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PHYLOGEOGRAPHY OF THE JUMPING SPIDER *HABRONATTUS PUGILLIS* (ARANEAE: SALTICIDAE): RECENT VICARIANCE OF SKY ISLAND POPULATIONS?

SUSAN E. MASTA[1]
Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, Arizona 85721

Abstract.—In island systems with diverging populations, the history of island formation and genealogical estimates of divergence dates can be mutually informative. In the ''sky islands'' of southeastern Arizona, climate-induced contraction of woodlands appears to have fragmented populations of woodland-dwelling species onto disjunct mountain ranges. Montane populations of the jumping spider, *Habronattus pugillis*, display striking amounts of phenotypic divergence among ranges. Paleoclimatic estimates date woodland fragmentation at approximately 10,000 years ago, suggesting that phenotypic divergence has been extraordinarily rapid in these spiders. This phylogeographic study of populations of *H. pugillis* attempts to clarify the species' history of isolation and divergence and to address the suitability of available paleoclimatic data for dating divergences among populations of the region's woodland-dwelling organisms. Mitochondrial sequence data of spiders from 13 mountain ranges was used to reconstruct genealogical relationships. Gene trees show that small mountain ranges tend to have populations whose sequences form monophyletic groups, whereas larger ranges do not. Paraphyly among genes from larger ranges could result from either recent migration or incomplete lineage sorting. I use phylogenetic and geographic information to test these alternatives, and conclude that incomplete lineage sorting best explains the observed paraphyly. Gene trees are concordant with some of the predictions of vegetation history generated by examination of topography. Dates estimated for divergence of populations vary from 30,000 years to more than 2 million years ago, suggesting multiple vicariance events that are older than would be inferred from paleoclimatic studies. These findings illustrate that use of any single paleontological dataset to calibrate molecular clocks can potentially greatly underestimate actual divergence times.

Key words.—*Habronattus pugillis*, lineage sorting, migration, mitochondrial DNA, molecular clock, phylogeography, sky islands.

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Studies of historical landscape change may provide a framework for examining vicariance events in populations of organisms, whereas genealogical studies of organisms may aid interpretation of the history of landscape change. In island-dwelling organisms, we may be able to place boundaries on divergence dates of populations if island formation can be dated (e.g., Carson and Clague 1995; Fleischer et al. 1998). This is the case both with true oceanic islands and with terrestrial ''islands'' of habitat, whose historical dynamics may sometimes be inferred from paleoclimatic data.

With terrestrial habitat islands, however, it may often be more difficult to reconstruct accurate phylogeographic histories of organisms. Whether an organism's history tracks island history may depend on a host of factors difficult to measure or infer. For instance, detailed knowledge of an organism's requirements in its current environment may not be adequate to deduce its needs in different environmental contexts in the past. Thus, habitat presently used by the organism may not be equivalent to habitat it has used historically. In addition, extrapolation of data on past landscape change over broad regions may overlook pockets of local variability that could have created habitat patches affecting the organism's evolutionary dynamics. Therefore, reliance on a single source of external data to date vicariance events or calibrate molecular clocks may often be inadequate, so multiple sources of information should ideally be used when reconstructing organismal histories.

The ''sky islands'' of southeastern Arizona are forested mountain ranges separated by low-lying desert and grassland. These isolated ranges feature the northernmost extent of Madrean evergreen woodland, which becomes contiguous as it extends south into northern Mexico (Brown and Lowe 1980; Brown 1982). Some organisms inhabiting Madrean evergreen woodland are known to show phenotypic differentiation among sky island populations. A particularly striking example is the jumping spider, *Habronattus pugillis* Griswold (Araneae: Salticidae). This ground-dwelling spider inhabits Madrean woodland habitats in southeastern Arizona, generally at elevations above 1400 m, and is absent from the lower-elevation desert and grassland (Maddison and McMahon 2000). *Habronattus pugillis* has been collected from Nayarit, Mexico (Griswold 1987), through the Sierra Madre Occidental of Sonora, to its northernmost limit in southeastern Arizona (G. Bodner, W. Maddison, and S. Masta, pers. obs.). Each Arizona sky island range contains a unique male phenotype. These phenotypes are uniform within most ranges and divergent among ranges (Maddison and McMahon 2000). Males vary among ranges both in morphological traits displayed to females during courtship and in their courtship dances. For example, males differ in the amount and coloration of the scales on their palps and first legs, in facial patterns, and in amount and type of leg and palp movement during courtship. This pattern of phenotypic divergence has been attributed to sexual selection (Maddison and McMahon 2000).

The differentiation seen in *H. pugillis* is reflected to a lesser extent in other taxa. Land snails in the genus *Sonorella* (Miller 1967; Bequaert and Miller 1973), the beetle *Scaphinotus petersi* Roeschke that feeds on these snails (Ball 1966), and

[1] Present address: Department of Biology, San Francisco State University, 1600 Holloway Avenue, San Francisco, California 94132; E-mail: smasta@sfsu.edu.

the plant *Castilleja austromontana* (Slentz et al. 1999) each display morphological divergence among montane populations in southeastern Arizona. However, only two studies (on the lily *Lilium parryi*: Linhart and Premoli 1994; on the tree frog *Hyla arenicolor*: Barber 1999a,b) have utilized this natural laboratory to examine genetic differentiation. Little is known of the patterns and rates of genetic evolution in these divergent populations.

The dearth of genetic studies is surprising because the region boasts well-documented paleoclimatic data from fossil packrat middens (Van Devender 1977, 1990; Van Devender and Spaulding 1979) that could potentially help illuminate the history of lineage splitting in woodland-dependent organisms. Although these data have been used to reconstruct the history of vegetation change in the Sonoran Desert and were not intended specifically to address evolution of habitat-restricted organisms, evolutionary biologists regularly use such information to make predictions about biogeographic patterns and to estimate divergence dates. The packrat midden data suggest that today's disjunct patches of woodlands had a greater degree of connectivity during the late Pleistocene to early Holocene, roughly 10,000 years ago, when the climate was cooler and more mesic (Van Devender 1990). Thus, woodland habitat may have spread contiguously across lower elevations at that time, effectively linking mountain ranges. A warming and drying climate has since caused woodlands to move upslope and has fragmented them on the isolated ranges. This climate-induced habitat fragmentation presumably subdivided populations of plants and animals dependent on the woodland habitat, resulting in their present islandlike distributions.

If isolation of *H. pugillis* populations occurred as woodlands retreated to higher elevations 10,000 years ago, then jumping spider populations appear to be phenotypically differentiating at a remarkably rapid rate. Alternatively, if population isolation occurred much earlier than the postulated end-Pleistocene vicariance—thus yielding a slower rate of differentiation—the suitability of midden data to guide conclusions on biogeography and evolution of woodland organisms becomes doubtful. Each of these vicariance scenarios predicts different patterns of genetic differentiation among sky island populations. If populations were isolated from one another within the past 10,000 years, there should be little differentiation of neutral genes among populations, and a genealogy of sequences may yield unresolved relationships. If fragmentation occurred longer ago, there should be greater differentiation and sequences may form monophyletic groups in a gene tree that correspond to mountain ranges of origin. Neigel and Avise (1986) have shown that following isolation, populations progress over time from polyphyly to paraphyly to reciprocal monophyly of sequences from each geographic location. If some ranges became isolated earlier than others due to differences in low-lying topography between them, amounts of genetic differentiation should vary among ranges, and consequently there may be varying amounts of paraphyly and monophyly seen among geographic populations. Therefore, a phylogeographic study (sensu Avise et al. 1987) of *H. pugillis* in these fragmented woodlands may shed light on both the phylogenetic history of *H. pugillis* and the history of the Madrean woodlands in southeastern Arizona. Such information might in turn be used to make phylogeographic predictions for other organisms inhabiting these island habitats.

One factor that can confound interpretation of historical vicariance events is ongoing gene flow between populations. Separating the effects of recurrent gene flow from those of historical association (incomplete lineage sorting) is problematic (e.g., Slatkin 1981; Felsenstein 1982; Strand et al. 1996). When populations have become isolated recently, parental genes may still be shared by the descendant populations. Therefore, incomplete lineage sorting may leave a genetic pattern similar to recent migration. One potential way to separate these two factors is to use a combination of phylogenetic and geographic information to infer whether populations may be exchanging migrants (e.g., Slatkin and Maddison 1989, 1990; Edwards 1993).

In this paper I use neutral mitochondrial variation to make inferences about the population fragmentation in *H. pugillis* that ultimately contributed to the impressive phenotypic divergence of this species discussed by Maddison and McMahon (2000). Mitochondrial DNA sequences coding for NADH dehydrogenase subunit 1 (ND1), $tRNA^{Leu(CUN)}$, and the large subunit ribosomal RNA gene (16S) from populations on 13 southeastern Arizona mountain ranges and three localities in the Sierra Madre Occidental of Mexico are used to construct a genealogy of *H. pugillis.* I then address three issues using data from the gene tree. First, geographic information is used to determine whether migration or incomplete lineage sorting best explains the paraphyletic relationships of sequences from certain mountain ranges. Second, predictions generated from topography are addressed with the phylogeographic patterns of the *H. pugillis* genealogy. Third, a molecular clock estimate is used to infer whether the timing of fragmentation of *H. pugillis* populations is concordant with historical vegetation patterns inferred from packrat midden studies.

Materials and Methods

Eighty-six individuals of *H. pugillis* were collected from 13 mountain ranges in southeastern Arizona (see Fig. 1) and three locations of continuous oak habitat in the Sierra Madre Occidental of Sonora and Chihuahua, Mexico. These locations represent the majority of ranges where *H. pugillis* is known to occur in Arizona (Maddison and McMahon 2000; S. Masta, pers. obs.). Individuals were collected from multiple locations within each range whenever possible. Four individuals of *H. oregonensis* and one individual of *H. geronimoi,* both of which also inhabit the Arizona sky islands, were used to provide polarity in the genealogy. A list of individuals and collection localities is available upon request.

Total genomic DNA was isolated from 89 of the 90 individuals using a modified SDS extraction protocol. Recent reports of mitochondrial fragments that have been transposed to the nucleus (e.g., Zhang and Hewitt 1996) illustrate the importance of verifying that sequences amplified with mitochondrial primers are indeed located in the mitochondria. Therefore, mitochondrial DNA was isolated from the eggs of one *H. pugillis* individual from the Sierrita Mountains

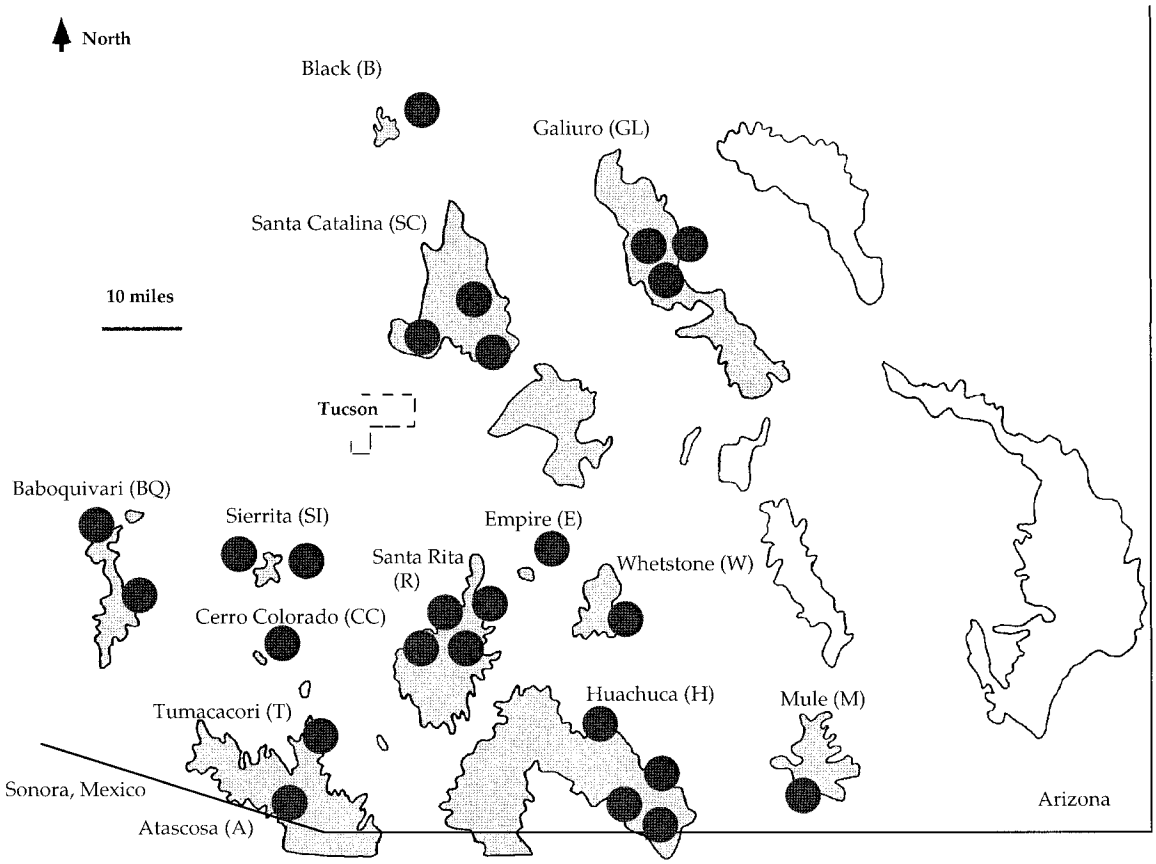

FIG. 1. Outlines of areas covered by Madrean oak vegetation (after Brown and Lowe 1980) on southeastern Arizona mountain ranges. Shaded areas indicate ranges that *Habronttus pugillis* is known to inhabit. Collecting locations of *H. pugillis* used in this study are depicted with circles. Abbreviations of mountain ranges follow their names.

(individual 1), as described by Masta (2000), to confirm similarity to sequences from the genomic DNA extracts.

The region of the mitochondrion spanning the 5′ end of ND1 through the 3′ half of 16S was amplified using the polymerase chain reaction (PCR) and sequenced using primers I designed specifically for *Habronattus*. The primer HbND1 (5′-TGAGCTACTCTTCGAATAGC-3′) corresponds to positions 12,257–12,276 on the J strand in *Drosophila yakuba* mitochondrial ND1 (Clary and Wolstenholme 1985). The primer Hb16S (5′-TTACGGAAGTGCACATATCG-3′) corresponds to positions 14,226–14,245 on the N strand of *D. yakuba*, located in the small subunit ribosomal RNA (12S) coding region.

Amplification by PCR was performed using final concentrations of 0.52 μM of each primer, 3 mM $MgCl_2$, 0.2mM of each dNTP, 1× PCR buffer (as supplied by the manufacturer of Taq polymerase), 1 unit of Taq polymerase, and 1 μl DNA (not quantified). Amplification conditions were: denaturation at 95°C for 30 sec, annealing at 58°C for 1 min, extension at 72°C for 1 min for 35 cycles. PCR products were purified either by gel purification or by spin-columns.

The PCR products were sequenced manually with the GibcoBRL dsDNA Cycle Sequencing system end-labeling kit (Life Technologies, Gaithersburg, MD) or on an ABI automated sequencer (Applied Biosystems, Foster City, CA) at the University of Arizona sequencing facility. The PCR amplification primer HbND1 was used to sequence ND1 through the 3′ half of 16S. Sequences were edited and aligned manually in SeqApp 1.9 (Gilbert 1994).

To determine if the sequences were evolving neutrally, and thus suitable for reconstructing intraspecific relationships, the McDonald-Kreitman (1991) test of neutrality was performed on the ND1 gene using the program DnaSP (Rozas and Rozas 1997). The ratio of synonymous:nonsynonymous polymorphisms within *H. pugillis* was compared to the ratio of synonymous:nonsynonymous substitutions fixed with respect to *H. geronimoi*. These two ratios are expected to be the same if the sequences have been evolving neutrally (McDonald and Kreitman 1991).

Prior to phylogenetic analysis, identical sequence haplotypes were merged using the search-and-merge function of MacClade 3.07 (Maddison and Maddison 1997). Phylogenetic analyses were conducted with PAUP* 4.0 (Swofford 1998) using several approaches. An unweighted heuristic parsimony analysis was performed with indels coded as missing data, starting trees obtained via stepwise addition, simple addition sequence, and TBR branch swapping. Due to the large number of most parsimonious trees, the search was limited to saving 5000 trees. Because a single heuristic search does not guarantee that the shortest tree will be found, multiple searches were conducted to search for islands of shorter trees as suggested by Maddison (1991). One hundred repli-

cate searches were conducted, each limited to saving 50 most parsimonious trees. Neighbor-joining analyses (Saitou and Nei 1987) were also performed with the HKY85 (Hasegawa et al. 1985) model of evolution. One hundred bootstrap replicates (Felsenstein 1985) were conducted to assess confidence in nodes of the trees, using both parsimony and neighbor-joining criteria. Because *H. geronimoi* lies outside the clade containing *H. pugillis* and *H. oregonensis* (M. Hedin and W. Maddison, pers. comm.), it was used to root all trees.

Models of evolution that incorporate rate heterogeneity have been found to yield the best estimates of sequence divergence (Yang 1994; Yang et al. 1994). Therefore, to estimate sequence divergence among individuals, maximum likelihood (ML) was used with PAUP*, using the following method. First, the neighbor-joining tree was used to estimate base frequencies and substitution rate-matrix parameters, using the general time reversible (Yang 1994) model of evolution, allowing for some sites to be invariable and variable sites to follow a gamma distribution (GTR + I + Γ) (Gu et al. 1995). These ML estimates were then used to calculate pairwise distances between all sequences and were used in a heuristic search to find the ML tree topology.

As a measure of long-term effective population size of spiders on each range, average nucleotide diversity values (π; Nei and Li 1979) within each mountain range were calculated from the above pairwise distances. For mitochondria, π is an estimator of $2N_e\mu$, where N_e is effective population size of females and μ is the neutral mutation rate. To date vicariance event(s) that subdivided populations of *H. pugillis*, two different calculations were performed. Takahata and Nei (1985) and Wakeley and Hey (1997) suggest that, in the absence of fixed differences between populations, one method for estimating minimum divergence time between populations is to calculate the smallest sequence divergence between individuals from each population. This method was used with the above ML calculated sequence divergences. It was found that this method yields a reasonable estimate of divergence time when the time since population splitting is short, but is an overestimate with older divergences (Takahata and Nei 1985). Therefore, the method of Nei and Li (1979) and Nei (1987) was also used to estimate divergence times. This method uses information from pairwise differences between lineages with a correction for differences due to ancestral polymorphisms to estimate the net nucleotide difference, such that $d = d_{xy} - 0.5(d_x + d_y)$, where d_{xy} is the mean number of nucleotide differences between populations x and y and d_x and d_y are the average number of nucleotide differences within populations x and y (Nei 1987, eq. 10.21). These calculations were performed with the program DnaSP (Rozas and Rozas 1997), with a Jukes and Cantor (1969) correction. The estimates of divergences from both the Takahata and Nei (1985) and Nei and Li (1979) methods were then divided by the molecular clock rate of 2.3% divergence per million years, as calibrated for mitochondria of arthropods (Brower 1994).

Methods employing phylogenetic and geographic distance information that can potentially distinguish between lineage sorting and isolation-by-distance migration have been developed by Slatkin and Maddison (1990) and Templeton et al. (1995). These methods require relatively large and equal (Slatkin and Maddison 1990) sample sizes from each locality. Given the realities of hand-collecting small, cryptic, seasonally active spiders from multiple ranges of disparate sizes, I was unable to collect enough individuals in all ranges to justify using these methods. However, it was possible to devise tests that could be used with unequal sample sizes.

If there is no migration between ranges, then ranges with smaller effective population sizes should show sequences that are more fully sorted than sequences from ranges with larger effective population size. Neigel and Avise (1986) and Tajima (1983) showed that populations isolated for $4N_e$ generations are expected to be reciprocally monophyletic, whereas those isolated less than $4N_e$ generations may be paraphyletic. To determine whether monophyly may be correlated with smaller N_e, I used area of *H. pugillis*' potential habitat as a rough indicator of their population size.

Because *H. pugillis* is closely associated with Madrean oak (*Quercus* spp.) woodlands, the geographic area these woodlands occupy on each mountain range may roughly reflect the population sizes of spiders on each range. In the absence of natural-history information adequate to provide reasonable population size estimates, the use of area as a rough measure of population size seems reasonable for these spiders. The current distribution of Madrean evergreen vegetation was measured from a scanned image of Brown and Lowe's (1980) vegetation map, and the area occupied by this habitat was calculated with NIH Image 1.61 (available at http://rsb.info.nih.gov/nih-image/download/html). If migration of *H. pugillis* is limited or absent, mountain ranges that are geographically most isolated should be no more or less likely to harbor populations of spiders whose DNA sequences form a single clade than mountain ranges that are geographically proximate. Therefore, distances between ranges were measured to determine closest distances between oak woodlands on different ranges. To examine possible historical woodland connections between ranges, I drew maps depicting the hypothetical distribution of woodlands, at elevations ~ 152 m (500 ft), ~ 305 (1000 ft), and ~ 457 m (1500 ft) below their current ~ 1523-m (5000-ft) elevation, using U.S. Geological Survey topographic maps.

Results

Approximately 815 bp of ND1-16S sequence was obtained for 87 individuals. This sequence encompasses the 5′ half of ND1, $tRNA^{Leu(CUN)}$, and the 3′ half of 16S. For another four individuals, 440 bp of ND1 was obtained (Empire 1 and 4, Tumacacori 1 and 2). GenBank accession numbers are AF239933–AF239941, AF239948, AF239952, AF239955 and AF255805–AF255883. Only a single 4- or 8-bp indel was present in the aligned sequences, located between $tRNA^{Leu(CUN)}$ and 16S (see also Masta 2000). Eighty-one unique haplotypes of *H. pugillis* were found among the 86 individuals sequenced, with a total of 160 variable sites. Three haplotypes were shared by more than one individual. Two such haplotypes were found in individuals from the same mountain range; the third occurred in individuals from neighboring mountain ranges. The sequence obtained from the mitochondrial DNA isolation was most closely related to sequences from individuals from the same mountain range, indicating that sequences obtained from total genomic DNA

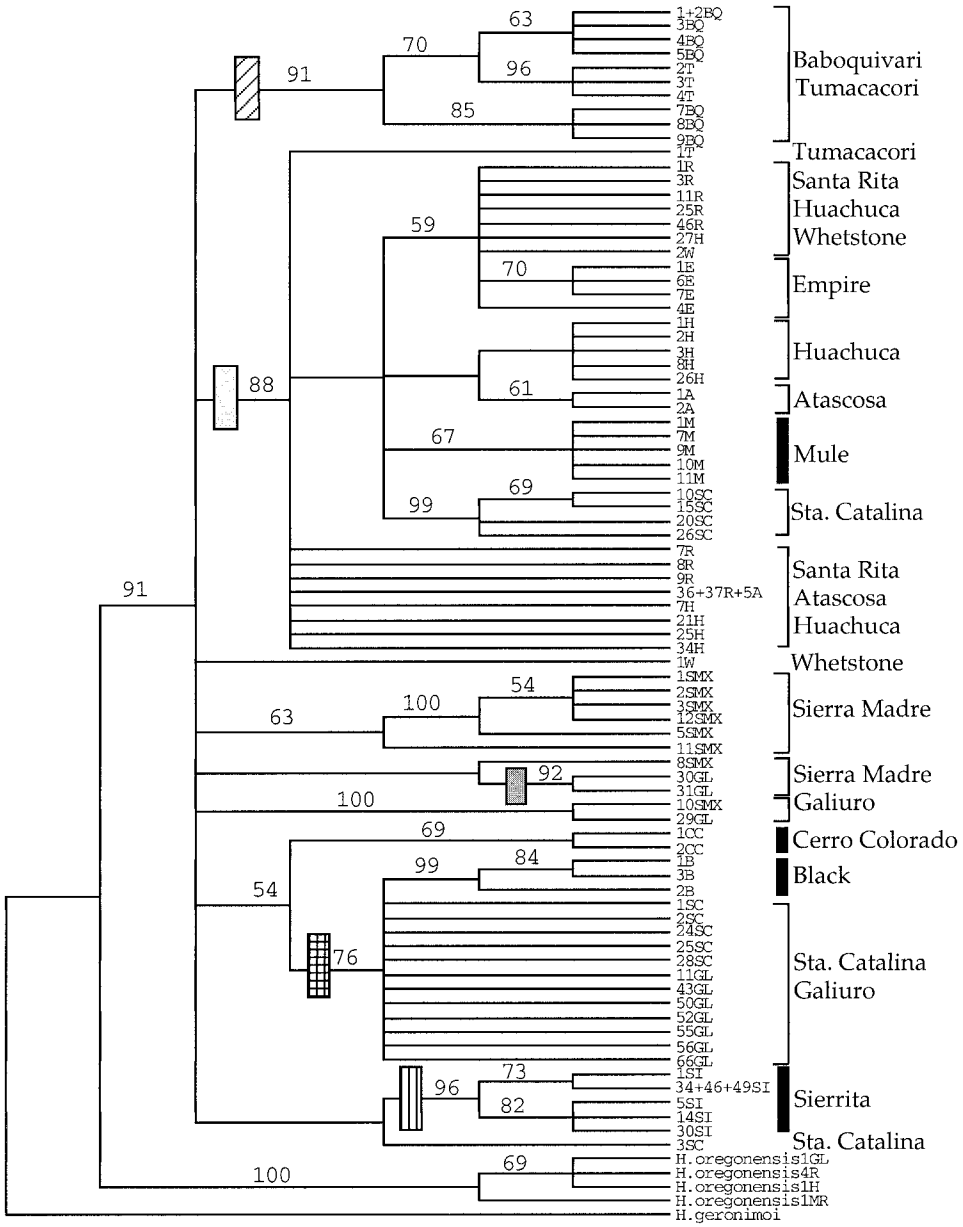

FIG. 2. Parsimony strict consensus of 5000 trees for *Habronattus pugillis* populations. Length = 368, consistency index (CI) = 0.6087, retention index (RI) = 0.8519 for all trees. Bootstrap values greater than 50% are shown above the branches. The patterned boxes across the branches indicate basal clades with > 50% bootstrap support referred to in the text. The dark bars marking terminal taxa along the right tree margin indicate clades corresponding to geographic location. Taxon names correspond to their collecting locations and are abbreviated as follows: B, Black; SC, Santa Catalina; GL, Galiuro; BQ, Baboquivari; SI, Sierrita; CC, Cerro Colorado; T, Tumacacori; A, Atascosa; R, Santa Rita; E, Empire; W, Whetstone; H, Huachuca; M, Mule Mountains; SMX, Sierra Madre Occidental.

isolations are not copies of mitochondrial genes transposed to the nucleus.

Results of the McDonald-Kreitman test were consistent with neutral evolution of the ND1 gene. The ratio of polymorphic synonymous:nonsynonymous mutations within *H. pugillis* was 71:7 versus 15:3 fixed substitutions with *H. geronimoi*. These ratios are not significantly different by Fisher's exact test ($P = 0.39$).

The single heuristic search (which was limited to saving 5000 most parsimonious trees) yielded trees of length 368, with the strict consensus tree depicted in Figure 2. The 100 replicate island searches also yielded only trees of length 368, suggesting islands with the shortest trees had been found. The strict consensus tree constructed from these replicate searches supports the same clades as the single heuristic search consensus tree. Neighbor-joining analysis with the HKY85 model of evolution yielded a tree (not shown) consistent with the parsimony consensus trees. The ML search was terminated when three trees of similar likelihood were found. All trees had identical topologies, differing only in the branch lengths of very similar sequences (Fig. 3).

Both parsimony and neighbor-joining trees show five basal

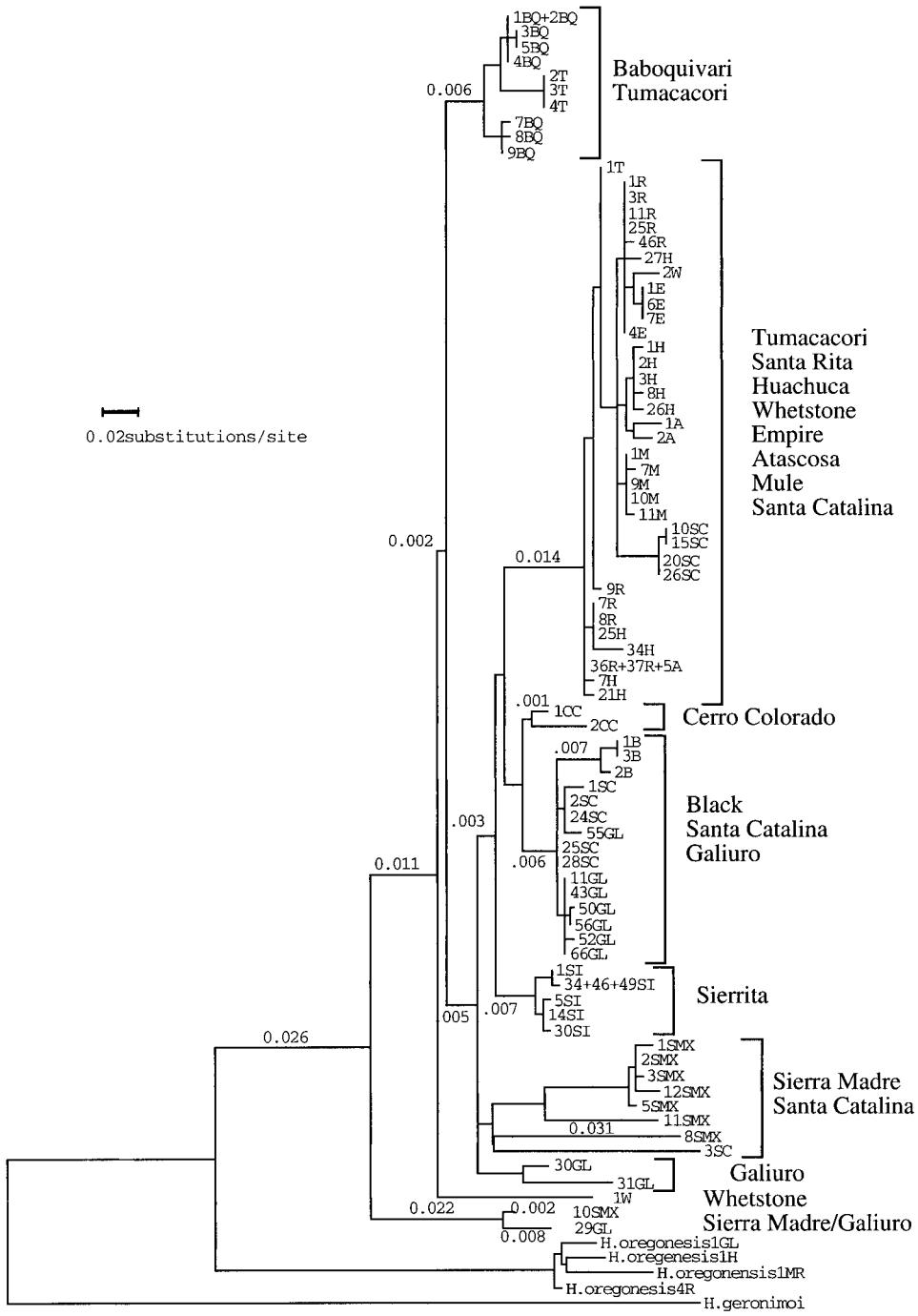

FIG. 3. One of three maximum-likelihood trees with the GTR + I + Γ model of evolution, for *Habronattus pugillis* populations. −Ln likelihood = 3075.94, % invariant sites = 0.613, gamma shape parameter = 1.292 estimated with four rate categories. Branch lengths referred to in the text are indicated next to the branches. Taxon names are abbreviated as in Figure 2.

clades with > 75% bootstrap support that consist only of individuals from Arizona. These clades are distinguished with crossbars in Figure 2. The remaining basal clades all contain individuals from Mexico. The map in Figure 4 depicts the geographic locations these clades occupy.

Of these five clades, only the Sierrita clade consists entirely of sequences collected from that range. All other basal clades consist of sequences from multiple mountain ranges or do not contain all sequences from a range. However, four of the more terminal clades consist entirely of sequences from a single mountain range (dark bars, Fig. 2), although there is low bootstrap support for some of these nodes.

Some geographic structuring is evident in the sequences, as certain clades are associated with northern, southern, and western mountain ranges (see Figs. 2, 4). This geographic structuring is most clear in the south, with all sequences

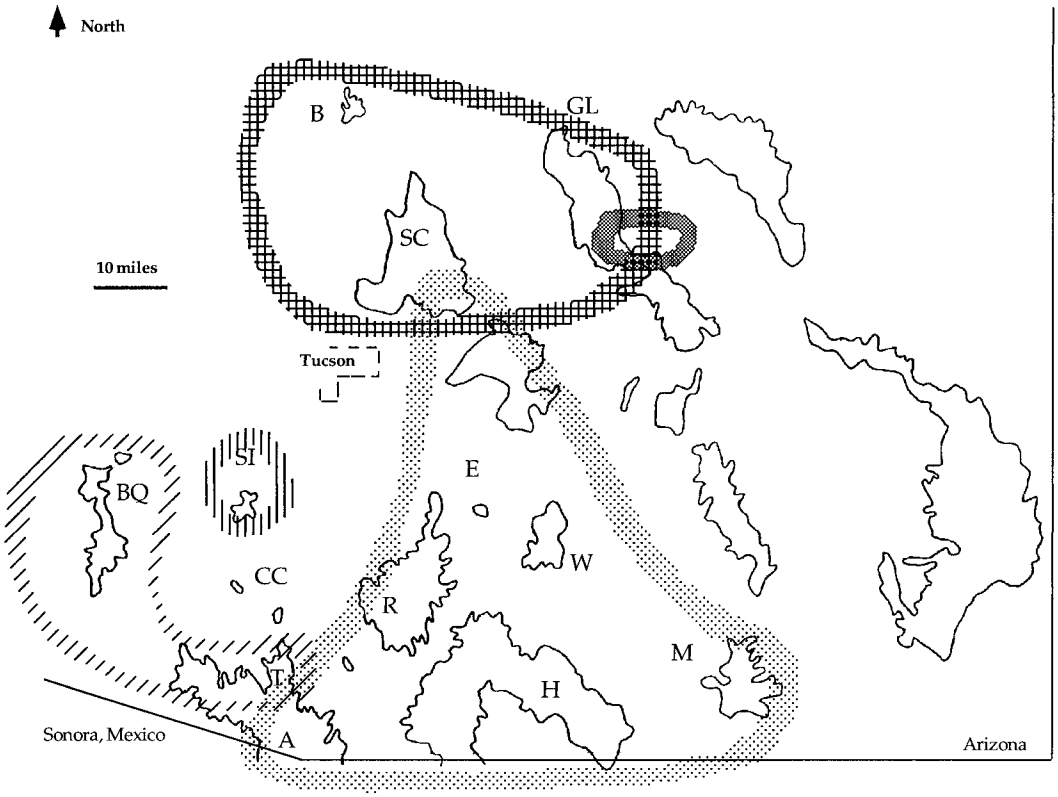

FIG. 4. Map showing outlines of montane Madrean oak vegetation as in Figure 1, overlaid with thick contours patterned to correspond to patterns of the boxes indicating the five basal clades in the parsimony tree in Figure 2. Ranges stretching through multiple delineated areas indicate that individuals collected from that range belong to multiple clades.

collected from southern ranges forming a large clade. However, this clade also contains some sequences from neighboring ranges. Many of the sequences collected from the northern ranges form a clade, with weak bootstrap support (54%) for a sister group relationship with the Cerro Colorado sequences to the southwest. Another basal clade is composed of sequences from the Tumacacori and Baboquivari Mountains in the southwestern region.

The area of oak woodlands on each mountain range is given in Table 1, along with whether each range has sequences that form geographically monophyletic clades. To test whether area of oak habitat (and therefore potentially N_e) differs between monophyletic and nonmonophyletic ranges, I performed a Wilcoxon-Mann-Whitney test (Siegel and Castellan 1988, p. 128). Results indicate that ranges with monophyletic sequences have significantly smaller areas of oak habitat ($P = 0.0098$). Because ranges have unequal sample sizes of spiders collected, I again performed a Wilcoxon-Mann-Whitney test to determine whether sample size differed between monophyletic and nonmonophyletic ranges. The relationship was not significant ($P = 0.1301$).

Table 2 shows the aerial distances between each mountain range and its nearest neighbors and whether individuals from each range form a clade in both the parsimony and neighbor-joining trees. I then asked whether ranges with sequences that formed monophyletic groups are more distant from other ranges. Average distance to the closest range for monophyletic ranges was 19.5 km and for nonmonophyletic ranges

TABLE 1. Geographic area covered by Madrean oak woodlands on each mountain range.

Mountain range	km² oak woodlands	*n*	π (%)	Monophyletic?
Cerro Colorado	1[1]	2	1.09	yes
Black	2.5[1]	3	0.272	yes
Empire	3.5	4	0.124	no
Sierrita	10	7	0.189	yes
Whetstone	20	2	4.563	no
Mule	30	5	0.108	yes
Baboquivari	35	8	0.485	no
Santa Catalina	44	10	2.498	no
Santa Rita	50.5	10	0.241	no
Atascosa	32.5[2,3]	3	0.843	no
Tumacacori	32.5[2,3]	4	2.874	no
Galiuro + Winchester	68[4]	10 + 0	2.179	no
Huachuca + Patagonia	150[2]	10 + 0	0.59	no

[1] The amount of oak habitat on these ranges estimated by personal observation, because presence of oak woodlands is not recorded on the Brown and Lowe (1980) map.

[2] These ranges are indicated as having continuous oak woodlands connecting them on the Brown and Lowe (1980) map, so the entire woodland was measured.

[3] Different morphological types exist in the Atascosa and Tumacacori Mountains. Where the range of one morphological type ends and the other begins is not known. Therefore the size of the range of each was estimated to be half the size of the entire oak woodlands; however, woodlands extend into Mexico and were not measured.

[4] These ranges are indicated as having oak woodlands connecting them on the vegetation map. They do not, but the map was measured for consistency of method.

TABLE 2. Distance to nearest Madrean woodlands.

Mountain range	Monophyletic[2]	Closest range (2 km)	Second closest range (km)	Third closest range (km)
Cerro Colorado	yes	Sierrita (15)	Atascosa (18)	Tumacacori (20)
Black	yes	Santa Catalina (23)	Galiuro (48)	Sierrita (100)
Empire	no	Santa Rita (10)	Whetstone (15)	Huachuca (38)
Sierrita	yes	Cerro Colorado (15)	Baboquivari (28)	Santa Rita (32)
Whetstone	no	Empire (15)	Santa Rita (20)	Huachuca (20)
Mule	yes	Huachuca (25)	Whetstone (50)	Santa Rita (70)
Baboquivari	no	Cerro Colorado (25)	Sierrita (28)	Atascosa (28)
Atascosa[1]	no	Tumacacori (0–10)	Cerro Colorado (18)	Santa Rita (25)
Tumacacori[1]	no	Atascosa (0–10)	Cerro Colorado (20)	Santa Rita (22)
Huachuca	no	Whetstone (20)	Mule (25)	Santa Rita (30)
Santa Rita	no	Empire (10)	Whetstone (20)	Tumacacori (22)
Santa Catalina	no	Black (23)	Galiuro (31)	Santa Rita (47)
Galiuro	no	Santa Catalina (31)	Black (48)	Whetstone (62)

[1] These mountain ranges are indicated as having Madrean woodlands connecting them on the Brown and Lowe (1980) map. Because different male morphological forms inhabit the different ranges, there is probably a distinct boundary to their respective ranges. However, this area has not been sampled thoroughly enough to know their exact distribution. Therefore, these two ranges were excluded from the analysis of whether ranges with monophyletic groups of sequences are more distant from other ranges.

was 19.1 km (excluding the Tumacacori and Atascosa ranges; see footnote in Table 2). These distances are not significantly different ($P = 0.3021$; Wilcoxon-Mann-Whitney test). Distances to second and third closest ranges also did not significantly differ between monophyletic and nonmonophyletic ranges ($P = 0.1476$ and $P = 0.1860$, respectively). Thus, ranges with sequences forming monophyletic groups are as geographically near neighboring ranges as those with sequences that do not form clades.

DISCUSSION

Migration Versus Incomplete Lineage Sorting

If the phylogenetic relationships among *H. pugillis* sequences are to be used to make inferences about the history of woodland fragmentation among the sky islands, it is important that genetic relationships are due to ancestral relationships predating the woodland fragmentation and not to ongoing migration. Not enough is known about the biology of *H. pugillis* to predict whether individuals can migrate between mountain ranges. Some spiders disperse aerially on silken threads, in a process known as ballooning (Bristowe 1939). Although this behavior has been well documented for other groups of spiders (e.g., Freeman 1946; Greenstone et al. 1987), salticids do not seem to show widespread ballooning ability (but for some documented cases, see Horner 1975) and there is no evidence for ballooning in *H. pugillis.*

Several lines of evidence indicate that incomplete lineage sorting is a better explanation than ongoing migration for paraphyly in the *H. pugillis* gene tree. First, the geographically distant distribution of two closely related haplotypes, with no similar haplotypes located between them, suggests retention of an ancestral polymorphism. Two individuals collected from the most distant localities (480 km apart), the Galiuro Mountains (individual 29) and the Sierra Madre (10), are closely related to one another, yet distantly related to individuals from mountain ranges between those two sites. If long-distance migration occurs regularly between locations such as these, one would expect little geographic structuring of the sequences. A more plausible explanation is that these individuals have retained genes from a common ancestor before the woodland habitat became fragmented, and that a large panmictic population of *H. pugillis* extended from southeastern Arizona to the Sierra Madre Occidental.

Another line of evidence suggesting retention of ancestral polymorphisms is the fact that geographic area of oak habitat predicts fairly well whether sequences from a range form a clade. Mountain ranges with oak habitats of < 32 km^2 generally contain sequences that form monophyletic lineages, whereas none of the sequences from larger ranges form exclusive clades. Incomplete lineage sorting predicts such a pattern; ongoing gene flow does not, because if small ranges either receive or send migrants, geographic monophyly will not occur. Lastly, mountain ranges harboring monophyletic groups of sequences are not geographically more distant from neighboring ranges than ranges harboring paraphyletic sequences. Thus, these lines of evidence indicate that gene flow via aerial ballooning does not seem to explain paraphyly of sequences in these populations of *H. pugillis.*

The observation of an identical haplotype shared between neighboring ranges (individual 5 of the Atascosa Mountains and 36 and 37 of the Santa Rita Mountains) may seem to suggest ongoing migration. However, it could also be that insufficient time has passed for substitutions to have accumulated in these sequences. If the rate of evolution of spider mitochondrial DNA is similar to other arthropods, we may expect 2.3% sequence divergence per million years (Brower 1994). Given 800 bp of sequence, we would expect to see a single nucleotide substitution only after 54,000 years—far longer than the postulated 10,000-year vicariance event.

It seems probable that repeated glaciation events have forced woodlands on at least some ranges through multiple cycles of fragmentation and reconnection. If this is the case, then spiders may have moved between ranges via the connected habitat, with consequent hybridization between populations. Indeed, patterns in the distribution of morphological traits found by Maddison and McMahon (2000) suggest that such hybridization may have formerly occurred between some ranges. Additionally, the geographic structuring of some sequences in the gene tree may indicate moderate historical gene flow between ranges.

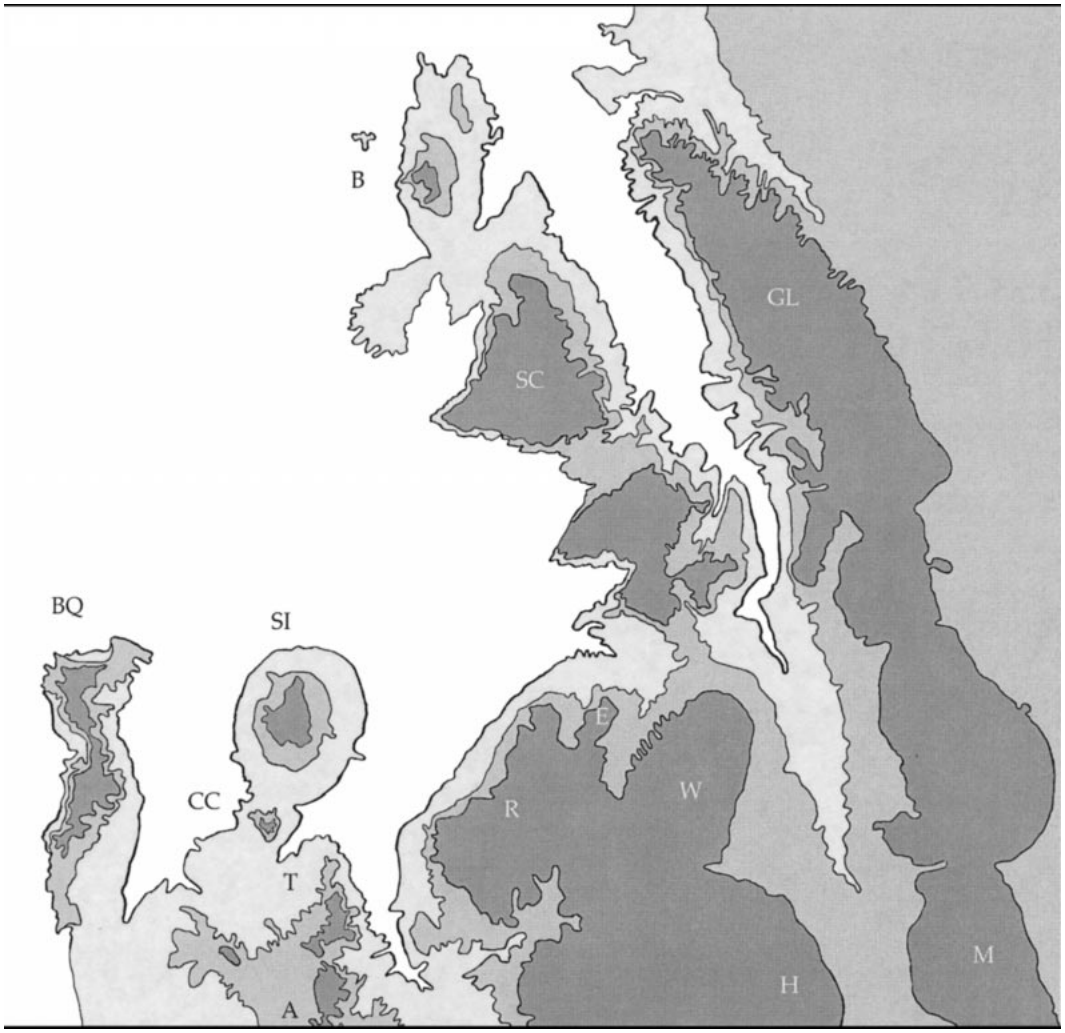

FIG. 5. Simplified topographic map of southeastern Arizona, with only selected elevation contours shown. Light gray areas are ~1067–1219 m (3500–4000 ft); medium gray areas are ~1067–1371 m (4000–4500 ft); dark gray areas are ~1371 m (4500 ft) and above. All unshaded areas are below ~1067 m (3500 ft) elevation.

Order of Fragmentation and Phylogeographic Connections

Examination of the topography of southeastern Arizona may allow predictions of both the order of fragmentation and phylogeographic connections among the region's montane populations. If observed phylogeographic patterns in *H. pugillis* are concordant with predictions generated by knowledge of local topography, then topography may be a useful predictor of the fragmentation history of other nonvagile, woodland-dependent organisms on these sky islands.

Elevations of land lying between sky islands vary, with some ranges separated by low desert and others by higher grassland. For this reason, it seems likely that some ranges were connected by corridors of continuous woodland more recently than others. Figure 5 illustrates that some southern mountain ranges would have been connected by habitat for *H. pugillis* if the oak zone began only 152 m below its current 1523-m elevation, whereas connections between others would have required the oak zone be substantially lower. By this logic, the ranges that should have become habitat islands first as the woodlands receded upslope are the Black, Sierrita, Cerro Colorado, and Baboquivari Mountains. Therefore, we may predict that sequences from these ranges would be the most differentiated from other ranges. This is in general true; however, this pattern could also result from these ranges' small population sizes. In addition, if these ranges were isolated first, we may predict a stepwise pattern of isolation in the gene tree, as has been found in other island systems (e.g., Shaw 1996; Roderick and Gillespie 1998). This pattern was not found, perhaps because relationships among the ranges are poorly resolved and collapse to a basal polytomy in the consensus tree (Fig. 2). A hard polytomy (Maddison 1989) may be expected if the woodlands fragmented within a very short period of time and populations diverged rapidly (Hoelzer and Melnick 1994). Alternatively, the number of molecular characters in this dataset (160 variable sites among the 81 *H. pugillis* haplotypes) may be too low to resolve an ordered series of vicariance events.

Some relationships among ranges can be resolved, however. All sequences from the Black, Mule, and Empire Mountians are nested within clades composed of sequences from

the adjacent ranges. This pattern of small (note small π-values in Table 1) peripheral populations derived from larger geographically adjacent populations is consistent with a peripheral isolates mode of differentiation (Mayr 1954). However, sample sizes from these ranges are small, and more thorough sampling might uncover more genetically distant haplotypes.

In contrast, populations sampled from the larger, north-central ranges (Santa Catalina and Galiuro Mountains) show deep divergences among individuals, with sequences widely distributed on the gene tree; this is reflected by their high nucleotide diversities (Table 1). These ranges likely harbored a large amount of old genetic diversity that existed before the woodlands contracted, possibly because of their large size (Fig. 5). The immense genetic diversity (π = 3.45%) of *H. pugillis* sequences from the continuous woodlands of the Sierra Madre Occidental hints that the ancestral population had a high genetic diversity. Two individuals collected here $<$ 10 m apart (8 and 10) show $>$ 6% mitochondrial sequence divergence. This much genetic diversity over such a small spatial scale is consistent with a very large effective population size.

Consideration of topography also generates a second prediction. Four north-to-south ridges or high-elevation corridors exist in southeastern Arizona (Fig. 5): a short corridor extending through the Baboquivari Mountains furthest west, one through the Atascosa and Sierrita Mountains, a long corridor through the Santa Rita and Santa Catalina Mountains in the center, and one through the Mule and Galiuro Mountains in the east. All these corridors are linked by elevations of at least 1067 m in southern Arizona and northern Mexico (not shown in figure). From this topographical perspective, I predicted the genealogy to show four clades, corresponding to these four corridors.

Indeed, there is evidence for close genetic connections between individuals in the central corridor. If oak woodland was only 152 m lower than it is at present, the Huachuca and Santa Rita ranges would be connected by continuous habitat for *H. pugillis.* Presently, individuals from these two ranges are genetically indistinguishable by their mitochondrial sequences, indicating that a woodland corridor probably existed relatively recently. There is not strong support for the other three high-elevation corridors, especially the easternmost corridor. However, *H. pugillis* has not been found to inhabit the intervening eastern mountain ranges, suggesting that other unidentified microhabitat factors may restrict its distribution.

The fact that individuals from the northernmost mountains are most closely related, despite the low-elevation topography separating the Galiuro and Santa Catalina Mountains, suggests an unexpected phylogeographic rift. Perhaps an early fragmentation event separated woodland ranges primarily along a latitude of 32°, thus cutting off the Whetstone from the Santa Catalina Mountains and the Mule from the Galiuro Mountains. This finding of a latitudinal phylogeographic divide is based only on a single mitochondrial locus and may not reflect patterns seen at other loci. However, the small effective population size of mitochondria, relative to nuclear loci, may make it more probable that mitochondrial loci have tracked population history accurately (Moore 1995). Additionally, there is evidence from other organisms to suggest a barrier may have prevented gene flow between northern and southern ranges. Phylogenetic analysis of mitochondrial sequences from *H. oregonensis* also groups Galiuro and Santa Catalina sequences together into a northern clade and Santa Rita and Huachuca sequences into a southern clade (Masta 1999). Additionally, evidence for a similar latitudinal divide has been found among populations of canyon treefrogs that inhabit these ranges (Barber 1999a,b). Ongoing work with multiple species of lizards and snakes (K. Zamudio, pers. comm.) on these sky islands may allow further assessment of the apparent divide.

Age of Population Fragmentation

The fossil packrat midden data imply that some southeastern Arizona woodlands fragmented approximately 10,000 years ago. For a biologist interested in the evolutionary history of extant populations of organisms here, the data provides either a scenario that can be used to generate hypotheses on vicariance and phylogeography or a date with which to calibrate a molecular clock, given genetic data from the organisms. In this paper, I have used the midden data to hypothesize that divergence of populations of *H. pugillis* may be as recent as 10,000 years. However, this date is based only on data from middens in the Santa Catalina Mountains and ranges outside of the Madrean sky islands (Van Devender and Spaulding 1979). If the scale of midden sampling was insufficient to represent patterns of vegetative change throughout the Madrean ranges, it is possible that many sky island woodlands actually became isolated from one another much longer ago than the midden data suggests.

Several lines of genetic evidence suggest a more ancient diversification for these spiders. First, the fully sorted mitochondrial sequences from some ranges suggest either that the effective population sizes of these spiders have been very small ($\leq 4N_e$ generations or $\leq$ 2500 individuals/range) or that the divergence is older than 10,000 years. Second, the morphological and behavioral distinctness of each mountain population suggests that sufficient time has passed to allow each population to evolve a unique phenotype. Although diversification of these spiders may indeed have been rapid due to the influences of sexual selection (Maddison and McMahon 2000; S. E. Masta and W. P. Maddison, unpubl. ms.), a post-Pleistocene divergence would require an extraordinary rate of phenotypic evolution.

Lastly, amounts of genetic divergence suggest older, and probably multiple, vicariance dates. The westernmost ranges that I postulated were isolated first, based upon the low-elevation topography between them (Fig. 5), show divergence estimates of 276,000 years ago (SD = $\pm$ 377,000 years) to 1.3 million years ago ($\pm$ 627,000 years), using the method of Nei and Li (1979). ML estimates of divergence from these same ranges are 390,000 years to 1.7 million years ago (see Fig. 3 branch lengths). The south-central mountain ranges, which were probably connected via high-elevation corridors most recently, tend to show more recent divergence times, with the Huachuca and Santa Rita Mountains having a divergence time of 30,000 years ago ($\pm$ 44,000 years). The oldest divergence among any pair of ranges (Black and Em-

pire Mountains) is more than 2 million years ago (± 700,000 years). The minimum divergence time between any of the Arizona populations and the Sierra Madre population is 642,000 years ago (± 322,000 years; ML estimate = 459,000 years). Such a date suggests that a high-elevation corridor may have connected the central Arizona and Sierra Madre populations long after some peripheral populations were isolated. Although all these estimates have large standard deviations, 57% of pairwise comparisons among the ranges (for which $n > 2$) give estimated divergence dates for which 10,000 years falls well beyond two standard deviations. Thus, it is clear that many populations of *H. pugillis* in Arizona were likely isolated far earlier than 10,000 years ago.

While such dates appear to thoroughly refute the idea of a single 10,000-year divergence driven by end-Pleistocene climate change, estimating divergence dates based upon a molecular clock calibrated for other arthropods is problematic for several reasons. First, the molecular clock (Brower 1994) was calibrated only with insects and crustaceans. Variation in the rate of mitochondrial evolution is known to exist both among taxa and among genes (reviewed in Simon et al. 1994). There is no clock calibration for arachnids, and it is possible that their mitochondrial DNA evolves at a very different rate than DNA of other arthropods. In fact, evidence suggests that molecular evolution of arachnid mitochondria may differ substantially from that of other arthropods (Masta 2000). Additionally, genes used in this study were selected to reconstruct intraspecific relationships and therefore may be evolving more rapidly than those used for interspecific phylogeny reconstruction. Finally, the dates used to calibrate the arthropod molecular clock were obtained from paleoclimatological, biogeographical, and geological data and may themselves be subject to error.

Even in the absence of the above issues, however, inferring absolute dates of divergence based upon a single mitochondrial locus would be problematic. These dating estimates have wide variances, due both to sampling variance and stochastic variance. Use of multiple loci generally reduces variance and therefore yields more accurate estimates (Takahata and Nei 1985). However, in the case of *H. pugillis*, caution must be exercised if nuclear loci are to be used to date divergence times. Accurate dating of vicariance events with a molecular clock requires the use of loci that are not under strong directional selection. If sexual selection is acting strongly on suites of traits in these populations, as suggested by Maddison and McMahon (2000) and S. E. Masta and W. P. Maddison (unpubl. ms.), we may expect directional selection to have decreased the coalescence time of the selected loci (e.g., Hudson 1990). If there are many such selected traits distributed throughout the genome, such that multiple other loci are physically linked to them, selective sweeps may have decreased genetic diversity across much of the nuclear genome. This would have the effect of decreasing the coalescence time of many nuclear loci, as compared to neutral, unlinked loci, thus making them inappropriate for dating vicariance events.

Even given the caveats of using a single locus and perhaps a poorly calibrated clock, it seems clear that divergences between some montane populations of spiders must be older than 10,000 years. It is also clear that caution must be exercised in extrapolating paleoclimatological or geological information to date divergence times of organisms in adjacent habitats. The paleontological history of vegetation patterns in the Sonoran Desert is very well documented (Betancourt et al. 1990) and therefore may seem to provide precise dates with which to calibrate a molecular clock for divergence of woodland organisms like these spiders. However, it appears that either the spatial scale of reconstruction of vegetation history was too coarse grained to reconstruct the fine-scale fragmentation of woodlands that affected *H. pugillis* or that earlier climate change had already effectively subdivided populations of these spiders.

Conclusions

This study has assessed the phylogeographic relationships of *H. pugillis* populations inhabiting sky-island mountain ranges in southeastern Arizona. It depicts a complex history of fragmentation and divergence only partially predicted by examination of the geography and vegetation patterns of the area. It also provides the first molecular-clock-based estimate for divergence dates of populations of Madrean woodland-dependent organisms of this region. Some divergence times are almost 200-fold greater than predicted from the assumption that fossil packrat midden data accurately depict times of woodland fragmentation and thus divergence of woodland organisms. Although these new estimates seem more in line with what we know of the amount of morphological differentiation of organisms on these ranges, it is clear that further improvement of molecular clock calibrations for arachnids is necessary before accurate estimates can be made.

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Corresponding Editor: S. Edwards