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► To cite this version:

Christophe Dufresnes, Simeon Lukanov, Sven Gippner, Johanna Ambu, Ilias Strachinis, et al.. Historical biogeography and systematics of yellow-bellied toads (*Bombina variegata*), with the description of a new subspecies from the Balkans. *Vertebrate Zoology*, 2025, 75, pp.1-30. <10.3897/vz.75.e138687>. <hal-04995210>

HAL Id: hal-04995210

<https://hal.science/hal-04995210v1>

Submitted on 18 Mar 2025

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Historical biogeography and systematics of yellow-bellied toads (*Bombina variegata*), with the description of a new subspecies from the Balkans

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<https://zoobank.org/10CFDFEC-8E1B-448E-9BFC-7A6C8B2AB776>

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Academic editor Deepak Veerappan | Received 6 October 2024 | Accepted 27 December 2024 | Published 23 January 2025

Citation: Dufresnes C, Lukanov S, Gippner S, Ambu J, Strachinis I, Arsovski D, Monod-Broca B, Cayuela H, Lymberakis P, Canestrelli D, Cogălniceanu D, Poyarkov NA, Litvinchuk SN, Suchan T, Denoël M, Jablonski D (2025) Historical biogeography and systematics of yellow-bellied toads (*Bombina variegata*), with the description of a new subspecies from the Balkans. *Vertebrate Zoology* 75 1–30. <https://doi.org/10.3897/vz.75.e138687>

Abstract

The Balkan Peninsula hosts a great proportion of Europe's biodiversity, and this is well illustrated by amphibian richness and endemism. Among them, the yellow-bellied toad *Bombina variegata* has been a model in ecology and evolution, but several aspects of its phylogeography and taxonomy remain surprisingly poorly understood. In this study, we combine cytochrome *b* DNA barcoding data (1238 individuals from 355 localities), mitogenome phylogenetics (17.2 kb), gene-based nuclear phylogenetics (3.7 kb from

four gene fragments) and multilocus phylogenomics (4759 loci / ~554 kb obtained by double digest Restriction Associated DNA sequencing; ddRAD-seq) to re-assess the diversification of *B. variegata*, and revisit its nomenclatural history to assign scientific names to phylogeographic lineages. The analyses support four major lineages, one assigned to *B. v. variegata* (Carpathians and northwestern ranges), one assigned to *B. v. pachypus* (Apennine Peninsula), and two assigned to *B. v. scabra* (Dinarides, Hellenides and Balkanides vs. the Rhodope mountains). Spatiotemporal patterns of diversification suggest a role for a Late Miocene marine incursion in the Pannonian Plain (Paratethys) as the initial trigger of divergence, followed by a vicariance event in the Apennines and a “sky island” process of Pleistocene differentiation in the Balkan Peninsula. As it reached the Dinarides during the Late Pleistocene, *B. v. variegata* potentially hybridized with *B. v. scabra* and captured its mitochondrial DNA, which resulted in a massive cyto-nuclear discordance across all northwestern European populations. Finally, we show that the two lineages of *B. v. scabra* significantly differ in morphology and ventral coloration patterns, and describe the Rhodope lineage as a new subspecies.

Keywords

Balkan Peninsula, cyto-nuclear discordance, glacial refugia, nomenclature, phylogeography, Rhodope Mountains

Introduction

Southern European peninsulas are considered hotspots of diversity and endemism for the terrestrial fauna of the Western Palearctic (e.g., Baquero and Tellería 2001; López-López et al. 2011; Sillero et al. 2014), a status largely owed to their key role as glacial refugia (Avice 2000; Hewitt 2004; Schmitt 2007). These refugia have multiple functions: (1) they represent shelters where species survived the Quaternary glaciations (Hewitt 1996, 2000; Taberlet et al. 1998; Recuero and García-París 2011); (2) they act as regional hubs of diversification of these species in situ (e.g., the refugia-within-refugia paradigm; Gómez and Lunt 2007; Feliner 2011); (3) they serve as melting pots of diversity as the lineages diverging in allopatry periodically exchange genes during recurrent periods of secondary contacts (Canestrelli et al. 2006, 2010; Dufresnes et al. 2016; Asztalos et al. 2021), sometimes even to the point of assimilating one another (lineage fusion; Garrick et al. 2019).

The Balkan Peninsula in its broad sense, defined as the landmass stretching from the Alps to the Black Sea in the north and to the Peloponnese in the south (Jablonski 2017a), is inhabited by a remarkable diversity of amphibians that perfectly illustrates its dynamic biogeography (Crnobrnja-Isailović 2007; Sillero et al. 2014; Dufresnes 2018). Nearly all amphibians found in the Balkans have been evolving on the spot for millions of years across topographically and environmentally complex landscapes that promoted the differentiation of multiple species and subspecies, only a few of which eventually expanded outside the peninsula e.g., the green toad *Bufo viridis viridis* (Dufresnes et al. 2019b), the tree frog *Hyla arborea* (Dufresnes et al. 2013), the water frog *Pelophylax ridibundus ridibundus* (Dufresnes et al. 2024; Litvinchuk et al. 2024), the crested newt *Triturus cristatus* (Wielstra et al. 2013), the alpine newt *Ichthyosaura alpestris* (Denœl et al. 2001; Robbemont et al. 2023) and the smooth newt *Lissotriton vulgaris* (Pabijan et al. 2015). As phylogeographic studies progressively unfold species diversifications, they reveal shared biogeographic subdivisions

among distinct species in Mediterranean areas such as the Adriatic (e.g., *Proteus anguinus*, *Pelophylax shqipericus*, *Bufo viridis longipes*; Dufresnes et al. 2019b, 2021c, 2024; Recknagel et al. 2024) and Ionian coasts (e.g., *Pelophylax epeiroticus*, *Pelobates balcanicus chloae*, *Lissotriton graecus*; Pabijan et al. 2017; Dufresnes et al. 2019a, 2024), the Dinarides (e.g., *Salamandra atra prenjensis*; Gippner et al. 2024) and Hellenides (e.g., *Rana graeca*; Jablonski et al. 2021), but also non-Mediterranean areas such as the Pannonian plain (e.g., *Triturus dobrogicus*; Wielstra et al. 2013), the Pirin (e.g., *Salamandra salamandra beschkovi*; Gippner et al. 2024) and the Carpathian Mountains (e.g., *Lissotriton montandoni*, *Lissotriton vulgaris ampelensis*; Rafiński et al. 2001; Pabijan et al. 2017; Herczeg et al. 2023). In parallel, the focus on multilocus nuclear DNA is demonstrating the role of past hybridization in the genetic diversity of these taxa, through phenomena such as mitochondrial capture, allele surfing and hybrid swarms that resulted from hybrid zone movements following range shifts during the Quaternary’s climatic oscillations and environmental fluctuations. This is exemplified by the foreign ancestry retrieved in many species, e.g., *L. montandoni* (Babik et al. 2005; Zieliński et al. 2013) and *B. v. viridis* (Dufresnes et al. 2019b), which carry mitochondrial DNA (mtDNA) from other species, or “*Triturus arntzeni*”, a taxon invalidated by the International Code of Zoological Nomenclature (ICZN) due to the introgressed nature of its type specimen (Wielstra and Arntzen 2014).

The yellow-bellied toad *Bombina variegata* is another case of the complex processes of population persistence, differentiation and reticulation that shape the historical biogeography of the Balkan Peninsula (Fijarczyk et al. 2011). In Central and Southern Europe, this small species inhabits the meadow and forest environments of reliefs, being replaced by its sister species the fire-bellied toad *Bombina bombina* in the lowlands (Speybroeck et al. 2016; Dufresnes 2019). Besides habitat and other morphological, reproductive, and developmental traits (Rafińska

1991; MacCallum et al. 1998), the two species are distinctive in their patterns of ventral coloration, which alternate colored and dark patches on the throat and belly (Speybroeck et al. 2016; Dufresnes 2019). The ventral coloration is also highly variable within species, enabling photographic-based individual recognition for mark-recapture studies (e.g., Cayuela et al. 2022), and contributing to the distinction of many subspecies and varieties since the early days of the Linnean classification system (Frost 2024).

Despite a relatively strong focus by zoologists and molecular ecologists, notably for studying the hybrid zones it forms with *B. bombina* (Szymura 1976; Szymura and Barton 1986; Dufresnes et al. 2021a), the evolution and systematics of *B. variegata* still feature many grey areas. Three subspecies are currently recognized, historically based on ecology, morphology and coloration patterns; (1) *B. v. variegata* inhabits the northern and western ranges, especially around the Pannonian plain and the Alps; (2) *B. v. pachypus* is isolated in the Apennine mountains of peninsular Italy; (3) *B. v. scabra* replaces *B. v. variegata* in the southern and western parts of the Balkan Peninsula. In contrast, mtDNA analyses recovered four major phylogeographic lineages with their respective divergence and distribution that differ between molecular markers, and conflict with the current taxonomy (Hofman et al. 2007; Fijarczyk et al. 2011; Pabijan et al. 2013). Both *B. v. variegata* and *B. v. scabra* are paraphyletic in the mitochondrial tree: *B. v. variegata* features a deeply diverged lineage restricted to the Carpathian Mountains (“Carpathian” lineage C) and a second lineage extending from the Dinarides to northwestern Europe (“Balkano-Western” lineage BW), shared with *B. v. scabra*. This BW lineage forms a clade with a second Balkan lineage restricted to the Rhodope Mountains (“Rhodope” lineage R) and with the lineage of *B. v. pachypus* (“Apennine” lineage A). In contrast, nuclear-encoding genes feature allele similarities and even sharing between the populations carrying these distinctive mtDNA lineages, which either suggests nuclear homogeneity, genetic introgression, or poor phylogenetic resolution due to slow evolution and ancestral polymorphism at the few markers analyzed (Zheng et al. 2009; Fijarczyk et al. 2011; Pabijan et al. 2013).

These discrepancies partly explain why the taxonomy of *B. variegata* has remained unsettled. For instance, some authors consider *B. v. pachypus* as a separate species (Lanza and Vanni 1991; Canestrelli et al. 2006), but this has been ignored given the phylogenetic placement of this taxon, nested within the two *B. v. variegata* lineages in mitochondrial analyses (Speybroeck et al. 2020). Likewise, the genetic similarities between the western populations of *B. v. variegata* and *B. v. scabra* cast doubts on the validity of the latter, which could then reflect a regional morphotype rather than an evolutionarily divergent taxon. Conversely, the deep divergence reported in the Carpathian and Rhodope mountains might represent cryptic evolutionary units worthy of specific conservation attention, and which would deserve taxonomic recognition. In parallel, the rich yet confusing repertoire of scientific names proposed for yellow- (and fire-) bellied toads since Linnaeus’ times (Lescure et al. 2011; Frost 2024)

calls for clarifications in order to enforce a stable taxonomy for these research models and emblematic species.

A comprehensive overview of the phylogeographic diversity of *B. variegata* would be timely to guide systematic revisions, and more generally to understand what shaped its evolution in the context of the historical biogeography of the Balkan Peninsula. We aim to provide such an overview by (1) updating the distributions of the main mitochondrial lineages, (2) re-examining their evolutionary relationships and timing of divergence, (3) assessing how these correspond to the nuclear diversity, and (4) to the taxa proposed in the historical zoological literature. As this framework suggests two unique lineages within the taxon *B. v. scabra*, we further quantify morphological and color pattern variation between them, and provide the taxonomic description of a new subspecies.

Material and Methods

Mitochondrial DNA barcoding

Mitochondrial lineages affiliated with *B. variegata* were identified for 1238 individuals sampled in 355 localities based on sequences of the cytochrome *b* (*cyt b*) gene, as gathered from previous studies (Szymura et al. 2000; Canestrelli et al. 2006; Hofman et al. 2007; Pabijan et al. 2008, 2013; Zheng et al. 2009; Fijarczyk et al. 2011; Cornetti et al. 2016; Talarico et al. 2020; Dufresnes et al. 2021a; Pröhl et al. 2021; Tzoras et al. 2023), and complemented by additional sequences for 17 individuals from 17 localities (methods in Dufresnes et al. 2021a). The new sequences were uploaded on GenBank under accessions PQ757961–PQ757977.

Sequences were manually aligned and trimmed (1096 bp) in Seaview 5 (Gouy et al. 2021), and unique haplotypes were identified with DnaSP 6 (Rozas et al. 2017). To visualize their relative divergence, a phylogenetic network was produced in SplitsTree 4 (Huson and Bryant 2006), based on uncorrected *p* distances. Haplotypes were assigned to the six lineages defined in the study of Fijarczyk et al. (2011) and lineage distributions were mapped in QGIS 3.24 (QGIS.org 2022). The list of localities is provided in Table S1.

For the purpose of a taxonomic description, which requires stating diagnostic characters, we further screened the *cyt b* haplotype alignment for substitutions unique to the Rhodope lineage using MOLD (Fedosov et al. 2022), as implemented in iTaxoTools (Vences et al. 2021); haplotypes with missing data were discarded for this analysis. The full gene sequence of *B. variegata* was used as reference (GenBank accession JX893177).

Mitogenome phylogeny

We gathered 19 full mitogenomes representative of the mitochondrial diversity of *B. variegata* ($n = 8$) and its

closest relatives *B. bombina* ($n = 9$) and *B. orientalis* ($n = 2$) sequenced in previous work (San Mauro et al. 2004; Jung et al. 2006; Pabijan et al. 2008, 2013; De Cahsan et al. 2021; sequence list in Table S2). Sequences were handled in Seaview for manual alignment, trimming (17,240 bp), and gene/tRNA partitioning using the annotations of JX893177 as a reference.

The dataset was used to infer time-calibrated Bayesian phylogenies with BEAST 2.6 (Bouckaert et al. 2019), using the birth-death tree model with $1/X$ priors for the birth and death rates, GTR+G+I models of sequence evolution for each partition, and a strict clock calibrated by two most recent common ancestor (MRCAs) priors, one on the root (prior 1) and one on the *B. variegata/bombina* divergence (prior 2). For those, we implemented the age estimates obtained by Pabijan et al. (2013) based on two fossil calibration schemes, which the authors considered equally plausible. The first calibration (calibration I) involved 21.3 Mya (95% Height Posterior Density [HPD]: 24.4–20.1 Mya) for prior 1 and 9.0 Mya (12.7–4.9 Mya) for prior 2, which was implemented with a log-normal prior of $M = 0.35$, $S = 0.73$ and offset = 19.7, and a normal prior of mean = 9.0 and $\sigma = 2.3$, respectively. The second calibration (calibration II) involved 14.1 Mya (13.1–17.4 Mya) for prior 1 and 6.5 Mya (8.9–4.2 Mya) for prior 2, which was implemented by a log-normal prior of $M = 0.35$, $S = 0.73$ and offset = 12.7, and a normal prior of mean = 6.5 and $\sigma = 1.4$, respectively. Short preliminary runs of 10 million iterations were conducted in order to optimize operator parameters as provided by the BEAST output. Final optimized runs were conducted for 100 million iterations with sampling every 50,000 and were monitored for stationarity and effective sample sizes of parameters in Tracer 1.7 (Rambaud et al. 2018). Consensus trees were produced with the TreeAnnotator module of BEAST, discarding the first 20% as burn-in.

In parallel, we also reconstructed a maximum-likelihood phylogeny in IQ-TREE 1.6 (Trifinopoulos et al. 2016), using the model finder option to find the best model of sequence evolution, and 1000 ultrafast bootstrap replicates.

Moreover, pairwise divergence between lineages was computed in MEGA 11 (Tamura et al. 2021) for the DNA barcoding genes *cyt b*, 16S ribosomal DNA (16S), and cytochrome *c* oxidase subunit I (*cox1*). These loci are frequently examined for amphibian species delimitation, notably to apply ad hoc speciation thresholds based on patterns of reproductive isolation (Dufresnes et al. 2021b; Dufresnes and Litvinchuk 2022).

Gene-based nuclear phylogeny

We built a supermatrix spanning four polymorphic fragments of nuclear genes previously sequenced in *Bombina*, namely sodium-calcium exchanger gene intron 1 (*ncx1*, 702 bp), recombination activating gene intron 1 (*rag-1*, 1061 bp), intron 2 (*rag-2*, 578 bp) and rhodopsin gene (*rho*, 1374 bp), for a subset of nine samples representative of the mitochondrial diversity of *B. variegata*

($n = 6$), as well as *B. bombina* ($n = 1$) and *B. orientalis* ($n = 2$) as outgroups (sequence list in Table S3). The samples considered are composite, as they combine sequences from two independent studies (*rag-2* and *rho*: Zheng et al. 2009; *ncx1* and *rag-1*: Fijarczyk et al. 2011), noting that correspondence was reliably established based on *cyt b* barcoding and geographic origins. In total, the dataset consists of 15 partitioned sequences (6 out of the 9 samples were heterozygous) for a total length of 3715 bp.

The supermatrix was analyzed with the multigene coalescent-based species tree method implemented in *BEAST (Heled and Drummond 2010), using independent site models (GTR+G+I), clock models (strict clocks) and trees for each gene, and a birth-death model for the species tree (with $1/X$ priors for the birth and death rates). Sequences were set in eight groups that distinguished the main mitochondrial lineages of *B. variegata* and the two outgroup species. As above, the analysis was run for 100 million iterations, sampling every 50,000, and monitored in Tracer. To see alternative topologies among sampled trees, the nuclear phylogeny was displayed as a cloudogram produced in DensiTree 2 (Bouckaert and Heled 2014).

For exploratory purposes, the concatenated alignment was also analyzed with IQ-TREE as for the mitogenome dataset above, but noting that unlike mitogenomes (where all genes are fully linked and share the same genealogy), such analysis can provide spurious topologies when the combined sequences are unlinked and form different gene trees (Maddison 1997).

Phylogenomics

To infer the nuclear diversity of *B. variegata*, we further analyzed double-digest Restriction Associated DNA sequencing (ddRAD-seq) data obtained for 10 samples representative of the mitochondrial lineages of that species, and one *B. bombina* sample to be used as outgroup (Table S4). These samples were included in a genomic library prepared with a custom protocol (<http://dx.doi.org/10.17504/protocols.io.kxygx3nzwg8j/v1>), as fully described in Dufresnes et al. (2025). In brief, this protocol involves enzyme restriction with *MseI* and *SbfI*, ligation of barcoded adapters, purification, PCR amplification with Illumina primers, size selection of fragments between 400–500bp, purification and concentration for paired-end sequencing on an Illumina NextSeq550 with the 2×75 bp kit. Raw reads were demultiplexed with STACKS 2.63 (Catchen et al. 2013) and uploaded on the NCBI Sequence Read Archive under BioProject PRJNA949685 (accessions SRR31792557–SRR31792567). Assembly and cataloging was performed with the *denovo_map.pl* pipeline with the *-rm-pcr-duplicates* flag and default *-m*, *-n*, and *-M* parameters. The catalog contained 230,498 loci with an average coverage of 15.3 per sample (9.9–19.6). Among these we called the loci polymorphic and sequenced among all samples with the module *populations* of STACKS and retaining invariable sites (*-p 11 -phylip-var-all*), which produced a supermatrix alignment

of 4759 loci totaling 553,608 bp. From this alignment, a maximum-likelihood tree was built with IQ-TREE as above.

Morphometric analyses in *Bombina variegata scabra*

Our analyses confirmed the occurrence of an evolutionarily unique population in the Rhodope mountains, previously confounded with its sister lineage *B. v. scabra* (see Results). To document morphological variation, we measured 58 adult specimens curated at the Museum Koenig Bonn (ZFMK), the Museum für Naturkunde Berlin (ZMB), and the Muséum d'histoire naturelle de Genève (MHNG), representing both sexes and of known geographic origin – so they could be assigned to each lineage. Twelve characters were measured with a digital caliper (0.1 mm precision) by one of us (CD) to avoid measurer bias: **L.** body length, from tip of snout to center of cloacal opening; **F.** femur length, from center of cloacal opening to distal end of the femur bone; **T.** tibia length, from knee to heel; **D.p.** length of the first toe, from the distal point of the base of the internal metatarsal tubercle to the end of the toe; **L.c.** head length, from the tip of the snout until the posterior edge of the mandible; **Lt.c.** head width, between the posterior edges of jaw articulations; **Sp.oc.** snout width, between the front edges of orbits; **D.r.o.** snout length, from the front edge of snout to the interior bony orbital border; **Lt.p.** maximum width of the upper eyelid; **Sp.p.** inter-orbital distance, as the minimum distance between orbits; **L.o.** maximum length of the upper eyelid; **Sp.n.** inter-nostril distance. Measured specimens are listed in Table S5. A schematic showing how each character was measured is available as Fig. S1.

To examine body shape without the effect of body size, we applied allometric corrections with the R package GroupStruct (Chan and Grismer 2022) using the multispecies method to perform lineage- and sex-specific adjustments by body length (L.). We then performed a Principal Component Analysis (PCA) on the corrected dataset using the R package FactoMineR (Lê et al. 2008) and built convex hulls to distinguish sexes and lineages (chull function of the R package grDevices; R Core Team 2024). The effect of lineage and sex (and their interaction) was assessed by a Multivariate Analysis of Variance (MANOVA) with the R package stats (R Core Team 2024).

To find characters diagnosable in the field, we further computed simple body ratios (characters/L.) and visualized differences between lineages using boxplots (R package ggplot2; Wickham 2016), testing for significance using Kruskal-Wallis rank sum tests (R package stats), and applying a Bonferroni correction for multiple testing.

Finally, we explored differences in body length (L.) by considering body length measurements of an additional 120 *B. v. scabra* specimens of known sex and origin, namely the 4 specimens of the type series of the new Rhodope taxon (see Results), 96 live specimens captured

in Bulgaria during monitoring surveys, and 20 specimens curated at the Zoological Institute of Russian Academy of Sciences, St. Petersburg (ZISP). The list is provided in Table S5. While these were measured by different persons, measurer bias is expected to be negligible for this standard character. The effects of sex and lineage were statistically tested on this extended dataset (n = 178) by a two-way Analysis of Variance (ANOVA), which statistical significance was assessed in a non-parametric way, namely by a permutation procedure in which the assignment of lineage and sex was reshuffled 1000 times. The variation was visualized using boxplots (R package ggplot2).

Ventral coloration pattern analysis in *Bombina variegata scabra*

We quantified variation in the proportions of yellow/orange vs. dark coloration on the ventral side of 197 specimens of *B. v. scabra* of known geographic origin and attributable to lineages. To this end, we gathered photographs where the throat and belly are visible for (1) field-caught specimens, taken from our own libraries and the citizen-science platform iNaturalist (2024), and (2) the museum specimens measured above. On each photo, the forelimbs, hindlimbs, and any foreign object were manually cropped out in Adobe Photoshop 2019 to retain only the head, thorax and abdomen.

Each photo was processed using a modified version of a Python script published by Sanchez et al. (2018), which was originally developed to quantify black-to-yellow color proportions in fire salamanders (*Salamandra salamandra*) and similarly colored amphibians. Here, three key adjustments were made to the script in respect to our target species: (1) the color range was expanded to include shades of orange and red, in addition to yellow; (2) the threshold for black pixels was adjusted and the spectrum was broadened to accurately capture even the faded dark patterns in preserved specimens; and (3) the “-background” argument was removed since the photos were cropped, and any pixels not classified as black or color were counted as “other”. The modified script outputs the absolute number of black, color and “other” pixels, the total number of pixels, the proportion of black and color pixels relative to the total (excluding “other” pixels), and the color-to-black ratio. It is made available on Zenodo (<https://doi.org/10.5281/zenodo.14359153>).

Differences in color-to-black ratios between the two lineages were analyzed with a Kruskal-Wallis test. Additionally, to account for the potential effect of museum preservation on coloration contrast, a two-way ANOVA was conducted (significance tested by a permutation procedure as above), treating specimen type (field-caught vs. museum) as a secondary explanatory variable.

Nomenclature overview

We examined the nomenclature with the goal of attributing available names to phylogeographic lineages. To this

end, we first mapped these lineages according to our results by updating the distribution shapefile of *B. variegata* designed by Dufresnes et al. (2021a) based on a synthesis of available atlases and occurrence datasets. We then researched the scientific names that could be associated with these lineages using the lists provided by Lescure et al. (2011) and Frost (2024) as starting references. We evaluated (1) the type material and information associated with each taxon; (2) to which populations they correspond to; and (3) whether they represent senior or junior synonyms in respect to the principle of priority of the ICZN.

The nomenclature of the yellow-bellied toad *B. variegata* is intertwined with the nomenclature of the fire-bellied toad *B. bombina*. Both were considered the same species for more than a century of Linnean classification (Lescure et al. 2011) and consequently, the affiliation of some historical names prior to their distinction remains questionable. Therefore, our nomenclature overview encompassed taxa proposed for both or either *B. variegata* and *B. bombina*. When necessary, we took actions to affirm the affiliation of taxa of uncertain origin to promote nomenclatural stability in present and future revisions.

Results

Mitochondrial phylogeography

Analyses of the *cyt b* gene (1096 bp) in 1238 toads from 355 localities revealed 202 *B. variegata* haplotypes grouped into the four main mitochondrial lineages retrieved by previous studies (Fig. 1):

- (1) the Carpathian lineage (C) is found in the *B. v. variegata* populations of the Carpathian Mountains and can be divided into two closely related mitogroups inhabiting the northern and southwestern ranges (C-W) and southeastern ranges (C-E);
- (2) the Apennine lineage (A) extends across all the populations of the Apennine *B. v. pachypus*;
- (3) the Rhodope lineage (R) is restricted to the easternmost *B. v. scabra* populations of the Balkan Peninsula;
- (4) the Balkano-Western lineage (BW) ranges from the southern to the northwestern edges of the distribution, and can be divided in two closely related mitogroups, one found in the southern populations of the Balkan Peninsula, attributed to *B. v. scabra* (BW-S), and one found across the populations stretching from the Dinarides to Western Europe, attributed to *B. v. variegata* (BW-N). The BW-S mitogroup is also found in Central Italy and the Aegean islands due to human-mediated introductions (Talarico et al. 2020; Tzoras et al. 2023).

The mitochondrial trees obtained with BEAST and IQ-TREE, based on full mitogenomes (17,240 bp), provided robustly supported topologies that recovered the four *B. variegata* lineages in two major clades, one including lineage C, and one regrouping lineages A, R, and BW

(Figs 2, S2). Calibration I expectedly featured slightly older node ages than calibration II: the split between lineage C and A/R/BW was estimated to 7.1 Mya vs. 4.7 Mya; the split between lineages A and R/BW was estimated to 3.4 Mya vs. 2.3 Mya; the split between lineages R and BW was estimated to 2.1 vs. 1.4 Mya; the diversification of lineage C was estimated to 0.6 vs. 0.4 Mya, and the diversification of lineage BW was estimated to 0.5 vs. 0.4 Mya.

Pairwise sequence divergences at barcoding genes (Table 1) were on average 3.6% at 16S, 6.7% at *cox1*, and 10.4% at *cyt b* between *B. variegata* and *B. bombina*. The highest intraspecific divergence in *B. variegata* involved the Carpathian vs. other lineages, with on average 2.8% at 16S, 5.0% at *cox1*, and 8.7% at *cyt b*. The Apennine *B. v. pachypus* featured an average divergence of 1.8% at 16S, 2.5% at *cox1*, and 4.6% at *cyt b* from its most closely related lineages (BW and R). The two lineages affiliated to *B. v. scabra* (BW-S and R) featured divergences of 1.3% at 16S, 1.6% at *cox1*, and 2.4% at *cyt b*.

Gene-based nuclear phylogeny

The nuclear species tree obtained with *BEAST based on the four gene fragments (3715 bp in total) recovered *B. variegata* as monophyletic and as the sister species of *B. bombina* (Fig. 3). Within *B. variegata*, this phylogeny differs from the mitochondrial phylogeny in two aspects. The first discordance is the position of Balkano-Western *B. v. variegata*, which branches with Carpathian *B. v. variegata* in the nuclear tree (vs. with Balkano-Western *B. v. scabra* as in the mitochondrial tree). This renders both *B. v. variegata* and *B. v. scabra* monophyletic: *B. v. variegata* populations carrying BW-N, C-W and C-E mtDNA form one nuclear clade, while *B. v. scabra* populations carrying BW-S and R mtDNA form a distinct nuclear clade. The second discordance is the position of *B. v. pachypus*, which is placed as the sister clade of *B. v. variegata* in the nuclear tree (vs. as the sister clade of *B. v. scabra*/Balkano-Western *B. v. variegata* as in the mitochondrial tree). Moreover, while the *B. v. variegata* samples feature shallow branches, the nuclear divergence within *B. v. scabra* rather compares to the divergence of *B. v. pachypus*.

Support of the gene fragment tree is low to moderate, with posterior probabilities of nodes ranging from 0.62 to 0.92 within the *B. variegata* clade, a typical issue given the much lower level of polymorphism at nuclear than mitochondrial sequences.

The IQ-TREE analysis confirms the homogeneity among the *B. v. variegata* sequences, as well as the distinctiveness between the two lineages of *B. v. scabra* and compared to other taxa (Fig. S3). The topology should however be interpreted with caution, both due to the low polymorphism of the dataset and because such analysis inappropriately assumes a single genealogy shared among the nuclear genes. Accordingly, the IQ-TREE topology appears uninformative, as seen from the nested placement of *B. bombina* within *B. variegata* (Fig. S3).

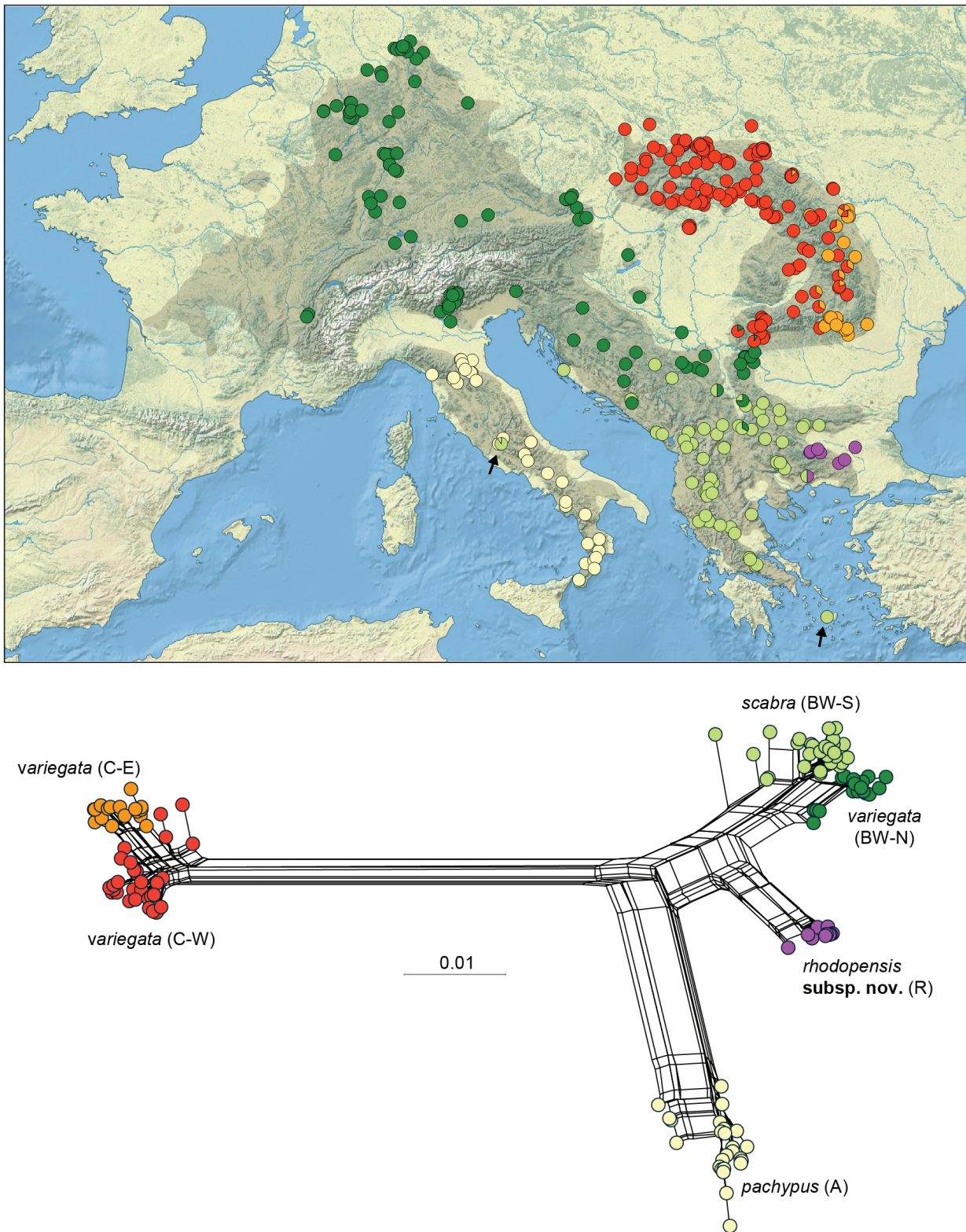


Figure 1. Relative divergence (network) and geographic distributions (map) of the mitochondrial lineages of *B. variegata* identified among 1096 bp sequences of the *cyt b* gene ($n = 1238$ individuals from 355 localities), defined as in Fijarczyk et al. (2011). Arrows indicate introduced populations.

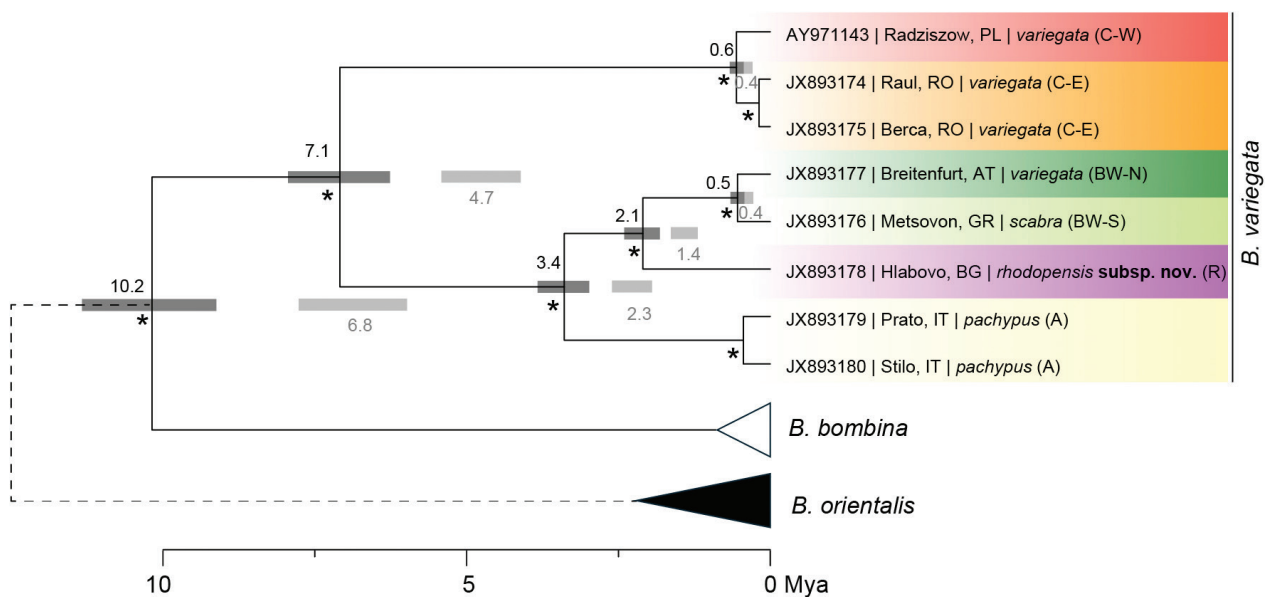
Phylogenomics

The ddRAD-seq data (4759 loci totaling 553,608 bp) provided a robust phylogenomic tree that distinguished the different phylogeographic lineages of *B.*

variegata (Fig. 4). In accordance with the mitochondrial phylogeny (Fig. 2), *B. v. pachypus* is more closely related to *B. v. scabra* than *B. v. variegata* in the phylogenomic tree, and *B. v. scabra* forms two distinct lineages (Fig. 4). As in the gene-based nuclear tree,

Table 1. Pairwise distances between mitochondrial lineages at the DNA barcoding genes 16S, *cox1* and *cyt b*. See Fig. 1 for the distribution of the *B. variegata* lineages.

		<i>B. variegata</i>							
16S		BW-N	BW-S	R	C-W	C-E	A	<i>B. bombina</i>	<i>B. orientalis</i>
<i>B. variegata</i>	BW-N (<i>variegata</i>)	—							
	BW-S (<i>scabra</i>)	0.2%	—						
	R (<i>rhodopensis</i> subsp. nov.)	1.2%	1.3%	—					
	C-W (<i>variegata</i>)	2.5%	2.6%	3.3%	—				
	C-E (<i>variegata</i>)	2.6%	2.6%	3.3%	0.3%	—			
	A (<i>pachypus</i>)	1.6%	1.7%	2.0%	2.7%	2.8%	—		
<i>B. bombina</i>	3.6%	3.5%	3.9%	3.5%	3.5%	3.5%	—		
<i>B. orientalis</i>	6.4%	6.4%	6.6%	6.6%	6.7%	6.2%	6.7%	—	
		<i>B. variegata</i>							
cox1		BW-N	BW-S	R	C-W	C-E	A	<i>B. bombina</i>	<i>B. orientalis</i>
<i>B. variegata</i>	BW-N (<i>variegata</i>)	—							
	BW-S (<i>scabra</i>)	0.3%	—						
	R (<i>rhodopensis</i> subsp. nov.)	1.6%	1.6%	—					
	C-W (<i>variegata</i>)	5.1%	4.8%	4.9%	—				
	C-E (<i>variegata</i>)	5.1%	4.8%	5.0%	0.3%	—			
	A (<i>pachypus</i>)	2.7%	2.5%	2.4%	5.1%	5.0%	—		
<i>B. bombina</i>	6.8%	6.6%	7.0%	6.3%	6.3%	7.2%	—		
<i>B. orientalis</i>	9.1%	9.1%	9.7%	9.3%	9.3%	10.3%	9.7%	—	
		<i>B. variegata</i>							
cyt b		BW-N	BW-S	R	C-W	C-E	A	<i>B. bombina</i>	<i>B. orientalis</i>
<i>B. variegata</i>	BW-N (<i>variegata</i>)	—							
	BW-S (<i>scabra</i>)	0.5%	—						
	R (<i>rhodopensis</i> subsp. nov.)	2.4%	2.4%	—					
	C-W (<i>variegata</i>)	8.3%	8.1%	8.2%	—				
	C-E (<i>variegata</i>)	8.6%	8.4%	8.5%	0.7%	—			
	A (<i>pachypus</i>)	4.7%	4.4%	4.8%	9.6%	9.7%	—		
<i>B. bombina</i>	11.3%	11.1%	10.2%	9.4%	9.4%	11.1%	—		
<i>B. orientalis</i>	17.7%	17.5%	17.1%	16.2%	16.0%	18.1%	15.7%	—	

**Figure 2.** Bayesian phylogeny obtained with BEAST based on full mitogenomes (17,240 kb). Nodes are annotated with dating estimates and their 95% HPD (node bars) obtained from two calibrations (dark grey: calibration I; light grey: calibration II). Sequence labels indicate accession numbers, geographic origins, taxa and mitochondrial lineages. Asterisks denote full branch support (posterior probabilities of 1.0).

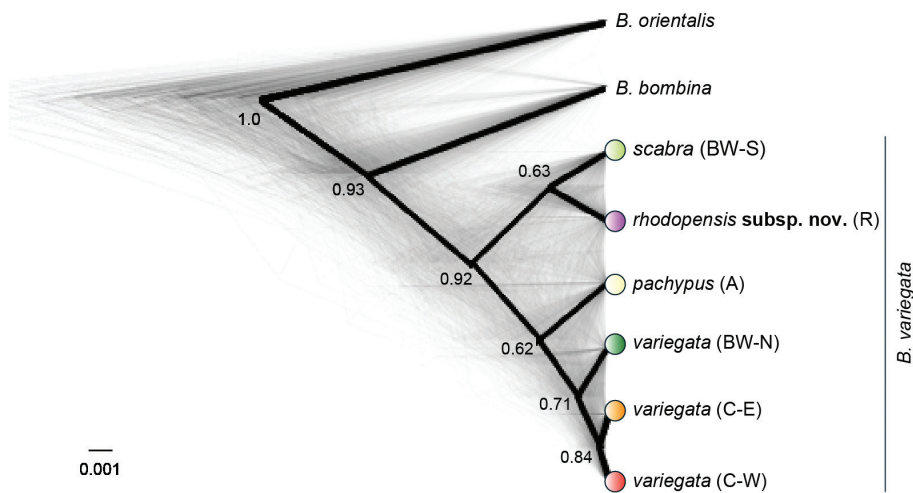


Figure 3. Cloudogram of the nuclear species trees obtained with *BEAST based on four gene fragments (3715 bp) and distinguishing samples representative of the different *B. variegata* mitochondrial lineages. Node labels indicate Bayesian posterior probabilities; sequence labels indicate taxa and mitochondrial lineages; the thick and thin lines show the average tree root and all the sampled trees, respectively.

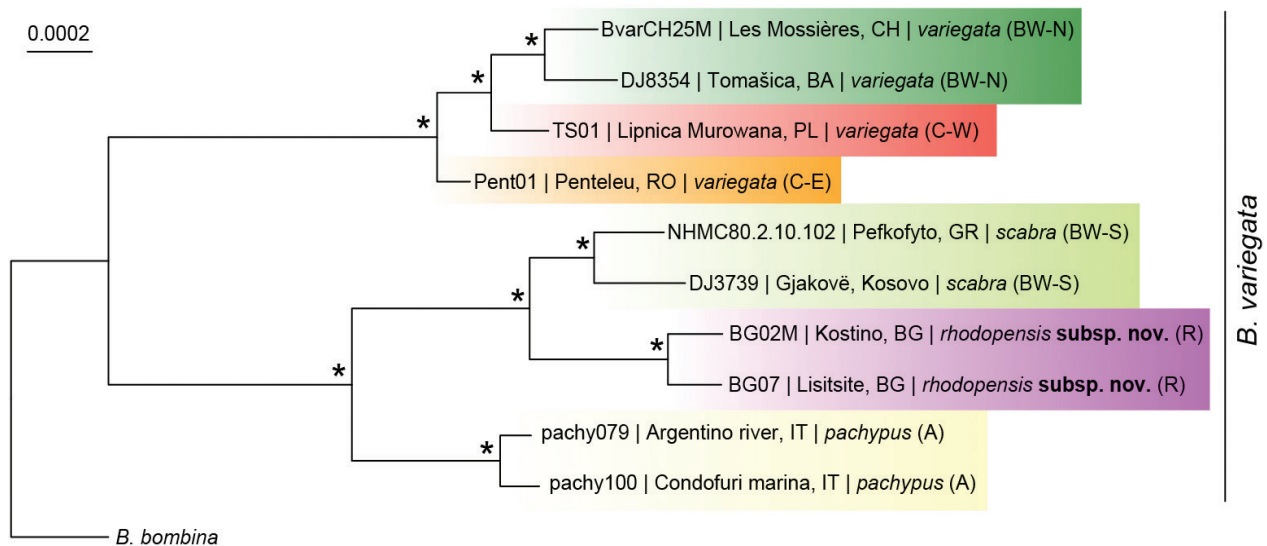


Figure 4. Maximum-likelihood phylogeny obtained with IQ-TREE of 4759 concatenated RAD loci (553,608 bp). Colors distinguish the different *B. variegata* mitochondrial lineages. Sequence labels indicate sample names, geographic origins, taxa and mitochondrial lineages. Asterisks denote robust branch support (bootstrap >95). The tree is rooted by *B. bombina*.

the analysis also retrieves *B. v. variegata* as monophyletic i.e., samples from the Carpathian ranges, the northern Balkans and Western Europe are grouped together despite their distinctive mitochondrial ancestry (BW-N and C; Fig. 4).

Morphological analyses in *Bombina variegata scabra*

Based on 11 morphological characters corrected by body length ($L.$), the two lineages of *B. v. scabra* differ in body shape (Fig. 5; variable loadings in Table S6). In the PCA, specimens attributed to the Balkano-Western vs. the Rhodope lineage show almost no overlap, which was particularly marked for females. Accordingly, lineage ($F = 25.9$, $P < 0.001$), sex ($F = 2.2$, $P = 0.03$) and their interaction ($F = 3.1$, $P = 0.004$) were all significant in the MANOVA.

Males and females from the Rhodope lineage were larger and smaller than their Balkano-Western counterparts, respectively, which resulted in a significant interaction between sex and lineage ($F = 7.2$, $P = 0.007$), but not for sex ($F = 1.0$, $P = 0.29$) and lineage ($F = 0.15$, $P = 0.691$) in the two-way ANOVA (significance tested by a permutation approach). Average raw measurements and their standard deviation are detailed per sex and lineage in Table 2.

Comparisons of body ratios (character/ $L.$) between the two *B. v. scabra* lineages suggest overlapping distributions for all characters (males and females combined), but four of them show significant differences (Kruskal-Wallis tests, $P < 0.05$) (Fig. 6). Specifically, the femur ($F.$), tibia ($T.$) and first toe ($D.p.$) are proportionally shorter, and the eyelid is proportionally longer ($L.o.$) in the Rhodope lineage. The eyelid also tends to be larger in the latter ($Lt.p.$), but this was marginally not significant ($P = 0.06$). After Bonferroni correction, only the difference for the femur ($F.$) remains significant ($P < 0.0045$).

Table 2. Average and standard deviation of the morphological characters measured in 58 specimens representing the Rhodope lineage and the south–northwestern Balkan populations of *B. v. scabra*, distinguishing females and males (in mm). Values for the extended body length dataset (178 specimens) are given on the last line. For character abbreviations, see Methods (section Morphometric analyses in *Bombina variegata scabra*).

	<i>rhodopensis</i> subsp. nov.		<i>scabra</i>	
	♀	♂	♀	♂
Morphological measurements				
n	4	6	26	22
L.	32.1 ± 2.3	36.6 ± 5.0	41.3 ± 4.5	40.5 ± 4.8
F.	10.1 ± 1.2	12.3 ± 2.6	15.9 ± 1.7	15.8 ± 2.0
T.	11.4 ± 1.0	13.4 ± 2.7	15.6 ± 1.4	16.0 ± 1.7
D.p.	2.7 ± 0.2	3.2 ± 0.7	3.9 ± 0.6	3.9 ± 0.7
L.c.	8.0 ± 0.3	9.2 ± 1.2	10.0 ± 1.1	10.5 ± 1.1
Lt.c.	10.2 ± 1.0	11.6 ± 1.6	12.8 ± 1.3	13.2 ± 1.4
Sp.oc.	4.4 ± 0.4	5.2 ± 0.6	5.7 ± 0.7	5.6 ± 0.6
D.ro.	6.5 ± 0.1	7.5 ± 1.3	8.6 ± 0.9	8.7 ± 0.7
Lt.p.	2.2 ± 0.1	2.2 ± 0.2	2.4 ± 0.3	2.5 ± 0.3
Sp.p.	2.8 ± 0.3	2.9 ± 0.2	3.1 ± 0.4	3.2 ± 0.6
L.o.	3.6 ± 0.4	4.1 ± 0.6	4.3 ± 0.4	4.3 ± 0.4
Sp.n.	2.0 ± 0.1	2.2 ± 0.3	2.4 ± 0.4	2.4 ± 0.3
Extended body length measurements				
n	12	36	72	58
L.	39.5 ± 7.5	45.1 ± 5.0	43.4 ± 6.1	43.01 ± 5.8

Ventral coloration pattern analysis in *Bombina variegata scabra*

Color/dark ratios range from 0.26 (almost entirely dark) to 10.3 (almost entirely colored) (Fig. 7). The Rhodope lineage is overall more colored (2.9 ± 2.0) than the Balkano-Western lineage of *B. v. scabra* (2.1 ± 1.4) and the difference is significant (Kruskal-Wallis test, $\chi^2 = 7.3$, $P = 0.007$). Whether specimens were field-caught or museum-preserved does not affect this pattern; in the two-way ANOVA, the lineage difference is significant ($F = 8.6$, $P = 0.003$) but the specimen type is not ($F = 0.20$, $P = 0.67$).

Nomenclature overview

Our analyses suggest that the species *B. variegata* features four genuine evolutionary lineages eligible for a taxonomic classification. First, the nuclear similarity among the two divergent mitochondrial lineages attributed to nominal subspecies *B. v. variegata* (mtDNA C and BW-N) implies the existence of a single taxon among these populations, which extends from the Carpathians to Western Europe; the mitochondrial discordance is discussed below (see Discussion). Second, we confirm the independent evolution of the Apennine populations known as *B. v. pachypus* (mtDNA A). Third and fourth, we documented the mitochondrial, nuclear and morphological differentiation of two lineages among the populations attributed to *B. v. scabra*, one in the Western

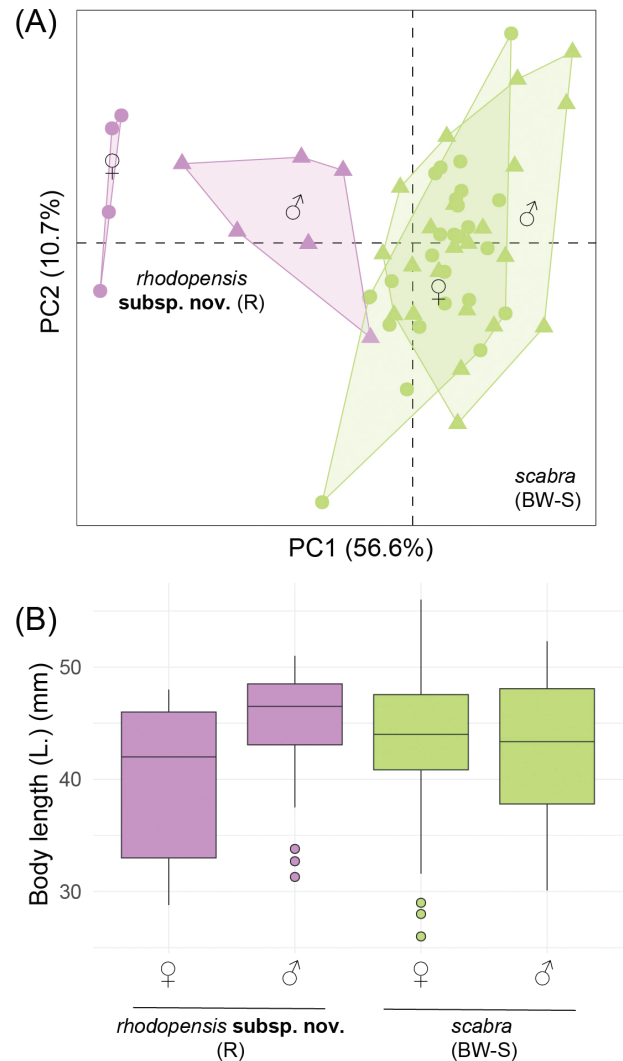


Figure 5. Morphological comparison of specimens attributed to the two lineages of *B. v. scabra*. **A** PCA on 11 characters corrected by body length distinguishing the males (triangles) and females (circles) of each lineage; **B** boxplots showing variation in body length (L.) among sexes and lineages. For the list of characters, see Methods (section Morphometric analyses in *Bombina variegata scabra*).

and Southern Balkans (mtDNA BW-S) and one in the Rhodope (mtDNA R). As argued below (see Discussion), we preliminarily consider these Northern, Apennine, Balkan and Rhodope lineages as four distinct subspecies of the yellow-bellied toad *B. variegata*. Their respective distributions are provided in Fig. 8, with the list of available names that apply to each of them according to the following nomenclature overview. Important illustrations are depicted in Fig. 9, along with a timeline summarizing the nomenclatural history of European *Bombina*.

***Rana variegata* Linnaeus, 1758** – First published in *Systema Naturae* 10th edition without mention of a specimen and a locality other than “Exteris regionibus [foreign countries]” (Linnaeus 1758: 211). There is no trace of specimens labelled *Rana variegata* in Linnaeus’s inventoried collections (Holm 1957). In his 12th edition of *Sys-*

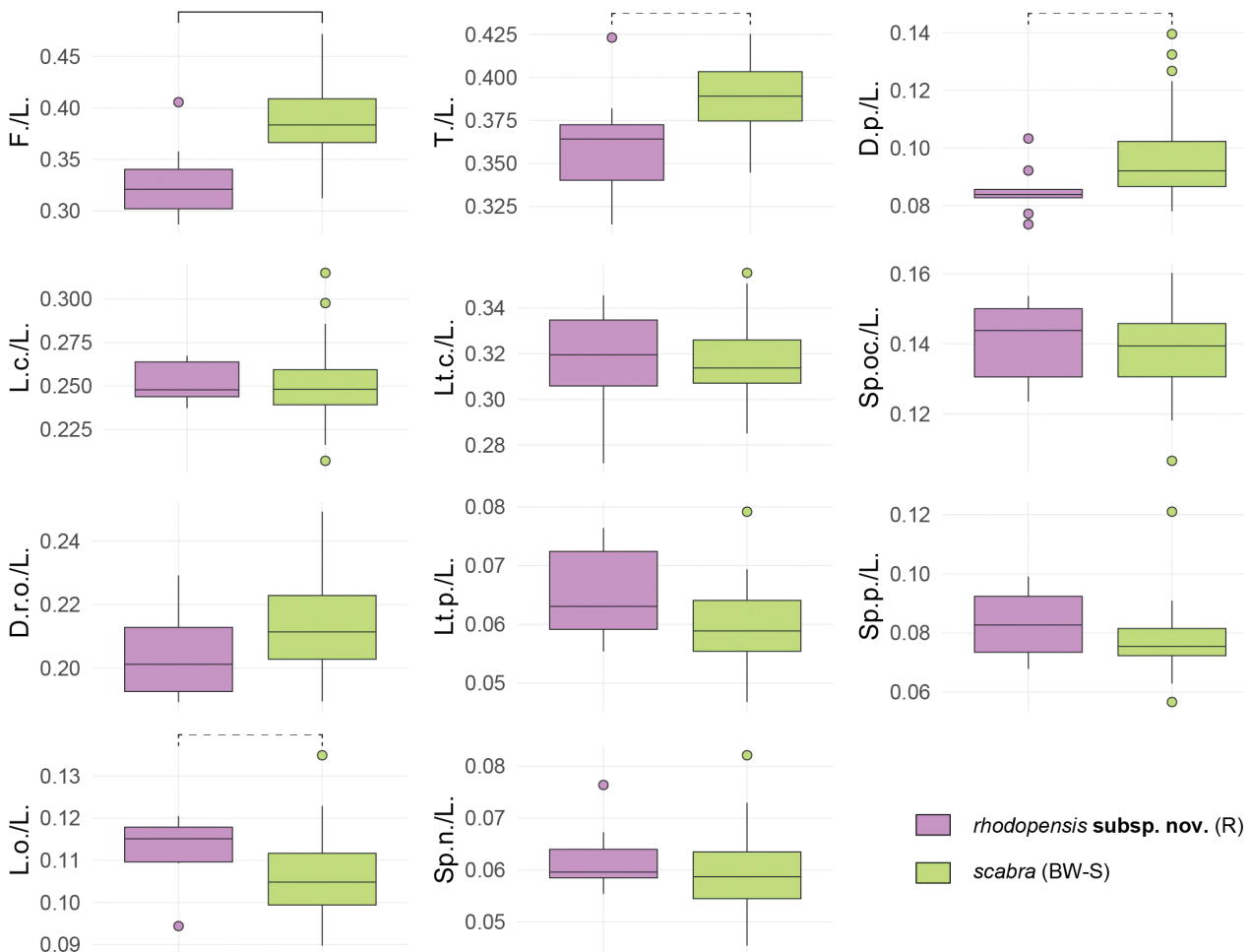


Figure 6. Body ratios (character/body length L.) between the Rhodope (purple) and Balkano-Western (light green) lineages of *B. v. scabra* (males and females combined). Brackets indicate significant differences (Kruskal-Wallis test) at $P < 0.05$ (dash) and at the adjusted $P < 0.0045$ for multiple testing (plain). For character abbreviations, see Methods (section Morphometric analyses in *Bombina variegata scabra*).

tema Naturae, Linnaeus (1766) had already replaced this name by *Rana bombina* Linnaeus, 1761 (now *Bombina bombina*), assuming that both represent the same species: the one depicted by Rösel von Rosenhof (1758: pl. XXII, XXIII), namely a yellow-bellied toad from Nuremberg, Germany (Fig. 9A). The nomen *variegata* remained in synonymy for more than a century and a half. In the late 18th and early 19th centuries, most naturalists continued to assume that there was only one species of yellow/fire-bellied toad in Europe, and favored other names for it, notably *Rana bombina* and *Bufo igneus* Laurenti, 1768 (e.g., La Cépède 1788; Latreille 1800; Daudin 1803; Fitzinger 1843; Duméril and Bibron 1841). Due to its unclear origin, *variegata* was unused or overlooked even after the yellow- and fire-bellied toads were distinguished (Boulenger 1886). Accordingly, its synonymy was frequently accompanied by question marks (e.g., Bedriaga 1890: 581; Boulenger 1897: 142), but it does appear in priority in a few works (e.g., *Bombinator variegatus* in Bedriaga 1881: 291). It was eventually resurrected by Mertens and Müller (1928), who affiliated it to the yellow-bellied toad by restricting its type locality to “Schweiz [= Switzerland]”, yet without any justification or taxonomic act. According to Gollmann et al. (2012), this association may be

linked to the account of the “Wasser Krott [water toad]” by the Swiss naturalist Gessner in his Thierbuch, which allegedly corresponds to the yellow-bellied toad based on the provided drawing (Gessner 1563: 170; perhaps the earliest naturalist illustration of the species, Fig. 9) and mentions “Demnach so ist auch ein geschlecht so in den wassere wohnt klein/mögen Wasserkrötlein genennet werden/in unseren landen Züger von ihrer stimm her/so ihnen angeboren ist [Accordingly, there is also a species that lives in the water, small, and could be called water toads, in our Zürich lands their sound is specific to them]” (Gessner 1563: 169). Gessner’s Thierbuch is an abridgement of his Historia animalium, the first and one of the most exhaustive pre-Linnean natural history work. However, unlike for some of his other taxa, Linnaeus did not refer to Gessner’s opus in his description of *Rana variegata*, hence any connection of this taxon with Switzerland, and by extension, with the yellow-bellied toad, is baseless. In addition, according to the ICZN, so-called restrictions of type locality (e.g., “terra typica restricta”) without providing any evidence to support this claim are nomenclaturally invalid for not being associated with a lectotype or neotype designation (Dubois 2011). There is no potential lectotype of *Rana variegata* from

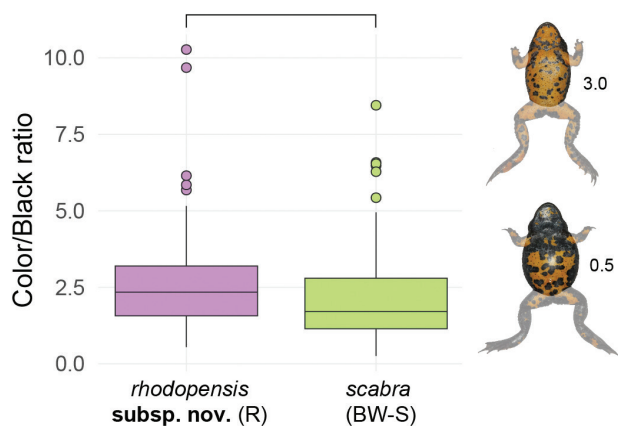


Figure 7. Proportion of color/dark coloration on the ventral side of specimens previously attributed to *B. v. scabra*. Bracket indicates significant difference (Kruskal-Wallis test) at $P < 0.05$. The body area analyzed is emphasized in the two colored (top) and dark (bottom) examples.

the information provided by Linnaeus (1758). A neotype is therefore necessary to unambiguously affiliate *Rana variegata* to the yellow-bellied toad and restrict its type locality to Switzerland, as it has been assumed for nearly a century. We here designate MZL 11988 as the neotype of *Rana variegata* Linnaeus 1768 (Fig. 9K). MZL 11988 is a male yellow-bellied toad curated at the Muséum Cantonal de Zoology of Lausanne (MZL); it was collected in 1966 in Promenthoux, Prangins, Vaud, Switzerland, which becomes the type locality of *Rana variegata*. Switzerland is only inhabited by the yellow-bellied toad, namely the Northern subspecies (mtDNA BW-N) in our figures. *Rana variegata* Linnaeus, 1758 thus applies to this lineage and is the oldest available name for it, *Bombina variegata variegata* (N^oA in Fig. 8).

***Rana bombina* Linnaeus, 1761** – Described from “Scaniae compestris fossis australibus [= southern Scania/Skåne]” in Sweden (Linnaeus 1761: 101), based on specimens curated in Uppsala (Boulenger 1888), at least two of which were featured in the Alströmer-Linné collection inventoried by Thunberg (Holm 1957: 43). For a century, it was widely used to designate all yellow-/fire-bellied toads (believed to be a single species), initially as *Rana bombina* (Linnaeus 1761) and *Bufo bombinus* (Latreille 1800). It was subsequently classified in a separate genus, as *Bombina bombina* (Oken 1816), *Bombinator bombinus* (Merrem 1820), or *Bombinator bombinus* (Wagler 1830; an emendation of Merrem’s *Bombinator*). The genus *Bombinator* was adopted for the rest of the 19th century. When distinguishing the yellow and fire-bellied toads, Boulenger briefly considered *Bombinator bombinus* as the yellow-bellied toad (Boulenger 1886), then as the fire-bellied toad under the synonymy of *Bufo igneus* Laurenti, 1768 (Boulenger 1888). Applying the law of priority, Stejneger (1905, 1907) affirmed both the genus *Bombina* and the name *Bombina bombina* to the fire-bellied toad, an arrangement that persists until today. Given that only the fire-bellied toad inhabits Sweden, *Rana bombina*

Linnaeus, 1761 accordingly applies to this species and is the oldest available name for it, as *Bombina bombina*.

***Bufo igneus* Laurenti, 1768** – Given as “Rösel. Tab XXIII. XXII.” and “Habitat in paludibus nostris Danubialibus ingenti in copia; autumnus omnis via, qua itur in Nussdorff, iisdem scaturit” [= lives in great abundance in our Danubian marshes; in the autumn every road that leads to Nussdorff is covered with it]” (Laurenti 1768: 29). Laurenti’s detailed description (Laurenti 1768: 129–133) corresponds to the fire-bellied toad and accordingly, this species used to occur along the Danube from Vienna to Nussdorff, now a suburb of Vienna (Schweiger et al. 2021). He further documented four experiments aimed at evaluating the species’ toxicity, implying that he had specimens at his disposal (Laurenti 1768: 131–133). At the same time, he specifically referred to Rösel’s plates and gave his species Rösel’s adjective *igneus* [fiery], in reference to the belly coloration; but Rösel’s drawings unambiguously represent the yellow-bellied toad (Fig. 9A). This confusion is understandable, as again, yellow- and fire-bellied toads were not distinguished in Laurenti’s time. In the 19th century, *igneus* was increasingly preferred to Linnaeus’s *bombina* (e.g., Wagler 1830; Fitzinger 1843), notably as *Bombinator igneus*, the name kept by Boulenger (1886, 1888) to differentiate the fire-bellied toad from the yellow-bellied toad. It was eventually replaced by *Rana bombina* Linnaeus, 1761 by application of the priority rule (Stejneger 1907). While *Bufo igneus* was unambiguously attributed to the fire-bellied toad, its type series is heterogeneous: it includes both yellow-bellied toads *B. variegata* (the Nuremberg specimens depicted by Rösel) and fire-bellied toads *B. bombina* (the Danubian specimens examined by Laurenti). To affirm this taxon solely to the fire-bellied toad, we here designate as the lectotype of *Bufo igneus* Laurenti, 1768 the specimen Laurenti kept in captivity after his experiment XII, narrated as “unamque ex iis fanam nuncin longam hyemem adhuc confervo [= one of them, in fact, I still keep healthy even now through the long winter]” (Laurenti 1768: 312). If they were ever curated, Laurenti’s specimens are not present at the Naturhistorisches Museum Wien (NHMW; G. Gassner pers. comm.). The lectotype is probably lost but its origin (the Danubian marches from Vienna to Nussdorff) is unambiguous. *Bufo igneus* Laurenti, 1768 thus belongs to *Bombina bombina*.

***Bufo salsus* Schrank in Schrank & Moll, 1785** – Documented from “Berchtesgaden” in the Bavarian Alps, with no mention of specimens (Schrank and Moll 1785: 308–309). The synonymy with *Rana bombina* Linnaeus, 1761 is questioned in the description, and was enforced by Latreille (1800). After the distinction of yellow and fire-bellied toads, early 20th century authors such as Stejneger (1905, 1907) and Nikolsky (1918) used *salsus* to designate the yellow-bellied toad, assuming that it was the oldest available name for this species. Only the yellow-bellied toad is present in Bavaria, namely the Northern lineage (carrying mtDNA BW-N). *Bufo salsus* Schrank in Schrank & Moll, 1785 thus applies to this lineage, and is

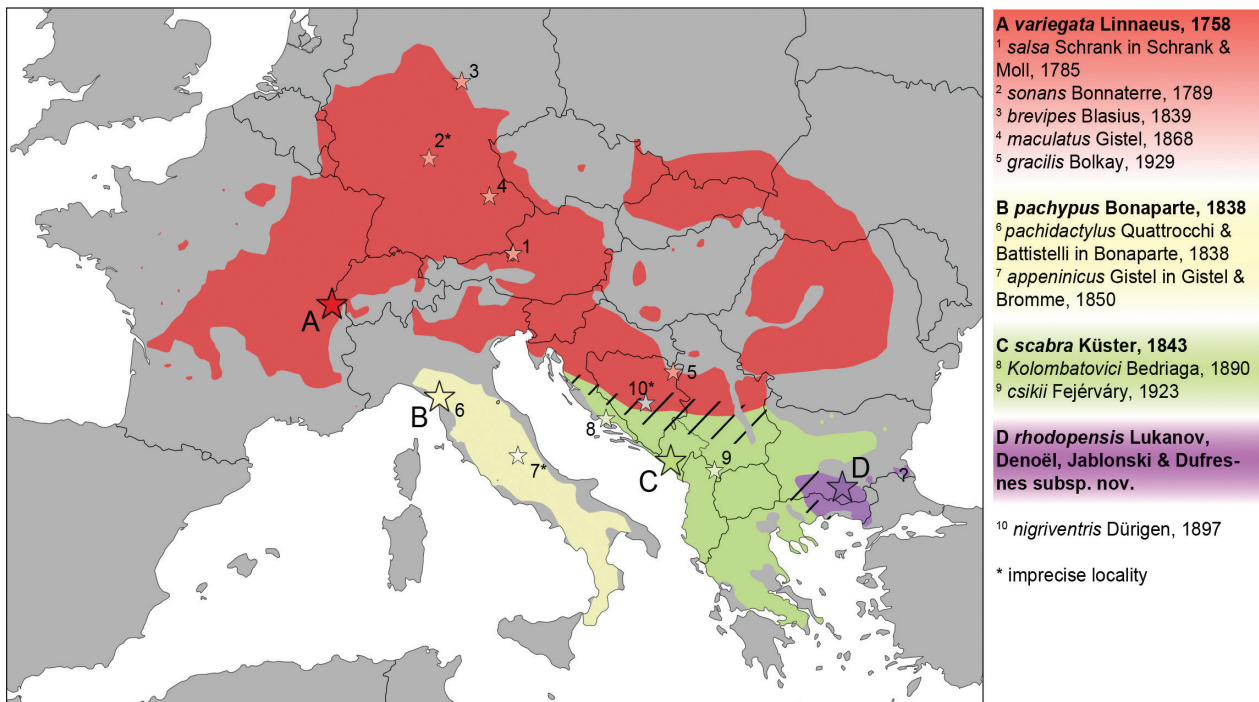


Figure 8. Geographic distributions of the four subspecies of *B. variegata* and location of the type localities of available names for each (stars). Red: Northern subspecies; yellow: Apennine subspecies; light green: Balkan subspecies; purple: Rhodope subspecies. The dashed and question-marked areas correspond to potential contact zones/introgression and regions of unclear subspecies assignment, respectively. Senior synonyms are shown by big stars and are labelled with capital letters; junior synonyms are shown by small stars and are labelled with numbers.

a junior subjective synonym of *Bombina variegata variegata* (N°1 in Fig. 8).

***Rana sonans* Bonnaterre, 1789** – Originally mentioned in La Cépède (1788) as a substitute name for *Rana campanisona* Laurenti, 1768, *Rana bombina* Linnaeus, 1761 and *Rana variegata* Linnaeus, 1758. The species was reported from Germany (“Allemagne”), with an illustration and a French name, “la grenouille sonnante” (La Cépède 1788: pl. XIII, 293–294; Fig. 9C). Although La Cépède lists the Latin name “*Rana sonans*” in his Synopsis Methodica (La Cépède 1788: 457), the name is not made available by his work, which has been considered invalid as non-binomial by the International Commission of Zoological Nomenclature (2005). Like many of La Cépède’s names, *Rana sonans* was made available by Bonnaterre (1789; see Dubois et al. 2021), who associated it to a reproduction of La Cépède’s illustration (Bonnaterre 1789: 7, pl. 2–fig. 3; Fig. 9D). However, Bonnaterre’s diagnosis of *Rana sonans* corresponds to the bull frog *Lithobates catesbeianus* (Shaw, 1802); this appears to be an unintentional mix-up, perhaps due to the presence of both species next to each other’s on the original plate of La Cépède (1788: pl. XIII). Instead, Bonnaterre referred to “la grenouille sonante” for *Rana bombina* and mentions the same illustration (Bonnaterre 1789: 4, pl. 2–fig. 3). To clarify this situation, we designate the specimen depicted in La Cépède (1788: pl. XIII) and Bonnaterre (1789: pl. 2–fig. 3) as the lectotype of *Rana sonans* (Fig. 9C–D). La Cépède (1788: 294) indi-

cated that several specimens of this species are kept at the “Cabinet du Roi [= now Muséum National d’Histoire Naturelle Paris, MNHN]”, thus potentially including the lectotype, but his collections are not present at MNHN (A. Ohler pers. comm.). The issue remains whether this taxon belongs to the yellow or the fire-bellied toad, since both species occur in Germany. La Cépède (1788) and Bonnaterre (1789) clearly distinguished *Rana sonans* (and its intended synonyms) from *Bufo igneus* Laurenti, 1768 (= the fire-bellied toad), referring to the latter with Laurenti’s description and including a different drawing – a partial reproduction of Rösel’s plate (Bonnaterre 1789: pl. 6–fig. 6; Fig. 9B). This distinction and classification (in different genera) tend to suggest that their *Rana sonans* was meant for a distinct species. Accordingly, La Cépède’s original diagnosis “ventre marbré de noir & de blanc [belly marbled with black and white coloration]” is consistent with the yellow-bellied toad, and the lectotype features a ventral coloration pattern also more typical of that species (Fig. 9C–D). Therefore, we conclude that the lectotype of *Rana sonans* is a yellow-bellied toad from Germany. Only the Northern lineage (mtDNA BW-N) inhabits this region, hence *Rana sonans* Bonnaterre, 1789 applies to this lineage and is a junior subjective synonym of *Bombina variegata variegata* (N°2 in Fig. 8).

***Bufo ignicolor* Fibig & Nau, 1789** – Originally mentioned in the Synopsis Methodica of La Cépède (1788: 460) as *Buffo ignicolor*, with the genus name “*Buffo*” intentionally misspelled to honor Buffon, La Cépède’s

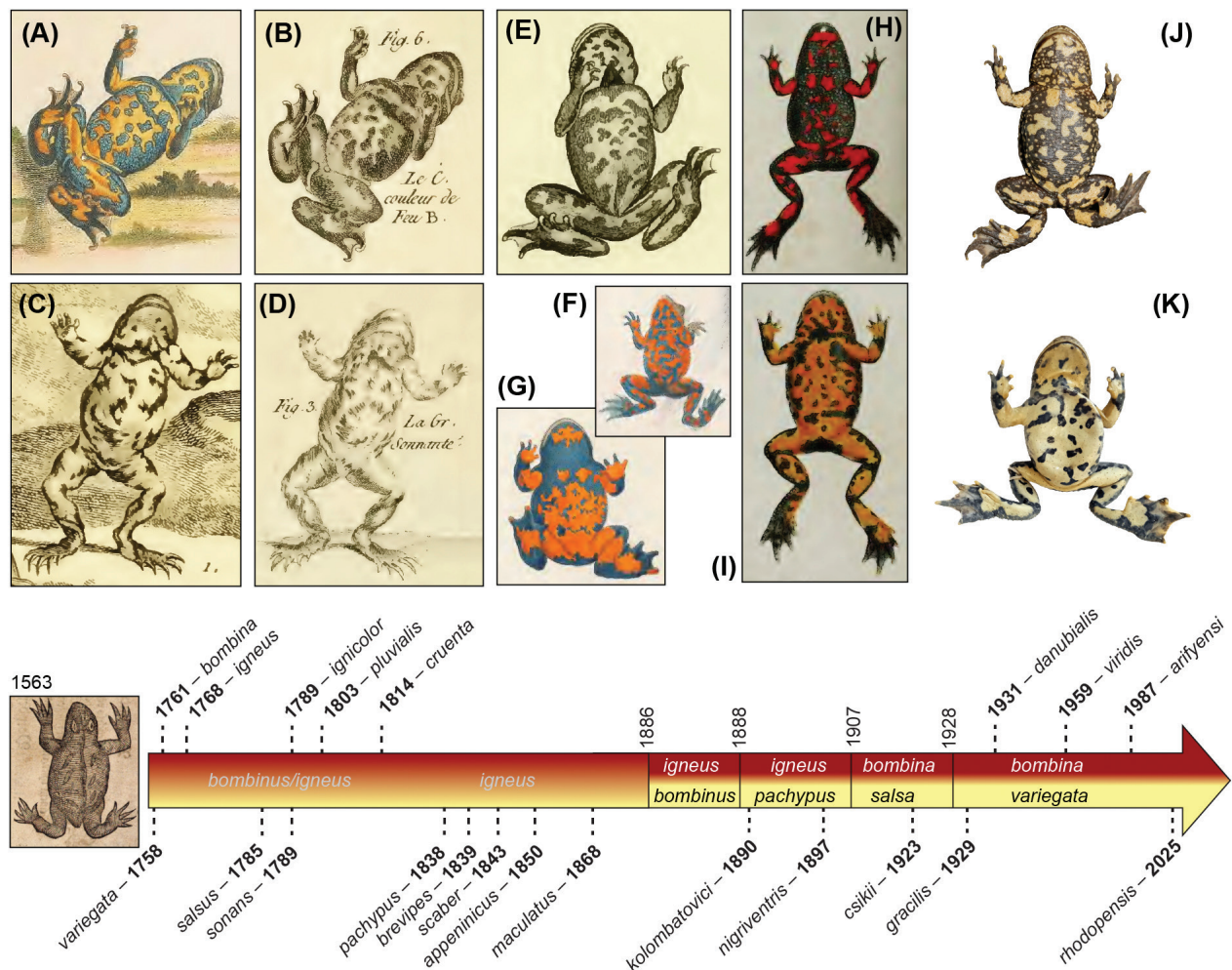


Figure 9. Illustrations of the ventral side of European *Bombina* toads in nomenclaturally important specimens (top) and summary of the nomenclatural history (bottom). **A** Bottom right specimen in plate XXVII of Rösel (1758, pl. XXII) considered by Linnaeus (1766) to represent both his *Rana variegata* and *Rana bombina*, and (partly) used by Laurenti (1768) to describe his *Bufo igneus*. The color pattern unambiguously corresponds to the yellow-bellied toad (now *B. variegata*), further noting that Rösel depicted the fauna of Nuremberg (Germany) where only this species is present. **B** Illustration of *Bufo igneus* in Bonnaterre (1789, pl. 6–fig. 6), which is clearly a reproduction of Rösel’s plate. **C** Illustration of La Cepède’s invalid *Rana sonans* (La Cepède 1788, pl. XIII) and **(D)** reproduction by Bonnaterre (1789, pl. 2–fig. 3) for *Rana sonans* Bonnaterre, 1789; the depicted specimen is designated as the lectotype of this taxon; **E** Illustration of *Bufo pluvialis* Daudin, 1803 (pl. XXVI); the depicted specimen is designated as the lectotype of this taxon; **F** *Bombinator igneus* (Laurenti, 1768) and **(G)** *Bombinator pachypus* Bonaparte, 1838 (labeled “*Bombinator pachidactylus*” on the plate), as distinguished by Bonaparte (1838). **H** The fire-bellied toad and **(I)** the yellow-bellied toad, as distinguished by Boulenger (1886, pl. L). **J** NHMW 6698/1, the neotype of *Bufo pluvialis* Daudin, 1803 (credit: DJ). **K** MZL 11988, the neotype of *Rana variegata* Linnaeus, 1758 (credit: CD). The timeline is annotated with years of descriptions (diagonal labels), years of important revisions (vertical labels), and commonly used names (inside labels) for *B. bombina* (top) or *B. variegata* (bottom). The left illustration is taken from what appears to be the earliest natural history account for a fire/yellow-bellied toad (Gessner 1563).

mentor, according to Dubois and Ohler (2009). In the account, La Cepède (1788: 362–364) used the French name “le couleur de feu” and explicitly referred to *Bufo igneus* Laurenti, 1768, citing the observations of Laurenti from the Danube. As mentioned above, La Cepède (1788) is considered an invalid work, and the name next appears in Fibig and Nau (1789: 297), where it is listed as *Bufo ignicolor*, with reference to “le couleur de feu” and to “Laur. n. 13” (= *Bufo igneus* Laurenti, 1768). *Bufo ignicolor* Fibig & Nau, 1789 thus appears as a junior objective synonym of *Bufo igneus* Laurenti, 1768, and by implication, it refers to *Bombina bombina*.

***Bufo pluvialis* Daudin, 1803** – Mentioned on a plate (Daudin 1803: pl. XXVI) and intended as a substitute name for *Rana bombina* Linnaeus, 1761 (and *Bufo bombinus*). The description does not state specific localities but mentions “assez frequent dans les eaux stagnantes et croupies des contrées méridionales de l’Europe [relatively frequent in the stagnant freshwaters of southern European countries]” (Daudin 1803: 75–76). It ends by referring to *Bufo igneus* and Rösel’s plates 22 and 23. The given geographic range could apply to either the yellow- or fire-bellied toad, and it is unclear which species is represented in Daudin’s drawing (Daudin 1803: Pl. XXVI;

Fig. 9E). The diagnosis tends to suggest the fire-bellied toad, according to details on the coloration: “dessous du corps presque lisse et d’un jaune-orangé, avec des taches bleuâtres [nearly smooth ventral side with yellow-orange coloration and blueish blotches]”; and the webbing extent: “cinq doigts presque entièrement palmés aux postérieurs [five toes almost entirely webbed]”. In addition, Daudin specifically distinguished a separate variety within his species, which features characteristics closer to the yellow-bellied toad: “blanchâtre à taches noires en dessous; pieds à bandes brunes, jaunes en dessous, ayant leurs doigts séparés [whitish underbelly with black blotches; feet with dark bands, yellow underneath, and with separated toes]”; he accordingly linked this variety to Schrank’s *Bufo salsus* (= *B. variegata variegata*, see above). To fix the name, we first designate the specimen illustrated by Daudin (1803: pl. XXVI) as the lectotype of *Bufo pluvialis* (Fig. 9E). The fate and exact nature of this specimen being unknown, we then restrict its application to the fire-bellied toad by designating NHMW 6698/1 as the neotype of *Bufo pluvialis* Daudin, 1803 (Fig. 9J). NHMW 6698/1 is a fire-bellied toad specimen curated at NHMW; it was collected in 1889 by Steindachner in “Jedlesees b. Wien [= Jedlesees, Vienna, Austria]”, which becomes the new type locality of *Bufo pluvialis*. Specifically, Jedlesees is located on the left bank of the Danube just a few hundred meters of Nussdorf (the village mentioned by Laurenti in its description of *Bufo igneus*), so this act stabilizes *Bufo pluvialis* as a synonym of *Bombina bombina* but also of *Bufo igneus*, as Daudin seems to have intended. This dual restriction is relevant for future revisions, given that *B. bombina* diversified into several lineages in southeastern Europe (Fijarczyk et al. 2011). *Bufo pluvialis* Daudin, 1803 now unambiguously refers to *Bombina bombina*.

***Rana cruenta* Pallas, 1814** – Mentioned from “Rossia media [= central Russia]” with no reference to any specimen (Pallas’s collections are not known to exist, Kuzmin 1996). Some elements of diagnosis and the mention “Forma *R. temporariae*, cujus et magnitudo [= shape and size of *Rana temporaria*]” (Pallas 1814: 12) led many authors to interpret this taxon as a member of the true frogs and it was consequently associated with several Ranid species in the past (reviewed by Kuzmin 1996). However, Pallas questioned his taxon as a variety of “*R. bombinae*”, did not refer to any other species corresponding to the fire-bellied toad in his work, and some details on the coloration consistently match that species e.g., “subtus cinerea, lituris sparsis rubris, punctis que rubris circa clunes [gray underneath, with scattered red stripes and red dots around the hips]”. It is thus parsimonious to believe that *Rana cruenta* is a *Bombina* toad (Kuzmin 1996). The fire-bellied toad is the only *Bombina* species inhabiting Central Russia and these populations all consist of a single shallow lineage (Fijarczyk et al. 2011). The other *Bombina* species occurring in Russia, *Bombina orientalis* (Boulenger 1890), is restricted to the Far-East (notably Primorsky Krai on the Pacific coast), which was part of the Chinese empire (Manchuria) in Pallas’ time, and was

not visited by him. Hence, *Rana cruenta* Pallas, 1814 quite likely refers to *Bombina bombina*.

***Bombinator pachypus* Bonaparte, 1838** – Identified in the Italian mountains and explicitly distinguished from *Bombinator igneus* (Laurenti, 1768) – the only accepted *Bombina* species at this time (see Fig. 9F–G). Bonaparte described this taxon following correspondences with Fitzinger from Vienna, who examined specimens from “Alpi Apuane [Apuan Alps]” sent to him by Salvi (Bonaparte 1838); these specimens are however absent from the herpetological collection of NHMW (G. Gassner pers. comm.). The description further mentions occurrence in “Monti Ascolani”. Bonaparte’s distinction was ignored until Boulenger (1886) separated the fire-bellied toad in Eastern Europe from the yellow-bellied toad in Western Europe (including Italy), based on detailed drawings (Boulenger 1886, pl. I; Fig. 9H–I). Initially, Boulenger attributed *Bombinator igneus* to the fire-bellied toad and *Bombinator bombinus* to the yellow-bellied toad, as he believed the latter to occur in Sweden given Linnaeus’ description and reference to Rösel’s drawings (see above). After being informed that Sweden was instead inhabited by the fire-bellied toad, Boulenger (1888) synonymized *Bombinator bombinus* with *Bombinator igneus* and used *Bombinator pachypus* for the yellow-bellied toad instead. In these arrangements, Boulenger disregarded the law of priority and voluntarily perpetuated *igneus* and *pachypus* rather than older names like *bombina*, *variegata* and *salsa*, attracting criticism and prompting further revisions (Stejneger 1907). Eventually, *Bombina salsa* and then *Bombina variegata* were used for the yellow bellied-toad, and the Apennine *pachypus* became one of its subspecies, as *Bombina salsa pachypus* (Stejneger 1907) and then *Bombina variegata pachypus* (Mertens and Müller 1928). More recent authors have considered it as a different species (e.g., *Bombina pachypus*; Lanza and Vanni 1991). The Apuan Alps are inhabited only by the Apennine lineage of *B. variegata* (mtDNA A), hence *Bombinator pachypus* Bonaparte, 1838 is the oldest available name for it, as *Bombina variegata pachypus* (N°B in Fig. 7).

***Bombinator pachidactylus* Quattrocchi & Battistelli in Bonaparte, 1838** – An alternative name for *Bombinator pachypus* that is mentioned only on the legend of the plate representing that species, signed by Quattrocchi, the illustrator, and by Battistelli, the lithographer. It is likely to be an error, which may have contributed to Boulenger’s open criticism of Bonaparte’s work (Boulenger 1886: 499). *Bombinator pachidactylus* Quattrocchi & Battistelli in Bonaparte, 1838 is an objective synonym of *Bombinator pachypus* and thus of *Bombina variegata pachypus* (N°6 in Fig. 8).

***Bombinator brevipes* Blasius, 1839** – Reported in Blankenburg [Blankenburg (Harz)] with locality given as “am Unterharze, namentlich bei Goslar, in einem Sumpfe am Fusse des Hartzensteins und bei Osterode vorkommend [occurring in the Lower Harz, especially near Goslar, in a swamp at the foot of the Hartzenstein, and near

Osterode]”, and no reference to any specimen (Blasius 1839: 667; see also Mertens and Wermuth 1960). It was subsequently viewed as a mountain form and given as a variety of *Bombinator igneus* (Koch 1872). Boulenger (1886) synonymized it with *Bombinator pachypus*, and later considered it as a variety of that species from outside the Apennines, namely in northern Italy and eastern Europe (Boulenger 1896). The Harz mountains are only inhabited by *B. variegata*, namely from the Northern lineage (carrying mtDNA BW-N), which makes *Bombinator brevipes* Blasius, 1839 a junior subjective synonym of *Bombina variegata variegata* (N°3 in Fig. 8).

***Bombinator scaber* Küster, 1843** – Discovered in Montenegro with locality given as “kleine Lache bei Cettigne [= small puddle near Cetinje]” and explicitly distinguished from *Bombinator igneus* (Laurenti, 1768), but with no reference to any specimen (Küster 1843: 656). It was kept as a subspecies of *B. variegata* by Mertens and Müller (1940). Cetinje, close to the Bay of Kotor, is only inhabited by *B. variegata*, namely the Balkan lineage (mtDNA BW-S), which makes *Bombinator scaber* Küster, 1843 the oldest available name for it, as *Bombina variegata scabra* (N°C in Fig. 8).

***Bombinator appenninicus* Gistel in Gistel & Bromme, 1850** – Distinguished from *Bombinator igneus* with distribution given as “Italien auf den Apenninen [Italy in the Apennine Mountains]”, without mention of a specific locality or any specimen (Gistel and Bromme 1850: 333). Stejneger (1907) considered it a synonym of *Bombina salsa pachypus* (= *Bombina variegata pachypus*). The Apennine Mountains are inhabited only by the Apennine lineage of *B. variegata* (carrying mtDNA A), so *Bombinator appenninicus* Gistel in Gistel & Bromme, 1850 indeed appears to be a junior subjective synonym of *Bombina variegata pachypus* (N°7 in Fig. 8).

***Bombina maculatus* Gistel, 1868** – Described from “Regensburg” in Bavaria, Germany, with no reference to any specimen (Gistel 1868: 162). It is synonymized by Mertens (1936). Bavaria is only inhabited by *B. variegata*, namely the Northern lineage (mtDNA BW-N), which makes *Bombina maculatus* Gistel, 1868 a junior subjective synonym of *Bombina variegata variegata* (N°4 in Fig. 8).

***Bombinator pachypus* var. *Kolombatovici* Bedriaga, 1890** – Given as a variety of *Bombinator pachypus* (= the yellow-bellied toad, now *B. variegata*) of large size and documented from “Dalmatien [Dalmatia]”, based on specimens from “Spalato [Split, Croatia]” (Bedriaga 1890: 568). It is subsequently considered a subspecies of the yellow-bellied toad, as *Bombina salsa kolombatovici* (Stejneger 1907) then *Bombina variegata kolombatovici* (Mertens and Müller 1928). The Dalmatian coast (including near Split) seems to be only inhabited by the Balkan lineage of *B. variegata* (mtDNA BW-S), so *Bombinator pachypus* var. *Kolombatovici* Bedriaga, 1890 appears to be a junior synonym of *Bombina variegata scabra* (N°8 in Fig. 8).

***Bombinator pachypus* var. *nigriventris* Dürigen, 1897** – Discussed as a black-bellied variety characteristic of “bosnischen Exemplaren [Bosnian specimens]” (Dürigen 1897: 546), without information on whether these were collected and curated. The author mentioned similarities with an (unnamed) local variety from Montenegro documented by Schreiber (1875: 96). This taxon was subsequently considered as a subspecies of the yellow-bellied toad by Stejneger (1907), namely as *Bombina salsa nigriventris*, but is explicitly rejected by Fejérváry (1923) and Mertens (1928), who qualified it as a local melanistic variation. The taxon corresponds to either the Northern lineage of *B. variegata* (mtDNA BW-N), which reaches the northern half of Bosnia, or the Balkan lineage of *B. variegata* (mtDNA BW-S), which potentially extends along the Adriatic coast of Bosnia. Their respective distributions in the area remain incompletely delimited and are potentially complex, especially as these lineages may hybridize and admix. *Bombinator pachypus* var. *nigriventris* Dürigen, 1897 is thus either a junior synonym of *Bombina variegata variegata*, or a junior synonym of *Bombina variegata scabra* (N°10 in Fig. 8).

***Bombina salsa* var. *csikii* Fejérváry, 1923** – Described based on 4 specimens from “Ipek” (Mus. Hung. Amph. [MNH] 2540/5), 1 specimen from “Mount Korab collected, at 1800 m.” (MNH 2540/6) and 1 specimen from “Kula Lums (from the banks of the Luma)” (MNH 2561/3), all near the border of present Albania, Kosovo/Serbia and North Macedonia. The author refers to a form with extremely developed horny spines and a black ventral coloration, which Schreiber (1912: 176) already noted in Montenegro. Partly similar observations and reference to Schreiber’s earlier edition of his work (Schreiber 1875) had previously motivated the distinction of the form *nigriventris* by Dürigen (1897) in Bosnia, but Fejérváry rejected the latter based on the examination of the Bosnian specimens he had at his disposal. Mertens and Müller (1928) considered Fejérváry’s taxon as the subspecies *Bombina variegata csikii*, until they synonymized it with *Bombina variegata scabra* (Mertens and Müller 1940). The area covered by the type locality (northern Albania) is only inhabited by the Balkan lineage (mtDNA BW-S), hence *Bombina salsa* var. *csikii* Fejérváry, 1923 is a junior subjective synonym of *Bombina variegata scabra* (N°9 in Fig. 8).

***Bombina variegata gracilis* Bolkay, 1929** – Described from “Zabrgje (Northeastern Bosnia) [=Zabrđe]” based on 9 males and 5 females (and “some younger larvae”), curated at the Bosnia-Herzegovina State Museum in Sarajevo (= now National Museum of Bosnia and Herzegovina, ZMBH). The author also mentions that specimens collected in the “Save [= Sava]” river belong to this form. The diagnosis emphasizes weakly developed warts compared to western Balkan populations attributed to *B. variegata kolombatovici* (Bolkay 1929: 2, pl. I). Northeastern Bosnia is only inhabited by the Northern lineage (mtDNA BW-N), so *Bombina variegata gracilis* Bolkay, 1929 is a junior subjective synonym of *Bombina variegata variegata* (N°5 in Fig. 8).

***Bombina bombina danubialis* Călinescu, 1931** – Described from several sets of unspecified specimens collected in south and southeastern Romania, namely 16 from “Lunca Mofleni, lângă Caiova, județul Dolj [Lunca Mofleni, near Craiova, Dolj county]”, 5 from “Lacul Herăstrău, lângă București [Herastrau Lake, near Bucharest]”, 1 from “Băneasa”, 3 from “Oltenița, județul Ilfov [Oltenița, Ilfov county]”, 13 from “un izvor în pădurea Comana (Vlașca) [a spring in the Comana forest (Vlasca)]”, 51 from “Craiova (canalul cald al moarei Mendel) și împrejurimi (Lunca Mofleni, pâraul Izvorului din Luncă și Balta Șerca) [Craiova (hot water canal of Mendel mill) and surroundings (Lunca Mofleni, Izvorului stream from Luncă and Balta Șerca)]”, 39 from “Tulcea (bălțile și smârcurile dinspre grădinării, sub dealul Monumentului) [Tulcea (the puddles and marshes from gardens, under the Monument hill)], 3 from “insula farului la Sf. Gheorghe, județul Tulcea [lighthouse island at Sf. Gheorghe, Tulcea county], 5 from “Periprava, județul Tulcea [Periprava, Tulcea county]” and 14 from “Vâlcov (canale părăsite), județul Ismail [Vâlcov (abandoned channels), Ismail county]” (now in Odesa Oblast, Ukraine). The author emphasized the distinctiveness of his taxon in terms of belly coloration (uneven yellow/yellow-orange blotches, never red), and the presence of dorsal spiny warts (Călinescu 1931: 135–140). From the geographic origin, description and drawings, *Bombina bombina danubialis* Călinescu, 1931 unambiguously refers to *Bombina bombina*.

***Bombina bombina* var. *viridis* Marián, 1959** – Given as a color variety characterized by a greenish dorsum and grey-pink flanks, sighted in “vielen Stellen der ungarischen Ebene [numerous places in the Hungarian plain]” and in the same biotopes as the nominal form. The author mentions the collection and terrarium breeding of specimens (perhaps subsequently curated in Móra Ferenc Múzeum, Szeged, Hungary). From the geographic origin and description, *Bombina bombina* var. *viridis* Marián, 1959 clearly refers to *Bombina bombina*.

***Bombina bombina arifiyensi* Özeti & Yilmaz, 1987** – Described as a subspecies inhabiting Anatolian Turkey, with holotype SZE 9/1983-11 from “Arifiye” [Sakarya Province, Turkey]. This taxon was noted for differences in color and morphology patterns compared to specimens collected from Thrace Province (European Turkey). From the geographic origin, description, and photographs, *Bombina bombina arifiyensi* Özeti & Yilmaz, 1987 refers to *Bombina bombina*.

Finally, a few non-*Bombina* taxa have occasionally been associated with fire/yellow-bellied toads in some historical work, e.g., *Rana Rubeta* Linnaeus, 1758 (a synonym of *Bufo bufo* (Linnaeus, 1758)) as the “Feuerkröte” [= the fire-bellied toad] in Lindaker (1791: 112), and listed with *Bombinator igneus* in Schreiber (1912: 178); *Rana campanisona* Laurenti, 1768 (a synonym of *Rana temporaria* Linnaeus, 1758) with *Rana sonans* in La Cépède (1788: 294).

There are, to the best of our knowledge, no other names that apply to European *Bombina* populations. While three of our *B. variegata* lineages delimited as subspecies have already been named (*B. v. variegata*, *B. v. pachypus*, *B. v. scabra*), the Rhodope populations seem to have never been the focus of any taxonomic work. We therefore describe it as a new subspecies in the next section.

***Bombina variegata rhodopensis* Lukanov, Denoël, Jablonski & Dufrenoyes subsp. nov.**

<https://zoobank.org/E61B7D1D-FF8D-48A4-93C9-DE43E-AC3B2B4>

Identity. Previously identified as a divergent mitochondrial (cyt *b*) lineage, attributed to the subspecies *Bombina variegata scabra* by Hofman et al. (2007) and Fijarczyk et al. (2011) and labeled as the “Rhodopean”, “R” or “RD”, in reference to its distribution in the Rhodope Mountains of southeastern Bulgaria and northeastern Greece. This new taxon corresponds to the Rhodope lineage (mtDNA R) that is purple-coded in our figures. Given its level of divergence (see Discussion), we describe this lineage as a new subspecies, *Bombina variegata rhodopensis* subsp. nov.

Holotype. BG-IBER-VER-000010561, adult male collected on 23 April 2024 by SL, MD, DJ and CD in a water fountain at the northeastern exit of Kostino, Kardzhali Municipality, Kardzhali Province, Bulgaria (41.7039°N, 25.3028°E; elevation: 563 m a. s. l.), and curated at the Institute of Biodiversity and Ecosystem Research of the Bulgarian Academy of Sciences (IBER-BAS), Sofia, Bulgaria. Measurements in mm: L. 42.7, F. 16.3, T. 17.3, Lt.c. 12.8, Sp.p. 4.8, L.o. 4.4, Sp.n. 2.4. The holotype and the type locality are depicted on Fig. 10.

Paratypes. BG-NMNHS-HER-00000000552 (L. 41.6 mm), adult male collected on 23 April 2024 by SL, MD, DJ and CD at the type locality and curated at the National Museum of Natural History of the Bulgarian Academy of Sciences (NMNHS-BAS), Sofia, Bulgaria; MNHN-RA-2024.0001 (L. 46.0 mm), adult female collected on 24 April 2024 by SL, MD, DJ and CD in Lisitsite, Kardzhali Municipality, Kardzhali Province, Bulgaria (41.6108°N, 25.4543°E; elevation: 233 m a.s.l.), and curated at MNHN. NHMW 41962 (L. 39.7 mm), adult male collected on 24 April 2024 by SL, MD, DJ and CD in Panichkovo, Chernoochene Municipality, Kardzhali Province, Bulgaria (41.8565°N, 25.1517°E; elevation: 753 m a.s.l.) and curated at NHMW. These three specimens are depicted in Fig. 11.

Diagnosis. General characteristics similar to those of the yellow-bellied toad *B. variegata*. It is the sister taxon of the Balkan subspecies *B. v. scabra*, from which it is distinguished by substantial mitochondrial, nuclear (especially phylogenomic) and morphological divergence.



BG-IBER-VER-000010561



Figure 10. The holotype and type locality of *Bombina variegata rhodopensis* subsp. nov. Credit: CD (top and middle) and DJ (bottom).

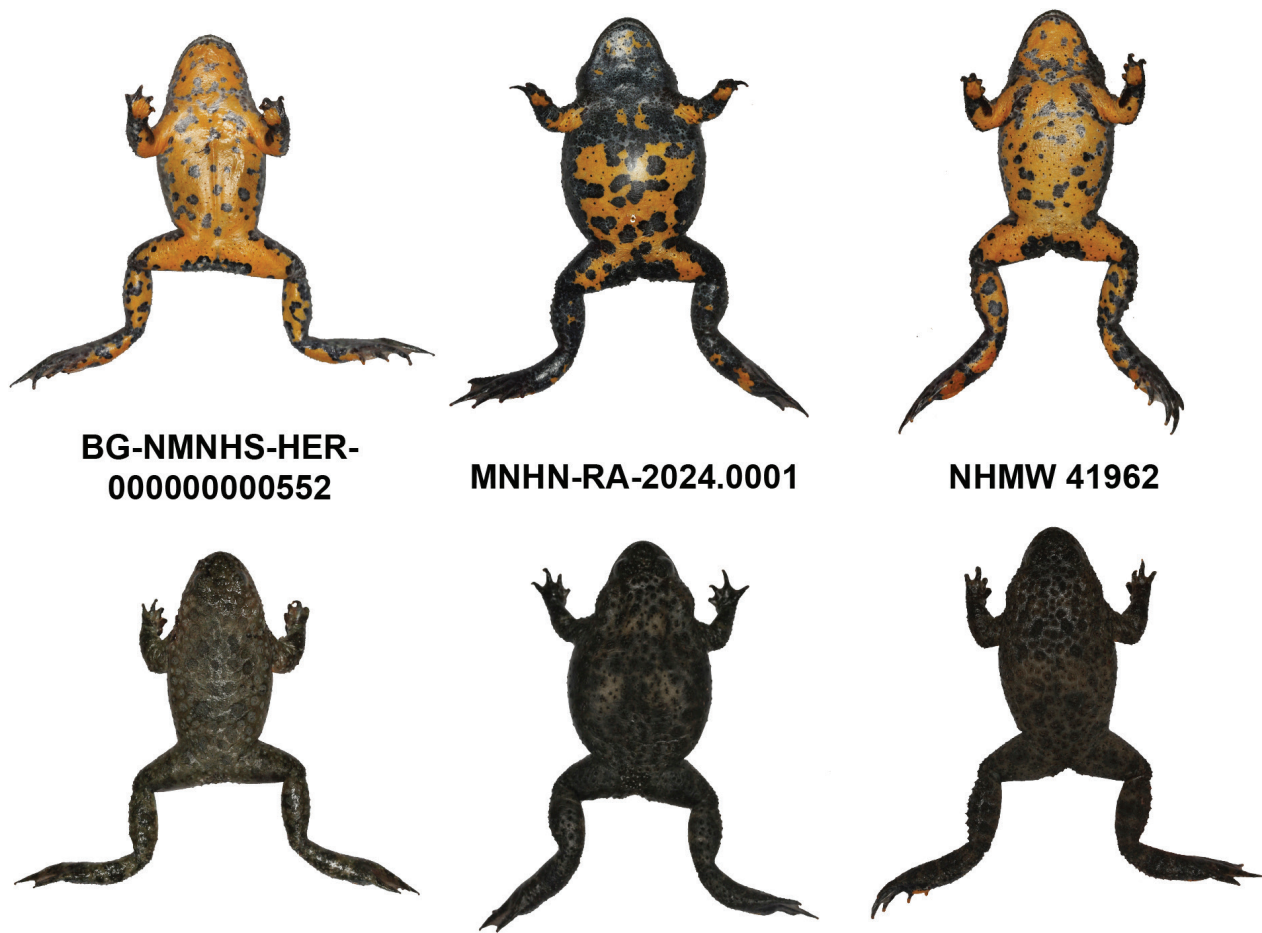


Figure 11. The paratypes of *Bombina variegata rhodopensis subsp. nov.* Credit: CD.

Specifically, *B. v. rhodopensis subsp. nov.* differs from *B. v. scabra* by 1.3% of sequence divergence at 16S, 1.6% of sequence divergence at *cox1*, and 2.4% of sequence divergence at *cyt b* (Table 1). Based on the mitochondrial time tree, the two taxa initiated their divergence during the Early Pleistocene, either around 2.1 or 1.4 Mya, depending on the calibration (Fig. 2). On the *cyt b* gene, the following nucleotides distinguish *B. v. rhodopensis subsp. nov.* from any other *B. variegata* subspecies: a “G” in the site 469, a “G” in the site 726 and an “A” in the site 801 (positions relative to the full gene sequence). The new subspecies also features a unique combination of alleles at the nuclear genes *ncx1*, *rag-1*, *rag-2* and *rho* (Figs 3, S3), as well as at 4759 RAD loci (Fig. 4). Externally, *B. v. rhodopensis subsp. nov.* differs from *B. v. scabra* by having on average a shorter femur, tibia, and first toe, as well as bigger eyes, noting that these rely on a small sample of *B. v. rhodopensis subsp. nov.* specimens (Table 2). Body sizes are on average similar (43.6 mm in *B. v. rhodopensis subsp. nov.* vs. 43.3 mm in *B. v. scabra*), with higher variation between the sexes in *B. v. rhodopensis subsp. nov.* (Fig. 5). From the examined specimens, the sexes appear morphologically dimorphic (Table 2, Fig. 5), as also seen in *B. v. scabra* (Radojčić et al. 2002; Fig. 5). Compared to *B. v. scabra*, *B. v. rhodopensis subsp. nov.* features a higher average proportion of yellow vs. dark coloration on the ventral side, although with wide overlap (Fig. 7).

Etymology. The name *rhodopensis* is a Latin toponymic adjective given in reference to the Rhodope Mountains in the southeastern part of the Balkan Peninsula (Bulgaria and Greece) where the new taxon is mostly distributed. It spotlights a rare case of Rhodope endemism in vertebrates – Rhodope endemics are so far known only from plants and invertebrates.

Vernacular names. Rhodope yellow-bellied toad (English), Родопска жълтокоремна бумка (Bulgarian), Κιτρινομπόμπινα της Ροδόπης (Greek), Rodop Sarılı Kurbağa (Turkish), Sonneur à ventre jaune des Rhodopes (French), Kunka žltobruchá rodopská (Slovak).

Distribution. *Bombina v. rhodopensis subsp. nov.* is essentially restricted to the Rhodope Mountains and their foothills (from sea level up to 1600 m a.s.l.) in southeastern Bulgaria, northeastern Greece, and the adjacent part of Turkish Thrace (Global Biodiversity Information Facility 2024). In Greece, it was documented eastward up to the National Forest Park of Dadia – Lefkimi – Soufli close to Evros (Maritsa) River which makes the border with Turkey (Petrov 2004; Valakos et al. 2008; Kret and Poirazidis 2015; Pafilis and Maragkou 2020; Strachinis 2024). In Bulgaria, population isolates exist east of the Rhodopes, namely in Sakar Mountain and perhaps Strandzha Mountain near the Black Sea (Boev et

al. 2008; Stojanov et al. 2011). In Turkey, it is very rare and restricted to Karacahasan Mountain (Enez District), close to the Evros River (Kariş et al. 2017). The eastern and northern margins of *B. v. rhodopensis* **subsp. nov.** correspond to the shifts from forest hills towards open lowland habitats colonized by the fire-bellied toad (*B. bombina*), where they probably form hybrid zones. The southern boundary of the range follows the coastal foothills of the Rhodopes (Valakos et al. 2008). According to the mtDNA barcoding, the transition with *B. v. scabra* in the west might follow the Nestos (Mesta) River valley, which separates the Rhodopes from the Rila massif in the north, and from the Pirin massif in the west; the mtDNA of both subspecies were reported in the middle part of the Nestos River in Greece (Platanias-Pteleas). Nevertheless, many sampling gaps remain in the Pirin, western Rhodopes and southern Rila, so the exact subspecies boundaries shall be fine-tuned by multilocus genotyping.

Natural history. The new subspecies inhabits similar habitats as *B. v. scabra*, being found in various aquatic sites such as mountain brooks, rivers, ponds, natural and artificial lakes or water-filled ruts and puddles (Petrov 2004). The type series was found in drinking throughs, a valuable habitat for this subspecies as it is for *B. v. scabra* in northwestern Greece (Denoël 2004). The breeding season starts in March and lasts until late July. Reports of several thousand eggs laid at the bottom of a slow-flowing stream in Eastern Rhodopes suggested possibly up to 200 per female (Stojanov et al. 2011). Toads are most active during the day and at dusk, and hibernation occurs on land. Diet studies indicate that Bulgarian populations are mostly insectivorous, preferring water beetles and winged insects, with arachnids and snails having a minor share (Donev 1984). *Bombina v. rhodopensis* **subsp. nov.** displays the anti-predator defense posture (“Unkenreflex”), well-known in *Bombina* toads (Baijger 1980), and which we could observe in Bulgaria (see also Kariş et al. 2017 for Turkey).

Conservation. *Bombina v. rhodopensis* **subsp. nov.** was reported widespread over the eastern Rhodopes of Greece and Bulgaria (Petrov 2004), but due to its much smaller distribution than that of *B. v. scabra*, it may be more vulnerable. In Greece, it is abundant above 200m (Strachinis 2024). In Turkey, it subsists only in a few isolated localities with potentially small population sizes (e.g., Kariş et al. 2017), and any potential pressure (habitat change, collection) might threaten its persistence in the country. Informed conservation management would benefit from assessing the continuity of the distribution of *B. v. rhodopensis* **subsp. nov.** across its range, in respect to known occurrence data (e.g., Valakos et al. 2008; Global Biodiversity Information Facility 2024) and from quantifying habitat loss and threats. As *B. variegata* is listed in the European Union Habitats Directive Annex 2 (= “Natura 2000”), dedicated protected areas could be declared for the conservation of *B. v. rhodopensis* **subsp. nov.**

Discussion

Our study confirmed the existence of four phylogeographic lineages within *B. variegata*, clarified their evolutionary relationships and distributions, and associated them with subspecies after an extensive overview of the nomenclatural history of European *Bombina* – no less than 21 existing names. These include *B. v. variegata* in northern ranges, *B. v. pachypus* in the Apennine Peninsula, *B. v. scabra* in the Balkans, and the newly described *B. v. rhodopensis* **subsp. nov.** in the Rhodopes. A tentative biogeographic scenario is presented in Fig. 12. As developed in the following, the patterns of diversification and their discordance between molecular markers illustrate well the biogeographic subdivisions of southeastern Europe and the recurrent connections between them, which contributed in concert to shape the high amphibian diversity found in this part of the Palearctic.

The phylogenetic position of the Apennine *B. v. pachypus*, which branches with the Balkan subspecies *B. v. scabra* and *B. v. rhodopensis* **subsp. nov.** in the mtDNA and phylogenomic trees suggests historical dispersal between the Apennine and Balkan Peninsula after the diversification of *B. variegata* was initiated (Fig. 12). The grouping of *B. v. pachypus* with *B. v. variegata* in the four-genes tree rather stems from the slow evolution and thus lower informativeness of these sequences, and was accordingly not robustly supported (Fig. 3). Sister relationships were retrieved in other amphibian taxa diverging on either side of the Adriatic Sea, such as water frogs (Balkan *P. shqipericus* and Apennine *Pelophylax lessonae*; Dufresnes et al. 2024), brown frogs (Balkan *R. graeca* and Apennine *Rana latastei* and *Rana dalmatina*; Jablonski et al. 2021) and crested newts (Balkan *Triturus macedonicus* and Apennine *Triturus carnifex*; Wielstra et al. 2019). Exchanges of terrestrial fauna potentially occurred along the coastlines or across the seabed during paleo-events involving water level subsidence, such as the Messinian salinity crisis 6.0–5.3 Mya (Krijgsman et al. 1999) and the Pleistocene climatic fluctuations (last 2.5 My), during which the Po Valley extended by 200 km into the Adriatic Sea – as far as between Ancona and Zadar (Monegato et al. 2015). These exchanges thus contributed to vicariance events, but also to historical secondary contact and hybridization between the diverging Balkan and Apennine lineages, as seen e.g., from mitochondrial introgression in slow worms (Balkan *Anguis cephalonica* and Apennine *Anguis veronensis*; Gvoždík et al. 2023).

The taxonomic rank of *B. v. pachypus* remains controversial (Speybroeck et al. 2020). Given its phylogenetic position, considering *B. v. pachypus* as a distinct species would render *B. variegata* paraphyletic, which could be solved by elevating *B. v. scabra/rhodopensis* **subsp. nov.** as a distinct species as well. Besides sequence divergence, it was suggested that *B. v. scabra* and *B. v. variegata* could represent two distinct species based on differences in genome size (Borkin et al. 2005), morphology (Vukov et al. 2006) and bioacoustic variation (Vasara et al. 1991). One way to test for these species hypothe-

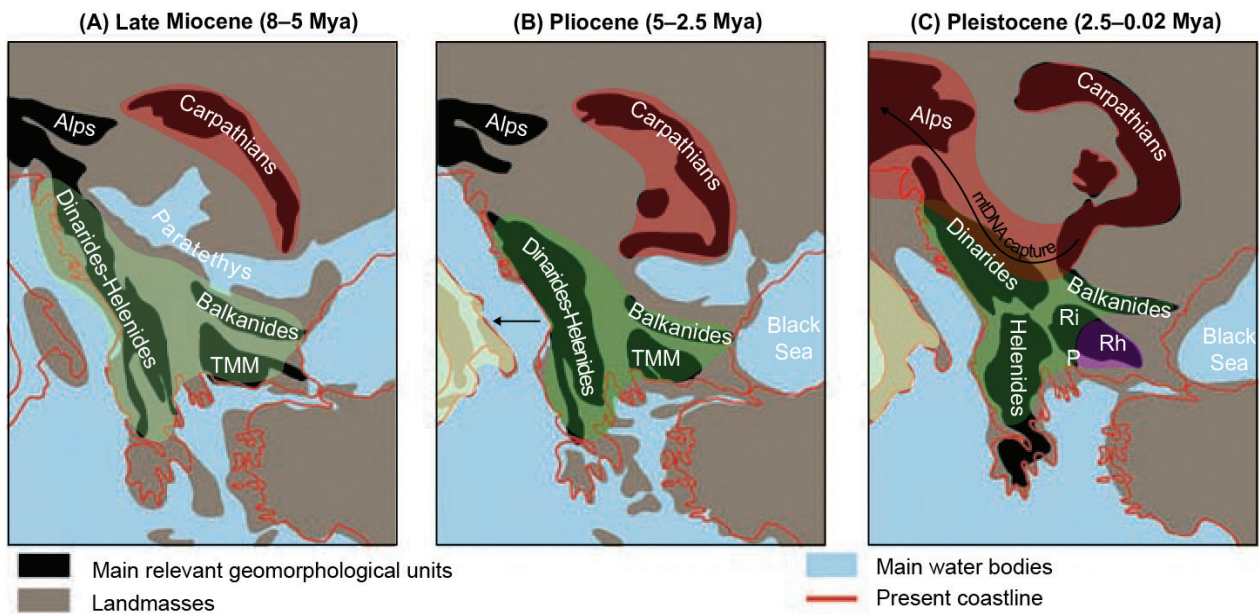


Figure 12. Hypotheses for the historical biogeography of *B. variegata* in the Balkan Peninsula in respect to paleo-mountain ranges and the time-calibrated phylogeny (Fig. 2). **A** The initial divergence separated the Carpathian (ancestral *variegata*, red) from the southern (ancestral *scabra*, green) populations, potentially as early as the Late Miocene. **B** The Apennine population (*pachypus*, yellow) subsequently originated from the latter. **C** During the Pleistocene, the southern population diversified into *scabra* (green) and *rhodopensis subsp. nov.* (purple); *variegata* (red) captured *scabra* mtDNA in the northern Dinarides and spread it across north-western Europe during the post-glacial colonization. TMM: Thrace-Macedonian massif, including Rila (Ri), Pirin (P) and Rhodope (Rh). Layout map modified from Jablonski (2017a) based on Popov et al. (2004).

ses would be to examine the degree of reproductive isolation of candidate taxa in their hybrid zones (Hillis et al. 2021; Dufresnes et al. 2021b, 2023; Chambers et al. 2023; Vences et al. 2024). Reproductively isolated taxa (= species) may show geographically restricted genetic introgression due to hybrid incompatibilities triggered by their genomic divergence, and the latter can thus be used as a cue for species delimitation (Dufresnes et al. 2021b). Here, the timeframe of diversification of the *B. variegata* subspecies potentially spans the Late Miocene (*B. v. variegata*/other subspecies in calibration I) to the Pleistocene (*B. v. scabra/rhodopensis subsp. nov.* in calibration II), which in Palearctic anurans generally correspond to either species (>6 My) and subspecies (<2 My) divergence (Dufresnes et al. 2021b). Likewise, the percentage of sequence differentiation at barcoding genes also fall in this so-called grey zone of speciation and species delimitation (Dufresnes et al. 2021b, 2023): 16S shows ~1–3 %, *cox1* shows ~2–5 % and *cyt b* shows 2–10 % of difference among the *B. variegata* subspecies (discarding the captured BW-N lineage), which corresponds to intermediate probabilities of speciation in divergence \times reproductive isolation correlations (see Fig. 2 in Dufresnes and Litvinchuk 2022). Whether *B. variegata* represents one, two or even three species should thus be addressed by relating the phylogenetic divergence of the target subspecies with the extent of genetic introgression across their hybrid zones, which exists and could be surveyed at least for the youngest pair *B. v. scabra/rhodopensis subsp. nov.* in the southeastern Balkans, and the oldest pair *B. v. variegata/scabra* in the Dinarides. For the time being, we continue

to consider the four taxa as subspecies of *B. variegata* and reiterate the relevance of this rank in the hierarchy of biological conservation (Kindler and Fritz 2018; Dufresnes et al. 2023).

The Balkano-western populations of *B. v. variegata* carry mtDNA derived from *B. v. scabra* (Fig. 1–2) despite the independent origin of these two subspecies suggested by the nuclear trees (Fig. 3–4). Such cyto-nuclear discordance likely results from a past mitochondrial introgression: *B. v. variegata* would have hybridized with *B. v. scabra* in the northern Balkans and locally captured its mtDNA, later spreading it northwestward following the post-glacial colonization of Western Europe (Fig. 12). Based on the mitochondrial timetree, the capture took place 0.4–0.5 Mya, which corresponds to the intensification of the climatic fluctuations of the Quaternary during the Middle Pleistocene (Andersen and Bjørn 1994). Accordingly, range contractions and expansions tracking the Quaternary glacial cycles potentially led to the formation of secondary contact zones and caused dramatic changes in effective population sizes (Avice 2000; Hewitt 2011), hence creating conditions that favored the acquisition of foreign genetic variants and their subsequent spread across vast geographic areas (e.g., Berthier et al. 2006).

This remarkable situation yet appears common among Balkan amphibians examined with both mitochondrial and nuclear data. For instance, mtDNA of the Anatolian green toad *Bufo viridis sitibundus* segregates in about half of the populations of the European green toad *B. v. viridis*, following refugial hybridization and post-glacial expansion (Dufresnes et al. 2019b, 2021c). The same pro-

cesses might explain some of the occurrences of mtDNA (as well as nuclear alleles) of the Balkan *Pelophylax ridibundus kurtmuelleri* across the northern ranges of *P. r. ridibundus* (Litvinchuk et al. 2020; Dufresnes et al. 2024). Moving hybrid zones, past species replacement and dynamic biogeography have also blurred the correspondence between nuclear and mitochondrial diversity in the crested newts *T. macedonicus* (Wielstra and Arntzen 2020) and *Triturus ivanbureschi* (Wielstra et al. 2017). Another well-known example is the Carpathian newt *L. montandoni*, which lost its mtDNA due to historical hybridization with the closely related *L. vulgaris* (Zieliński et al. 2013).

Cyto-nuclear discordance offers opportunities to detect past hybridization events, but it stresses the need to reconstruct mitochondrial and nuclear phylogenies independently in phylogeography and species delimitation. For the latter especially, it is often tempting to combine mitochondrial and nuclear sequences in order to obtain more robust species trees (e.g., with the multispecies coalescent), but these trees are “chimeric” if the mitogenome reflects a divergent evolutionary history than the nuclear genome. Accordingly, previous *Bombina* studies explored variation at only two nuclear fragments, either separately using haplotype networks (ncx1 and rag-1; Fijarczyk et al. 2011), or concomitantly with mitochondrial sequences in phylogenetic analyses (rag-2 and rho; Zheng et al. 2009; Pabijan et al. 2013), thus overlooking the mitochondrial capture in northwestern *B. v. variegata*.

Spatial patterns of diversification in the Balkans emphasize the Dinarides/Balkanides (*scabra/variegata*), and Pirin/Rila massifs (*scabra/rhodopensis subsp. nov.*) as major phylogeographic breaks (Fig. 12). Especially, the Dinarides correspond to the northern limits of other widespread Balkan amphibian lineages, such as the water frog *P. r. kurtmuelleri* (Dufresnes et al. 2024), the southern lineages of the tree frog *H. arborea* (Dufresnes et al. 2013), or the crested newt *T. macedonicus* (Wielstra et al. 2013, 2017). In the north, the fact that most of the original mitochondrial diversity of *B. v. variegata* is found in the Carpathian Mountains pleads for a Carpathian origin for this subspecies. Accordingly, the Carpathians are known to host endemic amphibian lineages that evolved on the spot for millions of years, especially in newts (e.g., *L. montandoni*, *L. v. ampelensis*, *I. alpestris*; Pabijan et al. 2017; Robbemont et al. 2023). Rather than topography, here the initial driver of divergence of *B. v. variegata* from the common ancestor of the other subspecies might have been remnants of the Paratethys, a large inland sea that used to stretch from the Alps to the Urals (Palcu and Krijgsman 2023) and still flooded the Pannonian plain during the Late Miocene (Popov et al. 2004; Fig. 12). Carpathian and Balkan ancestral populations would thus have been trapped north and south of this marine transgression, a scenario supported by the early split of *B. v. variegata* 7–5 Mya (Fig. 2).

In the south, the distribution of *B. v. rhodopensis subsp. nov.* shares similarities with the European ranges of the newts *Lissotriton vulgaris schmidtleri* (Pabijan et al. 2017) and *T. ivanbureschi* (Wielstra et al. 2013, 2017).

However, these newts are lowland species and both extend in nearby Anatolia, being affiliated to near-eastern taxa (namely the Caucasian *Lissotriton vulgaris lantzi* and the Anatolian *Triturus anatolicus*), which imply different biogeographic drivers of divergence (Pabijan et al. 2017; Wielstra et al. 2019). Instead, the *B. v. scabra/rhodopensis subsp. nov.* split (1–2 Mya) suggests a Pleistocene mountain diversification between the Thracio-Macedonian (Rhodope, Pirin and Rila mountains) and Hellenides-Dinarides massifs (Popov et al. 2004). The diverging subspecies may have remained isolated by the complex topography of their present area of contact, and the incapacity of *B. v. rhodopensis subsp. nov.* to escape the Rhodopes in the south (due to the Aegean Sea) and through the northern and eastern lowlands (due to ecological preferences and interspecific competition by *B. bombina*). Fine-tuning the relative distributions of *B. v. scabra* and *B. v. rhodopensis subsp. nov.* should help identify the landscape elements (notably the role of the Nestos River valley) that are currently mediating their respective distributions. The same phylogeographic pattern might be searched for in other understudied amphibians inhabiting these mountains, notably *S. salamandra*, which features several described and undescribed lineages in the Balkan Peninsula of yet largely unknown distributions (Gippner et al. 2024). Likewise, the slow worm *A. fragilis* features a unique mitochondrial haplogroup in the Rhodopes suggestive of regional population differentiation in a Late Pleistocene microrefugium (Jablonski et al. 2016). For the time being, the origin of *B. v. rhodopensis subsp. nov.* appears quite unique as we are not aware of Rhodope endemics among the European herpetofauna, or even among the vertebrate fauna.

Besides molecular divergence, *B. v. scabra* and *B. v. rhodopensis subsp. nov.* also quantitatively differ in morphology and in their ventral color patterns. In particular, we retrieved in both subspecies the sexual dimorphism in body shape previously established for *B. v. scabra* (Radojčić et al. 2002). The on-average darker belly of *B. v. scabra* echoes the historical literature reporting populations of darker specimens in the Balkan Peninsula (Schreiber 1875, 1912), which were eventually described as varieties (“*nigriventris*”, Dürigen 1897; “*csikii*”, Fejérváry 1923). Whether this variation conveys signals of long-term divergence vs. micro-evolutionary processes such as drift, local adaptation (e.g., Kang et al. 2017) or phenotypic plasticity (e.g., Preißler et al. 2021), in relation to extrinsic (environmental) factors, remain to be established, for instance, by quantifying the phenotypic diversity of additional *Bombina* taxa in a phylogeographically-explicit framework.

The various aspects surrounding the evolution of *B. variegata* (and their equivalents in other herpetofauna) illustrate well the processes by which the Balkan Peninsula generates and preserves phylogeographic diversity (e.g., Jablonski et al. 2016; Jablonski 2017a, 2017b; Mizsei et al. 2017; Psonis et al. 2018; Strachinis et al. 2021). As in “sky island” diversifications, the complex topography of the peninsula promoted the divergence of multiple allopatric lineages that survived the Quaternary glacia-

tions in both Mediterranean (Adriatic coast, Dinarides, Hellenides) and non-Mediterranean refugia (Pannonian plain, Carpathian Mountains), but at the same time, these geographic barriers were permeable enough during periods of milder environmental conditions to promote secondary contact, hybridization, and gene flow. Both attributes, formalized as the “refugia within refugia” (Gómez and Lunt 2007) and the “refugial melting pot” concepts (Canestrelli et al. 2010; Dufresnes et al. 2016; Wielstra and Arntzen 2020), fuel the two most common sources of genetic variation in species, namely novel mutations (resulting from divergence), and introgression (resulting from hybridization), hence boosting their adaptive potential and thus chances for survival in changing environments (Frankham 2005).

The generic term “refugium” traditionally designates specific geographic areas where populations survived the Pleistocene climatic fluctuations, either through range shifts from unsuitable areas (“true” refugium) or by persistence on the spot (“sanctuary” refugium, sensu Recuero and García-París 2011). The multiple evolutionary functions offered by large, composite refugia/sanctuaries such as the southern peninsulas of Europe call for a re-appraisal of these regions with a distinct, broader designation of “super-refugium”. Specifically, the diversification of *B. variegata* and other amphibians suggests three temporal phases in the buildup of the Balkan super-refugium within the last millions of years: (1) Miocene divergence within the peninsula and from other peninsular super-refugia (Anatolia, Apennines), initiated by major paleogeographic events such as isolation by the Paratethys and Adriatic Sea, and often corresponding to distinct species; (2) Plio-Pleistocene “sky islands” processes promoting the persistence and diversification of these species into multiple phylogeographic subspecies; (3) Late Pleistocene demographic fluctuations and spatial shifts resulting from the intensification of the climatic oscillations shaping the genetic diversity of these subspecies through dynamics of geographic isolation, expansions, and hybridization. These stages have set a “phylogeographic scene” that is likely to find equivalence across the three other southern peninsulas of the Western Palearctic (Iberian, Apennine, Anatolia), with deviations related to the specificities of each in terms of topography, paleogeography and paleoclimates (Macaluso et al. 2023). In turn, these super-refugia now represent hotspots of diversity and endemism in the Palearctic realm (Sillero et al. 2014; Dufresnes and Litvinchuk 2022) that should be prioritized for biodiversity protection.

Acknowledgements

We are grateful to A. Schmitz for access to the MNHG collection, to F. Tillack and M.-O. Rödel for access to the ZMB collection, to M. Fleck and W. Böhme for access to the ZFMK collection, to G. Gassner and S. Schweiger for access to the NHMW collection, to N.B. Ananyeva and K.D. Milto for access to the ZISP collection, to O. Glazot for access to the MZL collection, to M. Minařík, Z. Mačát, O. Kameniar, M. Szabolcs and M. Harzdorf for providing tissue samples, as well as J. Vörös, G. Gollmann and two anonymous reviewers for their useful

feedback on the manuscript. Specimen collections were conducted under a field permit issued by the Bulgarian ministry of environment and water (MOEW; N°861/13.01.2021). MD is a Research Director of Fonds de la Recherche Scientifique – FNRS. DJ was funded by the EU NextGenerationEU through the Recovery and Resilience Plan of the Slovak Republic (Project N°09I03-03-V04-00306). NAP was supported by the Russian Science Foundation (RSF grant N°22-14-00037). MD was funded by a Crédit de Recherche (CDR) grant of the F.R.S.-FNRS (N°J.0044.23). This study was based on specimens deposited in the vertebrate Collections of the Institute of Biodiversity and Ecosystem Research – Bulgarian Academy of Sciences, and the National Museum of Natural History – Bulgarian Academy of Sciences, upgraded in the frames of the project DiSSCo-BG (Upgrade of the Research Infrastructure “Distributed System of Scientific Collections – Bulgaria”) funded by the National Roadmap for Research Infrastructures, Ministry of Education and Science of the Republic of Bulgaria.

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Supplementary Material 1

Figures S1–S3

Authors: Dufresnes C, Lukanov S, Gippner S, Ambu J, Strachinis I, Arsovski D, Monod-Broca B, Cayuela H, Lymberakis P, Canestrelli D, Cogălniceanu D, Poyarkov NA, Litvinchuk SN, Suchan T, Denoël M, Jablonski D (2025)

Data type: .docx

Explanation notes: **Figure S1.** Schematic of the morphological characters measured. — **Figure S2.** Maximum-likelihood phylogeny of the mitogenome alignment obtained with IQ-TREE. — **Figure S3.** Maximum-likelihood phylogeny of the four nuclear gene fragments obtained with IQ-TREE.

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Supplementary Material 2

Tables S1–S6

Authors: Dufresnes C, Lukanov S, Gippner S, Ambu J, Strachinis I, Arsovski D, Monod-Broca B, Cayuela H, Lymberakis P, Canestrelli D, Cogălniceanu D, Poyarkov NA, Litvinchuk SN, Suchan T, Denoël M, Jablonski D (2025)

Data type: .docx

Explanation notes: **Table S1.** Locality information and number of *cyt b* sequences attributed of the *B. variegata* lineages. — **Table S2.** Information on the mitogenomes used in the mitochondrial phylogeny. — **Table S3.** Information on the nuclear sequences used in the gene-based nuclear phylogeny. — **Table S4.** Information on the samples used in the phylogenomic analysis. — **Table S5.** Information on the specimens used in the morphological and coloration analyses. — **Table S6.** Variable loadings of the PCA on morphological characters.

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