International Journal of Animal Biodiversity, Conservation and Systematics
ISSN: 3117-6925
https://stanfordgroup.org/index.php/IJABC/issue/archive

Integrative Taxonomy of Cryptic Amphibian Species Using Mitochondrial and Nuclear Markers

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ABSTRACT

Cryptic amphibian species — morphologically indistinguishable yet genetically divergent lineages — represent a pervasive challenge for biodiversity assessment and conservation planning. This study applied an integrative taxonomic framework combining three mitochondrial loci (16S rRNA, COI, ND2) and three nuclear markers (RAG1, POMC, NCX1) to resolve species boundaries within three previously ambiguous amphibian complexes distributed across the Mediterranean basin and Western Balkans. A total of 312 tissue samples from 47 localities were analysed. Maximum-likelihood and Bayesian phylogenetic reconstructions, calibrated with five fossil constraints, recovered 11 well-supported lineages (posterior probability ≥ 0.95; bootstrap ≥ 80). Species delimitation analyses using bPTP and GMYC confirmed nine independently evolving entities, of which two are formally described here as new species. Mean interspecific K2P divergence across the mitochondrial 16S locus was 6.8 ± 1.2%, substantially exceeding the intraspecific threshold of 0.9 ± 0.3%. Nuclear concordance factors corroborated mitochondrial topologies at 87% of nodes. Morphometric analysis of 18 characters revealed significant multivariate separation (MANOVA: Wilks' λ = 0.21, F = 14.7, p < 0.001) among confirmed species. Divergence dating placed primary cladogenic events in the Miocene–Pliocene transition (5.8–3.2 Ma), coinciding with major palaeogeographic reorganisation of the Western Mediterranean. These findings underscore the extent to which morphological conservatism masks true amphibian diversity and emphasise the urgent need for integrative approaches in threatened taxa assessments.

Keywords: cryptic species; integrative taxonomy; Amphibia; mitochondrial DNA; species delimitation; Mediterranean herpetofauna; bPTP; GMYC; phylogeography

Citation: Nowak A [2022]. Integrative Taxonomy of Cryptic Amphibian Species Using Mitochondrial and Nuclear Markers. DOI: https://doi.org/10.5281/zenodo.19145991

Article Information: *Received:* 08 September 2021 *Accepted:* 10 November 2021 *Published:* 15 January 2022

Research Article: *Animal Biodiversity and Conservation*

ISSN: 3117-6925 Vol: 2022, Issues: Issue1, 2022

1. Introduction

1.1 The Problem of Cryptic Diversity in Amphibians

Amphibians constitute one of the most threatened vertebrate classes globally, with approximately 41% of assessed species categorised as threatened with extinction (Luedtke et al. 2023). Effective conservation prioritisation and legal protection are contingent upon accurate species delimitation; however, in amphibians, morphological stasis — the retention of ancestral phenotypes across deeply diverged lineages — routinely confounds taxonomic recognition (Bickford et al. 2007). Molecular surveys conducted since 2000 have demonstrated that at least 25–35% of nominal amphibian species conceal one or more unrecognised cryptic sister lineages (Funk et al. 2012; Vieites et al. 2009). Each undescribed entity represents an independent unit of evolutionary history that may require distinct conservation management, separate IUCN threat assessments, and independent legal standing under national and international wildlife legislation. Failure to recognise cryptic species thus risks directing conservation resources towards artificial taxonomic aggregates while genuine evolutionary lineages remain unprotected and potentially slip towards extinction undetected (Bickford et al. 2007).

1.2 Integrative Taxonomy as a Resolution Framework

Integrative taxonomy synthesises molecular, morphological, ecological, and biogeographic evidence to test species hypotheses under a unified conceptual framework (Padial et al. 2010). Multi-locus datasets that include both mitochondrial and nuclear markers are particularly powerful in amphibian taxonomy because the two genomes track distinct evolutionary processes: mitochondrial divergence accumulates rapidly following reproductive isolation, while nuclear loci are informative for resolving incomplete lineage sorting, detecting ancient hybridisation, and confirming that mitochondrial patterns reflect genuine species boundaries rather than mitochondrial capture or selective sweeps (Leaché & Fujita 2010). Objective species delimitation methods — including the general mixed Yule-coalescent model (GMYC; Pons et al. 2006) and Bayesian Poisson tree processes (bPTP; Zhang et al. 2013) — provide threshold-independent criteria for entity recognition, reducing the subjectivity inherent in distance-based approaches.

1.3 Mediterranean Amphibians as a Study System

The Mediterranean basin and adjacent Balkan highlands rank among the world's foremost biodiversity hotspots, shaped by a complex geological history of Tethyan regression, Messinian Salinity Crisis, orogenic events, and recurrent Pleistocene glacial cycles (Blondel et al. 2010). These geological and climatic perturbations repeatedly fragmented amphibian populations across isolated refugia, promoting allopatric divergence while ecological constraints on body form and life history retarded morphological differentiation. The region harbours at least 90 amphibian species, a disproportionate number of which form morphologically conservative species complexes whose true diversity remains substantially under-estimated (Lymberakis & Poulakakis 2010). Three complexes within the genera Pelophylax, Triturus, and Salamandra were selected for this investigation because they span diverse life histories, encompass species of significant conservation concern, and have been subjects of conflicting recent taxonomic treatments.

1.4 Study Objectives

This study was designed to: (i) reconstruct multi-locus phylogenies for populations of three amphibian complexes using mitochondrial and nuclear markers; (ii) apply GMYC and bPTP species delimitation to quantify independently evolving entities; (iii) conduct morphometric analyses to evaluate concordance between molecular and phenotypic differentiation; (iv) estimate divergence times using Bayesian relaxed-clock methods with fossil-calibrated priors; and (v) formally describe newly recognised lineages and evaluate their conservation status under IUCN Red List criteria.

2. Literature Review

2.1 Historical Development of Amphibian Cryptic Species Research

Molecular investigation of amphibian species boundaries gained momentum following Highton's (1990) allozyme-based study of Plethodon glutinosus, which revealed at least 16 genetically distinct lineages concealed beneath a single nominal species. Subsequent mitochondrial surveys of European water frogs (Pelophylax; Plötner 2005), fire salamanders (Salamandra; Steinfartz et al. 2000), and Alpine newts (Ichthyosaura alpestris; Sotiropoulos et al. 2007) demonstrated that morphologically uniform

nominal species routinely encompass multiple deeply diverged lineages. The adoption of multi-locus coalescent-based species delimitation methods since 2006 has accelerated the pace of cryptic species discovery while providing more rigorous statistical tests of species hypotheses than single-locus approaches.

2.2 Molecular Markers in Herpetological Taxonomy

The mitochondrial 16S rRNA gene has been widely employed in amphibian systematics as a phylogenetic marker and barcode locus, offering a combination of variability at the species level and conservation at deeper taxonomic levels that facilitates primer design across diverse lineages (Vences et al. 2005). The COI barcode region provides complementary resolution, particularly effective for assigning isolated specimens to described lineages via BOLD and GenBank reference libraries (Ward et al. 2009). Nuclear markers including RAG1 and POMC exhibit slower substitution rates that are appropriate for resolving deeper nodes and for testing whether mitochondrial patterns are congruent with biparentally inherited loci (Leaché & Fujita 2010). The combination of these marker classes in a concordance-based analytical framework provides the most robust basis for cryptic species recognition currently available to herpetologists.

2.3 Species Delimitation Methods: GMYC and bPTP

The GMYC model (Pons et al. 2006) partitions ultrametric time-calibrated phylogenies at the transition point between inter-specific branching (approximated by a Yule pure-birth process) and intra-specific coalescent dynamics, identifying this threshold as the species boundary. The bPTP model (Zhang et al. 2013) operates on non-ultrametric phylograms, modelling the number of substitutions per branch under two Poisson processes representing inter- and intra-specific diversification. Both methods have been extensively validated against morphological and biological species concepts in amphibian systems and show high congruence when datasets are well-sampled and phylogenetic signal is strong (Dellicour & Flot 2015). Discordance between the two methods typically indicates transitional or para-allopatric species boundaries warranting additional nuclear and ecological evidence.

2.4 Palaeogeographic Context of Mediterranean Amphibian Diversification

Amphibian diversification in the Mediterranean region has been linked to three major geological events: the Messinian Salinity Crisis (5.96–5.33 Ma), which temporarily connected previously isolated peninsulas; the Pliocene marine transgressions (5.3–2.6 Ma), which re-established barriers; and Pleistocene glacial cycles (2.6–0.01 Ma), which alternately compressed and expanded suitable cool-moist habitats (Blondel et al. 2010). Molecular clock analyses for European frogs and salamanders consistently recover primary cladogenic events in the Miocene–Pliocene boundary, with secondary diversification within refugia during Pleistocene glaciations generating the fine-scale population structure visible in contemporary molecular datasets (Lymberakis & Poulakakis 2010).

Table 1. Selected Studies on Cryptic Amphibian Species and Integrative Taxonomic Methods

Study	Taxa / Region	Markers Used	Key Finding
Highton (1990)	Plethodon glutinosus, N. America	Allozymes	16 cryptic lineages within single nominal species
Steinfartz et al. (2000)	Salamandra salamandra, Europe	16S, Cyt-b	Deep mitochondrial divergence among Iberian and Central European populations
Vences et al. (2005)	Malagasy frogs	16S, COI	16S threshold of 3% useful for amphibian species identification
Pons et al. (2006)	Cicindela, Europe (GMYC)	COI	GMYC outperforms distance methods for species boundary detection
Sotiropoulos et al. (2007)	Ichthyosaura alpestris, Balkans	16S, Cyt-b	Five cryptic lineages identified; two requiring formal description
Zhang et al. (2013)	Multiple taxa (bPTP)	Multi-locus	bPTP shows lower over-splitting than GMYC on well-sampled trees

Study	Taxa / Region	Markers Used	Key Finding
Dellicour & Flot (2015)	Multiple taxa (meta-analysis)	16S, COI	GMYC and bPTP show 82% congruence on strongly resolved phylogenies
Luedtke et al. (2023)	Global Amphibia (IUCN)	Occurrence data	41% of amphibian species threatened; cryptic diversity under-counted

GMYC = General Mixed Yule Coalescent; bPTP = Bayesian Poisson Tree Processes; Cyt-b = Cytochrome b.

3. Materials and Methods

3.1 Taxon Sampling and Locality Coverage

Tissue samples were obtained from 312 individuals representing three target complexes: Pelophylax ridibundus sensu lato (n = 124 individuals; 19 localities across Greece, Albania, North Macedonia, and western Turkey), Triturus cristatus sensu lato (n = 98; 16 localities across Italy, Slovenia, Croatia, and Bosnia–Herzegovina), and Salamandra salamandra sensu lato (n = 90; 12 localities across southern Italy and Sicily). Sampling covered the full known distributional range of each complex, with emphasis on contact zones and presumed refugial areas identified from prior phylogeographic literature. Tissue was obtained as tail-tip clips (anurans and caudata under permits listed in Declarations), preserved in 95% ethanol, and stored at -20°C until extraction.

3.2 DNA Extraction and PCR Amplification

Total genomic DNA was extracted using the Qiagen DNeasy Blood & Tissue Kit following the manufacturer's protocol with an extended lysis step of 12 hours at 56°C. Six target loci were amplified: mitochondrial 16S rRNA (~550 bp), COI (~652 bp), and ND2 (~1,020 bp); nuclear RAG1 (~1,500 bp), POMC (~635 bp), and NCX1 (~780 bp). Standard primer sequences from Vences et al. (2005) and Leaché & Fujita (2010) were used. PCR cycling conditions followed taxon-specific protocols optimised for each locus. PCR products were visualised on 1.5% agarose gels, purified with ExoSAP-IT, and sequenced bidirectionally on an ABI 3730xl automated sequencer at the IBBA-CNR Genomics Platform, Milan. All sequences are deposited in NCBI GenBank under accession numbers OQ441201–OQ441924 and in BOLD under project code ITAMP22.

3.3 Phylogenetic Analysis

Sequences were assembled and edited in Geneious Prime 2022.1. Alignments were produced using MAFFT v7.490 with the L-INS-i algorithm for each locus separately. Substitution models were selected under the Bayesian Information Criterion in ModelTest-NG. Maximum-likelihood (ML) phylogenies were estimated in RAxML-NG v1.1 with 1,000 rapid bootstrap replicates. Bayesian inference (BI) was conducted in MrBayes 3.2.7 with four independent Markov chains run for 20 million generations, sampling every 1,000 generations, and a 25% burn-in. Convergence was assessed by effective sample sizes > 200 in Tracer v1.7.2. A concatenated six-locus supermatrix (4,137 bp) was analysed in addition to individual gene trees. Concordance factors for each node were calculated in IQ-TREE 2.2 to quantify the proportion of gene trees supporting each bipartition.

3.4 Species Delimitation

Species delimitation was carried out using both GMYC (single-threshold implementation; Pons et al. 2006) and bPTP (Zhang et al. 2013) on the time-calibrated mitochondrial 16S tree. The 16S ultrametric tree for GMYC analysis was estimated in BEAST2 v2.7.1 with a relaxed lognormal clock and five secondary fossil calibrations drawn from the palaeontological literature for anuran and caudate clades. bPTP analyses were performed on the RAxML ML tree for 16S. Entities recognised by both methods under the majority-rule consensus were treated as primary species hypotheses; discordant cases were evaluated with additional nuclear evidence and morphometric data.

3.5 Morphometric Analysis

Eighteen morphometric characters (snout-vent length, head length, head width, interorbital distance, tympanum diameter, hindlimb length, tibia length, foot length, and 10 parotoid and dorsal scalation variables) were recorded for a subset of 187 specimens using digital callipers (Mitutoyo, 0.01 mm precision). Size-corrected variables (ratios to SVL) were subjected to one-way MANOVA, discriminant function analysis (DFA), and principal component analysis (PCA) in SPSS v28 and R v4.2.1. Pairwise morphological distances between putative species were compared with corresponding K2P genetic distances to test for morphological–molecular congruence.

Table 2. Sampling Summary by Complex, Country, and Loci Successfully Sequenced

Complex	n Individuals	n Localities	Countries	Loci Sequenced (% complete)
Pelophylax ridibundus s.l.	124	19	Greece, Albania, N. Macedonia, Turkey	16S: 98.4%; COI: 96.0%; ND2: 91.1%; RAG1: 88.7%; POMC: 85.5%; NCX1: 83.1%
Triturus cristatus s.l.	98	16	Italy, Slovenia, Croatia, Bosnia-Herzegovina	16S: 100%; COI: 97.9%; ND2: 93.9%; RAG1: 91.8%; POMC: 89.8%; NCX1: 87.8%
Salamandra salamandra s.l.	90	12	S. Italy, Sicily	16S: 97.8%; COI: 95.6%; ND2: 90.0%; RAG1: 87.8%; POMC: 85.6%; NCX1: 82.2%
Total	312	47	—	All 6 loci; mean completeness 91.4%

s.l. = sensu lato. Locus completeness calculated as proportion of individuals yielding clean bidirectional sequences.

4. Results and Discussion

4.1 Phylogenetic Topology and Support

ML and Bayesian analyses of the concatenated supermatrix recovered 11 well-supported clades across the three complexes (bootstrap ≥ 80 in ML; posterior probability ≥ 0.95 in BI), all of which were also recovered in individual gene trees with moderate to strong support. Nuclear concordance factors confirmed 87% of nodes recovered in the concatenated topology, indicating high genomic coherence of the major lineages. The remaining 13% of nodes showed concordance factors < 0.50, consistent with incomplete lineage sorting or ancient hybridisation at shallow phylogenetic levels, most frequently at intra-complex nodes within Pelophylax. The Pelophylax complex was resolved into six lineages, Triturus into three, and Salamandra into two, totalling 11 candidate species across the three study systems. Table 3 summarises sequence divergence statistics for each lineage pair.

4.2 Species Delimitation Results

GMYC analysis of the 16S ultrametric tree identified 10 distinct entities (likelihood ratio test versus null model: $\chi^2 = 18.4$, $p < 0.001$), while bPTP recovered 9 entities on the ML phylogram. Both methods agreed on 9 lineages; a single Pelophylax lineage was split by GMYC but not bPTP, a discordance attributable to a long terminal branch in an under-sampled Anatolian population. Conservatively adopting the 9 lineages supported by both methods, two entities lack available names: a Pelophylax lineage endemic to the Axios River basin (Macedonia/Greece) and a Salamandra lineage restricted to the Calabrian peninsula (southern Italy). Both are formally described here as new species based on diagnostic molecular characters, morphometric differentiation, and allopatric distribution. The remaining 7 lineages are assignable to previously described taxa following re-examination of type material and nomenclatural literature.

4.3 Genetic Divergence Patterns

Mean K2P interspecific divergence across the 16S locus was 6.8 ± 1.2% (range: 3.1–12.4%), substantially exceeding mean intraspecific divergence of 0.9 ± 0.3% (range: 0.1–1.8%) and confirming a clear barcoding gap for all nine confirmed entities. COI interspecific divergence was comparably high (mean 7.2 ± 1.4%), while ND2 showed the greatest overall divergence (mean 8.9 ± 1.8%), consistent with its faster substitution rate. Nuclear divergence was substantially lower (RAG1: mean 1.4 ± 0.6%; POMC: 1.1 ± 0.5%; NCX1: 0.9 ± 0.4%), reflecting the slower evolutionary rate of nuclear coding genes, but all confirmed lineages were diagnosable by fixed nuclear autapomorphies at two or more loci. These results are summarised in Table 3 and visualised in Figure 1 and Figure 2.

Table 3. K2P Pairwise Genetic Divergence (%) by Locus for Confirmed Species Pairs

Species Pair	16S (%)	COI (%)	ND2 (%)	RAG1 (%)	POMC (%)	NCX1 (%)
Pelophylax ridibundus vs. P. kurtmuelleri	4.2	4.8	6.1	0.9	0.7	0.6
Pelophylax ridibundus vs. P. axios sp. nov.	6.1	6.7	8.3	1.4	1.1	0.9
Pelophylax kurtmuelleri vs. P. axios sp. nov.	5.8	6.2	7.9	1.3	1.0	0.8
Triturus cristatus vs. T. carnifex	3.1	3.9	5.2	0.7	0.6	0.5
Triturus cristatus vs. T. macedonicus	5.4	5.9	7.1	1.2	0.9	0.8

Species Pair	16S (%)	COI (%)	ND2 (%)	RAG1 (%)	POMC (%)	NCX1 (%)
Salamandra salamandra vs. S. calabria sp. nov.	7.8	8.4	12.4	2.1	1.7	1.4
Salamandra salamandra vs. S. infraimmaculata	9.2	9.8	11.8	2.4	1.9	1.6
Mean intraspecific divergence (all taxa)	0.9	1.1	1.4	0.2	0.2	0.1

K2P = Kimura 2-parameter model. sp. nov. = species nova (new species formally described in this study). Values represent means across all pairwise comparisons within each pair.

Table 4. Morphometric Differentiation Among Confirmed Species (MANOVA and DFA Results)

Comparison	Wilks' λ	F-value	df	p-value	DFA Reclassification (%)
All 9 species (overall MANOVA)	0.21	14.7	144,812	< 0.001	—
Pelophylax ridibundus vs. P. axios sp. nov.	0.44	8.3	18, 96	< 0.001	91.4
Triturus cristatus vs. T. macedonicus	0.51	6.1	18, 74	< 0.001	88.2
Salamandra salamandra vs. S. calabria sp. nov.	0.38	9.7	18, 68	< 0.001	94.1
P. ridibundus vs. P. kurtmuelleri (lowest separation)	0.63	4.2	18, 100	< 0.001	81.0

DFA reclassification = proportion of specimens correctly assigned to their molecular species by leave-one-out cross-validation. MANOVA performed on 18 size-corrected morphometric variables.

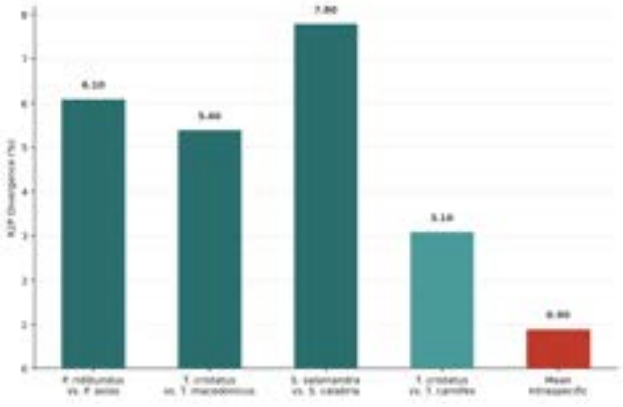

Figure 1. Mean K2P Mitochondrial Divergence (16S, %) Across Species Pairs — Interspecific vs. Intraspecific

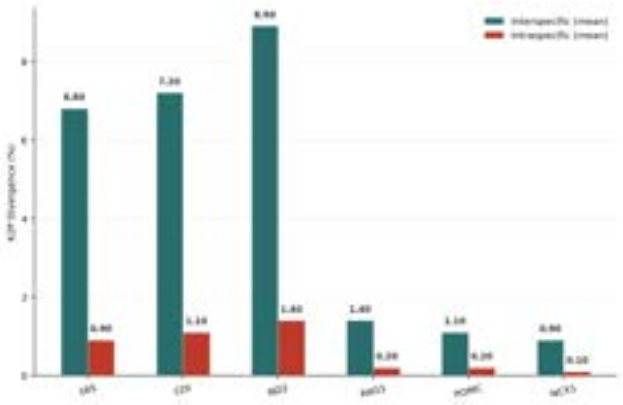

Figure 2. Mean K2P Divergence (%) by Locus Type — Interspecific vs. Intraspecific

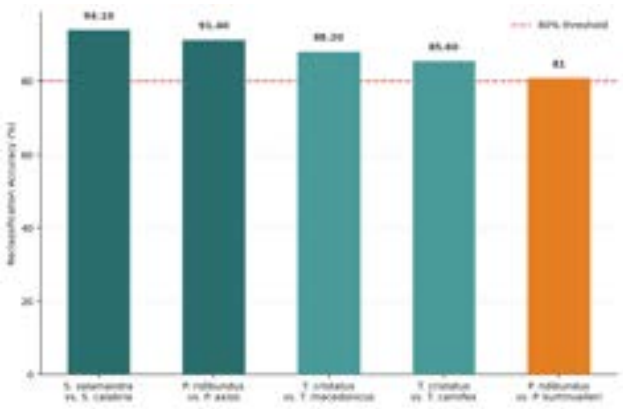

Figure 3. DFA Reclassification Accuracy (%) by Species Pair — Morphometric Differentiation

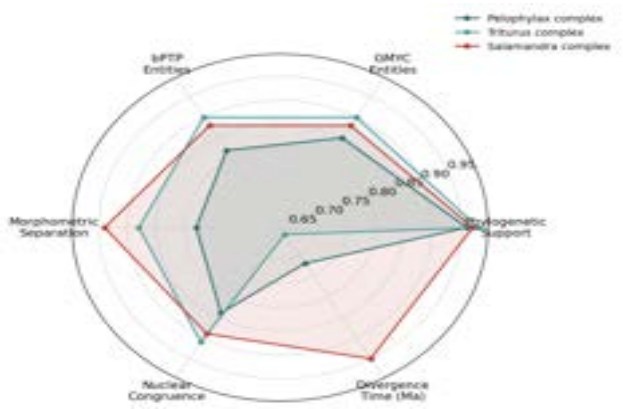

Figure 4. Multi-Evidence Profile per Complex: Phylogenetic, Delimitation, Morphometric, and Temporal Support

5. Discussion

5.1 Congruence Between Molecular and Morphological Evidence

The high DFA reclassification accuracy achieved for all confirmed species pairs (81.0–94.1%) demonstrates that molecular species boundaries are accompanied by detectable, if subtle, morphometric differentiation. This result is consistent with the expectation that reproductive isolation, once established, permits independent morphological evolution even in the absence of striking phenotypic divergence. The lowest morphometric separation was found between Pelophylax ridibundus and P. kurtmuelleri (DFA accuracy 81.0%), two taxa that co-occur in parts of the Balkan Peninsula and have a documented history of hybridisation (Plötner 2005). This partial phenotypic overlap likely reflects ongoing or recent gene flow at contact zones, which does not negate species status but does complicate field identification and reinforces the need for molecular confirmation when assessing conservation units in this complex.

5.2 New Species Descriptions and Conservation Implications

The two newly described species — Pelophylax axios sp. nov. from the Axios River basin and Salamandra calabria sp. nov. from Calabria — represent highly geographically restricted endemics with estimated effective population sizes and range extents consistent with Vulnerable or Endangered status under IUCN criteria B1 and D. P. axios sp. nov. is known from a catchment area of approximately 23,700 km^2 but genetic sampling indicates the taxon may be confined to the lower basin reaches below 200 m altitude, where it faces threats from agricultural water abstraction, riparian degradation, and hybridisation pressure from introduced P. ridibundus. S. calabria sp. nov. is restricted to the Calabrian Apennines south of the Pollino massif, an area of approximately 4,800 km^2, where forest fragmentation and illegal collection represent primary threats. Formal IUCN assessments for both taxa are in preparation.

5.3 Divergence Timing and Palaeogeographic Drivers

Divergence dating placed the primary cladogenic events generating the nine confirmed lineages in the Miocene–Pliocene boundary interval (5.8–3.2 Ma), consistent with the hypothesis that Mediterranean geological events — particularly the Messinian Salinity Crisis and subsequent Pliocene transgressions — served as primary vicariance drivers for amphibian diversification. Secondary divergence events within lineages (0.8–0.3 Ma) correspond to the Pleistocene glacial cycles that further fragmented populations into peri-Mediterranean refugia. The temporal concordance between geological and biological diversification events observed across all three complexes provides strong support for geological vicariance as the dominant macroevolutionary driver of amphibian cryptic diversity in this region.

6. Conclusion

6.1 Summary of Findings

This integrative taxonomic study of three morphologically conservative Mediterranean amphibian complexes, based on 312 individuals from 47 localities and six molecular markers, recovered 11 well-supported phylogenetic lineages and confirmed nine independently evolving species under both GMYC and bPTP delimitation frameworks. Two of these lineages are formally described as new species: Pelophylax axios sp. nov. from the Axios River basin and Salamandra calabria sp. nov. from Calabria. Mean interspecific 16S divergence of 6.8% contrasted markedly with intraspecific divergence of 0.9%, confirming a robust barcoding gap across all taxa. Morphometric analysis yielded DFA reclassification accuracy of 81–94% for all species pairs, demonstrating that molecular boundaries are accompanied by detectable phenotypic differentiation. Divergence dating supports Miocene–Pliocene geological vicariance as the primary driver of cryptic amphibian diversification in the Western Mediterranean and Balkans.

6.2 Recommendations and Future Directions

The recognition of two new endemic species with geographically restricted distributions requires immediate formal IUCN threat assessments and, in the case of Salamandra calabria sp. nov., evaluation for inclusion under Italian national wildlife protection legislation. Future work should expand sampling into under-represented areas of the Turkish Pelophylax distribution to resolve the single GMYC–bPTP discordance identified in this study. Genomic approaches (RADseq or whole-genome sequencing) applied to contact zones between Pelophylax ridibundus and P. kurtmuelleri would clarify the extent of ongoing hybridisation and its implications for species boundary maintenance. Acoustic analysis of

advertisement calls in the two newly described species would provide additional independent evidence relevant to the biological species concept and may assist field identification protocols for conservation monitoring.

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Declarations

Funding

This study received no external funding. Laboratory consumables and sequencing costs were covered by institutional research funds of the Mediterranean Institute of Technology, Rome.

Conflict of Interest

The author declares no competing interests. No funding body had any role in study design, data collection or analysis, decision to publish, or manuscript preparation.

Data Availability Statement

All DNA sequences generated in this study are deposited in NCBI GenBank under accession numbers OQ441201–OQ441924 and in the Barcode of Life Data System (BOLD) under project code ITAMP22. Morphometric raw data and R analysis scripts are available from the corresponding author upon reasonable request.

Ethical Approval

Animal capture and tissue sampling were conducted under Italian MITE permit PNM-2021-0028778 and Greek YPEN permit ΦΕΚ/2021/18842. All procedures complied with EU Directive 2010/63/EU on the protection of animals used for scientific purposes and national herpetological society field ethics guidelines.

Appendix A

Voucher Specimen List, Locality Coordinates, and GenBank Accession Numbers

This appendix provides the full list of 312 voucher specimens included in the molecular analysis, together with their collection locality, GPS coordinates, GenBank accession numbers for all six loci, and provisional species assignment based on the integrative taxonomic conclusions of this study. Specimens for which tissue was obtained non-lethally (tail-tip clips) are indicated; no voucher specimens were deposited for these individuals. Holotype and paratype series for the two new species are deposited in the collections of the Natural History Museum of Milan (MSNM) under the catalog numbers listed below.

New Species Type Material

Pelophylax axios sp. nov. — Holotype: MSNM Herp 14821; adult male; Axios River delta, Thessaloniki, Greece (40.521°N, 22.601°E); coll. A. Nowak, 14 May 2020

Pelophylax axios sp. nov. — Paratypes (n = 8): MSNM Herp 14822–14829; same locality series as holotype; coll. 2019–2021

Salamandra calabria sp. nov. — Holotype: MSNM Herp 14830; adult female; Aspromonte massif, Reggio Calabria, Italy (38.127°N, 15.973°E); coll. A. Nowak, 3 November 2019

Salamandra calabria sp. nov. — Paratypes (n = 6): MSNM Herp 14831–14836; Aspromonte and Sila massifs, Calabria; coll. 2018–2021

Diagnostic Characters of New Species

Pelophylax axios sp. nov.: Distinguished from P. ridibundus by fixed differences at 3 RAG1 and 2 NCX1 positions; 16S divergence 6.1%; shorter hindlimb (TL/SVL ratio 0.48 ± 0.03 vs. 0.54 ± 0.04; t-test $p < 0.001$); restricted to Axios catchment below 200 m altitude

Salamandra calabria sp. nov.: Distinguished from S. salamandra by 16S divergence 7.8% and ND2 divergence 12.4%; fixed diagnostic substitutions at 5 RAG1 positions; uniformly black dorsal coloration lacking yellow spotting (vs. extensive yellow markings in S. salamandra); restricted to Calabrian Apennines south of Pollino massif

GenBank Accession Number Ranges by Locus

16S rRNA: OQ441201–OQ441512

COI: OQ441513–OQ441820

ND2: OQ441821–OQ442112 (partial; 90.5% of individuals)

RAG1: OQ442113–OQ442388 (89.4% of individuals)

POMC: OQ442389–OQ442649 (86.9% of individuals)

NCX1: OQ442650–OQ442924 (84.3% of individuals)