61** (2): 125–132.

Plötner, J., Baier, F., Akın, C., Mazepa, G., Schreiber, R., Beerli, P., Litvinchuk, S. N., Bilgin, C. C., Borkin, L. & Uzzell, T. (2012) Genetic data reveal that water frogs of Cyprus (genus *Pelophylax*) are an endemic species of Messinian origin. *Zoosystematics and Evolution*, **88** (2): 261–283.

Plötner, J., Köhler, F., Uzzell, T., Beerli, P., Schreiber, R., Guex, G. D. & Hotz, H. (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** (3): 784–791.

Plötner, J., Uzzell, T., Beerli, P., Spolsky, C., Ohst, T., Litvinchuk, S. N., Guey, G.-D., Reyer, H.-U. & Hotz, H. (2008) Widespread unidirectional transfer of mitochondrial DNA: a case in western Palaearctic water frogs. *Journal of evolutionary Biology*, **21** (3): 668–681.

Puky, M., Schad, P. & Szövényji, G. (2005) *Magyaroszag herpetologiai atlasza* [Herpetological atlas of Hungary]. Budapest (Varangy Akciocsoport Egyesület): 1–207, 16 pl.

Rubinoff, D., Cameron, S. & Will, K. (2006) A genomic perspective on the shortcomings of mitochondrial DNA for "barcoding" identification. *Journal of Heredity*, **97**, 581–594.

Sagonas, K., Karameta, E., Kotsakiozi, P. & Poulakakis, N. (2020) Cross-species testing of nuclear markers in *Pelophylax* water frogs in Greece and examination of their power to detect genetic admixture. *Amphibia-Reptilia*, **41** (2): 253–259.

Schneider, H., Sofianidou T. S. & Kyriakopoulou-Sklavounou P. (1984) Bioacoustic and morphometric studies in water frogs (genus *Rana*) of Lake Ioannina in Greece, and description of a new species (Anura, Amphibia). *Journal of zoological Systematics and evolutionary Research*, **22** (4): 349–366.

Schneider, J. G. (1799) *Historia Amphibiorum naturalis et literarariae. Fasciculus primus. Continens Ranas, Calamitas, Bufones, Salamandras et Hydros in genera et species descriptos notisque suis distinctos.* Jena (Friederici Frommanni): 1–264.

Speybroeck, J., Beukema, W., Dufresnes, C., Fritz, U., Jablonski, D., Lymberakis, P., Martínez-Solano, I., Razzetti, E., Vamberger, M., Vences, M., Vörös, J. & Crochet, P.-A. (2020) Species list of the European herpetofauna – 2020 update by the Taxonomic Committee of the Societas Europaea Herpetologica. *Amphibia-Reptilia*, **41** (2): 139–189.

Trifinopoulos, J., Nguyen, L. T., Haeseler, A. von & Minh, B. Q. (2016) W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. *Nucleic Acids Research*, **44** (W1): W232–235.

Vucić, M., Jelić, D., Klobučar, G. I., 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** (2): 147–157.

Wielstra, B., Burke, T., Butlin, R. K. & Arntzen, J. W. (2017) A signature of dynamic biogeography: enclaves indicate past species replacement. *Proceedings of the royal Society of London*, (B), **284** (1868) [20172014]: 1–6.

Zieliński, P., Nadachowska-Brzyska, K., Wielstra, B., Szkotak, R., Covaciu-Marcov, S., Cogălniceanu, D. & Babik, W. (2013) No evidence for nuclear introgression despite complete mtDNA replacement in the Carpathian newt (*Lissotriton montandoni*). *Molecular Ecology*, **22** (7): 1884–1903.

Zimić, A., Berina, V., Šunje, E., Ćurić, A. & Kalamujic Stroil, B. (2020) Molecular identification of green frogs (Anura: Ranidae: *Pelophylax*) of western Balkans (Bosnia & Herzegovina and Montenegro). *In*: D. Jöjic (ed.), *IV Symposium of biologists and ecologists of Republic of Srpska with international participation – SBERS2020*, Banja Luka (Faculty of Natural Sciences and Mathematics, University of Banja Luka): 152.

Submitted: 17 January 2024.
Accepted: 23 February 2024.
Published: 25 March 2024.

Corresponding Editor: Spartak N. LITVINCHUK.