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Molecular Insights into Species Phylogeny, Biogeography, and Morphological Stasis in the Ancient Spider Genus *Hypochilus* (Araneae: Hypochilidae)

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The spider genus *Hypochilus* is currently restricted to cool, moist microhabitats in three widely separated montane regions of North America, providing an opportunity to study both deep (i.e., continental level) and shallow (within montane region) biogeographic history. Members of the genus also retain many plesiomorphic morphological characteristics, inviting the study of comparative rates of morphological evolution. In this paper, *Hypochilus* phylogeny and associated evolutionary problems are addressed using both new molecular (28S nDNA and CO1 mtDNA) and previously published (K. M. Catley, 1994, *Am. Mus. Nov.* 3088, 1–27) morphological data. Although the molecular data provide limited resolution of root placement within *Hypochilus*, most analyses are at least consistent with morphology-supported montane relationships of (Rockies (California, Appalachian)). The monophyly of *Hypochilus* species distributed in the California mountains is ambiguous, with several analyses indicating that this fauna may be paraphyletic with respect to a monophyletic Appalachian lineage. The montane regions differ in consistent ways in depths of both mitochondrial and nuclear phylogenetic divergence. Molecular clock analyses, in combination with arthropod-based mtDNA rate calibrations, suggest that the regional faunas are of different ages and that speciation in all faunas likely occurred prior to the Pleistocene. Limited intraspecific sampling reveals extraordinarily high levels of mtDNA cytochrome oxidase sequence divergence. These extreme divergences are most consistent with morphological stasis at the species level, despite preliminary evidence that *Hypochilus* taxa are characterized by fragmented population structures.

INTRODUCTION

Molecular data often provide unique, and perhaps otherwise inaccessible, insight into many phylogenetic problems. With respect to the thrust of this paper, such special problems include, for example, the resolution of phylogenetic patterns that are difficult to discern using other character systems. Such phylogenetic "crypsis" might apply to divergence between populations or recently evolved species (e.g., Hedin, 1997a) or, alternatively, to old taxa with slow rates of evolution in traditional systematic characters (Avise *et al.*, 1994). Another special problem lies in the assessment of character homology across taxa which are long-diverged (e.g., Metazoan phylogeny) or across taxa which exhibit character convergence due to shared lifestyles. Finally, molecular data are perhaps unique as systematic characters in providing a *potential* to distinguish temporal aspects of the phylogenetic process, either in a relative or in an absolute manner (Hillis *et al.*, 1996). Of course, molecular data can also be problematic for any one of several reasons, including paralogy/orthology determination, the gene tree/species tree problem, mutational saturation, biased base composition, etc. This means that as systematists we must be careful in our treatment and interpretation of molecular data—a principle that applies to all classes of character data.

This paper concerns the phylogenetic history and diversification patterns of spider species belonging to a genus (*Hypochilus*) with remarkable evolutionary, morphological, and biogeographical characteristics. The North American genus *Hypochilus*, along with the monotypic genus *Ectatosticta* of China, comprise the family Hypochilidae. Morphological analyses suggest that these spiders occupy a unique phylogenetic position as the most basal lineage of the araneomorph, or "true," spiders (Platnick, 1977; Forster *et al.*, 1987). This lineage is clearly old. Although fossil data do not exist for hypochilids, fossils of higher araneomorphs can be dated to the Jurassic (see Coddington and Levi, 1991), suggesting an even earlier divergence of the hypochilids (Catley (1994) suggests the Carboniferous). Morphologically, *Hypochilus* species share features representing a mosaic of "primitive" and autapomorphic character states, including a web architecture observed in no other extant spiders (summarized in Catley, 1994). Finally, *Hypochilus* species are distrib-

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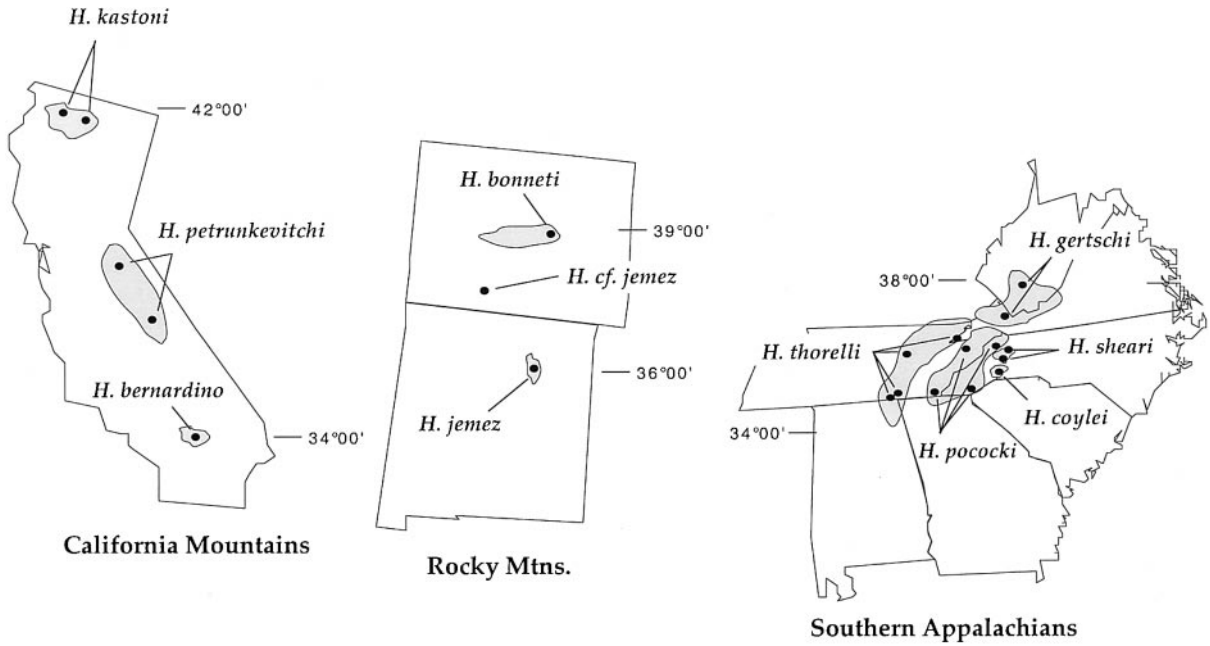

FIG. 1. The distribution of *Hypochilus* species in North America. Approximate geographic ranges are stippled (following Catley, 1994); continuity of stippling should not be interpreted as continuity in either habitat or species' distribution. Sampled populations (see Table 1) are indicated by dark circles. The population labeled as *H. cf. jemez* lies outside of the published distributional range of described species.

uted disjunctly over widely separated mountainous areas of North America, with groups of allopatric/parapatric species occurring in the southern Appalachians, the mountain ranges of California, and the southern Rockies of Colorado and New Mexico (Fig. 1). Given that *Hypochilus* taxa are restricted to mesic, rocky habitats and appear to be dispersal limited (reviewed in Catley, 1994), the observed regional endemicity suggests the ancient fragmentation of a once more-widespread ancestor.

To date, the majority of systematic work concerning *Hypochilus* has focused on the significance and placement of the genus in higher-level spider phylogeny (e.g., Platnick, 1977; Forster *et al.*, 1987), with much less attention directed at understanding species relationships within the genus. The exception is the work of Catley (1994), who inferred a phylogeny for the 10 described *Hypochilus* species based on analysis of 13 morphological characters. Catley's preferred phylogenetic hypothesis includes both Rockies and Appalachian faunas as monophyletic, but is interesting and perhaps surprising in suggesting a paraphyletic Californian fauna (see Fig. 5A legend). However, this result is not strongly supported, as alternative character optimizations resulting in parsimony trees of equal length include a monophyletic California clade. In addition, the morphological estimate is based on only 10 parsimony-informative characters (Appendix), 4 of which are perhaps nonindependent in being part of a single character system (the male palpus).

The implication is not that the morphology-based hypothesis is inaccurate, but rather that *Hypochilus* phylogeny is a problem in need of additional data to either corroborate or refute existing hypotheses. To this end, nuclear and mitochondrial DNA sequence data were gathered for a taxon sample which includes all described species of *Hypochilus* plus a "surrogate" outgroup taxon. These data are analyzed independently and in combination with existing morphological characters (Catley, 1994), paying particular attention to root placement and the possibility of California taxa paraphyly. The new data are also used to explore Catley's (1994) hypotheses regarding the temporal pattern of *Hypochilus* species divergence, including both within- and between-region divergence events. Finally, the molecular data are used to address the possibility of species-level morphological stasis in this ancient genus.

METHODS

Spiders from one or more localities were collected for all described *Hypochilus*, identified to species using the geographic and morphological criteria of Catley (1994). Geographically widespread taxa, including *H. kastoni*, *H. petrunkevitchi*, *H. thorelli*, *H. gertschi*, *H. sheari*, and *H. pococki*, are represented by multiple population samples (Fig. 1 and Table 1). Included in the sample are *Hypochilus* from a newly discovered population from southern Colorado (called *H. cf. jemez* in Table 1 and Appendix), a geographic position intermediate to the known species' ranges of *H. bonnetti* and *H. jemez*. The morphological characteristics and placement of this population are discussed below. Although EtOH-

preserved *Ectatosticta* specimens were made available by K. Catley, it was not possible to generate sequence from this material for outgroup purposes. As a potential replacement, DNA sequences were gathered for the atypid genus *Sphodros*. Most spider systematists agree that *Sphodros* is a relatively plesiomorphic member of the suborder (Mygalomorphae) most directly basal to the Hypochilidae, but also agree that these taxa are distantly related (see Platnick, 1977; Forster *et al.*, 1987; Coddington and Levi, 1991).

Entire legs were taken from spiders and preserved in either 100% EtOH or TE buffer and stored at −80°C. Otherwise-intact spiders are preserved as voucher specimens in the author's personal collection. Genomic DNA was extracted from leg tissue using the CTAB protocol of Shahjahan *et al.* (1995). Cytochrome oxidase (~1 kb) and 28S rDNA (800 bp) gene fragments were PCR amplified using primers and conditions described in Hedin and Maddison (2001). PCR products were polyacrylamide gel purified and sequenced directly using automated techniques (ABI 373 and 377 machines). Both strands were determined for all 28S sequences. Mitochondrial sequences were determined only for the "J" strand, with up to 60% sequence overlap. Hard copies of sequence chromatograms were checked by eye, compared to overlapping or complementary sequences, corrected as necessary, and transferred into the sequence editor of a beta version of MacClade V4.0 (Maddison and Maddison, 1999) for manipulation and manual alignment. *Hypochilus* sequences of both gene regions were easily aligned manually; alignment of the *Sphodros* 28S data to *Hypochilus* was achieved using the alignment program ClustalX (Higgins and Sharp, 1988) with default gap opening/extension costs and then adjusted manually.

Maximum likelihood (ML) was used to estimate phylogeny for nucleotide and amino acid data, using either PAUP* 4.0b2a (Swofford, 1999) or PUZZLE 4.0.2 (Strimmer and von Haeseler, 1999), respectively. Nucleotide-likelihood estimates included an initial evaluation of alternative models of DNA sequence evolution, with models differing in parameters associated with base composition (equal versus not), substitutional classes (one, two, or six parameter), and among-site rate variation (no rate variation, invariable sites method, four-category discrete gamma method). Nested models were compared using the likelihood ratio test (as implemented in MODELTEST (Posada and Crandall, 1998)), following the general suggestions of Cunningham *et al.* (1998). Using a best-fit model, likelihood estimates were conducted using heuristic searches (tree bisection–reconnection (TBR) branch swapping, multiple random addition sequence replicates), with simultaneous estimation of model parameter values. Parsimony analyses of nucleotide, amino acid, and morphological characters were conducted using exhaustive, branch-and-bound, or heuristic searches (as above), depending upon size of taxon sample. Relative support of reconstructed clades was evaluated using the nonparametric bootstrap (Felsenstein, 1985), based on analyses comprising 100–1000 replicates of a heuristic or branch-and-bound search. Tree comparison analyses were conducted using Kishino-Hasegawa (1989) and Templeton (1983) tests, with constraint trees built in MacClade. Phylogenetic congruence across data partitions was assessed using the PAUP* implementation of the incongruence length difference (ILD) test (Farris *et al.*, 1995).

RESULTS

28S phylogeny. 28S data were gathered for nine *Hypochilus* species, including at least two taxa from each of the three montane regions (Table 1). The distribution of site variation is biased across the region sequenced, the majority of 30 variable positions falling within proposed loop regions of either the D2 (20 sites) or the D3 (3 sites) domains (terminology of Schnare *et al.*, 1996). Four loop positions include indels. Although these positions were excluded in phylogenetic analyses, 2 of the positions are unambiguous in distinguishing Appalachian from western montane taxa. Observed base frequencies are statistically homogeneous across taxa ($\chi^2 = 0.2080$, $df = 24$, $P > 0.05$; PAUP* results), with mean frequencies of A, 0.1680; C, 0.2980; G, 0.3407; and T, 0.1933. Tests with constant sites removed also suggest homogeneity ($P > 0.05$).

Exhaustive parsimony analyses of the *Hypochilus* 28S data result in six most-parsimonious trees, one of which represents a resolution of the ML tree estimate (see Fig. 2A). All six trees share groupings of taxa corresponding to the California, Rockies, and Appalachian regional faunas. Maximum-likelihood analysis using a best-fit GTR + I model results in a tree with distinct clusters of taxa corresponding to regional faunas, each separated by relatively long branches with medium to high bootstrap support (Fig. 2A). Consistent with parsimony results, the ML estimate places *H. kastoni* as an embedded member of the California species cluster.

Alignment of the *Hypochilus* data to the longer *Sphodros* sequence requires the insertion of 42 positions unique to *Sphodros,* but does not influence the internal alignment of *Hypochilus* sequences. *Sphodros* base frequencies are similar to mean *Hypochilus* values. A heuristic ML search (using the best-fit model for the *Hypochilus* data alone, parameters reestimated) places the *Sphodros* sequence as sister to the *H. gertschi* sequence, suggesting that the root of the tree resides *within* a regional clade. Alternative root placements were examined by estimating likelihood scores of trees in which *Sphodros* was constrained to fall on one of the three internal branches of the 28S ML phylogeny. These alternative rootings result in likelihood scores very similar to the ML estimate (−ln likelihood ML tree = 1858.87; Rockies basal = 1862.29; Califor-

TABLE 1

***Hypochilus* Collecting Locality and Sequence Accession Number Information**

Taxon	Acronym	Locality	28S Accession No. (seq. length)	CO1 Accession No. (seq. length)
H. bonnetti	BONN	CO, Fly Cave	AF303497 (764)	AF303525 (1047)
H. jemez	JEME	NM, Pecos River	AF303498 (764)	AF303527 (1047)
H. cf. jemez	CFJE	CO, South Fork	—	AF303526 (1047)
H. kastoni	KAST_1	CA, West Boulder Lake	AF303499 (764)	AF303521 (1047)
—	KAST_2	CA, Ney Springs	—	AF303520 (1047)
H. petrunkevitchi	PETR_1	CA, Yosemite Falls	AF303500 (764)	AF303523 (1047)
—	PETR_2	CA, Big Fern Springs	—	AF303522 (1047)
H. bernardino	BERN	CA, Camp Creek	AF303501 (764)	AF303524 (1047)
H. pococki	POCK_1	NC, Chattooga River	—	AF303512 (1023)
—	POCK_2	NC, Nantahala River	AF303502 (766)	AF303513 (1047)
—	POCK_3	TN, GRSM, Chimneys	—	AF303511 (1023)
—	POCK_4	NC, Linville Gorge	—	AF303514 (1023)
H. coylei	COYL	NC, Rumbling Bald Mtn.	—	AF303517 (763)
H. sheari	SHEA_1	NC, Crabtree Falls	—	AF303515 (1047)
—	SHEA_2	NC, US 70 near Asheville	AF303504 (764)	AF303516 (1020)
H. gertschi	GERT_1	WV, Camp Creek SP	—	AF303518 (1020)
—	GERT_2	VA, Cascade Park	AF303503 (765)	AF303519 (1047)
H. thorelli	THOR_1	VA, Skylight Cave	—	AF303510 (853)
—	THOR_2	TN, Ozone Falls	AF303505 (765)	AF303509 (1047)
—	THOR_3	TN, Pitchfork Cave	—	AF303507 (1047)
—	THOR_4	TN, Signal Mountain	—	AF303508 (937)
Sphodros abbotti	SPHO	FL, Gainesville	AF303506 (802)	AF303528 (1047)

Note. Full locality information is available from the author upon request.

nia basal = 1862.29; Appalachians basal = 1859.70; all KH test P values > 0.19). Parsimony results are similar in suggesting a root placement within the Appalachian fauna (Fig. 2B), but the alternatives (as above) are only a single step longer than most parsimonious.

CO1 phylogeny. Cytochrome oxidase sequences corresponding to 349 amino acids were gathered from 21 populations of *Hypochilus*, representing all of the described species in the genus. Five sequences are lacking the first 9 amino acids, while three sequences lack 3′ data corresponding to 37 (THOR_4), 65 (THOR_1), or 95 (COYL) residues (Table 1). These sequences were included in phylogenetic analyses with incomplete sites scored as missing.

Heuristic parsimony analysis of CO1 nucleotides results in two MP trees. The trees include groups of taxa corresponding to Rockies and Appalachian faunas (monophyletic given certain root placements), but under no possible root placement are the California species monophyletic. At lower phylogenetic levels, multiple sequences from the same species group together in four of six cases (both *H. petrunkevitchi* and *H. pococki* cannot be monophyletic). The ML tree inferred under a best-fit GTR + I + Γ model has many similarities to the MP trees (Fig. 3), including the lack of a California clade, and deviations from species monophyly for both *H. pococki* and *H. petrunkevtchi*. Three alternative trees were compared to the ML estimate, each identical in topology to the ML estimate except that either California taxa, all species, or both California taxa and all species were constrained to form exclusive groupings. KH tests suggest that the alternative trees are not statistically different from the ML tree (−ln likelihood ML tree = 7752.08; California monophyly = 7752.29; All Species monophyly = 7761.22; California and All Species monophyly = 7762.51; all KH test P values > 0.10).

The CO1 data are extremely divergent at the nucleotide level. Estimated pairwise distances range up to 22% for sequences from the same species, with values ranging to 47% for comparisons across montane regions (distances estimates based on HKY85 + I model, see Table 2). Much of this variation is concentrated at third positions, which as a site class is characterized by marked cross-taxa variation in base composition (see Fig. 3). χ^2 analyses, uncorrected for phylogenetic relationship, indicate that this heterogeneity is statistically significant ($\chi^2 = 231.8954$, $df = 60$, $P < 0.01$; PAUP* results). Such base compositional bias, in violating assumptions of phylogeny estimation procedures (see Swofford *et al.*, 1996), might have potentially misleading effects on CO1 phylogeny estimation. This type of effect is perhaps evident in cases where taxa sharing similar values of percentage of A + T at third positions are also placed together on the ML tree (Fig. 3). Although some of this covariation is almost certainly attributable to common ancestry, the position of some sequences (e.g., KAST sequences) may be due to convergence in base composition.

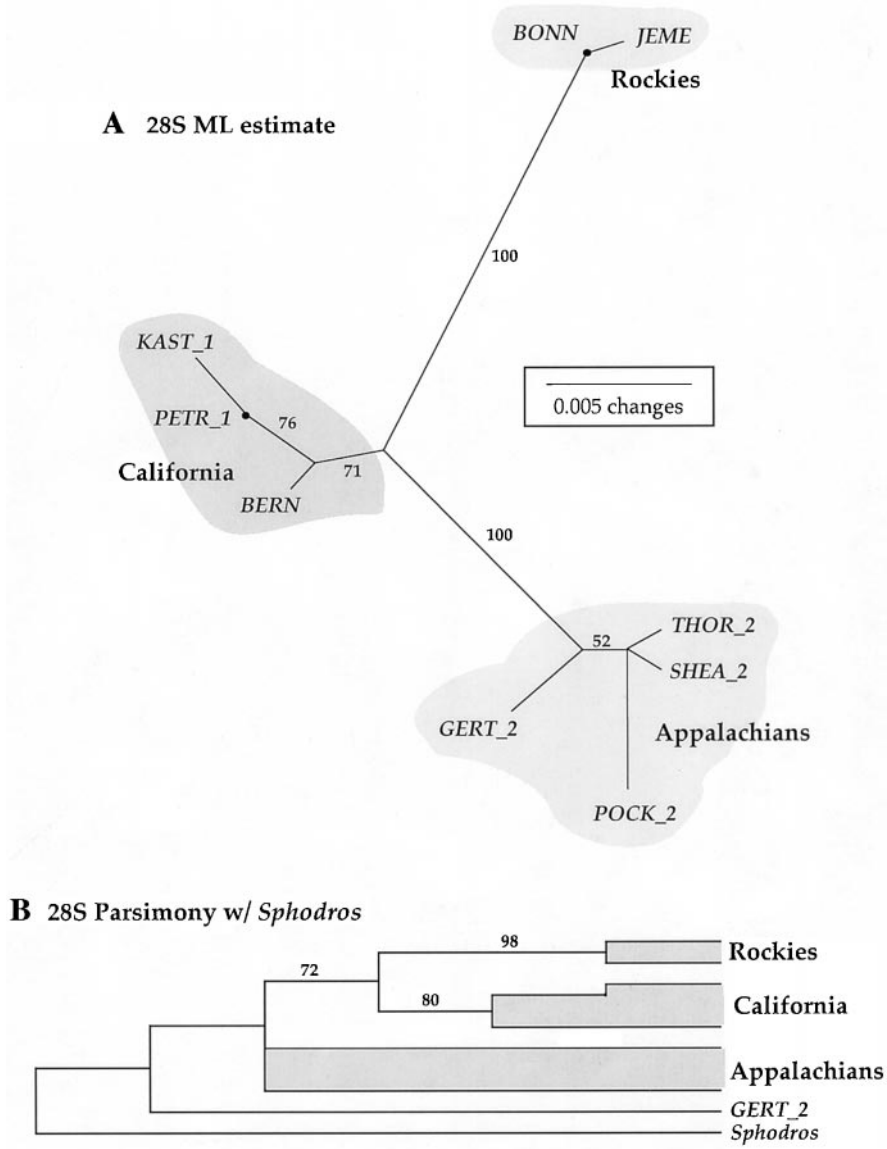

FIG. 2. (A) Unrooted 28S maximum likelihood tree, with branch lengths drawn proportional to their ML length estimates. Tree estimate is based on a heuristic search (five random addition sequence replicates), parameters of a GTR + I model estimated simultaneously. Bootstrap proportion values based on 100 replicates of a heuristic search (two random addition sequence replicates per pseudosample). GTR rate matrix and I parameter values were fixed to the ML estimate during bootstrap searches; nucleotide frequencies were estimated simultaneously with tree searches. Population acronyms as in Table 1. (B) Majority-rule consensus tree of eight MP trees resulting from a branch-and-bound search of 28S data, including *Sphodros* as outgroup. Bootstrap proportion values based on 1000 branch-and-bound search replicates.

Multiple analyses were conducted to explore the potentially misleading influence of high CO1 divergence and biased base composition on phylogeny estimation, including analysis of LogDet transformed data (with and without incorporation of among-site rate variation), and analyses of first and second position sites only (both ML and parsimony searches). These searches result in minor differences in the phylogenetic placement of sequences within either Appalachian or Rockies sequence clusters, with none of the trees including a branch supporting the monophyly of California taxa.

The CO1 data were also analyzed as amino acids, which are homogeneous in residue composition across taxa (5% χ^2 test, $P > 0.05$; PUZZLE results). The amino acid matrix included 19 *Hypochilus* sequences, after merging the redundant pairs SHEA_1/SHEA_2 and THOR_3/THOR_4. A representative most-parsimonious tree (1 of 84) is shown in Fig. 4A. All MP trees include clusters of sequences corresponding to the three montane faunas, including a California cluster in which *H. kastoni* is derived. Some of the MP trees (36 of 84) also include a cluster of *H. pococki* sequences, but

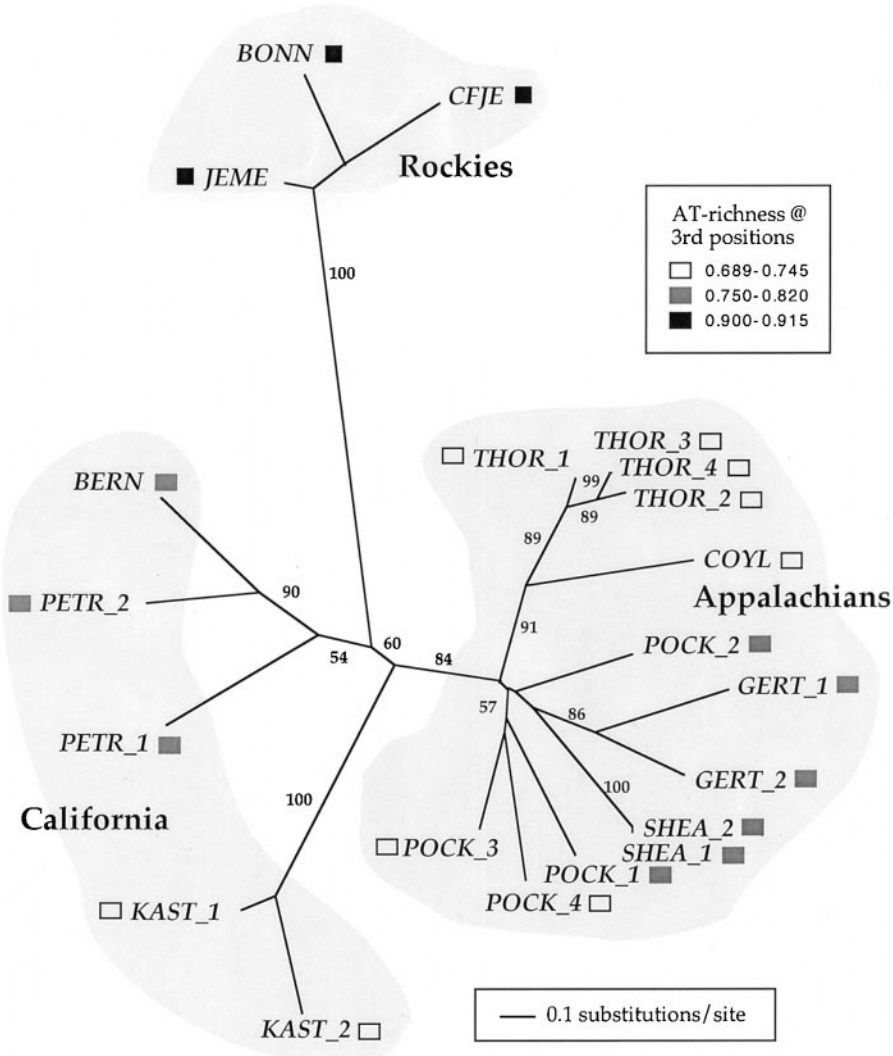

FIG. 3. Unrooted CO1 nucleotide maximum-likelihood tree. Branch lengths are drawn proportional to ML estimates. The tree estimate is based on a heuristic search (three random addition sequence replicates), parameters of the GTR + I + Γ model estimated simultaneously. Bootstrap proportion values based on 100 replicates of a heuristic search (two random addition sequence replicates per pseudosample), with all parameter values fixed to the ML estimate during bootstrap searches. Observed percentage A + T at CO1 third positions are indicated for each sample. Population acronyms as in Table 1.

none include a pairing of the *H. petrunkevitchi* sequences. The shape of the tree is similar to the 28S ML estimate (see Fig. 2A), with long branches subtending Appalachian and Rockies groupings, and a relatively shorter branch supporting a California grouping. In contrast to parsimony results, PUZZLE ML analyses using a best-fit mtREV24 + Γ model result in a tree in which California taxa cannot be monophyletic. However, an alternative tree in which the California taxa are grouped (holding all other relationships as in the ML estimate) is very similar to the ML tree (−ln likelihood ML tree = 1842.37; California monophyly = 1845.52; KH test $P > 0.05$).

CO1 root placement was assessed using the amino acid matrix, thus minimizing the genetic distance separating *Hypochilus* and *Sphodros* sequences. χ^2 analysis suggests that amino acid composition does not differ from ingroup to outgroup (5% χ^2 test, $P > 0.05$; PUZZLE results). Parsimony analyses place the *Sphodros* sequence on the longest branch of the tree, that separating the Rockies from the Appalachian plus California clusters, with high bootstrap support for the California plus Appalachian pairing (see Fig. 4B). Two alternative root placements were assessed, including California and Appalachian taxa basal hypotheses. Alternative trees included those MP trees consistent with (i.e., a resolution of) a strict consensus of the MP trees, except for the constrained placement of the *Sphodros* sequence. Such trees are not statistically different from the MP hypotheses as assessed by the Templeton

TABLE 2

DNA Distances

Clade	28S	CO1 nuc	CO1 AA
Rockies	**0.0013**	0.1073/**0.1183**/0.1324	0.0380/**0.0443**/0.0564
H. kastoni	—	**0.1289**	**0.0403**
H. petrunkevitchi	—	**0.2164**	**0.0561**
California	0.0027/**0.0046**/0.0069	0.1759/**0.2924**/0.3560	0.0565/**0.1076**/0.1489
H. pococki	—	0.1838/**0.1936**/0.2166	0.0174/**0.0311**/0.0447
H. sheari	—	**0.0104**	—
H. gertschi	—	**0.1744**	**0.0284**
H. thorelli	—	0.0040/**0.0795**/0.1124	0.0109/**0.0217**/0.0326
Appalachians	0.0027/**0.0054**/0.0072	0.1873/**0.2356**/0.3163	0.0159/**0.0543**/0.0771

Note. Distances shown include maximum, mean (bold), and minimum pairwise distance estimates if more than two sequences are compared. Regional values only include interspecific comparisons. Distance estimates are derived under "best-fit" ML models for 28S and CO1 amino acid data. Distances derived from an HKY85 + I model are presented for CO1 nucleotides, as a large number of the "best-fit" GTR + I + Γ distance estimates were undefined. All distance estimates are based on trees consistent with the monophyly of the named clades (see text for description of constraint trees).

(1983) test (California basal, pars length diff = 6, highest $T = 1.503$, lowest $P = 0.134$; App basal, pars length diff = 6, highest $T = 1.904$; lowest $P = 0.06$). ML analyses are similar in suggesting a basal Rockies clade, but again, KH tests suggest that this placement is not statistically different from alternative placements (−ln likelihood ML tree = 2078.14; California monophyletic *and* basal = 2087.15; Appalachian basal = 2083.50, KH test P values > 0.05).

Morphological phylogeny. The morphological matrix of Catley is recreated as the Appendix, with the addition of data from the new Colorado population (*H. cj. jemez;* number of adults scored = 1 male, 2 females). The matrix also includes data for the genus *Ectatosticta,* the hypothesized sister genus to *Hypochilus.* Branch-and-bound parsimony analysis, treating all but characters 5 and 10 as unordered (as in Catley, 1994) and with the PCT character of *H. cf. jemez* interpreted as polymorphism (see Appendix), results in 25 MP trees. The majority of MP trees include a monophyletic Rockies clade, whereas 10 of 25 trees include a monophyletic California clade (Fig. 5A). Analyses treating all characters as unordered result in more MP trees of equal length ($N = 42$), with a majority-rule consensus identical to that for the two-ordered matrix. Both sets of analyses place the root of the tree (outgroup *Ectatosticta*) on the branch separating the Rockies clade from the California plus Appalachian clades.

Combined evidence. Data combination was explored using a concatenation of four data partitions (28S, morphology, CO1 first and second positions, CO1 amino acids). For species in which multiple CO1 sequences were available, the included samples corresponded to those for which both 28S and CO1 data were collected from the same individual (9 taxa; see Table 1); two additional, CO1 only taxa, were also included (*H. cf. jemez* and *H. coylei*). This reduced 11-taxon molecular matrix was concatenated with the 11-taxon morphological matrix. Matrices excluding invariant sites were subjected to pairwise ILD tests (Farris *et al.,* 1995; Cunningham, 1997), treating all data as unordered. Test results suggest that nuclear versus mitochondrial molecular partitions provide congruent estimates of phylogeny (28S versus CO1 first and second, $P = 0.669$; 28S versus CO1 amino acids, $P = 1.000$). Morphology versus molecule comparisons were mixed, with evidence for both congruence (Morphology versus CO1 first and second, $P = 0.115$; Morphology versus 28S, $P = 0.540$) and significant incongruence (Morphology versus CO1 amino acids, $P = 0.032$).

Two combined matrices were analyzed using parsimony, both including morphological, 28S, and CO1 data (either amino acids or first and second positions only). Although the amino acid matrix included partitions judged as incongruent at the $P < 0.05$ level, some authors have suggested that this significance threshold level is too conservative, recommending data combination in cases where ILD significance values exceed $P > 0.01$ (see Sullivan, 1996; Cunningham, 1997). This advice was followed here. Exhaustive parsimony analysis of the amino acid matrix results in a single MP tree (Fig. 5B). The tree includes clusters of taxa corresponding to the regional faunas, each with high bootstrap support. Except for the sister relationship between *H. thorelli* and *H. coylei,* relationships within the Appalachian cluster are poorly resolved. Exhaustive analysis of the no-third matrix results in four MP trees. The strict consensus of these trees is identical to the amino acid tree, except for the collapse of the *H. pococki* plus *H. gertschi* branch to a polytomy (see Fig. 5B). Inclusion of *Sphodros* data in similar sets of combined analyses results in trees in which the Rockies clade is

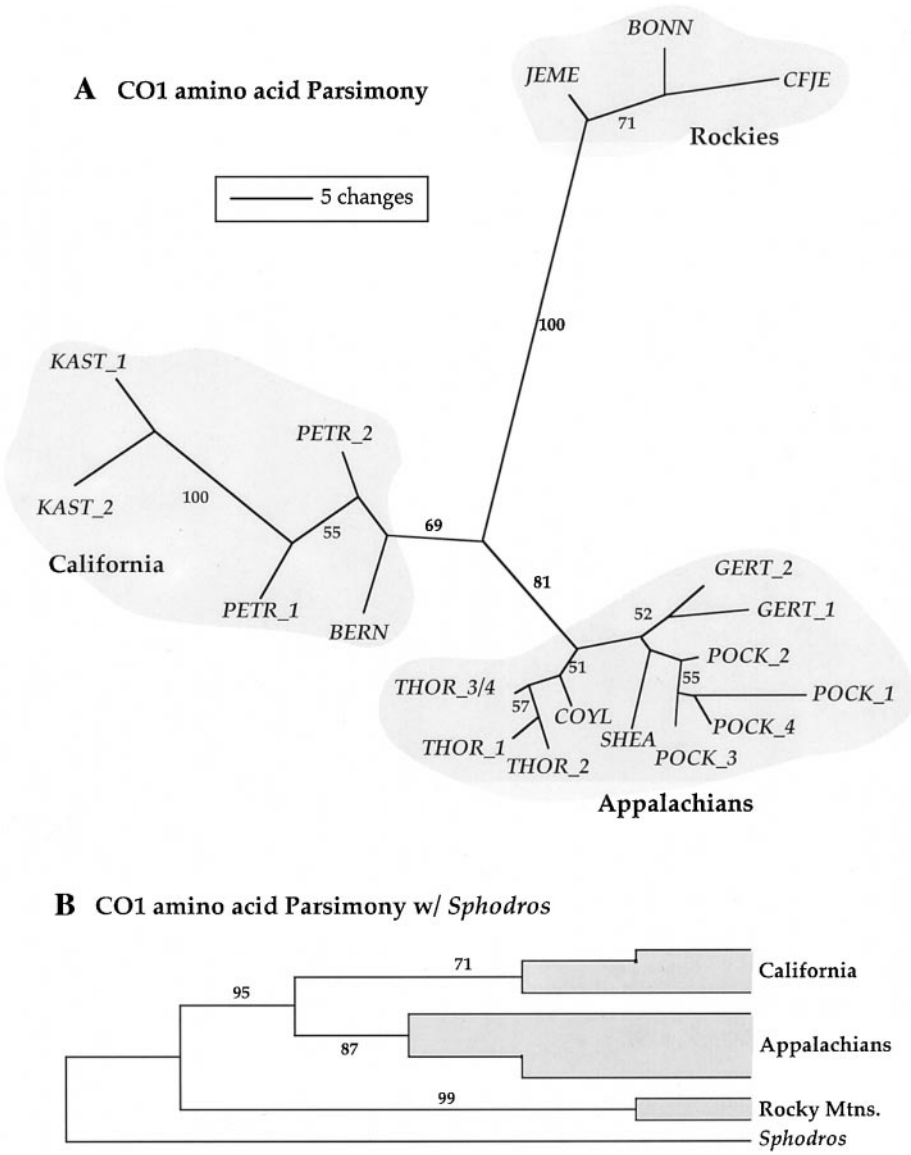

FIG. 4. (A) Unrooted CO1 amino acid parsimony phylogram, with branch lengths drawn proportional to parsimony estimates. The tree shown is one of 84 MP trees resulting from a branch-and-bound search. (B) Strict consensus of 31 MP trees resulting from branch-and-bound analysis of CO1 amino acid data, including *Sphodros* as outgroup. Bootstrap proportion values for both A and B trees based on 1000 replicates of a heuristic search, two random addition sequence replicates per pseudosample. Population acronyms as in Table 1.

sister to a well-supported California plus Appalachian clade (bootstrap proportion values > 92).

DISCUSSION

Regional phylogenetics. The placement of the root of *Hypochilus* is crucial in interpreting patterns of continental biogeography. In general, molecular-based root placements using the surrogate *Sphodros* sequence are ambiguous, suggesting that these data are effectively randomized with respect to the *Hypochilus* data. Aside from *Ectatosticta,* it might be argued that more appropriate outgroup taxa might have been sampled from other basal araneomorphs (e.g., Austrochiloids, see Forster *et al.,* 1987), rather than from a basal mygalomorph. Although this is potentially true, it might also be argued that any taxon chosen (including *Ectatosticta*) would be too divergent from *Hypochilus,* given a sample of molecular characters informative and divergent within *Hypochilus.* If a well-resolved molecular rooting is possible, it will likely come from a more slowly evolving character partition, perhaps sacrificing information content within the ingroup. This reasoning also suggests that morphological characters might provide the best resolution of root placement for the *Hypochilus* problem. Morphological tree estimates,

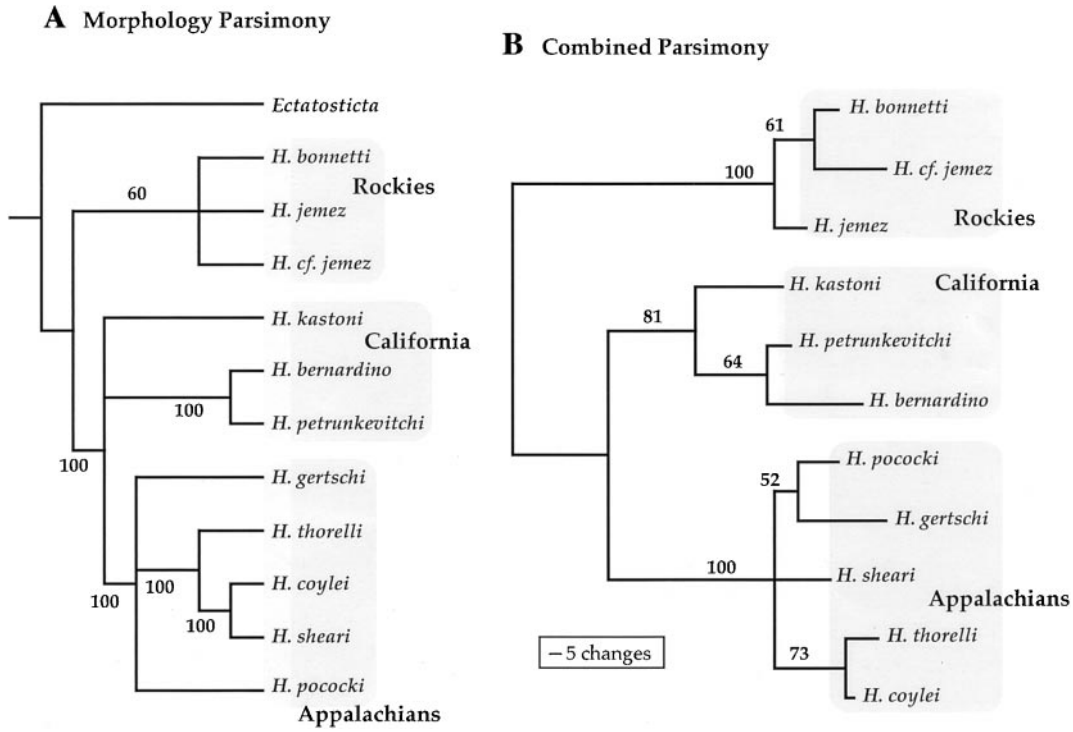

FIG. 5. (A) Majority-rule consensus tree of 25 MP trees resulting from a branch-and-bound search of morphological data, tree rooted with *Ectatosticta*. All data except characters 5 and 10 treated as unordered. Bipartitions found in >50% of the MP trees are indicated. Catley's (1994) preferred phylogenetic hypothesis differs from that figured in the placement of the *H. bernardino* + *H. petrunkevitchi* clade (sister to the Appalachian clade) and in the placement of *H. pococki* (sister to *H. thorelli* (*H. coylei, H. sheari*)). (B) Most-parsimonious phylogram resulting from an exhaustive search of a matrix including 28S, morphological, and CO1 amino acid data. All data were treated as unordered. Bootstrap proportion values are based on 1000 branch-and-bound search replicates.

rooted using *Ectatosticta,* indicate that the Rocky mountain species represent the most basal lineage of the genus, with a more recent split separating the California and Appalachian taxa (this paper and Catley, 1994). Despite ambiguity, molecular data are at least consistent with this rooting. Unrooted molecular trees generally include a long internal branch separating the Rockies from the Appalachian plus California taxa (e.g., Figs. 2A, 3, and 4A), and analyses of CO1 amino acids with *Sphodros* place the root on this long branch (Fig. 4B). It will be interesting to examine the root placement of other taxa that show distributional patterns similar to *Hypochilus* (e.g., *Aneides* salamanders (Wake, 1963), Spirobolid millipedes (Keeton, 1960)) to assess the generality of a (Rockies (California, Appalachian)) hypothesis.

The separation of *Hypochilus* taxa into widely separated areas of species endemism is striking (Fig. 1), and we might think that this distribution in itself would provide evidence as to species relationships within the genus. For example, one might expect the regional faunas to be monophyletic. Not surprisingly, this supposition apparently holds for species of the Rocky and southern Appalachian mountains. Phylogenetic analyses of both morphological and molecular data sets always recover clusters of taxa corresponding to these regional faunas, either independently or in combination, and regardless of data treatment or estimation procedure (Fig. 6). Molecular estimates of branch lengths subtending these clades are relatively high.

The monophyly of species distributed in the California mountains is less obvious. The possibility of a nonmonophyletic Californian fauna was first hinted at by Hoffman (1963), who noted morphological similarities between *H. petrunkevitchi* from central California and *H. gertschi* from the southern Appalachians. The phylogenetic work of Catley (1994) reinforced this possibility, suggesting that species of southern California (*H. petrunkevtchi* and *H. bernardino*) are perhaps more closely related to taxa in the Appalachians than they are to *H. kastoni* of northern California. The questionable monophyly of California taxa extends to the molecular data presented in this paper. Analysis of 28S data support a monophyletic California clade (Fig. 2A), but the branch subtending this node is relatively short, with only two unambiguous character changes. The CO1 data are more ambiguous, with conclusions about California monophyly sensitive to variation in data treatment and estimation procedure. Multiple analyses were attempted

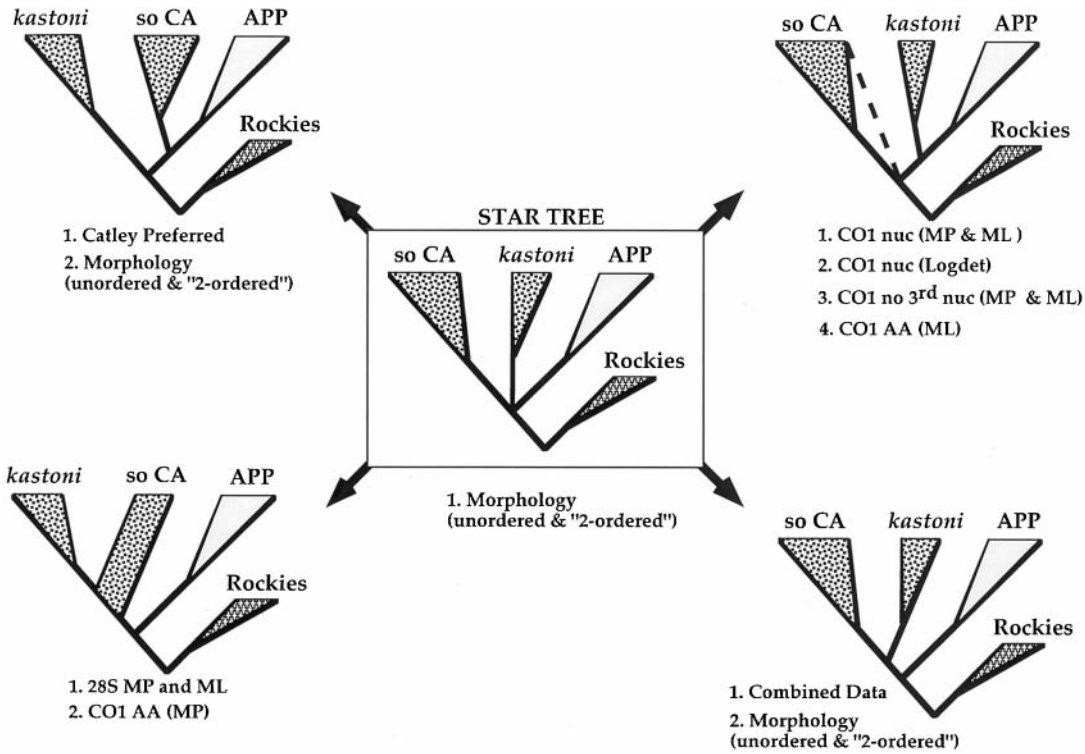

FIG. 6. Alternative resolutions of a polytomy involving members of the Appalachian clade (APP), *H. kastoni*, and species of southern California (*H. petrunkevitchi* and *H. bernardino*). Analyses reported in this paper result in trees that always include monophyletic Rockies, Appalachian, and *H. kastoni* clades, with differences in the placement of southern California species (monophyletic, paraphyletic, or sharing an unresolved node).

which included (either alone or in combination) CO1 data. Of these, only parsimony analyses of amino acids alone (Fig. 4A) and of combined matrices (Fig. 5B) indicate unequivocal California monophyly. Although some of the CO1 results are perhaps best excluded from consideration (e.g., analyses including third positions), other estimation/treatment combinations always recover a paraphyletic California fauna.

The observed paraphyly is not due to a consistently strong phylogenetic connection between certain California plus Appalachian species, as both molecular and morphological analyses indicating paraphyly also indicate ambiguity as to which California species is closest to the Appalachian clade. Instead, given a polarity hypothesis in which Rockies taxa are basal in the genus (see above), we might view the paraphyly problem as consistent with a "star tree" including terminal branches corresponding to the Appalachian lineage, the *H. kastoni* lineage, and the *H. petrunkevitchi* + *H. bernardino* lineage (Fig. 6). Because the short internal branches are relatively deep, additional difficulties such as signal convergence resulting in long-branch attraction (see Felsenstein, 1978) may compound the estimation problem, resulting in unstable relationships across analyses and character partitions.

In considering the California to Appalachians connection, Hoffman (1963) wrote "it seems to me possible that these two forms . . . represent the surviving remnants of a now largely extinct parental stock which (during the early or middle Tertiary) occurred in a crescent-shaped arc across the northern part of North America." Catley (1994) refined this vicariance hypothesis, suggesting that lineage separation was likely associated with either the marine continental submergence of the Lower Cretaceous or the drying of an Arcto-Tertiary vegetation during the late Tertiary (Miocene–Pliocene). As will be presented below, molecular clock calibrations result in divergence time estimates compatible with a more recent, Miocene, hypothesis. Analogous calibrations for the California species alone suggest similar divergence dates, consistent with a star-tree scenario in which between- and within-region divergences occur nearly simultaneously in time.

Within-region species phylogeny and divergence. The perception of species-level relationships within clades is, as above, dependent upon data treatment and estimation procedure. If one considers the combined-data phylogeny (Fig. 5B) as a "best available estimate," there appears to be consistent biogeographic signal within the western regional clades. The Rockies clade includes the basal southern *H. jemez* with a de-

rived pair of northern species (*H. bonnetti, H. cf. jemez*), while the California clade includes a basal northern *H. kastoni* with derived southern taxa (*H. bernardino, H. petrunkevitchi*). These biogeographically coherent relationships, in addition to patterns of discrete allopatry (Fig. 1), are consistent with a vicariance-dominated history of species diversification in the west. The situation in the Appalachians is less clear, as the combined-data estimate is unresolved (Fig. 5b) except for the sister relationship between the disjunct species *H. thorelli* and *H. coylei* (Fig. 1).

Catley (1994) forwards several alternative hypotheses on the timing of within-region species diversifications. In general, these hypotheses invoke vicariance in response to geological events or (associated) climatic fluctuations as a primary cause of lineage separation. For example, species diversification in southern California is alternatively related to either Oligocene movements of fault blocks, or to Pliocene/Pleistocene drying; divergence of the northern *H. kastoni* is linked to Quaternary volcanic activity. Species divergence in the Rockies is related either to the Cretaceous orogeny of the mountains themselves or, alternatively, to Pliocene drying. Finally, Appalachian diversification is linked to Pleistocene climatic fluctuations associated with glaciation (see also Huff and Coyle, 1992), a hypothesis generally consistent with temporal hypotheses forwarded for other cryophilic lineages of the region (see Hedin, 1997b). As such, it might be that speciation within each of the regions has occurred over roughly the same time frame (i.e., Pliocene/Pleistocene) or that diversification ages differ across clades, with a ranking from youngest to oldest corresponding to Appalachian, then California, and then Rockies.

The molecular data provide insight into these alternative temporal hypotheses in at least two ways. First, relative levels of within-region molecular diversity for both 28S and CO1 nucleotides are less in the Rockies than in either California or Appalachian clades, which share similar high levels of within-clade diversity (Table 2). Although this pattern is less obvious for CO1 amino acid data (Table 2), the trend is the same. It might be argued that inferences based on this pattern are weakened by differences in the number of populations sampled (fewest in Rockies) and the geographic scale of sampling (least extensive in Rockies; Fig. 1). However, it is also true that, at least for CO1 nucleotide data, the average pairwise divergence across Rockies taxa is less than that observed *within* most species from the other regions, each of which was sampled over a smaller geographic area (see Fig. 1; Table 2). Based on these relative comparisons, the data suggest that within-region species diversifications are of different ages. Although the Rockies lineage itself appears to be the oldest within the genus, species within this lineage are relatively young.

These relative age inferences are contingent upon an assumption of constancy in molecular evolutionary rates across different *Hypochilus* clades. The rate constancy hypothesis was tested for *Hypochilus* sequences by comparing likelihood scores of trees with and without the assumption of a molecular clock (Felsenstein, 1981), rooted assuming the Rockies taxa as outgroup. For the CO1 data, scores were compared over ML trees consistent with the monophyly of all regional faunas (see constraints as defined above); 28S comparisons were made over the unconstrained ML tree (Fig. 2A). Assuming a χ^2 distribution of the likelihood ratio test statistic with $n - 2$ degrees of freedom (n, number of taxa), this test indicates rate constancy across lineages for the 28S (model GTR + I, $df = 7$, $-\ln L_{\text{CLOCK}} = 1251.77$, $P > 0.010$) and CO1 nucleotide data (model GTR + I + Γ, $df = 19$, $-\ln L_{\text{CLOCK}} = 7766.0$, $P > 0.05$), but a lack thereof for CO1 amino acids (model mtREV24 + Γ, $df = 17$, $-\ln L_{\text{CLOCK}} = 1864.09$, $P = 0.0042$). Interestingly, rejection of amino acid rate constancy might be consistent with the revised relative age hypothesis. If the clades really are of different ages, this suggests that species of the hypothesized older clades (particularly Appalachian species) have *too little* amino acid divergence (see Table 2), perhaps due to constraints acting at the protein level.

How far back in absolute time might the within-region speciation events date? Because there are no known *Hypochilus* divergence dates, addressing this question requires a reliance on external rate calibrations. Such a calibration was applied exclusively to the CO1 data, as standard 28S rate calibrations could not be found in the literature. Based on comparisons of mitochondrial nucleotide divergence in a variety of recently diverged arthropod taxa, Brower (1994) estimated a rate of 2.3% pairwise divergence per million years. Applying this calibration to average interspecific CO1 nucleotide distances (see Table 2) results in estimates of within-Rockies diversification dates that span the Miocene/Pliocene boundary (5 to 6 Ma). However, under the same calibration Appalachian and California divergence estimates correspond to mid-Miocene dates ranging from 10 to 12 Ma, suggesting that most species-level diversification was well under way before the hypothesized Pliocene/Pleistocene dates of Catley (1994).

The absolute time inferences again rely upon assumptions about rate constancy across lineages, but here the comparison of interest lies between *Hypochilus* and other "landmark" taxa (i.e., those used in Brower's (1994) rate calibrations). For example, it might be that the *Hypochilus* lineage as a whole is characterized by accelerated rates of molecular evolution, perhaps explaining the generally deep estimates of divergence times. How fast would *Hypochilus* molecules have to be evolving to be consistent with *a priori* temporal hypotheses? Assume, for example, that Appalachian divergences actually occurred over an interval spanning the late Pliocene/early Pleistocene, an "average" event

occurring 2 Ma. Given an estimated average pairwise divergence of 22.3% (Table 2) for CO1 nucleotides requires a rate (ca. 11% pairwise per million years) approximately 5 times faster than that proposed by Brower (1994). Such a rate calibration would place species' divergence dates of all three regional faunas in the late Pliocene/Pleistocene, consistent with ideas forwarded by Catley (1994). Although accelerated rates have been reported for some mitochondrial genes in various invertebrates (e.g., Chiba, 1999), the accelerated rates proposed above are largely inconsistent with what is known about North American continental biogeography in that they imply interregional divergence dates (ca. 3 Ma between California and Appalachian clades) that are *too* recent. As discussed above, a standard rate provides time estimates of east–west divergences that are consistent with known paleoclimatic events. Ultimately, resolution of absolute rates of molecular evolution in *Hypochilus* will require more data, particularly those relating to more-recent, known divergence dates. But given available data, the most tenable hypothesis is that *Hypochilus* taxa have deep histories of differing ages within each of the disjunct montane regions.

Intraspecific phylogenetics. Cytochrome oxidase data relevant to intraspecific phylogeny are available for 6 of 11 *Hypochilus* species. Although the scope of intraspecific sampling is limited, some remarkable characteristics of the data require comment. A minority of intraspecific comparisons show little divergence, including those between geographically close populations of *H. sheari* and *H. thorelli* (Table 2). However, most population comparisons reveal extraordinarily high divergences, including a majority of values exceeding 10% average pairwise divergence (Table 2). These extreme values are best highlighted by comparison to other CO1 sequence data sets. In a recent summary of CO1 sequence data for a variety of terrestrial arthropods, Wilcox *et al.* (1997) cite several papers with reported intraspecific values of less than 6%. Although these values are considered standard (e.g., Vogler *et al.*, 1993; Simon *et al.*, 1994), there are examples of taxa that show CO1 intraspecific divergences that at least approach those observed in *Hypochilus.* These include tropical pseudoscorpions (max 14% between populations, see Wilcox *et al.*, 1997) and flightless Canary island beetles (max 12%, see Juan *et al.*, 1996), both of which represent cases of cryptic phylogenetic divergence, and at least for the pseudoscorpion data (Wilcox *et al.*, 1997), apparent cryptic speciation.

Given that we have no evidence for accelerated molecular rates in *Hypochilus* (see above), the perhaps more interesting alternative is that rather than being atypical at the molecular level, *Hypochilus* is instead somehow special at the morphological level. We know, in fact, that this is likely true. All *Hypochilus* species retain several plesiomorphic morphological characters that suggest extremely conservative rates of morphological evolution (summarized in Catley, 1994), prompting some authors to classify members of the genus as "living fossils" (Kraus, 1965). Extending this generic-level pattern to the species level, the data presented here suggest that *Hypochilus* species comprise groups of populations that are highly divergent at the DNA level, but share a "diagnostic" morphology. This DNA divergence is unlikely to reflect evolution in large, panmictic populations, as preliminary sampling indicates a complete lack of sequence variation within *Hypochilus* populations (unpublished data). Given the special habitat requirements of these spiders (see Catley, 1994), in combination with the generally discontinuous nature of such habitats, it is more likely that the observed DNA divergence reflects discrete-deme isolation. Understanding the reasons for morphological stasis, in the face of apparently *long-term* population fragmentation and isolation, provides an intriguing area for future studies of *Hypochilus.*

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APPENDIX

Morphological Character Matrix

Taxon	Character No.												
	0	1	2	3	4	5	6	7	8	9	10	11	12
Ectatosticta	0	0	0	0	0	0	0	0	0	0	0	0	0
H. gertschi	1	1	1	1	1	2	1	1	1	3	1	0	0
H. thorelli	1	2	1	1	1	1	0	1	1	4	1	0	0
H. pococki	1	1	1	1	1	1	0	1	1	3	1	0	0
H. coylei	1	2	1	1	1	1	0	1	1	4	2	0	0
H. sheari	1	2	1	1	1	1	0	1	1	4	2	0	0
H. kastoni	0	1	0	1	0	1	0	1	1	2	1	0	0
H. bernardino	0	1	0	1	0	2	1	1	1	2	1	0	1
H. petrunkevitchi	0	1	0	1	0	2	1	1	1	2	1	0	1
H. bonnetti	0	0	0	1	0	0	0	1	1	1	1	1	0
H. jemez	0	0	0	1	0	0	0	1	1	1	2	1	0
H. cf. jemez	0	0	0	1	0	1	0	1	1	1	1/2	1	0

Note. Morphological data matrix for *Hypochilus* and *Ectatosticta*. All of the data except that for *H. cf. jemez* are from Catley (1994). Characters are as follows: No. 0, PTaM (black "thumbprint mark" on retrolateral surface of male pedipalpal tarsus, 0 = absent, 1 = present); No. 1, SP (intensity of dark pigmentation on male sternum, 0 = absent, 1 = light, 2 = heavy); No. 2, CdL (length of male pedipalpal conductor in retrolateral view, 0 = 0.45 − 0.60 mm, 1 = 0.70–1.00 mm); No. 3, PTaL (length of male pedipalpal tarsus in retrolateral view, 0 = >2.5 mm, 1 = <1.3 mm); No. 4, AME (diameter of anterior eye pupil, 0 = 0.07–0.12 mm, 1 = 0.16–0.24 mm); No. 5, PTW/PTL (shape of male pedipalpal tibia, maximum width in retrolateral view in mm/length in retrolateral view in mm, 0 = 0.11–0.15, 1 = 0.17–0.22, 2 = 0.24–0.29); No. 6, SD (Spermathecal ducts, 0 = not convoluted, 1 = convoluted); No. 7, WEB (primary web construct, 0 = sheet, 1 = lampshade); No. 8, SIG (sigilla, 0 = three pairs clearly defined, 1 = poorly defined labial sigilla only); No. 9, APC (shape of apex of conductor, 0 = boat-shaped, 1 = hooked, 2 = twisted, 3 = corkscrewed, 4 = beak-shaped); No. 10, PCT (number of promarginal cheliceral teeth (0 = eight, 1 = five, 2 = six); No. 11, CTre (retromarginal cheliceral teeth, 0 = 1–6 large teeth distributed along entire length, 1 = group of denticles (6–15) situated distally); No. 12, PALP (palpal conductor distal apophysis, 0 = absent, 1 = present).

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