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# Genealogical exclusivity in geographically proximate populations of *Hypochilus thorelli* Marx (Araneae, Hypochilidae) on the Cumberland Plateau of North America

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**Abstract**

**The issue of sampling sufficiency is too infrequently explored in phylogeographical analysis, despite both theoretical work and analytical methods that stress the importance of sampling effort. Regarding the evolutionary pattern of reciprocal monophyly, both the probability of recovering this pattern and the possible inferences derived from this pattern, are highly contingent upon the density and geographical scale of sampling. Here, we present an empirical example that relates directly to this issue. We analyse genetic structure in the southern Appalachian spider *Hypochilus thorelli*, using an average sample of 5 mitochondrial DNA (mtDNA) sequences per location for 19 locations. All sampled sites are reciprocally monophyletic for mtDNA variation, even when separated by geographical distances as small as 5 km. For populations separated by greater geographical distances of 20–50 km, mtDNA sequences are not only exclusive, but are also highly divergent (uncorrected p-distances exceeding 5%). Although these extreme genealogical patterns are most seemingly consistent with a complete isolation model, both a coalescent method and nested cladistic analysis suggest that other restricted, but nonzero, gene flow models may also apply. *Hypochilus thorelli* appears to have maintained morphological cohesion despite this limited female-based gene flow, suggesting a pattern of stasis similar to that observed at higher taxonomic levels in *Hypochilus*.**

*Keywords*: *Hypochilus*, morphological stasis, nested cladistic analysis, phylogeography, population structure, reciprocal monophyly

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## Introduction

Biologists who gather and analyse gene tree data are often preoccupied with the evolutionary pattern of monophyly. We often ask questions such as 'Are sequences of species X a group exclusive of those of species Y?', or 'Do sequences sampled from populations in geographical area Y form a clade exclusive of those sampled from area Z?'. In the case of these pairwise comparisons, a pattern of *reciprocal* monophyly is strong evidence for a lack of gene flow at the genetic locus of interest, most often interpreted as a lack of gene flow among populations, geographical areas or species. This 'strong evidence' from monophyly differs from patterns of paraphyly and/or polyphyly, both of which can be ambiguous with respect to problems of evolutionary divergence. For example, a pattern of gene tree polyphyly is potentially simultaneously consistent with either panmixia (polyphyly arising from ongoing gene flow) or population isolation (polyphyly arising from shared ancestral polymorphism in recently separated, but now isolated, populations; e.g. Takahata & Slatkin 1990).

Evolutionary independence is necessary, but not sufficient, for monophyly to evolve (as indicated by the shared ancestral polymorphism example). Assuming selective neutrality, the other major factors influencing the probability of evolving to reciprocal monophyly include time since isolation and genetic effective size. It is now well established that longer divergence times and/or smaller population sizes (in both parent and daughter populations) increase the probability of reciprocal monophyly (e.g. Tajima 1983; Avise *et al*. 1984; Neigel & Avise 1986). Overlaid upon these evolutionary parameters is the issue of genetic sampling. Both intuition and theory tell us that the

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probability of recovered monophyly might depend upon both the number of sequences sampled per population, and the density and geography of population sampling. For example, if we randomly sample two sequences from each of two populations (of equal size) separated $N_e$ generations ago, the probability of genealogical exclusivity in one or both of the populations is ≈ 0.8 (Fig. 3 of Hey 1994). However, increasing the sample size to five sequences per population with the same demographic parameters drastically decreases this probability ($P \approx 0.3$). As such, sampling insufficiency might falsely indicate monophyly when, in fact, other gene tree patterns would be recovered with more samples (see also Takahata & Slatkin 1990). Similar arguments can be made regarding the geographical density of sampling, where sparse sampling might falsely imply genealogical exclusivity (e.g. Neigel & Avise 1993; Templeton *et al*. 1995; Templeton 1998). In both cases, sampling inadequacies have the potential to mislead evolutionary inferences, and patterns of monophyly may not be as unambiguous as initially perceived.

## Hypochilus

Eleven species of the genus *Hypochilus*, along with the monotypic genus *Ectatosticta*, constitute the early diverging spider family Hypochilidae (Forster *et al*. 1987; Catley 1994). *Hypochilus* species are distributed as three geographical clades of allopatric taxa occurring in the mountains of southern Appalachia, California and the southern Rockies of North America. Within a geographical clade, species are remarkably similar in both ecology and morphology, distinguished only by subtle differences in genitalia (summarized in Catley 1994). This conservative pattern of morphological and ecological variation also largely applies across clades. Molecular phylogenetic work suggests that this apparently limited divergence plus strict allopatry within montane areas is not due to recent common ancestry, as this work indicates deep species-level molecular divergences both within and among montane areas (Hedin 2001). Apparently, despite long-standing opportunity for divergence, different *Hypochilus* species have remained relatively similar in morphology and ecology (e.g. similar enough to preclude sympatry).

It would be interesting to know whether such patterns of stasis also apply at the species level. That is, do *Hypochilus* species comprise sets of populations that are divergent at some level (e.g. genetically), yet morphologically cohesive enough to be regarded as a single taxonomic species? The available data, although limited in scope, suggest that this may be the case. Previous molecular studies of *Hypochilus* were primarily directed at resolving interspecific phylogenetic relationships, but sparse sampling was also conducted within species. In general, this sampling reveals high levels of intraspecific mitochondrial DNA (mtDNA) sequence divergence, exceeding 10% for several *Hypochilus* species (Hedin 2001). Multiple population genetic/phylogeographical models are potentially compatible with this high divergence. For example, divergence may be regional (however defined), with gene flow between populations within a region but isolation between regions. Alternatively, divergence may be more finely structured, with divergence and isolation both within and between regions. Distinguishing among these (and other) alternatives is obviously crucial to an understanding of evolutionary dynamics within *Hypochilus* species, particularly stasis at the species level.

To further explore these issues we have gathered a geographically comprehensive sample of mtDNA sequences for the Appalachian species *Hypochilus thorelli* Marx. This species is distributed along a north–east-tending arc from northern Alabama and Georgia, through Tennessee west of the Tennessee River Valley, to near the confluence of the borders of Kentucky, Virginia and Tennessee (Fig. 37 of Forster *et al*. 1987; our Fig. 1). The major landform features of this region are the massive escarpments of the Cumberland Plateau, consisting of exposed sandstone, shale and carbonate Palaeozoic rocks. These rocky uplands (up to 1200 m in elevation) are some of the oldest in North America, having undergone erosional decay since a Mesozoic Alleghanian orogeny, with many current landforms essentially unchanged since the mid- to late Tertiary (Miller 1974). Despite this perhaps long-term temporal stability (i.e. the landscape may have been available for occupation by *Hypochilus* for a considerable amount of time), the landscape is spatially fragmented to varying degrees. Some rock outcrops are potentially continuous for hundreds of kilometres (e.g. Walden's Ridge), whereas other outcrops are isolated at small geographical scales, separated by rivers, valleys and/or mesic woodlands.

Similar to other Appalachian *Hypochilus* (see Hoffman 1963; Shear 1969; Fergusson 1972; Huff & Coyle 1992), the preferred web substrates and habitats for *H. thorelli* are vertical or overhanging rock faces, most often in sheltered, more mesic situations (e.g. Riechert & Cady 1983; Hodge & Marshall 1996). In this sense, these spiders appear to have relatively strict ecological requirements, as they are rarely found away from rocks. This, however, does not imply that the spiders are rare, as they can be quite common in appropriate habitats (Riechert & Cady 1983; Hodge & Marshall 1996). Not all rocky habitats have spider populations, however, as we visited several apparently suitable sites well within the range of *H. thorelli* that lacked these very noticeable spiders (personal observation). This last observation suggests that *H. thorelli* may be dispersal limited. Studies of other Appalachian taxa suggest that juvenile *Hypochilus* do not balloon, and that adult males searching for females may account for the majority of individual-based dispersal (see Fergusson 1972; Huff & Coyle 1992).

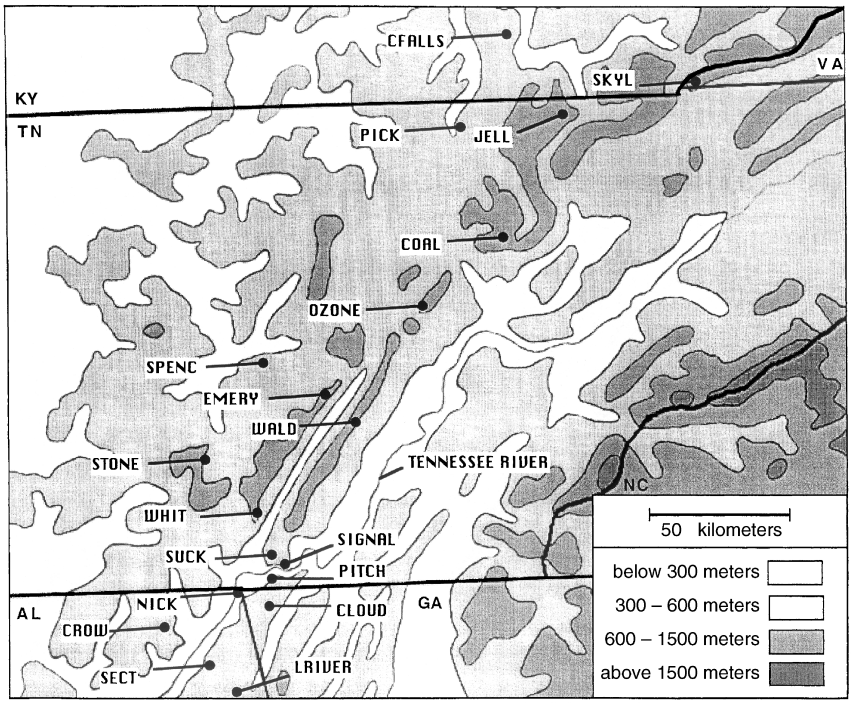


**Fig. 1** Collection locations of *Hypochilus thorelli* on the Cumberland Plateau of eastern North America. Population acronyms as in Table 1.

## Materials and methods

Specimens of *Hypochilus thorelli* were collected from locations throughout the known range of this species (following Forster *et al*. 1987). Although an effort was made to sample uniformly across this region, more locations at closer geographical distances were sampled towards the southern edge of the range (Fig. 1; Table 1). At each location a series of individuals was collected, including juveniles and adults of both sexes. Adults were used to confirm species identity (following Catley 1994) and are available as vouchers in the personal collection of MH. The strict allopatry of *Hypochilus* species allowed us to use juveniles for DNA extraction. At each location we made a concerted effort to disperse our collecting efforts over linear distances of 100–300 m, attempting to minimize the possibility of collecting siblings or close relatives.

Entire legs were removed in the laboratory from freshly sacrificed specimens. Legs and the remaining specimen were placed in cryotubes, preserved in 100% EtOH, and stored at –80 °C. Genomic DNA was extracted from leg tissue using the CTAB protocol of Shahjahan *et al*. (1995). Genomic templates were extracted and polymerase chain reaction (PCR)-amplified as sets of single animals from multiple populations, rather than sets of multiple individuals from the same population. We did this to maximize the possibility of detecting sequence contamination, although no such contamination was evident in our data (see below).

An ≈ 800 bp fragment of the cytochrome oxidase I gene was amplified via PCR using the *Hypochilus* devoted primers C1-J-1751HYPO (5′-GCGCCGGATATGGCGTTTC-3′) and C1-N-2568THOR (5′-GCCACAACGTAATAAGTATC-3′). PCR experiments were conducted on an MJ Research PTC-100 thermal cycler, with the following conditions: 92 °C initial denaturation for 30 s, 30 cycles of 92 °C for 30 s, 44 °C for 45 s +0.2 °C per cycle, 72 °C for 1 min 30 s, final extension at 72 °C for 5 min. PCR products were purified using either polyacrylamide gels or PEG, and sequenced directly using ABI Big Dye chemistry on an ABI 377 machine. Both strands were determined for a majority of templates using PCR primers; some sequences were found to be identical to other haplotypes in the same population (see Table 1), and thus were only determined in a single direction. MACCLADE Version 4.0 (Maddison & Maddison 2000) was used to edit, manually align and manipulate sequences. Cytochrome oxidase sequences from other *Hypochilus* species were used to root *H. thorelli* trees, including sequences of species from California (*H. kastoni*, AF303521), the Rocky Mountains (*H. bonnetti*, AF303525) and the Appalachians (*H. pococki*, AF303511, AF303512, AF303513; *H. sheari*, AF303515, AF303516; *H. gertschi*, AF303518, AF303519). These sequences were reported in Hedin (2001).

### *Selection*

In this study we used patterns in the geographical and genealogical structuring of mtDNA variation to make inferences about population history. These inferences are contingent upon the selective neutrality of mtDNA

**Table 1** Locality, haplotype and Accession no. information

| Locality | Acronym | *N* | Accession no. |
|---|---|---|---|
| VA: Lee Co., Skylight Cave | SKYL 1* | 5 | AF303510 |
| KY: Whitley Co., E of Cumberland Falls SP | CFALLS 1 | 4 | AY102038 |
| TN: Campbell Co., E of Jellico | JELL 1 | 3 | AY102039 |
| | JELL 2 | 1 | AY102040 |
| | JELL 3 | 1 | AY102041 |
| TN: Morgan Co., NW of Coalfield | COAL 1 | 5 | AY102042 |
| TN: Pickett Co., Pickett State Forest | PICK 1 | 2 | AY102043 |
| | PICK 2 | 3 | AY102044 |
| TN; Cumberland Co., Ozone Falls | OZONE 1 | 4 | AY102045 |
| | OZONE 2† | 1 | AF303509 |
| TN: Van Buren Co., E of Spencer | SPENC 1 | 2 | AY102046 |
| | SPENC 2 | 2 | AY102048 |
| | SPENC 3 | 1 | AY102047 |
| TN: Bledsoe Co., Emery Mill | EMERY 1 | 5 | AY102049 |
| TN: Rhea Co., Walden Ridge, W of Dayton | WALD 1 | 5 | AY102050 |
| TN: Grundy Co., Savage Gulf NA, Stone Door | STONE 1 | 3 | AY102051 |
| | STONE 2 | 1 | AY102052 |
| | STONE 3 | 1 | AY102053 |
| TN: Marion Co., NW of Whitwell | WHIT 1 | 4 | AY102054 |
| | WHIT 2 | 1 | AY102055 |
| TN: Marion Co., Hwy 27, along Suck Creek | SUCK 1 | 2 | AY102056 |
| | SUCK 2 | 2 | AY102057 |
| | SUCK 3 | 1 | AY102058 |
| TN: Hamilton Co., Pitchfork Cave | PITCH 1‡ | 2 | AF303507 |
| TN: Hamilton Co., Signal Mountain | SIGNAL 1§ | 3 | AF303508 |
| GA: Dade Co., Cloudland Canyon SP | CLOUD 1 | 2 | AY102059 |
| | CLOUD 2 | 1 | AY102060 |
| | CLOUD 3 | 1 | AY102061 |
| | CLOUD 4 | 1 | AY102062 |
| AL: Jackson Co., vic. Nickajack Cove | NICK 1 | 5 | AY102063 |
| AL: Jackson Co., Crow Mtn. Escarpment | CROW 1 | 2 | AY102064 |
| | CROW 2 | 3 | AY102065 |
| AL: Jackson Co., NE of Section | SECT 1 | 5 | AY102066 |
| AL: DeKalb Co., Little River Canyon | LRIVER 1 | 5 | AY102067 |

Called *THOR 1, †THOR 2, ‡THOR 3, §THOR 4 in Hedin (2001).

variation, an hypothesis that must be tested, particularly if patterns of mtDNA variation are apparently extreme (see Ballard *et al*. 2002). We tested for selection at the DNA level using the McDonald–Kreitman test (McDonald & Kreitman 1991). This test is based on the neutral prediction that the ratio of nonsynonymous to synonymous substitutions observed within and between 'species' should be roughly equal (McDonald & Kreitman 1991; Sawyer & Hartl 1992). Importantly, the test assumes that 'species' are panmictic and of constant size (see Wayne & Simonsen 1998), an assumption that does not apply to *H. thorelli* (see below). Clearly, some (most) of the molecular variation within *H. thorelli* is partitioned between populations (divergence), rather than within populations (polymorphism). As such, we used populations as the unit of analysis for tests of selection, counting fixed differences across populations as 'divergence', and segregating sites within populations as 'polymorphism'. Contingency tables were assessed for statistical significance using Fisher's exact test, as many cell values were small (following Sawyer & Hartl 1992).

### *Tree-based analyses*

The Templeton *et al*. (1992) network estimation procedure was used to resolve relationships between closely related haplotypes. This procedure is implemented in the TCS Version 1.13 software of Clement *et al*. (2000). Because levels of interpopulation sequence divergence in *H. thorelli* were generally high, we also used standard phylogenetic methods (parsimony and maximum likelihood) implemented in PAUP* Version 4.0b8 (Swofford 1999). Parsimony analyses were conducted using heuristic searches (TBR branch-swapping, 500 random addition

sequence replicates). Relative support of reconstructed clades was evaluated using the nonparametric bootstrap (Felsenstein 1985), based on analyses comprising 500 replicates of a heuristic parsimony search (TBR branch-swapping, 10 random addition sequence replicates per pseudoreplicate). Likelihood ratio tests were used to choose among nested models of molecular evolution, using the hierarchical framework implemented in the MODELTEST Version 3 software (Posada & Crandall 1998). Using a best-fit model, likelihood tree estimates included: (i) initial estimation of model parameters on parsimony trees, (ii) a heuristic likelihood search (TBR branch-swapping, 5 random addition sequence replicates) with fixed parameters, and (iii) reoptimization of parameters on maximum likelihood (ML) trees. This process was repeated until ML trees of approximately equal likelihood were found in successive searches.

### *Nonimmigrant ancestry*

Our results (see below) prompted us to ask whether the *Hypochilus* data were consistent with a 'population fragmentation' model, in which populations are descended from a common ancestral population at some time in the past and have since diverged in isolation. This isolation confines mutations that have arisen since fragmentation to the geographical region or population of origin. We tested this model using the coalescent approach of Slatkin (1989), which places an upper limit on the probability that all $n$ sequences randomly drawn from a population are descended from an ancestral sequence that was in that population, given some arbitrary level of gene flow ($N_m$). Slatkin (1989) termed this the 'probability of nonimmigrant ancestry' or $P(n, N_m)$. This probability is dependent upon both biology (i.e. levels of gene flow) and sampling effort. Finding patterns consistent with nonimmigrant ancestry (i.e. monophyly) with large $n$ is improbable, unless gene flow is very low (Fig. 1 of Slatkin 1989). However, we might expect to find patterns consistent with nonimmigrant ancestry by chance alone with small sample size, even with moderate levels of gene flow (Fig. 1 of Slatkin 1989).

### *Nested cladistic analysis*

A limitation of the Slatkin (1989) method is that it is nongeographical (i.e. the method assumes an island model). We therefore performed a more geographically explicit nested cladistic analysis (Templeton *et al*. 1995; Templeton 1998) on a subset of our data. Nested cladistic analysis (NCA) can be used to test for significant associations between genetic variation and geography, and to distinguish among alternative potential causes of such geographical associations. Haplotype relationships were reconstructed using a combination of parsimony and the TCS procedure, and the resulting network was 'nested' according to published rules (Templeton *et al*. 1987; Crandall 1996). The nested network was input into the software program GEODIS Version 2.0 (Posada *et al*. 2000) to calculate clade ($D_C$) and nested clade distances ($D_N$). Clade distances measure the geographical range of haplotypes in a given clade, whereas nested clade distances measure how a given clade is geographically distributed relative to related clades in a more inclusive 'nesting clade' (see Templeton *et al*. 1995; Templeton 1998). Patterns of contrast or coincidence in the structuring of these distances were used to make biological inferences, following general population genetic predictions outlined in Templeton *et al*. (1995).

## Results

### *Sequence data*

New CO1 sequences (804 bp) were gathered for 85 individuals from 19 populations of *Hypochilus thorelli* (Table 1); CO1 sequences from 4 *H. thorelli* individuals were reported previously (Hedin 2001). In total, these 89 individuals carry 34 unique haplotypes (Table 1). Sequences from animals collected at the same site are closely related. Of the 17 locations that include sample sizes greater than 4 individuals, 8 are fixed for the same mitochondrial sequence. Nine populations carry multiple haplotypes, with uncorrected sequence divergence values ranging up to 0.62% within such populations. In contrast, animals collected from different sites are typically genetically divergent. There are no haplotypes shared between any two populations, and interpopulation divergence values (as above, probably underestimates because of saturation) range from 0.25 to 11.1%, with a majority of values > 5%. The overwhelming pattern of low internal variation, combined with larger interpopulational distances, indicates that essentially all mitochondrial genetic variation in *H. thorelli* is apportioned among, rather than within, populations.

### *Selection*

Across the array of *H. thorelli* populations there exists variation at 12 amino acid positions. Most of this variation is conservative, involving substitutional interchange of the nonpolar residues isoleucine, valine and alanine. A character mapping of this variation onto a most-parsimonious tree suggests low homoplasy (Fig. 2), with single instances of apparent reversal (position 118) and parallelism (position 136) in different parts of the tree. Most nonsynonymous variation is fixed (i.e. accounts for population divergence), with only two populations

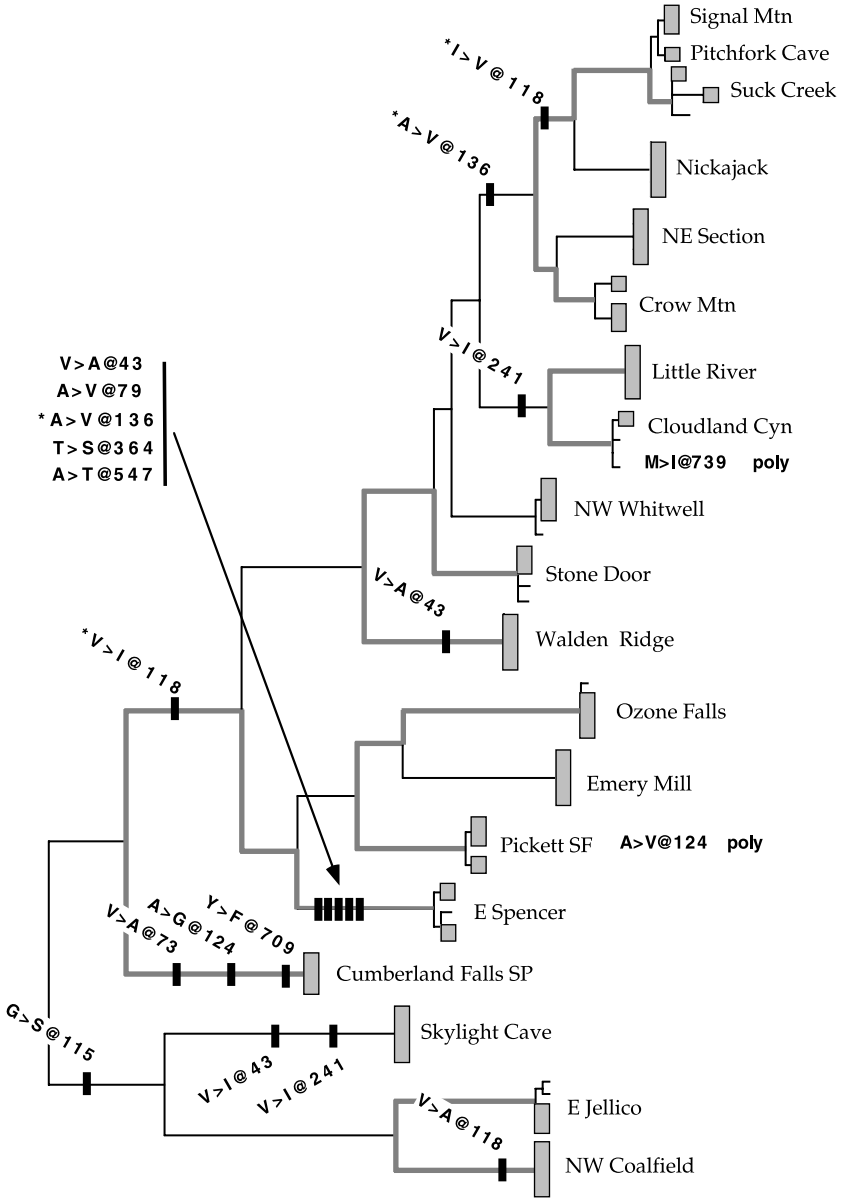


**Fig. 2** Parsimony reconstruction of protein evolution. Site numbers correspond to first codon position of each amino acid. Asterisks denote amino acid changes that are homoplastic on the tree. Population comparisons used in McDonald–Kreitman tests highlighted by grey shading. Tree same as that of Fig. 3, including scaled branch lengths.

carrying nonsynonymous polymorphism. The fixation of five nonsynonymous substitutions along the branch leading to the Spencer sequences is particularly striking (Fig. 2).

We used the phylogenetic distribution of fixed vs. polymorphic nonsynonymous variation (Fig. 2) to direct our choice of populations used in McDonald–Kreitman tests. We did this for two reasons. First, the phylogenetic topology suggests which pairwise comparisons are independent (i.e. do not share branches in common). In principle, we wanted to maximize this number of independent contrasts. However, many such contrasts precluded statistical analysis because of a lack of nonsynonymous variation, a lack of polymorphism or both (i.e. contingency tables included > 2 cells with zero values). As such, we constrained our analysis to phylogenetically independent population contrasts with both polymorphism (either nonsynonymous or synonymous) and nonsynonymous variation (either fixed or polymorphic). We could not reject the null hypothesis of neutrality for six such contrasts (see Fig. 2, Table 2), although sample sizes were low in all cases. Of course, other contrasts are possible given these constraints; tests using these alternatives are also nonsignificant (results not shown). To increase sample size we also analysed the entire array of populations, counting sites as either fixed between populations or polymorphic within populations, using the parsimony tree to identify site homoplasy. Contingency analysis of these counts fails to indicate a deviation from neutrality (Table 2).

**Table 2** Results of McDonald–Kreitman tests for selection. *P*-Values based on Fisher's exact test. Population comparisons as indicated in Fig. 2; see text for discussion

| Population comparison | | Fixed | Poly | *P*-value (2-tailed) |
|---|---|---|---|---|
| Crow Mtn — Suck Creek | Nonsynonymous | 1 | 0 | 1 |
| | Synonymous | 22 | 8 | |
| LittleRiver — Cloudland Cyn | Nonsynonymous | 0 | 1 | 0.1429 |
| | Synonymous | 18 | 2 | |
| Stone Door — Walden Ridge | Nonsynonymous | 1 | 0 | 1 |
| | Synonymous | 37 | 2 | |
| Ozone Falls — Pickett SF | Nonsynonymous | 0 | 1 | 0.0667 |
| | Synonymous | 42 | 2 | |
| E Spencer — Cumberland Falls | Nonsynonymous | 9 | 0 | 1 |
| | Synonymous | 51 | 3 | |
| E Jellico — NW Coalfield | Nonsynonymous | 1 | 0 | 1 |
| | Synonymous | 34 | 2 | |
| All Populations | Nonsynonymous | 17 | 2 | 0.6310 |
| | Synonymous | 278 | 20 | |

### *Tree-based analyses*

Haplotypes that differ by up to 12 observed differences can be connected under the 95% probability limits of the TCS estimation procedure. This allows intrapopulation haplotype connections (results not shown), and interpopulation connections of haplotypes from the geographically adjacent Suck Creek, Pitchfork Cave and Signal Mountain sites (Fig. 3 inset). All other interpopulation connections are beyond the divergence limits statistically justified by the TCS procedure. These haplotype relationships were reconstructed using standard phylogenetic methods.

Heuristic parsimony analysis of all *H. thorelli* sequences (including identical sequences) plus outgroup sequences results in 48 trees of length 1097. The tree shown in Fig. 3 is representative, as other most-parsimonious trees differ only in resolution of sequence relationships within populations. Sequences sampled from the same location almost always form monophyletic clades. These clades are well supported in bootstrap analyses, with 17 population clades recovered in > 93% of bootstrap replicates. The only exceptions involve sequences from the geographically adjacent Pitchfork Cave and Signal Mountain sites, with PITCH sequences paraphyletic with respect to SIGNAL sequences in some parsimony trees. Because the TCS method has more power at this low level of divergence (Crandall 1994; Posada & Crandall 2001), we prefer the TCS estimate that indicates reciprocal population monophyly (Fig. 3 inset). The deep structure of the parsimony tree suggests that northern populations are basal, and that populations can be arranged into three clusters (i.e. 'mostly southern', 'central + KY' and 'northern'), two of which are monophyletic under the parsimony criterion.

Likelihood analyses were conducted on a matrix of 34 unique *H. thorelli* sequences plus outgroups. Hierarchical likelihood ratio tests suggest that a five parameter model (four transversion rates, one transition rate) with unequal base frequencies and among-site rate variation (%I + Γ) explains the data as well as more complex models. Two rounds of the successive approximation approach were accomplished prior to settling on a single likelihood tree (–Ln likelihood = 5794.67) with the following parameter estimates: r-matrix = 0.782 (a), 9.22 (b), 0.677 (c), 1.021 (d), 1 (e); base frequencies = A:0.241, C:0.109, G:0.174, T:0.477; proportion of invariable sites = 0.531; gamma shape parameter = 0.738 (all notation following PAUP* 4.0b8). Similar to the parsimony result, the likelihood tree suggests that: (i) haplotypes from the same location always form monophyletic groups, (ii) northern populations are basal, and (iii) populations are arranged into three clusters, each separated by a relatively long internal branch from other clusters. Unlike the parsimony result, only the 'mostly southern' cluster is recovered as monophyletic under the likelihood criterion.

### *Nonimmigrant ancestry analyses*

Results of gene tree analyses indicate that *Hypochilus* collecting sites correspond directly to exclusive mitochondrial clades (Fig. 3). We assumed that this concordance indicates nonimmigrant ancestry within populations, and used equation 2 of Slatkin (1989) to find values of $N_m$ $(N_m)_{crit}$, for which $P(n\ (N_m)_{crit}) = 0.05$. If $N_m > (N_m)_{crit}$, the probability of finding this concordance is < 5%. For the *H. thorelli* data, given 3–5 sequences sampled per population $(N_m)_{crit}$ values range from ≈ 1.3 to > 2. This result implies that the *H. thorelli* data are consistent with low, but nonzero, levels of gene flow. If we had sampled

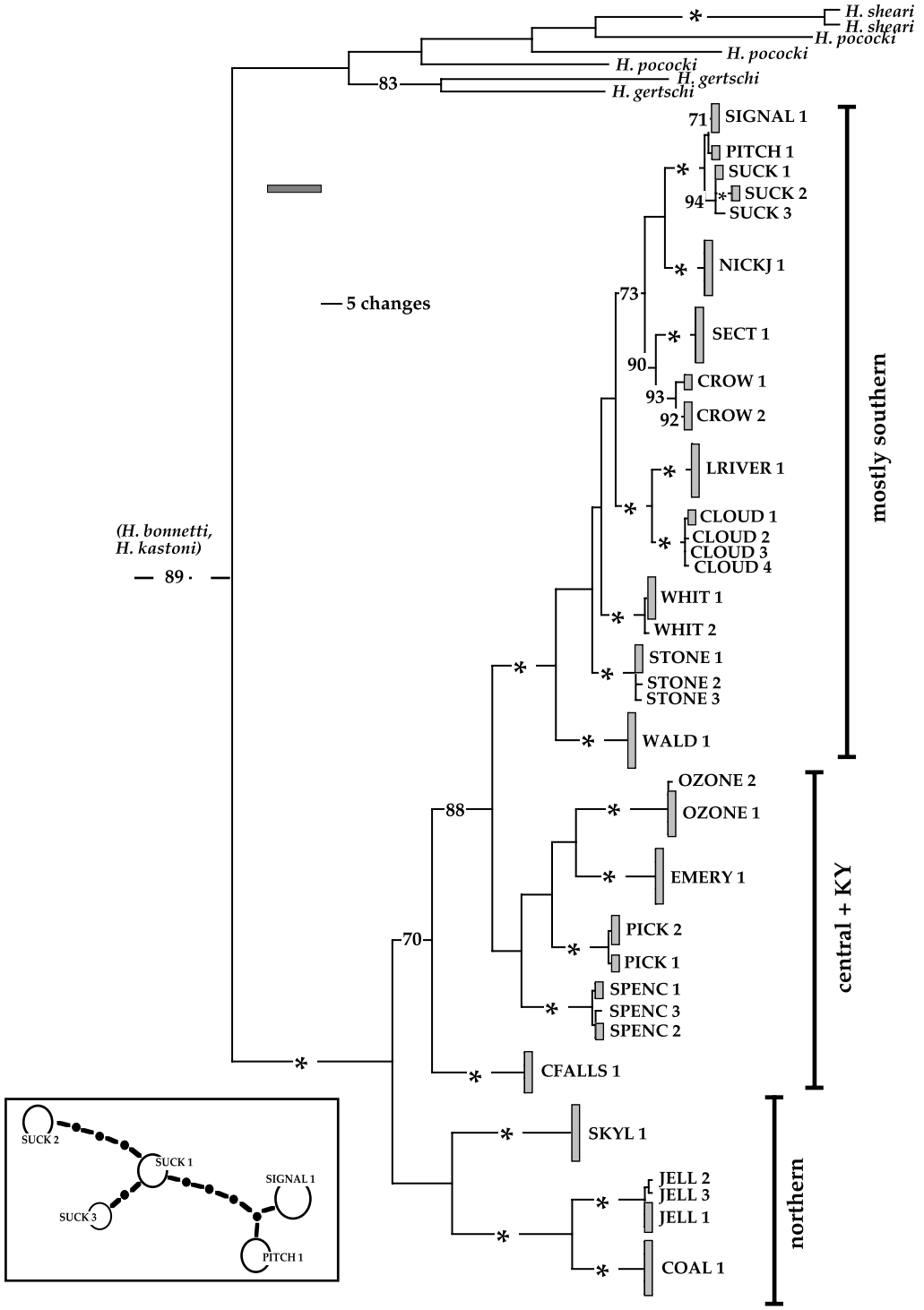


**Fig. 3** Representative maximum parsimony phylogram (tree shown is 1 of 48 MP trees, L = 1097). Bootstrap proportion values greater than 95 represented by asterisks, those > 70 indicated by number, those < 70 not shown. Haplotype acronyms as in Table 1; height of filled boxes associated with haplotype names roughly indicating haplotype frequency (see Table 1). Distant outgroup relationships to *Hypochilus kastoni*, then *H. bonnetti*, not shown. Inset: Network of haplotypes sampled from Pitchfork Cave, Signal Mountain and Suck Creek, estimated using TCS program of Clement *et al*. (2000). Circle size represents haplotype frequency. Small filled circles designate necessary intermediate haplotypes not found in the sample.

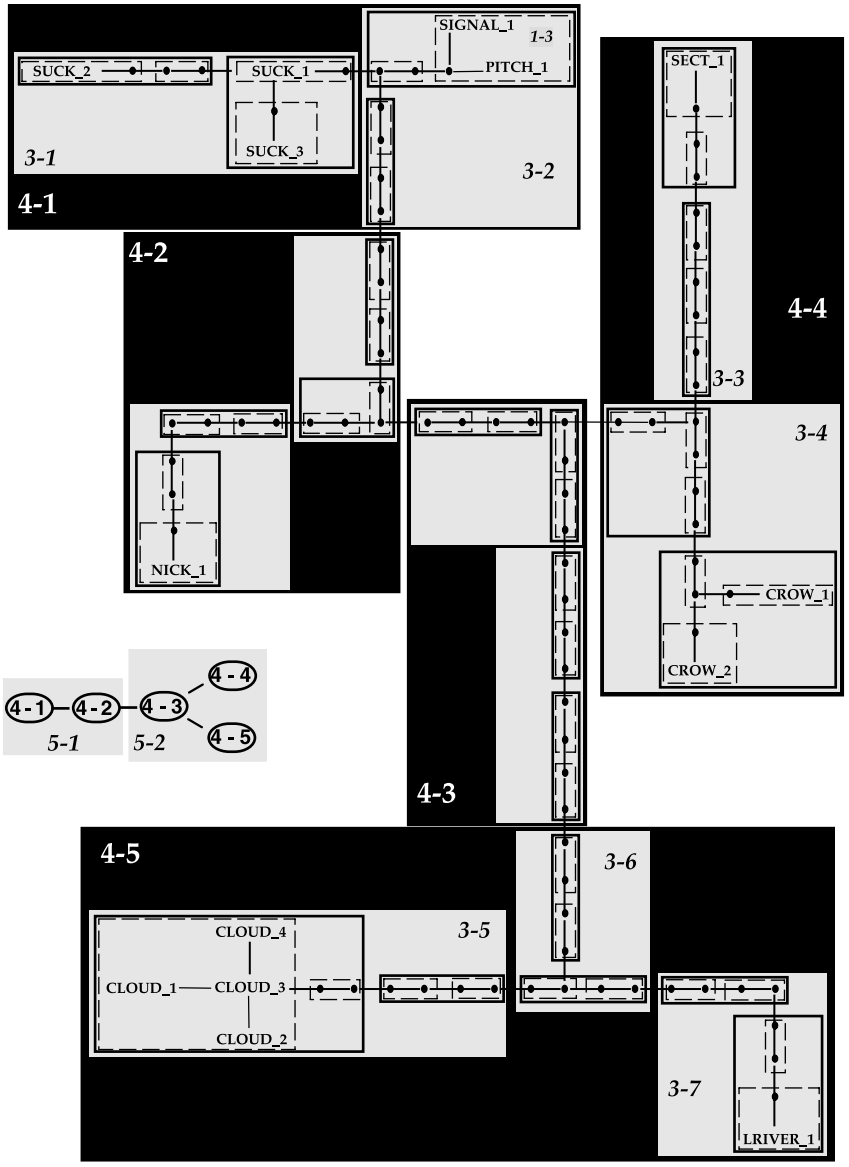


**Fig. 4** Input network used in nested cladistic analysis.

more sequences per population and recovered the same exclusive patterns we would have had stronger evidence for reduced gene flow.

### *Nested cladistic analysis*

We restricted NCA to a subset of the data for which we had *relatively* dense geographical sampling, corresponding to eight locations from the 'mostly southern' clade of populations. Of seven 'nesting clades' (haplotype groups that include subgroups) that include both geographical and genetic variation (clades 1-3, 4-1, 4-4, 4-5, 5-1, 5-2, entire network; Fig. 4), we can reject the null hypothesis of no statistical association between geography and genetic variation in six cases (all but 1-3; Table 3). Recurrent patterns of $D_C$ vs. $D_N$ values are evident for these six clades (Table 3). Essentially all clade distances ($D_C$) are significantly small (significance assessed by random permutation tests), reflecting an underdispersed geographical spread of haplotypes within any given clade. Many $D_C$ values are zero, meaning that all genetic variation within a clade is confined to a single geographical site. Nested clade distances show reversals from this pattern, where at least one of two $D_N$ values for all nesting clades is significantly large (Table 3). Large $D_N$ values for clades 3-1, 3-3, 3-7, 4-2, 4-5 and 5-2 all reflect instances in which a clade of geographically confined haplotypes is well separated from the geographical centre of the more inclusive nesting clade (Table 3). We pay less attention to interior vs. tip contrasts in our data, as most clades occupy tip positions. Also, the categorization of the few interior clades is ambiguous, as alternative nestings suggest alternative tip-interior status

**Table 3** Results of nested cladistic analysis. Nesting clades and clades as in Fig. 4

| Nesting clade | Chi-square* | Clades | $D_C$† | $D_N$† | GAP |
|---|---|---|---|---|---|
| 1-3 | 0.109 | | | | Inference chain‡ |
| 4-1 | 0.006 | 3-1 (Tip) | $0^S$ | $3.5^L$ | (1) Significant values for $D_C$ or $D_N$? YES |
| | | 3-2 (Int) | $1.29^S$ | 2.07 | (2) $D_C$ tip values significantly small? YES |
| | | I-T | $1.29^L$ | $0^S$ | (3) $D_N$ significantly reversed from $D_C$ values? YES |
| 4-4 | 0.006 | 3-3 (Tip) | $0^S$ | $11.9^L$ | (5) Do clades with restricted geographical distributions have |
| | | 3-4 (Int) | $0^S$ | $11.9^S$ | nonoverlapping ranges? YES |
| | | I-T | 0 | $0^S$ | (15) Are sampled sites separated by areas that have not been |
| 4-5 | 0.000 | 3-5 (Tip) | $0^S$ | $27.12^S$ | sampled? YES |
| | | 3-7 (Tip) | $0^S$ | $27.13^L$ | (16) Is the species absent in the nonsampled areas? NO |
| 5-1 | 0.001 | 4-1 (Tip) | $2.55^S$ | $7.22^S$ | (18) Are the clades found in the different geographical locations |
| | | 4-2 (Int) | $0^S$ | $20.19^L$ | separated by a branch length with a larger than average number of |
| | | I-T | 0 | $12.9^L$ | mutational steps? YES |
| 5-2 | 0.000 | 4-4 (Tip) | $11.9^S$ | $23.9^S$ | Inference: Geographical sampling scheme inadequate to |
| | | 4-5 (Tip) | 27.13 | $34.31^L$ | discriminate between fragmentation and isolation by distance |
| Entire | 0.000 | 5-1 (Tip) | $10.46^S$ | 29.92 | |
| | | 5-2 (Tip) | $29.08^S$ | $38.28^L$ | |

*Chi-square results based on exact permutational (1000 resamples) contingency analysis of categorical variation (clade × geographic location).
†Statistical significance of clade and nested clade distances determined by 1000 random permutations of clades against sampling location. Superscript S/L corresponds to significantly small distance/large distance at $P = 0.05$ level.
‡Inference chain from appendix of Templeton *et al*. (1995).

(e.g. clades 3-2, 3-4; Fig. 4 and Table 3). The statistical results of nested cladistic analysis were used to make population structure inferences using the inference key of Templeton *et al*. (1995). We followed a similar 1-2-3-5-15-16-18 path for all nesting clades, leading to the conclusion that our geographical sampling scheme is inadequate to discriminate fragmentation from isolation by distance (see Table 3). In this case, it is primarily the overdispersed geographical sampling, rather than the number of sequences sampled per site, that limits our evolutionary inference.

## Discussion

It is obvious that there exists a nonrandom association between genetic variation and geography in our *Hypochilus thorelli* sample, as all haplotypes are confined to single geographical locations. The question then becomes, what does this nonrandom association tell us about both current and historical population demographics in this species. The answer is not as obvious as one might assume. Taken at face value, the general pattern of genealogical exclusivity suggests an 'isolation' model. We applied the Slatkin (1989) method to place an upper bound on the level of gene flow consistent with genealogical exclusivity, but could not reject a *limited gene flow* model, given our level of per site sampling. That is, the data are potentially consistent with either no gene flow, or limited but undetected, gene flow.

However, the Slatkin (1989) method does not explicitly consider geography in estimates of gene flow. The method is based on the island model, in which the probability of gene exchange is independent of geography (i.e. all populations are equally likely to exchange migrants). This suggests that an alternative analytical approach, one that explicitly considers the geographical position of populations, might allow us to distinguish no gene flow from limited gene flow. We used nested cladistic analysis (Templeton *et al*. 1995; Templeton 1998) to address this possibility. Both population fragmentation (no gene flow) and isolation by distance (limited gene flow) models are incorporated into NCA, and are the most obvious alternatives for our data. Importantly, the method also considers sampling sufficiency, pointing out instances where the available sample is inadequate to distinguish alternative models (see Templeton *et al*. 1995; Templeton 1998).

Our use of NCA suggests such inadequacy, as we are unable to discriminate fragmentation from isolation by distance given our geographical sample (see Table 3). Two key characteristics of our data drive this inference. First, genetic variation is both genealogically and spatially clumped, such that individuals sampled from a particular geographical site are genetically similar, whereas individuals sampled from different geographical sites are genetically dissimilar. Obviously, such clumping is a prediction of population fragmentation, where *habitat discontinuities*

restrict gene flow and allow the accumulation of local genetic differences. Alternatively, consider a species that is *continuously distributed* (or nearly so) over an area that is large relative to individual dispersal distances, such that local genetic differences accumulate because of genetic isolation by distance. With mutation, such models also predict genealogical and spatial clumping (see Neigel & Avise 1993; Barton & Wilson 1995; Templeton *et al*. 1995), but this clumping is 'fuzzier' than that expected under a fragmentation model. This is because we expect the accumulation of local genetic differences, but we also expect populations separated by distance to share some genetic variation, given enough time.

Importantly, if we sample at geographical scales that greatly exceed individual dispersal distances, population fragmentation and isolation by distance predict similar genetic patterns (see Templeton *et al*. 1995). That is, the models become indistinguishable because of geographically overdispersed sampling. This is apparently the case for *H. thorelli*, and overdispersed sampling is the second key characteristic of our data that drives the ambiguous NCA inference. Most sampled sites are separated by unsampled areas of suitable, approximately continuous, habitat. Until we sample such intervening habitats we must consider both population fragmentation and isolation by distance as viable alternatives for our data, even for geographically close populations. The Signal Mountain, Suck Creek and Pitchfork Cave sites illustrate this point. Signal Mountain and Pitchfork Cave lie on opposite sides of the Tennessee River, which a priori might be considered a strong barrier to gene flow. However, these geographically close populations (≈ 2 km apart) are genetically similar (Fig. 3 inset). Alternatively, Signal Mountain and Suck Creek are slightly more geographically separated (≈ 4 km apart), but lack obvious intervening habitat barriers. These populations are more genetically diverged (Fig. 3 inset), consistent with a slightly greater geographical separation. These fine-scale patterns suggest that distance is the primary determinate of genetic divergence, but testing this hypothesis will require considerably more sampling at local geographical scales.

Of course, gene flow dynamics determine genetic structures in combination with other demographic (e.g. female family size) and nondemographic (e.g. selection on mtDNA) parameters. We tested for selection, but could not reject the null hypothesis of neutrality for our data. Other demographic factors are almost certainly impacting genetic structuring in *H. thorelli*, but these multiple factors are largely confounded in single gene analyses. Ambiguity in estimating any single parameter magnifies this problem. In our case, if gene flow in *H. thorelli* is limited only by distance, then arguments about factors that facilitate monophyly (e.g. small population size, high variance in female family size; Avise *et al*. 1984) in the face of a diffusive force (gene flow) are relevant and perhaps necessary to explain our data. Alternatively, if gene flow is currently nonexistent in *H. thorelli*, and has been so for some time, then isolation per se is perhaps enough to explain our data. Again, additional dense sampling will be needed before we can fully explore these alternatives.

### *Broader picture*

Our *H. thorelli* results might be considered by some as preliminary or pilot information. Two primary analyses that we have performed suggest an inadequate sample, indicating that more samples per site for more sites are needed to make conclusive evolutionary inferences. Although partially valid, this perspective is biased by our presentation of the data, as we have stressed the need for statistical evaluation of sampling adequacy, and have emphasized the inadequacies of our own data. Many studies in the phylogeographical realm fail to do this, conveniently side stepping the issue. We also feel that the 'preliminary data' stance understates the importance and potential significance of our results. The fact that sequences from *all* sample locations form monophyletic clades, *regardless* of geographical proximity is rather striking, and biologically significant for multiple reasons. We discuss two points of significance below.

First, although hundreds of studies have uncovered mtDNA 'phylogroup' structuring within species (summarized in Avise 2000), very few have discovered the combination of extreme structuring seen in *H. thorelli*. This combination includes many monophyletic units, most separated by relatively low geographical distances (e.g. 10–50 km), but high genetic divergences. The only vertebrates exhibiting such mtDNA structuring at geographical scales approaching that of *H. thorelli* are highly dispersal-limited (e.g. gophers, see Avise *et al*. 1979; salamanders, see Garcia-Paris *et al*. 2000; reef fishes, see Planes *et al*. 2001). Extreme mtDNA divergence and genealogical structuring is more often seen in small, dispersal-limited terrestrial arthropods, including spiders. Although very few published studies have considered spider phylogeography (fewer than five), three of these studies reveal mtDNA structuring similar to *H. thorelli*. Appalachian cave spiders (*Nesticus* sp.), California trapdoor spiders (*Aptostichus simus*) and Arizona jumping spiders (*Habronattus pugillis*) all show evidence for reciprocal mtDNA monophyly in geographically close populations, often with high divergence (Hedin 1997; Masta 2000; Bond *et al*. 2001). It will be interesting to see if future studies of dispersal-limited terrestrial arthropods continue to uncover such extreme genealogical structuring. Perhaps many such animals exist in a 'fine-grained' demographic world that has been under-appreciated and largely unexplored to date. If so, these animals will make wonderful systems for phylogeographical and comparative

biogeographical analyses, as exemplified by analyses of arthropods of New Zealand (e.g. Trewick *et al*. 2000; Trewick & Wallis 2001) and the Canary Islands (e.g. Brunton & Hurst 1998; Emerson *et al*. 1999; Salomone *et al*. 2002).

Second, the mtDNA data provide a potentially interesting perspective on patterns of morphological evolution in *Hypochilus*, particularly morphological stasis. As summarized in the Introduction, different *Hypochilus* species have remained relatively similar in morphology and ecology, despite long-standing opportunity for divergence. This is a classic pattern of 'decoupling', in which rates of evolution differ strongly across levels of organization (e.g. molecules neutral, morphology slow). We can potentially reveal the 'decoupling' pattern at the species level using a population genetic approach. To date, the various taxonomic works that have considered *H. thorelli* do not specifically mention geographical variation in morphology (Gertsch 1958; Forster *et al*. 1987; Catley 1994). In this sense, *H. thorelli* is morphologically 'cohesive'. This cohesion may reflect genetic exchange among populations, or perhaps, recent origin. Alternatively, this cohesion may be maintained in the absence of gene flow, either via stabilizing selection (e.g. Ehrlich & Raven 1969) or other constraint mechanisms (see Wake *et al*. 1983; Larson *et al*. 1984). Importantly, gene flow only confers cohesion within species, whereas the latter mechanisms potentially confer cohesion at multiple taxonomic levels. Unless we study populations we can never observe instances in which cohesion mechanisms differ across hierarchical levels (e.g. gene flow within species, selection between species).

We feel confident in rejecting the recent origin hypothesis for *H. thorelli*, as the mtDNA data are clearly inconsistent with such a scenario. We are less willing to reject gene flow as a cohesion mechanism, simply because of the female-limited nature of the mtDNA data. Clearly, there is little, if any, female-based gene flow in this system. However, this does not preclude male-based dispersal, as some authors suggest that adult males searching for females may account for the majority of individual-based dispersal in other Appalachian *Hypochilus* (Fergusson 1972; Huff & Coyle 1992). If males are acting as conduits of genetic exchange among populations, this gene flow may confer morphological cohesion at the species level. We are also hesitant to talk about *H. thorelli* as 'morphologically cohesive' until morphological variation across populations has been formally quantified. Fortunately, both male-based gene flow and population-level morphological variation can be measured in the future, allowing potential resolution of the stasis issue.

## Conclusions

We continue to be fascinated by these spiders. On the one hand, *Hypochilus* appears relictual and depauperate, a clade of only 11 species that lies sister to the remainder of spiders with > 38 000 species. The genus itself retains a large number of 'primitive' morphological characteristics compared with other spiders (see Catley 1994), leading some authors to conclude that these spiders are 'living fossils' (Kraus 1965). This viewpoint belies great diversity that occurs at the genetic level (at least at the mtDNA level), at the level of diverged female populations, and perhaps at the level of diverged bisexual populations. Remarkable advances in DNA technology now provide a window into this diverse world, but such technology is no substitute for population sampling. With sufficient sampling, we envision datasets comprising multiple hundreds of sequences for hundreds of populations, perhaps replicated for several *Hypochilus* taxa. Such datasets will tell us much about current and historical demographic processes in *Hypochilus* spiders, and will also provide detailed insight into the biogeographical history of the complex and biodiversity-rich Appalachian region. However, in conducting such genetic studies we cannot lose sight of patterns of morphological variation. Only through direct comparison of both morphological and molecular variation will we truly understand the special evolutionary characteristics of these spiders.

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