BioOne COMPLETE

Contemporary Methods and Evidence for Species Delimitation

Authors: Hillis, David M., Chambers, E. Anne, and Devitt, Thomas J.

Source: Ichthyology & Herpetology, 109(3) : 895-903

Published By: The American Society of Ichthyologists and Herpetologists

URL: https://doi.org/10.1643/h2021082

BioOne Complete (complete.BioOne.org) is a full-text database of 200 subscribed and open-access titles in the biological, ecological, and environmental sciences published by nonprofit societies, associations, museums, institutions, and presses.

Your use of this PDF, the BioOne Complete website, and all posted and associated content indicates your acceptance of BioOne's Terms of Use, available at www.bioone.org/terms-of-use.

Usage of BioOne Complete content is strictly limited to personal, educational, and non - commercial use. Commercial inquiries or rights and permissions requests should be directed to the individual publisher as copyright holder.

BioOne sees sustainable scholarly publishing as an inherently collaborative enterprise connecting authors, nonprofit publishers, academic institutions, research libraries, and research funders in the common goal of maximizing access to critical research.

Downloaded From: https://bioone.org/journals/Ichthyology-&-Herpetology on 03 Aug 2024
Terms of Use: https://bioone.org/terms-of-use

Ichthyology & Herpetology 109, No. 3, 2021, 895–903

Contemporary Methods and Evidence for Species Delimitation

David M. Hillis[1], E. Anne Chambers[1], and Thomas J. Devitt[1]

Over the last two decades, mitochondrial DNA (mtDNA) sequence data, as well as analyses of nuclear DNA based on the multispecies coalescent model, have increasingly been used to delimit species, sometimes based on limited sampling and without other supporting evidence. We have argued elsewhere that the uncritical use of these approaches has resulted in unnecessary and unwarranted taxonomic changes that have real and long-lasting consequences for science and society. Unfortunately, these arguments have been misrepresented by Burbrink and Ruane's Point of View on "contemporary" species delimitation in this issue. Here, we discuss the role of models in species delimitation research, and again argue that careful consideration of model assumptions (and their potential violation) is necessary when inferring population history and delimiting species. We echo recent calls for targeted, thorough geographic sampling across contact zones and into parapatric ranges to test for reproductive isolation and draw inferences about the evolutionary independence (or lack thereof) of populations that exchange genes. Finally, despite our very different views on how best to identify species and when to make taxonomic changes, we end by highlighting areas where we agree with Burbrink and Ruane's Point of View and offer suggestions for future research.

PERHAPS the most striking aspect of biological variation (at the genetic or phenotypic levels) across the Tree of Life is that it is not continuous. On one hand, we often observe continuous variation within species, even if they vary considerably from one part of their range to another. On the other hand, we also observe many genetic and phenotypic gaps between species, illustrating how species evolve on independent evolutionary trajectories from one another. At the genetic level, both of these facts are easily understood: continuous variation is a result of recombination of alleles within reproductive lineages, whereas reproductive barriers lead to the accumulation of independent mutations in different sister lineages, and thus distinct sets of genes in different species (which explains the gaps between species). The process of species delimitation seeks to identify these independently evolving lineages on the Tree of Life, and it is the genetic and phenotypic gaps between species that lead us to conclude that two lineages are evolving independently of one another.

Traditional species description is typically about the discovery of new and unexpected taxa—an undescribed species that exhibits character combinations never before observed. In contrast, species delimitation typically involves re-evaluation of the boundaries of previously known species. In the latter case, an investigator examines a known species or species group that exhibits considerable genetic or morphological variation and then asks if the variation represents geographic variation within a species, or instead, provides evidence that more species exist than were previously suggested. Species delimitation starts with a null hypothesis (such as an existing taxonomy that recognizes one geographically variable species) and asks if there is clear evidence that would allow us to reject that hypothesis. The evidence needed to reject a null hypothesis about a well-known and well-studied species is often far greater than the evidence needed to report the discovery of a new species that has never before been reported.

Many (perhaps most) species exhibit geographic variation in morphology and/or gene frequency, so how can we determine when a population lineage is evolving independently from other such lineages? Even though we argue that species are real, i.e., they are distinct entities that can be discovered by biologists (rather than arbitrary constructs along a continuum of hierarchical biodiversity; see Mallet, 2005), species delimitation is not always a simple process. As we expect populations in different parts of a species range to show genetic differentiation, the species delimitation problem becomes one of identifying where reproductive barriers (and thus, genetic and usually phenotypic gaps) exist between independent evolutionary lineages (Hillis, 2019). It is these reproductive barriers that result in the independent evolution of lineages, and hence the divergence of species.

WHAT DO WE SEE AS PROBLEMS OF RECENT SPECIES DELIMITATION STUDIES?

In a series of recent papers, we have argued that many contemporary species delimitation studies have been too quick to accept results based on particular genetic models and assumptions, even in the face of strongly conflicting information from other analyses (Hillis, 2019; Chambers and Hillis, 2020). This practice has resulted in many poorly supported (and often short-lived) proposals for changes in the names and limits of well-studied species, despite considerable conflicting evidence from prior analyses. In their Point of View on species delimitation in this issue, Burbrink and Ruane (2021; hereafter B&R) misconstrue or misrepresent many of our arguments in these papers. They argue that we "fall short of considering modern theory and application," "ignore that hybridization across the tree of life is common," and suggest that we fail to recognize that "incomplete gene (lineage) sorting is one expectation of the speciation process."

We find it difficult to believe that other readers of our papers would draw any of these same conclusions. Most importantly, nowhere in any of the aforementioned papers do we deny the importance of using any modern theory, models, or methods to aid in species delimitation. Instead, we urge caution at uncritically accepting output from species delimitation analyses, especially when the assumptions of

[1] Department of Integrative Biology and Biodiversity Center, 2415 Speedway, University of Texas at Austin, Austin, Texas 78712; Email: (DMH) dhillis@austin.utexas.edu. Send reprint requests to DMH.

Submitted: 5 May 2021. Accepted: 11 June 2021. Associate Editor: W. L. Smith.

© 2021 by the American Society of Ichthyologists and Herpetologists DOI: 10.1643/h2021082 Published online: 29 September 2021

Downloaded From: https://bioone.org/journals/Ichthyology-&-Herpetology on 03 Aug 2024
Terms of Use: https://bioone.org/terms-of-use

the underlying models are not tested or are likely to be strongly violated, or sampling is inadequate for appropriate application of the given method (for examples, see Barley et al., 2018; Chambers and Hillis, 2020).

Our papers have each explicitly acknowledged the existence of hybridization between species. Rather than deny that hybridization occurs in nature, we have emphasized the difference between broad zones of gradual intergradation across environmental gradients (where there is no evidence of selection against hybrids or hybridization) and hybrid zones between distinct species (where there is clear evidence of some degree of reproductive isolation and independent evolution of the two species; Fig. 1). We have explicitly discussed the implications of incomplete lineage sorting and have described why incomplete lineage sorting has been mischaracterized and methods to accommodate it misapplied in species delimitation papers by B&R (e.g., Ruane et al., 2014).

B&R further misrepresent a recent paper (Hillis, 2019) that includes recommendations for when and why systematists should make taxonomic changes, both to clade names as well as species boundaries. Below, we discuss the details of each of the case studies discussed by B&R and explain why their criticisms of our papers are unfounded or misdirected.

WHEN IS TAXONOMIC CHANGE APPROPRIATE?

Hillis (2019) presented an invited essay about the historical development of species delimitation in herpetology and changes in taxonomic practice over the course of his career. As requested, the essay was deliberately personal, relatively non-technical, and historical in nature. In the latter part of this essay, he argued that subjective nomenclatural changes to well-established clade and species names should be made conservatively, and only when required by clear evidence.

To emphasize his preference for conservative adoption of changes to scientific nomenclature, Hillis (2019) included a decision tree to indicate when subjective nomenclatural changes were justified. He supported nomenclatural changes in well-known groups only if they were consistently and strongly supported by evidence. For example, he argued against making changes to a well-established genus (or other clade name) if the evidence suggested that the genus was already monophyletic. Hillis (2019) argued that splitting a monophyletic genus, just to introduce nomenclatural changes or to name new (smaller) subgroups, was counter to responsible taxonomy (using the example discussed by Yuan et al., 2016, for Holarctic *Rana*). Likewise, Hillis (2019) did not consider the discovery of genetic variation within a species to be sufficient evidence for naming connected and interbreeding populations of the species as new species. He argued that we should split existing species into separate species only if there was clear evidence for some level of intrinsic or extrinsic reproductive isolation among the purported groups (however that was inferred—a hypothetical example is illustrated here in Fig. 1). If evidence indicated that a nomenclatural change was indeed justified (for example, the discovery that a recognized genus contains a polyphyletic group of species or the discovery of previously unrecognized reproductive isolation within a recognized species), then Hillis (2019) recommended that nomenclatural changes should involve the least disruption possible. All of these points were clarified in the accompanying text, so the source of B&R's confusion about the application or meaning of the decision tree is unclear.

***Species delimitation in North American Copperheads* (Agkistrodon contortrix).**—*Agkistrodon contortrix* presents a good example for illustrating the issue of taxonomic conservatism. Geographic morphological variation in *A. contortrix* has been examined extensively and thoroughly in a book-length monograph (Gloyd and Conant, 1990). That study showed that two major pattern classes of Copperheads gradually intergrade from one to the other across a several hundred-kilometer-wide region of eastern Texas, Oklahoma, and Kansas. In this zone of intergradation, snakes are phenotypically intermediate, and color pattern changes gradually along an east–west axis. Gloyd and Conant (1990) interpreted this as strong evidence that Copperheads in North America all belong to a single, geographically variable species, and they recognized distinct pattern classes as subspecies within *A. contortrix*.

Burbrink and Guiher (2015) examined the phylogeography of Copperheads using five nuclear loci and mitochondrial DNA (mtDNA) from Guiher and Burbrink (2008). Burbrink and Guiher's (2015) results show the same broad region of admixture that Gloyd and Conant (1990) had detected based on morphology, but, in contrast to Gloyd and Conant's (1990) conclusion, Burbrink and Guiher elected to split the species into two with an arbitrarily placed division through the middle of the zone of intergradation. Little had changed regarding what was known about the systematic biology of the snake, except for the nomenclature and the recognition of two species, rather than one. However, in the middle between the ranges of these two named "species" is a vast area where Copperheads cannot be identified by any means to either purported species; instead, they are all said to be "admixed" (meaning intermediate combinations of genotypes).

B&R justify the taxonomic division by Burbrink and Guiher (2015) by erecting and then discarding an inappropriate null model. They argue that the "hybrid zone" between the eastern and western forms is not "neutral," based on estimates of rates of dispersal potential. Their point is that if there is no selection for the observed geographic variation, then the dispersal potential of the snakes is high enough that complete homogenization of the species should have occurred by now. However, this represents the test of an inappropriate model to the question at hand. Geographic variation (whether genetic or morphological) is generally assumed to be driven by selection (May et al., 1975). Many of the alleles that have a selective advantage in the eastern temperate forest ecoregion are unlikely to be the same alleles that have a selective advantage in the Great Plains ecoregion.

The appropriate question is not whether there is selection for geographic variation; that is expected whether there is a single species or not. Rather, the appropriate questions are: what happens where the two forms come into contact (Endler, 1977)? Is there evidence of some level of reproductive isolation between the two groups, or do the two groups gradually intergrade from one to the other (Harrison, 1993; Hillis, 2020)? Testing the alternative hypotheses requires more than speculation about the fate of the hybrid zone based on rough estimates of dispersal distance, generation time, and unpublished data on how long these forms may have been in contact (as B&R have done). Drawing inferences

Downloaded From: https://bioone.org/journals/Ichthyology-&-Herpetology on 03 Aug 2024
Terms of Use: https://bioone.org/terms-of-use

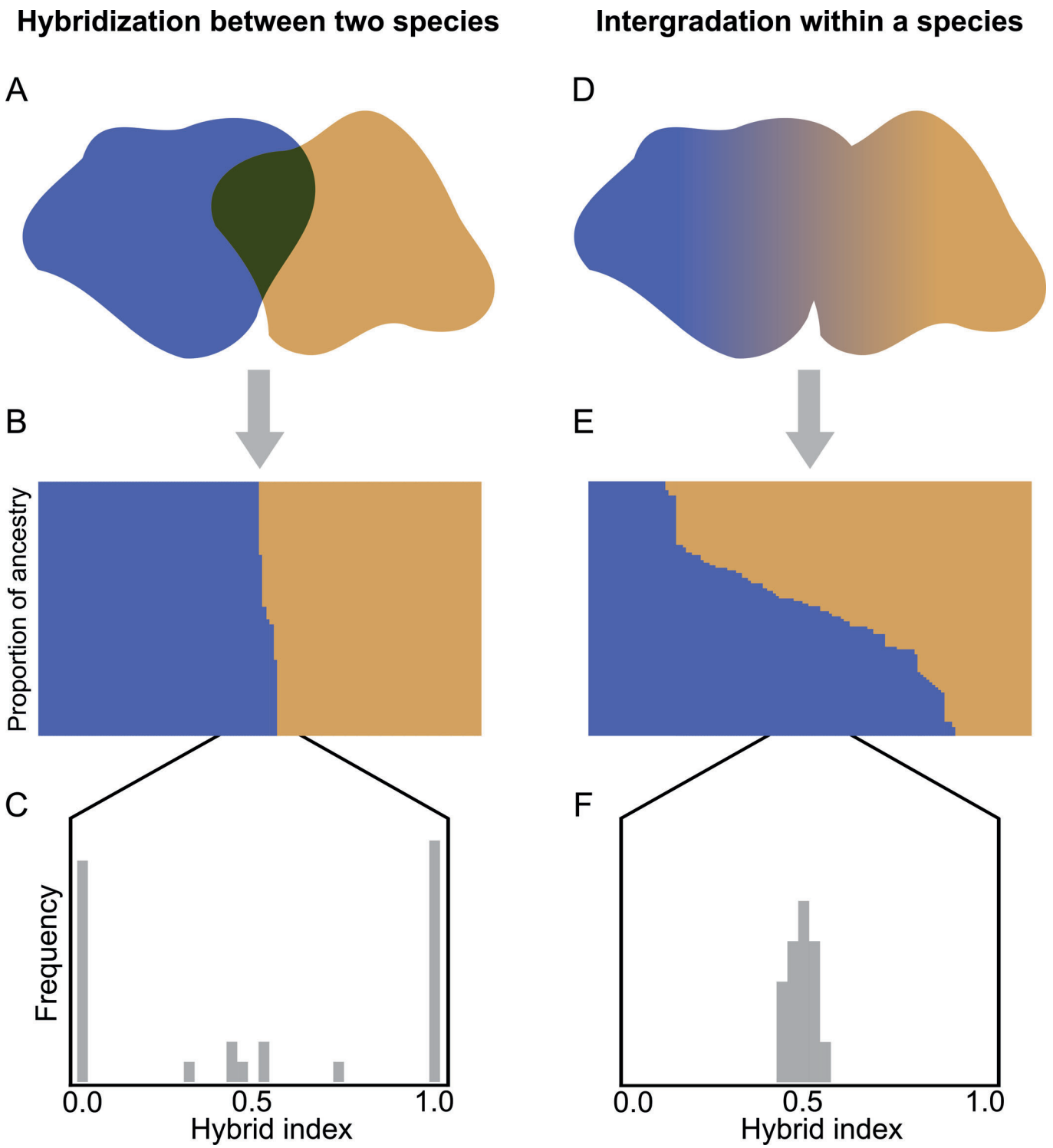

Fig. 1. A hypothetical example of distinguishing hybridization between two species (left column) from intergradation between two forms within a geographically variable species (right column). The two species or genotypes are represented in each case by blue and gold colors, with intermediate colors indicating intermediate genotypes. (A) The ranges of the two species are illustrated in blue and gold, respectively, with a zone of overlap in the middle. (B) A Structure plot showing the proportion of ancestry of an array of individuals (represented by vertical bars) that have been sampled across the corresponding ranges of the two species and their contact zone. (C) The genotype frequencies of individuals sampled from a single local population in the contact zone, as measured by a hybrid index (a hybrid index of 0 indicates a pure blue genotype; a value of 1 indicates a pure gold genotype; 0.5 is consistent with an F1 hybrid; and hybrid indices near 0.25 and 0.75 are consistent with the respective backcrosses). Note the U-shaped distribution. (D) Two geographically variable forms within a species (e.g., subspecies) are illustrated in blue and gold, respectively, with a broad zone of intergradation in the middle. (E) A Structure plot showing the proportion of ancestry of an array of individuals (represented by vertical bars) that have been sampled across the corresponding range of the species. (F) The genotype frequencies of individuals sampled from a single local population in the center of the intergrade zone, as measured by a hybrid index (as described in C, above). Note the intermediate values for all individuals, which are consistent with random mating among individuals in this sample.

Downloaded From: https://bioone.org/journals/Ichthyology-&-Herpetology on 03 Aug 2024
Terms of Use: https://bioone.org/terms-of-use

about hybrid zone dynamics and hence species boundaries requires thorough sampling of populations at the scale at which individuals meet, mate, and produce hybrids (Barton and Hewitt, 1985, 1989; Kruuk et al., 1999; Jiggins and Mallet, 2000; Linck et al., 2019; Mason et al., 2020; Cicero et al., 2021), and in the best-fit axis of sampling orientation (Macholán et al., 2008; Devitt et al., 2011; Dufková et al., 2011). This requires targeted fieldwork across the contact zone (Fig. 1), ideally with multiple east–west transects in different portions of the transition zone. Evidence for reproductive isolation can be tested in various ways, and Figure 1 illustrates just one example (using a test for significant departure from Hardy-Weinberg Equilibrium expectations; Hardy, 1908; Weinberg, 1908). Clines in allele frequencies can also be fit using appropriate cline models (Barton and Gale, 1993; Durrett et al., 2000) and estimates of multilocus linkage disequilibria (Barton, 2000; Slatkin, 2008). Although B&R acknowledge the importance of such studies, and ". . . recommend that future studies test all of these hypotheses and examine the width and nature of this hybrid zone with genomic data and adequate samples through the connecting ranges," we ask: Why change the taxonomy first, if nothing is known about contact zone dynamics and there is no evidence of anything other than gradual intergradation between these forms? Doing so leaves Copperheads throughout large portions of Texas, Oklahoma, Kansas, and Nebraska impossible to identify to species. If new evidence shows reproductive isolation between *A. c. contortrix* and *A. c. laticinctus*, then the taxonomic change may be appropriate. But in light of all available published evidence, we argue that Burbrink and Guiher's (2015) proposal for taxonomic change was premature. Taxonomic change without solid supporting evidence is a disservice to science and society because it causes confusion and taxonomic instability. Similar cases of premature species splitting (e.g., Burbrink, 2002) have now been reversed after examination and genetic analysis of the relevant (and previously unexamined) intergrade zones (Marshall et al., 2021).

***Species delimitation in* Eurycea *salamanders.*—**B&R state that the decision tree presented in Hillis (2019) ". . . rests entirely on the unqualified criterion that species should be sufficiently reproductively isolated to be considered independently evolving" and that our use of the word "sufficiently" implies that determining reproductive isolation is subjective. The point of the decision tree is that in a re-evaluation of an existing taxonomic hypothesis, the existing taxonomy (based on previous data and evaluation) should serve as the null hypothesis to be tested. Rejecting an existing hypothesis of geographic variation within a species, in favor of multiple distinct species, requires evidence for some level of barriers to gene exchange between the nominal groups (e.g., as shown in Fig. 1). Otherwise, there is no evidence for the existence of independent evolutionary lineages, and the null hypothesis of geographic variation within a species should stand. Different biologists may be willing to make the argument for multiple species at different levels of reproductive isolation and based on many different kinds of evidence. Those arguments should be made explicitly, so that other biologists can review the evidence and decide to accept or reject the proposal for taxonomic change. We do not wish to dictate those criteria for others; but neither can the question be simply ignored, nor contact zones left unexamined. Taxonomic decisions about lineages that are allopatric and weakly differentiated or lineages that exchange genes with other lineages will always require some level of debate and discussion (Wiens, 2004). Our point is that the evidence and tests for independently evolving lineages need to be made explicitly, so that others are free to agree or disagree with the conclusions. It is unclear why B&R find our acceptance of this biological reality problematic.

B&R go on to suggest that our criticisms (in Chambers and Hillis, 2020) of Bayesian Phylogenetics and Phylogeography (BPP; Yang and Rannala, 2010) analyses are inconsistent because another paper from Hillis's lab (Devitt et al., 2019) used BPP as one of several analytical approaches in identifying species boundaries in *Eurycea* salamanders. B&R state ". . . it is difficult to comprehend what philosophy and methods are approved given that Hillis recently co-authored a paper on the phylogeography of *Eurycea* using the MSC to delimit species, but without directly assessing degree of reproductive isolation or gene flow (Devitt et al., 2019)." This assertion is both inaccurate and misleading.

First, Devitt et al. (2019) used BPP to generate the posterior probabilities of different species delimitations based on support for recent coalescence, but they did not use the MSC to delimit species as B&R suggest. Devitt et al. (2019) performed BPP analyses separately for three clades, and found that the prior for θ had a large effect on the posterior probabilities of different species delimitations (p. 2628 and table S4). Had Devitt et al. (2019) relied on BPP results to delimit species, they would have recognized five species (rather than three) in the subgenus *Septentriomolge* and up to nine (rather than six) within the clade of eastern *Blepsimolge*, depending on the prior specification chosen. Only results for the clade of western *Blepsimolge* were consistent across prior specifications (three species; table S4, Devitt et al., 2019). In fact, the species delimitations inferred by Devitt et al. (2019) were based on multiple lines of evidence, including published morphology, allozymes, and mtDNA (Chippindale et al., 2000; Hillis et al., 2001), as well as hierarchical *F* statistics, molecular assessments of admixture, and Bayesian clustering methods conducted across hundreds of loci. BPP over-split species beyond what was supported by the data in Devitt et al. (2019), which is completely consistent with the results and conclusions of Chambers and Hillis (2020) regarding the performance of BPP as a species delimitation method.

Second, B&R's criticism that Devitt et al. (2019) delimited species "without directly assessing degree of reproductive isolation or gene flow" is difficult to understand. *Directly* identifying and measuring reproductive isolating barriers is fraught with difficulties, and has been performed in relatively few, well-studied systems (Coyne and Orr, 2004). For this reason, most species delimitation studies (including ours) use indirect tests to assess evidence for reproductive isolation (such as the absence or deficiency of hybrids in contact zones relative to the null expectations of random mating; e.g., see Fig. 1).

Directly assessing gene flow—e.g., using capture–recapture or radiotelemetry data to record the dispersal of individuals and document their reproductive success—is also impractical for many species (subterranean groundwater salamanders included). This is why methods have been developed that use allele frequencies to infer the exchange of genes between populations indirectly (Slatkin, 1985, 1987). However, Devitt et al. (2019) made no attempt to infer *Nm* (the number

Downloaded From: https://bioone.org/journals/Ichthyology-&-Herpetology on 03 Aug 2024
Terms of Use: https://bioone.org/terms-of-use

migrants per generation) because the methods for assessing this parameter require assumptions that were violated in their sampled populations (Whitlock and McCauley, 1999). We further note that it is even more difficult to interpret the meaning of calculations of *Nm* if they are based on small samples of individuals, with each individual sampled hundreds of kilometers apart (as in Ruane et al., 2014).

Instead, Devitt et al. (2019) examined population subdivision and identified admixed individuals using the Bayesian clustering algorithm implemented in Structure (Pritchard et al., 2000; Falush et al., 2003). The species delimited by Devitt et al. (2019) exhibit minimal hybridization (in most cases, none), which is strong evidence for reproductive isolation among species. Studies that directly identify the specific reproductive isolating mechanisms between species (e.g., Hillis, 1981) require intensive field studies in areas of sympatry. Most of the species Devitt et al. (2019) delimited are parapatric, and we observed little to no admixture between species. Where species are sympatric, hybridization is either rare (e.g., between *Eurycea waterlooensis* and *E. sosorum*) or absent (e.g., *E. rathbuni* and *E. nana*), and the species maintain distinct morphologies and gene pools. Therefore, the basis for species delimitation in Devitt et al. (2019) was clear, and it was consistent with our recommendations for species delimitation in our other papers (including our discussion here).

***The* Lampropeltis triangulum *complex*.**—Contrary to assertions made by B&R, Chambers and Hillis (2020) did not attempt or intend to produce a comprehensive revision of the *Lampropeltis triangulum* group. Rather, the objectives of Chambers and Hillis (2020) were to examine the limitations and potential problems of making taxonomic changes to a widely distributed group on the basis of limited sampling of specimens and genes, examined under the framework of the MSC model (specifically BPP; Yang and Rannala, 2010). To do so, Chambers and Hillis (2020) used the same dataset reported by Ruane et al. (2014) to explore how upstream alterations in population assignment would affect downstream results (species delimitation). Chambers and Hillis (2020) made these objectives very explicit, stating that they supported "an alternative hypothesis to that presented by Ruane et al. (2014)" and concluded that "the data presented by Ruane et al. (2014) are inadequate to fully examine the species boundaries in this group." Chambers and Hillis (2020) noted that (i) Ruane et al. (2014) uncritically accepted output from a single species delimitation model, despite strongly conflicting evidence from alternative models that they examined; (ii) they made no attempt to examine or consider the nature of the contact zones of their purported taxa; and (iii) they examined a limited set of loci that showed no consistent differences between some of their recognized taxa. Finally, when their combined analysis of genes indicated virtual genetic identity between individuals across some of their delimited taxa, Ruane et al. (2014) incorrectly implicated incomplete lineage sorting (ILS) as a possible explanation for the apparent contradiction.

B&R state that Chambers and Hillis (2020) used monophyly of gene trees as a species criterion, despite the clear statement by Chambers and Hillis (2020) that "we would not expect congruence among all gene trees, and some gene trees would not be expected to be monophyletic within species lineages." Chambers and Hillis (2020) did, however, present consensus trees for the nuclear genes examined by Ruane et al. (2014) to illustrate the lack of any support or divergence between some of the taxa they recognized, across all the loci that they examined. Within these "nuclear" trees, there was no evidence for any consistent differences or divergence between *L. triangulum* and *L. gentilis*, for example.

B&R go on to state that the Chambers and Hillis (2020) concept of *L. triangulum* was paraphyletic on all the consensus trees of nuclear genes. As Chambers and Hillis (2020) noted, apparent paraphyly of an individual gene tree is not unexpected for a species, particularly with the low levels of divergence observed in the loci examined by Ruane et al. (2014). However, B&R's statement that the consensus trees for Chambers and Hillis's (2020) preferred concept of *L. triangulum* were paraphyletic across all gene trees is false. In many of the trees, specimens of *L. triangulum* merely appear in an unresolved polytomy in the majority-rule consensus trees. These polytomies were largely a result of the minimal genetic differentiation of the genes examined by Ruane et al. (2014). In contrast, *all* the gene trees for some of Ruane et al.'s (2014) preferred taxa were demonstrably and significantly polyphyletic. If a polytomy of individuals in a consensus gene tree is evidence against recognizing a taxon (as argued by B&R), then surely unambiguous polyphyly and lack of divergence across all the gene trees (as in some taxa recognized by Ruane et al., 2014) should be a larger concern.

Isolation by distance (IBD) is, of course, a primary concern with a widespread taxon such as *L. triangulum* and has since received further attention. Given the limited sampling of nuclear loci by Ruane et al. (2014)—which was especially lacking at potential contact zones—no test of migration rates or IBD could be made with statistical confidence. However, although explicit tests for IBD (or migration) were not feasible, Chambers and Hillis (2020) did ask whether BPP was sensitive to population assignment under the sampling conducted by Ruane et al. (2014). Contrary to what B&R state, the divisions used to test the sensitivity of population assignment by Chambers and Hillis (2020) were not "random," but instead were systematically divided across geographic space. Chambers and Hillis (2020) found that all systematic divisions of geographically adjacent specimens across the range of the species were supported as distinct species by BPP. Clearly, all these divisions cannot represent different species, especially since the results based on different splits are incompatible with one another. Thus, Chambers and Hillis (2020) showed that the split preferred by Ruane et al. (2014) was no more or less supported by BPP than virtually any other geographic split of sampled individuals across the range of *L. triangulum*. Indeed, the patterns Chambers and Hillis (2020) observed in BPP's high support for multiple splits across the range of *L. triangulum* could certainly be due to IBD, and they did not argue against this. Their point was simply that all geographical splits within the continental range of *L. triangulum* produced the same result that was used to justify the split between *L. triangulum* and *L. gentilis* by Ruane et al. (2014). The role of IBD as an explanation for this problem has now been more thoroughly discussed in arguments against limited sampling in species delimitation studies (Mason et al., 2020). It seems an aimless and circular argument for B&R to criticize Chambers and Hillis (2020) for not having tested for IBD, when they themselves failed to do so with the exact same data in Ruane et al. (2014). The data reported by Ruane et al.

Downloaded From: https://bioone.org/journals/Ichthyology-&-Herpetology on 03 Aug 2024
Terms of Use: https://bioone.org/terms-of-use

(2014) were simply not sufficient to test for IBD. Moreover, IBD is ubiquitous in nature, and we would expect to see it in most dispersal-limited species.

Finally, B&R mention that the lack of reciprocal monophyly of their taxa in gene trees presented in Chambers and Hillis (2020) could be due to incomplete lineage sorting. Ruane et al. (2014) made a similar argument in stating that misplaced individuals in their summary SplitsTree (based on all their data) could be the result of incomplete lineage sorting. This explanation fails to explain the very similar genotypes (across all combined loci) that they reported between several purported species, such as between *L. triangulum* and *L. gentilis*, as well as *L. polyzona* and *L. abnorma*. Incomplete lineage sorting can result in individual genes being divergent within a species, but it cannot explain *all* genes being virtually identical across individuals of different species, if the species show any substantial level of divergence.

As stated several times in Chambers and Hillis (2020), we agree with Burbrink and Ruane (2021) that further evidence—and testing—is required to validate the species hypotheses that were preferred by Chambers and Hillis (2020). We did not argue that we know the correct answer to the species delimitation problems presented by the *L. triangulum* complex, but rather that the data presented by Ruane et al. (2014) do not support the conclusions in their paper, and that their data, furthermore, provide better support for an alternative (previously suggested) hypothesis. We noted two contact zones in the group that did exhibit evidence of sympatry, and thus reproductive isolation, and noted that all other "contact zones" between species delimited by Ruane et al. (2014) showed no evidence of any genetic divergence or reproductive isolation (based on the data they presented). We thus concluded that the evidence presented by Ruane et al. (2014) supported just three species in the group, rather than the seven they reported.

To summarize, Chambers and Hillis (2020) did not attempt to provide an integrative assessment of the *Lampropeltis triangulum* complex—which would require much more extensive sampling (including contact zones), more extensive sequencing of variable genes, and more thorough analyses of morphology—and they did not argue that it was their goal was to do so. Chambers and Hillis (2020) examined Ruane et al. (2014) as a case study to examine the potential problems of limited sampling when using multispecies coalescent-based species delimitation. Our findings were supportive of other studies that indicate potential oversplitting from multispecies coalescent-based species delimitation (Sukumaran and Knowles, 2017; Campillo et al., 2019). We agree that a full understanding of the species limits in this group will require considerably more data and more thorough geographic sampling than has been collected to date, especially at any purported contact zones. However, we also contend that several of the novel suggestions for species splits by Ruane et al. (2014) were unsupported by their data.

Describing species from limited specimens.—More than 30 years ago, Hillis (1990) conducted a systematic review of the snake genera *Synophis* and *Emmochliophis* and examined all specimens of the two genera that had been collected and deposited into museums since an earlier review of *Synophis* by Bogert (1964). These Neotropical snakes are extremely rare, and until the description of *Synophis calamitus* by Hillis (1990), every species of *Synophis* and *Emmochliophis* had been described from single specimens. To this day, one of the species is still known only from the holotype collected in the late 1800s (Boulenger, 1898), and it was 50 years after the description of *Emmochliophis fugleri* (Fritts and Smith, 1969) before a second specimen of that species was collected (Maynard et al., 2021). Hillis (1990) reconstructed the phylogeny of the group based on morphological characters (he collected tissues from the new species, but no material was available from any of the other species for comparison), and described the new species *Synophis calamitus* based on two specimens, one of which was a roadkill specimen.

B&R criticized several aspects of Hillis (1990), but their criticisms were largely untrue or unwarranted. They stated that "30 years ago Hillis described a new species of *Synophis* (Hillis, 1990) from only two specimens, one heavily damaged." Burbrink and Ruane (2021) failed to note the exceptionally rare nature of snakes in the studied genera, or the fact that the new species described by Hillis (1990) was the first species in the group to be based on multiple specimens. B&R go on to state that Hillis (1990) "did not account for morphological variation within the genus." Appendix I of Hillis (1990) listed all of the specimens of *Synophis*, *Emmochliophis*, and the related genus *Diaphorolepis* examined by Hillis (1990). This list included all known specimens of the subject genera that had been collected since Bogert's (1964) review; the variation of the specimens examined by Bogert (1964) was also considered and reported by Hillis (1990). Therefore, known morphological variation, from all specimens known at the time of the description, was described and evaluated in the paper. B&R state that Hillis (1990) "incorrectly scored material from the type specimens," which is apparently a reference to a different interpretation of one scalation character (number of postoculars) on one side of the damaged head of the paratype by Torres-Carvajal et al. (2015) compared to Hillis (1990). All of the scalation characters of the holotype reported by Hillis (1990) can be confirmed in his illustrations of that specimen. B&R further state that Hillis (1990) "could not reliably define the distribution of the new species," which was merely a function of the exceptionally rare nature of the snakes; the localities of all known specimens were reported. Despite these misleading criticisms, B&R then say that ". . . it would be pointless to . . . [criticize the paper and initial taxonomic decision] until additional new data were analyzed to refute or support the hypothesis (see Torres-Carvajal et al., 2015, for a more modern treatment of this genus)." This statement was made despite the fact that Torres-Carvajal et al. (2015) did indeed support the distinct species status of *S. calamitus* in describing three new species of *Synophis* (they found that *S. calamitus* was the most divergent species of *Synophis* that they examined, and they resolved it as the sister group of the three new species that they described). Torres-Carvajal et al. (2015) described these new species based on morphological variation and limited mitochondrial sequences. The sequence analyses of Torres-Carvajal et al. (2015) did not include all the species examined by Hillis (1990), which were still known only from a few preserved specimens, but they supported the comparable relationships found in the morphologically based phylogenetic analysis by Hillis (1990), including the distinctiveness of *S. calamitus*, the

Downloaded From: https://bioone.org/journals/Ichthyology-&-Herpetology on 03 Aug 2024
Terms of Use: https://bioone.org/terms-of-use

monophyly of the genus *Synophis*, and the close relationship of *Diaphorolepis* to *Synophis* (no specimens of either species of *Emmochliophis* were included in the phylogenetic analysis by Torres-Carvajal et al., 2015).

Is it a good idea to describe a rare species based on a very limited sample of specimens? Obviously, it is not ideal, and it should never be done when more extensive collection is possible. However, in the case of *Synophis*, a century of extensive collecting in the Ecuadorian Andes from the 1890s to 1990 had produced a very limited number of specimens. Based on morphological analysis of all available material, the specimens of *S. calamitus* described by Hillis (1990) were distinct from any previously described species. Given these circumstances, a new species description was warranted. Subsequent molecular analyses have confirmed the distinctiveness of this described species. The points that B&R intended to make in criticizing this example are unclear, and their criticisms are false or misleading. Sometimes describing a distinct new species from limited morphological comparisons is justified, especially when it is the only option available.

The role of mitochondrial DNA in species delimitation.—B&R argued that statements made by Hillis (2019) concerning the use of mtDNA alone to revise species boundaries undermine decades-old research studies. For clarification, this interpretation of Hillis (2019) is clearly incorrect; Hillis (2019) simply mentioned progress that had been made from a time when only mtDNA was accessible and, that in a genomic era, we must expand on this knowledge and explore the genome further. In fact, Hillis (2019) also explored the merits of DNA barcoding approaches, a method which relies on a fragment of a single mitochondrial gene (e.g., Chambers and Hebert, 2016). Mitochondrial DNA does often support the same boundaries and provide the same conclusions as genome-level nuclear data (Devitt et al., 2019), although numerous counterexamples also exist (e.g., Leaché and McGuire, 2006; Toews and Brelsford, 2012; Marshall et al., 2021). We certainly do not wish to undermine past research when genetic studies were technologically or logistically limited and drew conclusions based only on mtDNA, and we hope that no researchers interpret the language in Hillis (2019) as such. Hillis (2019) made a point of noting that his own species delimitation studies had made use of many different emerging technologies over the years, including studies of morphology, behavior, allozymes, karyotypes, mitochondrial DNA, nuclear genes, and complete genomes, and he emphasized that no one approach or technology should be viewed as giving the "final answer" in systematic studies. Rather, each approach offers advantages and disadvantages, and the best species delimitation studies make use of the entire systematic toolbox. It is, however, important to re-examine past studies and conclusions, and Hillis (2019) emphasized that past taxonomic revisions that were based solely on a mitochondrial gene tree should be re-examined with more comprehensive data (see Marshall et al., 2021, for a recent example from the *Pantherophis guttatus* complex).

WHERE DO WE AGREE WITH BURBRINK AND RUANE (2021)?

Despite our objections to false or unwarranted criticisms of our work, there are points in B&R with which we agree. In general, B&R provide a reasonable summary of some recent developments in species delimitation, including advances that allow for measuring aspects of population genetics that are crucial to our understanding of species boundaries. They suggest a framework upon which researchers can implement newly developed tools and software to answer questions related to the speciation process. We agree that these advancements and their refinement will result in new insights into the formation and maintenance of species boundaries in any taxonomic group. We especially agree with B&R's statements regarding the necessity for thorough and comprehensive analyses, both at the genomic and organismal level, to fully understand species limits. We agree with them as well that there are many new and worthwhile analyses related to contact and hybrid zones, and we believe this progress shows much promise for the future of species delimitation. Indeed, these are the exact conclusions that we emphasized in several of our papers that were criticized by B&R.

CONCLUSIONS

Although B&R claim that we "fall short of considering modern theory and application," we argue instead that our differences lie in fundamental disagreements about (i) the evidence needed to delimit species, and (ii) when taxonomic change is warranted. Changing one's mind in the face of new evidence is a hallmark of science, and we should always be willing to state the evidence that would lead us to do so, which we hope to have done here. We argue that this approach is far more interesting and appropriate than making unjustified attacks on efforts to improve the field of species delimitation practices.

Science is an iterative process, and it is always good for researchers to critique others' work with the hopes of improving it (Gerwing et al., 2020). However, such critique is useful only if it is objective, constructive, and true. In addition, it is important for the products of taxonomic study—including the discovery, delimitation, and naming of clades and species—to be as useful as possible to other biologists. Unnecessary and poorly supported changes to the taxonomy of well-studied groups can be highly confusing and problematic, rather than informative and helpful. Thus, subjective taxonomic and nomenclatural changes demand thorough evidence, testing, and justification before implementation, especially in groups where sampling is not a serious obstacle. These were the primary points of our papers that were criticized by B&R. Although B&R unfortunately misrepresented much of our work, we are grateful to the editor of this journal for the opportunity for make public our response. Honest critique and the correction of errors are at the heart of improving scientific knowledge and our understanding of the natural world, and this in turn propels any scientific field forward.

DATA ACCESSIBILITY

Unless an alternative copyright or statement noting that a figure is reprinted from a previous source is noted in a figure caption, the published images and illustrations in this article are licensed by the American Society of Ichthyologists and Herpetologists for use if the use includes a citation to the original source (American Society of Ichthyologists and Herpetologists, the DOI of the *Ichthyology & Herpetology*

Downloaded From: https://bioone.org/journals/Ichthyology-&-Herpetology on 03 Aug 2024
Terms of Use: https://bioone.org/terms-of-use

article, and any individual image credits listed in the figure caption) in accordance with the Creative Commons Attribution CC BY License.

ACKNOWLEDGMENTS

We would like to thank Anthony Barley, Prosanta Chakrabarty, Harry Greene, and Greg Pauly for comments on initial drafts of this manuscript as well as helpful discussion regarding species delimitation.

LITERATURE CITED

Barley, A. J., J. M. Brown, and R. C. Thomson. 2018. Impact of model violations on the inference of species boundaries under the multispecies coalescent. Systematic Biology 67: 269–284.

Barton, N. H. 2000. Estimating multilocus linkage disequilibria. Heredity 84:373–389.

Barton, N. H., and K. S. Gale. 1993. Genetic analysis of hybrid zones, p. 13–45. *In*: Hybrid Zones and the Evolutionary Process. R. G. Harrison (ed.). Oxford University Press, Oxford.

Barton, N. H., and G. M. Hewitt. 1985. Analysis of hybrid zones. Annual Review of Ecology and Systematics 16:113–148.

Barton, N. H., and G. M. Hewitt. 1989. Adaptation, speciation and hybrid zones. Nature 341:497–503.

Bogert, C. M. 1964. Snakes of the genera *Diaphoralepis* and *Synophis* and the colubrid subfamily Xenoderminae (Reptilia, Colubridae). Senckenbergiana Biologica 45:509–531.

Boulenger, G. A. 1898. An account of the reptiles and batrachians collected by Mr. W.F.H. Rosenberg in western Ecuador. Proceedings of the Zoological Society of London 1898:107–126.

Burbrink, F. T. 2002. Phylogeographic analysis of the cornsnake (*Elaphe guttata*) complex as inferred from maximum likelihood and Bayesian analyses. Molecular Phylogenetics and Evolution 25:465–476.

Burbrink, F. T., and T. J. Guiher. 2015. Considering gene flow when using coalescent methods to delimit lineages of North American pitvipers of the genus *Agkistrodon*. Zoological Journal of the Linnean Society 173:505–526.

Burbrink, F. T., and S. Ruane. 2021. Contemporary philosophy and methods for studying speciation and delimiting species. Ichthyology & Herpetology 109.

Campillo, L. C., A. J. Barley, and R. C. Thomson. 2019. Model-based species delimitation: Are coalescent species reproductively isolated? Systematic Biology 69:708–721.

Chambers, E. A., and P. D. N. Hebert. 2016. Assessing DNA barcodes for species identification in North American reptiles and amphibians in natural history collections. PLoS ONE 11:e0154363.

Chambers, E. A., and D. M. Hillis. 2020. The multispecies coalescent over-splits species in the case of geographically widespread taxa. Systematic Biology 69:184–193.

Chippindale, P. T., A. H. Price, J. J. Wiens, and D. M. Hillis. 2000. Phylogenetic relationships and systematic revision of central Texas hemidactyliine plethodontid salamanders. Herpetological Monographs 14:1–80.

Cicero, C., N. A. Mason, R. A. Jiménez, D. R. Wait, C. Y. Wang-Claypool, and R. C. K. Bowie. 2021. Integrative taxonomy and geographic sampling underlie successful species delimitation. Ornithology 138:1–15.

Coyne, J. A., and H. A. Orr. 2004. Speciation. Sinauer Associates, Inc., Sunderland, Massachusetts.

Devitt, T. J., S. J. Baird, and C. Moritz. 2011. Asymmetric reproductive isolation between terminal forms of the salamander ring species *Ensatina eschscholtzii* revealed by fine-scale genetic analysis of a hybrid zone. BMC Evolutionary Biology 11:245.

Devitt, T. J., A. M. Wright, D. C. Cannatella, and D. M. Hillis. 2019. Species delimitation in endangered groundwater salamanders: implications for aquifer management and biodiversity conservation. Proceedings of the National Academy of Sciences of the United States of America 116: 2624–2633.

Dufková, P., M. Macholán, and J. Piálek. 2011. Inference of selection and stochastic effects in the house mouse hybrid zone. Evolution 65:993–1010.

Durrett, R., L. Buttel, and R. Harrison. 2000. Spatial models for hybrid zones. Heredity 84:9–19.

Endler, J. A. 1977. Geographic Variation, Speciation, and Clines. Princeton University Press, Princeton, New Jersey.

Falush, D., M. Stephens, and J. K. Pritchard. 2003. Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. Genetics 164:1567–1587.

Fritts, T. H., and H. N. Smith. 1969. A new genus and species of snake from western Ecuador. Transactions of the Kansas Academy of Science 72:60–66.

Gerwing, T. G., A. M. A. Gerwing, S. Avery-Gomm, C.-Y. Choi, J. C. Clements, and J. A. Rash. 2020. Quantifying professionalism in peer review. Research Integrity and Peer Review 5:9.

Gloyd, H. K., and R. Conant. 1990. Snakes of the *Agkistrodon* complex—a monographic review. Contributions to Herpetology No. 6. Society for the Study of Amphibians and Reptiles, Oxford, Ohio.

Guiher, T. J., and F. T. Burbrink. 2008. Demographic and phylogeographic histories of two venomous North American snakes of the genus *Agkistrodon*. Molecular Phylogenetics and Evolution 48:543–553.

Hardy, G. H. 1908. Mendelian proportions in a mixed population. Science 28:49–50.

Harrison, R. G. 1993. Hybrids and hybrid zones: historical perspective, p. 3–12. *In*: Hybrid Zones and the Evolutionary Process. R. G. Harrison (ed.). Oxford University Press, Oxford.

Hillis, D. M. 1981. Premating isolating mechanisms among three species of the *Rana pipiens* complex in Texas and southern Oklahoma. Copeia 1981:312–319.

Hillis, D. M. 1990. A new species of xenodontine colubrid snake of the genus *Synophis* from Ecuador and the phylogeny of the genera *Synophis* and *Emmochliophis*. Occasional Papers of the Museum of Natural History, University of Kansas 135:1–9.

Hillis, D. M. 2019. Species delimitation in herpetology. Journal of Herpetology 53:3.

Hillis, D. M. 2020. The detection and naming of geographic variation within species. Herpetological Review 51:52–56.

Hillis, D. M., D. A. Chamberlain, T. P. Wilcox, and P. T. Chippindale. 2001. A new species of subterranean blind salamander (Plethodontidae: Hemidactyliini: *Eurycea*: *Typhlomolge*) from Austin, Texas, and a systematic revision of central Texas paedomorphic salamanders. Herpetologica 57:266–280.

Downloaded From: https://bioone.org/journals/Ichthyology-&-Herpetology on 03 Aug 2024
Terms of Use: https://bioone.org/terms-of-use

Jiggins, C. D., and J. Mallet. 2000. Bimodal hybrid zones and speciation. Trends in Ecology & Evolution 15:250–255.

Kruuk, L. E., S. J. Baird, K. S. Gale, and N. H. Barton. 1999. A comparison of multilocus clines maintained by environmental adaptation or by selection against hybrids. Genetics 153:1959–71.

Leaché, A. D., and J. A. McGuire. 2006. Phylogenetic relationships of horned lizards (*Phrynosoma*) based on nuclear and mitochondrial data: evidence for a misleading mitochondrial gene tree. Molecular Phylogenetics and Evolution 39:628–644.

Linck, E., K. Epperly, P. V. Els, G. M. Spellman, R. W. Bryson, J. E. McCormack, R. Canales-del-Castillo, and J. Klicka. 2019. Dense geographic and genomic sampling reveals paraphyly and a cryptic lineage in a classic sibling species complex. Systematic Biology 68:956–966.

Macholán, M., S. J. Baird, P. Munclinger, P. Dufková, B. Bímová, and J. Piálek. 2008. Genetic conflict outweighs heterogametic incompatibility in the mouse hybrid zone? BMC Evolutionary Biology 8:271.

Mallet, J. 2005. Hybridization as an invasion of the genome. Trends in Ecology & Evolution 20:229–237.

Marshall, T. L., E. A. Chambers, M. V. Matz, and D. M. Hillis. 2021. How mitonuclear discordance and geographic variation have confounded species boundaries in a widely studied snake. Molecular Phylogenetics and Evolution 162: 107194.

Mason, N. A., N. K. Fletcher, B. A. Gill, W. C. Funk, and K. R. Zamudio. 2020. Coalescent-based species delimitation is sensitive to geographic sampling and isolation by distance. Systematics and Biodiversity 18:269–280.

May, R. M., J. A. Endler, and R. E. McMurtrie. 1975. Gene frequency clines in the presence of selection opposed by gene flow. American Naturalist 109:659–676.

Maynard, R., J. Culebras, S. Kohn, and J. M. Guayasamin. 2021. Finding a shadow in the dark: rediscovery of Fugler's shadow snake (*Emmochliophis fugleri* Fritts & Smith, 1969) after 54 years, with comments on its conservation status, distribution, and the tribe Diaphorolepidini. Check List 17: 239–245.

Pritchard, J. K., M. Stephens, and P. Donnelly. 2000. Inference of population structure using multilocus genotype data. Genetics 155:945–959.

Ruane, S., R. W. Bryson, R. A. Pyron, and F. T. Burbrink. 2014. Coalescent species delimitation in milksnakes (genus *Lampropeltis*) and impacts on phylogenetic comparative analyses. Systematic Biology 63:231–250.

Slatkin, M. 1985. Gene flow in natural populations. Annual Review of Ecology and Systematics 16:393–430.

Slatkin, M. 1987. Gene flow and the geographic structure of natural populations. Science 236:787–792.

Slatkin, M. 2008. Linkage disequilibrium—understanding the evolutionary past and mapping the medical future. Nature Reviews Genetics 9:477–485.

Sukumaran, J., and L. L. Knowles. 2017. Multispecies coalescent delimits structure, not species. Proceedings of the National Academy of Sciences of the United States of America 114:1607–1612.

Toews, D. P. L., and A. Brelsford. 2012. The biogeography of mitochondrial and nuclear discordance in animals. Molecular Ecology 21:3907–3930.

Torres-Carvajal, O., L. Y. Echevarría, P. J. Venegas, G. Chávez, and J. D. Camper. 2015. Description and phylogeny of three new species of *Synophis* (Colubridae, Dipsadinae) from the tropical Andes in Ecuador and Peru. ZooKeys 2015:153–179.

Weinberg, W. 1908. Über den Nachweis der Vererbung beim Menschen. Jahreshefte des Vereins Varterländische Naturkunde in Württemberg 64:369–382.

Whitlock, M. C., and D. E. McCauley. 1999. Indirect measures of gene flow and migration: $F_{ST} \neq 1/(4Nm+1)$. Heredity 82:117–125.

Wiens, J. J. 2004. What is speciation and how should we study it? American Naturalist 163:914–923.

Yang, Z. H., and B. Rannala. 2010. Bayesian species delimitation using multilocus sequence data. Proceedings of the National Academy of Sciences of the United States of America 107:9264–9269.

Yuan, Z.-Y., W.-W. Zhou, X. Chen, N. A. Poyarkov, Jr., H.-M. Chen, N.-H. Jang-Liaw, W.-H. Chou, N. J. Matzke, K. Iizuka, M.-S. Min, S. L. Kuzmin, Y.-P. Zhang, D. C. Cannatella, D. M. Hillis, and J. Che. 2016. Spatiotemporal diversification of the true frogs (genus *Rana*): a historical framework for a widely studied group of model organisms. Systematic Biology 65:824–842.

Downloaded From: https://bioone.org/journals/Ichthyology-&-Herpetology on 03 Aug 2024
Terms of Use: https://bioone.org/terms-of-use