



Shedding the mitochondrial blinkers: A long-overdue challenge for species delimitation in herpetology

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Abstract

The advent of molecular methods has revolutionised the field of species delimitation and description, one of the key tasks of systematic biology. In animal taxonomy, one marker, the mitochondrial DNA (mtDNA) molecule, has acquired and retained disproportionate influence. This is despite its uniparental, clonal mode of inheritance, as a result of which the entire molecule acts as a single locus, and that precludes its use as a test for admixture between putative lineages, a key consideration in species delimitation. To establish the extent to which the limitations of mtDNA affect present-day taxonomy in non-avian reptiles, I surveyed species descriptions and delimitations published during the years 2023–2024, determined the markers used, and whether analyses of different markers were set up to critically test or just to confirm mtDNA-inspired candidate species. Mitochondrial DNA remains the dominant molecular marker in reptile taxonomy, being used in 84% of species descriptions and delimitations, and as the sole molecular marker in 44%. Despite the immense progress in next generation sequencing (NGS) technologies and their increasing affordability, only 3.4% of descriptions used NGS approaches. In 61% of descriptions, taxa were identified primarily through mtDNA divergence, and additional data (morphology, single-copy nuclear gene sequences) were used as confirmatory evidence rather than as rigorous tests of mitochondrially inferred species limits. I reiterate the importance of truly integrative species delimitation that critically tests species limits first hypothesised from mtDNA, and suggest ways of improving the robustness of species delimitations by optimising the allocation of resources to more appropriate markers and through analytical approaches that critically test the evolutionary independence of putative species.

Keywords

Herpetology, integrative taxonomy, mitochondrial DNA, morphometrics, multilocus analyses, Reptilia, species delimitation, taxonomy

Introduction

The advent of the molecular age in systematics has resulted in a rapid increase in the rate of species descriptions in many taxa, including reptiles (Uetz 2010; Guedes et al. 2024), and also in an increase in the number of lines of evidence underpinning species delimitations, including the increasing use of molecular data and multilocus ana-

lyses (Guedes et al. 2024). However, while the development of molecular methods has expanded the possibilities for species discovery and delimitation, some approaches have become the topic of ongoing controversy. In particular, the mitochondrial DNA molecule has revolutionised animal taxonomy, but many authors have failed to

fully consider the limitations of this marker, resulting in multiple controversial and unstable species delimitations (Hillis et al. 2021; Marshall et al. 2021; Reyes-Velasco 2024; Wüster et al. 2024; Reynolds 2025) that have detracted from the urgent need to inventory the biodiversity of our planet as we progress into the Anthropocene mass extinction.

The enumeration of the biodiversity of our planet is one of the key tasks of systematic biology. The fundamental unit of this biodiversity is the species. Species not only constitute the taxonomic rank that is closest to being objectively definable, but also form the basis of many conservation metrics and efforts, such as IUCN Red Lists, as well as of regulatory instruments, including both international agreements, such as CITES, and national legislation. Despite the need for accurate species lists for these purposes (Garnett et al. 2020; Lien et al. 2023; Guedes et al. 2024), our knowledge of the world's alpha taxonomy remains woefully inadequate, a phenomenon known as the Linnaean shortfall (Brown and Lomolino 1998). Given the ongoing rate of global biodiversity loss, assembling the inventory of the world's species is more urgent than ever, especially given that undescribed species remain overlooked in conservation planning (May 1990) and may be more likely to be under threat than named species (Liu et al. 2022). Herpetology is in no way exempt from this trend: A steadily increasing rate of new species descriptions over the last 35 years illustrates the incompleteness of our current knowledge (Uetz and Stylianou 2018; Guedes et al. 2024).

However, despite the pressure of time, species descriptions and delimitations are only useful if they are rigorous and provide the evidence required to support their case, thereby reducing the likelihood of instability from later corrective changes (Garnett and Christidis 2017; Hillis 2019; Thiele et al. 2021; Wüster et al. 2021, 2024; Braby et al. 2024). Unconvincing species delimitations and descriptions lead to instability, which hinders rather than enhances communication, conservation efforts and regulation (Garnett and Christidis 2017; Jiménez-Mejías et al. 2024). There is therefore a strong onus on taxonomists to follow best possible practice within the constraints of the resources available to them.

Background: Systematics and the role of mitochondrial DNA

What is a species?

While there is a long history of controversy over what species are and how they should be delimited, the General Lineage Concept of species (de Queiroz 1998, 2007), defining species as the smallest metapopulation lineages on independent evolutionary trajectories, encapsulates what most systematists would regard as the essence of what a species is. As noted by de Queiroz (1998, 2007), most prior disputes about "species concepts" disagreed

more about the criteria by which such lineages might be diagnosed (e.g., reproductive isolation vs. diagnosable character states) than about the actual concept of what a species is.

Hennig (1950, 1966) defined the point of speciation as the point where tokogenetic relationships, where individuals mate and their genes recombine within a lineage, are replaced by phylogenetic lineages, where there is no such exchange or recombination between lineages on an evolutionary timescale, and the lineages show bifurcating, hierarchical relationships amongst themselves. Within this framework, it becomes the central task of species delimitation to identify the point where tokogenetic patterns of genetic exchange cease and lineages become independent. Where potential species are in geographical contact, more or less complete reproductive isolation remains a key test of whether two lineages are indeed on independent evolutionary trajectories, and thus a fundamental criterion for species delimitation (Hillis 2019; Hillis et al. 2021; Jarrett et al. 2025), even though newer sequencing technologies have highlighted the persistence of varying degrees of introgression and hybridisation even among well-defined separate species (Schield et al. 2019; Edelman and Mallet 2021; Aguillon et al. 2022; Schöneberg et al. 2023; Myers et al. 2024).

Molecular systematics and the role of mitochondrial DNA

Until the second half of the 20th century, morphology and other phenotypic traits (e.g., behaviour, physiology), as well as crossbreeding experiments, were the only sources of evidence available to systematists to support their inferences. The molecular revolution in systematics gained momentum with increasing ease of access to DNA sequence information. In particular, mitochondrial DNA (mtDNA) provided a relatively accessible route to genetic information, which moreover evolves at a rate that makes it suitable for systematics at low taxonomic levels (Avice et al. 1979). Sequencing mtDNA fragments became the default approach following the development of the Polymerase Chain Reaction, the use of thermostable primers (Mullis and Faloona 1987; Saiki et al. 1988), and direct sequencing of PCR products using conserved priming sites (Kocher et al. 1989). Since then, despite multiple technological and conceptual developments, mitochondrial DNA has retained a perhaps disproportionate importance in herpetological systematics and species delimitation.

The properties that first made mitochondrial DNA popular in systematics include its high rate of evolution, making it useful at low taxonomic ranks, including for intraspecific phylogeography (Avice et al. 1987), and its clonal, matrilineal mode of inheritance. The lack of recombination means that the entire mtDNA molecule is inherited as a single locus, allowing multiple gene sequences to be concatenated into larger datasets to provide greater support for the mitochondrial gene tree, although care must be taken to avoid the accidental inclusion of

nuclear integrations (Numts – Zhang and Hewitt 1996). The smaller effective population size of mitochondrial DNA (1/4 that of nuclear autosomal genes) results in more rapid coalescence times. As a result, other things being equal, a mitochondrial gene tree is more likely to track organismal phylogeny than any single autosomal gene fragment (Moore 1995). Moreover, as the gene content of the mtDNA molecule is largely conserved across all Metazoa, orthologous gene sequences can be obtained from a phylogenetically broad spectrum of animal life. Due to these unique properties and the insights they provide, mtDNA remains a key component of systematic research in animals (Blair 2023).

The role of mitochondrial DNA in species delimitation was further emphasised by the advent of the barcoding initiative (Hebert et al. 2003a, 2003b), whose central aim is to establish a standard mitochondrial marker for both specimen identification and biodiversity discovery, including species delimitation (Hebert et al. 2004b). The formulation of the concept of a “barcoding gap” (Hebert et al. 2003b) separating intraspecific from interspecific divergences (Hebert et al. 2004a) triggered the development of multiple approaches to species delimitation from single locus (usually mtDNA) data. Some of these methods are based on the detection of a barcode gap (e.g., ABGD, ASAP; Puillandre et al. 2012a, 2021), whereas others are tree based (e.g., GMYC, PTP; Pons et al. 2006; Zhang et al. 2013). The use of barcoding for species delimitation was taken to extremes in entomology, where some researchers described hundreds of species based on nothing but a consensus barcode sequence and a photograph (e.g., Sharkey et al. 2021), causing extensive controversy (Ahrens et al. 2021; Ahrens 2024). This extreme approach has so far not been extended to vertebrate taxonomy.

The limits of mtDNA

While its properties, mode of inheritance and rapid rate of sequence evolution make mtDNA logistically and economically attractive, these very features are responsible for the many limitations that make it unsuitable as a sole source of evidence in species delimitation (Miralles et al. 2024). Its solely matrilineal mode of inheritance, contrasting with the biparental inheritance of the nuclear genome, precludes the detection of admixture between lineages from mtDNA alone (Fig. 1) and can generate conflict between mitochondrial phylogeographic structure and genomic (nuclear) genetic structure in multiple ways. This phenomenon, variously referred to as mitonuclear or cytonuclear discordance, can potentially cause mitochondrial phylogeography to be grossly incongruent with patterns of genomic population structure. In the following paragraphs, I review the mechanisms underlying mitonuclear discordance in a number of herpetological examples.

Do phylogeographic breaks necessarily reflect population genomic breaks? A common assumption in phy-

logeographic and mtDNA-led systematic studies is that phylogeographic breaks correspond to past or present barriers to gene flow and reflect discontinuities in the genomic make-up of the phylogroups. However, this need not be the case. Irwin (2002) demonstrated that phylogeographic structure can evolve in situ in a continuously distributed taxon, without past or present range fragmentation. Contrasting population structures suggested by mitochondrial and nuclear markers have long been documented (Palumbi and Baker 1994): Female stasipatry combined with male vagility can result in strong mitochondrial phylogeographic structure within a single gene pool that misleadingly suggests population differentiation or speciation, potentially resulting in gross overestimates of species numbers (Eberle et al. 2019; Schultze et al. 2020).

Inability to assess introgression and hybridisation.

Since the entire mtDNA molecule is (usually) inherited matrilineally and clonally, it follows that it cannot show admixture. Even in the presence of rampant hybridisation, each hybrid will carry the mtDNA haplotype inherited from its mother and her mother, etc. (Fig. 1). Consequently, a mtDNA phylogeny of two hybridising species will identify each specimen by its matrilineal ancestry only, and the topology of the mitochondrial gene tree alone will give no indication of the occurrence of hybridisation (e.g., Zancolli et al. 2016). Where mitochondrial haplotype clades overlap geographically, the mtDNA phylogeny alone cannot reveal whether these haplogroups represent independently evolving, sympatric organismal lineages or relictual mitochondrial haplotype clades within a single gene pool, e.g., after secondary contact (Castoe et al. 2007; Schield et al. 2015).

Since detecting admixture is key to distinguishing between tokogenetic and phylogenetic processes, the central task of species delimitation, it follows that on this basis alone, mtDNA cannot be used as the sole source of evidence for species delimitation. The North American ratsnake (*Pantherophis obsoletus*) complex is a prominent example where parapatric contact zones inferred from mtDNA led to the delimitation of four separate species (Burbrink 2001), whereas later genomic analyses (Burbrink et al. 2021a) showed broad zones of admixture between three of the lineages. Similarly, whereas the main mitochondrial genetic break in western rattlesnakes (*Crotalus oreganus*) along the North American Pacific coast coincides with the Transverse Ranges of southern California (Schield et al. 2019; Pook et al. 2000), a recent genomic study (Holding et al. 2021) placed the main genetic break in the species near the Sacramento and San Joaquin River Delta on San Francisco Bay.

Selection pressure versus population history. Especially in the presence of male-biased gene flow, changes in selection pressures, such as across ecotones, may drive population genomic breaks reflecting major barriers to gene flow that are entirely independent of mitochondrial phylogroups that reflect past lineage fragmentation. In some cases, patterns of genomic differentiation can cut

orthogonally across the distribution of mtDNA haplotype clades (Smith et al. 1997; Ogden and Thorpe 2002). In these situations, the mitochondrial phylogeographic structure is almost completely unrepresentative of the genomic and phenotypic differentiation of these populations, and thus taxonomically irrelevant.

“Lost” mitochondrial species identity. Mitochondrial introgression can lead to the loss of species-specific mitochondrial haplotype clades. For instance, the Carpathian newt (*Lissotriton montandoni*) is a well-supported and universally recognised species of newt. However, due to mitochondrial introgression from *L. vulgaris*, it “disappears” into the latter species in a mtDNA-only phylogeny (Babik et al. 2005), whereas there is little evidence of matching nuclear gene flow (Zieliński et al. 2013).

Mitochondrial “ghost” species. Matrilineally inherited haplotype clades can persist in gene pools despite extensive gene flow with other populations, even when most of the genome has been replaced by that of the introgressing species. *Vipera walser* was described from northwestern Italy by Ghielmi et al. (2016), based on mtDNA sequences associating these populations with the *V. kaznakovii* complex from the Caucasus, rather than with the widespread *Vipera berus* to which they had previously been assigned. However, Doniol-Valcroze et al. (2021) and Dufresnes et al. (2024) found that the taxon instead shares most genomic similarity with the northern Italian clade of *V. berus* (*V. berus marasso*), and Dufresnes et al. (2024) recognised it as a subspecies of *V. berus*, *V. b. walser*.

The origin of the Caucasian mtDNA haplotype clade of these populations remains an intriguing biogeographical enigma, but has little or no bearing on their overall affinities. In a similar vein, based on a mitochondrial phylogeny, Pyron and Burbrink (2009a, 2009b) recognised the black and speckled kingsnakes, previously subspecies of *Lampropeltis getula*, as separate species, *L. nigra* and *L. holbrooki*. However, a later genomic study showed both to be part of a single organismal lineage together with *L. getula* sensu stricto, leading to their synonymy with the latter (Harrington and Burbrink 2022).

The problematic role of mtDNA in species delimitation. While single-locus species delimitation algorithms claim increased precision in inferring species limits from mitochondrial sequence data (e.g., Pons et al. 2006; Puillandre et al. 2012a; Zhang et al. 2013), it must be remembered that all rely on the assumption that the mitochondrial tree reflects organismal phylogeny: No degree of sophistication of these methods can control for or overcome the problem of cytonuclear discordance (Miralles et al. 2024).

In summary, while mitochondrial DNA sequences remain invaluable as tools to generate species hypotheses, and may well reflect aspects of population history such as past range fragmentation, numerous potential confounding factors preclude their use as the sole or dominant source of evidence for species delimitation. Critical testing of mtDNA-derived species hypotheses using independent marker sets, which can be either nuclear genetic markers or phenotypic characters, and analytical approaches capable of detecting admixture between

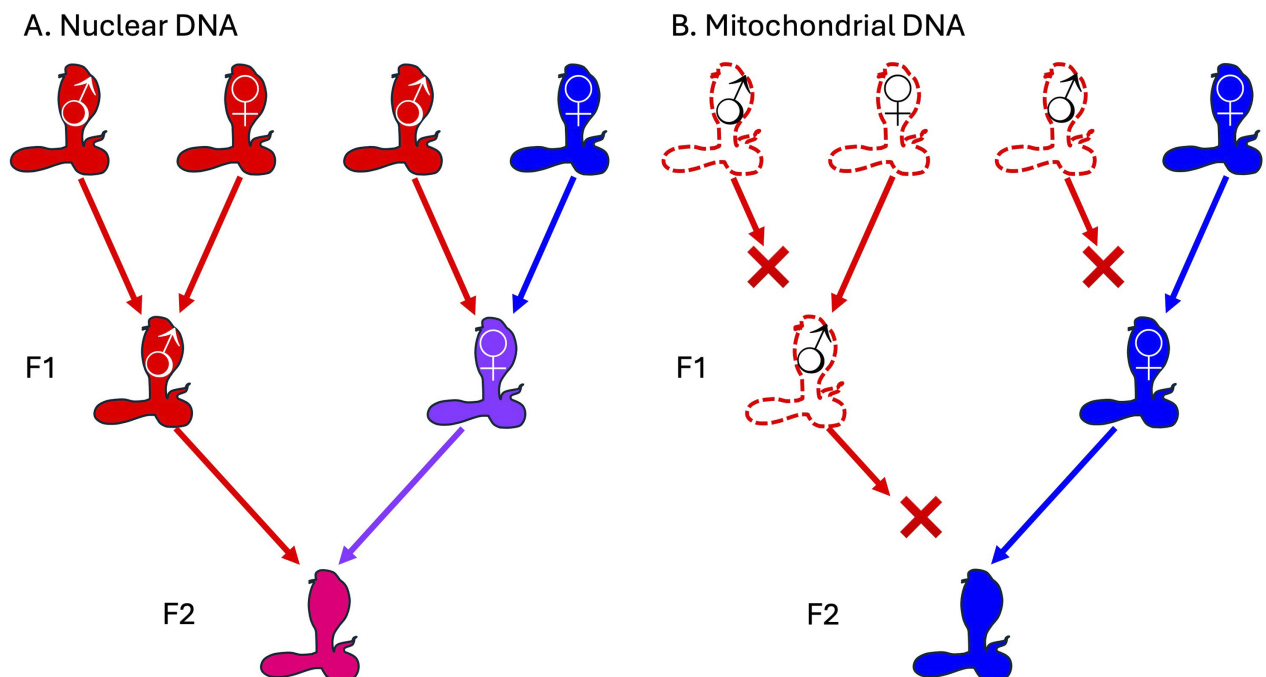


Figure 1. Schematic illustration of the impact of the different modes of inheritance of nuclear and mitochondrial DNA on the ability to detect admixture between a hypothetical red and a blue species. **A** Nuclear DNA is inherited biparentally and reflects admixture in hybridization. In this case, the nuclear markers of the F2 offspring will reflect a genome originating to 75% from the red species. **B** Mitochondrial DNA is inherited matrilineally only. Therefore, the information on lines of descent involving male ancestors (indicated by dashed outlines) is lost from the mitogenome of the offspring. The mtDNA of the F2 offspring will reflect the matrilineal ancestry of the blue species only, without any indication of the 75% admixture from the red species.

putative taxa must be considered a fundamental and critical part of any species delimitation attempt (Padiál et al. 2010; Miralles et al. 2024; Vences et al. 2024).

Integrative taxonomy to the rescue

The formulation of the General Lineage Concept of species (de Queiroz 1998, 2007) emphasised the multiplicity of criteria that can be used to delimit species, and spawned the framework of integrative taxonomy. Authors such as Dayrat (2005), Will et al. (2005) and Padiál et al. (2010) advocated for an integrative approach to taxonomy, whereby different sources of evidence are used to test species hypotheses formulated on the basis of one marker. In particular, Padiál et al. (2010) formulated a workflow where mitochondrial haplotype clades are treated as testable hypotheses termed Candidate Species (Padiál et al. 2010) or Primary Species Hypotheses (Puillandre et al. 2012b; Miralles et al. 2024), which can then be critically tested with additional evidence (Padiál et al. 2010; Miralles et al. 2024). Where such additional lines of evidence support species status for a Candidate Species or Primary Species Hypothesis, these achieve the status of Confirmed Candidate Species (Padiál et al. 2010) or Secondary Species Hypotheses (Puillandre et al. 2012b), which form the basis for taxonomic decisions.

Although de Queiroz (2025) re-emphasised that evolutionary lineages should be treated as species without recourse to additional properties, such as reproductive isolation, most treatises on species delimitation continue to place considerable emphasis on the need to demonstrate a considerable degree of reproductive isolation (Padiál et al. 2010; Miralles et al. 2024). Where gene flow between putative lineages is high, it becomes increasingly difficult to support the argument that there are indeed multiple lineages in existence. Consequently, where candidate species occupy contiguous ranges in parapatry or sympatry, understanding the extent of reproductive exchange between them remains a critical test of these hypotheses, even though there remains ample legitimate disagreement in the taxonomic treatment of incipient lineages with incomplete reproductive isolation (e.g., Burbrink et al. 2021b; Burbrink and Ruane 2021; Hillis et al. 2021; Thiele et al. 2021; Hillis 2022).

What constitutes a critical test of mitochondrially defined candidate species?

Mitochondrial DNA sequences will unquestionably continue to be an important source of data in animal systematics in the foreseeable future. However, the main role of mtDNA should be seen as the crucial and often underrated function of hypothesis generation rather than that of hypothesis testing, or, worse, as a sole source of evidence in species delimitation (Blair 2023). However, framing the role of mitochondrial DNA in this manner then begs the question of what approaches would be appropriate to

provide the critical tests of mitochondrially defined candidate species, as recommended by recent works (Padiál et al. 2010; Miralles et al. 2024).

The key requirement for any delimitation analysis is that it must be designed to critically test the status of mitochondrial candidate species as independent evolutionary lineages. A critical test must be able to explicitly reject such a primary species hypothesis, not just fail to support it. For example, failure to find statistically significant differences in a phenotypic trait would fail to support a primary species hypothesis, but not reject it; in contrast, if a trait displays a pattern of clinal variation that crosses the putative boundary between the candidate species, this would constitute explicit evidence favouring rejection of the species hypothesis.

I therefore distinguish between two types of approaches: (i) critical approaches that can test and actively reject species hypotheses; and (ii) confirmatory approaches that can at best fail to actively support a species hypothesis, but cannot refute it. Broadly, methods that simply treat each of the candidate species as pre-defined units of comparison cannot critically test them, and are therefore confirmatory. To illustrate the importance of the distinction between critical tests and confirmatory approaches, Figure 2 shows a hypothetical scenario where two mitochondrial candidate species are distributed parapatrically along a geographic and environmental gradient. Genomic data and morphology both show continuous clinal variation across the gradient, including across the boundary between the mitochondrial lineages. These patterns should lead to rejection of the two candidate species hypotheses, as the mitochondrial haplotype clades clearly exist within a single panmictic gene pool. Any species delimitation analysis intended to offer a critical test must therefore be capable of revealing these patterns. Any analysis that simply compares the two pre-defined candidate species as single units of comparison would mask the clinal pattern of variation, and lead to the misinterpretation of the data as confirming the two candidate species. Consequently, for contiguously distributed candidate species, such approaches are essentially tautological.

This scenario is unlikely to be of relevance in situations where mitochondrial candidate species are allopatric and restricted to small, isolated ranges, such as oceanic islands or isolated mountains, where continued genetic exchange is highly improbable. However, structure within candidate species and continuing genetic exchanges between them must be critically evaluated in any situation where the candidate species are contiguously distributed, or likely to have been recently, prior to Pleistocene climatic and sea level fluctuations.

Population genetic approaches to the analysis of nuclear loci. The critical analysis of nuclear loci is a key component of species delimitation, especially where candidate species were first formulated from mtDNA sequence data. A multitude of approaches that do not require a priori grouping of samples into candidate species are available. Algorithms that can detect the number of distinct populations in a set of individual specimen data,

such as STRUCTURE or STRUCTURAMA (Pritchard et al. 2000; Huelsenbeck et al. 2011), or analogous programs for next-generation sequencing data, such as NGSADMIX (Skotte et al. 2013), have clear potential for species delimitation and independent testing of mitochondrial candidate species, although even here, interpretation must be cautious (Wiens and Colella 2025). Haplotype networks generated by software such as NETWORK (Fluxus Technology Ltd. – www.fluxus-engineering.com) can visualise the extent of haplotype sharing between individuals, which can be sufficient to document reproductive isolation in some cases (e.g., Ratnarathorn et al. 2023). Multilocus genetic distances can be calculated through programmes such as POFAD (Joly and Bruneau 2006), and the resulting matrix of between-individual distances visualised through a principal coordinates analysis (Barlow et al. 2013; Gowri Shankar et al. 2021). Genetic distance matrices can also be tested for the relative degree of association with geographic distance (IBD) and candidate species membership using Mantel tests (Jensen et al. 2005). All these approaches can visualise or explicitly test the degree of reproductive isolation between candidate species without the biases often associated with a priori grouping of specimens.

Testing candidate species with phenotypic characters.

Most phenotypic traits are likely to be under polygenic control, and the analysis of multiple traits can be used to provide a proxy for overall genomic variation, either

in conjunction with or instead of nuclear genetic markers. Candidate species would be confirmed by fulfilling the prediction that they constitute phenotypically discrete entities distinct from other such entities. Similar considerations apply here as to nuclear markers: Only methods that consider variation both within and between candidate species can positively reject these primary species hypotheses. Suitable approaches include PCA and derivatives such as MFA and NMDS, as well as the use of Mantel tests to determine whether a given pattern of geographic variation is better explained by IBD or assignment to candidate species (Puerto et al. 2001).

What does not constitute appropriate tests of mitochondrial candidate species?

Analyses of concatenated multilocus sequence data.

The key reason for generating multilocus datasets should be to test for cytonuclear discordance, thereby challenging mitochondrial candidate species. However, phylogenetic analyses of concatenated multilocus datasets shoehorn all loci into a single tree topology, and thereby entirely negate their ability to reveal cytonuclear discordance. Concatenating multiple highly variable mitochondrial sequences with a few relatively conserved nuclear loci generates a mitochondrial phylogeny with added noise, not a multilocus phylogeny in any meaningful sense (Wüster

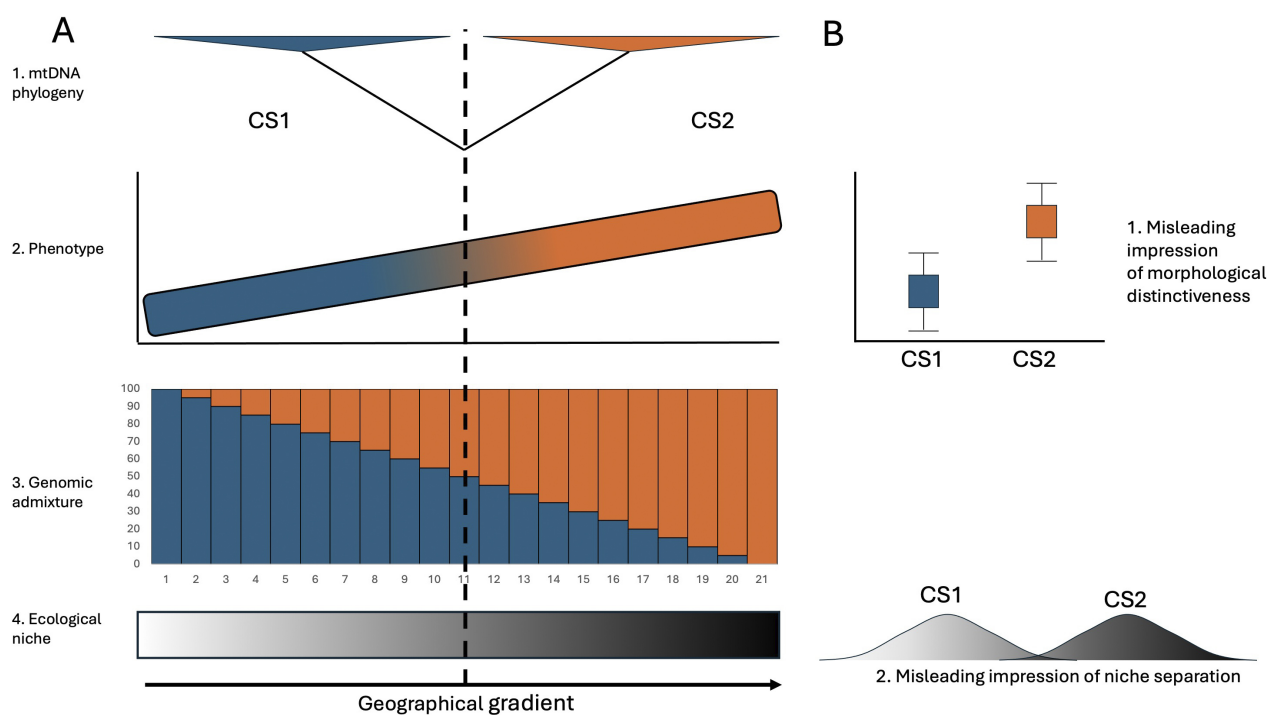


Figure 2. Hypothetical scenario of two mitochondrial candidate species distributed along a geographic and ecological gradient. **A** observed patterns: 1) parapatrically distributed mitochondrial candidate species CS1 and CS2; 2) clinal phenotypic variation along geographical gradient; 3) genomic signature of isolation by distance along geographical gradient as revealed by software such as STRUCTURE (Pritchard et al. 2000); 4) continuous distribution through ecological gradient. Patterns 2–4 reject the candidate species hypothesis derived from 1. **B** consequences of analyses treating the candidate species CS1 and CS2 as a priori units of comparison: 1) misleading impression of confirmation of candidate species from phenotypic differences; 2) misleading impression of confirmation of candidate species from ecological niche differences.

et al. 2024). Concatenated analyses may be appropriate for inferring a species tree where there is no significant conflict between different markers but have no place in species delimitation.

Some multispecies coalescent (MSC) approaches. Bayesian species delimitation analyses such as those implemented in the popular programs BPP and BPP3 (Yang and Rannala 2010, 2014) have become popular in systematics (Leaché and Fujita 2010). Their ability to account for conflict between gene trees in both species tree reconstruction and species delimitation represents a major step forward compared to concatenated analyses of multilocus data. However, important limitations of the models as originally conceived include (i) the requirement for specimens to be grouped a priori into candidate species, (ii) assumption of lack of genetic structure within the candidate species, and (iii) assumption of a lack of migration (i.e., gene flow) between candidate species (Barley et al. 2018). Since the extent or lack of gene flow between candidate species is exactly what should be tested rather than assumed, this makes the use of such methods problematic in cases of candidate species with contiguous distributions (Chambers and Hillis 2020). Similarly, the methods are sensitive to isolation by distance, especially in conjunction with sparse geographic sampling, which is liable to lead to oversplitting (Sukumaran and Knowles 2017; Chambers and Hillis 2020; Mason et al. 2020). While MSC approaches are being refined to take gene flow into account (Jackson et al. 2016; Kornai et al. 2024), users must remain critically aware of the assumptions of the specific analyses they are using and how these may be violated by their data.

Analyses of phenotypic variation treating candidate species as units of comparison. As shown in Figure 2, it is essential to test critically for congruence between phenotypic variation and mitochondrial phylogeography. Any methods that require grouping individuals into a priori Operational Taxonomic Units (OTUs) cannot do so if each candidate species is treated as a single OTU. That includes simple tables of morphological comparisons between candidate species, t-tests or ANOVAs treating candidate species as units of comparison. Approaches that seek to maximise separation between a priori-defined groups, such as canonical variate or discriminant function analyses, are especially problematic if they treat the candidate species as single OTUs. These procedures effectively cherry-pick those traits that happen to approximately coincide with the candidate species while ignoring those that show different patterns of variation and could potentially reject these hypotheses. They are thus effectively tautological. A work-around for many of these approaches can be dividing each candidate species into multiple OTUs, which then allows testing of the prediction that multiple OTUs of each candidate species should form homogeneous groups distinct from the OTUs of other candidate species (Thorpe 1983).

It is also important to be aware of approaches that rely on a priori grouping of specimens as a preparatory step

prior to further analyses, such as corrections for size or the estimation of missing data. These can be problematic even if the final analysis itself does not require a priori grouping. For instance, correcting for overall size to identify differences in shape requires grouping into OTUs (Thorpe 1975, 1976). At this point, grouping artefacts can be introduced inadvertently. For instance, if, as suggested for multispecies analyses in the description of the popular R package GroupStruct (Chan and Grismer 2022), dependent variables are regressed to a different adjusted SVL in each OTU, then the impacts of size differences between OTUs will be magnified, not controlled, which will eclipse the differences in shape and artificially inflate the distinctness of the OTUs in downstream analyses. Moreover, the use of different regression slopes β in different OTUs, rather than the pooled within-OTU regression slope (Thorpe 1975, 1976), to adjust a dependent variable could lead to misleading downstream results (Reist 1986). While grouping specimens for preparatory analyses is often unavoidable, care must be taken to avoid grouping artefacts that will impede the usefulness of the data for critically testing species boundaries.

Ecological niche modelling. The notion of ecological divergence as an indicator of speciation has a long history in systematics (Van Valen 1976). The advent of ecological niche modelling (Guisan and Thuiller 2005) has allowed a much more rigorous circumscription of the ecological niches of a taxon, and has attracted the attention of systematists as an additional tool for species delimitation. However, Meik et al. (2015) cautioned against the use of climatic data as surrogates for physiological adaptations, and hence as indicators of speciation. It is important to understand that the observed distributions of taxa reflect their realised niches, not the fundamental niche within which they could exist without constraints imposed by biotic interactions, such as competition with close relatives. Consequently, the occupation of different niches by geographic segments of contiguously distributed candidate species could reflect either shifts in the fundamental niche (i.e., adaptive evolution of likely inherited traits), or simply the spatial division of a wider range by taxa with a single broad fundamental niche, perhaps through competition or as a result of historical contingency (Tingley et al. 2014; Meik et al. 2022). While tests like that proposed by Raxworthy et al. (2007) can help distinguish between these hypotheses, the simple comparison of realised niches of candidate species is likely to confound realised and fundamental niches and thus lead to an overestimate of species numbers. In particular, where candidate species occur along an environmental gradient, constructing niche models for each and projecting them back onto a map will inevitably result in the model suggesting different climate niches, and would be tautological (Fig. 2).

Aims and objectives

This paper was prompted by multiple recent cases where species limits in high-profile taxa were primarily inferred

using an mtDNA-led approach, without critical independent testing of the putative taxa, and the results were later found to be misleading once a multilocus dataset was brought to bear on them. Further recent controversial species delimitation studies and the ensuing criticism (Dubois et al. 2024; Reyes-Velasco 2024; Vásquez-Restrepo et al. 2024; Wüster et al. 2024; Reynolds 2025) have again focussed attention on the problems of over-reliance on mtDNA for species delimitation. However, beyond these high-profile cases, it is clear that mtDNA-led species delimitation remains widespread in herpetology, despite the well documented intrinsic limitations of this marker. This paper is thus an expression of the desire to put the mtDNA molecule into perspective and to encourage more rigorous approaches to species delimitation that capitalise on the strengths of mtDNA while avoiding its pitfalls. I approach this by analysing the use of different marker systems and analytical approaches in recent species delimitation studies in non-avian reptiles, assessing to what extent different markers are used to critically test, rather than just confirm, mtDNA-based species limits, and how currently widespread approaches can be improved to achieve more convincing species delimitations, while remaining mindful of the resource limitations afflicting much of systematics.

Methods

To understand current practices in species delimitation in reptiles, I conducted a survey of the herpetological literature in the years 2023 and 2024. I searched the Reptile Database (Uetz et al. 2024) for all species described in 2023 and 2024. In addition, I searched the spreadsheets listing changes since previous updates also available from the database website for species that were revalidated or elevated from subspecies status during the same period. In almost all cases, I obtained the full text version of the papers. I was unable to obtain the full text version of six publications containing seven species descriptions due to a combination of journal paywalls and authors not responding to PDF requests. These taxonomic acts were not considered further. Fossil taxa were not considered.

Only taxonomic decisions resulting from new data and a genuine attempt at species delimitation were included. Taxonomic changes resulting from procedural issues, such as Code-mandated changes, rediscovered senior synonyms, replacement names for *nomina nuda*, etc., were not considered further. In accordance with widely agreed practice in herpetology (Kaiser et al. 2013; Wüster et al. 2021), certain taxa described or revalidated outside the peer-reviewed herpetological literature were not considered.

For each taxonomic decision, I recorded the markers used and whether the data from different markers were used to critically test or merely to confirm the validity of the hypothesised new species. Genetic markers were divided into mtDNA, single-copy nuclear DNA genes (scnDNA), for both of which I recorded the number of

genes used, and next-generation sequencing data, such as ddRAD-seq, ultraconserved elements (UCEs), etc. I then noted whether any nuclear markers were used in a manner allowing the critical testing of candidate species, or in a confirmatory manner that could at best fail to confirm species status.

For morphology, I again noted whether this was used in an approach allowing critical testing of mitochondrial candidate species, or in a confirmatory or solely descriptive manner. I also noted whether any newly defined or redefined species is likely to be in geographic contact with closely related congeneric taxa.

Results

I collected data on 440 species descriptions and revalidations published in 253 individual papers. The compilation of the descriptions and the list of references are provided in Files S1 and S2. All the taxonomic decisions analysed here represent bona fide attempts at species delimitation: None could be described as “*en passant*” taxonomy, i.e., casual taxonomic decisions taken on the basis of superficial exploration of mitochondrial gene trees or distances (Wüster and Tillack 2023), or as provocatively minimalist (e.g., Sharkey et al. 2021). The results are summarised in Figure 3. Out of 440 species delimitations, only 65 (14.8%) were based solely on morphological data without molecular support. Five (1.1%) were based on a combination of morphological data and ddRAD-seq NGS data. In one instance, the markers used were not clearly stated. All remaining 370 (84.1%) taxonomic decisions were partly based on mtDNA sequence information as well as morphology. Of these, 179 (47.3%) also used nuclear DNA data in the shape of ddRAD-seq data (7 descriptions, 1.9%) or one or several single-copy nuclear loci (168, 45.4%), leaving 195 (45.4% of all descriptions, 52.7% of those using mtDNA) based on morphology and mitochondrial DNA only.

Both morphological and nuclear sequence data were predominantly used in a confirmatory manner rather than as critical tests, precluding rigorous independent testing of mitochondrially defined candidate species. Overall, only 71 out of 179 delimitations (39.7%) including nuclear sequences used these as critical tests of mitochondrially-derived species hypotheses, and only 112 out of 440 delimitations (25.5%) using morphological data used them as critical tests of species hypotheses rather than as confirmatory evidence. These proportions did not differ significantly between taxa with likely contact zones with close relatives and taxa without such contact zones (Tables 1, 2). Moreover, the interpretation of a number of the 112 analyses seemingly using morphology as a critical test is affected by the frequent use of preliminary analyses requiring grouping of specimens to adjust for allometric growth (Chan and Grismer 2022). Overall, of the 370 assessed studies using mitochondrial DNA, 229 (61.9%) used neither morphology nor nuclear markers as critical tests of mitochondrial lineages.

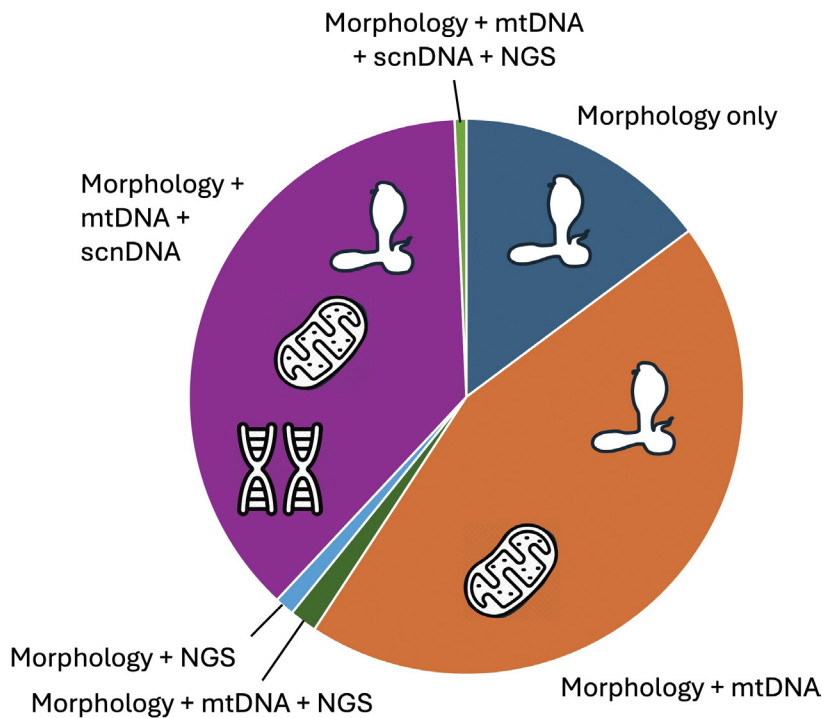


Figure 3. Frequencies of different sources of data in reptile species delimitations, 2023–2024.

Table 1. Proportion of species descriptions using nuclear data as critical tests versus in a confirmatory approach. The proportions of each do not differ significantly between species with and without contact zones with close relatives ($X^2 = 1.8684$, d.f. = 1, $p = 0.171661$; contact unknown category excluded).

	N	Nuclear data used as test	Nuclear data used as confirmation
Contact	104	37 (35.6%)	67 (64.4%)
No contact	72	33 (45.8%)	39 (54.2%)
Contact unknown	3	1	2

Table 2. Proportion of species descriptions using morphological data as critical tests versus in a confirmatory approach. The proportions of each do not differ significantly between species with and without contact zones with close relatives ($X^2 = 2.1536$, d.f. = 2, $p = 0.340684$; contact unknown category included).

	N	Morphology used as test	Morphology used as confirmation
Contact	194	43 (22.2%)	151 (77.8%)
No contact	214	61 (28.5%)	153 (71.5%)
Contact unknown	31	8 (25.8%)	23 (74.2%)

Where both mtDNA and single copy nuclear gene sequences were used, most studies used as many or more mitochondrial loci than nuclear loci (Fig. 4). The data reveal a weak positive correlation between the number of mtDNA and scnDNA loci used (Pearson correlation, $R = 0.256$, $P < 0.001$).

Discussion

The results of our survey document the extent to which mitochondrial DNA still dominates reptile systematics, and that, despite numerous conceptual and technological

advances (Padial et al. 2010; Hillis et al. 2021; Miralles et al. 2024; Vences et al. 2024), many studies still fail to compensate for the well-documented limitations of mtDNA as a sole source of evidence for species delimitation. The overwhelming majority (98.7%) of descriptions using molecular markers used mtDNA, either alone or in conjunction with single copy nuclear markers. In contrast, despite the increasing availability and affordability of next generation sequencing (NGS) technology, this has so far made very little impact on species delimitation in reptile systematics. More than likely, the lack of sequencing facilities and bioinformatic infrastructure in many settings remain greater obstacles than the falling cost of the molecular work itself. This will be a key area of capacity-building in the future, especially for the ben-

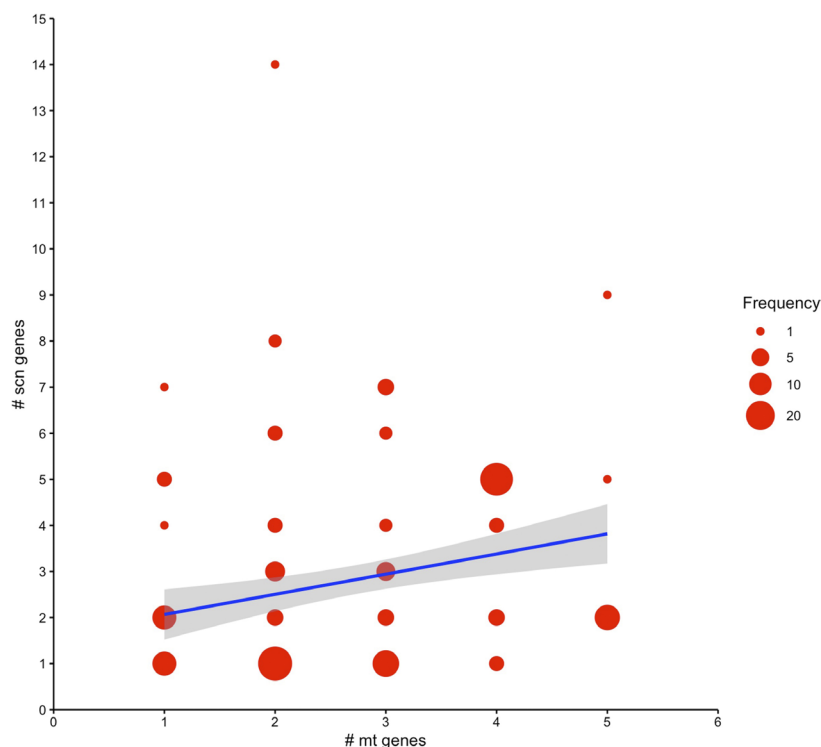


Figure 4. Relationship between the number of mitochondrial and single-copy nuclear genes used in multilocus reptile species delimitations. Symbol size indicates frequency of the combination of mitochondrial and nuclear gene numbers. Note that the numbers of specimens sequenced for mitochondrial and nuclear genes are not considered! Many studies only sequence a subset of specimens for the nuclear genes.

efit of taxonomists in resource-poorer settings (Carneiro et al. 2025).

Startlingly, most (61.9%) species descriptions and revalidations using mtDNA still do not critically test whether mitochondrially-defined candidate species are indeed independently evolving lineages. This matters less in taxa restricted to small, isolated ranges without contact with closely related species (making ongoing gene flow very unlikely). However, the proportion of studies using confirmatory-only approaches in their analysis of scnDNA or morphological data is as high in descriptions of more widespread taxa with contact zones as in isolated taxa. This trend contradicts the broader conclusions of Guedes et al. (2024), that the evidential basis underlying reptile descriptions has improved over the decades, and highlights a major crisis of evidence quality in much of present-day reptile systematics. While Guedes et al. (2024) document an increase in the number of genes sequenced, it is important not to confuse quality with quantity when assessing the robustness of taxonomic research: The use of a greater number of genes in a purely confirmatory approach, rather than as a critical test, adds nothing to the quality of the evidence supporting a species description.

Sequencing strategies in multilocus studies also betray a lack of awareness of the need for independent data: Most multilocus studies sequence as many or fewer nuclear than mitochondrial loci (Fig. 4), although there is extensive variation in current practice. Since all mitochondrial genes are part of a single linkage unit, the addition of further mitochondrial genes can potentially increase branch support in the mitochondrial gene tree, but cannot help to independently test species limits. Only the addition of further nuclear loci can enhance the rigour and power of species delimitations. Overall, this suggests a need for a greater awareness of the limitations of mtDNA and of the

analytical approaches needed to capitalise on the potential of this molecule while avoiding the pitfalls.

I stress that nothing in this paper is intended as criticism of any specific study: The approaches used by different author teams may have been dictated by a wide range of technical, economic, logistic or historical reasons or constraints, which may not be apparent to the reader. Instead, the focus should be on the broad pattern of the data, which document continued high levels of reliance on mitochondrial DNA and insufficient testing of its conclusions, and the consequent lack of stringency of the taxonomic conclusions. Below, I offer suggestions for improving the rigour and reliability of species delimitations in herpetology, particularly where mtDNA is used as a primary tool for hypothesis generation.

Do better with no more: Optimising resource use to rigorously test species hypotheses

Many or most of the problems highlighted above could be prevented through the use of next-generation sequencing methods, potentially coupled with increasing standardisation of the markers used (Eberle et al. 2020). However, their minimal uptake in herpetological species delimitation suggests that significant barriers continue to exclude most herpetologists from using these techniques (Carneiro et al. 2025). Consequently, my aim in the following paragraphs is to stimulate taxonomists from all resource settings to make the most of whatever means are at their disposal. My comments will therefore be based on the methodological status quo, where the overwhelming majority of taxonomic herpetology is restricted to morphological analyses and Sanger sequencing of a small number of loci.

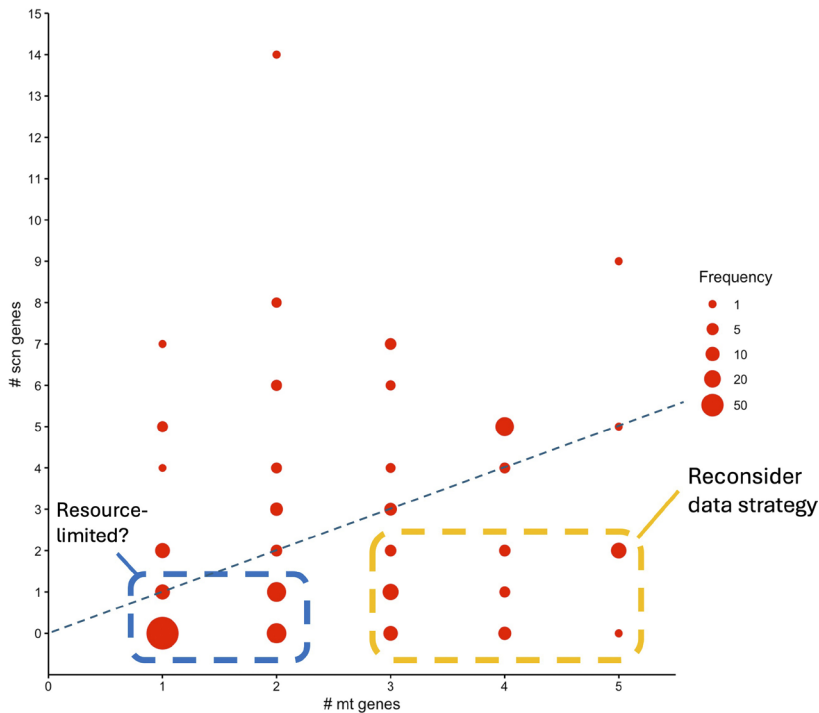


Figure 5. Allocation of sequencing effort between mitochondrial and single copy nuclear genes in reptile species descriptions. While the use of few loci overall (blue box) may be an inevitable consequence of resource limitation, the sequencing of numerous mitochondrial gene fragments but few or no nuclear markers (orange box) constitutes a suboptimal allocation of resources for the purposes of species delimitation.

Sequence smarter, not harder

The need for more genuinely multilocus analyses inevitably raises questions about the cost of additional sequencing, especially in resource-poor settings. However, the data collected here offer only very partial support for this concern: In many studies, researchers sequence multiple mitochondrial genes for numerous specimens, presumably to enhance the resolution of the resulting mitochondrial gene tree, but only a small subset of specimens is sequenced for fewer nuclear genes (Fig. 5). At least for the purposes of species delimitation, this is a misallocation of effort. Sequencing multiple mitochondrial genes is important if the main goal is an accurate mitochondrial phylogeny. However, the formulation of primary species hypotheses only requires the monophyly of the haplotypes of each candidate species to be strongly supported in the mtDNA tree, not so much the phylogenetic relationships within or between them. This is often achievable through the sequencing of a single mitochondrial gene. Indeed, that was the entire starting point of the barcoding initiative (Hebert et al. 2003a, 2003b, 2004b). While a second mitochondrial gene may be advisable for greater resolution and also to guard against misleading phylogenetic signals due to nuclear integrations (Numts - Zhang and Hewitt 1996), the use of further mitochondrial genes provides rapidly diminishing returns in species delimitation. Diverting the resources spent on additional mtDNA sequences towards sequencing nuclear genes would enable genuine multilocus analyses and more rigorous testing of barcode-inferred species boundaries for the same outlay (Fig. 5).

As an example, one recent study later criticised for excessive reliance on mtDNA (Arteaga et al. 2024; Reyes-Velasco 2024) generated 206 new gene sequences for 70 specimens across 7 gene fragments. However,

only 18 of these sequences, from 11 individuals of 5 candidate species (out of 10 recognised in the paper), were from two nuclear loci, while the remaining 188 sequences were distributed across 5 mitochondrial loci! An alternative strategy for the same outlay would have been to sequence a single mitochondrial gene for all specimens, and to allocate the remaining 136 sequences to the two nuclear markers for virtually all specimens. If an accurate mitochondrial phylogeny was deemed essential, the same sequencing effort would have allowed all 70 specimens to be sequenced for one mitochondrial gene, one specimen of each candidate species for the remaining four mitochondrial genes, and 48 specimens (4–5 for each species) for the nuclear NT3 and RAG1 genes. Both approaches would have allowed more rigorous tests of the evolutionary independence of the mitochondrial candidate species and forestalled later criticisms of the work.

Choose appropriate nuclear markers

Where researchers use single copy nuclear (scn) genes to test mitochondrial candidate species, the choice of loci is of key importance. Most scn loci are slower-evolving and more conserved than most mitochondrial genes. As a result, many contain few or no positions that vary at or around the species level. However, evolutionary rates vary widely between loci (Townsend et al. 2008), so that the choice of appropriately informative loci can make the difference between success and failure. However, even then the informativeness of individual loci can also vary unpredictably between taxa. For instance, the C-MOS oncogene, one of the first scnDNA loci used in herpetological phylogenetics (Saint et al. 1998), and now almost the default nuclear marker in squamate systematics, is generally relatively slow-evolving (Townsend et al. 2008), and yielded limited or no useful data in a number of snake

species delimitation studies (Anderson and Greenbaum 2012; Folt et al. 2019; Carbajal-Márquez et al. 2020). However, the same gene was instrumental in convincingly demonstrating the validity of *Naja foxi* (Ratnathorn et al. 2023, their fig. 4B), which had previously been described using only mtDNA and a confirmatory approach to morphological analysis (Shi et al. 2022). Yet, in the same study, this gene was invariant across more distantly related cobra species such as *N. kaouthia* and *N. siamensis*. To avoid disappointment and a waste of resources, taxonomists would therefore be well advised to test multiple potential scnDNA loci for informativeness on a subset of their samples before embarking on a fuller sequencing programme (Malhotra et al. 2025). I also urge all researchers to publish details of nuclear markers found to be uninformative in their study system, even if excluded from formal analyses, and to submit the sequences to public databases. Single-copy nuclear loci are often grossly underrepresented in higher level phylogenies, and sequences that are uninformative for species delimitation may nevertheless be useful for higher-level phylogenetic analyses (Wüster et al. 2024).

Choose appropriate morphological analyses

While molecular work inevitably involves significant costs, morphology remains an accessible and appropriate source of data for testing species boundaries that requires only very limited financial investment. As a proxy for overall genomic variation, detailed studies of morphology can be used to test whether mitochondrial candidate species represent discrete evolutionary lineages. Unfortunately, the analyses above show that this potential of morphological data is rarely realised. Only around one quarter of all descriptions analysed used morphology to critically test species boundaries. Often, morphological data are only used in comparative tables showing the range of values in the suggested candidate species, without further analysis. As noted earlier, approaches based on the a priori grouping of morphological data into OTUs by mitochondrial candidate species (e.g., comparative tables, ANOVA, DFA) assume what should be tested, that each candidate represents a homogeneous taxon and thus a suitable unit for comparison. Instead, methods not involving a priori grouping should be used, such as Principal Components Analysis or conceptually similar approaches. In the case of widespread taxa, dividing each candidate species into several OTUs based on collecting gaps, and then using DFA or similar to test the prediction that the OTUs of each candidate species should be homogeneous but distinct from the OTUs of others, constitutes a suitable alternative solution (e.g., Wüster et al. 2018).

Ensure adequate geographical coverage

For candidate species with broader geographic ranges, thorough coverage of the entire range of the candidate species and its closely related neighbours is essential. In particular, sampling likely contact zones between candidate species is crucial, since these are likely to contain

evidence on the degree of genetic isolation between them. Inadequate sampling of contact zones is likely to lead to an overestimate of the number of species present (Chambers and Hillis 2020; Mason et al. 2020; Hillis et al. 2021).

Provide key distributional and natural history information

The aim of any scientific publication is to provide the reader with the information required to independently assess the validity of the conclusions. In the case of species delimitation studies, a number of additional factors can affect the assessment of the validity of a paper's conclusions. In particular, understanding the likely nature of contact zones is key to interpreting the evidence for evolutionarily independent lineages. Ideally, papers dealing with species delimitation should provide maps with all known records of the focal species and its near relatives. Maps showing just type localities are of little use in assessing the likelihood of contact between taxa. Similarly, a list of sympatric species, particularly any congeneric species, is valuable to assess the status of the species.

A sense of proportion

As in any scientific endeavour, where multiple lines of evidence are available, great strength in one can compensate for weakness in another. In species delimitation, the most complex cases are those of widespread candidate species in geographic contact with each other and low or intermediate levels of mtDNA sequence divergence: Here, a genuinely integrative approach with critical testing of mtDNA-defined species limits is essential. Equally, allopatric populations separated by low levels of mtDNA divergence require strong additional evidence to justify recognition as species. In contrast, where entirely allopatric candidate species occupying isolated ranges differ by very high levels of mtDNA divergence or are clearly non-monophyletic, a confirmatory approach to morphological data is likely to be adequate.

Conclusions

This study documents the continued disproportionate influence of mitochondrial DNA in reptile systematics. Many researchers still use mitochondrial DNA as the main source of evidence for species delimitation and fail to critically test whether their mitochondrial candidate species really denote independently evolving organismal lineages. The result of this excessive reliance on mtDNA and lack of rigorous testing of mitochondrial species hypotheses is that the rapid increase in the number of recognised reptile species (Guedes et al. 2024) may partly be a result of oversplitting (Hillis 2019; Hillis et al. 2021). I therefore argue that the long-standing reliance on mtDNA has blinkered the perspective of herpetological taxonomy

against the consideration of tokogenetic processes that would refute many species hypotheses, and may thereby have led to an inflation of our estimates of reptile species diversity. Casting aside these mitochondrial blinkers and focussing on the use of multiple sources of data to critically test putative species limits in a genuinely integrative framework is essential if we are to obtain a realistic estimate of reptile diversity at the onset of the Anthropocene.

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Supplementary Material 1

Files S1, S2

Authors: Wüster W (2025)

Data type: .zip

Explanation notes: **File S1.** Species descriptions and delimitations analysed in this paper, with bibliographic source, number of mitochondrial and single copy nuclear genes used, whether next-generation sequencing methods were used, whether nuclear genetic data and morphological data were used as critical tests of mitochondrial candidate species or in a confirmatory approach, and whether the species is likely to have a contact zone with a closely related species. — **File S2.** List of references of all species descriptions and delimitations analysed in this paper, from File S1.

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