

Species Delimitation in Herpetology

DAVID M. HILLIS

Department of Integrative Biology and Biodiversity Center, University of Texas at Austin, Austin, Texas USA; E-mail: dhillis@austin.utexas.edu

ABSTRACT.—The discovery and delimitation of species has changed dramatically over time. Species delimitation practices became more thorough and formal in the 1900s with the introduction of detailed studies of geographic variation, contact zones, and reproductive isolating mechanisms. In the 1960s, genetic methods for examining the allelic composition across many loci began to be used to test for gene flow and to delimit species boundaries. Methods for DNA sequencing were invented in the late 1970s, just as I started graduate school, when I set my sights on applying the vast stores of information in genomes to understanding biodiversity. In the late 1980s, a new method for rapid amplification of mitochondrial DNA led to “barcoding” of species and the subsequent splitting of species into mitochondrial haplotype groups. By the 1990s, widespread sequencing of nuclear genes led to the development of models that incorporated multispecies coalescent theory (MSC). Molecular-based methods provide new insights and opportunities for species delimitation, but many species delimitation studies do not adequately consider violations of underlying model assumptions before making taxonomic changes. Inadequate sampling and a lack of attention to contact zones often leads to the over-splitting of species into geographically proximate groups of populations. I predict the future will bring a synthesis of many older practices (careful sampling, with attention to reproductive isolation, contact zone analysis, and geographic variation) with the new powerful analysis of genomic data sets, leading to a reevaluation and reversal of much of the recent overly enthusiastic splitting of geographically variable species.

Biologists have long understood that the diversity of life is not distributed as a continuum. Rather, there are distinct entities, which we term species, within which individuals share genes and traits through genetic exchange and reproduction. Between species, there are reproductive gaps and barriers that lead to morphological, behavioral, physiological, and genetic differences, which make the identification of species boundaries an important and useful endeavor.

In this essay, I briefly review the history of methods and practices that biologists have used to distinguish and delimit species of reptiles and amphibians. I also give my personal perspective on the changes that I’ve witnessed since the 1970s, when I began studying species boundaries. Over my career, as new approaches have been introduced, I’ve seen many proponents of new methods argue that they have “solved” the problem of species delimitation and sometimes propose wholesale changes in classifications from the limited view of a new data set. I argue here that species delimitation is a complex problem and that no one method or model or approach provides a comprehensive picture of the boundaries between species. Instead, species delimitation should take into consideration evidence from all sources, and changes in classifications should be made only when it is clear that the existing classification does not appropriately reflect evolutionary history.

This paper is not a review of species concepts in herpetology (see Frost and Hillis, 1990). Although species concepts are often considered controversial, this view stems largely from a confusion of species concepts with methods of species delimitation. Although different researchers might identify different species concepts by name (e.g., biological species concept, evolutionary species concept, general lineage species concept), one would be hard-pressed to find significant conceptual differences in the kinds of entities practitioners of these concepts wish to identify. By using the name of a particular species concept, researchers are usually just emphasizing a slightly different point in the noninstantaneous process of speciation (de Queiroz, 1998). In delimiting species, most biologists wish to identify the distinct evolutionary lineages—isolated through

time and space from other such lineages—that are described in the first paragraph of this essay. Hence, in this paper, I accept that species can be recognized by two general features: 1) sexual species represent cohesive evolutionary lineages, within which individuals mate and share genes; and 2) species remain distinct from one another through space and time, typically as a result of some means of reproductive isolation. This general view of species as reproductively isolated, independently evolving lineages on the Tree of Life is compatible with most modern concepts of species (Simpson, 1961; Mayr, 1969; Ghiselin, 1974; Wiley, 1978; Templeton, 1989; Frost and Hillis, 1990; Mayden, 1997; de Queiroz, 1998). For a discussion of asexual lineages, which are conceptually distinct from sexual species (even though they are often formally treated as species), see Frost and Hillis (1990). Delimitation practices for asexual lineages are distinct, and I will consider them only briefly here because of space limitations.

I argue that robust delimitation of species requires two stages: a “grouping” stage, whereby specimens are grouped into putative evolutionary lineages (the hypothesis-generation step); and a second stage, where the groups found in the first step are tested to see whether they are sufficiently reproductively isolated from one another to be evolving independently of one another and, hence, considered species (the hypothesis-testing step). Biologists may disagree about the degree of reproductive isolation that is needed to recognize two taxa as distinct species, but all modern concepts of species require (either explicitly or implicitly) some degree of reproductive isolation between species. Lineages can still diverge with limited gene flow (Hey, 2006), but some degree of extrinsic or intrinsic reproductive isolation is necessary to recognize two groups as different species. Otherwise, there would be no distinction between the concept of species and the concept of local populations of a species (which are not reproductively isolated from other such populations). Therefore, in recognizing a species split, a systematist should describe the degree of reproductive isolation between the proposed species and make an argument as to why the observed degree of reproductive isolation is viewed as sufficient to maintain separately evolving and diverging evolutionary lineages.

The view of species as distinct evolutionary lineages, evolving independently from other such lineages through space and time, has developed gradually over the past half century and especially during my professional career. For a historical perspective on recent developments in this field, we need to go back even farther, and consider how species delimitation and recognition practices have changed over the past few centuries.

Species Names by Typology.—Prior to the publication of Charles Darwin's (1859) *On the Origin of Species*, most systematic herpetology was limited to the description and naming of species, with occasional cursory studies of their geographic distributions. Systematic thinking was influenced strongly by Aristotelian essentialism. Species were viewed typologically, and variation was seen as deviation from perfection. Higher taxa usually were defined based on a few "essential" characters. There were some notable exceptions: for instance, the French natural historian Buffon (1753), in the fourth volume of his *Histoire Naturelle*, suggested that the common morphological features revealed by comparative anatomy were an expression of genealogical relationships. Other authors, most notably Lamarck (1809), in his book *Philosophie Zoologique*, were even more explicit about their theories of evolution and the connection of these theories to systematics. The lack of a believable mechanism for evolution, however, meant that relatively few biologists (or others) accepted evolution as an explanation for biological diversity. Exploration of the Earth was quite incomplete; therefore, naturalists (primarily from Europe) set forth to discover and describe the diversity of organisms that existed worldwide.

The formal beginnings of binomial nomenclature, in which species were assigned to a genus with a species epithet, date to the Swedish naturalist Carolus Linnaeus, whose 10th edition of *Systema Naturae* (1758), established the system of binomial nomenclature that is most widely used for animals today. Although Linnaean nomenclature and the Aristotelian logic it incorporated originally had some formidable opposition from people like Buffon, evolution was far from the mainstream at the time. Phylogeny was a concept given little thought (and not yet given a name), and it played virtually no direct role in the activities of most systematists.

Natural Classifications.—Despite the lack of a unifying evolutionary theory, herpetologists of the early to mid-1800s attempted to create "natural classifications." Although natural classification today is equated with a classification that directly reflects phylogenetic relationships, such was obviously not the case before evolution became widely accepted and studied. Just before publication of Darwin's *On the Origin of Species*, the widely influential biologist Louis Agassiz (who never accepted evolutionary ideas, even in later years) wrote *An Essay on Classification* (1859). In this book, Agassiz discussed the "Natural System" of classification (1859, 8):

The divisions of animals according to branch, class, order, family, genus, and species, by which we express the results of our investigations into the relations of the animal kingdom, and which constitute the primary question respecting any system of Zoology, seem to me to deserve the consideration of all thoughtful minds. Are they the devices of the human mind to classify and arrange our knowledge in such a manner as to bring it more readily within our grasp and facilitate further investigations, or have they been instituted by the Divine Intelligence as the categories of his mode of thinking? Have we, perhaps, thus far been only the unconscious inter-

preters of a Divine conception, in our attempts to expound nature?

To Agassiz, these questions were rhetorical; for him, systems of classification could be natural only if they were a reflection of the mind of God. Nonetheless, in a footnote, Agassiz (1859, 8) conceded that

. . . a system may be natural, that is, may agree in every respect with the facts in nature, and yet not be considered by its author as the manifestation of the thoughts of the Creator, but merely as the expression of a fact existing in nature—no matter how—which the human mind may trace and reproduce in a systematic form of its own invention.

The only problem was that Agassiz could not imagine what that "fact existing in nature" could be if it were not the "thoughts of the Creator." The understanding of evolution changed that. It quickly became clear to many systematists that phylogeny was the underlying cause of the organization they had so long detected and that classifications, therefore, should be based directly on phylogeny. Species were the lineages that evolved on the Tree of Life, and groups of related species formed a hierarchy of life that could be described by higher ranks in classification.

The Ebb and Flow of Phylogenetic Thinking.—In the few decades after the publication of Darwin's *On the Origin of Species*, study of phylogeny was a primary concern of systematists. Haeckel's (1866) drawings of the Tree of Life were outstanding examples of the results of this interest in phylogeny (in fact, it was Haeckel who coined the term phylogeny). Because no clear and repeatable methods existed for inferring phylogeny, however, research in phylogenetics began to fade into the background. During the first half of the 20th century, the emphasis in systematics turned to studies of speciation and geographic variation. The word "phylogeny" does not even appear in the index to Huxley's *Evolution: The Modern Synthesis*, published in 1942. In *Methods and Principles of Systematic Zoology*, Mayr et al. (1953, 9) considered "modern" systematics to be concerned with the "study of the evolution within species" (emphasis in original). Mayr et al. (1953, 45) accepted that ". . . it should remain the ultimate aim of the taxonomist to devise a phylogenetic classification," but they did not consider any of the existing methods for inferring phylogenies to be particularly reliable. Some other systematists of the first half of the 20th century, such as the German systematist Walter Zimmerman (1931; see Donoghue and Kadereit, 1992), did argue for phylogenetic classifications. But many systematists were just as skeptical as Mayr was of systematists' abilities to reconstruct phylogenies, and the emphasis in systematics was largely on intraspecific geographic variation.

In the early 1900s, several herpetologists set new standards for studying geographical variation within species, and in developing methods for morphological species delimitation. Ruthven (1908) introduced statistical methods for analyzing geographic variation and species limits in *Thamnophis*. His monograph set a new standard for others to follow. Ruthven's model of careful geographic sampling and morphological analysis was followed by Blanchard (1921) in his monograph of the genus *Lampropeltis*, as well as by many other authors of the 1920s to 1940s. That these monographs are still useful and important systematic references today is testimony to the enduring value of these authors' careful and comprehensive

approaches; however, any discussion of phylogeny in these papers was highly speculative, rather than analytical.

Then, a renaissance of phylogenetics occurred in the 1950s and 1960s. Several research groups began to develop objective, quantitative, reliable methodologies to infer phylogenies and to apply phylogenetic information to classifications. For example, in *Grundzüge einer Theorie der Phylogenetischen Systematik*, Willi Hennig (1950) presented an argument for the central role of phylogeny in classifications, as well as a method for inferring phylogenies from the characters of organisms. His book reached its greatest influence after being translated into English in 1966 (as *Phylogenetic Systematics*).

Hennig's thoughts about phylogeny also strongly influenced his thinking about species. Hennig (1950, 1966) emphasized that central to the idea of a species is the concept of a *lineage*—a series of ancestor–descendant relationships through time. Hennig (1950, 1966) noted that the nature of ancestor–descendant relationships is different within species (where individuals mate and, thus, mix and recombine their genes; Hennig called these *tokogenetic* relationships) versus between species (where the lineages evolve independently from one another and show hierarchical, bifurcating relationships, to which Hennig limited the term *phylogenetic* relationships).

But even though all species are lineages, all lineages are not species. To understand this important point, consider a particular nucleotide in one of your genes. This nucleotide descended (through replication) from another nucleotide at the same location in the same gene in one of your parents. If you have an adenine at this location, the ancestral nucleotide in your parent's gene was probably an adenine, too; if so, then no mistake was made during DNA replication. On the other hand, there is some small chance that a mutation occurred during replication of your parent's gene and that the ancestral nucleotide in your parent was actually a guanine. If so, then even though the nucleotide was different in your parent compared to you, the guanine in your parent's gene was still the ancestor to the adenine in your gene, because it was the template upon which the replication was based. This imperfect replication of ancestors is what produces evolution, which can be defined simply as change in a lineage through time. Although an ancestor–descendant series at a particular nucleotide position constitutes a lineage, it is also part of several other lineages that are organized at higher levels. For instance, adjacent nucleotides within your DNA are usually inherited together as a block. If the nucleotide we considered earlier was located in the gene that encodes the alpha unit of hemoglobin, then it is likely that all the nucleotides in that gene are descended from a single copy of that gene in one of your parents. Therefore, there is a lineage of α -hemoglobin genes, which if we trace backward in time goes from you to one of your parents to one of your grandparents and so on. Of course, because you are diploid, you also have a second lineage of α -hemoglobin genes that has a descent back through your other parent.

There are also lineages of blocks of genes, including chromosomes, but these tend to be disrupted through time via recombination in meiosis. Biparental inheritance and meiotic recombination produce a network of reticulation within sexual lineages. Thus, sexual reproduction produces reticulations of genomes within species. Within sexually reproducing species, individual relationships form a reticulating network through time, which is what Hennig (1950, 1966) called tokogenetic relationships (parent–offspring relationships of individuals

within a species). The sexual lineages themselves evolve independently of one another, however, if they are reproductively isolated. Therefore, we can consider phylogenetic relationships among species.

Speciation and Other Processes of Lineage Division.—Sexual reproduction clearly has boundaries, even if they are sometimes fuzzy. Humans do not reproduce with other mammals, for example. Consequently, individuals that interact reproductively together form a lineage that rarely (if ever) reticulates with other such lineages. We call these larger, independently evolving lineages “species.” When one such lineage splits into two lineages, which no longer interact reproductively, we call this process speciation.

Many other processes besides speciation can lead to the division of gene lineages, however (Fitch, 1970). New alleles arise through mutation, and genes or even whole genomes can be duplicated. Genes can also be horizontally transferred between species. Therefore, we see a complex history of gene lineages within species lineages—speciation gives rise to some similarities between gene trees and species trees, but other processes lead to many differences as well (Maddison, 1997).

Other processes besides sex may also hold species lineages together. For instance, selection for particular morphotypes through time may constrain the divergence of a lineage. Even asexual organisms may be organized into groups of closely related individuals that fill distinct regions of ecological and morphological space (Uzzell, 1964; Cole, 1985; Darevsky et al., 1985; Hillis, 2007b; Fontaneto and Barraclough, 2015). Although these asexual lineages may be conceptually somewhat different from lineages that are held together through sexual recombination, the two entities often fill similar roles in communities and ecosystems. Therefore, most herpetologists recognize both sexual as well as ecologically and morphologically cohesive asexual lineages as species (Frost and Hillis, 1990).

My own interests in herpetological diversity began just as phylogenetic approaches and methods were being rapidly developed in the 1960s. During this decade, my family lived in equatorial Congo and India, where my father was investigating tropical diseases. There was no television, nor any of the other usual distractions of youth; therefore, I turned my attention to the frogs, lizards, and snakes that I'd find on my daily forays in tropical paradise. I was particularly fascinated by the species of *Chamaeleo* that I found around our house and spent hours catching insects so that I could watch the lizards feed with their projectile tongues. I didn't yet know how, but I knew that somehow I wanted to spend my life studying these diverse and fascinating animals.

Laboratory Crossing Experiments.—In the 1940s–1960s, biologists began to emphasize the importance of intrinsic postmating reproductive isolating mechanisms in maintaining species boundaries, which led to a wealth of laboratory crossing experiments. For example, Moore (1941, 1944, 1946, 1965) made many crosses of leopard frogs from different geographic localities, all of which had been assigned to a single species, *Rana pipiens*. His studies showed numerous reproductive incompatibilities between populations, which led herpetologists to reexamine the taxonomic status of this nominal species (see review in Hillis, 1988). Others, including one of my eventual graduate coadvisors (John Frost), continued these crossing experiments in *Rana* (e.g., Mecham, 1969; Frost and Bagnara, 1977; Frost, 1982), thus uncovering and describing new species. These experiments detected many reproductively isolated, but cryptic, species. Similar crossing studies in other groups

(especially the genus *Bufo*; summarized in Blair, 1972) revealed the reproductive incompatibilities that allowed many species in the same genus to exist sympatrically.

Behavioral Studies of Reproductive Isolation.—Crossing experiments demonstrated postmating reproductive isolating mechanisms between many pairs of species but found none among others. And yet, morphological analysis suggested that these latter pairs of species did not mate where they occurred sympatrically. Hence, biologists began to study the premating reproductive isolating mechanisms that kept reproductively compatible species from merging with one another. In particular, frog calls were shown to be important in premating isolation of anuran species (Littlejohn, 1960, 1965; Littlejohn and Oldham, 1968; Brown and Brown, 1972; Foquette, 1975).

In the 1960s, I owned three phonographic records: a Beatles album, and two records of frog calls, Bogert's (1958) *Sounds of North American Frogs and Toads*, and Kellogg and Allen's (1953) *Voices of the Night: The Calls of 34 Frogs and Toads of the United States and Canada*. I memorized those records, and when I learned to drive, I made tapes and played them wherever I went. In fact, when I first met my wife-to-be, Ann, in 1976, I invited her up to my dorm room to listen to my records of frog calls. Ann declined my invitation but her roommate accepted it. Ann was later surprised to learn that I really did play records of frog calls for her roommate.

In addition to the calls of frogs, visual displays were shown to be important in premating isolation of many reptiles and amphibians (Carpenter and Ferguson, 1977; Houck and Verell, 1993). Furthermore, olfaction was found to be an important reproductive cue in many reptile species (Manton, 1979; Boiko, 1984; Ford, 1986). Consequently, analysis of behavioral reproductive isolation became an important component of many species delimitation studies.

As an undergraduate, my research was focused on studying the various behavioral premating isolating mechanisms that allowed closely related species of the *Rana pipiens* complex to coexist in contact zones (Hillis, 1981). But I soon realized that I needed to be able to measure the degree of hybridization that was occurring in the contact zones. I was fortunate that many people were just beginning to apply genetic approaches to systematic herpetology at the time.

Chromosomal Studies of Reproductive Isolation.—Analysis of chromosomal karyotypes had a big impact on species delimitation studies of reptiles and amphibians beginning in the 1960s. The widespread *Hyla versicolor* was already known to exhibit considerable variation in its mating calls (e.g., listen to the calls and discussion by Bogert, 1958). Yet, no diagnostic morphological differences could be found between individuals of different call types. The answer to this riddle was revealed by chromosomal analysis, which showed there were reproductively isolated haploid and diploid species (Wasserman, 1970). More recent molecular analyses have confirmed multiple origins of polyploids, all of which breed with one another but are reproductively isolated from their diploid ancestors (Holloway et al., 2006).

Studies of many other species identified chromosomal rearrangements that resulted in partial or complete reproductive isolation among morphologically similar species. For example, chromosomal studies by Hall and Selander (1973) and Sites (1983) of the *Sceloporus grammicus* complex revealed numerous parapatrically distributed species, partially reproductively isolated from one another by chromosomal rearrangements.

Incorporation of Allelic Genetic Data into Systematics.—With the rapid development of molecular biology in the 1950s and 1960s, some systematists became interested in inferring phylogenies and species boundaries using allelic genetic data sets. Initially, the products of genes (proteins) proved much easier to study than the genes themselves. The simplicity of DNA sequences, with building blocks of only four nucleotides, initially made them much harder to study than the more complex proteins they encoded. Biologists discovered that they could separate similar alleles of a given protein in a gel subjected to an electric current and then visualize the location of the different alleles using histochemical staining (Smithies, 1955; Hunter and Markert, 1957; Harris, 1966; Hubby and Lewontin, 1966). The different alleles, which often differ in size, shape, and charge, move through the gel at different rates. Suddenly, biologists had a method to study the genetic makeup of individuals, populations, and species and, thus, study their reproductive interactions. As an undergraduate, I used allozyme electrophoresis to study the degree of hybridization in contact zones of closely related species of leopard frogs—a project that I would eventually expand into my dissertation in graduate school.

Herpetologists were quick to adopt methods of allozyme electrophoresis to study species delimitation problems. The resulting data could be used to group individuals into lineages, and they also provided a direct way to detect gene flow and hybridization (e.g., Gorman and Yang, 1975; Sage and Selander, 1979). Although most new species descriptions continued to emphasize morphological diagnoses, many morphologically conservative species complexes were now reevaluated using allozyme data, with a resultant rapid expansion in the number of recognized species.

The application of allozyme electrophoresis to systematic studies had many positive effects on the field, but it generated some problems as well. Tensions sometimes arose between systematists who had spent a lifetime collecting detailed morphological and/or behavioral data in a group and some of the new “gel jocks” who rejected their findings on the basis of new genetic data. Nonetheless, most species delimitation studies emphasized the need to combine and incorporate data from multiple sources (e.g., Wake and Schneider, 1998).

My graduate school experience provides a glimpse of the early days of molecular systematics, and the morphological–molecular tensions that developed at this time. When I started graduate school, I knew that I wanted to develop genetic approaches for studying biodiversity. But I also wanted to get classical training in herpetology. Therefore, I chose two coadvisors—the late geneticist John Frost, and the classical systematist Bill Duellman—who fortunately were both at the University of Kansas. John was working mostly on crossing experiments and chromosomal investigations in *Rana*, but he let me build a laboratory for DNA investigations; however, I had to design, build, or acquire all my own equipment. I assembled gel rigs from scrap Plexiglas and expensive platinum wire that I had purchased with precious funds supplied by SSAR student grants, built my own power supplies from kits, and scrounged freezers from university surplus. After a few years of collecting tissues throughout North, Central, and South America, I went to work cloning ribosomal RNA genes from *Rana*.

While I was developing genetic approaches for my dissertation, Bill Duellman approached me about taking a semester-long trip to the Ecuadorean Andes, where he was studying marsupial frogs (*Gastrotheca*). Bill had long been puzzled about the species limits in the *Gastrotheca riobambae* complex, and he

thought that genetic data might help clarify the species boundaries. I jumped at the opportunity to explore another tropical country. After four delightful months in the field and two wrecked field vehicles, I returned to Kansas with a liquid nitrogen tank full of samples and started examining the genetics of *Gastrotheca*. Bill thought there were likely two cryptic species that had been confused as *G. riobambae*, plus another couple of related species he had already described. My analyses suggested there were actually nine species in the group in the Andes of Ecuador and that one of the species Bill had described was not actually distinct from *G. riobambae*. At first, Bill was highly doubtful of my results, but as he examined the osteology of the group in light of the species boundaries my analyses had suggested, Bill found that the morphological variation he had so long puzzled over quickly fell into place. Bill was convinced, and we eventually presented the combined data analyses along with the description of the new Ecuadorian species (Duellman and Hillis, 1987). Meanwhile, I became an enthusiastic proponent of integrating data from different approaches into systematic studies (Hillis, 1987).

Mitochondrial DNA, PCR, and Barcoding.—The development of DNA sequencing methods (particularly the method of Sanger et al., 1977) opened a new door for systematics and species delimitation. But an early barrier to the use of DNA sequences in systematics was the enormous size of genomes. Cloning individual genes from nuclear genomes, as I did for my dissertation, was tedious and time consuming. In the 1980s, the most easily isolated portion of the genome was mitochondrial DNA (mtDNA), which could be separated from the much more extensive nuclear genome by high-speed centrifugation. After isolation, mtDNA could be studied by restriction-site analysis (Lansman et al., 1981) or sequencing (Brown et al., 1982). Studies of mtDNA began to replace allozyme electrophoresis, as DNA restriction-site analysis and sequencing proved more repeatable and comparable across studies relative to allozyme studies (Avice, 2004).

Downsides of mtDNA analyses, however, include the facts that mtDNA is inherited (at least in reptiles and amphibians) from a single parent, and as a single genetic locus. These are advantages from the standpoint of building a gene tree, because there is no recombination or lineage sorting across multiple genes to worry about. From the standpoint of species delimitation, however, it presents numerous problems. Single species can exhibit deep divergences of mtDNA, especially in widely distributed species with many local populations (as is true for many reptiles and amphibians). In addition, the uniparental inheritance of mtDNA means that it is not very useful for detecting hybridization, and is completely uninformative about genetic contributions of the male parent. Using mtDNA to understand species relationships is like using family names (in a paternal system of name descent) to understand family relationships: it works for one parent but not for the other.

Another problem with using mtDNA for species delimitation is that many cases of mtDNA “capture” are now known (e.g., Sullivan et al., 2004; Linnen and Farrell, 2007; Hedtke and Hillis, 2011; Ruane et al., 2014). When mtDNA is captured from another species, it can introgress into the capturing species, providing signal about the past introgression event, rather than about phylogenetic or ongoing reproductive relationships. A “foreign” mtDNA haplotype can spread throughout populations of a species, or it may result in a deep divergence of

mtDNA haplotypes within a species (Ballard and Whitlock, 2004).

Development of the Polymerase Chain Reaction (Mullis and Faloona, 1987) made amplification and isolation of specific genes (both in the mitochondrial and nuclear genome) much faster and easier. By this time, however, databases of mtDNA had become so widespread that many people began using short “barcodes” of mtDNA sequences to identify species (Hebert et al., 2003). Barcoding using mtDNA is a fast and easy way to identify species when an appropriate database exists (or can be created) for the potential target species, and many species have diagnostic mtDNA haplotypes. Barcoding has proved valuable in many applications, such as identifying larval life stages and associating them with adult forms, rapid forensic identification of species, and environmental-DNA applications. Unfortunately, the deep divergences of mtDNA gene trees within many species of reptiles and amphibians (e.g., Burbrink et al., 2000; Zamudio and Savage, 2003; Baird et al., 2006; Ruane et al., 2014), as well as the propensity of mtDNA to introgress across species boundaries, severely limits its use in robust species delimitation, unless it is combined with information from other sources. In addition, the move from allozyme studies to mtDNA studies was often accompanied by a shift from population-level sampling to sampling one or a few individuals per locality. The combination of a uniparentally inherited single gene, sampled from limited numbers of individuals, severely restricted the ability of systematists to consider gene flow and reproductive isolation.

High-Throughput Sequencing, Nuclear Genomes, and Coalescent Theory.—The development of PCR also opened up the use of nuclear DNA sequences to species delimitation studies; however, gene-by-gene amplification and sequencing of genes can be a laborious process (Hillis et al., 1996). Automated sequencing approaches helped with the sequencing of individual genes (Prober et al., 1987), and the development of high-throughput (“next-generation”) sequencing methods allowed the simultaneous sequencing of many nuclear loci (Margulies et al., 2005) or even of whole genomes (e.g.: *Alligator*: Wan et al., 2013; *Ambystoma*: Nowoshilow et al., 2018; *Anolis*: Alföldi et al., 2011; *Chelonia* and *Pelodiscus*: Wang et al., 2014; *Nanorana*: Sun et al., 2015; *Ophiophagus*: Vonk et al., 2013; *Python*: Castoe et al., 2013; *Thermophilis*: Li et al., 2018; *Xenopus*: Hellston et al., 2010). These new genomic approaches provide a wealth of new data that can be applied to species delimitation studies, but with these developments, systematists now need to grapple with the reality of many individual gene histories within a species lineage. Hence, multispecies coalescent theory (MSC) often is applied to the species delimitation problem (Leaché and Fujita, 2010; Yang and Rannala, 2010; Zhang et al., 2011; Leaché et al., 2014; Yang, 2015).

MSC-based methods examine the histories of multiple gene lineages within putative species, and ask whether a given species hypothesis is consistent with the coalescence of a series of reconstructed gene histories. Conceptually, this makes sense; however, one must understand two major limitations of this approach. First, a set of gene trees can be “consistent with” many different species hypotheses, often with little power to discriminate among the different possibilities. Therefore, MSC-based methods are one of several ways of identifying genetic structure among samples (Sukumaran and Knowles, 2017), which is the first step in the species delimitation process. However, the same set of gene trees can be expected in geographically proximate samples of individuals taken from

across the range of a widely distributed species or among distinct species. Therefore, the geography of sampling must be considered in evaluating species boundaries. For example, Jackson et al. (2017) showed that analysis of human populations based on a widely used MSC-based approach (BPP; Yang and Rannala, 2010) supported the division of *Homo sapiens* populations from around the world into four different species, which clearly is inconsistent with our knowledge of human biology. Without adequate sampling, or direct examination of contact zones, or methods that take the geography of sampling into account, MSC-based approaches can easily misidentify population structure within species as boundaries between species.

Second, all MSC-based approaches have many underlying population and methodological assumptions, many of which are not even closely met for most species. For example, these methods assume that all incongruence among genes is the result of independent lineage sorting (Yang and Rannala, 2010) even though many other biological processes can lead to gene tree incongruence. Furthermore, gene flow is assumed not to exist across test populations; when gene flow is present, it can severely affect species tree estimation under the MSC (Solís-Lemus et al., 2016). In addition, many MSC-based methods also make unrealistic assumptions about the population structure, constant rates of evolution, and methods for reconstructing individual genes. Assumptions about these and other factors rarely are tested or considered in applications of MSC studies (Zhang et al., 2011; Olave et al., 2014; Barley et al., 2018). Limited sampling of specimens is especially likely to result in clusters of geographically proximate samples being identified as “species” (Schwartz and McKelvey, 2008; Rittmeyer and Austin, 2012; Barley et al., 2018), even if the only genetic structure within the samples represents classic isolation by distance (Wright, 1943; Slatkin and Maddison, 1990).

Some recent authors have used MSC-based analyses to divide wide-ranging species into multiple named forms, despite the limitations of the methods (e.g., Leaché and Fujita, 2010). In some cases, wide-ranging species have been split into clusters of geographically proximate samples that are re-named as species, even though genetic analyses of adjacent populations suggest continuous gene flow across the range of samples. MSC-based methods often support a split of a continuous geographic cline into multiple species (Barley et al., 2018). For example, Burbrink and Guiher (2015) proposed splitting both *Agkistrodon contortrix* and *Agkistrodon piscivorus* into two species each, dividing eastern and western populations of both species in the middle of the species range, with broad geographic regions of “hybrids” on either side of the putative species boundaries. In contrast, other studies of *A. contortrix* and *A. piscivorus* (Gloyd and Conant, 1990; Strickland et al., 2014) have indicated that both of these species consist of a continuous series of reproductively connected populations, with very broad and gradual regions of intergradation between eastern and western populations. The eastern and western populations may well have been geographically isolated at some point the past, but they are clearly not currently geographically or reproductively isolated, and the central portion of the range of each species consists of a continuum of genetic and morphological intermediates. Dividing such reproductively continuous populations at an artifactual intermediate boundary, without evidence of any reproductive isolation at that boundary, is not delimiting species; instead, it is the arbitrary slicing of a continuum.

Despite these limitations, MSC-based analyses can be highly informative about species delimitation, especially when they

integrate information from other analyses, and when sampling is adequate to consider potential contact zones and gene flow among connected populations (e.g., Chan et al., 2017; Nieto-Montes de Oca et al., 2017; Devitt et al., 2019). The depth of information from genomic studies can be combined with the breadth of data from better-sampled morphological, ecological, and behavioral studies to produce comprehensive, robust, and stable species delimitation.

The Future of Species Delimitation.—Biological classifications are one of the most visible products of systematics, even though many people argue that biologists should pay more attention to the primary results of systematic analyses (phylogenies and analyses of reproductive boundaries) than to the classifications that result from these studies (e.g., see Felsenstein, 2004). Realistically, however, most biologists do not have the training, time, or skills to interpret systematic data for themselves. Therefore, they rely on the classifications produced by experts in a particular group of organisms. Thus, systematics, and especially classification, is a service provided to the rest of the biological community, as well as to human society at large, to inform comparative studies of biology and the public about biodiversity.

The service aspect of systematics makes it imperative for systematists to propose changes to classifications only when the existing classifications are clearly misleading about the evolutionary history of the corresponding organisms. Unnecessary or premature nomenclatural changes confuse the literature, especially in widely studied species with extensive bibliographies (Hillis, 2007a). Each new methodology that has been applied to species delimitation has brought new information and insights, but each approach also has its limitations and disadvantages. No method or data set provides the final answer in science, and careful species delimitation requires consideration of all accumulated evidence. Morphological, behavioral, reproductive, chromosomal, and genetic approaches each have distinct advantages, and each provide important information about the complexities of species boundaries and interactions. Thus, it makes little sense to ignore relevant information from any of these sources in considering nomenclatural changes.

Unfortunately, a recent trend in species delimitation studies has been to change the nomenclature for a group of species with each new data set collected. This trend is especially problematic when the new approaches merely address the first stage in species delimitation: that of grouping individuals into putative lineages without testing whether the identified groups are evolving independently of one another. Thus, “species” names get assigned to local populations or artifactual slices of continuous geographic clines. This does not serve the biological community or inform other biologists about the actual reproductive breaks between species. This has been especially problematic in studies that have subdivided broadly distributed geographic species largely on the basis of mtDNA haplotypes (e.g., *Bufo valliceps*: Mulcahy and Mendelson, 2000; *Pantherophis obsoletus*: Burbrink, 2001; *Pantherophis guttatus*: Burbrink, 2002; *Drymarchon*: Krysko et al., 2016 [see Folt et al., 2018]). These hypotheses about species boundaries associated with mtDNA haplotypes need to be tested to see whether there are any reproductive barriers between the respective divergent mtDNA haplotype lineages. If there are none, then these are simply cases of deep divergence of mtDNA within species rather than evidence of multiple species.

An additional disruption to the meanings of species names comes from the splitting of well-established monophyletic

genera into many smaller genera, which needlessly changes the meaning of generic names (an essential part of species binomials). The drive to make these changes is largely sociological rather than scientific: some systematists argue that their work will not be recognized or cited by the scientific community unless it involves name changes. This largely self-serving approach to systematics runs counter to the idea that nomenclature and classification are services to the broader scientific community and the public and that systematics strives to inform biology about the boundaries and relationships of species.

I do not mean to imply that the problems I have outlined in this paper with some recent species delimitation studies of reptiles and amphibians apply to all species delimitation studies. First, I acknowledge that in rarely sampled and poorly known groups, we often have to do the best we can with minimal data in making species delimitation decisions. In these cases, it is better to describe a new species based on the limited samples and make the presence of the species known to other biologists than to wait for extensive sampling and genetic analysis that may never be possible. Second, there are, indeed, many excellent species delimitation studies in well-known groups that exhibit none of the problems I described (some of these are cited above in the section on MSC analyses). A non-MSA example is the delimitation and description of a new species of *Contia* by Feldman and Hoyer (2010) that was accompanied by thorough geographic sampling and analysis, consideration of multiple sources of evidence, and careful examination of contact zones. There have also been previous pleas for more integrative studies of systematics (e.g., Hillis, 1987; Dayrat, 2005; de Queiroz, 2007; Padiol et al., 2010; Schlick-Steiner et al., 2010). Integration of information should be a standard practice in all well-sampled and well-studied taxa, especially if nomenclature changes are proposed (Hillis, 2007a).

I present a modest proposal for sensible nomenclatural practice in Figure 1. Systematics should be first and foremost about discovery, description, and explanation of biological diversity. Nomenclature is a tool that can be used to present those findings to other biologists. But when should we change scientific names? Unnecessary changes can confuse and disrupt the literature, and can make older literature obsolete or difficult to interpret (see discussions in Hillis, 2007a; Pauly et al., 2009; Yuan et al. 2016). Nonetheless, name changes are needed when the existing nomenclature clearly misinforms other biologists about species boundaries and relationships. I suggest that before systematists propose a change in names, they should follow a sensible taxonomic process (Fig. 1). If all accumulated data on a species or group is consistent in showing that the existing nomenclature is misleading about evolutionary relationships or species boundaries, then a systematist revising the group should fix the problem with as minimal disruption to the existing taxonomy as possible. But when existing named taxa represent monophyletic groups and reproductively connected species lineages, we should leave them alone. Change for change's sake is disruptive and counter-productive.

As an illustration of application of the principles shown in Fig. 1, consider the case of the proposed splitting of the Holarctic genus *Rana* into two genera, as discussed by Yuan et al. (2016). Splitting *Rana* would change the meaning of a well-established and well-studied monophyletic group, as well as change the names of many species that have been the subject of thousands of biological studies of morphology, physiology, behavior, development, and genetics. Furthermore, splitting out

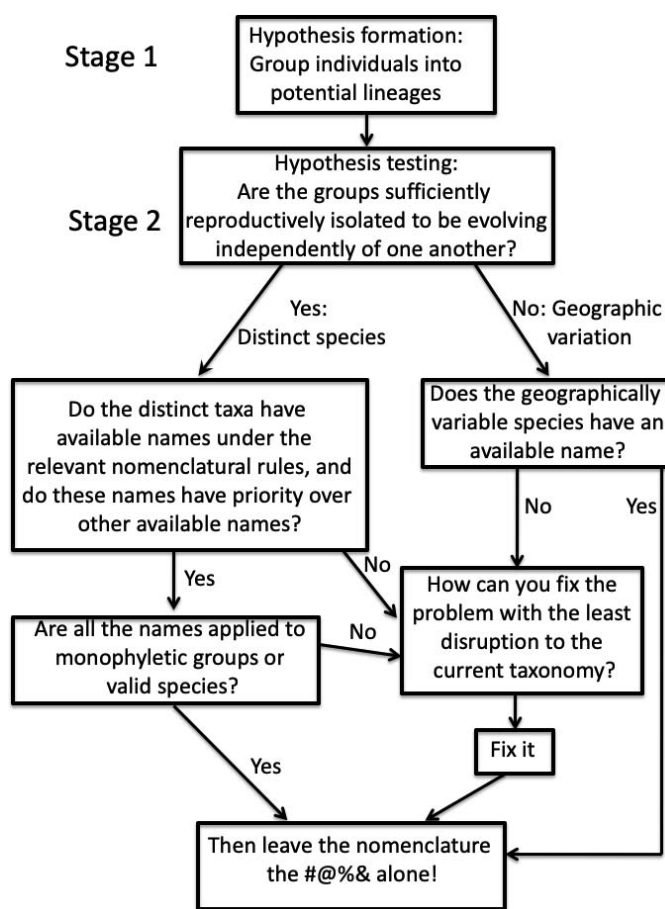


FIG. 1. Flow chart of a proposal for sensible nomenclatural practice in species delimitation studies. Stage 1 and Stage 2 refer to the two primary steps of the species delimitation process.

some species into a new genus leaves the rest of *Rana* paraphyletic (Yuan et al. 2016). We need to stop equating name changes with systematic “progress.” There are other mechanisms for naming new clades within existing genera (subgeneric names), which do not change the meaning of well-established names but still allow biologists to name and discuss newly discovered subdivisions (Hillis, 2007a). Subjectively changing the meaning of established names distracts from legitimate progress in species and clade discovery and advancement of our understanding of the Tree of Life.

Similarly, I see no reasonable justification for the current trend of dividing widespread, geographically variable species into artificial slices of continuous clines (e.g., the division of *Agkistrodon* species discussed above). Such splitting is misinformative about reproductive boundaries, and it has little utility for the rest of biology. I predict that the pendulum has swung too far and that as systematists begin to integrate previous studies and methodologies with the new genomic analyses, we will see a reversal of many of the recently proposed splits of widely distributed species.

The purpose of systematics is to inform humanity about biodiversity. I am a systematist because I am excited by biodiversity, and I want to inform others about the diversity and evolution of life. One of our ways of doing that is through scientific nomenclature. We have many tools in our systematic toolbox, and more power to understand species boundaries than ever before in human history. We need to use all the

information at our disposal and apply our findings judiciously and conscientiously. Science is impeded when we change names needlessly, or without giving full consideration to all the available data. It is time for all responsible systematists to insist that we do a better job of serving and informing the rest of the biological community.

Acknowledgments.—I thank Carole Baldwin, David Cannatella, E. Anne Chambers, Tom Devitt, Kevin de Queiroz, Chris Feldman, Brain Folt, Harry W. Greene, Craig Guyer, Elizabeth Jockusch, Lacey Knowles, Katie Lyons, Thomas Marshall, Dan Mulcahy, Erin Muths, Analisa Shields-Estrada, Jack Sites Jr., Ruben Tovar, C. Richard Tracy, David Wake, Brittney White, and Kelly Zamudio for comments and discussions about species delimitation.

LITERATURE CITED

- AGASSIZ, L. 1859. *Essay on Classification*. Museum of Comparative Zoology, USA.
- ALFÖLDI, J., F. DI PALMA, M. GRABHERR, C. WILLIAMS, L. KONG, E. MAUCELLI, P. RUSSELL, C. B. LOWE, R. E. GLOR, J. D. JAFFE ET AL. 2011. The genome of the green anole lizard and a comparative analysis with birds and mammals. *Nature* 477:587–591.
- AVISE, J. C. 2004. *Molecular Markers, Natural History, and Evolution*. Sinauer Associates, USA.
- BAIRD, A. B., J. K. KREJCA, J. R. REDDELL, C. E. PEDEN, M. J. MAHONEY, AND D. M. HILLIS. 2006. Phylogeographic structure and color pattern variation among populations of *Plethodon albagula* on the Edwards Plateau of central Texas. *Copeia* 2006:760–768.
- BALLARD, J. W. O., AND M. C. WHITLOCK. 2004. The incomplete natural history of mitochondria. *Molecular Ecology* 13:729–744.
- 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.
- BLAIR, W. F. (ed.). 1972. *Evolution in the Genus Bufo*. University of Texas Press, USA.
- BLANCHARD, F. N. 1921. A revision of the king snakes: genus *Lampropeltis*. *Bulletin of the United States National Museum* 114: 1–260.
- BOGERT, C. M. 1958. *Sounds of North American Frogs: The Biological Significance of Voice in Frogs*. Smithsonian Folkways, USA.
- BOIKO, V. P. 1984. The participation of chemoreception in the organization of reproductive behavior in *Emys orbicularis* (Testudines, Emydidae). *Zoologicheskii Zhurnal* 63:584–589.
- BROWN, L. E., AND J. R. BROWN. 1972. Call types of the *Rana pipiens* complex in Illinois. *Science* 176:928–929.
- BROWN, W. M., E. M. PRAGER, A. WANG, AND A. C. WILSON. 1982. Mitochondrial DNA sequences of primates: tempo and mode of evolution. *Journal of Molecular Evolution* 18:225–239.
- BUFFON, G.-L. L. 1753. *Histoire naturelle, générale et particulière, avec la description du Cabinet du Roi*. Texte établi, introduit et annoté par Stéphane Schmitt avec la collaboration de Cédric Crémère. Vol. 4. Honoré Champion, France.
- BURBRINK, F. T. 2001. Systematics of the eastern ratsnake complex (*Elaphe obsoleta*). *Herpetological Monographs* 15:1–53.
- . 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., R. LAWSON, AND J. B. SLOWINSKI. 2000. Mitochondrial DNA phylogeography of the polytypic North American ratsnake (*Elaphe obsoleta*): a critique of the subspecies concept. *Evolution* 54: 2107–2118.
- CARPENTER, C., AND G. W. FERGUSON. 1977. Variation and evolution of stereotyped behavior in reptiles. Pp. 335–354 in C. Gans and D. W. Tinkle (eds.), *Biology of the Reptilia*, Vol. 7. Academic Press, USA.
- CASTOE, T. A., A. P. J. DE KONING, K. T. HALL, D. C. CARD, D. R. SCHIELD, M. K. FUJITA, R. P. RUGGIERO, J. F. DEGNER, J. M. DAZA, W. GU, J. REYES-VELASCO ET AL. 2013. The Burmese python genome reveals the molecular basis for extreme adaptation in snakes. *Proceedings of the National Academy of Sciences USA* 110:20645–20650.
- CHAN, K. O., A. M. ALEXANDER, L. L. GRISMER, Y.-C. SU, J. L. GRISMER, E. S. H. QUAH, AND R. M. BROWN. 2017. Species delimitation with gene flow: a methodological comparison and population genomics approach to elucidate cryptic species boundaries in Malaysian torrent frogs. *Molecular Ecology* 26:5435–5450.
- COLE, C. J. 1985. Taxonomy of parthenogenetic species of hybrid origin. *Systematic Zoology* 34:359–363.
- DAREVSKY, I. S., L. A. KURRIYANOVA, AND T. UZZELL. 1985. Parthenogenesis in reptiles. Pp. 411–526 in C. Gans (ed.). *Biology of the Reptilia*, Vol. 15. John Wiley and Sons, USA.
- DARWIN, C. 1859. *On the Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life*. John Murray, U.K.
- DAYRAT, B. 2005. Towards integrative taxonomy. *Biological Journal of the Linnean Society* 85:407–415.
- DE QUEIROZ, K. 1998. The general lineage concept of species, species criteria, and the process of speciation: a conceptual unification and terminological recommendations. Pp. 57–75 in D. J. Howard and S. H. Berlocher (eds.) *Endless Forms: Species and Speciation*. Oxford University Press.
- . 2007. Species concepts and species delimitation. *Systematic Biology* 56:879–886.
- 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, USA* 116. <https://doi.org/10.1073/pnas.1815014116>.
- DONOGHUE, M. J., AND J. W. KADEREIT. 1992. Walter Zimmermann and the growth of phylogenetic theory. *Systematic Biology* 41:74–84.
- DUELLMAN, W. E., AND D. M. HILLIS. 1987. Marsupial frogs (Anura: Hylidae: *Gastrotheca*) of the Ecuadorian Andes: resolution of taxonomic problems and phylogenetic relationships. *Herpetologica* 43:141–173.
- FELDMAN, C. R., AND R. F. HOYER. 2010. A new species of snake in the genus *Contia* (Squamata: Colubridae) from California and Oregon. *Copeia* 2010:254–267.
- FELSENSTEIN, J. 2004. *Inferring Phylogenies*. Sinauer Associates, USA.
- FITCH, W. F. 1970. Distinguishing homologous from analogous proteins. *Systematic Zoology* 19:99–113.
- FOLT, B., J. BAUDER, S. SPEAR, D. STEVENSON, M. HOFFMAN, J. OAKS, C. JENKINS, D. STEEN, AND C. GUYER. 2018. Phylogenetic, population genetic, and morphological analyses reveal evidence for one species of eastern indigo snake (*Drymarchon couperi*). *bioRxiv* doi:10.1101/318766.
- FONTANETO, D., AND T. G. BARRACLOUGH. 2015. Do species exist in asexuals? Theory and evidence from bdelloid rotifers. *Integrative and Comparative Biology* 55:253–263.
- FOQUETTE, M. J., JR. 1975. Speciation in chorus frogs. I. Reproductive character displacement in the *Pseudacris nigrita* complex. *Systematic Zoology* 24:16–22.
- FORD, N. B. 1986. The role of pheromone trails in sociobiology of snakes. Pp. 261–278 in D. Duvall, D. Müller-Schwarze, and R. M. Silverstein (eds.), *Chemical Signals in Vertebrates*. Vol. 4. Plenum Publishing, USA.
- FROST, D. R., AND D. M. HILLIS. 1990. Species in concept and practice: herpetological applications. *Herpetologica* 46:87–104.
- FROST, J. P. 1982. Functional genetic similarity between geographically separated populations of Mexican leopard frogs (*Rana pipiens* complex). *Systematic Zoology* 31:57–67.
- FROST, J. P., AND J. BAGNARA. 1977. An analysis of reproductive isolation between *Rana magnaocularis* and *Rana forreri* (*Rana pipiens* complex). *Journal of Experimental Zoology* 202:291–306.
- GHISELIN, M. T. 1974. A radical solution to the species problem. *Systematic Zoology* 23:536–544.
- GLOYD, H. K., AND R. CONANT. 1990. *Snakes of the Agkistrodon Complex: A Monographic Review*. Society for the Study of Amphibians and Reptiles, USA.
- GORMAN, G. C., AND S. Y. YANG. 1975. A low level of backcrossing between the hybridizing *Anolis* lizards of Trinidad. *Herpetologica* 31:196–198.

- HAECKEL, E. 1866. *Generelle Morphologie der Organismen: allgemeine Grundzüge der organischen Formen-Wissenschaft, mechanisch begründet durch die von Charles Darwin reformirte Descendenz-Theorie*. Georg Riemer, Germany.
- HALL, W. P., AND R. K. SELANDER. 1973. Hybridization of karyotypically differentiated populations in the *Sceloporus grammicus* complex (Iguanidae). *Evolution* 27:226–242.
- HARRIS, H. 1966. Enzyme polymorphism in man. *Proceedings of the Royal Society of London Series B* 164:298–310.
- HEBERT, P. D., S. CYWINSKA, S. L. BALL, AND J. R. DEWAARD. 2003. Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London Series B* 270:313–321.
- HEDTKE, S. M., AND D. M. HILLIS. 2011. The potential role of androgenesis in cytoplasmic–nuclear phylogenetic discordance. *Systematic Biology* 60:87–109.
- HELLSTON, U., R. M. HARLAND, M. J. GILCHRIST, D. HENDRIX, J. JURKA, V. KAPITONOV, I. OVCHARENKO, N. H. PUTNAM, S. SHU, L. TAHER ET AL. 2010. The genome of the western clawed frog *Xenopus tropicalis*. *Science* 328: 633–636.
- HENNIG, W. 1950. *Grundzüge einer Theorie der phylogenetischen Systematik*. Deutscher Zentralverlag, Germany.
- . 1966. *Phylogenetic Systematics*, D. Davis and R. Zangerl (trans.). University of Illinois Press, USA.
- HEY, J. 2006. Recent advances in assessing gene flow between diverging populations and species. *Current Opinions in Genetics and Development* 16:592–596.
- HILLIS, D. M. 1981. Premating isolating mechanisms among three species of the *Rana pipiens* complex in Texas and southern Oklahoma. *Copeia* 1981:312–319.
- . 1987. Molecular versus morphological approaches to systematics. *Annual Review of Ecology and Systematics* 18:23–42.
- . 1988. Systematics of the *Rana pipiens* complex: puzzle and paradigm. *Annual Review of Ecology and Systematics* 19:39–63.
- . 2007a. Constraints in naming parts of the Tree of Life. *Molecular Phylogenetics and Evolution* 42:331–338.
- . 2007b. Asexual evolution: can species exist without sex? *Current Biology* 17:R543–R544.
- HILLIS, D. M., B. K. MABLE, A. LARSON, S. K. DAVIS, AND E. A. ZIMMER. 1996. Nucleic acids IV: sequencing and cloning. Pp. 321–381 in D. M. Hillis, B. K. Mable, and C. Moritz (eds.), *Molecular Systematics*. 2nd. ed. Sinauer Associates, USA.
- HOLLOWAY, A. K., D. C. CANNATELLA, H. C. GERHARDT, AND D. M. HILLIS. 2006. Polyploids with different origins and ancestors form a single sexual polyploid species. *American Naturalist* 167: E88–E101.
- HOUCK, L. D., AND P. A. VERRELL. 1993. Studies of courtship behavior in plethodontid salamanders: a review. *Herpetologica* 49:175–184.
- HUBBY, J. L., AND L. H. LEWONTIN. 1966. A molecular approach to the study of genic heterozygosity in natural populations. I. The number of alleles at different loci in *Drosophila pseudoobscura*. *Genetics* 54:577–594.
- HUNTER, R. L., AND C. L. MARKERT. 1957. Histochemical demonstration of enzymes separated by zone electrophoresis in starch gels. *Science* 125:1294–1295.
- HUXLEY, J. 1942. *Evolution: The Modern Synthesis*. Allen and Unwin, U.K.
- JACKSON N. D., B. C. CARSTENS, A. E. MORALES, AND B. C. O'MEARA. 2017. Species delimitation with gene flow. *Systematic Biology* 66:799–812.
- KELLOGG, P. P., AND A. A. ALLEN. 1953. *Voices of the Night: The Calls of 34 Frogs and Toads of the United States and Canada*. Cornell University Laboratory of Ornithology, USA.
- KRYSKO, K. L., M. C. GRANATOSKY, L. NUÑEZ, AND D. L. SMITH. 2016. A cryptic new species of indigo snake (genus *Drymarchon*) from the Florida platform of the United States. *Zootaxa* 4138:549–569.
- LAMARCK, J.-B. 1809. *Philosophie Zoologique ou exposition des considérations relatives à l'histoire naturelle des animaux*. Museum d'Histoire Naturelle, France.
- LANSMAN, R. A., R. O. SHADE, J. F. SHAPIRA, AND J. C. AVISE. 1981. The use of restriction endonucleases to measure mitochondrial DNA sequence relatedness in natural populations. *Journal of Molecular Evolution* 17:214–226.
- LEACHÉ, A. D., AND M. K. FUJITA. 2010. Bayesian species delimitation in West African forest geckos (*Hemidactylus fasciatus*). *Proceedings of the Royal Society B* 277:3071–3077.
- LEACHÉ, A. D., M. K. FUJITA, V. N. MININ, AND R. R. BOUCKAERT. 2014. Species delimitation using genome-wide SNP data. *Systematic Biology* 63:534–542.
- LI, J.-T., Y.-D. GAO, L. XIE, C. DENG, P. SHI, M.-L. GUAN, S. HUANG, J.-L. REN, D.-D. WU, L. DING ET AL. 2018. Comparative genomic investigation of high-elevation adaptation in ectothermic snakes. *Proceedings of the National Academy of Sciences USA* 115: 8406–8411.
- LINNAEUS, C. 1758. *Systema naturæ per regna tria naturæ, secundum classes, ordines, genera, species, cum characteribus, differentiis, synonymiis, locis*. 10th ed. Laurentius Salvius, Sweden.
- LINNEN, C. R., AND B. D. FARRELL. 2007. Mitonuclear discordance is caused by rampant mitochondrial introgression in *Neodiprion* (Hymenoptera: Diprionidae) sawflies. *Evolution* 61:1417–1438.
- LITTLEJOHN, M. J. 1960. Call discrimination and potential reproductive isolation in *Pseudacris triseriata* females from Oklahoma. *Copeia* 1960:370–371.
- . 1965. Premating isolation in the *Hyla ewingi* complex (Anura: Hylidae). *Evolution* 19:234–243.
- LITTLEJOHN, M. J., AND R. S. OLDHAM. 1968. *Rana pipiens* complex: mating call structure and taxonomy. *Science* 162:1003–1005.
- MADDISON, W. P. 1997. Gene trees in species tree. *Systematic Biology* 46: 523–536.
- MANTON, M. L. 1979. Olfaction and behavior. Pp. 289–301 in M. Harless and H. Morlock (eds.), *Turtles: Perspectives and Research*. Wiley and Sons, USA.
- MARGULIES, M., M. EGHOLM, W. E. ALTMAN, S. ATTIYA, J. S. BADER, L. A. BEMBEN, J. BERKA, M. S. BRAVERMAN, Y. J. CHEN, Z. CHEN ET AL. 2005. Genome sequencing in open microfabricated high density picoliter reactors. *Nature* 437:376–380.
- MAYDEN, R. L. 1997. A hierarchy of species concepts: the denouement in the saga of the species problem. Pp. 381–423 in M. F. Claridge, H. A. Dawah, and M. R. Wilson (eds.), *Species: The Units of Biodiversity*. Chapman and Hall, U.K.
- MAYR, E. 1969. *Principles of Systematic Zoology*. McGraw-Hill, USA.
- MAYR, E., E. G. LINSLEY, AND R. L. USINGER. 1953. *Methods and Principles of Systematic Zoology*. McGraw-Hill, USA.
- MECHAM, J. S. 1969. New information from experimental crosses on genetic relationships within the *Rana pipiens* species group. *Journal of Experimental Zoology* 170:169–180.
- MOORE, J. A. 1941. Developmental rate of hybrid frogs. *Journal of Experimental Zoology* 86:405–422.
- . 1944. Geographic variation in *Rana pipiens* Schreber of eastern North America. *Bulletin of the American Museum of Natural History* 82:345–370.
- . 1946. Incipient intraspecific isolating mechanisms in *Rana pipiens*. *Genetics* 31:304–326.
- . 1965. Diploid and haploid hybridization of different populations of the *Rana pipiens* complex. I. Experiments with females from Mexico. *Journal of Experimental Zoology* 165:1–20.
- MULCAHY, D. G., AND J. R. MENDELSON III. 2000. Phylogeography and speciation of the morphologically variable, widespread species *Bufo valliceps*, based on molecular evidence from mtDNA. *Molecular Phylogenetics and Evolution* 17:173–189.
- MULLIS, K. B., AND F. A. FALOONA. 1987. Specific synthesis of DNA *in vitro* via a polymerase catalyzed chain reaction. *Methods in Enzymology* 155:335–350.
- Nieto-Montes de Oca, A., A. J. BARLEY, R. N. MEZA-LÁZARO, U. O. GARCÍA-VÁZQUEZ, J. G. ZAMORA-ABREGO, R. C. THOMSON, AND A. D. LEACHÉ. 2017. Phylogenomics and species delimitation in the knob-scaled lizards of the genus *Xenosaurus* (Squamata: Xenosauridae) using ddRADseq data reveal a substantial underestimation of diversity. *Molecular Phylogenetics and Evolution* 106:241–253.
- NOWOSHILOW, S., S. SCHLOISSNIG, J.-F. FEI, A. DAHL, A. W. C. PANG, M. PIPPEL, S. WINKLER, A. R. HASTIE, G. YOUNG, J. G. ROSCITO ET AL. 2018. The Axolotl genome and the evolution of key tissue formation regulators. *Nature* 554:50–55.
- OLAVE, M., E. SOLA, AND L. L. KNOWLES. 2014. Upstream analyses create problems with DNA-based species delimitation. *Systematic Biology* 63:263–271.
- PADIAL, J. M., A. MIRALLES, I. DE LA RIVA, AND M. VENCES. 2010. The integrative future of taxonomy. *Frontiers in Zoology* 7:16.
- PAULY, G. B., D. C. CANNATELLA, AND D. M. HILLIS. 2009. Taxonomic freedom and the role of official lists of species names. *Herpetologica* 65:115–128.

- PROBER, J.M., G. L. TRAINOR, R. J. DAM, F. W. HOBBS, C. W. ROBERTSON, R. J. ZAGURSKY, A. J. COCUZZA, M. A. JENSEN, AND K. BAUMEISTER. 1987. A system for rapid DNA sequencing with fluorescent chain-terminating dideoxynucleotides. *Science* 238:336–41.
- RITTMAYER, E.N., AND C. C. AUSTIN. 2012. The effects of sampling on delimiting species from multi-locus sequence data. *Molecular Phylogenetics and Evolution* 65:451–463.
- RUANE, S., R. W. BRYSON JR., 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.
- RUTHVEN, A. G. 1908. Variations and genetic relationships of the garter snakes. *Bulletin of the United States National Museum* 61:1–201.
- SAGE, R.D., AND R. K. SELANDER. 1979. Hybridization between species of the *Rana pipiens* complex in central Texas. *Evolution* 33:1069–1088.
- SANGER, F., S. NICKLEN, AND A. R. COULSON. 1977. DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences USA* 74:5463–5467.
- SCHLICK-STEINER, B.C., F. M. STEINER, B. SEIFERT, C. STAUFFER, E. CHRISTIAN, AND R. H. CROZIER. 2010. Integrative taxonomy: a multisource approach to exploring biodiversity. *Annual Review of Entomology* 55:421–438.
- SCHWARTZ, M., AND K. MCKELVEY. 2008. Why sampling scheme matters: the effect of sampling scheme on landscape genetic results. *Conservation Genetics* 10:441–452.
- SIMPSON, G. G. 1961. *Principles of Animal Taxonomy*. Colombia University Press, USA.
- SITES, J. W., JR. 1983. Chromosomes evolution in the iguanid lizard *Sceloporus grammicus*. I. Chromosomal polymorphisms. *Evolution* 37:38–53.
- SLATKIN, M., AND W. P. MADDISON. 1990. Detecting isolation by distance using phylogenies of genes. *Genetics* 126:249–260.
- SMITHIES, O. 1955. Zone electrophoresis in starch gels: group variations in the serum proteins of normal individuals. *Biochemical Journal* 61:629–641.
- SOLIS-LEMUS, C., M. YANG, AND C. ANÉ. 2016. Inconsistency of species-tree methods under gene flow. *Systematic Biology* 65:843–851.
- STRICKLAND, J. L., C. L. PARKINSON, J. K. MCCOY, AND L. K. AMMERMAN. 2014. Phylogeography of *Agkistrodon piscivorus* with emphasis on the western limit of its range. *Copeia* 2014:639–649.
- SUKUMARAN, J., AND L. L. KNOWLES. 2017. Multispecies coalescent delimits structure, not species. *Proceedings of the National Academy of Sciences USA* 114:1607–1612.
- SULLIVAN, J.P., S. LAVOUÉ, M. E. ARNEGARD, AND C. D. HOPKINS. 2004. AFLPs resolve phylogeny and reveal mitochondrial introgression within a species flock of African electric fish (Mormyroidea: Teleostei). *Evolution* 58:825–841.
- SUN, Y.B., Z.-J. XIONG, X.-Y. XIANG, X.-L. TU, W.-W. ZHOU, S.-P. LIU, L. ZHONG, L. WANG, D.-D. WU, Y. LI ET AL. 2015. Whole-genome sequence of the Tibetan frog *Nanorana parkeri* and the comparative evolution of vertebrate genomes. *Proceedings of the National Academy of Sciences USA* 112:E1257–E1262.
- TEMPLETON, A. 1989. The meaning of species and speciation: a population genetics approach. Pp. 3–27 in D. Otte and J. A. Endler (eds.), *Speciation and its Consequences*. Sinauer Associates, USA.
- UZZELL, T. 1964. Relations of the diploid and triploid species of the *Ambystoma jeffersonianum* complex (Amphibia, Caudata). *Copeia* 1964:257–300.
- VONK, F. J., N. R. CASEWELL, C. V. HENKEL, A. M. HEIMBERG, H. J. JANSEN, R. J. R. MCCLEARY, H. M. E. KERKAMP, R. A. VOS, I. GUERREIRO, J. J. CALVETE ET AL. 2013. The king cobra genome reveals dynamic gene evolution and adaptation in the snake venom system. *Proceedings of the National Academy of Sciences USA* 110:20651–20656.
- WAKE, D. B., AND C. J. SCHNEIDER. 1998. Taxonomy of the plethodontid salamander genus *Ensatina*. *Herpetologica* 54:279–298.
- WAN, Q.-H., S. K. PAN, L. HU, Y. ZHU, P. W. XU, J. Q. XIA, H. CHEN, G. Y. HE, J. HE, X. W. NI ET AL. 2013. Genome analysis and signature discovery for diving and sensory properties of the endangered Chinese alligator. *Cell Research* 23:1091–1105.
- WANG, Z., J. PASCUAL-ANAYA, A. ZADISSA, W. LI, Y. NIIMURA, Z. HUANG, C. LI, S. WHITE, Z. XIONG, D. FANG ET AL. 2014. The draft genomes of soft-shell turtle and green sea turtle yield insights into the development and evolution of the turtle-specific body plan. *Nature Genetics* 45:701–706.
- WASSERMAN, A. O. 1970. Polyploidy in the common tree toad *Hyla versicolor* Le Conte. *Science* 167:385–386.
- WILEY, E.O. 1978. The evolutionary species concept reconsidered. *Systematic Zoology* 27:17–26.
- WRIGHT, S. 1943. Isolation by distance. *Genetics* 28:114–138.
- YANG, Z. 2015. The BPP program for species tree estimation and species delimitation. *Current Zoology* 61:854–865.
- YANG, Z., AND B. RANNALA. 2010. Bayesian species delimitation using multilocus sequence data. *Proceedings of the National Academy of Sciences USA* 107:9264–9269.
- YUAN, Z.-Y., W.-W. ZHOU, X. CHEN, N. A. POYARKOV, H.-M. CHEN, N.-H. JANG-LIAW, W.-H. CHOU, K. IZUKA, M.-S. MIN, S. L. KUZMIN ET AL. 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.
- ZAMUDIO, K.R., AND W.K. SAVAGE. 2003. Historical isolation, range expansion, and secondary contact of two highly divergent mitochondrial lineages in spotted salamanders (*Ambystoma maculatum*). *Evolution* 57:1631–1652.
- ZHANG, C., D. X. ZHANG, T. ZHU, AND Z. YANG. 2011. Evaluation of a Bayesian coalescent method of species delimitation. *Systematic Biology* 60:747–761.
- ZIMMERMAN, W. 1931. Arbeitsweise der botanischen Phylogenetik und anderer Crupperungswissenschaften. Pp. 941–1053 in E. Abderhalden (ed.), *Handbuch der biologischen Arbeitsmethoden*. Urban and Schwarzenberg, Germany.

Accepted: 4 December 2018.

Published online: 12 February 2019.