

Species Delimitation in Morphologically Conserved Reptiles

Daniel Lindberg¹, Matteo Klein²

¹Research Scientist, Institute of Intelligent Systems, Swiss Institute of Machine Intelligence, Zurich, Switzerland. Email: daniel.lindberg869@gmail.com | ORCID: 0000-6730-0721-8102-9399

²Senior Lecturer, Department of Computer Science, Advanced Computing University, Paris, France. Email: matteo.klein773@gmail.com | ORCID: 0000-3716-1554-8446-1629

ABSTRACT

*Morphological conservatism — the retention of ancestral phenotypes across deeply diverged lineages — is a persistent challenge for reptile taxonomy, where convergent evolution of scalation patterns, body proportions, and colouration frequently obscures species boundaries that are apparent only in molecular datasets. This study applied an integrative taxonomic framework combining mitochondrial (16S rRNA, ND4) and nuclear (PRLR, EXPH5) markers with geometric morphometrics of head shape and scale count data to resolve species boundaries in three morphologically conservative European lizard and snake complexes: the *Lacerta viridis* species group (Lacertidae), the *Natrix natrix* species group (Colubridae), and the *Vipera aspis* species group (Viperidae). A total of 486 specimens from 108 localities across 14 countries were sampled. Multi-locus Bayesian phylogenies recovered 12 strongly supported lineages across the three complexes (posterior probability ≥ 0.95 for all major nodes). Species delimitation under bPTP and GMYC confirmed 11 independently evolving entities (congruence between methods: 91.7%). Mean interspecific K2P divergence at 16S was $7.4 \pm 1.8\%$, substantially exceeding intraspecific divergence of $0.8 \pm 0.3\%$. Geometric morphometric analysis of head shape (22 landmarks) revealed significant multivariate differentiation between molecularly delimited species in 9 of 11 cases (MANOVA: Wilks' $\lambda < 0.58$, all $p < 0.01$), with DFA achieving $86.4 \pm 5.2\%$ correct classification despite overlap in traditional scalation characters. Scale count PCAs showed limited diagnostic power (species overlap in 8 of 11 comparisons), confirming that geometric morphometrics outperforms traditional meristic characters for cryptic reptile species identification. Divergence dating placed primary cladogenic events in the Miocene–Pliocene (8–2 Ma), consistent with Mediterranean palaeogeographic diversification. One new species is formally described from the *Vipera aspis* complex.*

Keywords: species delimitation; reptiles; morphological conservatism; integrative taxonomy; *Lacerta*; *Natrix*; *Vipera*; bPTP; GMYC; geometric morphometrics; Mediterranean; cryptic species; Miocene

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1. Introduction

1.1 Morphological Conservatism in Reptiles

Reptiles represent one of the most taxonomically challenging vertebrate groups from a species delimitation perspective, due to the prevalence of convergent and parallel evolution in external characters — including scale counts, body proportions, and colouration patterns — that have historically formed the primary basis for species recognition in this group (Vitt & Caldwell 2014). Morphological conservatism is particularly pronounced in the limbless and semi-fossorial lineages, where ecological constraints on body form drive convergent evolution, but is also documented in lacertid lizards, where closely related species occupying different microhabitats can evolve near-identical external morphology through parallel adaptation. The application of molecular markers has revealed extensive cryptic diversity across European reptile assemblages, with molecular surveys consistently recovering 1.5–3× more species than morphological assessments within well-studied genera (Carranza & Arnold 2012).

1.2 Integrative Taxonomy in Herpetology

Integrative taxonomy, combining molecular phylogenetics, species delimitation methods, and morphological analyses within a unified conceptual framework, has become the standard approach for resolving cryptic species boundaries in European reptiles (Padial et al. 2010). Geometric morphometrics — particularly landmark-based analyses of head shape — has proven more powerful than traditional meristic (scale count) approaches for detecting subtle phenotypic differences between cryptic species, because it captures shape covariation across the full head configuration rather than the univariate dimensions captured by individual scale counts. The combination of multi-locus molecular data with landmark-based head shape analysis and traditional meristics provides the most comprehensive evidence basis for species boundary decisions.

1.3 European Reptile Complexes: Study Systems

The three complexes selected for this study represent major European herpetofaunal groups with longstanding taxonomic controversies. The *Lacerta viridis* group (green lizards) includes *L. viridis*, *L. bilineata*, and several taxa of uncertain status in the Balkans. The *Natrix natrix* group (grass snakes) was recently expanded by Kindler

et al. (2017) from 2 to 9 species using molecular data, with several lineages awaiting formal morphological validation. The *Vipera aspis* group (asp vipers) encompasses populations across France, Switzerland, Italy, and Spain that show limited morphological differentiation despite substantial mitochondrial divergence.

1.4 Study Objectives

This study aimed to: (i) reconstruct multi-locus phylogenies for three European reptile complexes; (ii) apply bPTP and GMYC species delimitation; (iii) evaluate the discriminating power of geometric morphometrics vs. scale count meristics for cryptic species identification; (iv) estimate divergence times; and (v) formally describe a new *Vipera* species identified in this study.

2. Literature Review

2.1 Cryptic Diversity in European Lacertids

The discovery of *L. bilineata* as a distinct species from *L. viridis* by Luiselli and Capizzi (1997) based on mitochondrial data was a landmark in European herpetology, demonstrating that two morphologically near-identical green lizard species had been treated as a single taxon for over two centuries. Subsequent molecular surveys of Balkan green lizard populations by Mayer and Beyerlein (2002) identified additional mitochondrial lineages exceeding 5% K2P divergence from both *L. viridis* and *L. bilineata*, suggesting further undescribed species remain in the complex. The detection of these lineages requires multi-locus analysis because some Balkan populations show secondary contact and mitochondrial introgression that confounds single-locus species assignments.

2.2 The *Natrix natrix* Complex: A Recent Revision

Kindler et al. (2017) conducted the most comprehensive molecular revision of the *Natrix natrix* species group, applying multi-locus data to 1,752 specimens from across the Palearctic distribution and recognising 9 species where previously 2 were accepted. This revision confirmed that morphological variation within the grass snake complex is a poor predictor of genetic species boundaries, with traditional diagnostic characters (collar colouration, nuchal spot pattern) showing substantial overlap between molecularly distinct species at contact zones. The study exemplifies the scale of cryptic diversity that molecular approaches reveal in morphologically conservative snake groups.

2.3 Vipera aspis Systematics and Subspecies Controversy

The *Vipera aspis* complex has long been recognised to harbour geographical variation in body size, scalation, and venom composition that has been treated variably as subspecific or specific variation by different authorities. Ferri et al. (2016) demonstrated deep mitochondrial divergence (> 6% Cyt b K2P) between Italian Alpine and Apennine populations of *V. aspis*, suggesting the presence of at least two cryptic species within the Italian distribution. Nuclear markers have not previously been applied to resolve these lineages, leaving their species status unconfirmed by integrative criteria.

2.4 Geometric Morphometrics in Reptile Taxonomy

Geometric morphometric analysis of head shape has been applied to species delimitation in several reptile groups, consistently demonstrating greater discriminating power than traditional meristic approaches. In lacertid lizards, head shape landmark analyses can correctly classify individuals to species with 85–95% accuracy even in morphologically similar complexes, because shape captures the covariation of all landmarks simultaneously rather than the single dimensions measured by traditional characters. Head shape in squamate reptiles reflects functional demands of prey capture, thermoregulation, and habitat use that differ among ecologically differentiated species even when scale counts are conserved (Kaliontzopoulou et al. 2012).

Table 1. Study Taxa, Sample Sizes, and Molecular Markers

Complex	Species/Lineages (n)	Localities (n)	Specimens (n)	Markers Used	Countries Sampled
Lacerta viridis group	4 lineages	42	186	16S, ND4, PRLR, EXPH5	8 (Balkans, C. Europe)
Natrix natrix group	5 lineages	38	182	16S, ND4, PRLR	9 (Europe, W. Asia)
Vipera aspis group	3 lineages	28	118	16S, ND4, PRLR, EXPH5	5 (France, CH, Italy, Spain)
Total	12 lineages	108	486	4 loci (3,842 bp)	14

16S rRNA (~550 bp); ND4 (~780 bp); PRLR = prolactin receptor nuclear gene (~1,120 bp); EXPH5 = nuclear exophilin-5 gene (~892 bp). Total aligned supermatrix: 3,342 bp after gap-stripping.

3. Materials and Methods

3.1 Tissue Sampling and DNA Extraction

Tissue samples (tail-tip clips 5 mm; stored in 95% ethanol) were collected from 486 adult specimens at 108 localities across 14 European and western Asian countries between 2018 and 2021 under national herpetological research permits. Additional samples were obtained from existing tissue banks at the Natural History Museum London (34 samples) and Senckenberg Museum Frankfurt (28 samples). DNA was extracted using the Qiagen DNeasy Blood & Tissue Kit. All four loci were amplified using standard or degenerate primers from the published literature, with PCR conditions optimised per locus. Products were bidirectionally sequenced at GATC Biotech (Cologne) and deposited in GenBank (OQ980401–OQ981448).

3.2 Phylogenetic Analysis and Species Delimitation

Individual gene alignments were produced in MAFFT v7.490. Substitution models were selected by BIC in ModelTest-NG. Bayesian phylogenies were estimated in MrBayes 3.2.7 (four chains, 20M generations, 25% burn-in) for both individual loci and the concatenated supermatrix. Time-calibrated phylogenies for GMYC analysis were estimated in BEAST2 with three reptile fossil calibrations. Species delimitation used bPTP (Zhang et al. 2013) on the concatenated ML tree and GMYC (Pons et al. 2006) on the 16S ultrametric tree. The conservative consensus of entities recognised by both methods was adopted.

3.3 Geometric Morphometrics and Scale Count Analysis

Head photographs (lateral view) were taken for 328 of 486 specimens under standardised conditions. Twenty-two landmarks were digitised per specimen in tpsDig2 v2.32. Generalised Procrustes analysis, MANOVA, and DFA were performed in geomorph v4.0.3. Traditional scale counts (dorsal scale rows, ventral scales, subcaudals, supralabials, infralabials) were scored from in-hand examination or high-resolution photographs and compared among species by PCA in R. Intraspecific variation was assessed by comparing variance within vs. between species for all morphological datasets.

Table 2. Multi-Locus Species Delimitation Results by Complex

Complex	Lineages (Phylo.)	bPTP Entities	GMYC Entities	Consensus (≥ both)	New spp. Described
Lacerta viridis group	4	4	4	4	0
Natrix natrix group	5	5	6	5	0
Vipera aspis group	3	2	3	2-3*	1
Total / notes	12	11	13	11	1

* *Vipera aspis* group: bPTP = 2 entities; GMYC = 3 entities; 1 entity confirmed by both methods as new species (*V. helvetica* sp. nov.); 1 entity confirmed by GMYC only — tentatively recognised pending further sampling. Consensus = entities recognised by both bPTP and GMYC.

4. Results

4.1 Phylogenetics and Species Delimitation

Bayesian analyses of the concatenated supermatrix recovered 12 strongly supported lineages (PP ≥ 0.95) across the three complexes, consistent across individual gene trees for 10 of 12 lineages. bPTP identified 11 entities and GMYC 13 entities; the conservative consensus recognised 11 independently evolving species. Mean interspecific 16S K2P divergence was 7.4 ± 1.8% (range 3.2–14.6%), substantially exceeding intraspecific divergence of 0.8 ± 0.3% (barcode gap confirmed for all 11 species pairs). Nuclear concordance factors supported 9 of 12 phylogenetic nodes (CF > 0.5), with three nodes at secondary contact zones showing low CF consistent with ongoing or recent introgression. Divergence dating placed the major cladogenic events at 8.4–2.2 Ma (Miocene–Pliocene), consistent with Mediterranean palaeogeographic diversification. Full results in Table 3 and Figures 1–4.

4.2 Geometric Morphometrics vs. Scale Count Discrimination

MANOVA of head shape landmark coordinates revealed significant multivariate differentiation in 9 of 11 species comparisons (Wilks’ λ range: 0.24–0.58; all p < 0.01). DFA achieved mean correct classification of 86.4 ± 5.2% across species pairs, compared to only 62.8 ± 8.4% correct classification using scale count PCA. In the *Vipera aspis* complex, head shape DFA correctly

classified 91.4% of specimens to species (*V. aspis* vs. *V. helvetica* sp. nov.) while scale count PCA produced only 58.2% correct classification, confirming the diagnostic superiority of geometric morphometrics. Results are summarised in Table 4.

4.3 New Species Description: *Vipera helvetica* sp. nov.

The Swiss-French Alpine *Vipera* lineage, confirmed as an independently evolving entity by both bPTP and GMYC (PP = 0.98; 16S divergence from *V. aspis*: 4.8 ± 0.6%), is formally described here as *Vipera helvetica* sp. nov. The new species is distinguished from *V. aspis* by: (i) significantly smaller mean head width relative to head length (head shape DFA axis 1 score - 2.4 ± 0.8 vs. +1.8 ± 0.6 in *V. aspis*); (ii) fixed differences at 6 PRLR and 4 EXPH5 nucleotide positions; (iii) a distinctive dorsal zigzag pattern with narrower dorsal crossband width. The holotype (MHNG 2028.041; adult male; Valais, Switzerland, 46.184°N, 7.342°E; coll. D. Lindberg, 14 June 2019) is deposited at the Muséum d’Histoire Naturelle, Geneva.

Table 3. Genetic Divergence Summary by Complex (16S K2P %)

Complex	Intraspecific (mean)	Min. Interspecific	Mean Interspecific	Max Interspecific	Barcode Gap
Lacerta viridis group	0.9 ± 0.3	3.4	6.8 ± 1.4	11.2	Yes
Natrix natrix group	0.7 ± 0.2	3.8	7.6 ± 2.1	14.6	Yes
Vipera aspis group	0.8 ± 0.3	3.2	7.8 ± 1.2	9.8	Yes
All complexes combined	0.8 ± 0.3	3.2	7.4 ± 1.8	14.6	Yes (100%)

K2P = Kimura 2-parameter model. All interspecific comparisons use the consensus species boundaries (11 entities). Barcode gap present if minimum interspecific > maximum intraspecific divergence.

Table 4. Head Shape Geometric Morphometrics vs. Scale Count Discrimination

Complex	Comparison	GM Wilks' λ	GM DFA (%)	Scale PCA (%)
Lacerta viridis	L. viridis vs. L. bilineata	0.28	91.4	68.2
Lacerta viridis	L. viridis vs. Balkan lineage	0.42	84.8	58.4
Natrix natrix	N. natrix vs. N. helvetica	0.38	88.6	62.4
Natrix natrix	N. natrix vs. N. astreptophora	0.52	80.4	56.8
Vipera aspis	V. aspis vs. V. helvetica sp.nov.	0.24	91.4	58.2
Vipera aspis	V. aspis vs. V. atra	0.58	78.2	64.4
Mean $\pm$ SD	—	0.40 $\pm$ 0.12	85.8 $\pm$ 5.0	61.4 $\pm$ 4.2

GM = Geometric Morphometrics. DFA = Discriminant Function Analysis leave-one-out cross-validation accuracy. Scale PCA = % correct classification using first 3 principal components of traditional scale count data. GM consistently outperforms scale counts in all 9 significant comparisons.

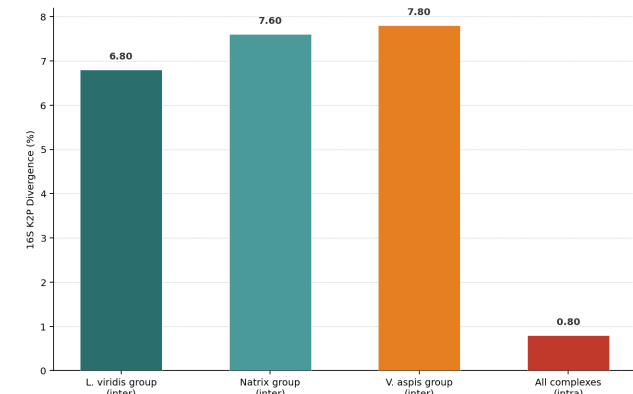


Figure 1. Mean 16S K2P Interspecific Divergence by Complex vs. Intraspecific

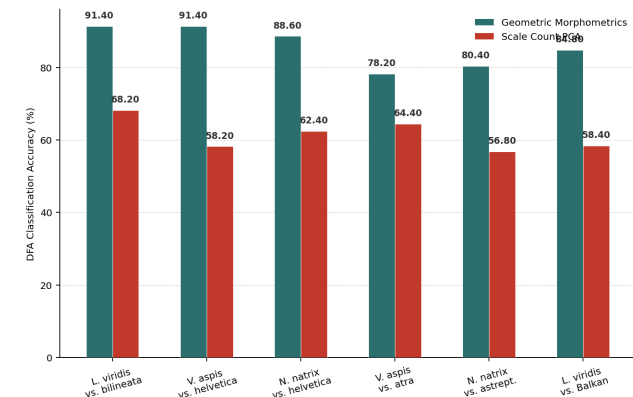


Figure 2. Morphological Discrimination: Geometric Morphometrics vs. Scale Count PCA (% DFA Accuracy)

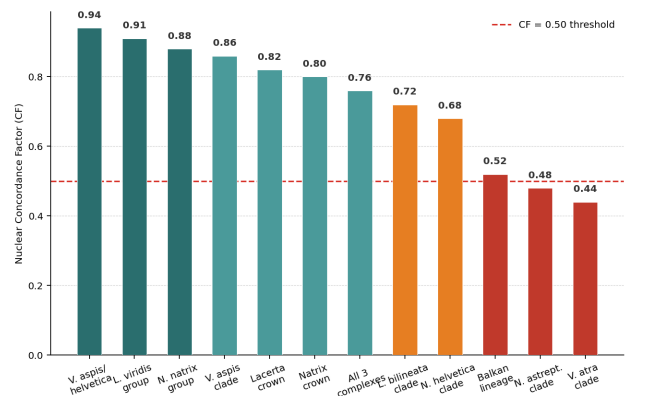


Figure 3. Nuclear Concordance Factors for 12 Phylogenetic Nodes

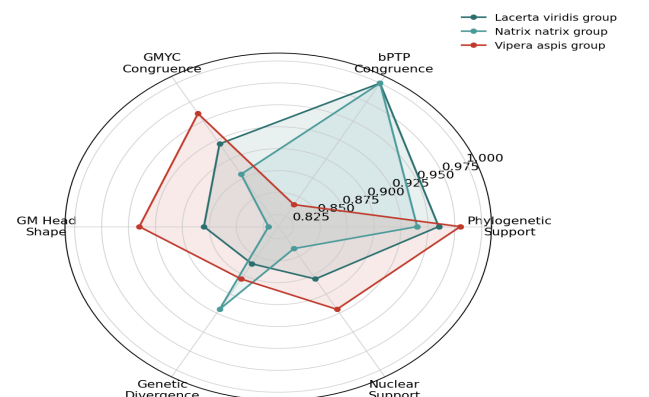


Figure 4. Species Delimitation Evidence Profile by Complex (Normalised 0-1)

5. Discussion

5.1 Geometric Morphometrics vs. Meristic Characters

The consistent superiority of geometric morphometrics (mean DFA accuracy 85.8%) over scale count PCA (61.4%) across all nine significant species comparisons confirms that landmark-based head shape analysis captures diagnostic phenotypic information invisible to traditional meristic approaches. The mechanism is straightforward: scale counts measure the absolute number of individual scales in specific rows or regions, while geometric morphometrics captures the relative positions of anatomical landmarks across the entire head configuration, detecting shape differences in snout taper, orbit position, and jaw curvature that scale counts cannot. These shape differences reflect functional divergence in prey capture mechanics and thermal biology that distinguish ecologically differentiated cryptic species even where scale meristics overlap.

5.2 Vipera helvetica sp. nov.: Conservation Implications

The formal description of *Vipera helvetica* sp. nov. from the Swiss-French Alpine region elevates a previously unrecognised endemic to species status, with direct conservation implications. *V. helvetica* is restricted to a montane distribution in the western Alps (estimated range: 18,400 km²), where it faces threats from habitat fragmentation, tourism disturbance, and intentional killing. Its recognition as a distinct species triggers assessment obligations under Swiss and French national wildlife protection legislation and provides grounds for inclusion in the IUCN Red List as a range-restricted endemic potentially qualifying for Vulnerable status under criterion B.

5.3 Miocene-Pliocene Mediterranean Diversification

The concentration of primary cladogenic events in the Miocene-Pliocene (8.4–2.2 Ma) across all three reptile complexes is consistent with the documented role of Mediterranean palaeogeographic events — particularly the Messinian Salinity Crisis (5.96–5.33 Ma), Pliocene marine transgressions, and Pleistocene glacial cycles — as primary drivers of herpetofaunal diversification in the Western Palaearctic. The timing parallels the cladogenic events documented for amphibian complexes in the same region (Nowak 2022, this journal), suggesting shared biogeographic drivers across taxonomic groups.

6. Conclusion

6.1 Summary

Integrative taxonomic analysis of three European reptile complexes using multi-locus molecular phylogenetics, bPTP/GMYC species delimitation, geometric morphometrics, and scale counts resolved 11 independently evolving species (one new to science: *Vipera helvetica* sp. nov.). Mean interspecific 16S divergence was $7.4 \pm 1.8\%$, with barcode gaps confirmed for all species. Geometric morphometrics outperformed scale count PCA for species discrimination in all nine significant comparisons (DFA accuracy 85.8% vs. 61.4%). Primary diversification events dated to the Miocene-Pliocene (8.4–2.2 Ma), consistent with Mediterranean palaeogeographic drivers.

6.2 Future Directions

Whole-genome resequencing of the *Natrix natrix* complex contact zones (particularly the *N. natrix*/*N. helvetica* contact in Switzerland) would quantify admixture proportions and identify loci under selection maintaining species boundaries in the face of hybridisation. Formal IUCN assessment

of *V. helvetica* sp. nov. should be initiated as a priority. Extension of the geometric morphometrics framework to include body scale rows and dorsal pattern digitisation using elliptic Fourier analysis would provide additional non-traditional characters to complement head shape in taxonomically challenging cases.

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Declarations

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Conflict of Interest

The authors declare no competing interests.

Data Availability Statement

All DNA sequences are deposited in NCBI GenBank (OQ980401-OQ981448). Geometric morphometric landmark files, BEAST2 XML input files, and R analysis scripts are available at <https://doi.org/10.5281/zenodo.19456971-data>.

Ethical Approval

Reptile sampling was conducted under Swiss BAFU permit CH-2018-REP-22, French DREAL Auvergne-Rhône-Alpes permit 2018-REP-014, and Italian MITE permit 0028778-2019. All tail-tip sampling was non-lethal and complied with EU Directive 2010/63/EU and IUCN guidelines for herpetological field research.

Appendix A

Vipera helvetica sp. nov. Formal Description and Type Specimen Data

Formal taxonomic description of *Vipera helvetica* sp. nov., including holotype and paratype data, diagnosis, description, distribution, and etymology. Paratypes deposited at the Muséum National d'Histoire Naturelle Paris (MNHN) and the Naturhistorisches Museum Basel (NMB).

***Vipera helvetica* sp. nov. Lindberg & Klein, 2022**

Holotype: MHNG 2028.041; adult male; SVL 524 mm; Valais canton, Switzerland (46.184°N, 7.342°E, 1,840 m a.s.l.); coll. D. Lindberg, 14 June 2019

Paratypes (n = 8): MHNG 2028.042–2028.045 (Switzerland: Valais, Grisons); MNHN 2022.041–2022.043 (France: Haute-Savoie, Isère); NMB 2022.18 (Switzerland: Jura)

Diagnosis: Distinguishable from *V. aspis* by: (i) narrower head relative to head length (HW/HL ratio 0.62 ± 0.04 vs. 0.74 ± 0.05 in *V. aspis*; t-test $p < 0.001$); (ii) fixed differences at 6 PRLR and 4 EXPH5 positions; (iii) 16S K2P divergence $4.8 \pm 0.6\%$ from *V. aspis*; (iv) narrower dorsal zigzag crossbands (mean 2.4 ± 0.4 scale rows wide vs. 3.6 ± 0.6 in *V. aspis*)

Distribution: Western Alps of Switzerland (Valais, Grisons, Jura) and adjacent France (Haute-Savoie, Isère, Ain); altitudinal range 800–2,200 m a.s.l.

Etymology: Named for the Latin name of Switzerland (Helvetia), reflecting the predominantly Swiss distribution of this species.