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Mitochondrial phylogeny and molecular-based species delimitation illuminate cryptic diversity in saw-scaled vipers (Viperidae: *Echis*)

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Molecular species delimitation can clarify species limits and uncover morphologically cryptic taxa, particularly in groups that have conflicting taxonomies based on morphology alone. We reassess the phylogenetic relationships within *Echis*, a viperid genus widely distributed in the Eastern Hemisphere with a complicated taxonomic history. Using 134 mitochondrial cytb sequences sampled from ten of the twelve currently recognised species, we applied gene-tree and distance-based species delimitation methods to delineate evolutionary significant units within *Echis*. Our results validate current taxa, adding additional support to recently described species, and uncover cryptic genetic diversity within *Echis*. Although single-locus delimitation methods may not fully capture species diversity, congruent support between methods can identify lineages in need of future investigation. We emphasise that the lineages identified here can be used as a guide for further geographic sampling and in an integrative taxonomic treatment of *Echis* that combines genomics, morphometrics and ecological data in a unified framework.

Keywords: diversification, phylogenetics, population genetics, species delimitation, Viperinae

Species are a primary unit of study in all fields of biological research, offering insights into organismal ecology, evolution and physiology. Therefore, applying consistent criterion for identifying species is important for all fields of biology (de Queiroz, 2007). Historically, taxonomists have delimited species based on morphological characteristics and largely non-overlapping geographic distributions (Wiens & Penkrot, 2002). With advances in DNA sequencing technologies, molecular data are now commonly included as evidence when delimiting species (Sites & Marshall, 2003; Flot, 2015; Luo et al., 2018; da Silva et al., 2023). These molecular based approaches have the goal of objectively determining the boundary between species (Fujisawa & Barraclough, 2013; Dellicour & Flot, 2018). While large genomic datasets are informative for understanding speciation, analysing these data can be computationally demanding (Bernstein et al., 2024), whereas single-locus molecular data are more efficient to acquire and analyse, particularly given the large datasets that have been accessioned in databases like NCBI's GenBank (Dellicour & Flot, 2018; Guo & Kong, 2022).

The genus *Echis* (Schneider, 1801), commonly called saw-scaled vipers, are true vipers (subfamily Viperinae) widely distributed across the arid regions of North Africa throughout the Middle East into Southwest and South Asia. Despite the attention this group of snakes has received from taxonomists, no consensus exists on species limits (Pook et al., 2009; Mallow et al., 2003). Much of the taxonomic work has been based on morphological variation, with many subspecies and species being named in the late 20th century (e.g. Stemmler, 1969; 1970; Stemmler & Sochurek, 1969; Roman, 1975; Cherlin, 1981; 1983; 1984; 1990; Babocsay, 2004; Trape, 2018). The most extensive revision recognised 12 species and 13 subspecies (Cherlin, 1990), yet this proposed taxonomy was questioned by subsequent authors (Auffenberg & Rehman, 1991; David & Ineich, 1999; Pook et al., 2009). To further complicate these taxonomic hypotheses, scale counts may be associated with climatic variation and local adaptation (Cherlin, 1983; Auffenberg & Rehman, 1991; Mallow et al., 2003); therefore, such characters may be of little utility in assessing species limits. Molecular phylogenetic analyses have recognised four species complexes (*E. carinatus, E. ocellatus, E. pyramidum* and *E. coloratus* complexes; Pook et al., 2009; Arnold et al., 2009), confirming the species status of many, but not all, morphologically delimited taxa, and found unexpected diversity within the *E. pyramidum* complex (Pook et al., 2009). Taxonomic authorities currently recognise 12 species within the genus (Uetz et al., 2024; Wallach et al., 2014).

High phenotypic variation in *Echis* is observed not only in external morphology, but also in phenotypic traits such as venom. Most notably, there is extensive variation in venom composition within *Echis*, which is

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thought to be a result of adaptation to diet (Barlow et al., 2009; Casewell et al., 2009; Richards et al., 2012). To understand the evolution of these traits, a clear understanding of species limits and a well-supported phylogeny is necessary (Fry et al., 2003). Moreover, accurate species delimitation within *Echis* have applications to medicine and snakebite treatment (Alshalah et al., 2024).

Here, we generate mtDNA sequence data for vouchered specimens, combining these with GenBank accessions, to create a time-calibrated phylogeny, sampling ten currently recognised species. This gene-tree was then used for multiple tree-based species delimitation approaches in conjunction with two distance-based delimitation methods, revealing several novel Evolutionary Significant Units (ESUs; Moritz, 1994). These ESUs are compared to the current taxonomy, and we suggest that the lineages identified here can be used as a guide for future geographic sampling and may be the most fruitful for an integrative taxonomic assessment.

We extracted genomic DNA from 24 tissue samples (Table S1 in supplementary materials) using a DNeasy Blood & Tissue kit (Qiagen Inc.) following manufacturer protocols. The Cytochrome b (cytb) gene was amplified using the H16064 and L14910 primers (Burbink et al., 2000). We performed a standard PCR in 25 µL reactions for each sample (Kreader, 1996; supplementary materials). PCR products were sent to ELIM Biopharmaceuticals (Hayward, CA, USA) for sequencing in both forward and reverse directions. We downloaded 110 cytb gene accessions from GenBank for *Echis* and 14 outgroup taxa for which node calibrations could be applied for divergence dating (Pook et al., 2009; Alencar et al., 2016; Šmíd & Tolley, 2019; Zaher et al., 2019; Table S1 in supplementary materials). We georeferenced all *Echis* accessions based on locality data provided in the original publication. The newly generated sequences were edited and trimmed in Geneious Prime v2023.2.1 (Biomatters Ltd.). All sequences were then aligned using Muscle v3.11 (Edgar, 2004) in Geneious. The mean final length of our cytb sequences was 1052 base pairs (GenBank ID PV599738-PV599761); our final alignment is available as a supplementary file (OSF ID https://osf.io/e6unw/). To estimate a maximum likelihood gene-tree, we used IQTREE v 2.2.2.7 (Minh et al., 2020; supplementary materials). We also estimated a time calibrated gene-tree using BEAST v2.7.5 (Bouckaert et al., 2019; supplementary materials). We note that the divergence times estimated here are gene divergences and may be overestimates of the species divergence times (Edwards & Beerli, 2000).

We used several tree- and distance-based, single-locus species delimitation methods on the 134 individual cytb dataset to identify ESUs that may warrant further taxonomic investigation. Tree-based delimitation methods use the topology and/or branch lengths of a phylogeny in favour of relying on threshold values of sequence divergence between two groups (Ahrens et al., 2016). We implemented several tree-based methods, including Multi-rate Poisson tree processes (mPTP; Kapli et al., 2017), Bayesian Poisson tree processes (bPTP; Zhang et al., 2013), and the General Mixed Yule Coalescent (GMYC; Fujisawa & Barraclough, 2013). By contrast, distance-based methods assume the amount of genetic variation within species is smaller than the amount of variation between species (Meyer & Paulay, 2005); they do not consider phylogenetic relationships between samples. For distance-based methods, we implemented the Automatic Barcode Gap Discovery (ABGD; Puillandre et al., 2012) and the Assemble Species by Automatic Partitioning methods (ASAP; Puillandre et al., 2021). Specifics regarding each method and parameters can be found in Methods in the supplementary materials. Resulting delimitations were compared using three metrics: match ratio (Ahrens et al., 2016), taxonomic index of congruence (C_{tax}), and relative taxonomic resolving power index (R_{tax}; Miralles & Vences, 2013). The match ratio statistic measures congruence between the current taxonomy and proposed ESUs. The C_{tax} metric measures congruence among species delimitation methods, where a value of 1 indicates complete congruence between two methods. R_{tax} quantifies the strength of a method to identify all estimated speciation events, where a value of 1 is indicative of all speciation events inferred by the delimitation method.

The *E. coloratus* complex is the oldest lineage within *Echis*, diverging ~20.1 mya (million years ago; HPD range: 13.7–29.4 mya; Fig. 1). All methods support the currently recognised *E. omanensis* species group, and two tree-based methods suggest additional diversity within this taxon. The crown age of *E. coloratus* is estimated at ~7.9 my (million years) and the two early diverging lineages within this species are supported as distinct ESUs by six methods (Fig. 1); these delimitations may be support for the taxonomic designations of Babocsay (2003). However, the specimens within these ESUs are located across Oman and Yemen, with geographically overlapping distributions (Table S1 in supplementary materials).

The stem age of *E. carinatus* is estimated at ~18.2 my (HPD range: 12.0–26.7 mya; Fig. 1). While current taxonomy recognises only a single species within this lineage, our results suggest an early diverging ESU (~9.7 mya; Fig. 1) distributed in the eastern portion of this taxon's range. This ESU is geographically distinct, occurring in southern and central India (Fig. S1 and Table S1 in supplementary materials). While some of the support for this ESU comes from tree-based methods that tend to over split lineages (Esselstyn et al., 2012; Talavera et al., 2013; Blair & Bryson, 2017) it is also supported by distance-based methods that are more conservative. We suggest that this ESU deserves further taxonomic investigation (Fig. 1).

The *E. ocellatus* and the *E. pyramidum* complexes were recovered as sister groups and diverged ~16.3 mya (HPD: 10.3–23.6 mya; Fig. 1). All *E. ocellatus* samples used here were GenBank accessions and we found support across all methods for the recently described *E. romani* by cross-referencing locality information

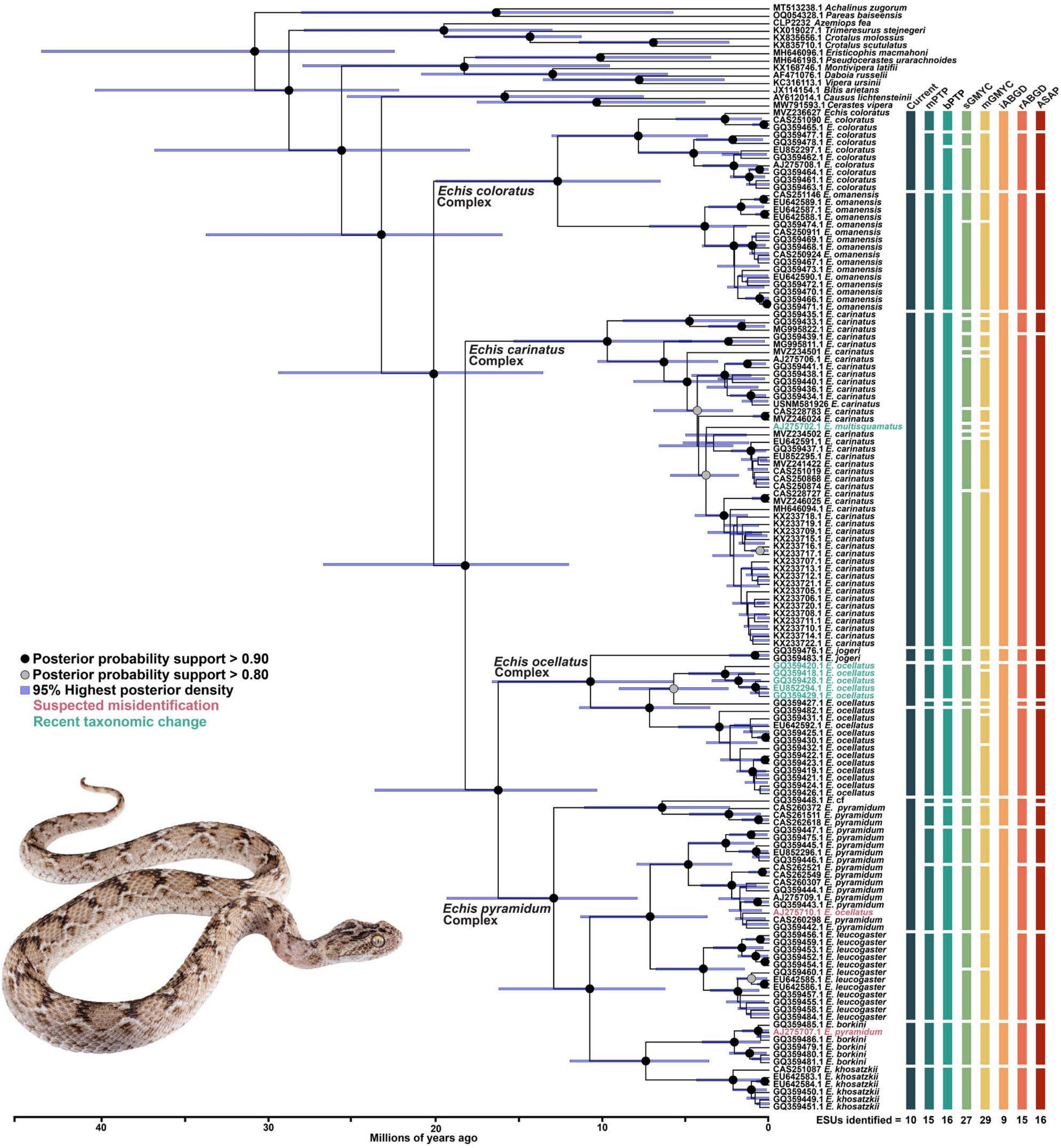

Figure 1. The Bayesian inference phylogeny containing 134 Cytochrome b sequences of the genus *Echis*. Posterior probability support > 0.90 is represented by black circles and > 0.80 with grey circles). Blue bars represent error around estimated gene divergence times. Each coloured rectangle to the right of the phylogeny represents the species limits drawn from seven delimitation methods, as well as the current taxonomy. Note that *Echis romani* is recently described, and therefore not labelled in the sequences (these names were kept the same as they appear in GenBank). *Echis ocellatus* specimens highlighted in green are estimated to be *E. romani* based on their geographic locality. Photo inset is of *Echis carinatus*.

from NCBI with range maps provided in Trape (2018). Six of the methods used here suggest an additional ESU in West Africa; however, this ESU is represented by a single specimen on a long branch (Fig. 1). The currently recognised *E. pyramidum* is paraphyletic with up to four ESUs (Fig. 1). The earliest divergence within this complex occurred ~12.9 mya (HPD range: 7.9–19.3 mya; Fig. 1). This ESU is considered distinct by all delimitation methods, and is located across Ethiopia, Sudan and Eritrea (Table S1 in supplementary materials). Five of our analyses also distinguish the Ethiopian specimen sequenced by Pook et al. (2009) as an ESU separate from this clade. Lastly, six methods support an additional split in *E. pyramidum* that produced two ESUs, diverging ~4.9 mya (Fig. 1). One of these ESUs is comprised of specimens from Kenya, whereas the rest of the samples

Table 1. Values for three metrics used to compare species delimitation methods used in this study. The match ratio statistic measures discrepancies between an empirical species delimitation method and the currently recognised taxonomy. The Relative taxonomic resolving power index (R_{tax}) infers the strength of a method to identify all estimated speciation events, where a value of 1 indicates a method identifies all speciation events that are estimated across all methods. The taxonomic index of Congruence (C_{tax}) compares the delimitations made between individual methods, with 1 indicating complete congruence.

	Match ratio	R_{tax}
mPTP	0.56	0.4667
bPTP	0.5385	0.5
sGMYC	0.2162	0.8667
mGMYC	0.1538	0.9333
iABGD	0.6316	0.2667
rABGD	0.48	0.4667
ASAP	0.4615	0.5

C^{tax}	mPTP	bPTP	sGMYC	mGMYC	iABGD	rABGD	ASAP
mPTP							
bPTP	0.8667						
sGMYC	0.3846	0.4231					
mGMYC	0.3214	0.2759	0.7586				
iABGD	0.2857	0.2667	0.0769	0.0714			
rABGD	0.7333	0.625	0.3077	0.25	0.2857		
ASAP	0.8667	0.75	0.3846	0.3214	0.2	0.8667	

span Eritrea, Sudan, Egypt and Mali. We suspect that one of these ESUs may represent *E. pyramidum leakeyi* (Stemmler & Sochurek, 1969).

Comparisons between the current taxonomy and molecular delimitation results reveal high variance in congruence, number, and structure of ESUs (Table 1). Due to species delimitation model assumptions regarding intra- and interspecific variation, diversity within a group is highly dependent upon the method applied to the dataset. The GMYC methods recovered the highest number of ESUs, with high R_{tax} values, where the likelihood of over splitting is highest (Miralles & Vences, 2013). Previous work has shown that GMYC models tend to over split (Esselstyn et al., 2012; Talavera et al., 2013; Blair & Bryson, 2017), and our results corroborate these findings. This tendency is largely due to the sensitivity of the model to variation in effective population sizes and uneven sampling intensity across populations (Esselstyn et al., 2012; Ahrens et al., 2016). While we generated new sequence data from *Echis* specimens collected in three countries not previously included in phylogenetic analyses (Afghanistan, Eritrea and Iran; Fig. S1 in supplementary materials) large sampling gaps exist, as do disparities in sampling intensity between species.

Both Poisson Tree Process (PTP) methods suggest similar levels of ESU diversity. The R_{tax} values of these methods indicate minimised potential for delimitation errors and may provide more realistic views of species diversity (Table 1; Miralles & Vences, 2013). The discrepancies in number of estimated ESUs between these two methods could result from mPTP's model assumptions that account for differences in coalescent times, effective population sizes and sampling intensity within the data set; bPTP does not account for these factors and may lead to oversplitting (Kapli et al., 2016). Because mPTP is designed to account for variation in the data, it may be best suited for empirical data sets, as it produces a realistic view of species limits and has previously been considered the most reasonable tree-based delimitation method (Blair & Bryson, 2017; Hofmann et al., 2019).

The distance-based method, rABGD, is largely congruent with mPTP in terms of R_{tax} and C_{tax} (Table 1). These methods differ in two ways: 1) rABGD delimited an ESU within *E. carinatus*, estimated to have diverged from the rest of the species complex ~9.71 mya, and 2) mPTP, and all methods other than rABGD and iABGD, recognise a singleton in *E. pyramidum* that diverged ~6.43 mya. These differences may be due to how tree- and distance-based methods interpret genetic data in delimiting ESUs. Distance-based methods do not rely upon relationships between samples or branch lengths in estimated gene-trees and have been suggested to outperform tree-based methods in some scenarios (Guo & Kong, 2022). The results for iABGD and rABGD differ in the hypothesised ESU diversity by a wide margin (Fig. 1). This is because of the reapplication of the barcode gap in rABGD that continues to split groups until no further partitions are inferred (Puillandre et al., 2012). Though rABGD has been found to delimit more groups than iABGD and is known to manage higher levels of genetic variation, it is more stable, often suggesting species limits supported by morphological taxonomies (Puillandre et al., 2012). We find a similar result in our data, with iABGD having the greatest congruence with the current taxonomy, though this may be a result of iABGD being overly conservative (Table 1; Puillandre et al., 2021). Lastly, ASAP results are largely congruent with that of rABGD, baring one singleton ESU in *E. pyramidum*. These methods show high C_{tax} congruency with mPTP and bPTP, which all hypothesise undescribed diversity within *Echis*. This consistency across tree- and distance-based delimitation methods supports the need for taxonomic revision in this medically significant genus by providing candidate species in need of further validation through integrative analyses.

Our results support studies based solely on morphology (Trape, 2018) and revealed several lineages that warrant further investigation for potential taxonomic revision across *Echis* (Fig. 1). For example, the Ethiopian specimen within *E. pyramidum* previously suggested to be a novel lineage (Pook et al., 2009), was supported as a distinct ESU from *E. pyramidum*, and may be part of a more widespread, undescribed species. This group, among other ESUs within *Echis*, further exemplify the need to generate genomic-scale datasets and apply coalescent methods in determining species diversity within the genus, despite the previous underutilisation of these techniques to describe species limits within Viperinae (Dufresnes et al., 2024).

This study was conducted using only mtDNA sequence data, which is inadequate as the sole basis for taxonomic revision (Moritz, 1994; Puorto et al., 2001; Sites & Marshall, 2004). However, due to the uniparental inheritance of mtDNA resulting in a rapid coalescence of haplotypes, single-locus analyses are often a good starting point for directing future research efforts (Wiens & Penkrot, 2002; Zink & Barrowclough, 2008; Pelletier et al., 2022). Here, we have used an mtDNA marker and congruency across multiple species delimitation methods to emphasise areas of remaining uncertainty within *Echis* taxonomy that should be clarified. The contribution of newly sequenced geographic regions, confirmation of recently described species, and identification of ESUs across the *Echis* phylogeny highlights the need for extensive future effort in better understanding the true diversity within these vipers regarding distribution, ecology and phenotypic evolution.

Regarding further taxonomic analysis of *Echis*, we suggest the implementation of an integrative taxonomy through the combination of coalescent-based speciation models that incorporate both genomic and morphological data (Padial et al., 2010; Solís-Lemus et al., 2015). For example, genetic, morphological and ecological data can be used in conjunction via an integrative machine learning approach for species delimitation (Derkarabetian et al., 2022; Pyron, 2023; Martin et al., 2021). Such integrative approaches should be implemented to better understand species diversity within *Echis*. Through integrating multiple lines of evidence, inferences regarding the speciation history of these ESUs can be made, such as distinguishing between true species and locally adapted populations (Burbrink et al., 2024). Our findings highlight the potential for significant unresolved diversity within *Echis*, emphasising the need for integrative approaches that combine genetic, morphological and ecological data. Determining species boundaries, particularly within the ESUs we have proposed, is essential not only for clarifying taxonomy, but for understanding the evolutionary history of these medically significant vipers.

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Author contributions

Dylan Koalani Highland (DKH) and Edward A. Myers (EAM) wrote the manuscript. DKH performed lab analysis and extracted sequences. DKH and EAM performed delimitation analysis. DKH and EAM edited and reviewed the manuscript.

Data accessibility

Newly generated sequences are publicly available on NCBI under GenBank accession numbers PV599738-PV599761. Alignments, trees, and additional data are available on OSF (https://doi.org/10.17605/OSF.IO/E6UNW).

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