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Delimiting Species Using DNA and Morphological Variation and Discordant Species Limits in Spiny Lizards (*Sceloporus*)

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Abstract.—Haplotype phylogenies based on DNA sequence data are increasingly being used to test traditional species-level taxonomies based on morphology. However, few studies have critically compared species limits based on morphological and DNA data, and the methods used to delimit species using either type of data are only rarely explained. In this paper, we review three approaches for species delimitation (tree-based with DNA data and tree-based and character-based with morphological data) and propose explicit protocols for each. We then compare species limits inferred from these approaches, using morphological and mtDNA data for the Yarrow's spiny lizard (*Sceloporus jarrovii*), a traditionally polytypic species from the southwestern United States and Mexico. All three approaches support division of *S. jarrovii* into five species, but only two species are the same among the three approaches. We find the greatest support for the five species that are delimited based on mtDNA data, and we argue that mtDNA data may have important (and previously unappreciated) advantages for species delimitation. Because different data and approaches can disagree so extensively, our results demonstrate that the methodology of species delimitation is a critical issue in systematics. [Mitochondrial DNA; molecular systematics; morphological systematics; nested-clade analysis; phylogeography; population genetics; species limits; taxonomy.]

The two major goals of systematics are delimiting species and reconstructing their phylogenetic relationships. Although species are fundamental units in studies of evolution, ecology, phylogeny, and conservation biology, surprisingly little attention has been paid to the methods and data used to recognize and delimit them (Wiens, 1999). This trend is particularly apparent when the meager literature on the methodology of species delimitation is contrasted with the extensive body of work on the theory and methods of phylogenetic analysis. Few specific criteria or methods for species delimitation have been proposed (e.g., Avise and Ball, 1990; Davis and Nixon, 1992; Baum and Donoghue, 1995; Mallet, 1995; Brower, 1999; Wiens, 1999; Wiens and Servedio, 2000; Puorto et al., 2001; Templeton, 2001), and these criteria are rarely stated explicitly by empirical workers. Morphological data traditionally have been used to delimit species and continue to be widely used today, but many recent studies have used DNA sequence data to test traditional, morphology-based taxonomies, particularly analyses of mitochondrial DNA (mtDNA) variation in wide-ranging, polytypic animal species (e.g., Sperling and Harrison, 1994; Shaffer and McKnight, 1996; Sullivan et al., 1997; Zamudio et al., 1997; Zamudio and Greene, 1997; Steppan, 1998; Parkinson et al., 2000; Rodríguez-Robles and de Jesús-Escobar, 2000; Serb et al., 2001). These studies have revealed both discordance and concordance with previous taxonomies. However, few studies have critically compared species limits inferred from morphological and DNA sequence variation (e.g., Puorto et al., 2001).

Disagreement between species boundaries inferred from different data types raises several important questions. How common are these disagreements, and under what conditions are they most likely to occur? Are disagreements between species limits from DNA and morphological data generally caused by deviations between gene and species trees (e.g., Pamilo and Nei, 1988; Maddison, 1997)? Or is discordance more frequently caused by convergence in morphological characters? Will DNA sequence phylogenies give a very different picture of species diversity and patterns of speciation from that obtained from traditional morphological characters? What approach will give us the most accurate picture of the true species boundaries? Addressing these questions requires explicit methods for species

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delimitation and critical comparisons of morphological and DNA variation in empirical case studies.

The use of mtDNA sequence data in species delimitation has been particularly controversial, and some authors have argued that species should not be delimited based on these data alone (e.g., Moritz et al., 1992; Moritz, 1994; Sites and Crandall, 1997; Puorto et al., 2001). MtDNA data may be problematic in that all mitochondrial genes are inherited as a single linkage group; as a result, any mismatch between gene and population histories caused by ancestral polymorphism or gene flow between species will simultaneously affect all mitochondrial genes (Moore, 1995). Furthermore, because mtDNA is maternally inherited, mtDNA haplotype phylogenies will reflect only patterns of female gene flow and dispersal, which may be quite different from those patterns in males (Avise, 1994). Despite these potential problems, we argue that mtDNA has an important advantage in species delimitation relative to nuclear-based markers that has not been widely appreciated. As pointed out by Moore (1995) in his defense of mtDNA data in phylogeny reconstruction, the smaller effective population size (N_e) of the mitochondrial genome will cause mtDNA haplotypes of a given species to coalesce (i.e., become "monophyletic") four times more quickly than will nuclear markers (given some assumptions). We suggest that this property is important in species delimitation because newly formed species should become distinct in their mtDNA haplotype phylogenies long before they become distinct in nuclear-based markers. Thus, analysis of mtDNA data should allow resolution of species limits in many groups that are difficult to resolve with nuclear-based markers, such as morphology. We present an empirical study of spiny lizards (*Sceloporus*) that may illustrate this phenomenon.

The Yarrow's spiny lizard (*Sceloporus jarrovii*) has traditionally comprised seven subspecies, distributed in the mountains and deserts of northern Mexico and the southwestern United States. Wiens et al. (1999) presented a phylogeny for 30 populations of *S. jarrovii* (including the type localities of all seven subspecies) that was based on parsimony and maximum likelihood analyses of 1.8 kb of mtDNA sequence data from the 12S and ND4 genes (and adjacent tRNA genes). They found *S. jarrovii* to be paraphyletic within the monophyletic *torquatus* species group (sensu Wiens and Reeder, 1997), and divided *S. jarrovii* into five species. These species correspond to five allopatric clades of haplotypes, none of which are sister taxa. Wiens et al. (1999) mentioned some diagnostic morphological characters for these species but did not present a critical analysis of morphological variation in the group. Because we wish to evaluate the partitioning of *S. jarrovii* more carefully, we herein revert to the traditional taxonomy and refer to these taxa collectively as *S. jarrovii*.

In this paper, we discuss three approaches for species delimitation using DNA and morphological variation. We propose and review specific protocols for delimiting species using these general approaches and then apply these methods in an empirical case study of the traditionally recognized *Sceloporus jarrovii*. We find that these methods and data give surprisingly discordant results, which may reflect a problematic pattern of morphological variation in this group. We suggest that mtDNA data may generally be a very powerful tool in species delimitation, especially in groups that are difficult to resolve with morphological data.

Approaches to Species Delimitation

Concepts and Overview

Before we attempt to recognize species, we need a clear concept of what species are. de Queiroz (1998) suggested that, despite the long history of dispute over species concepts, most species concepts agree fundamentally that species are lineages (Simpson, 1961; Wiley, 1978; Cracraft, 1983; de Queiroz and Donoghue, 1988; Frost and Kluge, 1994; Baum and Shaw, 1995), and for sexual organisms, they are lineages that are united through the process of gene flow (Mayr, 1942; Dobzhansky, 1950; Templeton, 1989). What previous authors have generally disagreed about are the best criteria for recognizing these lineages (de Queiroz, 1998).

In this study, we compare the results of three approaches (or criteria) to species delimitation: (1) tree-based delimitation (sensu Baum and Donoghue, 1995) using DNA haplotype phylogenies, (2) tree-based delimitation using morphological data, and (3) character-based delimitation (sensu Baum and Donoghue, 1995) using morphological data. We discuss these three

approaches below. However, species are real entities that exist in nature regardless of whether they are supported by any, some, or all of these approaches, and we acknowledge that in some cases all approaches are likely to fail or give ambiguous results (Frost and Kluge, 1994).

We do not apply character-based delimitation to the DNA sequence data because we find the arguments of Brower (1999) against this approach to be compelling. Character-based delimitation is concerned only with the distribution of alleles and ignores their phylogenetic history, and implicitly requires that the potentially diagnostic characters evolve relatively slowly. Brower (1999) showed a plausible hypothetical example in which two species each have many distinct haplotypes for a rapidly evolving marker, with no fixed differences between them, but a phylogenetic analysis of these haplotypes easily separates them into two distinct lineages. For morphological characters (to which character-based delimitation is usually applied), there are generally few "alleles" (i.e., character states) and their history is relatively difficult to estimate.

In the following discussion we use the term "exclusive" instead of monophyletic to discuss haplotype phylogenies, because the term monophyly may not be applicable below the species level (following de Queiroz and Donoghue [1990] and subsequent authors).

Tree-Based Species Delimitation with DNA Data

Overview.—We propose an explicit protocol for delimiting species based on DNA haplotype phylogenies, which can be integrated with nested-clade analysis (NCA; Templeton et al., 1995). Despite the widespread use of DNA data in evolutionary, conservation, and taxonomic studies of population-level differentiation (Avise, 2000), the methodology by which haplotype phylogenies are used to delimit species is only rarely explained (e.g., Baum and Shaw, 1995; Brower, 1999; Templeton, 2001). Our approach is essentially a dichotomous key for making species-level decisions, which we outline both verbally (below) and graphically (Fig. 1). For our approach, we assume a phylogeny of nonrecombining DNA haplotypes of known locality and taxonomic designation. We also assume that the failure of haplotypes from a given locality to cluster together is potential evidence for gene flow with other populations (Slatkin and Maddison, 1989), as is the general discordance between haplotype clades and the geographic area from which the haplotypes are found (e.g., some haplotypes from Australia cluster with some from New Guinea, rather than the haplotypes from each region being mutually exclusive). Alternative explanations for this discordance include incorrect estimation of the gene tree or incomplete lineage-sorting of ancestral polymorphisms. Incorrect estimation of the gene tree may be unlikely if there is strong statistical support for discordant clades (e.g., as assessed by bootstrapping; Felsenstein, 1985). The effects of incomplete lineage sorting may be difficult to distinguish from gene flow. Because of the smaller N_e of the mitochondrial genome, the use of mtDNA data may greatly reduce the possibility of discordance caused by ancestral polymorphisms (Moore, 1995), and we generally assume in this paper that discordance is caused by gene flow. Given that retained ancestral polymorphisms are most likely in populations that have split very recently (Neigel and Avise, 1986), mistaking retained polymorphism for gene flow should only rarely lead to mistakes in species delimitation using our approach, given our emphasis on the older lineages within a putative species.

Methodology.—Our approach is as follows. Given a haplotype phylogeny for a set of populations currently classified as a species (the focal species of the study) and one or more closely related species, the haplotype tree may show the focal species to be either exclusive or not. If the haplotypes of the focal species are exclusive, the presence of multiple species that are hidden by previous taxonomy is suggested by well-supported basal lineages (by "basal" we mean the oldest split or splits within the species) that are concordant with geography (Fig. 1a). The presence of a single species (Fig. 1b) is suggested by evidence for gene flow among the basal lineages (i.e., haplotypes from a given locality appear in both of the basal lineages and/or there is overall discordance between the haplotype phylogeny and geography). If the haplotypes of the focal species are not exclusive (the focal species is "paraphyletic" or "polyphyletic") with respect to one or more species that are each distinct and exclusive, then the focal species may represent multiple

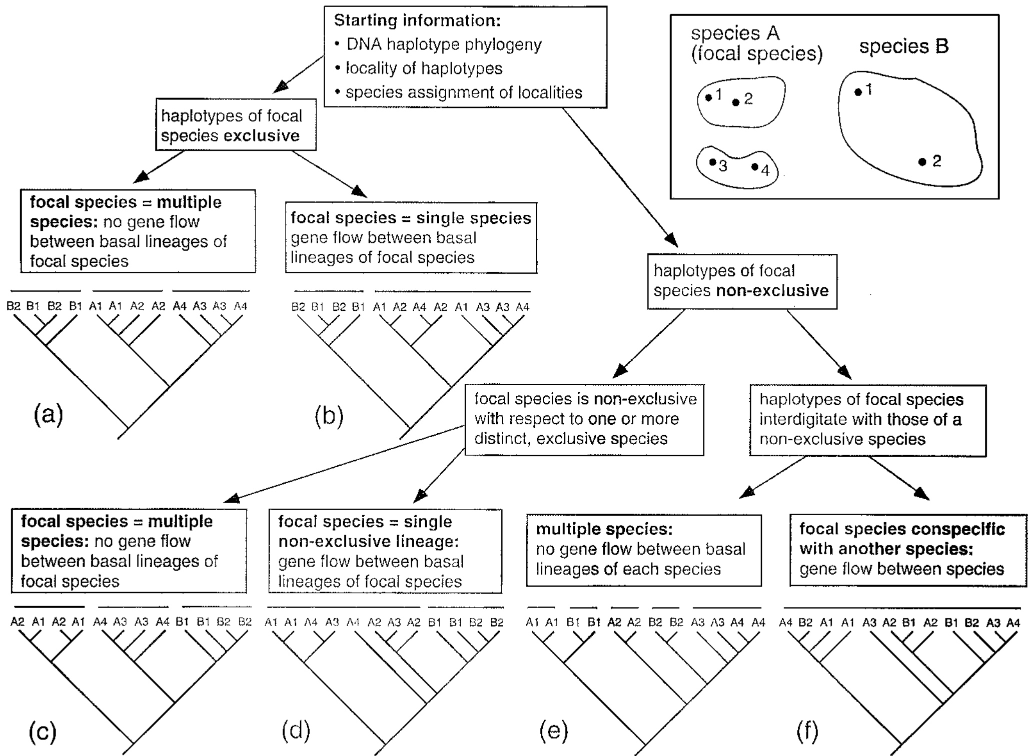

FIGURE 1. Simplified flow chart for species delimitation based on a DNA haplotype phylogeny, illustrated with a hypothetical example. The hypothetical example involves two species, one with two disjunct populations (species A), the other with a single contiguous population (species B). Two individuals are sampled from each population (e.g., A1 = a haplotype from locality 1 of species A). The line above the terminal taxa indicates species limits.

species if there is no evidence of gene flow between the basal lineages (Fig. 1c). If there is gene flow among the basal lineages (Fig. 1d), the focal species may represent a single nonexclusive species ("ferespecies" [Graybeal, 1995] or "plesiospecies" [Olmstead, 1995]). If the focal species is nonexclusive with respect to a species that is itself not exclusive (the haplotypes of the focal species interdigitate with those of another species), and if there is no evidence of gene flow between the basal lineages of each species, then the focal species and the other species may each represent multiple species, disguised by traditional taxonomy (Fig. 1e). Alternatively, the focal species and the other species may be conspecific if there is evidence of extensive gene flow between them (Fig. 1f)—for example, if some of the haplotypes from a given locality of the focal species cluster with some of the haplotypes from a given locality of the other species. In all of these cases, the support for the hypothesis that multiple species are present will be strengthened if the putatively conspecific populations are not merely nonexclusive but are also relatively distantly related.

Assumptions and extensions.—Two aspects of sampling design are critical to our approach: (1) including as many species as possible that are closely related to the focal species, to thoroughly test the exclusivity of the focal species, and (2) including two or more individuals from as many localities as possible, to evaluate gene flow among populations (Slatkin and Maddison, 1989). Including closely related species is crucial, because several previous studies have found that the focal species is nonexclusive and consists of multiple species that are not each other's closest relatives (e.g., Zamudio et al., 1997; Steppan, 1998; Rodríguez-Robles and de Jesús-Escobar, 2000; Serb et al., 2001). This pattern can be detected only by including the additional (nonfocal) species in the analysis. If multiple individuals cannot be obtained from each population, then delimitation of species will depend on the relationship

(i.e., exclusivity) of populations of the focal species to other species and on the general concordance between phylogeny and geography within the focal species (which can be objectively tested using NCA). Our approach can be applied without extensive sampling of individuals from each species, but is more prone to error if sample sizes are small (e.g., failing to detect putative gene flow between populations).

Although our method emphasizes the basal lineages of a focal species as potentially distinct species, it is theoretically possible that each of these basal lineages might contain multiple species (which could be exclusive or nonexclusive), and we would use the same reasoning outlined above to detect such cases. However, at these progressively lower levels, small sample sizes may limit our ability to confidently rule out gene flow with other lineages (e.g., if only two individuals are sampled from a locality, they are likely to appear to be exclusive, even if there is ongoing gene flow with other populations). Our approach does not determine when splitting is no longer justified by the available data, but this can be evaluated statistically by using NCA.

We have assumed that all the putative species are allopatric or parapatric with respect to each other. This seems to be true for most of the case studies we have cited, but sympatric species may be present that have been unrecognized by previous taxonomy, and the focal taxon might even consist of a mixture of sympatric and allopatric species. If a focal species contains morphologically cryptic, sympatric species that occur together at several localities, we expect that the haplotypes from each species will phylogenetically segregate into basal clades containing haplotypes from each locality. However, distinguishing between gene flow and the presence of sympatric species may be difficult when divergent haplotypes of the same putative species occur at the same locality.

We have implicitly assumed that any gene flow between haplotype clades is evidence that these clades are conspecific. In fact, species may be distinct and still engage in limited gene flow, but the exact level of gene flow that determines whether taxa are conspecific is unclear (and will probably always remain so). Templeton (1994) suggested that species could be delimited despite the presence of hybridization by using random permutation methods to test for a significant association between the phylogenetic structure of the haplotype tree and prior taxonomic categories. This application of NCA seems promising, even if it is not a universal solution. In general, we consider the critical factors in determining the species status of hybridizing taxa to be the geographic extent of gene flow relative to the geographic range area of the species and relative to the area of sympatry between species—hybridizing taxa might still be distinct species if gene flow is limited in extent and/or is restricted to a small portion of their ranges and/or to a small part of their area of sympatry.

We have also assumed that the haplotype phylogenies are inferred from a single gene or linked set of genes (e.g., mtDNA). When multiple genes are available, one expects that gene histories will generally be concordant between species (and with regard to species exclusivity) and discordant within species. These expected patterns of concordance and discordance may be an additional line of evidence for species delimitation when using haplotype phylogenies (Avise and Ball, 1990; Baum and Donoghue, 1995; Baum and Shaw, 1995).

Comparison with other approaches.—Our approach differs from those of several other authors (e.g., Avise and Ball, 1990; Baum and Shaw, 1995) in that we do not require species to be exclusive (e.g., Paetkau, 1999; Templeton, 2001). Furthermore, we do not consider nonexclusive species to be fundamentally different from exclusive species (contra Graybeal, 1995; Olmstead, 1995), following Frost and Kluge (1994). Because of incomplete lineage sorting of ancestral polymorphisms, all species may be nonexclusive at some point in their history (Neigel and Avise, 1986), and species may be distinct and even morphologically diagnosable from each other but still have nonexclusive gene genealogies. This scenario may be common when a species with a large geographic range and a large population size gives rise to a diagnosably distinct species with a much smaller range (i.e., a peripheral isolate), such that the latter species quickly becomes exclusive, whereas the former species does not (e.g., Funk et al., 1995; Talbot and Shields, 1996; Hedin, 1997; Paetkau, 1999).

Our approach is similar to the cladistic haplotype aggregation method of Brower

(1999) but differs fundamentally in that we explicitly consider estimated patterns of gene flow in inferring species limits. According to Brower (1999:202), "each population is a phylogenetic species if the haplotypes of all of its members are joined in a contiguous section of an unrooted network." However, without utilizing estimated patterns of gene flow, it is unclear how exactly one divides up these networks (trees) into species. For example, applying Brower's method to the tree in our Fig. 1a, it appears that populations A1, A2, and A3 could each be recognized as distinct species, or all four populations (A1–A4) might collectively represent a single species. Similarly, when populations of a putative species fail to "aggregate," the method seems to give little basis for distinguishing the presence of a single species rather than multiple species. Given the idea that species differ fundamentally from higher taxa because of gene flow, it is unclear how any method can hope to delimit species successfully without taking into account this process.

Templeton (2001) recently advocated application of his NCA method (Templeton et al., 1995) to the problem of species delimitation. NCA uses permutation tests to determine whether there is a nonrandom association of haplotype clades with their geographic locations, given the latitude and longitude of each locality and the position of each haplotype within the tree. NCA then applies a dichotomous inference key to distinguish among different causes for these significant associations (e.g., range fragmentation [as in allopatric speciation], isolation-by-distance, contiguous range expansion, long-distance colonization). This method requires sampling from throughout the range of all included species and may therefore be inapplicable to many species or groups of species. Furthermore, NCA does not distinguish among different causes for the lack of significant association between haplotype clades and their geographic location (e.g., insufficient sampling, gene flow, ancestral polymorphism). However, NCA has at least two important advantages relative to our approach: (1) it takes into account all of the available information on the geographic and phylogenetic position of haplotypes and statistically tests for their association, and (2) it can be applied to many different levels within a clade to determine whether a speciation (fragmentation) event can be inferred with significant statistical support given the data available.

Combining our approach with NCA.—We suggest the strengths of our method and NCA are complementary, and we propose using them together. Our method can be used to delimit species at the highest levels of divergence, even when some of the included species are represented by only one or two individuals (as in our study of *Sceloporus*). NCA can then be used to determine whether further splitting is statistically justified within well-sampled clades and to more rigorously test the limits of taxa that are closely related and extensively sampled. We demonstrate this integrated approach using our data from spiny lizards (*Sceloporus*).

Tree-Based Delimitation with Morphological Data

We propose an explicit protocol for delimiting species using population-level trees based on morphology. Tree-based delimitation with morphology, although advocated by some authors (e.g., Baum and Donoghue, 1995), has rarely been used by empirical systematists (e.g., Hollingsworth, 1998), and the precise methodology for its use has never been thoroughly explained. This approach is facilitated by recent methods that allow continuous quantitative characters and polymorphic characters to be included in phylogenetic analyses with little loss of information (e.g., Thiele, 1993; Wiens, 1999, 2001). We use populations rather than individuals as terminal units (following Hollingsworth, 1998) because morphological characters are generally thought to be genetically unlinked, and using individuals will inappropriately treat all polymorphisms shared between populations as homoplasies rather than potential synapomorphies (Wiens, 2000). We consider sets of populations that are strongly supported as exclusive and are geographically coherent to be potentially distinct species (e.g., for a given taxon, all populations from Australia form one well-supported clade, and the populations from New Guinea form another well-supported clade). We emphasize clades that are strongly supported—for example, as assessed by bootstrapping—because it is theoretically possible that a clade of populations could be united by slight differences in qualitative trait frequencies or quantitative trait means but still be engaging in

gene flow with other populations. However, continuing gene flow seems unlikely if the clade of populations is well differentiated and strongly supported. Methods for statistically determining the congruence between morphology-based clades and their geographic locations (equivalent to NCA) have not yet been developed.

As with tree-based delimitation using DNA data, we present our method for morphological data verbally and graphically as a dichotomous key (Fig. 2). If the populations of the focal species appear as exclusive, the focal species may represent a single species if it contains no strongly supported basal lineages that are concordant with geography (Fig. 2b); whereas, it may represent multiple species if it contains basal clades of populations that are strongly supported and geographically coherent (Fig. 2a). If the focal species is not exclusive, the basal lineages of the focal species may represent distinct species if they are strongly supported as exclusive and concordant with geography (Fig. 2c), and especially if they are not on adjacent branches, such that the focal species is polyphyletic. The focal species may be a single, nonexclusive species if the basal clades of populations are weakly supported, are not concordant with geography, and appear on adjacent branches of the phylogeny, such that the focal species is paraphyletic and not polyphyletic (Fig. 2d). The basal lineages of a nonexclusive species could appear to be polyphyletic and still be conspecific (freely interbreeding), but this seems less likely the more distantly related these populations are on the tree. The focal species may be conspecific with another species if their populations interdigitate on the tree, if relationships among them are weakly supported, and if the clades of populations are not concordant with geography (Fig. 2e).

Some authors have stated that phylogenetic analysis should not be attempted below the species level (e.g., Davis and Nixon,

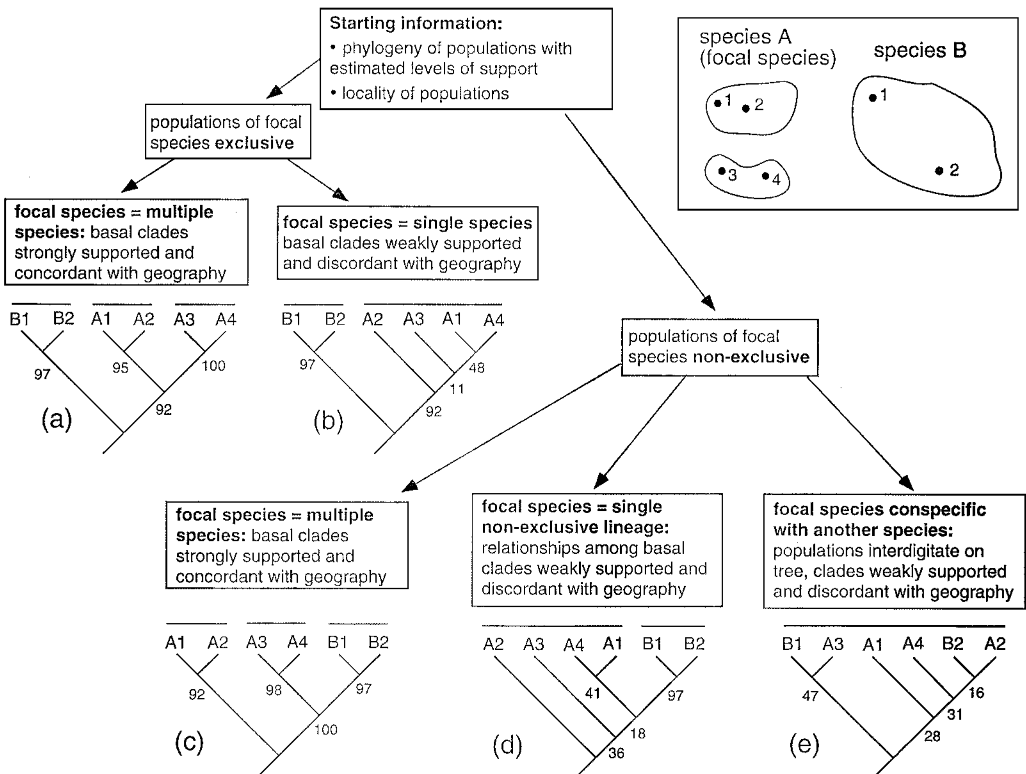

FIGURE 2. Simplified flow chart for species delimitation using a population-level phylogeny based on morphological data illustrated with a hypothetical example. The hypothetical example involves two species, one with two disjunct populations (species A), the other with a single contiguous population (species B). The line above the terminal taxa indicates species limits. Numbers at nodes indicate hypothetical bootstrap proportions.

1992), and few researchers have attempted to reconstruct phylogenies for populations using morphology. Because gene flow among populations and recombination among characters should break up hierarchical patterns within species inferred from morphological data, there is unlikely to be a clear phylogenetic signal within species using these data. Our method rests on the idea that this lack of intraspecific signal will lead to weakly supported population-level trees that are discordant with geography and, moreover, that we can detect this lack of signal and use it to infer species limits in cases where these limits are unknown. Finally, we note that the tree-based approach we outline for morphological data should be equally applicable to allozyme and microsatellite data.

Tree-based species delimitation can also be attempted with combined DNA and morphological data. We expect strong phylogenetic signal within species from the DNA data and weak intraspecific signal from morphological data. Thus, combining these data may simply yield the DNA haplotype tree, or some slight modification thereof and this is basically the pattern we observe in our results from spiny lizards (*Sceloporus*). We do not see any clear advantage to delimiting species using trees from such combined analyses.

Character-Based Delimitation with Morphological Data

Character-based species delimitation involves finding diagnostic character states that represent seemingly fixed differences between the putative species (e.g., frequency of the diagnostic state in species A = 100% vs. frequency in species B = 0%), or differences that are at least nonoverlapping (e.g., range in number of vertebrae in species A = 10–12 vs. range in species B = 14–15). This approach has been formalized as population aggregation analysis (Davis and Nixon, 1992). The approach is advantageous in that there is a clear relationship between fixed differences and gene flow—if the diagnostic traits are genetically based and are truly fixed in each species, there is unlikely to be any gene flow between the species. However, given finite sample sizes, determining with certainty whether traits are truly fixed is virtually impossible (Wiens and Servedio, 2000). Even allowing some low level of polymorphism in the diagnostic characters (e.g., a frequency of 10%) requires very large sample sizes to achieve statistical confidence (using the method of Wiens and Servedio, 2000), and the best frequency cutoff to use remains uncertain. This approach should be applicable to other types of character data as well (e.g., allozymes).

Many systematists utilize statistical analyses of quantitative morphological characters to test species boundaries, often evaluating the extent to which individuals of a putative species cluster together using principal components or canonical variates analysis. This approach may be useful in inferring species limits but lacks the clear relationship to estimated patterns of gene flow that the character-based approach offers or the phylogenetic component of the tree-based approach.

Puorto et al. (2001) recently developed an approach to species delimitation that involves testing for significant association between quantitative variation in morphology and haplotype trees from DNA data using Mantel tests. Their method tests whether morphological data significantly confirm groupings based on DNA by testing for significant association between matrices of Euclidean distances based on morphological data and matrices of patristic distances based on the haplotype tree.

Concordance Between Approaches and Advantages of mtDNA Data

We make the following predictions about patterns of concordance between the three approaches. In general, we predict that the distinctness of a species based on all three approaches will be related to how long the species has been isolated from gene flow with other species. Thus, given enough time, distinct species should: (1) have exclusive DNA haplotype phylogenies relative to other species (Neigel and Avise, 1986); (2) have one or more diagnostic morphological characters (either fixed or at high frequency); and (3) form strongly supported clades of populations based on morphology. We consider the strongest evidence for distinct species to be concordance between these different approaches (e.g., Avise and Ball, 1990; Sites and Crandall, 1997). Conversely, for species that have diverged very recently, we predict that species will

have nonexclusive haplotype phylogenies (i.e., the individuals or populations will be paraphyletic or polyphyletic with respect to one or more other species; Neigel and Avise, 1986), will lack diagnostic characters, and will have nonexclusive population-level phylogenies from morphology.

For species that are intermediate in the amount of time they have been reproductively isolated, we predict that species will generally become exclusive in their mtDNA haplotype phylogenies long before becoming exclusive in morphology-based phylogenies and before acquiring diagnostic morphological characters (see also Frost et al., 1998). We expect this pattern largely because mitochondrial genes have an N_e that is one-fourth that of a given nuclear gene and because N_e strongly influences the rate at which the haplotypes of a species become exclusive (Neigel and Avise, 1986; Moore, 1995). Thus, all else being equal, a species will appear distinct (exclusive) based on a mitochondrial gene in a quarter of the time it will take for its nuclear gene haplotypes to become exclusive (Moore, 1995). Similarly, it will take four times as long for a given nuclear marker (such as a morphological or allozyme character) to become fixed within a species as it will take for the mtDNA haplotypes of that species to become exclusive (Kimura, 1983). Furthermore, for conditions where a single mitochondrial gene has a 95% probability of correctly resolving a species as exclusive, 16 or 40 nuclear genes (depending on the null hypothesis used) must be sampled to have the same probability of success (based on Moore, 1995). In summary, mitochondrial markers should be able to correctly resolve species boundaries for many species that are too recently diverged to be resolved using nuclear markers alone and should do so with much greater efficiency and probability of success.

These advantages of mtDNA data depend on certain assumptions. Hoelzer (1997), responding to Moore (1995), suggested that mtDNA gene trees would coalesce less rapidly (relative to nuclear-gene trees) when there is polygyny, female philopatry, and male-biased dispersal. Moore (1997) pointed out that the level of polygyny would have to be extreme to offset the advantages of mitochondrial markers, and showed that female philopatry and male dispersal would only lengthen the coalescence times for mitochondrial markers if speciation occurred without geographic subdivision. Apart from their effect on population size, male dispersal and female philopatry can bias estimates of gene flow that are based on mitochondrial markers (Avise, 1994; Moritz, 1994), which in some cases might influence estimates of species boundaries (see studies on *Ensatina* salamanders for a possible example: Moritz et al., 1992; Jackman and Wake, 1994; Wake, 1997; Wake and Schneider, 1998). Some authors have also suggested that delimiting species based on mtDNA data may be problematic because the smaller N_e of the mitochondrial genome may lead to coalescence of haplotype lineages in populations that are only temporarily isolated (e.g., Moritz, 1994; Sites and Crandall, 1997; Paetkau, 1999). The problems of male-biased dispersal, female philopatry, and coalescence of temporarily isolated populations all involve potential overresolution of intraspecific mtDNA trees (i.e., mtDNA clades appear as distinct lineages even though they are not) and are most likely to affect the more recent branches of the haplotype tree. Our emphasis on recognizing basal haplotype lineages (i.e., the oldest splits) within a taxon as distinct species should greatly reduce these problems.

MATERIALS AND METHODS

DNA Haplotype Phylogeny and Nested-Clade Analysis

The mtDNA haplotype phylogeny for *S. jarrovii* and related species was based on a maximum likelihood analysis of the combined mitochondrial 12S and ND4 gene regions (for details, see Wiens et al., 1999). The 12S data consist of 883 bp and 133 parsimony-informative characters. The ND4 data consist of 889 bp and 248 parsimony-informative characters. The data matrix is given in Appendix 1 (available at the website of the journal [www.systbiol.org]). The maximum likelihood tree is very similar to trees based on weighted and unweighted parsimony. Maximum likelihood analyses used the GTR + I + Γ model (selected using the model-testing procedure outlined by Huelsenbeck and Crandall, 1997), and the weighted parsimony analyses used a 5:1 transition:transversion weighing (based on the ratio estimated by maximum likelihood).

We applied NCA (Templeton et al., 1995) to two well-sampled haplotype clades revealed

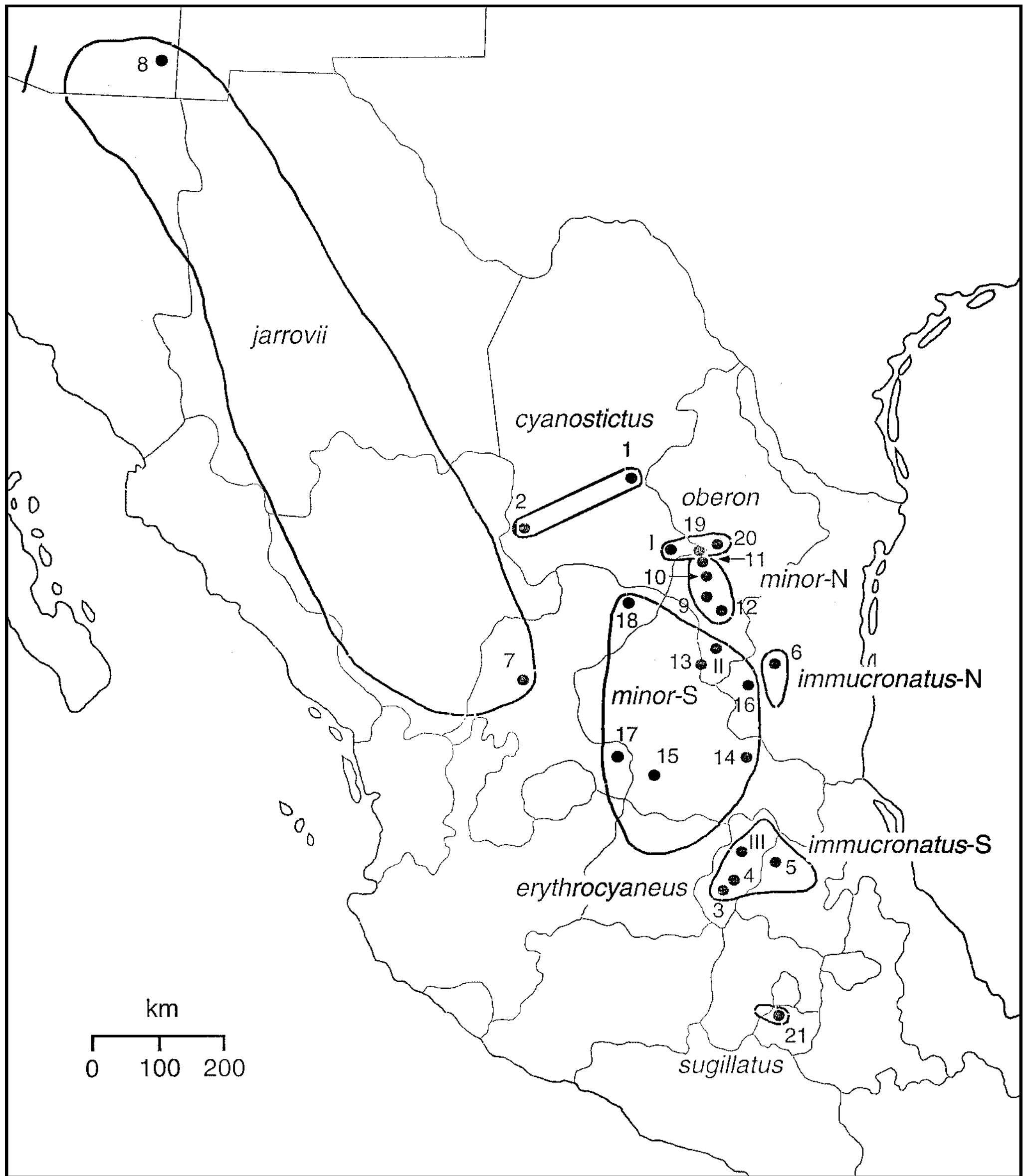

FIGURE 3. Map of northern Mexico and adjacent United States, showing geographic location of populations of *Sceloporus jarrovii* (sensu lato) used in this study. See Appendix 3 for explanation of localities. Three populations included in the molecular analysis are not included in the morphological analysis because of inadequate sampling of specimens: I = *S. j. oberon*, Coahuila, N of El Diamante; II = *S. j. minor*, Nuevo Leon, 7.1 km north of Doctor Arroyo; C = *S. j. immucronatus*, Queretaro, near Pinal de Amoles. Figure is modified from Wiens et al. (1999). Lines indicate inferred range limits of each taxon based on a broader sampling of museum specimens. The "N" and "S" indicate the northern and southern sets of populations of the two subspecies with highly disjunct ranges (*S. j. immucronatus* and *S. j. minor*). Distribution maps for other included members of the *torquatus* group are provided by Sites et al. (1992) and are summarized as follows: *S. cyanogenys*: southern Texas, eastern Nuevo Leon, and western Tamaulipas; *S. dugesii*: southern Nayarit to Guanajuato; *S. insignis*: narrowly distributed in southwestern montane Mexico, in Jalisco and Michoacan; *S. lineolateralis*: just east of the range of *S. j. jarrovii* in the state of Durango; *S. mucronatus*: montane southern Mexico from southern Hidalgo to western Oaxaca and Guerrero; *S. ornatus*: north and west of *S. j. oberon* and south of *S. j. cyanostictus* in southern Coahuila; *S. poinsettii*: northern Chihuahuan desert, from Arizona, New Mexico, and Texas south to Durango; *S. torquatus*: widely distributed in central Mexico from Nuevo Leon to Puebla.

in the mtDNA trees (B and C in Fig. 4) in order to test for the presence of additional species and to further test the distinctness of these two lineages from each other. We used the nesting algorithms described by Templeton et al. (1987) and Templeton and Sing (1993), with modifications for DNA sequence data described by Crandall (1996), to divide the maximum likelihood tree within each of these lineages into nested clades of haplotypes. The nesting designs for clades B and C are shown graphically in Appendix 2. The number of mutational steps separating clades was estimated using equally weighted parsimony. Permutation tests were performed using GeoDis version 2.0 (Posada et al., 2000), and the causes of significant association of haplotype clades with geography were evaluated using the inference key given by Templeton et al. (1995). The NCA was considered to potentially support the presence of distinct species if the inference chain for a given clade ended in "range fragmentation" or "allopatric fragmentation" (Templeton, 2001). The status of clades as "interior" versus "tip" was determined using outgroup rooting. In the analysis that included both clades B and C, we picked clade B to be interior because it is much more geographically widespread than clade C and is therefore more likely to be ancestral based on coalescent theory (Crandall and Templeton, 1993).

Morphology-Based Phylogeny

Sampling of populations.—We used 21 populations of *S. jarrovii* as terminal units in our phylogenetic analysis (see Fig. 3 for map and Appendix 3 for localities and specimens examined). Wiens et al. (1999) examined 30 populations for their molecular phylogenetic analyses. Some of these populations were separated by only a few kilometers and were combined for our analysis to increase sample sizes, and a few populations that lacked adequate material (i.e., one or more adult males) were not included. In addition to specimens obtained through fieldwork by Wiens, A. Nieto, T. Reeder, and R. Reyes-Avila, most specimens of *S. jarrovii* from all major U.S. herpetological collections were examined during the course of the study. Only presumed adults were included. Institutional abbreviations follow Leviton et al. (1985).

Eight other species of the *torquatus* species group that were included in the molecular analysis of Wiens et al. (1999) were also included. A representative of the sister taxon of the *torquatus* group (*S. grammicus* of the *grammicus* group) was included to root the tree (Wiens and Reeder, 1997). Apart from *S. jarrovii*, all species were treated as a single terminal taxon in the phylogenetic analyses, because these species were generally represented by a single individual in the molecular analyses and most are morphologically distinct (e.g., Smith, 1939).

Character sampling.—Morphological characters consisted of those used previously in diagnosing subspecies of *S. jarrovii* (e.g., Smith, 1939), those used in a prior phylogenetic analysis of *Sceloporus* (Wiens and Reeder, 1997), and new characters found during the course of the study. Many of the morphological characters used by Wiens and Reeder (1997) showed little or no variation among populations of *S. jarrovii* or other members of the *torquatus* group and were not included.

Characters were derived largely from squamation, coloration, and morphometric features. Because of limited sample sizes for many populations, osteological preparations and characters were not used, and these characters show relatively little variation in the *torquatus* group (Wiens and Reeder, 1997). Characters were not excluded because of intraspecific variability or overlap in quantitative trait values between populations, because both polymorphic (Wiens, 1995, 1998a; Wiens and Servedio, 1997) and continuous traits (Thiele, 1993; Wiens, 2001) have been shown to contain useful phylogenetic information. A few morphometric and meristic characters were excluded because of probable correlations with included characters. The characters used are listed in Appendix 4. Terminology for squamation and coloration features follows Smith (1939) and Wiens and Reeder (1997).

Character coding and weighting.—Most of the qualitative characters showed extensive variation within populations. Qualitative polymorphic characters were coded using the frequency-based step-matrix approach (Wiens, 1995, 1999; Berlocher and Swofford, 1997). Studies using simulations (Wiens and Servedio, 1997, 1998), congruence between data sets (Wiens, 1998a), and statistical criteria (Wiens, 1995) suggest that frequency-based methods may be the most accurate methods for coding polymorphic data (see review and discussion of controversy by Wiens, 1999, 2000).

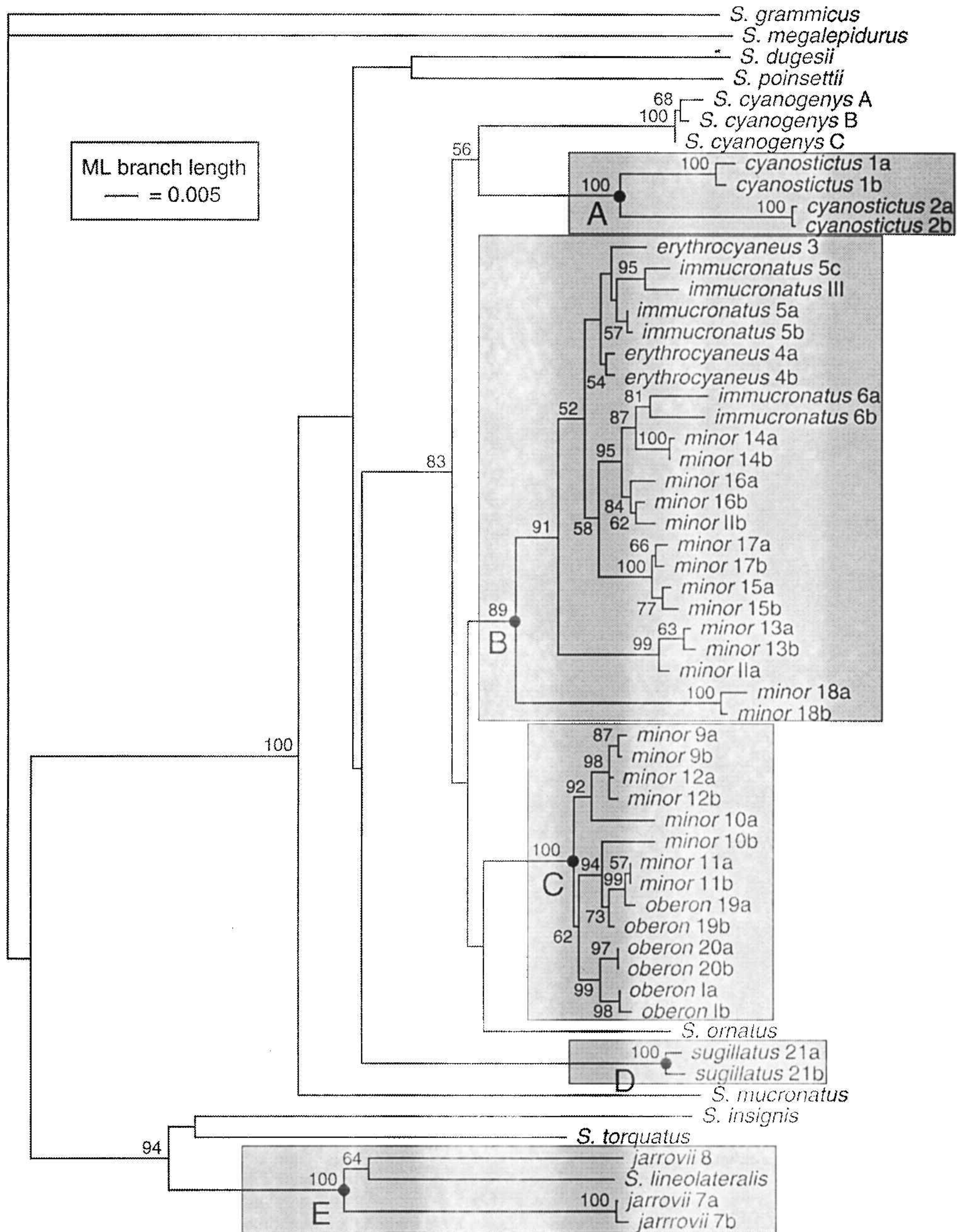

FIGURE 4. Molecular phylogeny for populations of *Sceloporus jarrovii* (sensu lato) and related species of the *torquatus* species group, based on maximum likelihood analysis (GTR + I + Γ model) for the combined ND4 and 12S gene regions (Wiens et al., 1999). The lengths of branches are proportional to the maximum likelihood branch length estimates (the expected number of changes per site; the scale bar is roughly equal to 10 substitutions). Numbers at branches indicate bootstrap values >50%, based on the same maximum likelihood model (100 pseudoreplicates with TBR-branch swapping). Figure is modified from Wiens et al. (1999). Letters indicate the five putative species inferred from tree-based delimitation using DNA data, and the five species we recognize in this study. A = *S. cyanostictus (S. j. cyanostictus)*, B = *S. minor* (*S. j. erythrocyaneus*, *S. j. immucronatus*, and southern *S. j. minor*), C = *S. oberon* (northern *S. j. minor* and *S. j. oberon*), D = *S. sugillatus (S. j. sugillatus)*, E = *S. jarrovii (S. j. jarrovii* and possibly *S. lineolateralis*). Lowercase letters associated with population numbers indicate individual specimens from a given locality, corresponding to those in Table 1 of Wiens et al. (1999), with the following exceptions (designation in this paper = locality or specimen designation in Wiens et al., 1999): I = 27; II = 18; III = 7; 5a = 6a; 5b = 6b; 5 = 5c; 6a = 8; 6b = 9; 7 = 11; 8 = 10; 9 = 21; 10a = 22; 10b = 23; 11 = 24; 12a = 19; 12b = 20; 13a = 16; 13b = 17; 14 = 15; 15 = 14; 16a = 25; 16b = 26; 17 = 12; 18 = 13; 19 = 28; 20 = 29; 21 = 30.

For a given character, each terminal taxon was given a unique character state in the data matrix. The cost of a transition between each pair of character states was then entered into a step matrix, and the cost was based on the Manhattan distance between the frequencies of each pair of taxa. One character (dorsal color in life) was coded using the scaled method (Campbell and Frost, 1993), implemented using a step matrix (Mabee and Humphries, 1993). The scaled method was used because estimating the frequencies of the different conditions was difficult for this character. Meristic and morphometric characters were coded using step-matrix gap-weighting (Wiens, 2001), a modification of the gap-weighting method of Thiele (1993). Conversion of trait frequencies and "scores" to step matrices was performed with a program written in C by J.J.W. The raw data are presented in Appendix 5.

Three morphometric characters were included. Populations differ considerably in overall body size and maximum male snout-to-vent length (SVL) was used as a character. Maximum SVL was used rather than mean SVL because of the difficulty in determining sexual maturity with certainty, and only male size was considered to reduce biases caused by the combination of sexual-size dimorphism and unequal sex ratios in the sample of a given population. The other morphometric characters were the length of the hindlimb relative to the SVL and the length of the head relative to SVL. These measures of shape were obtained by using the residuals of hind limb length and head length regressed against SVL, and were scored for males only to avoid possible sex-biased differences in shape within species.

We scaled all quantitative characters to have the same maximum cost as a fixed, binary character (between-character scaling; Wiens, 2001). This is a common "default" approach and may be appropriate for our study because many of the meristic characters had very large ranges of trait values. Large ranges of trait values may be problematic for between-state scaling (see Wiens, 2001).

Phylogenetic analysis.—Phylogenetic analyses of the data matrix (Appendix 6) were performed using PAUP* (Swofford, 1998). Shortest trees were sought using the heuristic search option with 100 random-addition sequence replicates. Relative support for individual branches was assessed using nonparametric bootstrapping (Felsenstein, 1985), with 100 bootstrap pseudoreplicates. Each bootstrap pseudoreplicate used a heuristic search with five random-addition sequence replicates to find the shortest tree for that matrix. Branches with bootstrap values >70% were considered to be strongly supported, following Hillis and Bull (1993; but see their caveats).

Combined Analysis of Morphological and Molecular Data

We performed a combined analysis of the mtDNA and morphological data for the 21 populations analyzed using both data sets. Although we did not use the combined analysis in species delimitation, it may provide the best estimate of species or population phylogeny. We performed two combined-data analyses. In the first, the DNA sequence data were weighted equally (transitions and transversions were given equal weight), as were the morphological and molecular characters, such that the maximum cost of a morphological character-state change was equivalent to a nucleotide substitution (see matrix in Appendix 7). In the second analysis (Appendix 8), transversions were weighted five times as much as transitions (see Wiens et al., 1999), and the DNA sequence data were weighted such that the maximum cost of a morphological change was equivalent to that of a transversion (assuming that morphological changes are equivalent to the rarer class of molecular changes, because many molecular changes may contribute to a single morphological change). Before combining the data sets, we examined trees from the separately analyzed datasets to identify regions of strongly supported incongruence (following Wiens, 1998b).

Character-Based Species Delimitation

The character-based approach was implemented by comparing the frequencies of qualitative characters and the ranges of trait values for quantitative characters (Appendix 5) across all populations to seek potentially diagnostic characters. Characters were considered to diagnose one species or set of populations from others if those characters were invariant for alternative character states or showed no overlap in trait values.

We used the method of Wiens and Servedio (2000) to evaluate statistical confidence in the distinctness of sets of populations that were delimited using the character-based

approach. Specifically, we used Equation 3 of that paper, which tests whether sample sizes (for a given set of populations) are large enough to argue that at least one of the diagnostic characters is not polymorphic above a selected frequency cutoff. For this test, we used 10% as the frequency cutoff (i.e., the maximum frequency of the alternative character state allowed in the diagnostic character). Thus, failing this test means that there is a >5% probability that all of the diagnostic characters for a given taxon are actually polymorphic, with the unobserved character state occurring at a frequency of 10% or greater. We treated overlap in trait values between populations as equivalent to polymorphism in qualitative traits.

RESULTS

DNA Haplotype Phylogeny and Nested-Clade Analyses

Phylogenetic analysis of the mtDNA sequence data using parsimony and likelihood shows that the haplotypes of *S. jarrovii* are not exclusive, and that they instead interdigitate among the other species of the *torquatus* group (Fig. 4). These haplotypes are partitioned among five lineages, none of which are sister taxa. Each of these five lineages is allopatric and concordant with geography (Fig. 3). Application of our protocol to tree-based delimitation using DNA data suggests that the focal species is nonexclusive and consists of five basal lineages with no gene flow among them (Fig. 1d) and supports the division of *S. jarrovii* into five species, as suggested by Wiens et al. (1999).

Application of NCA to the entire ingroup would be impractical because extensive geographic sampling was undertaken only for *S. jarrovii* and not the other eight species of the *torquatus* group that were included (and which interdigitate among populations of *S. jarrovii*). We applied NCA within the two lineages of *S. jarrovii* that were sampled most extensively (B and C in Fig. 4). Our results (Figs. 5, 6a) suggest that there is a significant association between haplotype clades and their geographical locations

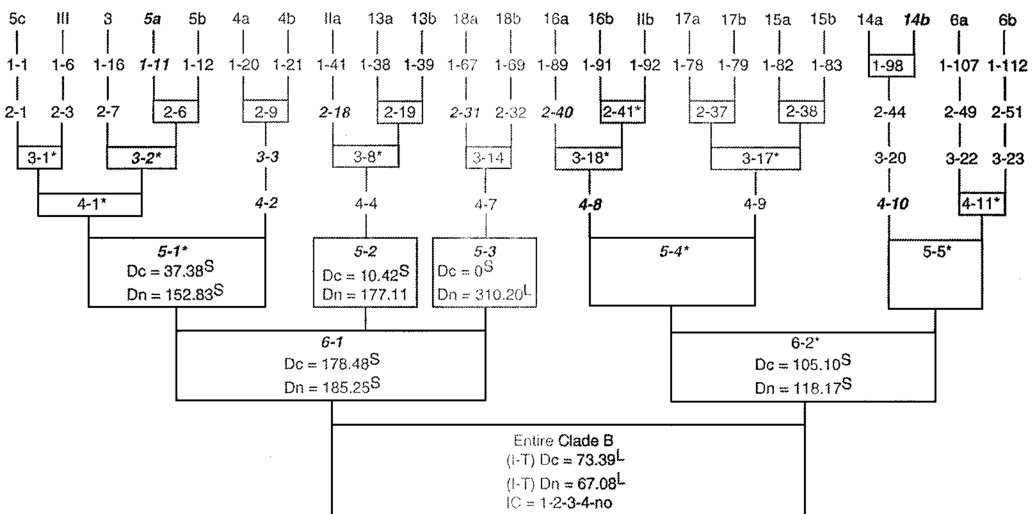

FIGURE 5. Graphical summary of results of NCA for haplotype clade B (Fig. 4), which shows restricted gene flow caused by isolation-by-distance. Nesting levels are indicated from top to bottom, starting with the individual haplotypes (0-step clades) at the top. The numbering of clades accounts for empty clades (clades containing inferred ancestral haplotypes but no observed haplotypes), which are not shown, and the complete nesting design is given in Appendix 2. For clades that show a significant ($P < 0.05$) probability of association between geography and clade distribution based on an exact contingency test (Templeton et al., 1995), the clade distance (Dc) and the nested clade (Dn) distances are given. Clades that contain two or more clades and two or more localities but do not show a significant association are asterisked. Distances significantly smaller than expected are indicated with a superscripted S and distances significantly larger than expected are indicated with a superscripted L (given a null expectation of random geographical distribution of haplotypes). Distances for a few clades (5-1, 6-2) that do not show overall significant association are included for interpretation of results from higher-level clades. Results contrasting interior versus tip clades are indicated by I-T, and the numbers of interior clades are boldfaced and italicized. The inference chain (IC) from the key provided by Templeton et al. (1995) is used to interpret the statistical results.

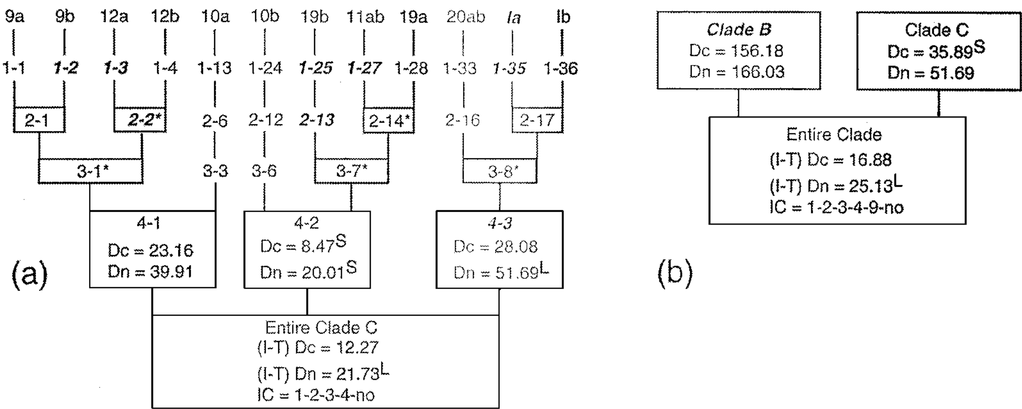

FIGURE 6. Graphical summary of results of NCA for (a) haplotype clade C (Fig. 4), which shows restricted gene flow caused by isolation-by-distance, and (b) a clade including clades B and C, which shows range fragmentation (speciation). See Figure 5 for explanation and Appendix 2 for nesting design.

within each of these clades at the highest clade levels. However, within each of these two clades, restricted gene flow appears to be the result of isolation-by-distance within the range of a single lineage, rather than range fragmentation by speciation. When these two lineages are analyzed together (combined analysis of the molecular and morphological suggests they might be sister taxa; Fig. 8a), NCA indicates that the geographical association of haplotypes within each lineage is caused by range fragmentation, supporting the hypothesis that these are two distinct species (Fig. 6b).

Combined application of our approach with NCA supports division of *S. jarrovii* into five lineages, and suggests that further division of these five lineages into additional species is currently unwarranted. One of these five lineages contains haplotypes from both *S. j. jarrovii* and an individual of *S. lineolateralis*—a taxon that some consider to be morphologically indistinguishable from *S. j. jarrovii* (Sites et al., 1992). Two likely possibilities are that *S. lineolateralis* is conspecific with *S. j. jarrovii*, or *S. j. jarrovii* is a nonexclusive species relative to a distinct *S. lineolateralis*. Although our sampling was too limited to resolve the separation of *S. j. jarrovii* and *S. lineolateralis*, it is clear that *S. j. jarrovii* is not conspecific with other populations of *S. jarrovii*.

Sceloporus j. cyanostictus is known only from two populations that are well-separated geographically. Two individuals were sequenced from each of these populations, and the haplotypes of each are exclusive and relatively divergent. Although these two populations might represent distinct species, further sampling from these localities and geographically intermediate localities is needed to distinguish speciation from isolation-by-distance. *Sceloporus j. sugillatus* was sampled from the type locality and is known only from there and a nearby locality. Because of the extreme geographic proximity of these populations, they most likely are members of a single species.

Sceloporus j. oberon and the northern populations of *S. j. minor* form a clade (clade C) that is weakly supported as the sister taxon of *S. ornatus*. The populations of *S. j. oberon* and northern *S. j. minor* are not mutually exclusive, and the haplotype phylogeny suggests geographically widespread gene flow among these populations (e.g., population 10 appears in two disparate locations on the haplotype tree; Fig. 4). It is not possible to delimit any basal, exclusive lineages within this clade, and NCA does not support further division of this lineage into additional species.

The most extensively sampled clade of *S. jarrovii* haplotypes (B) contains *S. j. erythrocyaneus*, *S. j. immucronatus*, and the southern populations of *S. j. minor*. None of these three taxa is exclusive in the haplotype tree, and the disparate placements of the haplotypes from near Doctor Arroyo, Nuevo León (population II, Fig. 3), suggest geographically widespread gene flow in this lineage (Fig. 4). Although there is a well-supported basal split within this clade, between the two haplotypes from Concepción

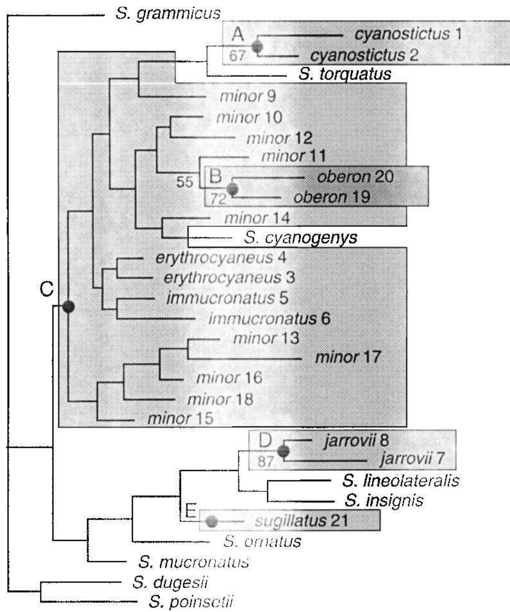

FIGURE 7. Population-level phylogeny of *Sceloporus jarrovii* and related species of the *torquatus* group, based on parsimony analysis of morphological data. Branch lengths are drawn proportional to estimated character change, and numbers at branches indicate bootstrap values >50%. Letters indicate the five species suggested by tree-based delimitation. A = *S. j. cyanostictus*; B = *S. j. oberon*; C = *S. j. erythrocyaneus*, *S. j. immucronatus*, and *S. j. "minor"*; D = *S. j. jarrovii*; E = *S. j. sugillatus*.

del Oro (population 18) and all other populations, NCA does not support recognition of additional species within this lineage.

Tree-Based Morphology

A total of 44 characters were scored (43 parsimony-informative; Appendix 4), consisting of 24 scale characters (11 qualitative and polymorphic, 13 meristic), 17 coloration characters (15 qualitative and polymorphic, 1 qualitative and fixed, and 1 meristic), and 3 morphometric characters (Appendix 5).

Phylogenetic analysis of the morphological data matrix (Appendix 6) yielded a single shortest tree with a length of 171.404, a consistency index of 0.268 (excluding uninformative characters), and a retention index of 0.400. Like the mtDNA phylogeny, the morphology-based phylogeny shows *S. jarrovii* to be nonexclusive, with populations of *S. jarrovii* interdigitating among other species of the *torquatus* group. In the morphological phylogeny, the populations of *S. jarrovii* form eight basal clades (Fig. 7), none of which are sister taxa. In other words, eight lineages would have to be elevated to distinct species to avoid paraphyly of *S. jarrovii*. However, only two of these basal clades are well-supported (*S. j. jarrovii* and *S. j. cyanostictus*), and many of the weakly supported clades are obviously

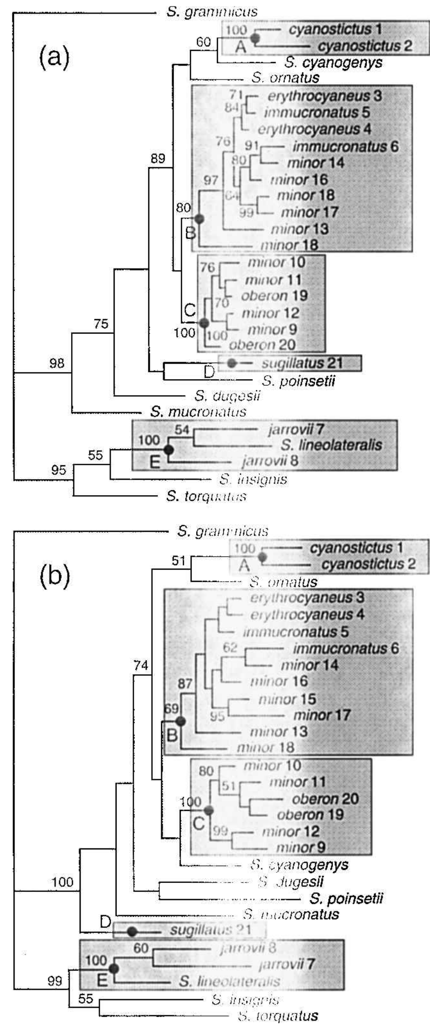

FIGURE 8. Population-level phylogeny of *Sceloporus jarrovii* and related species of the *torquatus* group, based on parsimony analysis of combined molecular and morphological data. Branch lengths are drawn proportional to estimated character change, and numbers at branches indicate bootstrap values >50%. (a) Unweighted analysis with all DNA substitutions weighted equally to each other and to maximum cost of each morphological character change. (b) Weighted analysis, with transversions weighted five times as much as transitions, and weighted equal to the maximum cost of each morphological character change. Clades A–E are essentially the same as in Figure 4.

discordant with the geographic proximity of populations. For example, the populations of southern *S. j. minor* fail to cluster together (i.e., population 14 clusters with *S. cyanogenys,* instead of with populations 13 and 15–18), as do the populations of northern *S. j. minor* (population 9 clusters with *S. torquatus* and *S. j. cyanostictus,* instead of with populations 10–12). The two populations of *S. j. oberon* also form a strongly supported clade that is concordant with geography (suggesting that *S. j. oberon* also represents a distinct species), although this clade seems to emerge from among the northern populations of *S. j. minor.*

Using our criteria, the morphological results suggest that *S. j. minor, S. j. immucronatus,* and *S. j. erythrocyaneus,* form a single, nonexclusive species (clade C; Fig. 7), from which arises the seemingly distinct species *S. torquatus, S. cyanogenys, S. j. oberon,* and *S. j. cyanostictus*. The supporting evidence is the nonexclusive nature of these populations on adjacent branches of the phylogeny, the weakly supported relationships among them, and the discordance of many of these clades with geography (Fig. 2d). Our criteria also support *S. j. jarrovii, S. j. sugillatus,* and *S. j. cyanostictus* as distinct species, because each is phylogenetically separated from other *S. jarrovii* populations, each is strongly supported as exclusive, and each is concordant with geography (although the inclusion of *S. j. sugillatus* is based on a single population). In summary, application of our protocol for tree-based delimitation using morphology suggests that *S. jarrovii* represents four exclusive species and one nonexclusive species.

Combined Data Analysis

Phylogenetic analysis of the combined data using the two weighting schemes (matrices in Appendices 7 and 8) yields phylogenies that are similar to the tree based on the molecular data alone (Fig. 8), as predicted above. Nevertheless, there is a strongly supported conflict between the morphological and DNA data that is apparent from examining bootstrap values on the separately analyzed trees, involving the exclusivity of *S. j. oberon* (supported by the morphological data, rejected by the mtDNA data). The combined-data trees, although not fully congruent between weighting schemes, support the exclusivity of the same five clades based on the mtDNA data alone. However, the equally weighted analysis shows weak support for placing the two clades that contain *S. j. minor* populations as sister taxa, a result not seen in separate analyses of either the molecular or morphological data. The combined-data trees were not used in species delimitation.

Character-Based Morphology

The character-based approach using morphological data supports recognition of five sets of populations as distinct species: (1) *S. j. sugillatus,* (2) *S. j. cyanostictus,* (3) *S. j. immucronatus* + *S. j. erythrocyaneus,* (4) population 18 of *S. j. minor* (Concepción del Oro), and (5) all the remaining populations of *S. jarrovii,* including *S. j. jarrovii, S. j. minor,* and *S. j. oberon.* However, three of these five (2–4) are consistently diagnosed by only a single character (male dorsal coloration), which can be difficult to score, and the last species is simply an amalgam of the populations that lack the diagnostic characters seen in the other species. *Sceloporus j. sugillatus* have unique dark, transverse stripes on the flanks of adult males and a wider black collar than other populations of *S. jarrovii* (6–8.5 scales in *S. j. sugillatus* vs. 2–5.5 in other *S. jarrovii* populations). Furthermore, the large number of scales around the forelimb in *S. j. sugillatus* (14–18) shows only minimal overlap with other populations of *S. jarrovii. Sceloporus j. cyanostictus* has a unique dorsal coloration in adult males that ranges from green to blue-green. *Sceloporus j. erythrocyaneus* and *S. j. immucronatus* are diagnosed by blue dorsal coloration. The population of *S. j. minor* from Concepción del Oro (population 18) has a distinct yellow dorsal coloration, but this was scored from only a few individuals and may not be diagnostic for the population as a whole.

Applying the test of Wiens and Servedio (2000; their Equation 3) shows that sample sizes are insufficient to argue that any of these five sets of populations are statistically distinct, given a frequency cutoff of 10% and a P-value of 0.05. This test was not applied to the composite species (*S. j. jarrovii, S. j. minor, S. j. oberon*) because the other species had already failed.

Contrary to our predictions, *S. j. jarrovii* and *S. j. oberon* have strong bootstrap support in the morphology-based phylogeny but lack seemingly fixed diagnostic characters. There

are two variable features that distinguish *S. j. jarrovii* from most other *S. jarrovii* populations. First, *S. j. jarrovii* usually have three or more interpostanals (one of the six individuals from Arizona had only two) versus two or fewer in the other populations (except for the single male *S. j. minor* from Saldana). Second, the reduced blue gular blotch distinguishes *S. j. jarrovii* from most populations, although this was observed at low frequencies in *S. j. sugillatus* and *S. j. minor* from population 18. The clade of *S. j. oberon* populations is supported by several polymorphic characters, including the distinctive black dorsal coloration. The lack of diagnostic characters may be related to hybridization between this taxon and geographically adjacent populations of *S. j. minor*.

Summary of Concordance, Conflict, and Taxonomic Conclusions

Given that these three approaches are not fully congruent (Table 1), what are the species limits in *S. jarrovii*? *Sceloporus j. cyanostictus* and *S. j. sugillatus* are distinct species using all three approaches. The morphology- and mtDNA-based phylogenies agree that *S. j. jarrovii* is not closely related to other *S. jarrovii* populations; the only question is whether *S. lineolateralis* is inside or outside the clade of *S. j. jarrovii* populations. Furthermore, although *S. j. jarrovii* lacks seemingly fixed diagnostic morphological characters, some morphological characters do strongly differentiate these populations, even though these characters exhibit some intraspecific variation in *S. j. jarrovii* and other populations.

There is conflict over the relationships of *S. j. oberon* populations, with the morphological tree strongly suggesting exclusivity and the mtDNA tree strongly suggesting nonexclusivity with respect to *S. j. minor.* The obvious explanation for this conflict seems to be lateral transfer of mitochondrial genes between *S. j. oberon* and adjacent *S. j. minor;* these two taxa share a contact zone where there are morphologically intermediate specimens and mixing of mtDNA haplotypes (Wiens et al., 1999). Thus, we consider *S. j. oberon* to be conspecific with northern *S. j. minor.*

The character-based approach to morphology provides weak evidence that *S. j. immucronatus* and *S. j. erythrocyaneus* together represent a single species that is distinct from other *S. jarrovii* populations based on the blue dorsal coloration. In contrast, the mtDNA data place these two subspecies among the southern populations of *S. j. minor* and suggest that the blue *immucronatus* morph has evolved independently in Tamaulipas and in a geographically distant region in Queretaro and Hidalgo. The morphology-based phylogeny can be interpreted as favoring either the distinctness of these populations (because they cluster together) or their conspecificity with *S. j. minor* (because the clade is weakly supported and interspersed among geographically disparate populations of *S. j. minor*). Because of its stronger statistical support, greater concordance with the geographic proximity of populations, and possible support from the morphology-based phylogeny, we favor the interpretation that *S. j. erythrocyaneus, S. j. immucronatus,* and southern *S. j. minor* are conspecific. The morphology-based phylogeny also suggests that *S. j. minor* represents a single, nonexclusive species (including *S. j. immucronatus* and *S. j. erythrocyaneus*). In contrast, the mtDNA tree and NCA show strong support for division of *S. j. minor* into two clades: a northern one (including *S. j. oberon*) and a southern one (including *S. j. immucronatus* and *S. j. erythrocyaneus*). This division is

TABLE 1. Comparison of the results of three different approaches to species delimitation applied to mtDNA and morphological variation for populations of *Sceloporus jarrovii.* Listed is each taxon that is considered a distinct species by each approach as listed.

Tree-based DNA	Tree-based morphology	Character-based morphology
S. j. cyanostictus	*S. j. cyanostictus*	*S. j. cyanostictus*
S. j. sugillatus	*S. j. sugillatus*	*S. j. sugillatus*
S. j. jarrovii (+ *S. lineolateralis*?)	*S. j. jarrovii*	*S. j. jarrovii* + *S. j. minor* + *S. j. oberon*
S. j. oberon + northern *S. j. minor*	*S. j. oberon*	population 18 of *S. j.minor* (Concepción del Oro)
S. j. erythrocyaneus + *S. j. immucronatus* + southern *S. j. minor*	*S. j. erythrocyaneus* + *S. j. immucronatus* + all *S. j. minor*	*S. j. erythrocyaneus* + *S. j. immucronatus*

not supported by any diagnostic morphological characters, but some of the clades of the morphology tree are concordant—namely, a clade including most northern populations of *S. j. minor* plus *S. j. oberon,* and a clade containing most southern populations of *S. j. minor*. We hypothesize that *S. j. minor* does represent two species (as suggested by the mtDNA tree) and that these two species have not yet become mutually exclusive in the morphology-based, population-level phylogeny.

In summary, based on the data and arguments presented above, we support division of *S. jarrovii* into five species: *S. cyanostictus* (for *S. j. cyanostictus*), *S. jarrovii* (for *S. j. jarrovii,* which may also include *S. lineolateralis*), *S. minor* (for *S. j. erythrocyaneus, S. j. immucronatus,* and southern populations of *S. j. minor*), *S. oberon* (for *S. j. oberon* and northern populations of *S. j. minor*), and *S. sugillatus* (for *S. j. sugillatus*). These are the same species recognized by Wiens et al. (1999). For each species, the oldest available name was chosen, and because of the absence of sympatry among these species and the collection of material from at or close to the type localities, there is no question that the type specimen pertains to the appropriate species (type specimens examined by Wiens for *S. cyanostictus, S. minor, S. oberon,* and *S. sugillatus*).

Discussion

In this paper, we outline explicit protocols for species delimitation using DNA and morphological data and provide the first critical comparison of species delimitation based on mtDNA haplotype phylogenies with tree-based and character-based species delimitation based on morphology. We found the results to be surprisingly discordant (Table 1). Although all three approaches support recognition of five species, only two of these species are the same between the three. This discordance is important, because it suggests that at least two of these approaches are giving a misleading picture of species boundaries in this group.

Our study differs from many previous analyses in showing real discordance between species limits suggested by DNA and morphology, and not merely disagreement with traditional taxonomy. Previous studies have shown incongruence between the species limits suggested by mtDNA and morphology-based taxonomy (e.g., Zamudio et al., 1997; Steppan, 1998; Serb et al., 2001). Specifically, those authors found mtDNA evidence suggesting that one or more subspecies should be recognized as distinct species. However, on closer inspection, these distinct subspecies were found to be diagnosed by one or more morphological characters (using data from the literature), thus demonstrating concordance between the mtDNA phylogenies and the character-based approach to morphology. One previous study (Hollingsworth, 1998) analyzed morphological variation using both tree-based and character-based approaches for a group (the lizard genus *Sauromalus*) in which there was also an mtDNA haplotype phylogeny (Petren and Case, 1997), although there was no explicit comparison of the three approaches. Despite some incongruence over interspecific relationships, the three approaches can be interpreted as being highly concordant in terms of the species limits they suggest (our interpretation of species limits in *Sauromalus* matches that of Hollingsworth, 1998). The results of these previous studies contrast with those of our study of *Sceloporus jarrovii,* in which there is real conflict between species limits inferred by the three approaches.

An obvious explanation for discordance between morphological and mtDNA species limits is failure of the mtDNA gene tree to match the species tree. This may explain one case of small-scale incongruence in our study—namely, the strongly supported conflict between DNA and morphological data over the exclusivity of *S. j. oberon*. However, two mtDNA clades that have little support from morphology (northern and southern *S. j. minor* and their relatives) are each highly concordant with geography, whereas the species boundaries implied for these populations by the two morphological approaches are not concordant with each other, and those implied by the character-based approach show some discordance with the geographical proximity of the populations. We would not expect lateral transfer of genes between taxa or stochastic retention of ancestral polymorphisms to generate large-scale patterns that are concordant with geography. Thus, mismatch between gene and species trees seems to explain relatively little of the discordance in our study. Recent studies have also shown little evidence to suggest that this phenomenon is generally

problematic in species delimitation with mtDNA data (e.g., Zamudio et al., 1997; Steppan, 1998; Parkinson et al., 2000; Serb et al., 2001).

We propose that much of the discordance involving *S. jarrovii* is caused by a particular pattern of morphological variation in the group, in which between-species differentiation is small relative to within-species variation in some taxa. Interspecific clades are generally weakly supported by morphology in the *torquatus* group (Fig. 7). Bootstrap values for interspecific clades are generally low, as was found in a previous, more comprehensive analysis of *Sceloporus* phylogeny (Wiens and Reeder, 1997). The number of parsimony-informative characters within the group is also relatively small, and most of these characters show extensive variation within populations and within putative species. Some of the species also show striking within-species divergence. For example, two mtDNA clades were recognized as species that lacked diagnostic morphological characters and were not supported as clades by morphological data. These two putative species (*S. minor* and *S. oberon*) show remarkable within-species divergence in dorsal coloration, possibly driven by sexual selection or an interaction between sexual selection and habitat features (Wiens et al., 1999). Thus, instead of showing strong differentiation in morphology between species and weak differentiation within species, some species in the *torquatus* group show striking differentiation within species and limited differentiation between species, a "worst-case scenario" for morphology-based species delimitation. This hypothesis is also supported by qualitative inspection of the branch lengths in the morphology-based phylogeny (Fig. 7), which suggests that intraspecific branch lengths are similar to interspecific branch lengths (although this obviously depends on which branches are considered to be intraspecific versus interspecific). Whether the specific pattern seen in *S. jarrovii* is common remains unclear, but we suggest there may be many cases in which species have split too rapidly to allow time for many diagnostic morphological differences to evolve. Haplotype phylogenies from mtDNA may be particularly useful in these cases because of the relatively fast rate at which species become differentiated.

An alternative view of the discordance between the results from morphology and mtDNA is that morphological species delimitation in the group has been biased or compromised by small sample sizes (for some populations) or problematic phylogenetic methods. Although small sample sizes can reduce the accuracy of phylogenetic analysis when intraspecific variation is extensive (e.g., Wiens, 1998a; Wiens and Servedio, 1997, 1998), small sample sizes should increase the probability of finding diagnostic characters that appear to be fixed. Yet, only two of the five species that we consider distinct have any "fixed" diagnostic characters. The method that we used for coding polymorphic data (frequency-based coding) has been found to perform well relative to other methods (Wiens, 1999). Methods for coding quantitative data have not been tested thoroughly. Both methods use fine-grained continuous data and should extract the maximum information possible from the morphological data. Furthermore, although the morphological phylogeny was generally weakly supported and somewhat discordant with the mtDNA tree, the topology was far from random. The morphology-based phylogeny supported the exclusivity of six of the seven subspecies of *S. jarrovii,* whereas the mtDNA tree supported the exclusivity of only two. We suggest that our analysis accurately summarized morphological variation in the group, but that morphological and molecular evolution have not been concordant.

We also found surprising discordance between the tree-based and character-based approaches for delimiting species using morphology, with only two of five species agreed on by both approaches (Table 1). As far as we know, this is the first time such incongruence has been shown for morphological data (but see Brower [1999] for an example with molecular data). This discordance may also be related to the problematic nature of morphological variation within the *torquatus* group. The character-based approach to morphological data has been widely used to delimit and describe species for the past 200 years or more, whereas the tree-based approach has been argued for on theoretical grounds (Baum and Donoghue, 1995) but has rarely been applied to morphology. Both approaches are somewhat problematic. The character-based approach is questionable

without some statistical test (otherwise, it is possible to describe a species based on a single individual with a single diagnostic character). The only such test currently available (Wiens and Servedio, 2000) requires setting a frequency cutoff and requires large sample sizes to achieve statistical significance. A general problem of the character-based approach is that it ignores all but a few of the characters that differ between species, using only those that show no within-species variation. On the other hand, the tree-based approach can potentially use all characters, if methods that incorporate polymorphic and quantitative characters are employed. In our study, the tree-based approach appears to give results that are marginally more congruent with the mtDNA results than does the character-based approach (Table 1). However, the theory behind using population-level trees from morphology to infer species limits remains poorly explored. Despite the long history of morphology-based taxonomy, the best way to delimit species using morphological data remains an open and largely unexplored question, and one that critically impacts the issue of congruence between species limits from morphology and DNA data.

Appendices for this paper are available at the website of the journal (www.systbiol.org).

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