Evolution, 60(5), 2006, pp. 1050–1065

DELIMITING SPECIES: COMPARING METHODS FOR MENDELIAN CHARACTERS USING LIZARDS OF THE *SCELOPORUS GRAMMICUS* (SQUAMATA: PHRYNOSOMATIDAE) COMPLEX

JONATHON C. MARSHALL,[1,2,3] ELISABETH ARÉVALO,[4] EDGAR BENAVIDES,[5] JOANNE L. SITES,[6] AND JACK W. SITES, JR.[5]

[1]*Department of Ecology and Evolutionary Biology, P.O. Box 208105, Yale University, New Haven, Connecticut 06520*
[2]*Department of Biology, Southern Utah University, Science Center 105, Cedar City, Utah 84720*
[3]*E-mail: jonathon_c_marshall@hotmail.com*
[4]*Biology Department, 549 River Avenue, Providence College, Providence, Rhode Island 02918*
[5]*Department of Integrative Biology and M. L. Bean Museum, 401 Widtsoe Building, Brigham Young University, Provo, Utah 84602*
[6]*229 West, 255 South, Orem, Utah 84058*

Abstract.—Species form the fundamental units of analysis in many areas of biology and, therefore, rigorous delimitation of this unit is important to a broad array of researchers. Recently, many new empirical methods have been proposed to delimit species in nature, and a large literature exists on the theoretical merit and superiority of each method. However, few empirical studies actually compare the results of these methods applied in the same study system. We used a large allozyme and chromosome dataset to apply a number of genetic-distance, character-based, and tree-based methods to a well-studied, data-rich system: the *Sceloporus grammicus* lizard complex of central Mexico. We hypothesized species boundaries under a general lineage or evolutionary species conceptual framework in an a priori fashion using mapped restriction-site data (mitochondrial DNA and nuclear rDNA), allozymes, and morphology. We then compared the ability of different methods to recover the ''hypothesized evolutionary species'' (HES). Highton's genetic-distance method and a tree-based method consistently recovered all four HES, although sometimes with weak support. With two exceptions, other methods recovered the same HES, but additional groups were weakly delimited and nested within the HES. Given the apparent recent divergence of some of the chromosome races and distinct populations in this complex, these are encouraging results. We emphasize the value of specifying testable criteria as clearly as possible and testing these with methods that make use of different properties of a single dataset.

Key words.—Allozymes, chromosomes, field for recombination, Highton's genetic distance method, population aggregation analysis, species delimitation.

Received September 27, 2005. Accepted February 23, 2006.

Species are routinely used as fundamental units of analysis in biogeography, ecology, macroevolution, and conservation biology (Blackburn and Gaston 1998; Barraclough and Nee 2001; Agapow et al. 2004), and a deep understanding of evolutionary processes, as well as biodiversity assessments, requires that systematists employ methods objectively and rigorously to delimit species in nature. Biologists endeavoring to delimit species in natural populations are often confronted with an intimidating number of alternative species concepts from which to choose. Mayden (1997) identified 22 distinct species concepts, and this number is incomplete (Pigliucci 2003). Such a large number of concepts suggests that there is no general agreement among biologists on what species are, but some have argued that, in fact, most biologists do agree on the ontological meaning of the species entity and have for roughly the last 50 years (Miller 2001). De Queiroz (1998, p. 60) noted that ''All modern species definitions either explicitly or implicitly equate species with segments of population level evolutionary lineages.'' This revised version of Simpson's evolutionary species concept (ESC) as ''a lineage (an ancestral-descendent sequence of populations) evolving separately from others and with its own evolutionary role and tendencies'' (Simpson 1961, p. 153) was labeled the general lineage concept (GLC) of species. De Queiroz (1998, p. 65) then suggested that most species concepts are merely different criteria or empirical approaches used to judge ''whether a particular entity qualifies as a member of the species category'' under the ESC/GLC ontological framework. The different criteria are reflective of the various common but contingent properties (sometimes associated with different evolutionary processes in play during speciation) that species often possess (de Queiroz 2005a). Common properties may include such things as interconnectedness of populations by gene flow (de Queiroz 2005b), unbranched pattern of descent from a common ancestral population, morphological similarity, shared adaptive zones or ecological niches, or shared mate-recognition systems, among others (Sites and Marshall 2003, 2004). This idea, the separation of an ontological definition of species from empirical methods of delimiting them, has been proposed in similar terms by other researchers. For instance, Mayden (1997) argued that the ESC represents a primary description of species in a theoretical sense and that all other concepts, as secondary concepts, should be considered operational tools for discovery of entities in accord with the primary ESC.

An emerging consensus among evolutionary biologists is that data gathered from various methodologies, based on different common properties, can be useful in delimiting distinct lineage segments (the ontological species). No single method or dataset will always give the ''right'' answer, but this should not be expected given the many contingencies associated with speciation (Frost and Kluge 1994). Hey et al. (2003, p. 600) insisted that investigators should not simply decide ''whether or where to draw lines of demarcation, but

rather to present the full picture that research has revealed, and to do so in its full complexity rather than to reduce that complexity artificially.'' The use of a single criterion to delimit species artificially reduces the complexity of evolving lineages (de Queiroz 2005b). Only when a more eclectic approach is taken, by the use of several criteria, can this complexity begin to be ordered and described (see examples in Wiens and Penkrot 2002; Dettmann et al. 2003a,b; Fukami et al. 2004; Johnson et al. 2004; Cardoso and Vogler 2005; Ross and Shoemaker 2005).

Operationally, the obvious question then becomes: when confronted with the practicality of limited resources, which combination of methods and data is generally most useful and reliable in delimiting lineages consistent with the ESC/GLC framework? In this study, we use different species-delimitation methods to gauge the relative independence of proposed evolutionary lineages within a well-studied polytypic lizard complex in central Mexico. We test the idea that several groups of chromosome races (see below for description) represent entities consistent with the ESC/GLC of species, and as such should be diagnosable by multiple species-delimitation methods based on distinct common properties of species.

The Sceloporus grammicus Complex

Lizards of the *Sceloporus grammicus* complex are found throughout much of mainland Mexico and comprise at least eight distinct chromosome races in which diploid numbers range from $2n = 31/32$ (male/female) to $2n = 45/46$ (Hall 1973; Sites 1983; Porter and Sites 1986; Arévalo et al. 1991). The differences between the sexes are due to a $X_1X_2Y/X_1X_1X_2X_2$ (male/female) sex chromosome heteromorphism (Cole et al. 1967), but for simplicity we use female $2n$ numbers in this paper. Seven of these races were originally described by Hall (1973, 1980) from the morphology of the six pairs of macroautosomes (hereafter called ''macrochromosomes''; these are numbered 1–6 in order of decreasing size; Fig. 1). The inferred ancestral state for all six macrochromosomes is the meta- or submetacentric morphology (Hall 1980; arguments reviewed by Sites et al. 1992), and Hall (1973) referred to the presumed ancestral race ($2n = 32$) as the ''standard'' (S) race. All other races are named on the basis of the macrochromosomal rearrangement(s) that diagnose each (usually centric fissions). Hall (1973) identified a P1 race diagnosed by a fission polymorphism for pair 1, an F5 race diagnosed by a fixed fission for pair 5 ($2n = 34$), an F6 race fixed for a pair 6 fission ($2n = 34$), an F5+6 race fixed for fissions at both of these pairs ($2n = 36$), and two multiple fission (FM) races that were polymorphic or fixed for fissions at most macrochromosome pairs (FM1 and FM2, Fig. 1). Hall's (1973, 1980) original surveys identified six of these races (all but F5) in a small region of central Mexico, and subsequent work by the Sites group has discovered the F5 race and a new multiple-fission race (FM3) in this same small region (Porter and Sites 1986). Figure 1 illustrates the basic chromosome morphology of these eight races.

The *S. grammicus* complex has provided a model group for studies focused on interrelated issues of chromosome evolution, hybrid-zone dynamics, and speciation potential (White 1978; Hall 1980, 1983; King 1993). In most habitats where they live these lizards are abundant and easy to collect, facilitating the widespread geographic sampling necessary to map distributions of chromosome races (Sites 1983; Porter and Sites 1986; Arévalo et al. 1991). Detailed distributional studies have provided basic information on some zones of parapatric hybridization between different combinations of these races (Hall and Selander 1973; Arévalo et al. 1993; Sites et al. 1993), and one of these (Tulancingo transect) has been studied extensively (summarized in Marshall and Sites 2001).

This group represents a formidable taxonomic challenge due to extensive chromosomal and morphological variation (Smith 1939) as well as the uncertain application of earlier names (Günther 1885–1902; Smith and Taylor 1950). Smith and Laufe (1945) described three subspecies, *S. grammicus disparilis*, *S. grammicus microlepidotus*, and *S. grammicus grammicus*, based chiefly on dorsal scale counts, but the boundaries of some of these subspecies are not concordant with the distribution of chromosome races in at least some parts of the range (Sites 1983). Lara-Góngora (1983) described two new species, *S. anahuacus* and *S. palaciosi*, from small regions in central Mexico, based on a combination of morphological characters. *Sceloporus anahuacus* was described from relatively open, high-elevation pine forests (generally 3000 m and higher; Lara-Góngora 1983) on mountain peaks surrounding the Valley of Mexico. This distribution matches the known range of Hall's (1973) P1 race ($2n = 32$; here the high standard [HS] race; Fig. 1). *Sceloporus palaciosi* was described from fir forests below 3000 m on the same and adjacent mountain ranges, a distribution coincident with the some populations of the F6 (fission 6) race ($2n = 34$). To avoid confusion, we call *S. palaciosi* the F6 race and *S. anahuacus* the HS race.

Independent evidence also supports species recognition of HS and F6 races on the basis of allozyme data (Sites et al. 1988). Sites et al. (1988) scored 145 LS, 25 F6, and 76 HS lizards from across central Mexico for 38 allozyme loci and found significant genetic divergence of the F6 lizards and lower but consistent divergence of the HS lizards from the LS lizards. Sites and Davis (1989) used allozymes and rDNA plus mitochondrial DNA (mtDNA) mapped restriction-site data to estimate phylogenetic relationships among 72 samples from all but the F5 chromosome races described above. We reanalyzed a dataset of combined markers from Sites and Davis (1989; Tables 2, 3; Appendix available online only at http://dx.doi.org/10.1554/05-545.1.s1) under maximum parsimony with PAUP* (Swofford 1999) and estimated nodal support by bootstrapping (Fig. 2), which was not implemented by Sites and Davis (1989). We used these results coupled with the morphological data described by Lara-Góngora (1983) and the allozyme data from Sites et al. (1988) to hypothesize four a priori groups consistent with the ESC/GLC ontological framework of species (Fig. 2) and labeled them hypothesized evolutionary species (HES). In this study, we used these a priori HES groups to compare the results of five methods of species delimitation based on a more complete sampling of allozyme and chromosome data.

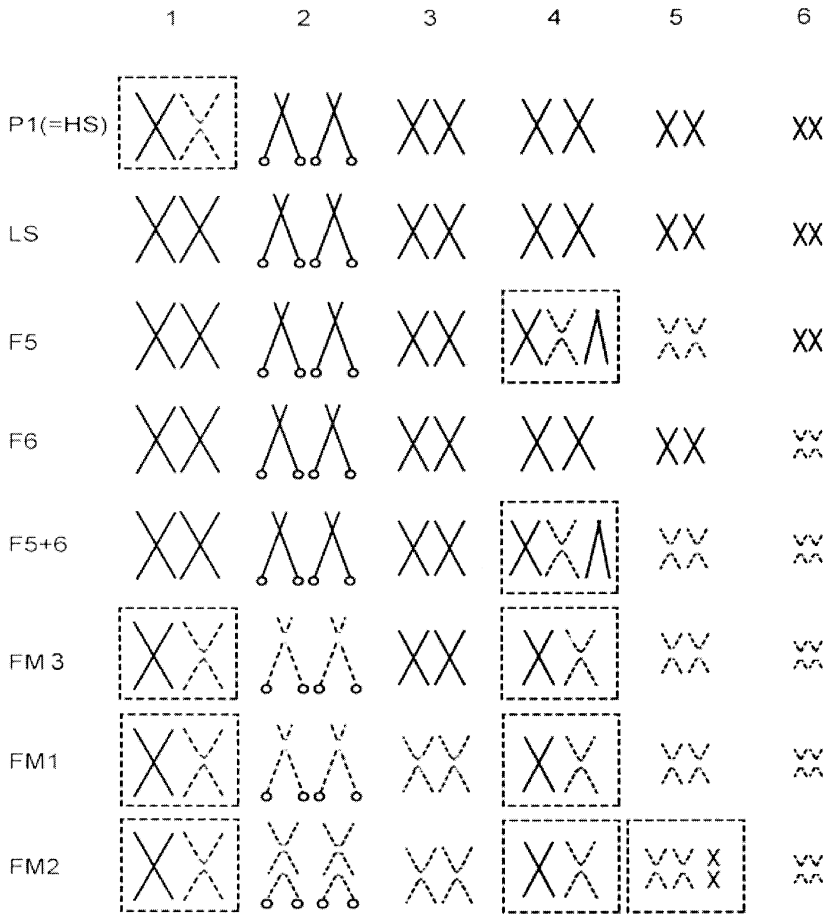

FIG. 1. Diagrammatic representation of eight *Sceloporus grammicus* chromosome races known from the study areas, with macrochromosomes identified by number and arranged in order of decreasing size (pairs 1–6). Solid chromatids denote metacentric (pairs 1, 3–6) and submetacentric (pair 2) morphologies, and dashed-line chromatids represent fission products. Dotted rectangles surrounding some chromosomes identify pairs segregating for polymorphisms (fission and pericentric inversion) in some populations (absence of rectangles denotes presumed fixation for the morphologies illustrated), and open circles on the long arm of pair 2 represent the single pair of nucleolar organizing regions (NORs). Note that pairs 3 and 4 are identical in size and morphology; the designation of some polymorphisms in pair 4 is a matter of convention (Sites 1983). Biarmed and fissioned chromosomes are scored as A and B alleles, occasional pericentric inversions are scored as C, and the unique pair 2 morphology in the FM2 race is scored as D. Novel rearrangements in chromosome 5 (the small metacentrics) are interpreted as pericentric inversions of the acrocentric fission products; one of these metacentrics is scored as allele E, and two of these are registered as F. Race designations are self-evident (F, fission; F5, fission pair 5; F5+6, fission pairs 5 and 6; FM, multiple fission) except that LS is the low-elevation form of Hall's (1973) ''standard'' (S) race, and HS identifies the high-elevation standard race (Hall's [1973] ''polymorphic one'' [P1] race).

MATERIALS AND METHODS

Data Collection

Samples for this study were selected from a subset of the 93 localities in central Mexico originally mapped by Arévalo et al. (1991), based on lizards collected from 1983 to 1991. The subset of samples selected for this study was chosen by two criteria: (1) to represent multiple localities (4–14 per race) of each of the eight recognized chromosome races, and (2) to represent locations removed from all known and suspected zones of parapatric hybridization (seven known and four possible hybrid zones between various combinations of chromosome races were mapped by Arévalo et al. 1991, fig. 5). There are explicit methods for delimiting species across hybrid zones (i.e., the hybrid zone barrier method described by Porter 1990), and although we have studied the structure of some of these zones (summarized in Marshall and Sites 2001), we do not have the sampling design necessary to implement Porter's test.

A total of 662 lizards for which both chromosome and allozyme genotypes are available, representing 55 localities, is included in this study. These are summarized by locality and chromosome race in Table 1, and sampling localities are plotted in Figures 3 and 4. One locality, Sierra del Tigre (Jalisco, Mexico) contains F6 individuals as well as *S. heterolepsis* (a morphologically distinct member of the *S. grammicus* group as defined by Smith 1939); these are separated by altitude. GPS coordinates for all populations were gathered either by taking coordinates on site with a hand-held GPS unit (Garmin Inc., Olathe, KS; eMap model, www.garmin.com), by locating sites on an electronic map with mapping software MapSource (Garmin), or taken from plotted locality data compiled by Falling Rain Genomics, Inc. (http://www.fallingrain.com/world). GPS coordinates were

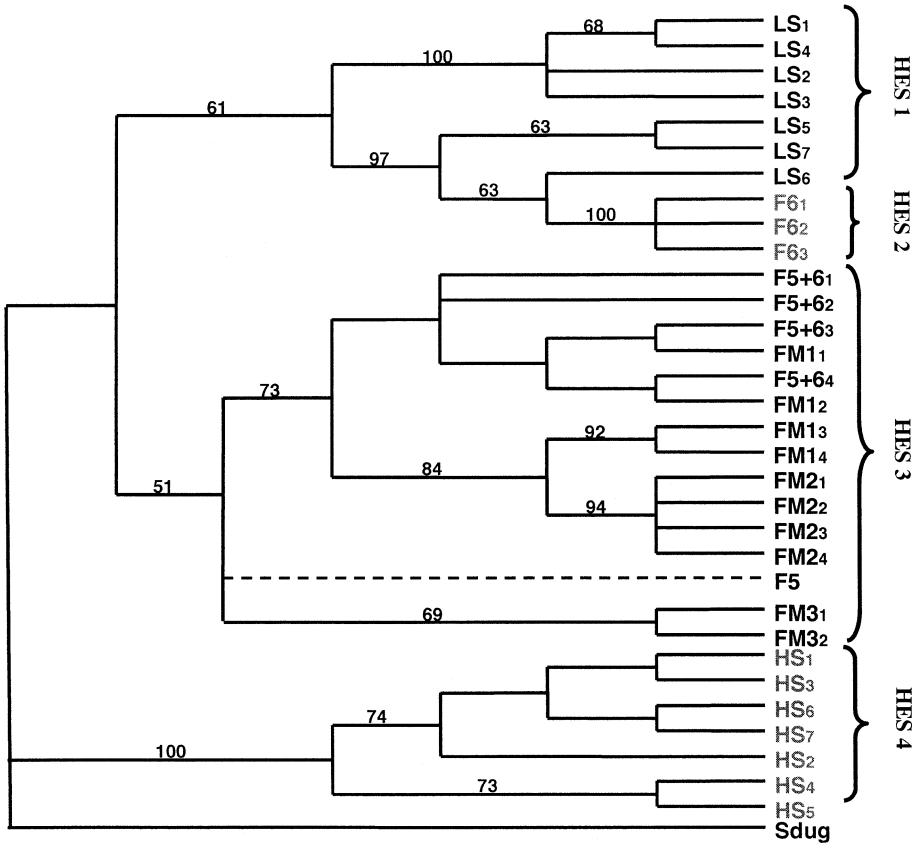

FIG. 2. Genealogical lineage concept (HES) taxa inferred from a 50% majority tree based on 900 unweighted maximum parsimony trees (tree length = 177) obtained from a combined reanalysis of the nuclear and mitochondrial markers presented by Sites and Davis (1989). Dashed line represents placement of F5 race as indicated by a later study (Arévalo et al. 1994) of a mtDNA sequences–based phylogenetic study of all central Mexico races (albeit with single exemplars of most races), and this race was recovered within the FM clade (F5+6, FM1, FM2, and FM3 races) with strong support. We therefore included the F5 race here in an unresolved position within the HES3 clade because the F5 race had not been identified when data were collected for Sites and Davis (1989). The separation of the F6 race (HES1) from the LS race (HES2) is not evident in our reanalysis but is based on the morphological distinctions reported by Lara-Góngora (1983) and allozyme data from Sites et al. (1988). Terminals are labeled by chromosome race, with subscripts denoting separate localities for each sample, bootstrap values (1000 replicates) are presented above the line, and Sdug is the outgroup *Sceloporus dugesii*.

recorded as degrees latitude or longitude to four decimal points for all localities (Table 1).

Karyotypes were scored as diploid genotypes following Arévalo et al. (1991), but with one modification. For each macrochromosomal pair, nonfissioned and fissioned chromosomes was scored as alleles A and B, respectively (homozygotes and heterozygotes were AA, BB, and AB), and inversions as illustrated by Arévalo et al. (1991, fig. 2). Our modification here is to score the ''double fission'' rearrangement of submetacentric pair 2 in the FM2 race (see Reed et al. 1992) as a single allele, D (this arrangement was unknown by Porter and Sites [1986] and Arévalo et al. [1991], and was originally scored as an extra microchromosome, following Hall 1973). This coding simplifies the complexity of the pair 2 rearrangement, because recombinant morphologies for this chromosome are known from the Tulancingo hybrid zone (Reed et al. 1995), but these and all known hybrid zone localities are excluded from this study.

A total of 38 allozymes was resolved by starch-gel electrophoresis from tissues cryopreserved for molecular studies, as described by Sites et al. (1988); these characters represent the subset of polymorphic characters identified from a total of 38 markers resolved in the original study. Table 2 summarizes the 18 polymorphic allozyme characters resolved for all population samples included in this study; polymorphic allozyme and chromosome genotypes are summarized for all lizards by locality (see Appendix available online only).

Data Analyses

We implemented four different empirical methods to delimit species, using various combinations of the allozyme and chromosome datasets, including genetic-distance, character-based, and tree-based approaches. Although some of these methods were developed specifically for allozyme data and might be considered largely irrelevant in modern laboratories devoted to screening high-resolution molecular markers (AFLPs, SNPs, microsatellites, and nuclear gene sequences), we make two observations that suggest that the data and methodological comparisons evaluated in this study are broadly relevant. First, allozyme data are still widely used to delimit species or to estimate population structure when

TABLE 1. Localities, sample sizes (*n*), and chromosome races (as defined in Fig. 1; the S for both outgroup taxa denotes a $2n = 32$ karyotype identical to LS) for the lizards of the *Sceloporus grammicus* complex used in this study. Locality numbers correspond to those plotted in Figures 2 and 3. Mexican states are given in parentheses (abbreviated as in Fig. 3). GPS coordinates are given in degrees north and west. Superscripts 1 and 2 identify populations named as *S. anahuacus* and as *S. palaciosi* (Lara-Góngora 1983) respectively, all other ingroup samples belong to *S. g. microlepidotus*. Museum voucher numbers are given for samples in the last column; however, voucher specimens were not available in some instances. BYU, Brigham Young University; MZFC, Museo de Zoologia-Facultad de Ciencias, Universidad Nacional Autonoma de Mexico (UNAM); IBH, Instituto de Biologia, UNAM; EDHEM, Ecologia de la Herpetofauna del Estado de Mexico, UNAM (some numbers for Mexican collections reference series of specimens from some localities); MCZ, Museum of Comparative Zoology, Harvard University.

	Localities	GPS coordinates (N, W)	*n*	Race	Museum voucher numbers
1	Texcoco (MEX)	19.5633, 98.7917	8	LS	EDHEM 1377–84
2	Xochimilco (DF)	19.2500, 99.0667	15	LS	BYU 38445, 38447–48, EDHEM 1461
3	Sn. M. Ajusto (DF)	19.2330, 99.1833	15	LS	BYU 38486–92, MZFC 1939
4	Lindavista (DF)	19.4833, 99.1167	37	LS	BYU 38407–15, 38417–18, EDHEM 0638–39, 0641–47
5	Tepotzotlan (MEX)	19.7248, 99.2217	10	LS	EDHEM 1536–1545
6	Cuautlalpan (MEX)	19.8042, 99.0021	9	LS	EDHEM 1920–28
7	Presa Iturbide (MEX)	19.5204, 99.4716	25	HS	BYU 38424–27, 38429, 38431–51
8	Monte Alegre[1] (DF)	19.2204, 99.2659	19	HS	BYU 38480–85, MZFC 1938A-J, 1946
9	Popo-Ixta[1] (MEX)	19.0667, 98.6333	19	HS	BYU 38455–73
10	San Francisco (MEX)	19.6754, 98.9945	6	LS	EDHEM 1942–47
11	Tlalnepantla (MEX)	19.5500, 99.2000	12	LS	BYU 38398–404, EDHEM 0627–31
12	Pachuca (HGO)	20.1105, 98.7517	11	LS	BYU 38573–74, 38597–98, MZFC H945A-C, 1984A,B, 4240A,B
13	El Chico (HGO)	20.1950, 98.6900	5	HS	BYU38575, 38578, 38607–08
14	Vol. Malinche (TLAX)	19.3106, 98.0394	19	LS	IBH 6874
16	Cahuacan (MEX)	19.6310, 99.3988	15	F6	EDHEM 1423–28, 1430–38
17	Nev. de Toluca[2] (MEX)	19.1618, 99.8088	15	F6	BYU37460, 37469–81
18	Acambay (MEX)	19.9966, 99.8878	6	F6	BYU 38555, MZFC H942A-E
19	El Capulin[2] (MEX)	19.0500, 99.8333	16	F6	BYU 38493–38501; MZFC 1941A-G
20	Cerro Burro (MICH)	19.3621, 101.5254	15	F6	MCZ 127289–90
21	Nev. de Colima (JAL)	19.5508, 103.6277	15	F6	BYU 39759, 39765–39772; IBH 07171
22	Sierra del Tigre (JAL)	19.9167, 103.0333	12	F6	BYU 39773–39778; IBH 07174
23	Sn. Lorenzo (HGO)	19.9827, 98.2962	9	F5	MZFC 1992A-H
24	Presa Tejocotal (HGO)	20.1406, 98.1451	2	F5	EDHEM 1847
25	Zoquizoquipan (HGO)	20.6500, 98.7167	16	F5	BYU 38566–70, 38572
26	Toto. El Grande (HGO)	20.1798, 98.4419	7	F5	EDHEM 1657–63
27	Zacualtipan (HGO)	20.6443, 98.6373	13	F5	EDHEM 1833–44
28	Zimapan (HGO)	20.7416, 99.3891	2	F5+6	EDHEM 2001–2
29	Amealco (QRO)	20.1833, 100.1500	13	F5+6	BYU 38558–65, MZFC H943A-E
30	San Joaquin (QRO)	20.9159, 99.5588	23	F5+6	BYU 37433–35, 37441–48, 37345–53 MZFC H939A-C
31	Rio Verde (SLP)	22.4016, 101.3576	10	F5+6	—
32	E Huasca (HGO)	20.2031, 98.5917	4	FM3	EDHEM 2060–63
33	E. Omitlan (HGO)	20.1707, 98.6413	8	FM3	MZFC 4232A-H
34	N. Huasca (HGO)	20.2385, 98.5701	12	FM3	EDHEM 1814–25
35	Capula (HGO)	20.2395, 98.5873	11	FM3	EDHEM 1666–72
36	Atotonilco (HGO)	20.2415, 98.6498	15	FM3	EDHEM 1848–62
37	Chapa Mota (HGO)	19.8233, 99.5248	9	FM1	EDHEM 1462–70
38	Huichapan (HGO)	20.3833, 99.6500	10	FM1	BYU 38535–37, 38541–42, MZFC H940A-E
39	Amealco (HGO)	20.2333, 99.5500	13	FM1	BYU 38545–47, 38549, MZFC H941A-I
40	Villa Carbon (HGO)	19.71162, 99.4350	12	FM1	EDHEM 1445–57
41	2 Tep. Rio (HGO)	19.9247, 99.3642	12	FM2	EDHEM 1493–1504
42	Santa Matilde (HGO)	20.0544, 98.8029	5	FM2	BYU 37505–09
43	Sn. Agustin (HGO)	20.0027, 99.4727	4	FM2	EDHEM 1646–49
44	W. Pachuca (HGO)	20.1263, 98.8171	8	FM2	EDHEM 1756–63
45	P. Estancuela (HGO)	20.1697, 98.7502	14	FM2	BYU 38586, 38588–90, MZFC H948A-J
46	Ajacuba (HGO)	20.1025, 99.1219	18	FM2	BYU 38689–96, 38699–702, 38704–06
47	Tizayuca (HGO)	19.8575, 98.9649	16	FM2	BYU 38542, 38545, 38655–61, 38664–5
48	Int. 30/32 (HGO)	19.8618, 98.6209	17	FM2	BYU 38670–75, 38677–78, 38680–88
49	Es. CONASEP (HGO)	20.0808, 98.7433	15	FM2	BYU 37517–23, 37536, 38628–34
50	17 Tep. Rio (HGO)	19.9615, 99.4127	8	FM2	EDHEM 1625–32
51	N. Tepotzotlan (MEX)	19.8011, 99.2350	2	FM2	EDHEM 1552–53
52	Rd. To Tula (HGO)	19.9052, 99.2511	14	FM2	BYU 38504, 38724, MZFC H937A-L
53	Actopan (HGO)	20.2840, 98.9718	15	FM2	EDHEM 1726–40
54	S.L. Taxhimay (MEX)	19.8423, 99.3712	8	FM2	EDHEM 1478–85
Outgroups					
15	Igualatlaco (GRO) *S. g. grammicus*	17.4833, 99.6516	11	S	BYU 39787, 39740 IBH 07177, 07178
55	Sierra Tigre 2 (JAL) *S. heterolepsis*	19.9507, 103.0167	2	S	BYU 39783, 39784
	Total (N) = 662				

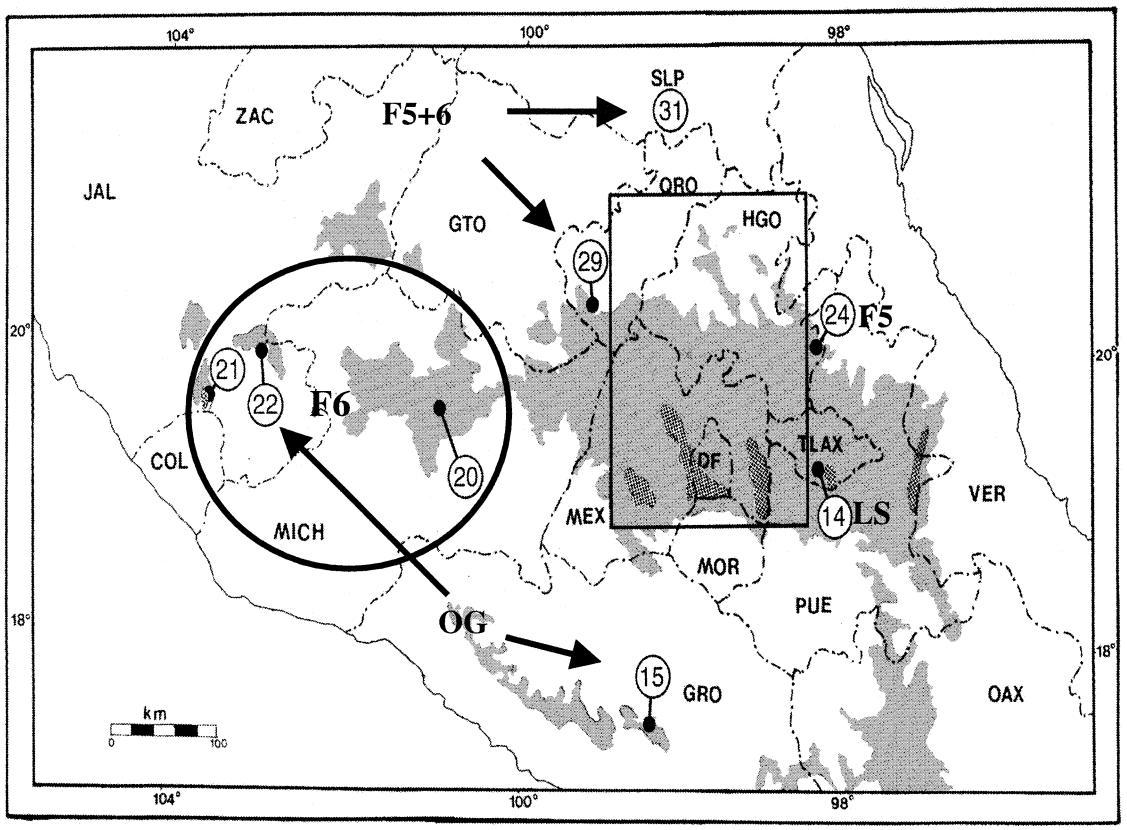

FIG. 3. Localities sampled for outlying populations of the *Sceloporus grammicus* complex (place names are given for each number in Table 1); the central region outlined by the rectangle is enlarged in Figure 4. OG indicates localities where outgroup individuals were sampled and used in appropriate analysis. Locality 22 consists of an F6 population and *S. heterolepis* (locality 55). Political units are the following states: Colima (COL), Distrito Federal (DF), Guanajuato (GTO), Guerrero (GRO), Hidalgo (HGO), Jalisco (JAL), Mexico (MEX), Michoacan (MICH), Morelos (MOR), Oaxaca (OAX), Puebla (PUE), Queretero (QRO), San Luis Potosi (SLP), Tlaxcala (TLAX), and Veracruz (VER), and Zacatecas (ZAC). Light gray and darker shading delimits land areas above 2000 m and 3000 m, respectively.

inexpensive nuclear markers must be screened for large samples (Highton and Peabody 2000; Mead et al. 2001; Jockusch and Wake 2002; Gabor and Nice 2004). Second, several of these methods have recently been extended to include other classes of markers (e.g., the population aggregation analysis of Davis and Nixon [1992] has been extended by Brower [1999] to include DNA haplotype data), but the original methods have yet to be empirically evaluated in detailed comparative studies. We therefore consider all methods worthy of comparative study, regardless of the original basis of their development, until they have been empirically shown to be of limited utility.

Distance method.—We used the genetic-distance method employed by Highton (2000) for multilocus allozyme data, and originally implemented in a group characterized by extremely slow rates of morphological evolution (e.g., salamanders of the family Plethodontidae). Highton (1989) suggested that groups of samples differing by a Nei (1978) genetic distance of 0.15 or higher should be hypothesized to be distinct species. Highton recognized that this value was arbitrary, but noted that most (97%) pairwise Nei identity values (Nei I) between well-defined species of vertebrates are <0.85, whereas most (98%) values within species are >0.85 (a Nei I of 0.85 is $\approx$ a genetic distance [D] of 0.16; Thorpe 1982). Highton (1989) also found this cutoff concordant with geographically cohesive units that clustered together on UPGMA (unweighted pair-group method with arithmetic mean) trees. These patterns are general enough across nonavian vertebrates to suggest that, as a rule of thumb, the divergence needed to complete speciation by attainment of reproductive isolation is correlated with a $D \approx 0.15$–0.16.

Operationally, the method is implemented by plotting a histogram of D-value frequencies for pairwise comparisons between populations (Highton 2000); the distribution should be approximately unimodal with values clumping below $D \approx 0.15$ under the hypothesis that all samples are drawn from conspecific populations interconnected by gene flow (Highton 2000). If the samples are drawn from different species, then the distribution of D-values is expected to be bimodal, with a second peak above $D \approx 0.15$. In this study, unbiased Nei (1978) genetic distances were calculated using the computer program POPGENE (Yeh et al. 2001).

Character-based methods.—We implemented two different character methods here, both of which are strictly nontopological. One was the multilocus field for recombination (mlFFR) approach described by Doyle (1995), which uses codominant nuclear characters to identify gene pools (distinct fields for recombination [FFRs], as described by Carson 1957) that are inferred to be coincident with the boundaries

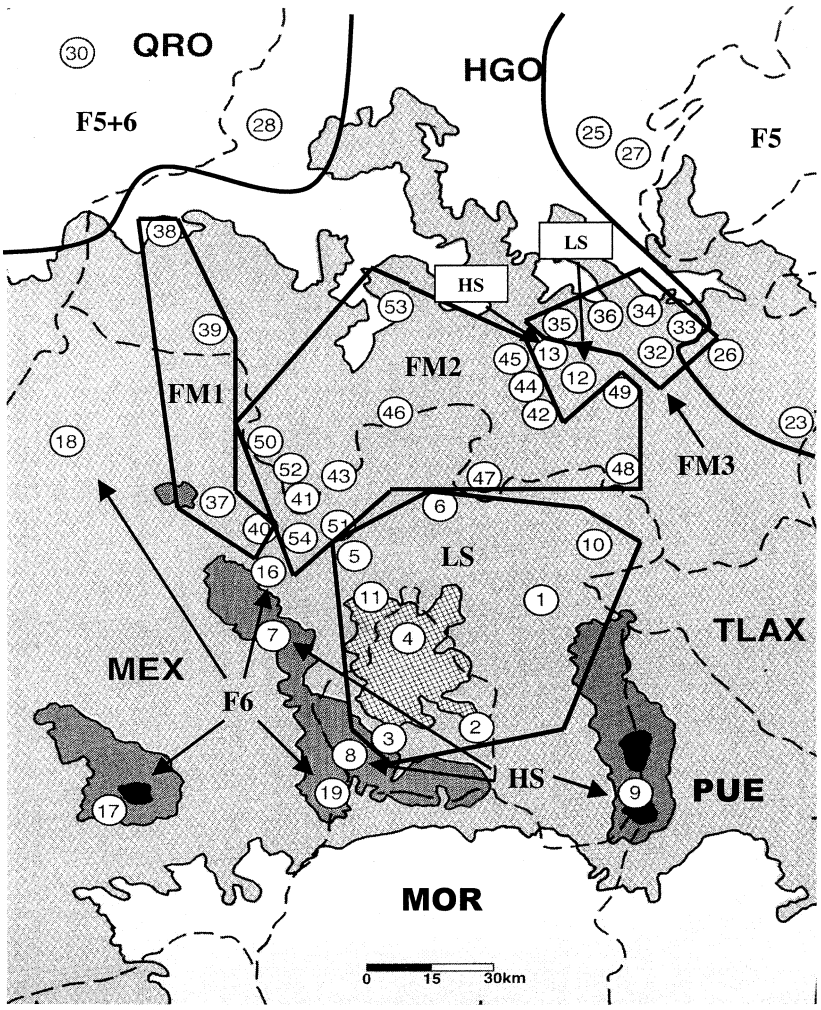

FIG. 4. Central Mexico distribution of localities sampled for chromosome races (as quantitatively defined by Arévalo et al. 1991) of the *Sceloporus grammicus* complex used in this study. Place names are given in Table 1 for each number, and the solid lines delimit the races on the basis of the grouping of samples in a UPGMA dendrogram derived from pairwise genetic distances (Arévalo et al. 1991, fig. 4). Light thatched area in the bottom center of the map represents Mexico City (DF in Fig. 3). Shading reveals elevation contours of 2000 m (gray), 3000 m (dark gray), and 4000 m (black ''island'' volcanic peaks). State names are abbreviated as in Figure 3. Localities 12 and 13 represent isolated populations of the LS and HS races, respectively.

of species in which constituent populations are interconnected by gene flow. The method identifies discontinuities between FFRs on the basis of nonoverlapping sets of heterozygous individuals (heterozygotes provide evidence for recombination within a single allele pool), using multiple, presumably unlinked characters to identify the FFRs (Doyle 1995). Here we used both allozyme and chromosome data as appropriate nuclear markers to delimit mlFFRs.

Second, we implemented the population aggregation analysis (PAA) as described by Davis and Nixon (1992); this is a formal codification of the traditional methodology for delimiting species based on one or more diagnostic character differences. The PAA requires that character states be summarized for all individuals in a sample to estimate a population profile for those states. Samples with identical population profiles, or ones that show no fixed characters differences, are then combined. This process is iterated until the only remaining sample aggregates are those separated from each other by at least one fixed character-state difference, and these aggregates are taken to be species.

One limitation of the PAA is the requirement that diagnostic character states must be ''fixed'' (present at 100% frequency) in a population, and documentation of fixation will normally be unattainable at accepted levels of statistical confidence with finite sample sizes (Wiens and Servedio 2000). We therefore extended the PAA analysis by using the test developed by Wiens and Servedio (2000), which identifies populations for which sample sizes are inadequate to identify diagnostic characters with confidence. In developing this methodology it was necessary for Wiens and Servedio (2000) to relax the requirement of fixation in the ''true'' population (where the character state for every individual is known) to some level of allowable polymorphism (generally 5–10%). In this study, all Wiens-Servedio tests were performed at a 10% polymorphism level. However, it is important to remember that even when the requirement of fixation

TABLE 2. Enzymes, loci examined (abbreviations follow Murphy et al. 1996), enzyme commission (EC) numbers (International Union of Biochemistry, 1984), and buffer conditions for protein electrophoretic assays used in this study. M and S prefixes indicate mitochondrial and supernatant loci, respectively, and superscripts 1 and 2 identify presumed locus duplications. Buffer 1, Tris-citrate pH 8.0 (run for 20 h, 35 mA); 2, Tris-borate-EDTA-I (10 h, 250 V); and 3, lithium-borate (20 h, 200 V).

	Enzyme	Locus	EC number	Buffer
1	Isocitrate dehydrogenase	Icdh-1	1.1.1.42	1
2	Isocitrate dehydrogenase	Icdh-2	1.1.1.42	1
3	Malic enzyme	Mdhp	1.1.1.40	1
4	Aconitate hydratase	M-Acon-A	4.2.1.3	1
5	Aconitate hydratase	S-Acon-A	4.2.1.3	1
6	Lactate dehydrogenase	Ldh-A	1.1.1.27	1
7	Lactate dehydrogenase	Ldh-B	1.1.1.27	1
8	α-Glucosidase	α-Glus-A	3.2.1.20	1
9	Phosphoglucomutase	Pgm-A	5.4.2.2	1
10	Esterase	Est-1	—	2
11	Esterase	Est-2	—	2
12	Purine-nucleoside phosphorylase	Pnp	2.4.2.1	3
13	Superoxide dismutase	S-Sod-A[1]	1.15.1.1	2
14	Superoxide dismutase	S-Sod-A[2]	1.15.1.1	2
15	Aspartate aminotransferase	M-Aat-A	2.6.1.1	3
16	Aspartate aminotransferase	S-Aat-A	2.6.1.1	3
17	Glycerol-3-phosphate dehydrogenase	G3pdh[1]	1.1.1.8	3
18	Glycerol-3-phosphate dehydrogenase	G3pdh[2]	1.1.1.8	3

in the ''true'' population is relaxed, fixation is still a requirement in the sampled population (for details see Wiens and Servedio 2002). We call this extension the ''statistical PAA'' (stPAA), treat it as a separate method, and again use the combined allozyme and chromosomal Mendelian characters as population attributes.

Tree-based methods.—We implemented one tree-based method based again on the combined allozyme and chromosome characters. Allozyme data are often used to infer phylogenetic relationships among conspecific populations and closely related species, but coding and analysis of these kinds of data have been controversial (Wiens 2000). Wiens (2000) used a congruence approach to compare the performance of 13 phylogenetic methods based on eight datasets across divergent animal taxa. His results showed that distance and likelihood methods generally outperformed parsimony approaches, and that neighbor joining (NJ) and UPGMA clustering of Nei's (1972) distances performed reasonably well with no other methods scoring significantly better. The size of our dataset (55 populations, 44 characters) and the fact that not all characters were scored for all individuals made it difficult to implement the continuous maximum-likelihood method, so here we estimate phylogenetic relationships from the matrix of Nei's unbiased (1978) *D*-values using the NJ method implemented in PAUP* (Swofford 1999), and a weighted step-matrix frequency-based parsimony method (Wiens 1999). Bootstrap values for NJ analysis were estimated in PHYLIP (Felsenstein 1993) by generating 1000 replicate allele-frequency datasets using the subroutine SEQBOOT for the NJ and using PAUP* for the weighted parsimony trees to evaluate levels of nodal support.

Trees in which terminals were concordant or discordant with geography and/or HES taxa were then used qualitatively to delimit species on the basis of criteria described by Wiens and Penkrot (2002). In the present study we have few nonfocal species (taxa closely related but distinct from the populations under investigation in the present study) because the sampling design for this study was implemented before the Wiens-Penkrot method was described. As a surrogate for nonfocal species we use reciprocal focal/nonfocal relationships between all focal entities (HES taxa) and the two outgroup taxa listed in Table 1. For instance, to test the species status of the HES3 (one of the focal species) we used the other three HES groups as nonfocal species.

RESULTS

Patterns of Variation

Individual genotypes ($n = 662$) across the 18 allozyme characters and six macrochromosomes resolved for all population samples from the 55 named localities (Table 1) included in this study are available (see Appendix available online only).

A Distance Method

Nei (1978) genetic **D** matrices (available from J. C. Marshall) calculated for the allozyme data set were used to plot unimodal and bimodal distributions of pairwise values between four groups: LS race (HES1), F6 race (HES2), FM2, FM1, FM3, F5, F5+6 races (HES3), and HS race (HES4), following Highton (2000). All within-group comparisons had approximately unimodal (although slightly skewed left) frequency distributions with most pairwise values clustering below a $D \approx 0.15$ (solid bars in each comparison in Fig. 5). However, separation between the groups, as inferred by a bimodal pattern, was evident in several between-group comparisons. For instance, comparisons between HES1/HES2, HES1/HES4, and HES2/HES4 all showed well-defined bimodal distributions, albeit below the arbitrary $D \approx 0.15$ cutoff, and the HES2/HES3 and HES3/HES4 comparisons showed moderate bimodality. The HES1/HES3 comparison also showed a right shift in the distribution of the between-group comparisons.

Character-Based Methods

We implemented Doyle's (1995) test for estimating the number of mlFFRs with both the combined and separated

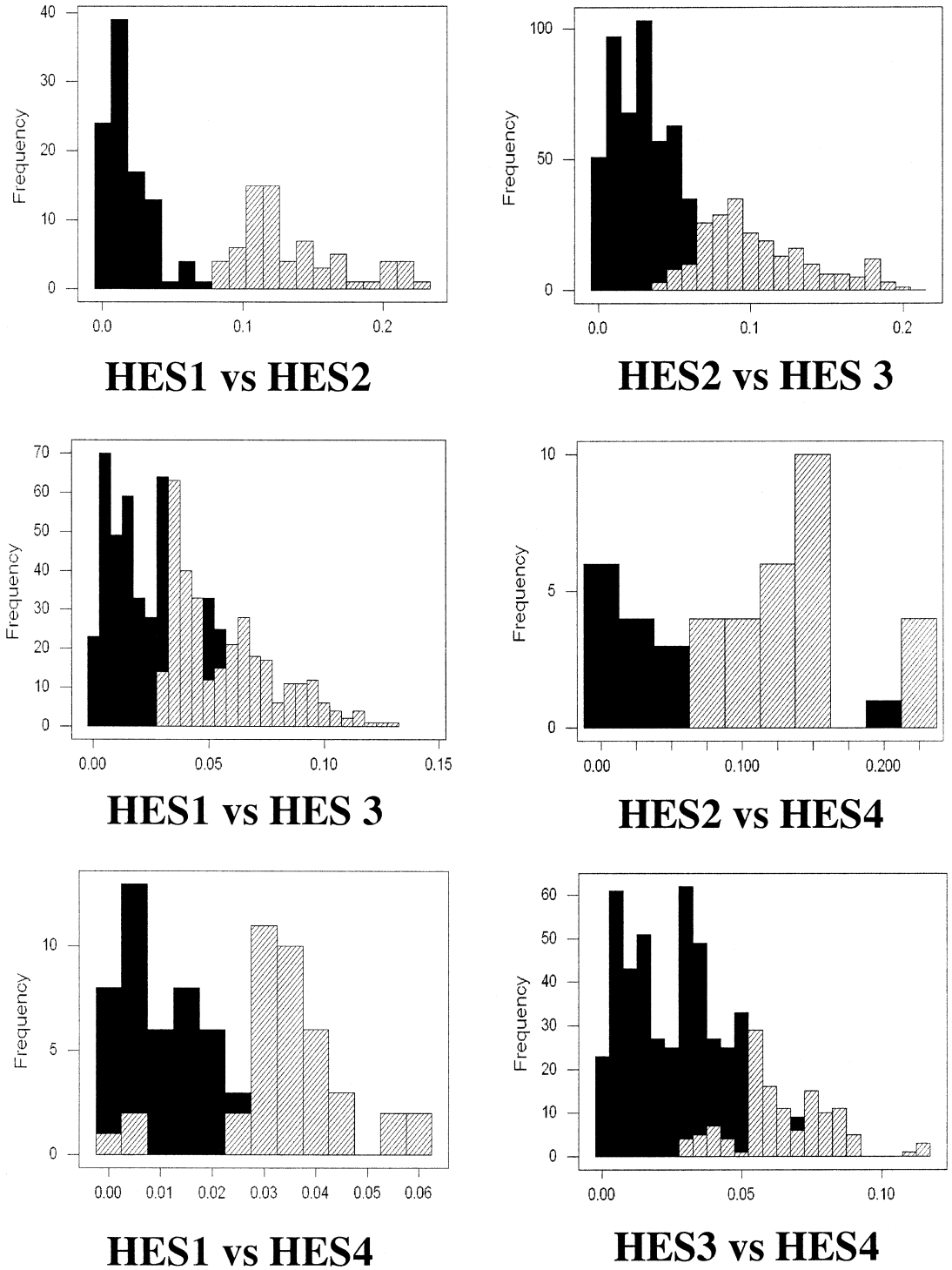

FIG. 5. Frequency histograms for all pairwise comparisons between HES taxa based on unbiased Nei's (1978) genetic distances. Genetic distances (*D*-values) are given on the x-axis of each comparison. Solid bars represent pairwise genetic distances for population pairs within each of the two groups (each HES taxon), and light shaded bars represent pairwise genetic distances for population pairs between HES taxa.

allozyme and chromosome datasets. Our results demonstrate the low resolution of this method and its poor ability to identify distinct lineages; only four allozyme characters, Icdh-1, Mdhp, Est-2, and G3pdh-A[1], delimited more than a single FFR for all of the individuals from this study (see Appendix available online only). Icdh-1 and Mdph both delimited population 17 (Nevado de Toluca, F6 race) as a separate FFR. Likewise, Est-2 delimited population 37 (Chapa del Mota, FM1 race) as a separate FFR, and G3pdh-A[1] delimited all LS and HS race populations together as a separate

single-locus FFR (the G3phd-A^1 locus actually represents the presence/absence of a gene duplication [Hall and Selander 1973; Sites and Murphy 1991], rather than a fixed difference of alternative electromorphs at a single locus). However, heterozygous individuals were found for a sufficient number of allelic combinations across all other allozyme characters (see Appendix available online only) that incorporating any one of the remaining 14 characters collapses all separate single-locus FFRs into a single mlFFR.

Chromosome 2 delimited three separate single-locus FFRs, one containing all samples from the FM2 race, another containing all FM1 and FM3 samples combined, and a third containing all other populations. Consideration of all chromosomes, however, collapses all samples into one mlFFR, and, across all nuclear markers, 19 of the 24 characters contained heterozygous individuals for enough allelic combinations to collapse all individuals into a single mlFFR. A strict interpretation of this result would support the recognition of only a single species.

Population aggregation analysis (Davis and Nixon 1992) was implemented for the combined allozyme and chromosome data, and iterative pairwise comparisons aggregated all samples into 11 distinct populations (Table 3). The PAA recovered one distinct aggregate consisting of the HS and LS races combined (aggregated population profile 1; APP1), another with the FM1 and FM3 races combined (APP9), distinct aggregates for the F5 (APP7), F5+6 (APP8), FM2 races (APP10), and the outgroups (APP11) and five distinct aggregates for the F6 race (APPs 2–6).

Table 4 summarizes the number of fixed character differences between all pairwise profile comparisons. Excluding the outgroup taxa (APP11), the greatest number of fixed character differences, six, separates APP3 (locality 17, F6 race) from APP9 (FM1, FM3 localities) and APP10 (FM2 localities). Notably, APP3 (Nevado de Toluca) contains the only fixed allozyme electromorphs unique to a single population (Icdh-1, Mdhp; see Appendix available online only). Table 3 shows that the F5, F5+6, and the FM2 races were recovered as distinct ''species'' (APPs) upon completion of iterated aggregation, whereas the F6 race was split into five distinct APPs, of which four are represented by single localities (16–19; APP 2–5). All of the single localities were collected from either isolated volcanic peaks (localities 16, 17, and 19) or a geographically peripheral area (locality 18) in central Mexico (Fig. 3). The fifth species (APP6) represents all F6 localities from the westernmost parts of the range (sites 20–22, Fig. 2). PAA collapses the following four chromosome races into two species; LS and HS (APP1), and FM1 and FM3 (APP9; Table 3).

Partitioning the characters between allozymes and chromosomes revealed which markers are diagnostic for these groups. For instance, performing PAA on only the chromosomes resulted in five APPs: one consisting of the F5 race; another of the LS, HS, F6 races and the outgroup samples; a third of the F5+6 race; a fourth of the FM2 race; and finally one consisting of the FM1 and FM3 races. Performing PAA on only the allozyme data united the races separated by the chromosome data and partially separated the groups that the chromosomes aggregated together. For instance, the allozymes separated the LS, HS, F6, OG chromosome group into seven separate APPs, one consisting of the LS and HS races, another of the two outgroup populations, and five separate APPs from various F6 populations. The allozymes also united the remaining chromosome APPs (F5, F5+6, FM1, FM3, and FM2) into a single APP corresponding to our HES4.

Statistical PAA (Wiens and Servedio 2000) is designed to be applied to one comparison in one direction, and the evidence for diagnostic differences between two samples must be evaluated in each of the samples separately in each direction. Therefore, to assess the confidence in our initial PAA ''species'' (APPs) decisions, we made pairwise comparisons between all 11 APPs (Table 4). It should also be noted that comparing diagnostic differences between multiple groups would generate wide numbers of fixed differences depending on which two APPs are being compared. For example, in this study when APP8 ($n = 48$) is reciprocally compared to APP1 ($n = 210$) and APP9 ($n = 94$), inadequate sampling was found for APP8 but not for APP1 and APP9 because of their large sample sizes. In contrast, when APP8 was reciprocally compared to APP3 ($n = 15$), sample size was adequate for APP8 but insufficient for APP3 even though four possible diagnostic markers were identified by the uncorrected PAA (Tables 3, 4). After making all comparisons and tabulating failure rates, we could then determine which APPs had not been sampled well enough based on some a priori statistical cutoff point.

Given these possibilities, we have approached statistical PAA by comparing multiple APPs and reporting the percentage of all pairwise comparisons that indicate more sampling is necessary for a given APP. For example, Table 4 indicates that APP8 (the F5+6 race) failed adequate sampling in 40% of its comparisons ($\alpha = 0.05$), which in every case occurred when only one diagnostic marker was possible in the comparison (when comparing APP8 to APPs 4, 6, 7 and 9). In this study we did not consider, as a candidate species, any APP that failed adequate sampling tests in more than 50% of the pairwise comparisons at an $\alpha = 0.05$ or 25% of the time at an $\alpha = 0.10$.

Table 4 summarizes the results of the Wiens-Servedio tests at two probability levels. Inadequate sampling was inferred for APPs 2, 3, 4, 5, and 11 at both probability levels. APPs 2–5 are all F6 populations and APP11 is the profile of the outgroup taxa. Adequate sampling was inferred in all pairwise comparisons for APP1 (LS, HS), APP9 (FM1, FM3), and APP10 (FM2), all of which had $n > 90$ individuals (Table 4). APP7 (F5) failed adequate sampling in one comparison at $\alpha = 0.05$, but had adequate sample sizes in all other comparisons. APPs 6 and 8 represent more intermediate cases; at $\alpha = 0.05$ they failed 40% of the comparisons, respectively, but passed adequate sample tests for all comparisons at $\alpha = 0.10$. Comparing APP7 to APP8 reveals that the total number of individuals in APP8 is greater than in APP7 (47 vs. 46), but APP8 comparisons failed much more frequently than APP7 comparisons, due to the fact that APP7 has on average 2.6 potentially diagnostic character differences, whereas APP8 has only 1.9.

A Tree-Based Method

All frequency-based parsimony analyses resulted in very low-resolution trees. The NJ tree in Figure 6 summarizes the

TABLE 3. Aggregation results of population aggregation analysis: combined population profiles result in separate phylogenetic species identified here as aggregated population profiles (APP). Locals are the localities in Table 1 and *N* is total individuals in aggregated populations. Allozyme characters (columns 1–18) correspond to numbered loci in Table 2, and columns C1–C6 represent the six macrochromosomes in the order presented in the Appendix (available online only). The uppercase letters represent alleles present for each APP at each locus; for example, AA represents a locus segregating only for A alleles in the identified APP, whereas ABCD represent a locus segregating for alleles A, B, C, and D in the APP indicated.

	Locals	Races	*N*	Aggregated population profiles								
				1				5	6			
APP1	1–14	HS, LS	210	AB	BB	ABD	AB	ABC	AA	ABC	AB	ABCD
APP2	16	F6	15	AA	BB	AA	AA	AA	AA	AB	AA	AA
APP3	17	F6	15	AB	AA	EE	AB	AA	AA	ABC	AA	AA
APP4	18	F6	6	AA	BB	AA	AB	AA	AA	AA	AB	AC
APP5	19	F6	16	AA	BB	AA	AB	AA	AA	AA	AA	AC
APP6	20–22	F6	42	AA	BB	ABC	AB	AB	AA	AB	AB	AB
APP7	23–27	F5	47	AB	BB	AB	ABC	AB	AB	AB	AB	ABC
APP8	28–31	F5+6	48	AB	BB	AA	ABC	ABCD	AA	ABD	AB	AA
APP9	32–40	FM1/3	94	AB	BB	ABD	AB	ABC	AB	AB	AB	AB
APP10	41–54	FM2	156	AB	BB	AA	AB	ABC	AA	ABC	AB	ABC
APP11	15, 55	OG	13	AA	BB	AC	ABC	AA	AA	BB	AB	AA

results of the tree reconstruction based on the combined dataset, which is better resolved but did not recover many strongly supported clades. Although several tree-construction approaches were undertaken, weak bootstrap support for all groups and inadequate sampling of nonfocal species precluded a rigorous application of the Wiens and Penkrot (2002) method. However, a ''qualitative'' implementation of this method using the HES taxa and outgroups as reciprocal focal/nonfocal groups to each other reveals five nodes of interest (numbered in Fig. 6).

Node 1 groups all LS populations (HES1), and these samples are geographically proximal to each other in the central part of the study area (Fig. 3), with the exception of isolates from localities 12 and 14. Node 2 groups all F6 populations (HES2), which are isolated on volcanic peaks scattered throughout the study area (Figs. 2 and 3), but several of these correspond to the species *S. palaciosi*, which can be diagnosed by morphological characters (Lara-Góngora 1983); the other F6 samples have not been studied morphologically. Node 3 recovers the geographically proximal and chromosomally interdigitated populations from the F5, F5+6, FM2, FM1, and FM3 races (HES3), and node 4 consists of all HS populations (HES4). The HES4 samples are also isolated on high volcanic peaks (Fig. 3), but three of the four (all but locality 13) comprise the morphologically distinct species *S. anahuacus* (Lara-Góngora 1983), and the fourth sample has not been studied. Finally, node five recovers all F5+6 populations that, although nested within the large multiple-fission group, represent a geographically cohesive set of populations in the north-northeastern part of the study area (Figs. 2 and 3). The low bootstrap support for each of these groups could indicate significant levels of gene flow and thus require that all populations be considered single species under a strict application of the Wiens-Penkrot method. However, the absence of strong nodal support at any level suggests that this observation may simply reflect limited signal in the datasets.

DISCUSSION

Overview of Patterns

Table 5 summarizes results of our empirical comparisons relative to the HES species hypothesized from our reanalysis of morphology, allozyme, and mapped restriction site data (Fig. 2). In the analyses presented here, the LS populations (HES1) were recovered as separate species by the Highton's (2000) genetic-distance and tree-based methods. Both PAA analyses group HES1 with HES4 and the mlFFR groups HES1 with all other HES groups.

The F6 race (HES2) was found to be the most distinct of all; it was recovered as a separate species by all analyses except the mlFFR (Table 5). Specifically, when performing group comparisons using Highton's genetic-distance method, the most distinct bimodal distributions resulted when F6 samples were paired with the other HES groups (Fig. 5). The PAA analysis identified five separate species within the F6 races (Table 3), but the stPAA suggests that sampling is too limited at many of these localities to make this inference with statistical confidence (Table 4). One population, Nevado de Toluca (APP3 in Table 4), showed a large number of ''fixed'' character differences relative to the other APPs, a result strongly suggesting that this sample represents a distinct species. Finally, the NJ tree (Fig. 6, node 2) recovered the F6 samples as an exclusive group. The emerging pattern here is that possibly multiple evolutionary lineages exist within the F6 race, and that future research should target more intense sampling of this race throughout the central Mexico region and in the disjunct parts of its range farther north (Sites 1983). Again, if our a priori assumption that the F6 populations represent an independent genealogical lineage (HES2) is true, all methods except mlFFR proved effective in clearly identifying it as such.

Moderate support for separate species delimitation of the HES3 group (F5, F5+6, and all FM races) resulted with Highton's genetic-distance and the tree-based methods. The PAA methods showed support for separate species status; however, this evidence is equivocal because both delimited multiple species within the HES3 group. Some samples within the HES3 group were also delimited as separate species by other methods. For instance, the F5+6 race qualifies as a species by both PAA (Table 3) and stPAA (Table 4) criteria, and is recovered as an exclusive group in the NJ tree (Fig. 6, Node 5). The F5 and FM2 races are also delimited as separate species by PAA and stPAA criteria.

TABLE 3. Extended.

Aggregated population profiles														
10					15			18	C1	C2	C3	C4	C5	C6
AA	AA	AB	AA	AB	AB	AB	AA	AB	AB	AA	AA	AA	AA	AB
BB	AA	BB	BB	AB	AA	BB	CC	AA	AA	AA	AA	AA	AA	BB
AB	AA	AA	BB	BB	AA	BB	CC	AA	AA	AA	AA	AA	AA	BB
AA	AA	AB	AA	BB	AA	BB	CC	AA	AA	AA	AA	AA	AA	BB
AA	AA	AB	BB	BB	AB	BB	CC	AA	AA	AA	AA	AA	AA	BB
AB	AB	AB	AA	AA	AB	AB	CC	AB	AA	AA	AA	AA	AA	BB
AA	AA	AB	AA	AB	AB	AB	CC	AA	AB	AA	AA	ABC	BB	AA
AB	AC	AB	AA	AB	AB	AB	CC	AA	AA	AA	AA	ABC	BB	BB
AB	AD	AB	AA	AA	AB	AB	CC	AB	AB	BB	AB	AB	BB	BB
ABC	AB	AB	AA	AA	AA	AB	CC	AA	BB	DD	BB	AB	AB	AB
BB	AA	AB	AA	AA	AA	AB	AA	AA	AA	AA	AA	AA	AA	AA

Lastly, the HS populations (HES4) are recovered as distinct by Highton's genetic-distance method, and they formed an exclusive group (weakly supported) in the NJ tree (Fig. 6, node 4). On the contrary, HS populations were found to be conspecific with LS populations by PAA and stPAA and conspecific with all other populations by the mlFFR method. If our a priori assessment that the HS populations represent an independent genealogical lineage (HES4) is true, Highton's genetic-distance and the tree-based method provided strong to moderate support for evolutionary independence of the HS populations.

Comparing Methods

Comparing our results with the a priori defined HES species revealed that methods performed with varying degrees of accuracy in recovering the genealogical lineages. Highton's genetic-distance method consistently recovered the HES species when the bimodality of pairwise *D* distributions was used as the distinguishing criterion, and the $D \approx 0.15$ cutoff criterion was relaxed. Of all comparisons made here, the HES1 × HES2 reflects a distinctly bimodal genetic distance distribution on both sides of $D \approx 0.09$, the closest approximation to $D \approx 0.15$ used for plethodontid salamanders (Highton 2000). Two other bimodal patterns are evident in the HES1 × HES3 and HES1 × HES4 histograms (Fig. 5), although both show some overlap in within-HES versus between-HES pairwise *D* distributions; more importantly, both are bimodal across much smaller *D* values (≈ 0.03). This pattern would be expected if lineages had been isolated for enough time to accumulate allele frequency differences at multiple nuclear characters, even if reproductive isolation was not absolute (i.e., some hybridization persisted), or had only recently attained closure (incomplete allele sorting). We suggest that this is the case here, and that bimodality of pairwise *D* distributions may be among the earliest attributes to emerge in the speciation process, and thus provide signal for the delimitation of species lineages independent of the absolute value of *D*. Additionally, we see no reason why other

TABLE 4. Results of the Wiens-Servedio test. Columns 3–12 represent a matrix of the number of fixed character differences (18 allozymes and six chromosomal rearrangements; $k = 0$–24) for pairwise aggregated population profile comparisons between APPs (Table 3) of the *Sceloporus grammicus* complex. Races and HES taxa are given next to each profile, as are samples sizes for each aggregate (*n*), and the last two columns represent results for the Wiens-Servedio test at two probability levels (0.05 and 0.10, respectively). Tests are applied to each pairwise comparison, and the figures in the last two columns represent percentage of comparisons for each profile in which the Wiens-Servedio test indicates more sampling may be needed to determine whether the characters that appear to be fixed really are fixed. APP profiles highlighted in bold indicate groups that would not be recognized as separate species; that is, the five F6 profiles. These would remain conspecific until a better sample was achieved.

		APPs										Wiens-Servedio tests	
APPs (race) HES	*n*	1	2	3	4	5	6	7	8	9	10	$\alpha = 0.05$	$\alpha = 0.10$
APP1 (LS, HS) 1, 4	210	—										0%	0%
APP2 (F6) 2	15	3	—									100%	90%
APP3 (F6) 2	15	4	1	—								100%	100%
APP4 (F6) 2	6	1	2	3	—							100%	100%
APP5 (F6) 2	16	2	1	2	1	—						90%	90%
APP6 (F6) 2	42	1	1	4	1	2	—					40%	0%
APP7 (F5) 3	47	2	4	5	2	3	2	—				10%	0%
APP8 (F5+6) 3	48	2	2	4	1	2	1	1	—			40%	0%
APP9 (FM1/3) 3	94	3	3	6	3	4	2	2	1	—		0%	0%
APP10 (FM2) 3	156	3	4	6	4	5	3	2	3	1	—	0%	0%
APP11 (OG)	13	1	3	6	5	6	2	3	3	4	4	100%	100%

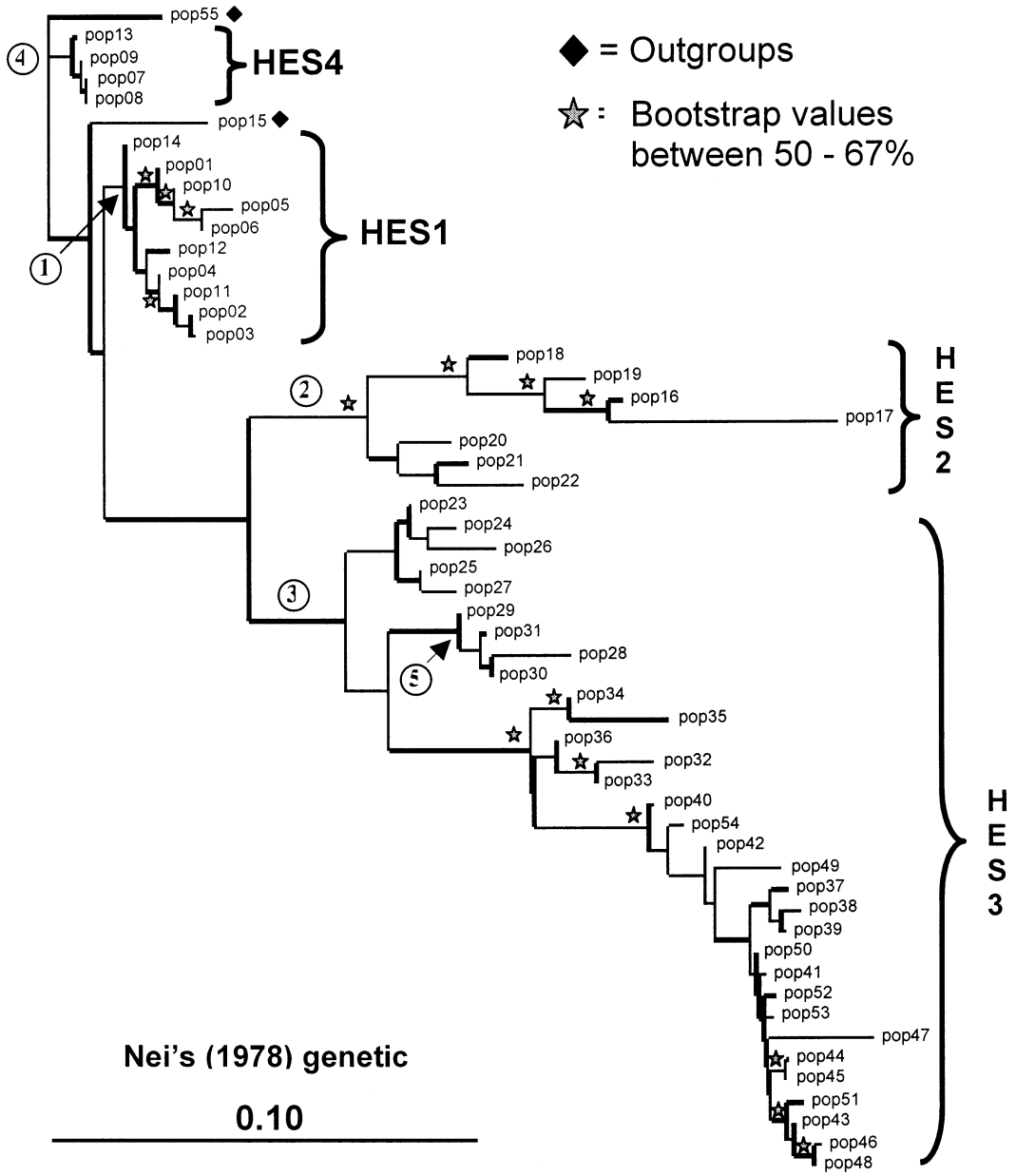

FIG. 6. Neighbor-joining tree and bootstrap values based on unbiased Nei's (1978) genetic distances for a combined allozyme and chromosome dataset. Population samples are numbered as in Table 1 and Figures 2 and 3, and corresponding HES taxa are shown with brackets. Number nodes (circles) indicate exclusive groups of interest.

measures of genetic differentiation, such as a comparisons of pairwise F_{ST} values, should not also show bimodal distribution patterns emerging early in the speciation process.

PAA was more discriminating when delimiting the HES2 and HES3 species (several species in each, and many single populations within HES2), and was less discriminating for the other two species; the HES1 and HES4 species were merged by this method (Table 5). These results are partially corrected by stPAA (Wiens and Servedio 2000), but even under these conditions, some groups are delimited as valid species that are not recovered by any other method. However, the generous ''species splitting'' of PAA and stPAA did not really conflict with other methods in the sense of delimiting different combinations of populations: with the exception that the HES1 and HES4 merge, the species delimited by PAA and stPAA are nested within more inclusive species delimited by the other methods and our a priori HES taxa. The PAA and stPAA methods are likely the most sensitive to the number of markers that would be identified as fixed by conventional criteria (present in some state at 100% frequency in one sample, and totally absent in another); thus, the F5 and FM2 races are identified as separate species by the PAA methods because both are unique in the combinations of fixed chromosomal rearrangements by which they were first identified (Fig. 1). Even when the chromosome and allozyme data are combined, allozyme electromorph frequencies are suffi-

TABLE 5. A general summary of the results for each of our assumed independent genealogical lineages (HES 1–4) from the species delimitation methods applied in this study. Asterisks indicate that the method divided the group further into separate species. mlFFR, multilocus field for recombination; PAA, population aggregation analysis; stPAA, statistical PAA.

	Highton's genetic distance	mlFFR	PAA	stPAA	Tree-based
HES1	strong support for separate species	conspecific with all other HES	grouped with HES4	grouped with HES4	moderate support of separate species
HES2	strong support for separate species	conspecific with all other HES	strong support of separate species*	strong support of separate species	moderate support of separate species
HES3	moderate support for separate species	conspecific with all other HES	moderate support of separate species*	moderate support of separate species*	moderate support of separate species
HES4	strong support for separate species	conspecific with all other HES	grouped with HES1	grouped with HES1	moderate support of separate species

ciently idiosyncratic across some races to permit recovery of the chromosomal ''signal'' by both methods. PAA and stPAA might be good indicators of incipient species when only a single character appears fixed by conventional definition.

The tree-based method provided limited support for the resolution of species boundaries in this study, but if the requirement of strong nodal support is relaxed and more weight given to the fact that some geographically widespread chromosome races form phylogenetically separate clades (for example, F6 and HS), then delimitation of species is possible. The combined chromosome and allozyme NJ tree (Fig. 6) generated from a genetic-distance matrix (Nei 1978) recovered all four HES taxa, but with low nodal support for all of these clades. This result reflects weak phylogenetic signal in the data, no doubt because within-race allozyme polymorphism was high relative to between-race divergence for most races.

The least discriminatory method used was the mlFFR approach (Doyle 1995), which, if strictly applied, collapses all eight chromosome races. Doyle argued philosophically that a nontopological method should be used to delimit the basal units for phylogenetic inference, and therefore tree reconstruction should proceed only after species delimitation (see also Davis and Nixon 1992). The justification for his method is that codominant nuclear characters can identify gene pools or distinct fields for recombination (in gonochoristic species) from which no individual can be selected and represented on more that one branch of the tree. If tree reconstruction is performed below the species level, then it is possible for the two alleles from a heterozygous individual to be recovered at different places on the tree.

Doyle (1995) was aware of the sensitivity of his method to marker resolution (it requires identification of all alleles at all loci), and recognized that allelic proteins with similar electrophoretic mobility (electromorphs) will often be divergent at the DNA level, and this will cause them to have a higher probability of being shared between distinct gene pools. Because of their limited resolution, electromorphs might group populations even when they are not connected by gene flow; however, all other methods in this study were able to identify lineages based on the same ''low resolution'' datasets, and therefore the failure of mlFFR in our study is mostly likely not due to marker resolution but rather the extremely conservative nature of this method. This limitation coupled with the availability of newer multilocus assignment and clustering approaches that group individual genotypes into the most likely populations based on Hardy-Weinberg and linkage equilibrium assumptions (Pearse and Crandall 2004), renders the mlFFR approach obsolete.

General Conclusions

Although the Highton genetic-distance and the tree-based methods showed moderate support for all HES taxa, no single method used in this study strongly delimited all of these lineages. This study is one of only a few that has used several methods to delimit species boundaries. Other studies, including Dettmann et al. (2003a,b), Johnson et al. (2004), Wiens and Penkrot (2002), Cardoso and Vogler (2005), and Ross and Shoemaker (2005) also found varying degrees of agreement between methods. Where different boundaries were delimited with different methods, the likely causes for the discordance could be inferred. Dettmann et al. (2003a,b) found that species delimited by tests of reproductive isolation in the fungal genus *Neurospora* were highly congruent to species delimited as phylogenetically separate clades. Similarly, Johnson et al. (2004) demonstrated complete concordance among mtDNA and nuclear-gene tree topologies, morphological clusters, and ecological traits, in two species of the fish genus *Lepidomeda*. These patterns suggest that the target species have diverged sufficiently to show complete concordance of datasets sampled in these studies.

Results of the Wiens and Penkrot (2002) study were more discordant; five species were delimited in the *Sceloporus jarrovii* complex with each of three approaches: a morphological tree-based method, a DNA tree-based method, and a morphological character-based method. Only two species were fully congruent among all methods, and most of the discordance was attributed to high within-species coupled with low between-species variation in morphological characters. A similar pattern was evident in the allozyme variation observed across many of the chromosome races of the *S. grammicus* complex sampled in this study, particularly those included in the HES3 lineage. This may be due in part to a relatively recent origin for these races; the fixation and spread of chromosomal rearrangements may occur on a time scale of a few thousand years, and thus allow little time for other classes of characters to diverge. Britton-Davidian et al. (2000, 2002), for example, described the rapid origin of multiple chromosome races of *Mus musculus domesticus* in isolated

areas of Madeira island in possibly less than 600 years, so a rapid and recent origin of some *S. grammicus* chromosome races, coupled with historical and/or ongoing hybridization, makes this a challenging system in which to explore issues of species delimitation.

In this context, we are encouraged by the degree of concordance between several methods and the HES taxa (notably the Highton genetic distance method, qualitative tree-based method, and the nested PAA results). Our results are especially gratifying when compared to the small, isolated populations of fishes in desert springs studied by Johnson et al. (2004), an ideal system in which coalescence of many different character attributes would be expected to occur rapidly, as was indeed the case for nuclear and mitochondrial genes, morphology, and some ecological attributes. We conclude that codominant nuclear markers are likely to be successful at delimiting species by a number of methods even in complexes where taxa have recently diverged, and will explore these issues further with additional datasets.

Acknowledgments

This manuscript was improved by helpful comments and discussions with K. Crandall, E. Jellen, D. Rogers, and M. Whiting. We also thank J. Wiens for his help with frequency-based parsimony analysis. This project was supported by grants from the National Science Foundation (BSR 85-09092 and 88-22751 to JWS and doctoral dissertation improvement grant DEB 03-09103 to JCM and JWS), the Society for the Study of Amphibians and Reptiles (to JCM), and several awards from Brigham Young University (Graduate Research Fellowship, Graduate Mentoring Fellowship, Julia Greenwell Award, J. R. and Shauna Larsen scholarships, and the M. L. Bean Life Science Museum, to JCM). We would also like to thank R. Waples and two anonymous reviewers for their very helpful comments.

Literature Cited

Agapow, P. M, O. R. P. Bininda-Edmonds, K. A. Crandall, J. L. Gittleman, G. M. Mace, J. C. Marshall, and A. Purvis. 2004. The impact of species concept on biodiversity studies. Q. Rev. Biol. 79:161–179.

Arévalo, E., C. A. Porter, A. Gonzalez, F. Mendoza, J. L. Camarillo, and J. W. Sites, Jr. 1991. Population cytogenetics and evolution of the *Sceloporus grammicus* complex (Iguanidae) in central Mexico. Herpetol. Monogr. 5:79–115.

Arévalo, E., S. K. Davis, G. Casas, G. Lara, and J. W. Sites, Jr. 1993. Parapatric hybridization between chromosome races of the *Sceloporus grammicus* complex (Phrynosomatidae): structure of the Tulancingo transect. Copeia 1993:352–372.

Arévalo, E., S. K. Davis, and J. W. Sites, Jr. 1994. Mitochondrial DNA sequence divergence and phylogenetic relationships among eight chromosome races of the *Sceloporus grammicus* complex (Sauria, Phrynosomatidae) in Mexico. Syst. Biol. 43: 387–418.

Barraclough, T. G., and S. Nee. 2001. Phylogenetics and speciation. Trends Ecol. Evol. 16:391–399.

Blackburn, T. M., and K. J. Gaston. 1998. Some methodological issues in macroecology. Am. Nat. 51:6814–6883.

Britton-Davidian, J., J. Catalan, M. da Graca Ramalhinho, G. Ganem, J. Auffray, R. Capela, M. Biscoito, J. B. Searle, and M. da Luz Mathias. 2000. Rapid chromosomal evolution in island mice. Nature 403:158.

Britton-Davidian, J., J. Catalan, K. Belkhir. 2002. Chromosomal and allozyme analysis of a hybrid zone between parapatric Robertsonian races of the house mouse: a case of monobrachial homology. Cytogenet. Genome Res. 96:75–84.

Brower, A. V. Z. 1999. Delimitation of phylogenetic species with DNA sequences: a critique of Davis and Nixon's population aggregation analysis. Syst. Biol. 48:199–213.

Cardoso, A., and A. P. Vogler. 2005. DNA taxonomy, phylogeny and Pleistocene diversification of the *Cicindela hybrida* species group (Coleoptera: Cincidelidae). Mol. Ecol. 14:3531–3546.

Carson, H. L. 1957. The species as a field for recombination. Pp. 23–38 *in* E. Mayr, ed. The species problem. American Association for the Advancement of Science, No. 50. Washington DC.

Cole, C. J., C. H. Lowe, and J. W. Wright. 1967. Sex chromosomes in lizards. Science 155:1028–1029.

Davis, J. I. and K. C. Nixon. 1992. Populations, genetic variation, and the delimitation of phylogenetic species. Syst. Biol. 41: 421–435.

De Queiroz, K. 1998. The general lineage concept of species, species criteria, and the process of speciation. Pp. 57–75 *in* D.J. Howard and S.H. Berlocher, eds. Endless forms: species and speciation. Oxford Univ. Press, Oxford, U.K.

———. 2005a. Ernst Mayr and the modern concept of species. BioEssays 27:1263–1269.

———. 2005b. Different species problems and their resolution. Proc. Natl. Acad. Sci. USA 102(Supp. 1):6600–6607.

Dettman, J. R., D. J. Jacobson, and J. W. Taylor. 2003a. A multilocus genealogical approach to phylogenetic species recognition in the model eukaryote *Neurospora*. Evolution 57: 2703–2720.

Dettman, J. R, D. J. Jacobson, E. Turner, A. Pringle, and J. W. Taylor. 2003b. Reproductive isolation and phylogenetic divergence in *Neurospora*: comparing methods of species recognition in a model eukaryote. Evolution 57:2721–2741.

Doyle, J. 1995. The irrelevance of allele tree topologies for species delimitation, and a non-topological alternative. Syst. Bot. 20: 574–588.

Felsenstein, J. 1993. PHYLIP (phylogeny inference package). Ver. 3.5c. Distributed by author. Department of Genetics, University of Washington, Seattle.

Frost, D. R, and A. G. Kluge. 1994. A consideration of epistemology in systematic biology, with special reference to species. Cladistics 10:259–294.

Fukami, H., A. F. Budd, D. R. Levitan, J. Jara, R. Kersanach, and N. Knowlton. 2004. Geographic differences in species boundaries among members of the *Montastraea annularis* complex based on molecular and morphological markers. Evolution 58: 324–337.

Gabor, C. R., and C. C. Nice. 2004. Genetic variation among populations of eastern newts, *Notophthealmus*: a preliminary analysis based on allozymes. Herpetologica 60:373–386.

Günther, A. C. L. G. 1885–1902. Biologia Centrali-Americana. Reptilia and Batrachia. Porter, London.

Hall, W. P. 1973. Comparative population cytogenetics, speciation, and evolution of the iguanid lizard genus *Sceloporus*. Ph.D. diss. Harvard University, Cambridge, MA.

———. 1980. Chromosomes, speciation, and evolution of Mexican iguanid lizards. Natl. Geogr. Soc. Res. Rep. 12:309–329.

———. 1983. Modes of speciation and evolution in the sceloporine iguanid lizards. I. Epistemology of comparative approach and introduction to the problem. Pp. 643–679 *in* A. G. J. Rhodin and K. Miyata, eds. Advances in herpetology and evolutionary biology. Museum of Comparative Zoology, Cambridge, MA.

Hall, W. P., and R. K. Selander. 1973. Hybridization of karyotypically differentiated populations in the *Sceloporus grammicus* complex (Iguanidae). Evolution 27:226–242.

Hey, J., R. S. Waples, M. L. Arnold, R. K. Butlin, and R. G. Harrison. 2003. Understanding and confronting species uncertainty in biology and conservation. Trends Ecol. Evol. 18:597–603.

Highton, R. 1989. Biochemical evolution in the slimy salamanders of the *Plethodon glutinosus* complex in the eastern United States. Part I. Geographic protein variation. Illinois Biol. Monogr. 57: 1–78.

———. 2000. Detecting cryptic species using allozyme data. Pp. 215–241 *in* R. C. Bruce, R. G. Jaeger, and L. D. Houck, eds.

The biology of plethodontid salamanders. Kluwer Academic/Plenum Publishers, New York.

Highton, R., and R. B. Peabody. 2000. Geographical protein variation and speciation in salamanders of the *Plethodon jordani* and *Plethodon glutinosus* complexes in the southern Appalachian mountains with the description of four new species. Pp. 31–94 *in* R. C. Bruce, R. G. Jaeger, and L. D. Houck, eds. The biology of plethodontid salamanders Kluwer Academic/Plenum Publishers, New York.

Jockusch, E. L., and D. B. Wake. 2002. Falling apart and merging classification of slender salamanders (Plethodontidae: *Batrachoseps*) in the American West. Biol. J. Linn. Soc. 76:361–391.

Johnson, J. B., T. E. Dowling, and M. C. Belk. 2004. Neglected taxonomy of rare desert fishes: congruent evidence for two species of leatherside chub. Syst. Biol. 53:841–855.

King, M. L. 1993. Species evolution: the role of chromosome change. Cambridge Univ. Press, NY.

Lara-Góngora, G. 1983. Two new species of the lizard genus *Sceloporus* (Reptilia, Sauria, Iguanidae) from the Ajusco and Ocuilan Sierras, Mexico. Bull. Mayland Herpetol. Soc. 19:1–14.

Marshall, J. C., and J. W. Sites, Jr. 2001. A comparison of nuclear and mitochondrial cline shapes in a hybrid zone in the *Sceloporus grammicus* complex (Squamata; Phrynosomatidae). Mol. Ecol. 10:435–449.

Mayden, R. L. 1997. A hierarchy of species concepts: the denouement in the saga of the species problem. Pp. 381–424 *in* M. F. Claridge, H. A. Dawah, and M. R. Wilson, eds. Species: the units of biodiversity. Chapman and Hall, London.

Mead, L. S., S. G. Tilley, and L. A. Katz. 2001. Genetic structure of the blue ridge dusky salamander (*Desmognathus orestes*): inferences from allozymes, mitochondrial DNA, and behavior. Evolution 55:2287–2302.

Miller, W. III. 2001. The structure of species, outcomes of speciation and the ''species problem'': ideas for palaeobiology. Palaeogeogr. Palaeoclimatol. Palaeoecol. 176:1–10.

Murphy, R. W., J. W. Sites, Jr., D. G. Bluth, and C. H. Haufler. 1996. Proteins: isozymes electrophoresis. Pp. 51–120 *in* D. M. Hillis, C. Moritz, and B. K. Mable. eds. Molecular systematics. Sinauer, Sunderland, MA.

Nei, M. 1972. Genetic distance between populations. Am. Nat. 106: 283–292.

———. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89: 583–590.

Pearse, D. E., and K. A. Crandall. 2004. Beyond F_{ST}: analysis of population genetic data for conservation. Conserv. Genet. 5: 585–602.

Pigliucci, M. 2003. Species as family resemblance concepts: the (dis-) solution of the species problem? BioEssays 25:596–602.

Porter, A. H. 1990. Testing nominal species boundaries using gene flow statistics: the taxonomy of two hybridizing admiral butterflies (*Limenitis*: Nymphalidae). Syst. Zool. 39:131–147.

Porter, C. A., and J. W. Sites, Jr., 1986. Evolution of the *Sceloporus grammicus* complex (Sauria: Iguanidae) in central Mexico: population cytogenetics. Syst. Zool. 35:334–358.

Reed, K. M., J. W. Sites, Jr., and I. F. Greenbaum. 1992. Synapsis, recombination, and meiotic segregation in the mesquite lizard, *Sceloporus grammicus* complex. II. Fission heteromorphism of the FM2 cytotype and the evolution of chromosome 2. Cytogenet. Cell Genet. 61:46–54.

Reed, K. M., I. F. Greenbaum, and J. W. Sites, Jr. 1995. Cytogenetic analysis of chromosomal intermediates from a hybrid zone between two chromosome races of the *Sceloporus grammicus* complex (Sauria, Phrynosomatidae). Evolution 49:37–47.

Ross, K. G., and D. D. Shoemaker. 2005. Species delimitation in native South American fire ants. Mol. Ecol. 14:3419–3438.

Simpson, G. G. 1961. Principles of animal taxonomy. Colombia Univ. Press, New York.

Sites, J. W., Jr. 1983. Chromosomes evolution in the iguanid lizard *Sceloporus grammicus*. I. Chromosomal polymorphisms. Evolution 37:38–53.

Sites, J. W., Jr., and S. K. Davis. 1989. Phylogenetic relationships and molecular variability within and among six chromosome races of *Sceloporus grammicus* (Sauria, Iguanidae) based on nuclear and mitochondrial markers. Evolution 43:296–317.

Sites, J. W., Jr., and J. C. Marshall. 2003. Delimiting species: a Renaissance issue in systematic biology. Trends Ecol. Evol. 18: 462–470.

Sites, J. W., Jr., and R. W. Murphy. 1991. Isozyme evidence for independently derived, duplicated G3PDH loci among squamate reptiles. Can. J. Zool. 69:2381–2396.

———. 2004. Operational criteria for delimiting species. Annu. Rev. Ecol. Evol. Syst. 35:199–227.

Sites, J. W., Jr., J. L. Camarillo, A. Gonzalez, F. Mendoza, L. Javier, M. Mancilla, and G. Lara-Góngora. 1988. Allozyme variation and genetic divergence within and between three cytotypes of the *Sceloporus grammicus* complex (Sauria: Iguanidae) in central Mexico. Herpetologica 44:297–307.

Sites, J. W., Jr., J. W. Archie, C. J. Cole, and O. Flores-Villela. 1992. A review of phylogenetic hypotheses for lizards of the genus *Sceloporus* (Phrynosomatidae): implications for ecological and evolutionary studies. Bull. Am. Mus. Nat. Hist. 213: 1–110.

Sites, J. W., Jr., S. K. Davis, D. W. Hutchinson, B. A. Maurer, and G. Lara. 1993. Parapatric hybridization between chromosome races of the *Sceloporus grammicus* complex (Sauria: Iguanidae) in central Mexico. Herpetologica 44:297–307.

Smith, H. M. 1939. The Mexican and Central American lizards of the genus *Sceloporus*. Field Mus. Nat. Hist. Zool. Ser. 26:1–397.

Smith, H. M. and L. E. Laufe. 1945. Mexican amphibians and reptiles in the Texas Cooperative Wildlife Collections. Trans. Kans. Acad. Sci. 48:325–354.

Smith, H. M., and E. H. Taylor. 1950. An annotated checklist and key to the reptiles of Mexico exclusive of snakes. Bull. U.S. Nat. Mus. 199:1–253.

Swofford, D. L. 1999. PAUP*: phylogenetic analysis using parsimony (*and other methods). Ver. 4. Sinauer, Sunderland, MA.

Thorpe, J. P. 1982. The molecular clock hypothesis: biochemical evaluation, genetic differentiation and systematics. Annu. Rev. Ecol. Syst. 13:139–168.

White, M. J. D. 1978. Modes of speciation. W. H. Freeman, San Francisco, CA.

Wiens, J. J. 1999. Polymorphism in systematic and comparative biology. Annu. Rev. Ecol. Evol. 30:327–362.

———. 2000. Reconstructing phylogenies from allozyme data: comparing method performance with congruence. Biol. J. Linn. Soc. 70:613–632.

Wiens, J. J., and T. A. Penkrot. 2002. Delimiting species using DNA and morphological variation and discordant species limits in spiny lizards (*Sceloporus*). Syst. Biol. 51:69–91.

Wiens, J. J., and M. R. Servedio. 2000. Species delimitation in systematics: inferring diagnostic differences between species. Proc. R. Soc. Lond. B. 267:631–636.

Yeh, F., R. Yang, and T. Boyle. 2001. POPGENE Ver. 1.31. Available from the authors via http://www.ualberta.ca/~fyeh/.

Corresponding Editor: J. Hey