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Testing Species Boundaries in an Ancient Species Complex with Deep Phylogeographic History: Genus *Xantusia* (Squamata: Xantusiidae)

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ABSTRACT: Identification of species in natural populations has recently received increased attention with a number of investigators proposing rigorous methods for species delimitation. Morphologically conservative species (or species complexes) with deep phylogenetic histories (and limited gene flow) are likely to pose particular problems when attempting to delimit species, yet this is crucial to comparative studies of the geography of speciation. We apply two methods of species delimitation to an ancient group of lizards (genus *Xantusia*) that occur throughout southwestern North America. Mitochondrial cytochrome *b* and nicotinamide adenine dinucleotide dehydrogenase subunit 4 gene sequences were generated from samples taken throughout the geographic range of *Xantusia*. Maximum likelihood, Bayesian, and nested cladogram analyses were used to estimate relationships among haplotypes and to infer evolutionary processes. We found multiple well-supported independent lineages within *Xantusia*, for which there is considerable discordance with the currently recognized taxonomy. High levels of sequence divergence (21.3%) suggest that the pattern in *Xantusia* may predate the vicariant events usually hypothesized for the fauna of the Baja California peninsula, and the existence of deeply divergent clades (18.8%–26.9%) elsewhere in the complex indicates the occurrence of ancient sundering events whose genetic signatures were not erased by the late Wisconsin vegetation changes. We present a revised taxonomic arrangement for this genus consistent with the distinct mtDNA lineages and discuss the phylogeographic history of this genus as a model system for studies of speciation in North American deserts.

Keywords: species boundaries, phylogeography, mtDNA, maximum likelihood, Bayesian analysis, nested clade analysis, *Xantusia*.

Morphologically conservative species complexes with deep phylogeographic structure are likely to pose particular problems when attempting to delimit species boundaries. Limited morphological variation may mask considerable evolutionary diversity in some groups (e.g., Highton 2000; Wake and Jockusch 2000), yet reliance on the mtDNA locus alone may overresolve this diversity (Irwin 2002) or provide misleading results (e.g., Shaw 2002). Several new methods attempt to combine morphological and molecular data (Puorto et al. 2001; Wiens and Penkrot 2002) or to integrate these with ecological, physiological, or life-history data (Templeton 1998, 2001) to delimit species. Species are obviously fundamental to virtually all studies in evolutionary biology and biodiversity assessment, and rigorous delimitation of their boundaries clearly impacts inferences made in all areas (Brown et al. 1996; Blackburn and Gaston 1998; Peterson and Navarro-Sigüenza 1999). Rigorous delimitation of species coupled with detailed distributional data is also required for phylogenetic study of the geography of speciation (Losos and Glor 2003).

Species biology lies at the interface between population genetics and systematics; relationships above the species level are of a hierarchical nature (and hence traditional phylogenetic methods can be used for tree reconstruction), while relationships below the species level are tokogenetic, and nonhierarchical network-based approaches are more

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appropriate (Davis 1996; Posada and Crandall 2001). Given the nature of these relationships, we propose that a reasonable approach to identifying species boundaries might incorporate traditional phylogenetic methods to recover deep lineages and nonhierarchical methods to understand shallower (unresolved) relationships within lineages and to infer the processes responsible for their genetic diversity. Here, we adopt the concept of species as independent evolutionary lineages (Mayden 1997; de Queiroz 1998) and apply two recently developed methods to delimit species boundaries. Wiens and Penkrot (2002) propose an explicit (qualitative) protocol for delimiting species boundaries based on haplotype phylogenies integrated with the nonhierarchical network-based approach of Templeton et al. (1995; nested clade analysis [NCA]). The NCA was recently extended explicitly to test species boundaries within the context of the cohesion species concept (Templeton 1989, 2001). Both methods should provide the same result relating to the identification of independent lineages, but inferences from the NCA may also provide information about the processes leading to and following speciation. We are aware of the limitations of the NCA (Knowles and Maddison 2002; Petit and Grivet 2002; Masta et al. 2003) and return to some of these points in the discussion.

The night lizards (genus *Xantusia*) provide an excellent study system for cryptic-species diagnosis due to the large number of allopatric populations that are deeply divergent genetically (Bezy and Sites 1987; Lovich 2001; Papenfuss et al. 2001) but difficult to diagnose on the basis of morphology, when individual and geographic variation is fully appraised (Bezy 1967*a*, 1967*b*; Bezy and Flores-Villela 1999). *Xantusia* occurs throughout the southwestern desert region of North America (fig. 1; fig. A1 in the online edition of the *American Naturalist*), an area with unusually complex geological and vegetational structure and history. *Xantusia vigilis* is the most widespread of seven currently recognized species (Bezy 1982; Bezy and Flores-Villela 1999; Lovich 2001; Papenfuss et al. 2001); however, its distribution is extremely patchy due in part to its limited vagility (Zweifel and Lowe 1966) and close association with particular structural niches, such as rock crevices or decaying yuccas and agaves (Bezy 1989). These life-history characteristics may make this genus particularly attractive as a model system for studies of the geography of speciation because historical range changes may have been sufficiently slow that the mode of speciation remains detectable (Barraclough and Vogler 2000; Losos and Glor 2003). This attribute is particularly attractive in view of the current interest in using multiple clades of vertebrates in comparative biogeographic studies of the Baja California peninsula and warm deserts of southwestern North America (Riddle et al. 2000*a*, 2000*b*, 2000*c*).

Here, we take a molecular-based phylogenetic approach to hypothesize species boundaries and biogeographic patterns in *X. vigilis* using partial DNA sequences from the mitochondrial cytochrome *b* (cyt *b*) and nicotinamide adenine dinucleotide dehydrogenase subunit 4 (ND4) gene regions. Extensive sampling was conducted across the entire range of the genus to delimit species boundaries based on the Wiens and Penkrot (2002) molecular approach and contrast the biogeographic pattern reflected in the *Xantusia* phylogeny with those for other North American desert vertebrates. We draw attention to general issues of testing species boundaries in morphologically conservative, low-vagility species and to geographic sampling and limitations of the NCA. Because these limitations can now be accommodated in future research designs, we suggest that *Xantusia* offers many attributes as a model system for further study of the geography of speciation in southwestern North America.

Material and Methods

Data Collection

We obtained mtDNA sequence data from a total of 122 individuals of *Xantusia* from 87 localities. All localities are summarized in table A1 and figure A1 in the online edition of the *American Naturalist* or are available from the authors on request. Our sampling locations closely reflect the known distribution of *Xantusia vigilis* (map in Bezy 1982), but not all known localities were sampled, and new localities continue to be reported (e.g., Feldman et al. 2003). All named taxa were sampled, including *Xantusia henshawi gracilis* ($n = 1$), *Xantusia bolsonae* ($n = 1$), *Xantusia sanchezi* ($n = 2$), *Xantusia riversiana* ($n = 1$), and the seven described subspecies of *X. vigilis* ($n = 117$), including populations recently assigned to *Xantusia bezyi* and *Xantusia arizonae* by Papenfuss et al. (2001). In addition, 15 *X. henshawi* cyt *b* sequences were included (accession nos. AF313374–AF313386; Lovich 2001). For outgroup rooting, we generated sequences for five *Lepidophyma* species, the sister group to *Xantusia* (Hedges et al. 1991; Hedges and Bezy 1993; Vicario et al. 2003).

Genomic DNA was extracted using chloroform/phenol with isopropanol precipitation (Sambrook et al. 1989) from fresh frozen liver, heart, or muscle tissue maintained in liquid nitrogen (−194°C). Two partial mitochondrial gene regions were amplified via polymerase chain reaction (PCR) using cyt *b* L14841 (Bickham et al. 1995) and H15149 (Kocher et al. 1989) and ND4 (11165–11196) and Leu (12086–12111; Arévalo et al. 1994). Details of PCR amplification and sequencing protocols can be found in appendix B in the online edition of the *American Naturalist*. Sequences were edited and aligned using ClustalX

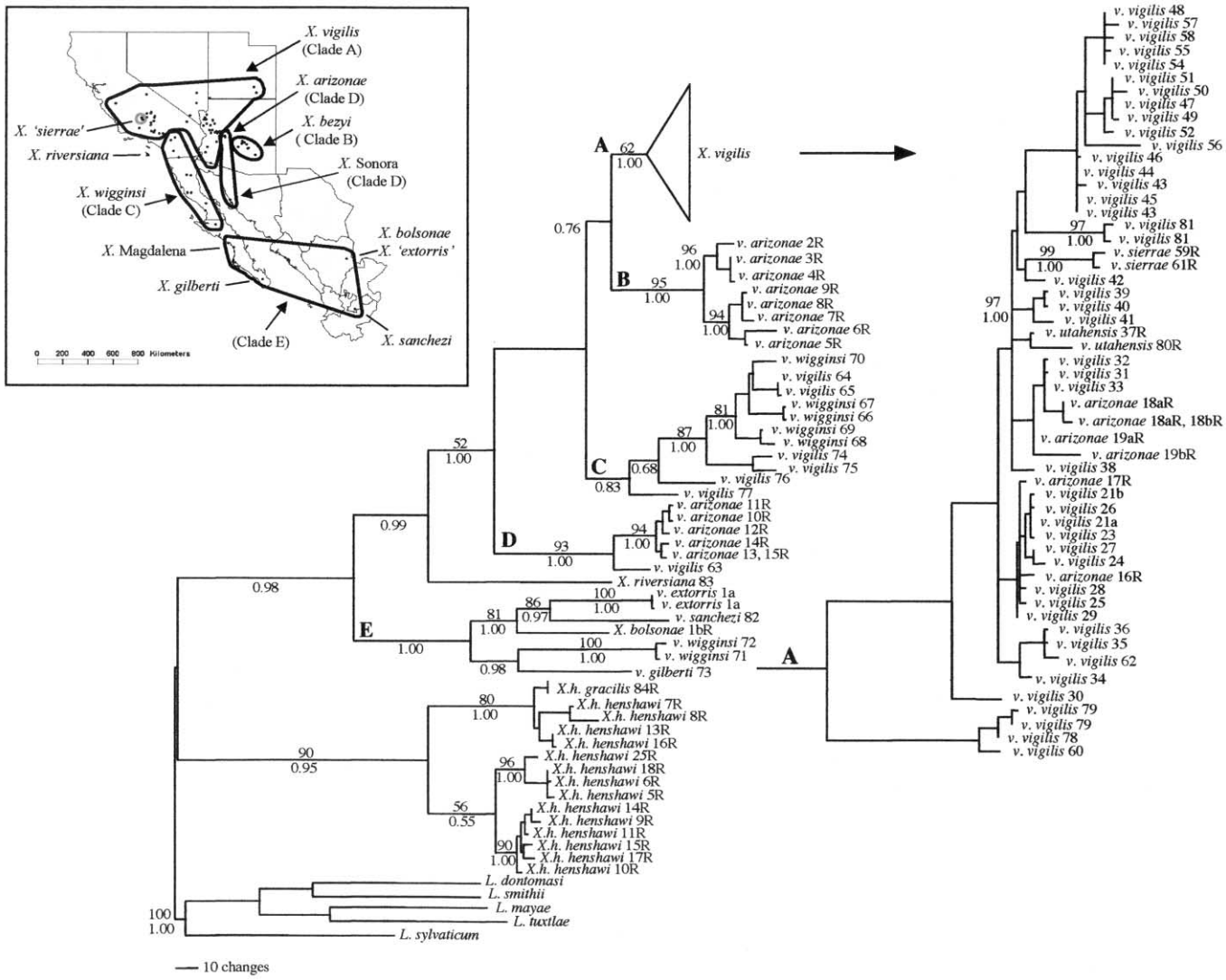

Figure 1: Maximum likelihood (ML) tree reconstructed from both gene regions under the GTR + I + Γ model of nucleotide substitution. Numbers above the branches are ML bootstrap values based on 1,000 replicates (if >50%), and numbers below are posterior probabilities from the Bayesian analysis. Specific clades have been labeled for discussion (*A*–*E*). Terminal branches are labeled according to current taxonomy, the sample location number in table A1 in the online edition of the *American Naturalist*, and whether they inhabit rock-crevice habitat (*R*). *Inset*, Geographic range of the five major clades. *Xantusia henshawi* has been omitted because its range extensively overlaps with clade C and it is not the focal species.

(Thompson et al. 1997). Alignments were checked by eye. Both protein-coding regions were translated into amino acid sequences using MacClade 4.0 (Maddison and Maddison 2000). Sequence-divergence estimates were based on the combined data set incorporating the best-fit model of evolution (see below).

Phylogenetic Approaches

We used both maximum likelihood (ML) and Bayesian methods to reconstruct single and combined gene phylogenies; ML analyses were performed using PAUP* 4.0b10 (Swofford 2002) and the program Modeltest 3.06 (Posada and Crandall 1998) to select the best-fit model of evolution for each gene region and for the combined data set. Tree searches were performed (heuristic search with 100 random additions and tree bisection/reconnection branch swapping) using unique haplotypes and implementing the best-fit model. *Lepidophyma* sequences were designated as outgroups, and all *Xantusia* sequences were defined as ingroup taxa. Confidence in the nodes was assessed using the bootstrap procedure (Felsenstein 1985) with 1,000 resampling replicates, and bootstrap values ≥70% were considered well supported (Hillis and Bull 1993). We evaluated potential conflict between topologies recovered from the individual gene regions using the qualitative method suggested by Wiens (1998), which prevents a combined analysis from being strongly misled by conflicting phylogenetic histories in two data sets (if these exist) and allows regions of shared history to be corroborated by the maximum number of characters.

Bayesian analyses were performed for the combined data set using the computer program MrBayes 2.0 (Huelsenbeck and Ronquist 2001). Bayesian inference of phylogeny is a probability-based method in which prior beliefs about the probability of a hypothesis are combined with the likelihood. The posterior probability of a phylogeny is approximated by sampling trees from the distribution of posterior probabilities. A model of evolution is incorporated, although the model parameters were treated as unknown variables with uniform priors and are subsequently estimated as part of the analysis (rather than given before the search as in our ML analysis). Markov chain Monte Carlo (MCMC) was used to sample the phylogenies according to their posterior probabilities and was initiated with a random tree and run for 1×10^6 generations, with sampling every 1,000 generations. To ensure the Markov chain has reached stationarity (ln-likelihood values of the sample points reach a stable equilibrium; Huelsenbeck and Ronquist 2001), the ln-likelihood scores for sampling points were plotted against generation time. All sample points before stationarity (burn in) were removed before generating posterior probabilities. Three replicate runs were performed, and stationarity levels were compared for convergence (Huelsenbeck and Bollback 2001); independent analyses (which have different starting trees) were considered to have converged if their ln-likelihood scores approach similar mean values (Leaché and Reeder 2002). Four incrementally heated Markov chains were used to enable a more thorough search of the parameter space (and avoid getting stuck on local optima). A 50% majority rule consensus tree was generated in PAUP*, and the percentage of samples recovering a particular clade was taken as that clade's posterior probability (Huelsenbeck and Ronquist 2001). Convergence among the independent runs was evaluated by comparing tree topology and support for comparable nodes across the three consensus trees. We consider Bayesian posterior probabilities >0.95 well supported (Leaché and Reeder 2002).

To test for monophyly of the *X. vigilis* complex, we implemented the Shimodaira-Hawegawa test (S-H test; Shimodaira and Hawegawa 1999) in PAUP*. The tree obtained from our ML search was compared with a constrained tree obtained from a second heuristic search using the search criteria described above. The S-H test is an appropriate nonparametric test to compare tree topologies when at least one of the trees being tested is selected a posteriori from analysis of the data being tested (Goldman et al. 2000). One thousand bootstrap replicates were run using the RELL methods option.

Nested Clade Analysis

A statistical parsimony network was constructed from cyt *b* sequences from one densely sampled clade (see below) using the approach of Templeton et al. (1992). This method has been shown to outperform parsimony analyses when few characters are available to differentiate between haplotypes (Crandall et al. 1994; Posada and Crandall 2001). This algorithm was implemented using the computer program TCS v1.13 (Clement et al. 2000), which estimates the number of substitutions among haplotypes that could be linked in the cladogram with a probability of 0.95 or greater and then obtains a minimum connecting network of genealogical relationships. With the resulting network, we then implemented the nesting algorithm of Templeton and Sing (1993) as modified by Crandall (1996) for sequence data and followed Templeton et al. (1995) to test the association between the nested cladograms and geographic distances among the sampled populations. Two statistics were estimated: the clade distance Dc, which measures the geographical spread of a clade, and the nested clade distance Dn, which measures how a clade is geographically distributed relative to other clades in the same higher-level nesting category. Ten thousand permutations were performed to discriminate among nonindependent

geographic distributions of haplotypes at the 95% confidence level using the computer program GeoDis v2.0 (Posada et al. 2000). The computer programs and a revised inference key (from Templeton 1998) used to interpret patterns of population structure and historical events are available at http://inbio.byu.edu/faculty/kac/crandall_lab/computer.html. We also included the modification to the inference key suggested by Masta et al. (2003) and performed Tajima's D-test (Tajima 1989) and Fu's *F*s-test (Fu 1997), using Arlequin version 2.0 (Schneider et al. 1996), to cross-verify inferences of recent population expansion.

Delimiting Species Boundaries

The diagnosis of taxa within *Xantusia* in the past has been ad hoc and ambiguous, and in this study we present a priori criteria in a hypothesis-testing framework (Sites and Crandall 1997; Sites and Marshall 2003) to delimit species boundaries. We used the approach described by Wiens and Penkrot (2002), which requires two sampling design components: inclusion of as many closely related reference species as possible to increase the strength of the exclusivity test for the focal species of interest, and inclusion of at least two individuals from as many localities as possible to increase the strength of the between-population gene-flow inferences (Slatkin and Maddison 1989). To implement the method, we constructed a haplotype sequence tree (using the combined data set) for all population samples of the focal species (*X. vigilis* complex), along with all nonfocal species (all other *Xantusia*), and followed the chain of inference given by Wiens and Penkrot (2002; fig. 1).

At shallow levels of divergence, we implemented the NCA to test the boundaries of cohesion species in a single densely sampled exclusive group (clade A; figs. 1, 2), following the method described by Templeton (2001). The method is implemented by testing two hypotheses: H_1, organisms sampled are derived from a single evolutionary lineage; and H_2, populations of lineages identified by rejection of H_1 are genetically exchangeable and/or ecologically interchangeable. For inferring species boundaries, the relevant outcome is rejection of H_1 with statistical support for historical fragmentation at some clade level. Hypothesis two can be tested by direct statistical contrasts of the lineages previously identified or through the NCA with other types of data. Hypothesis two is rejected by a significant association between geography and the trait or traits associated with genetic exchangeability or ecological interchangeability and the concordant phylogenetic position of this change with the previously identified fragmentation of evolutionary lineages (i.e., when both are identified at the same clade level). Although data limitations do not permit us to implement the statistical test required by H_2, we can for many samples qualitatively assess concordance between the distinct mtDNA clades identified by rejection of H_1 and independent data sets (allozymes, chromosomes, and morphological characters). Here, we recognize candidate species from the NCA analysis if the clades identified with significant support for past fragmentation events are also distinct in at least one other data set and if the distribution of unique combinations of characters in the second data set is also concordant with geography.

Results

Phylogeny Estimation

Cytochrome b *(cyt* b*).* Three hundred seven base pairs (bp) of cyt *b* sequence data were obtained from 122 individuals of *Xantusia*, and these resolved 71 unique haplotypes representing 68 localities. There were 147 variable sites across ingroup haplotypes (47.8%). Despite collecting multiple samples for single localities and the fine-scale sampling, only 14 haplotypes were observed at more than one location, and in every case these were geographically close. Twelve individuals were sequenced from population 23; 11 of these were identical, and one differed by a single base. The general time reversible model plus gamma distribution rate heterogeneity (GTR + Γ) was the best-fit model of evolution for the cyt *b* data. The base frequencies were A = 0.3364, C = 0.3288, G = 0.0626, and T = 0.2722; transition rates were (A-G) 6.0842 and (C-T) 3.8196; transversion rates were (A-C) 0.2571, (A-T) 0.2103, (C-G) 0.5427, and (G-T) 1.0000; and a gamma distribution shape parameter (G) was 0.2988. The ML analysis based on this model resolved a single tree (not shown) with $-$lnL = 3,686.7432. The Bayesian analysis recovered the same topology as the ML tree but with higher nodal support. Three independent Bayesian analyses converged on similar ln-likelihood scores (mean = $-$3,891.8016; range = $-$3,798.5574 to $-$3,971.3222) and generation times (first 80,000 generations were discarded as burn in).

Nicotinamide Adenine Dinucleotide Dehydrogenase Subunit 4 (ND4). Six hundred fifteen bp of ND4 sequence (including 100 bp of the histidine and serine tRNAs) were obtained from 96 individuals of *Xantusia*; there was less variation among ND4 sequences, with 272 variable sites among ingroup sequences (44.2%) resolving 91 unique haplotypes. All sequences translated into amino acids and ended with a TAA or TAG stop codon. The transversion model plus invariable sites plus gamma distribution rate heterogeneity (TVM + I + Γ; Posada and Crandall 1998)

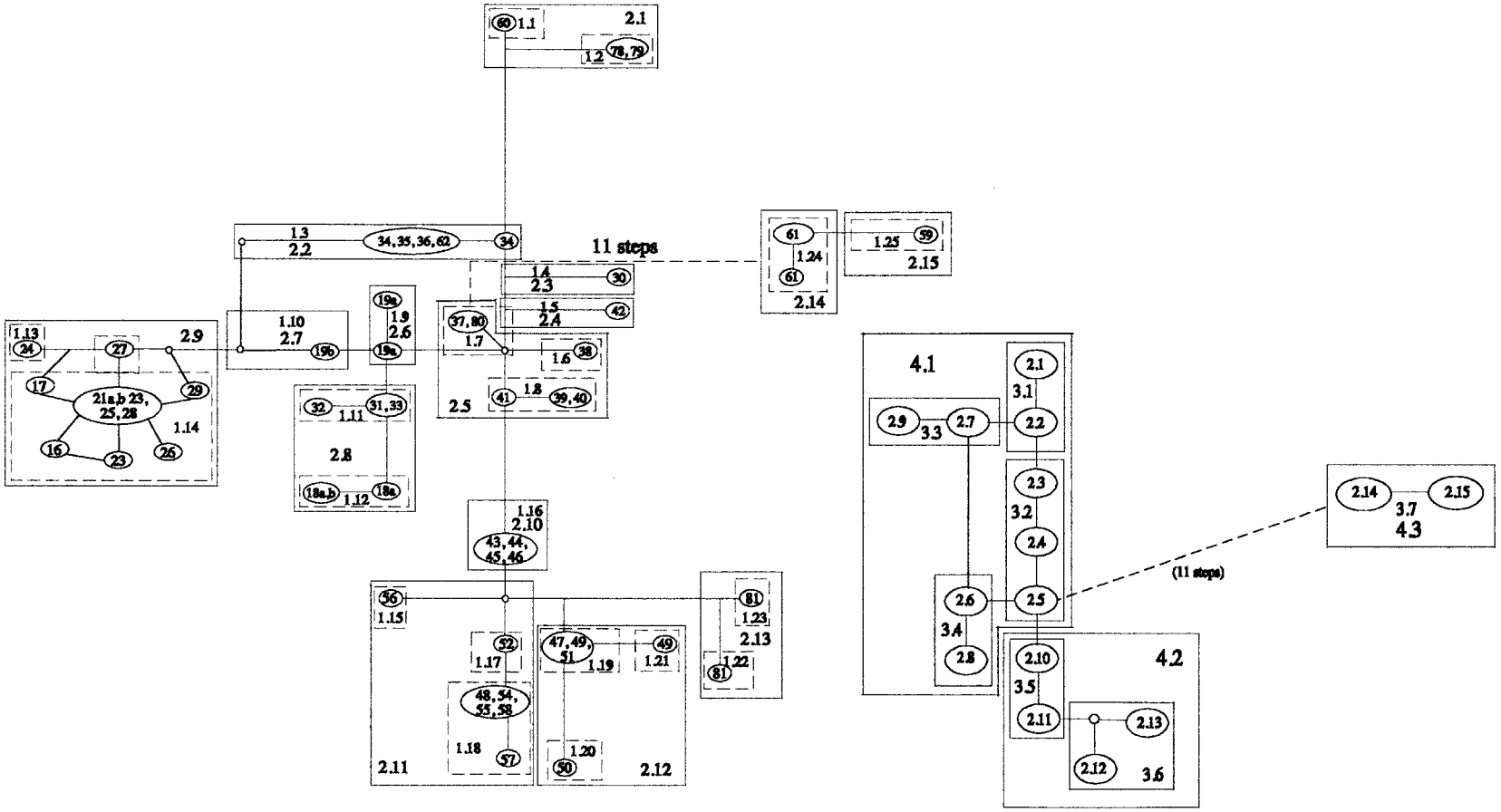

Figure 2: Haplotype network for the 65 cyt *b* sequences from clade A in figure 1. This network represents the most parsimonious connections within the limits of statistical parsimony, using the algorithm of Templeton et al. (1992). Inferred intermediate haplotypes that were not sampled are indicated by a circle, lines are proportional to the number of mutational steps, and clade 4.3 is provisionally joined to this network by 11 steps. Haplotypes are numbered according to their sampling locality (see table A1 and fig. A1 in the online edition of the *American Naturalist*).

was the best-fit model of evolution for the ND4 data. Base frequencies were A = 0.3909, C = 0.3168, G = 0.0797, and T = 0.2126; transition rates were (A-G) 9.3621 and (C-T) 9.3621; transversion rates were (A-C) 0.7714, (A-T) 0.9099, (C-G) 0.9617, and (G-T) 1.0000; the proportion of invariable sites was 0.3532; and a gamma shape parameter was 1.0759. The ML analysis based on this model resolved four most likely trees with −lnL = 6,052.8001. Differences among these four trees (not shown) were restricted to haplotypes from three geographically proximal localities (21a, 23, and 26) that were within the largely unresolved clade A (see below). The Bayesian analyses recovered the same topology as the ML tree but with higher nodal support, particularly at the deeper nodes. Three replicate runs for the Bayesian analyses converged on similar ln-likelihood scores (mean = −6,188.9586; range = −6,154.8470 to −6,225.8354), with consensus trees for the independent runs differing only in the arrangement of haplotypes from three localities (60, 78, and 79).

Combined Phylogeny. The qualitative test of Wiens (1998) revealed no major conflicts between the trees obtained for cyt *b* and ND4. For 94 *Xantusia* terminals (excluding *Xantusia henshawi*) for which we had sequences for both genes, there were 91 unique haplotypes, including one that was shared by two individuals from geographically proximal locations (18a and b; app. A). For phylogenetic analyses we included 15 *X. h. henshawi/gracilis* sequences, although ND4 sequence was available for only two of them (13 left as missing data). The general time reversible model plus invariable sites plus gamma distribution rate heterogeneity (GTR + I + Γ; Rodríguez et al. 1990) was the best-fit model of evolution for the combined gene regions. Base frequencies were A = 0.3660, C = 0.3267, G = 0.0743, and T = 0.2330; transition rates were (A-G) 9.9283 and (C-T) 9.0450; transversion rates were (A-C) 0.6545, (A-T) 0.7639, (C-G) 0.9102, and (G-T) 1.0000; the proportion of invariable sites was 0.3636; gamma distribution shape parameter was 1.0632. A single most likely tree was found, with a ln-likelihood score of −9,785.6682 (fig. 1). For the Bayesian analyses, the first 130,000 generations were discarded as burn in. The mean ln-likelihood score for the consensus tree was −9,939.3337 (range = −9,904.1019 to −9,976.3290). The 50% majority rule consensus tree supported 59 of 73 (ingroup) nodes with posterior probabilities ≥0.95.

The ML and Bayesian trees were very similar to each other and to the single-gene trees (not shown). All major clades present in the individual gene trees were retained in the combined data tree but with slightly higher bootstrap support for most nodes. Differences in the topologies among the gene trees are reflected by lower ML bootstrap values (all <50) at the deeper nodes. Posterior probabilities for all nodes were higher than the ML bootstrap values, but there was a significant correlation between these values ($r^2 = 0.5078$, $P = .0049$). Phylogenetic analysis of the combined data recovered five major clades within the *Xantusia vigilis* complex (fig. 1), all of which have geographically cohesive ranges. All but one of the clades is strongly supported, and three also contain strongly supported geographic subclades. In order of branching sequence, from basal to most nested, these are *X. henshawi* (including *X. h. gracilis*) in the northern peninsular ranges of Baja California and southern California; clade E composed of five deeply divergent lineages found below 24°N latitude in mainland Mexico and Baja California; *Xantusia riversiana*, which is restricted to the California Islands and is the sister species to all populations of the complex above 27°N latitude; clade D composed of two deeply divergent lineages on the coast of Sonora and in west central Arizona; clade C on the Baja California peninsula from 27°N latitude into southern California; clade B in east central Arizona; and a weakly supported, widely distributed clade A found from central and southern California east through southern Nevada, southern Utah, and western Arizona. Thus, the *X. vigilis* complex (clades A–E) is deeply paraphyletic with respect to *X. riversiana.* When the *X. vigilis* complex is constrained to be monophyletic (−lnL = −9,827.6207), the resulting tree is rejected as significantly less likely than our ML tree (S-H test: $P < .004$). There is thus considerable discordance between the clades recovered in figure 1 and the taxa as presently defined within the genus.

All haplotypes from populations inhabiting rock-crevice habitats are indicated with an *R* in figure 1. The transition from plant-litter to rock-crevice habitats appears to have occurred in a total of four major clades (*henshawi*, A, B, and D). Within clade A, there have also been several younger independent origins of the rock-crevice morphotype (fig. 1; *v. sierrae*, *v. utahensis*, northwest Arizona), indicating repeated convergent evolution to this structural niche.

Nested Cladogram Analysis

A statistical parsimony network was constructed from 65 cyt *b* sequences only (fig. 2) within the poorly resolved clade A (fig. 1) because variation at the ND4 region was lower and less informative for examining phylogeographic patterns. The most parsimonious connections were made to a maximum of seven mutational steps. The most distant clade (4.3) was included in the network only by invoking the conditions of pars prob + 1 (Posada et al. 2000). The lower bootstrap support for clade A (62%; fig. 1) is consistent with the tentative inclusion of these haplotypes.

There was significant geographic structure associated with the pattern of genetic diversity, particularly at the

higher nesting levels (fig. 3). A geographic overlay of the statistically significant nesting levels in this cladogram is given in figure 4, and NCA inferences for all significant associations are given in figure 3. At the two-step level, contiguous range expansion was inferred for populations across Nevada and into southern Utah (clade 2.5), and past fragmentation was inferred within clade 2.8 (figs. 3, 4). At the three-step level, contiguous range expansion was inferred for clades 3.1, 3.4, and 3.6, and a long-distance colonization event was inferred for clade 3.5 (figs. 3, 4). Contiguous range expansion is seen across the majority of the southwestern desert region (clade 4.1), while past fragmentation was observed across the total cladogram, reflecting the existence of some historically geographically isolated lineages within the clade A, such as the *X. v. sierrae* haplotypes (clade 4.3; 59 and 61) and the *X. vigilis* haplotypes from Panoche (81) separated by >65 km from other localities (bootstrap = 97; posterior probability = 1.00).

Tests for population growth using Tajima's D were nonsignificant for both clade 3.5 and the total cladogram ($D = -0.0764$ and -0.6959, respectively). However, clade 3.5 and the total cladogram showed significant population growth (highly negative values) using Fu's *F*s-test ($Fs = -13.728$, $P = .000$; $Fs = -11.3046$, $P = .003$, respectively), consistent with population growth in clade A.

Discussion

Species Delimitation in Xantusia

Delimiting species boundaries in the *Xantusia vigilis* complex (table 1; hereafter, the complex) presents difficulties arising largely from the predominant ecological features of the group: low vagility and microhabitat specialization (Bezy 1989). These lizards occupy specific structural niches (e.g., rock crevices, decaying yucca logs), and the available demographic data suggest that both males and females are highly philopatric (Zweifel and Lowe 1966). Correlated with these characteristics is an exceptionally patchy spatial distribution (map in Bezy 1982). Degrees of spatial isolation may range from scales as small as adjacent Joshua trees/logs (*Yucca brevifolia*) to yucca stands within valleys separated by apparently unoccupied mountain ranges to the most isolated populations of the southern Bolson de Mapimi of Durango, Mexico (fig. A1), hundreds of kilometers from the nearest known occurrence of members of the group (Webb 1970). The mtDNA data reported in this study reflect this temporal and spatial disjunction for the members of this complex. Ninety-one unique *Xantusia* haplotypes were found at 81 localities from which we obtained data for both genes, with only a single haplotype shared between two localities (separated by <5 km of nearly continuous habitat). The patchy geographic distribution, presence of unique allozyme alleles (Bezy and Sites 1987), and exclusivity of mtDNA haplotypes in most of the populations (table 1) suggest that ongoing gene flow is not a major cohesive force for members of this complex. Conversely, there is little evidence for the existence of intrinsic reproductive isolation mechanisms, because only two instances of sympatry are known: between *Xantusia henshawi* (*X. h. henshawi*) and *X. vigilis* (*X. v. vigilis*) in southern California (Klauber 1931) and between *Xantusia bolsonae* and *X. v. extorris* in southern Durango, Mexico (Webb 1970). The two sympatric species in these two pairs differ by 27.8% and 15.5% sequence divergence, respectively.

More than the usual word of caution is in order before applying any criterion for delimiting species in this group. Based on the large genetic distances present in the mtDNA data (this study) and allozymes (Bezy and Sites 1987), it might be assumed that more than sufficient time has lapsed for lineage assortment to have erased any evidence of polyphyly or paraphyly generated by the initial divergence. However, small isolated populations may present somewhat of a paradox in this regard. These are unlikely to harbor extensive polymorphism, and their time to monophyly is expected to be relatively short, making deep coalescence less likely (Maddison 1997; Paetkau 1999). At the metapopulation level, however, fixation of alternative haplotypes in small isolated populations may result in retention of evidence of polyphyly and paraphyly until extinction of some of the populations (Patton and Smith 1994). In other words, lineage sorting within small allopatric populations may result in relatively rapid fixation of mutations, but sorting among the populations may involve the relatively slow process of population extinction. Furthermore, the deep branches in several clades that separate many morphologically conservative populations (fig. 1) suggest that speciation probably occurs initially by isolation of populations to small geographic areas, with ecological divergences developing later, if at all. This pattern is known in many low vagility, ecologically and morphologically conservative invertebrates (Bond et al. 2001). However, the presence of recently evolved/ecologically and morphologically distinct populations with low sequence divergence (fig. 1; *X. h. gracilis* and *X. v. sierrae*) suggests that ecologically driven divergence may precede allopatric divergence in other parts of the complex. This dichotomy presents challenges to species delimitation that are not fully resolved in this study, but our results do permit formulation of more focused hypotheses about species boundaries and speciation processes in this group.

In delineating species, we followed the basic protocol described by Wiens and Penkrot (2002) where possible and used the statistical parsimony NCA test of the co-

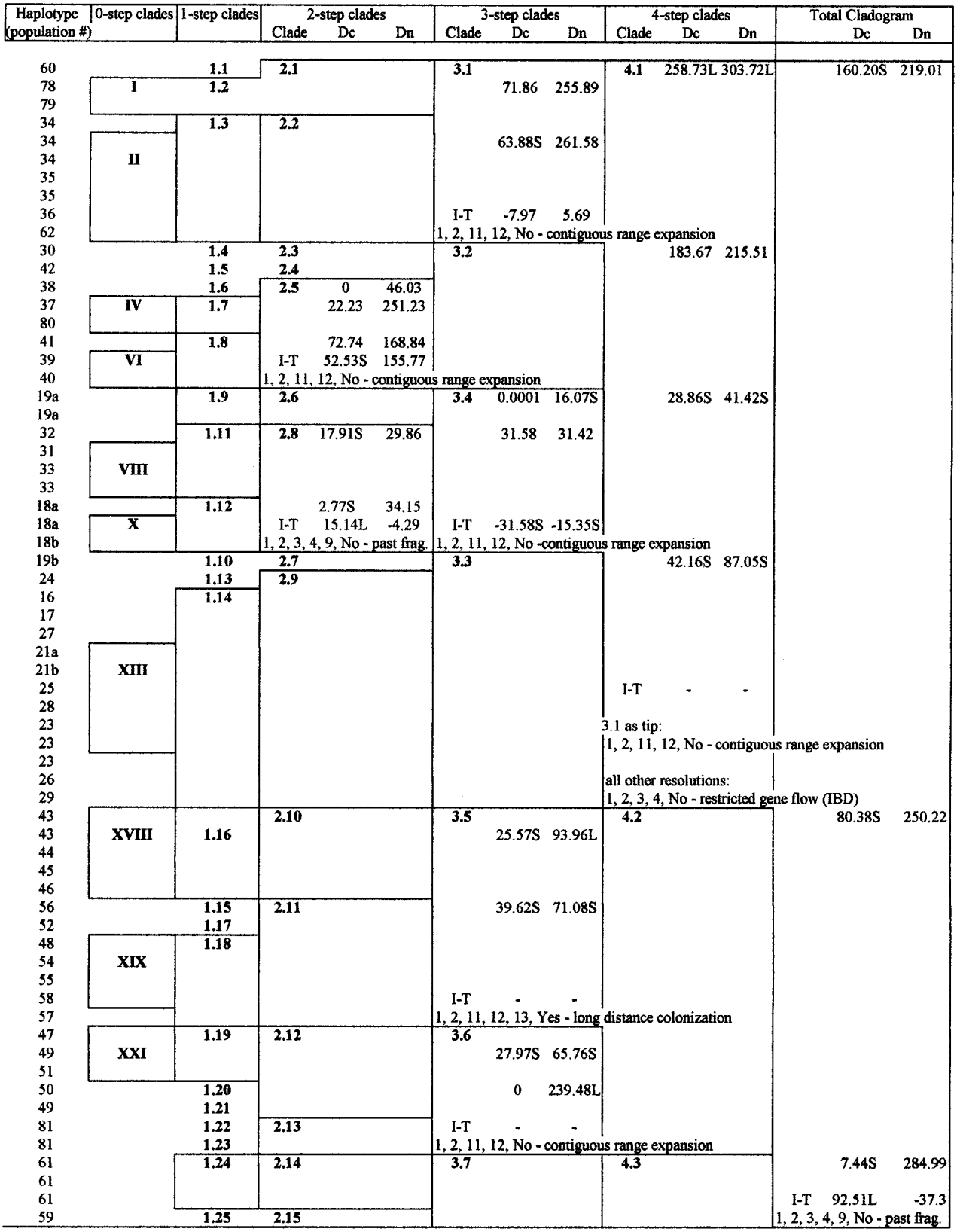

Figure 3: Results from the nested geographical analysis of *Xantusia* cyt *b* haplotypes from clade A (fig. 1). Boxes indicate the nesting structure. Clade (*Dc*) and nested clade (*Dn*) distances are given; *S*, the distance is significantly small at the 5% level; *L*, the distance is significantly large. In those clades containing both tip and interior nested clades, the average distance I–T is given.

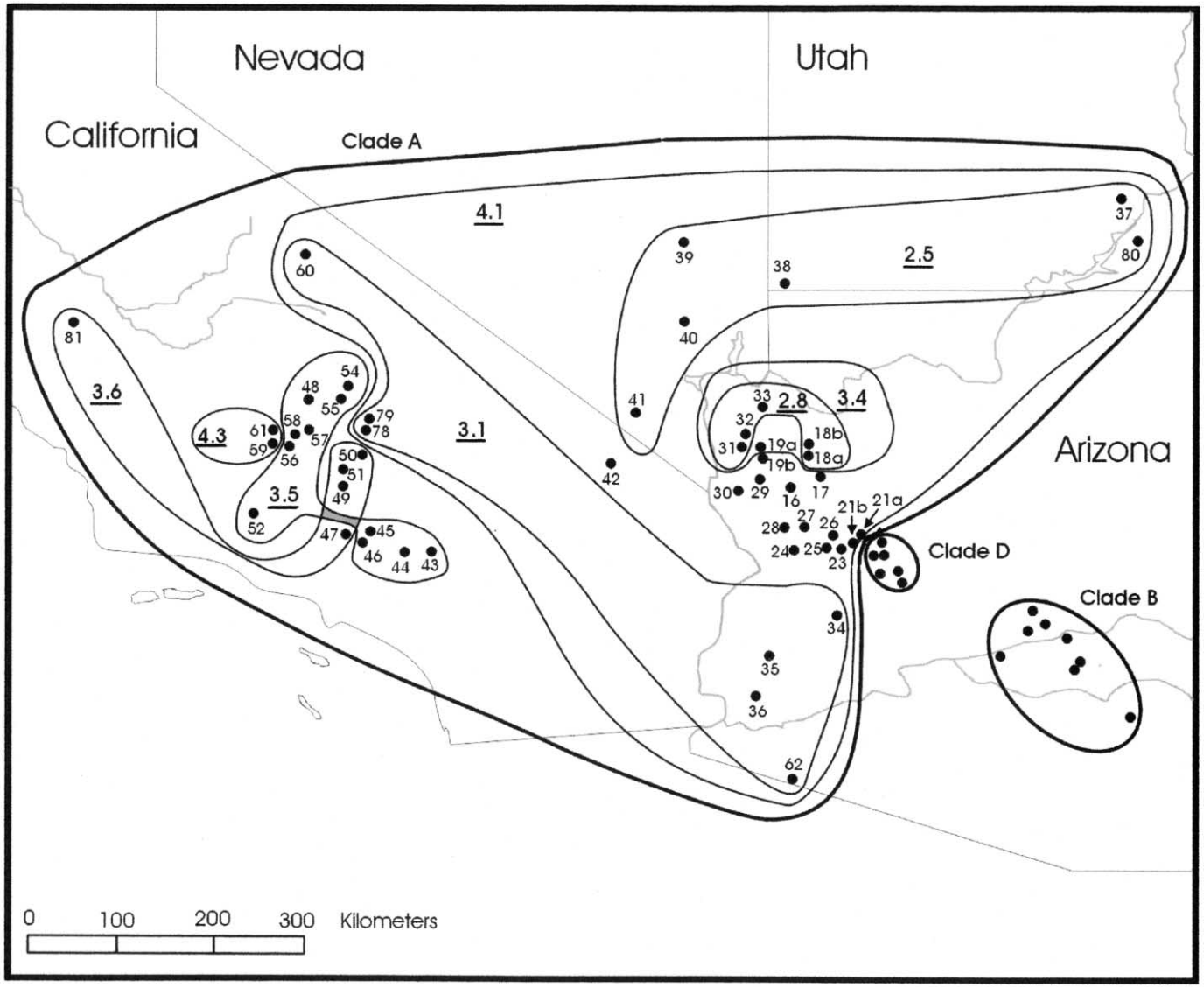

Figure 4: Geographic overlay of the nested clades from figure 2 for terminals in clade A of the phylogenetic analyses (fig. 1). Only those nested clades that were significant (see fig. 3) are indicated. Clades B and D are indicated for phylogeographic completeness of this part of the geographic range.

Table 1: Taxa recognized in the genus *Xantusia*

Previous taxa		Proposed taxa							
Name	Phyly	Name	Phyly	Clade	Morphologically diagnosable	Haplotype divergence (%)	Alleles	Karyotype (2n = 40)	Microhabitat
henshawi:	P	…	…	…	…	…	…	…	…
h. henshawi	P	*henshawi*	P	…	+	5.0	4 (4)	2	Rock-crevices
h. gracilis	M	*'gracilis'*	M	…	+	5.0	2 (2)	…	Rock-crevices
h. bolsonae[a]	M	*bolsonae*	M	E	+	15.5	2 (2)	1b	Rock-crevices
riversiana	M	*riversiana*	M (?)	…	+	15.7	5 (4)	1b	Rocks
sanchezi[a]	M	*sanchezi*	M	E	+	14.1	…	1a	Plants
vigilis:[a]	P	…	…	…	…	…	…	…	…
v. vigilis[a]	P	*vigilis*	P	A	?	3.3	0	1a	Rocks/plants
v. sierrae[a]	M	*'sierrae'*	M	A	+	3.3	2 (2)	1a	Rock-crevices
v. utahensis[a]	M	*vigilis*	M	A	?	1.0	…	…	Rocks/plants
(*v. arizonae*)[a]	P	*vigilis*	P	A	?	.4	0	…	Rock-crevices
(*v. vigilis*)[a]	…	Sonora	M (?)	D	+	4.7	…	1b	Plants (cardon)
v. arizonae[a]	P	*arizonae*	M	D	?	4.7	7 (7)	1b	Rock-crevices
(*v. arizonae*)	…	*bezyi*	M	B	?	9.2	3 (3)	…	Rock-crevices
v. extorris[a]	M	*'extorris'*	M	E	?	14.1	0	1b	Plants
v. gilberti[a]	M	*gilberti*	M (?)	E	+	18.8	…	…	Rocks/plants
v. wigginsi[a]	P	*wigginsi*	M	C	?	9.8	1 (1)	1a	Plants
(*v. wigginsi*)[a]	…	Magdalena	M	E	+	18.8	…	…	Plants

Note: Taxa previously recognized on the basis of morphology are given, followed by our proposed arrangement in which species are based on monophyletic haploclades and corroborative evidence. Listed for each taxon is the nature of the contained haplotype lineage (phyly: M = monophyletic; P = polyphyletic or paraphyletic; ? = single haplotype); whether the entity is diagnosable using current data for scalation (morphologically diagnosable: plus sign = all individuals can be separated from those of other taxa; question mark = slight overlap with individuals of other taxa [Bezy and Flores-Villela 1999]); mean maximum likelihood distance from nearest clade/lineage (haplotype divergence %); the number of allozyme alleles that are unique within *Xantusia* followed (in parentheses) by the number of loci at which these segregate (Bezy and Sites 1987); karyotype (Bezy 1972; 1b differs from 1a by one pericentric inversion; 2 differs from 1a by two pericentric inversions [one of which is shared with 1b]); and microhabitat (plants are agave and/or yucca logs).

[a] Members of the *Xantusia vigilis* complex as treated in this article.

hesion species concept (CSC) for the poorly resolved terminals in clade A. For the CSC only the first hypothesis relating to the sampled organisms being derived from a single evolutionary lineage can be tested here. Other data were not available for each population to permit testing of the second hypothesis of whether the lineages are genetically exchangeable and/or ecologically interchangeable, although general microhabitat associations and morphotypes can offer provisional evidence. In addition to morphological and microhabitat data, we examine the available data for allozymes and karyotypes (table 1) for the presence of corroborating evidence that exclusive or nonexclusive lineages (evidenced by haploclades) meet the criteria we proposed for species recognition.

Detailed discussion of individual clades and taxa is given in appendix C in the online edition of the *American Naturalist*, and results of all inferences are summarized in table 1. Under the original taxonomy, four species and 10 subspecies were recognized, but we suggest that 11 species and three candidate species be recognized on the basis of the criteria used in this study. The candidate species are presented as hypotheses (identified by single quote marks in table 1) in need of further testing, while sampling gaps are evident in figures 1 and A1. One of the candidate species, *Xantusia 'extorris,'* is deeply divergent in mtDNA but is not fully diagnosable on the basis of existing data for allozymes, karyotypes, and scalation. Recognition of the other two candidate species (*Xantusia 'gracilis'* and *Xantusia 'sierrae'*) renders *X. henshawi* and *X. vigilis* (respectively) as nonexclusive units, and stronger evidence of the genetic connectedness of the populations within these latter two units is needed. Directed field sampling is being targeted for the poorly known lineages to permit more rigorous comparative study of the geography of speciation in these clades (see below).

Biogeography and Speciation within the Xantusia vigilis *Complex*

Populations of the complex are scattered across all four major North American deserts but are nearly continuous only in areas of the Mojave Desert with moderate elevations, high winter precipitation, low maximum summer temperatures, and an abundance of yucca habitat (Bezy 1988). Climate and vegetation similar to that of the Mojave prevailed across much of the North American Desert re-

gion in the Late Wisconsin (summaries in Betancourt et al. 1990). Even in the Lower Colorado Desert, the hottest and driest region in North America, there is clear evidence of the presence of Joshua trees (*Y. brevifolia*) and California junipers (*Juniperus californicus*) during the Middle Wisconsin (43,000 bp), with the latter persisting to 8,900 bp, indicating that greater winter precipitation and cooler summer temperatures prevailed until the Middle and Late Holocene (Van Devender 1990). This suggests that mtDNA differences between samples should be relatively small throughout much of the range, an expectation met by most samples within clade A (*X. vigilis*) from the Mojave Desert, and extending into portions of the southern Great Basin and northern Sonoran Deserts (figs. 1, 2).

The existence of deeply divergent mtDNA clades (18.8%–26.9%) elsewhere in the complex indicates the occurrence of ancient sundering events whose genetic signatures were not erased by these late Wisconsin vegetation changes. The divergence between the northern (A–D) and southern (E) clades (22.2%) is consistent with the Miocene rise of the Sierra Madre Occidental, which today isolates *X. bolsonae*, *X. 'extorris'*, and *Xantusia sanchezi* from all other members of the genus. *Xantusia gilberti* presumably has occurred throughout its history in the Sierra La Laguna, a granitic block initially located along the Pacific coast of Mexico south of the landmass that became the remainder of the Baja California peninsula (Gastil et al. 1983).

Both *X. gilberti* and *X.* Magdalena conform to the southern Miocene vicariant complex of Baja California (Grismer 1994) based on their lack of contact with other species to the north, the depth of mtDNA divergence, and the presence of sister species in mainland Mexico below latitude 25°N (fig. 1). The nearest relatives of *X. gilberti* and *X.* Magdalena are other members of the southern clade in mainland Mexico rather than the populations of *Xantusia wigginsi* found to the north on the peninsula. High levels of sequence divergence (21.3%) suggest that the pattern in *Xantusia* may be much older than the vicariant events usually hypothesized for the fauna of Baja California peninsula, namely the formation of the Gulf of California (~5.5 Ma), the isthmus of La Paz (~3 Ma), and the mid-peninsula seaway (~1 Ma; Grismer 1994; Upton and Murphy 1997). *Xantusia gilberti* is restricted to high elevations of the Sierra La Laguna (Galina-Tessaro et al. 1995), and the Magdalena plains populations are isolated from it and from *X. wigginsi* to the north by substantial distribution gaps (table A1). The northern gap appears to correspond with an abrupt transition in climate, vegetation, and substrate, a conclusion independently reached by Grismer (2002) for other taxa in this region.

The distributional gaps between the three clades on the Baja California peninsula resemble those of their host plants *Yucca vallida* and *Agave* spp., which have been attributed to ancient seaways (Gentry 1978; Turner et al. 1995) and suggest the possibility of shared vicariant events. However, *Xantusia* appears to be absent from *Y. vallida* forests in the entire cape region (R. L. Bezy, personal observation) and vast areas of the Vizcaino peninsula (Grismer et al. 1994). Clearly, identification of shared biogeographic history for the biota on the peninsula requires a closer examination of the precise magnitude and geographic position of genetic signatures of hypothesized seaways for a greater variety of animal and plant taxa (Grismer 2002).

The third clade on the Baja California peninsula (clade C, *X. wigginsi*) occurs north of ~27°N latitude, and its mtDNA divergence (10.9%) from other members of the northern clade is consistent with a vicariant event involving the late Miocene formation of the Gulf of California and the rifting of the peninsula to the northwest. Samples that are clearly members of clade C extend into the lower Colorado Desert of southern California to at least 33°N latitude (fig. 1). Two enigmatic haplotypes (76, 77) found at the northern end of the clade, near the transition to the Mojave Desert, differ extensively from each other (7.2%) and from other *X. wigginsi* (8.9%) and are recovered as basal to clade C (fig. 1). The two have no closer relationship with the Mojave haplotypes just to the northwest (9.7% difference), and they may be the result of a vicariant event related to the northward extension of the Gulf of California into the San Gorgonio Embayment (Gastil et al. 1983). The distribution and relationships of the haplotypes in this and other areas in southern California remain unclear, and further sampling is in progress.

Xantusia bezyi (clade B) and *Xantusia arizonae* (clade D, excluding locality 63) are found in predominately granitic rock-crevice habitats of central Arizona (Klauber 1931; Bezy 1967*b*; Papenfuss et al. 2001). The known populations of the two clades are separated by a distribution gap of about 160 km, which remains despite extensive fieldwork (fig. A1). This gap consists of the low-lying Verde and Agua Fria river valleys and mountain ranges dominated by basaltic lava flows that may have isolated these two haploclades. Data from allozymes (Bezy and Sites 1987) and mtDNA indicate that *X. arizonae* is restricted to a small area (localities 10–15).

One of the more surprising phylogenetic relationships recovered in the gene trees is that a population restricted to cardon cactus (*Pachycereus*) on the coast of Sonora (*X.* Sonora, 63; Felger 1965) is the sister group to *X. arizonae* found in rock crevices in west central Arizona (fig. 1). The two differ markedly in habitat and morphology, although at least one morphological feature appears to support this relationship (R. L. Bezy, personal observation). The occurrence of isolated populations of *Xantusia* and *Idria* (the

Boojum tree) in the same region on the coast of Sonora has long been considered evidence of transgulf dispersal from the Baja California peninsula (Humphrey 1974), a hypothesis that can be rejected for *Xantusia* on the basis of the mtDNA evidence.

The existence of deep divergences among the biota of regional deserts (e.g., Grismer and McGuire 1996; Hafner and Riddle 1997; Zamudio et al. 1997; Orange et al. 1999; Riddle et al. 2000*a*, 2000*b*, 2000*c*; Rodríguez-Robles and De Jesús-Escobar 2000) has been interpreted as a shared response across taxa to vicariant events (Riddle et al. 2000*c*). However, the pattern in *Xantusia* does not match those summarized in Riddle et al. (2000*b*), possibly due to the much older age of this group. Like Murphy (1983*a*, 1983*b*), Grismer (1994), and Upton and Murphy (1997), we find that Miocene and Pliocene vicariant events probably played the dominant role in lizard speciation in the deserts of North America. The data for *Xantusia* indicate that divergences among lineages (particularly within clade E) that differ only slightly in morphology are ancient and probably predate the development of both the Sierra Madre Occidental and the Baja California peninsula. Pleistocene climatic oscillations emphasized by earlier authors (Orr 1960; Savage 1960) may have played a major role in some groups, (e.g., *Gambelia wislizenii*; Orange et al. 1999), but at least for *Xantusia* they did not erase the deeper phylogeographic signals obtained from populations found at lower latitudes or in the isolated rock-crevice habitats of the northern part of the range. Further, the microhabitat specificity, especially for rock crevices, and extremely low vagility of *Xantusia* populations suggest that many of the clades recovered in this study may not have experienced high rates of historical range shifts and that the genetic signatures of the deep history of this group might still permit valid inferences to be made about the geographic details of speciation (Losos and Glor 2003).

The separate and combined analyses of the mitochondrial data support a minimum of seven independent origins for morphotypes associated with occurrence in rock-crevice habitats (fig. 1). The morphological features associated with this habitat are a large flat body, long limbs, boldly spotted color pattern, and certain features of scalation. The occurrence of similar features among such a large number of lineages, including the basal clade of the genus (*X. henshawi*), could be interpreted as indicating that the rock-crevice morphotype is primitive within the genus. We believe that this is not the case because the morphotypes occur in widely scattered regions of the range, and it seems unlikely that these regions were formerly connected by continuous rock-crevice habitat. The limited distribution of this habitat suggests rather that these are all independent ecological and morphological transitions. The species boundaries we hypothesize in this article suggest that this transition has been associated with speciation in at least four cases: *X. henshawi* (84–87), *X. bolsonae* (1), *X. bezyi* (2–9), and *X. arizonae* (10–15). However, in the case of *X. vigilis* such transitions have occurred within a single species (clade A), and should further study support species recognition for *X. 'sierrae'*, it may then represent a well-documented example of recent speciation (as suggested by low mtDNA divergence) within or peripatric to the range of its ancestral species (clade A, fig. 1). This parallels the situation seen in *X. 'gracilis'* and *X. henshawi* (Lovich 2001). Distinguishing between speciation via ecological/morphological shifts into rock-crevice microhabitat and similar transitions occurring at the intraspecific level is ultimately important for understanding the origin and spread of novel traits (Coyne et al. 1997) and the general causes of speciation (Barraclough and Nee 2001); linking pattern to process will in turn depend on how rigorously species are delimited (Harrison 1998).

Xantusia *as a Model System: Issues Relevant to Delimiting Species and Inferring Speciation Processes*

We recognized a total of 11 *Xantusia* species as defined by the Wiens-Penkot (W-P) criteria and hypothesize three candidate species (table 1). With the exception of *X. henshawi* and *X. vigilis*, the species hypothesized here are exclusive (although this is not a requirement of the W-P method) and correspond to deeply divergent (all but three are >8.7% uncorrected sequence divergence) haploclades or lineages with distinct allopatric distributions. All have statistically significant differences in morphology (Bezy and Flores-Villela 1999), but only six are strictly (100%) diagnosable with available data on scalation. Karyotypes, unique allozyme alleles, and unique microhabitats provide corroborative evidence for species recognition of many of these (table 1). Two of the candidate species, *X. 'sierrae'* and *X. 'gracilis'*, have distinctive morphology, unique allozyme alleles, and occur in close proximity to their nearest relatives, but neither represents basal mtDNA clades, and their recognition renders *X. henshawi* and *X. vigilis* as nonexclusive entities. *Xantusia 'extorris'* is deeply divergent genetically but lacks corroborative evidence. Further corroboration of our hypotheses depends on well-designed population and character sampling, and these are briefly considered here in the context of the distinctive biological attributes of *Xantusia*.

Widely distributed species are frequently composed of many distinct populations exhibiting recognizable genetic differences (Hughes et al. 1997), and available molecular technologies can resolve unique genetic differences to the level of small local demes and even individuals (Hedrick 1999). Empirically testing the boundaries of species by sampling natural populations will then in part be a func-

tion of the spatial scale of sampling for a given focal species, the geographic density of sampling points within the defined scale, and ultimately the number of individuals sampled per locality. Confounding these points, Masta et al. (2003) have suggested that species whose ranges have been subjected to some historical cycles of expansion and contraction may experience random extinction of haplotypes (via a number of processes) in isolated populations, and that this kind of demographic history would produce a patchwork geographic distribution of haplotypes (Nichols and Hewitt 1994; Ibrahim et al. 1996). Such a pattern is expected to be magnified in low vagility organisms, and the NCA will produce a large Dn and small Dc. Under these conditions the NCA inference key is misled to infer a long-distance colonization event.

Our results produced one case of inferred long-distance colonization in clade 3.5 (figs. 3, 4), which we suspect is a false positive when we apply the modifications to the inference key suggested by Masta et al. (2003). First, is it biologically realistic that the *X. vigilis* could have undergone long-distance movements? Mark-recapture data show that individual *X. vigilis* may remain under the same yucca plant for a decade (Zweifel and Lowe 1966), suggesting that it is extremely unlikely that these lizards would undertake long distance movements. We answer "no" to this question. Second, are the nested haplotypes inferred to have undergone long-distance colonization within a clade that shows evidence of population growth by other methods? Third, at the level of the entire cladogram, does the clade not inferred to have produced long-distance colonization not show evidence of past population growth with other methods? For the second and third questions, Tajima's D was nonsignificant while Fu's *F*s was significantly negative, consistent with a recent range expansion, but both tests assume mutation-drift and migration-drift equilibria. Furthermore, there are museum records for intermediate localities (in clade 3.5) that were not sampled for this study, so cross-validation tests of the NCA inference of long-distance colonization are equivocal at best. We suggest that the more conservative inference is "insufficient evidence to discriminate between long-distance colonization and past fragmentation followed by range expansion" (Masta et al. 2003, p. 1552). This region is the focus of further sampling.

On a related point, an overdispersed sampling design will yield patterns of genetic variation consistent with both population fragmentation and restricted gene flow/isolation-by-distance in the NCA inference key (Hedin and Wood 2002), and estimating the exclusivity of population samples (and hence making a distinction between low gene flow and no gene flow) is heavily dependent on sample sizes and assumptions of coalescent theory (Morando et al. 2003). In this study we collected 12 cyt *b* sequences from a single locality (23), which under the most restrictive assumptions of coalescent theory (reviewed by Nordborg 2001) give a probability of sampling the deepest coalescent in the population (what Morando et al. [2003] called a "probability of exclusivity" [P_{excl}]) at an $\alpha = 0.05$ level of certainty of 0.85 (and 0.50 and 0.67). Hedin (1997) has suggested that a dense sampling of many geographically close populations and the inclusion of three to five individuals per locality would be adequate to discriminate between an absence of gene flow and very limited gene flow in low vagility taxa (see also Hedin and Wood 2002). Sample sizes of three or five give P_{excl} values of 0.50 and 0.67, respectively, but many of the restrictive assumptions of coalescent theory will not be met in natural populations, and with few exceptions these violations will reduce the number of individuals needed to capture the deepest coalescent in a sample (Morando et al. 2003).

Our sampling was geographically dense within clade A (where the NCA was performed), with some of the apparent gaps reflecting an absence of populations rather than inadequate sampling, and while this design has probably included a large portion of the genetic diversity within this clade, most localities are represented by one or two haplotypes, so from the perspective of coalescent theory, we cannot with any confidence distinguish between low gene flow and no gene flow among many of these localities. Given the extreme site philopatry of *Xantusia* (Zweifel and Lowe 1966) and its close associations to patchily distributed microhabitats (Bezy 1989), we suspect our sampling is too overdispersed in some regions to detect all relevant phylogeographic signal in this complex, but with the exception of the above qualification for long-distance colonization, at no nesting level did we reach an inference of "inconclusive outcome" or "geographic sampling design inadequate." This suggests that collecting a few animals from many locations (rather than many animals from fewer locations) was the best approach to maximize the genetic variation sampled in this complex, an approach that would be relevant to any species with a similar biology. For further consideration of character sampling see appendix C.

We recognize that there are larger statistical issues, such as the accuracy of the NCA method (Knowles and Maddison 2002), that cannot be considered here, but we would emphasize that the NCA is one of the most assumption-free methods currently available for inferring demographic histories from DNA sequences (Emerson et al. 2001). Although intensive effort will be required to rigorously test demographic and distributional histories of *Xantusia* populations in habitats that probably have shifted over time, we suggest that the biology of these lizards makes them ideal for further comparative study of ecological and geographic aspects of speciation.

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