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# Multigenic phylogeographic divergence in the paleoendemic southern Appalachian opilionid *Fumontana deprehendor* Shear (Opiliones, Laniatores, Triaenonychidae)

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**Abstract**

The paleoendemic opilionid *Fumontana deprehendor* is restricted to a small area of mid-elevation forested habitats in the southern Blue Ridge province of the Appalachian Mountains. In a recent study we reported on the discovery of 22 new montane populations of this monotypic genus, specimens from which exhibit remarkably little morphological divergence despite their separation by intervening lowlands and large riverine barriers. Here, we further explore spatial and temporal patterns of divergence in this taxon using DNA sequence data from a portion of the mitochondrial cytochrome c oxidase subunit I gene (~1000 bp) and full-length sequences of both nuclear ribosomal internal transcribed spacer regions, including the intervening 5.8S rRNA region (~700 bp total). Bayesian phylogenetic analyses of these independent data sets reveal congruent genealogical patterns, with all data partitioning and combination strategies consistently recovering five allopatric, geographically cohesive genetic clades. These clades show an almost complete lack of internal genetic divergence, with most individuals sharing a clade-specific, regionally widespread haplotype. The geographic distribution of these clades corresponds to patterns seen in other upland taxa of the region, possibly indicating coincident vicariance. Because of a lack of quantifiable morphological divergence and relatively modest levels of genetic divergence, we conservatively refer to the geographically cohesive genetic clades as "phylogeographic units", although these may actually represent cryptic species. Conservation implications and the prospect for future comparative arachnid phylogeography in the southern Appalachians are discussed in light of the results presented here.


*Keywords:* Cryptic species; Cytochrome *c* oxidase subunit I (COI); Genealogical concordance; Internal transcribed spacer (ITS); Montane divergence

## 1. Introduction

The uplands at the southern end of the Appalachian Mountains in eastern North America include some of the most biodiverse temperate habitats in the northern hemisphere (Stephenson et al., 1993; Stein et al., 2000). The region comprises several distinct physiographic provinces, including, from west to east, the Appalachian Plateau, Valley and Ridge, Blue Ridge, and Piedmont provinces (Fenneman, 1938). Together, this landscape includes dense forests, many high-elevation mountains (particularly in the Blue Ridge province), a multitude of spring and river systems, and the largest concentration of limestone caves in the United States. The southern Appalachians include a tremendous diversity of surface-dwelling arthropods, including, for example, species-rich radiations of xystodesmid millipeds (Shelley and Whitehead, 1986; Marek and Bond, 2006), phalangodid opilionids (Thomas, 2007), *Hypochilus* spiders (Catley, 1994), and *Nesticus* spiders (Hedin, 1997). The evolution and persistence of this biodiversity can be attributed to the region's ancient and complex topographic landscape, where upland habitats have persisted throughout the Cenozoic. And although the southern uplands were never glaciated (Hack, 1989), shifting climatic regimes during the Plio-Pleistocene have lead to a

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fine-grained, heterogeneous assemblage of habitats (Delcourt and Delcourt, 1998), and have provided considerable opportunity for vicariance.

Given this rich biodiversity and heterogeneous landscape, the southern Appalachians provide a model setting for investigating biogeographic divergence in terrestrial habitats. A recent review by Soltis et al. (2006) on phylogeographic patterns in the eastern United States highlights two important patterns with respect to southern Appalachian phylogeography. First, these authors showed that phylogeographic studies in the region are dominated by vertebrate taxa—only four arthropod studies were reviewed (of over 390 studies total), and no arachnid studies were included. Second, these authors documented very few examples of *in situ* divergence in the southern Blue Ridge highlands. Most known examples involve habitat-specialized, non-vagile salamanders, which almost universally show evidence for *both* fine-scale phylogeographic divergence and localized species radiation in the region (Highton, 1995; Crespi et al., 2003; Kozak and Wiens, 2006; Weisrock and Larson, 2006). These non-vagile taxa clearly retain both deep and shallow biogeographic signal. We argue that many cryophilic arachnid taxa will show similar patterns of *in situ* phylogeographic divergence and species radiation (e.g., see Hedin and Wood, 2002; Hendrixson and Bond, 2005), and that studies of such taxa will prove exceedingly valuable in formulating a comprehensive, unbiased understanding of southern Appalachian biogeography and biodiversity.

Despite comprising more than 6300 described species, the arachnid order Opiliones (commonly known as ''harvestmen'' or ''daddy long-legs'') is one of several ''neglected cousins'' (Harvey, 2002) of the much better-studied and well-known arachnid orders, such as Araneae (spiders) and Acari (mites and ticks). Opiliones is currently divided into four major taxonomic groups: the Cyphophthalmi, Laniatores, Dyspnoi, and Eupnoi, with the latter two groups comprising the historically recognized ''Palpatores''. Although the inter-relationships and monophyly of certain opilion subgroups is an area of debate and active research, Laniatores monophyly is universally accepted (reviewed in Giribet and Kury, 2007). Laniatoreans comprise the majority of described opilion diversity with more than 4000 described species, with most laniatorean species endemic to tropical regions. In North America, Laniatores is represented by nine families (Kury, 2003; Pinto-da-Rocha and Giribet, 2007), many of which display an affinity towards cool, dark, moist habitats (e.g., under rocks and logs) in upland or montane forests (e.g., Briggs, 1971; Shear, 1977; Ubick and Briggs, 1989; Thomas and Hedin, 2006).

Molecular phylogenetic data have been used to study higher-level relationships in Opiliones (Giribet et al., 1999, 2002; Shultz and Regier, 2001), and a recent study examined species-level relationships in the Balkan cyphophthalmid genus *Cyphophtalmus* (Boyer et al., 2005). However, we are unaware of published molecular studies that analyze phylogeographic or species-level relationships in any laniatorean species. High potential for fine-scale geographic divergence and speciation in laniatoreans is expected given their small size and typically strict habitat preferences. In North America, this pattern is exemplified by western genera of the family Phalangodidae (although other western families show similar patterns), which contain many morphologically unique species (diagnosed principally by genitalic features) restricted to very small areas (e.g., Ubick and Briggs, 1989). We anticipate that fine-scale divergence will be particularly apparent in genetic data, and expect phylogeographic analyses to become an important tool in characterizing relatively ''shallow'' phylogenetic patterns in laniatoreans.

*Fumontana deprehendor* (Shear, 1977) is a laniatorean endemic to the Blue Ridge province of the southern Appalachians (Shear, 1977; Thomas and Hedin, 2006). This monotypic genus is the sole representative of the family Triaenonychidae in eastern North America, as other nearctic taxa currently classified as ''triaenonychids'' (the family is likely polyphyletic, see below) are known only from west of the Rocky Mountains in the United States and Canada (Briggs, 1971). The ''Triaenonychidae'' currently includes 479 described species, with only 18 of these endemic to the Nearctic (Kury, 2003, 2006). The bulk of triaenonychid diversity is found in the southern hemisphere, including Australia, New Zealand, South Africa, and southern South America (Pinto-da-Rocha and Giribet, 2007). Despite the presence of con-familial taxa in North America, *Fumontana* exhibits several distinctive morphological attributes that suggest phylogenetic affinities to Gondwanan triaenonychids (Shear, 1977), including, for example, the South African genus *Monomontia*, and the South American genera *Triaenonyx* and *Valdivionyx* (Kury, 2004). This placement would be consistent with the recent classification of Giribet and Kury (2007), who classify *Fumontana* into the superfamily Triaeonychoidea with austral triaenonychids, to the exclusion of other north temperate triaenonychids classified as Travuniodea (i.e., these authors hypothesize that triaenonychids are polyphyletic).

In a previous study (Thomas and Hedin, 2006), we reported on the discovery of 22 new *Fumontana* populations in the southern Blue Ridge (previously known only from two sites; Shear, 1977, 1978), demonstrating that this taxon is not as rare as previously believed. *Fumontana* is most consistently found in well-decayed logs in hemlock-dominated forest, and all known populations are found between elevations of approximately 400–1200 m above sea level. Qualitative assessments of somatic and genitalic morphology, and multivariate morphometric analyses of somatic characters, revealed little divergence between disjunct populations (Thomas and Hedin, 2006). This result was surprising, given the obvious habitat and topographic complexity of the region, and evidence for divergence in other regional taxa (references above). Thus, we were motivated to explore the possibility of morphologically-cryptic phylogeographic divergence in this unique Appalachian taxon.

We examined molecular phylogenetic divergence in *Fumontana* via Bayesian analysis of DNA sequence data, collected from independently segregating loci representing both the nuclear and mitochondrial genomes. We also present haplotype networks inferred using the Templeton, Crandall, and Sing parsimony algorithm (TCS; Templeton et al., 1992) to better visualize intra-clade divergence. Emphasis is placed on demonstrating phylogenetic concordance between independently segregating genes. Finding genealogical concordance increases the probability that inferred gene trees accurately represent the true population history (Maddison, 1997). Additionally, congruent multilocus data strengthens hypotheses regarding the geographic position of inferred genealogical breaks, as single-gene phylogeographic breaks can arise "haphazardly" in low-dispersal species, even when geographic barriers to dispersal do not exist (Kuo and Avise, 2005).

## 2. Materials and methods

### 2.1. Taxon sampling

Field methods and population sampling for this study are described in Thomas and Hedin (2006). Of 141 specimens originally collected, 58 were preserved in 100% ethanol and stored in a −80 °C freezer for DNA preservation (Vink et al., 2005). These DNA-preserved specimens are from 22 localities distributed across the uplands of the southern Blue Ridge province, including habitats in western North Carolina and adjacent Tennessee (Fig. 1). The two previously published (i.e., before Thomas and Hedin, 2006) localities were also sampled for this study, including the type locality in Greenbrier Cove, Sevier Co., Tennessee (Shear, 1977), and a second published locality in the Joyce Kilmer Memorial Forest of western North Carolina (Shear, 1978). These two locations are indicated on Fig. 1 by the acronyms GREENBR and JOYCE, respectively. An attempt was made to sample multiple individuals (2–3) per location; however, this was not always possible as *Fumontana* specimens were very rare at many sites. Locality and voucher information is provided in Table 1; voucher specimens representing all genetic clades (see Section 3) have been preserved in 80% ethanol and deposited at the California Academy of Sciences.

The systematic placement of *Fumontana* within the Triaenonychidae is uncertain (Kury, 2003; Pinto-da-Rocha and Giribet, 2007), making it difficult to conduct targeted sampling for close outgroups. We included several

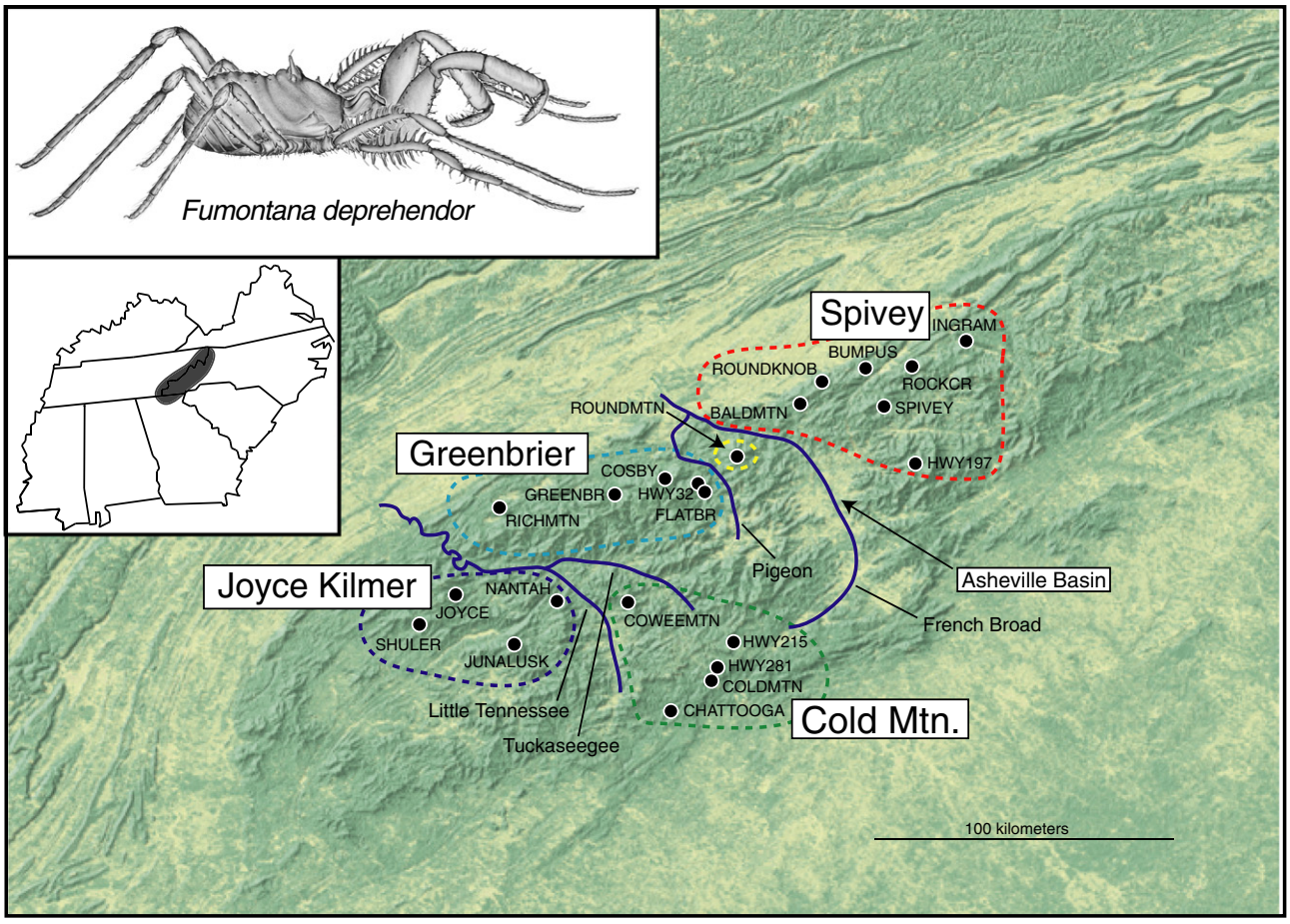


Fig. 1. Map of the southern Appalachians showing the distribution of *Fumontana deprehendor* and the geographic position of genetic clades. Clade names are based on prominent geographic features. Approximate position of major rivers possibly acting as barriers to gene flow are shown.

Table 1
Taxon identity, population acronym, lab voucher number, locality information (including latitude/longitude in decimal degrees and elevation in meters), and GenBank accession numbers for all samples included in this study

| Taxon | Acronym | Lab No. | Locality information | Lat/Long | Elev. | COI | ITS |
|---|---|---|---|---|---|---|---|
| *F. deprehendor* | COWEEMTN | OP268, 269 | NC: Macon Co., S of Cowee Mtns. Lookout | N 35.3168/ W 83.3735 | 955 | EU162768, EU162769 | EU162818 |
| | CHATTOOGA | OP271, 272, 273 | NC: Macon/Jackson Co. line, Chattooga River | N 35.0160/ W 83.1265 | 755 | EU162770-72 | EU162819 |
| | COLDMTN | OP275, 276, 278 | NC: Transylvania Co., Cold Mtn. Rd. | N 35.1611/ W 82.9791 | 1100 | EU162773-75 | EU162820 |
| | HWY281 | OP281, 282, 283 | NC: Transylvania Co., Hwy 281 | N 35.1950/ W 82.9603 | 965 | EU162776-78 | EU162821 |
| | HWY215 | OP284 | NC: Transylvania Co., Hwy 215 | N 35.2575/ W 82.9204 | 1217 | EU162779 | EU162822 |
| | HWY32 | OP006 | TN: Cocke Co., Hwy 32 @ Stateline | N 35.7705/ W 83.1112 | 605 | EU162780 | EU162823 |
| | GREENBR | OP007 | TN: Sevier Co., Greenbrier Cove | N 35.7154/ W 83.3831 | 490 | EU162781 | EU162824 |
| | FLATBR | OP254, 256, 257 | NC: Haywood Co., Flat Branch Rd. | N 35.7526/ W 83.0895 | 588 | EU162782-84 | EU162825 |
| | RICHMTN | OP260, 261, 262 | TN: Blount Co., Rich Mtn. Rd. | N 35.6508/ W 83.7979 | 412 | EU162785-87 | EU162826 |
| | COSBY | OP621, 622 | TN: Cocke Co., GSMNP, road to Cosby CG | N 35.7633/ W 83.2115 | 615 | EU162788, EU162789 | EU162827 |
| | JOYCE | OP065 | NC: Graham Co., Joyce Kilmer Forest | N 35.3585/ W 83.9291 | 685 | EU162790 | EU162828 |
| | SHULER | OP263, 264, 265 | NC: Cherokee Co., Shuler Creek | N 35.2424/ W 84.2227 | 515 | EU162791-93 | EU162829 |
| | JUNALUSK | OP267 | NC: Cherokee Co., Junaluska Rd. | N 35.1760/ W 83.7680 | 700 | EU162794 | EU162830 |
| | NANTAH | OP270 | NC: Swain Co., Nantahala River | N 35.3305/ W 83.5921 | 545 | EU162795 | EU162831 |
| | ROUNDMTN | OP611, 612 | TN: Cocke Co., S. Round Mtn. | N 35.8350/ W 82.9519 | 982 | EU162796, EU162797 | EU162832, EU162833 |
| | SPIVEY | OP046, 047 | NC: Yancey Co., E Spivey Gap | N 36.0342/ W 82.4043 | 931 | EU162798, EU162799 | EU162834 |
| | BUMPUS | OP241, 242, 243 | TN: Washington Co., Bumpus Cove | N 36.1424/ W 82.5079 | 598 | EU162800-02 | |
| | ROCKCR | OP245, 246 | TN: Unicoi Co., Rock Creek Rec. Area | N 36.1379/ W 82.3482 | 727 | EU162803, EU162804 | |
| | INGRAM | OP247, 248 | TN: Carter Co., Ingram Branch Rd. | N 36.2140/ W 82.1456 | 840 | EU162805, EU162806 | EU162835 |
| | ROUNDKNOB | OP249 | TN: Greene Co., Bald Mtns., Round Knob Rd. | N 36.0799/ W 82.6859 | 860 | EU162807 | EU162836 |
| | BALDMTN | OP250, 251 | TN: Greene Co., Bald Mtn. Rd. | N 36.0284/ W 82.7253 | 1079 | EU162808, EU162809 | EU162837 |
| | HWY197 | OP252, 253 | NC: Buncombe Co., Hwy 197 | N 35.8036/ W 82.3536 | 1160 | EU162810, EU162811 | EU162838 |
| *Bishopella laciniosa* | | OP004 | TN: Sevier Co., Elkmont Area | N 35.6536/ W 83.5802 | | EU162812 | |
| *Chinquipellobunus* sp. | | TX327 | TX: Bexar Co, Camp Bullis, Hold Me Back Cave | Withheld | | EU162813 | |
| *Equitius doriae* | | — | Australia | From GenBank | | AY744908 | |
| *Monomontia* sp. | | OP052 | RSA: KwaZulu-Natal, Ngome SF | S 27.8199/ E 31.4175 | | EU162814 | |
| *Sclerobunus nondimorphicus* | | OP120 | WA: Kings Co., Rattlesnake Lake | N 47.4345/ S 121.7722 | | EU162815 | |
| *Texella reyesi* | | TX316 | TX: Williamson Co., Temples of Thor Cave | Withheld | | EU162816 | |
| *Zuma acuta* | | OP750 | CA: San Mateo Co., Pescadero Rd. | N 37.2999/ S 122.6959 | | EU162817 | |

*Notes:* Population acronyms correspond to those in Fig. 1. Specific coordinates of two outgroups are withheld to maintain confidentiality of cave locations.

outgroup taxa, including representatives of two (of five total) other triaenonychid genera from North America (*Sclerobunus* and *Zuma*), and triaenonychids from South Africa (*Monomontia* sp.) and Australia (*Equitius doriae*—sequence from GenBank; Giribet et al., 2005). We also included other North American non-triaenonychid laniatoreans (*Bishopella laciniosa* (Phalangodidae), *Texella reyesi* (Phalangodidae), and *Chinquipellobunus* sp. (Stygnopsidae)) (Table 1); these latter taxa are not expected to be particularly close to *Fumontana*, but are clearly outside the group of interest. We also sampled and sequenced other laniatoreans from eastern North America (e.g., *Theromaster* (Cladonychiidae) and *Vonones* (Cosmetidae)), but preliminary phylogenetic analyses reveal these as relatively distant relatives of *Fumontana* (results not shown); these taxa were excluded from this study in order to streamline phylogenetic analysis.

### 2.2. Sequence data collection

Genomic DNA was extracted using the Qiagen DNeasy Kit, per manufacturer's protocol. For adult specimens, two or three legs provided sufficient amounts of material for DNA extraction. For a few small immature specimens, the entire individual was used. From genomic templates, we amplified a 945–1140 bp fragment of the mitochondrial cytochrome c oxidase subunit I gene (COI). For amplification of mtDNA, we used primers developed and used in other arachnid taxa (see Hedin, 2001; Hedin and Maddison, 2001), or modifications thereof. Primer combinations used were forward "C1-J-1517" (5′-AATCATARGGATATTGGAAC-3′) with reverse "C1-N-2568" (5′-GCTACAACATAATA AGTATCA-3′) or "C1-N-2776spider" (5′-GGATAATCA GAATANCGNCGAGG-3′). COI PCR cycle conditions were 94 °C, 3 min; 30× (94 °C, 45 s; 45 °C, 45 s; 72 °C, 90 s); 72 °C, 5 min. We also amplified a 689–712 bp (depending on length variation) fragment spanning both nuclear ribosomal internal transcribed spacer regions (ITS1 and ITS2) and the intervening 5.8S rRNA region. ITS sequences have been successfully used in species-level studies of other arthropods (e.g., Collins and Paskewitz, 1996; Hedin, 1997). For amplification of the ITS regions, the primer combinations used were forward "CAS18sF1" with reverse "CAS28sB1d" (Ji et al., 2003); PCR cycle conditions were 94 °C, 3 min; 40× (94 °C, 30 s; 60 °C, 60 s; 72 °C, 60 s); 72 °C, 5 min. All PCR reactions included 0.8 μL genomic DNA with 0.08 μL (0.4 U) Ex Taq polymerase (Takara Bio Inc.), 2.5 μL of manufacturer provided dNTP mixture (25 pmol each dNTP), 2.5 μL Ex Taq buffer ($Mg^{2+}$), and tissue culture water (Sigma–Aldrich Co.) for a final volume of 25 μL.

PCR amplification products were purified via polyethylene glycol (PEG) precipitation, or via agarose gel extraction using an *IsoPure* PCR Purification and Gel Extraction Kit (Denville Scientific, Inc.). PCR templates were sequenced directly using Big Dye 3 dye chemistry (ABI) on an ABI 377 machine, or were sequenced at the SDSU Microchemical Core Facility on an ABI Prism 3100 capillary machine. Contigs were assembled and sequences edited using Sequencher 4.5.

### 2.3. Sequence alignment and molecular phylogenetic analyses

Alignment of *Fumontana* COI sequences was mainly accomplished manually using MacClade 4.07 (Maddison and Maddison, 2001). However, the presence of several codon indels relative to the various outgroup taxa (in the third inter-membrane loop of the COI gene) required algorithmic alignment. Similar COI length variation has been shown between genera of cyphophtalmid opilionids (Boyer et al., 2005). We translated the matrix to amino acids and aligned the matrix using Clustal X (Thompson et al., 1997) with default parameters (gap opening and extension parameters of 10 and 0.10, respectively). This aligned amino acid matrix was then used as a guide to realign the nucleotide matrix.

Aligning the ITS sequences, and utilizing them in phylogenetic analyses required additional effort. We observed relatively small amounts of sequence divergence among *Fumontana* populations (seven variable, non-indel, nucleotide positions). However, all ITS sequences included a length-variable stretch of repeated adenines found in the ITS1 region, which is sporadically interrupted by relatively rare, individual thymines (see Appendix). To utilize this potential phylogenetic information in a Bayesian framework, we used the thymines as anchors for alignment (positional homology justified based on their relative rarity), and recoded the data as presence/absence characters. Additionally, two gaps (one 1-base and one 12-bases in ITS1 and ITS2, respectively) in the sequences from ROUNDMTN were coded as present or absent in the matrix in the same manner. In total, 40 nucleotide positions were recoded as 13 presence/absence characters (see Appendix).

Bayesian analysis was used to simultaneously infer tree topologies and estimate model parameters using the computer program MrBayes 3.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The Akaike information criterion (AIC) implemented in MrModeltest 2.2 (Nylander, 2004) was used to determine the appropriate model of DNA substitution for both data sets, following Posada and Buckley (2004). We experimented with various data combination and partitioning strategies following Brandley et al. (2005). We also performed several analyses on the ingroup alone, to explore how the removal of distant outgroups affected nodal support values (Buckley et al., 2006). Recoded ITS indel characters were added to the end of the data file, assigned to a separate partition and modeled using an F81-like restriction site (binary) model (following Ronquist et al., 2005). These two ITS-specific partitions (variable nucleotide + recoded indel sites) were included in all analyses that incorporated the ITS data set. In total, we conducted nine analyses (see Table 2), including

Table 2
Data combination and partitioning strategies used in Bayesian analyses

| Analysis No. | Taxa | Genes | Partitions | Generations | Average $-\ln L$ |
|---|---|---|---|---|---|
| 1 | All-taxa | COI | Unpartitioned | 2.0E+06 | −7405.37 |
| 2 | | | Codon partitions | 3.0E+06 | −7077.38 |
| 3 | | COI and ITS | Partition by gene | 4.0E+06 | −8601.05 |
| 4 | | | Gene and COI codon | 4.0E+06 | −8300.07 |
| 5 | Ingroup only | COI | Unpartitioned | 1.0E+06 | −2463.66 |
| 6 | | | Codon partitions | 2.0E+06 | −2367.25 |
| 7 | | ITS | None | 1.0E+06 | −1148.90 |
| 8 | | COI and ITS | Partition by gene | 3.0E+06 | −3641.85 |
| 9 | | | Gene and COI codon | 3.0E+06 | −3600.49 |

"all-taxa" codon-partitioned and -unpartitioned COI analyses (analyses 1 and 2), "all-taxa" codon-partitioned and codon-unpartitioned combined COI and ITS analyses (analyses 3 and 4), "ingroup only" codon-partitioned and -unpartitioned COI analyses (analyses 5 and 6), "ingroup only" ITS analysis (analysis 7), and "ingroup only" codon-partitioned and codon-unpartitioned combined COI and ITS analyses (analyses 8 and 9).

All Bayesian analyses consisted of two simultaneous independent runs, each consisting of four independent Markov Chain Monte Carlo (MCMC) chains (Metropolis-Coupled MCMC) with default hot and cold chain temperatures. Trees with associated branch lengths were saved every 100 generations. All analyses were permitted to run until the standard deviation of split frequencies was observed to be below 0.01, assuring convergence of the two analyses on a posterior distribution of tree topologies (following Ronquist et al., 2005). Majority rule consensus trees, average branch lengths, and average likelihood scores based on the post burn-in tree sets (discarding the first 40%) were computed using the *sumt* and *sump* commands in MrBayes 3.1 (Table 2).

### 2.4. Haplotype networks

We reconstructed COI haplotype networks using the computer program TCS 1.21 (Clement et al., 2000), which implements the statistical parsimony estimation procedure of Templeton et al. (1992). The inferred haplotype networks should allow for better visualization of geographic patterns of divergence within genetic clades, and provide an additional criterion for recognizing divergent genetic lineages (e.g., Cardoso and Vogler, 2005). A few ambiguities in the data set required that we manually modify our haplotype network; incomplete sequences or sequences with ambiguous calls were assumed to be identical to complete/unambiguous sequences sampled from the same site, consistent with observations for more complete intra-site sample sets. We did not perform TCS analyses on the ITS data due to an almost complete lack of intra-clade variation (maximum intra-clade ITS divergence was only a single mutational step).

## 3. Results

### 3.1. Sequence characteristics

Mitochondrial COI sequences were generated for 44 *Fumontana* specimens from 22 locations, including the type locality. There was no evidence for nuclearized mtDNA (pseudogenes), as no stop codons were observed in the data and homology was assigned without issue. Twenty-two unique COI haplotypes were recovered, all of which have been deposited in GenBank (see Table 1). Most sampled sites were fixed, or nearly fixed, for a single haplotype (maximum intra-site divergence was three mutational steps). Average uncorrected pairwise sequence divergence was 4.1% within *Fumontana*, 31.0% between *Fumontana* and the triaenonychid outgroup sequences, and 32.2% between *Fumontana* and all outgroup sequences. The extremely high levels of divergence between *Fumontana* and outgroup taxa further supports the phylogenetic isolation of *Fumontana* (see Giribet and Kury, 2007).

Nuclear ribosomal sequences were collected from specimens representing 20 of 22 locations included in the mitochondrial data set. Preliminary sequencing efforts showed that it was difficult to align ITS between *Fumontana* and outgroup taxa (i.e., ITS is too fast-evolving); as such, ITS data were collected for *Fumontana* only. Only a single individual was sequenced per site, due to the observed lack of sequence variation between sites. However, two individuals were sequenced from ROUNDMTN to confirm the presence of several unique deletions in this sequence, and to include an additional representative of this unique genetic clade. In total, the ribosomal sequences represent seven unique haplotypes, which have been deposited in GenBank (see Table 1). The 5.8S rRNA region was invariant except for a single substitution in individuals from the ROUNDMTN site.

### 3.2. Molecular phylogenetic analyses

Support for the monophyly of *Fumontana* was high (posterior probability = 100%) for all analyses that included outgroups. In addition, there was strong

support (posterior probability ⩾98%) for a clade including *Fumontana*, *Equitius*, and *Monomontia* (the latter from Australia and South Africa, respectively), to the exclusion of the North American *Zuma* and *Sclerobunus*. Therefore, these molecular data tentatively support previous morphological hypotheses suggesting that *Fumontana* is more closely-related to austral triaenonychids than to other North American taxa (Shear, 1977). These results are also consistent with the Triaenonychoidea hypothesis of Giribet and Kury (2007), although a much larger and globally-comprehensive taxon sample will be necessary to definitively reveal triaenonychid polyphyly implied by this hypothesis.

Bayesian analyses indicate that *Fumontana* is fragmented into five, genealogically distinct geographic clades, which we have tentatively designated (1) the

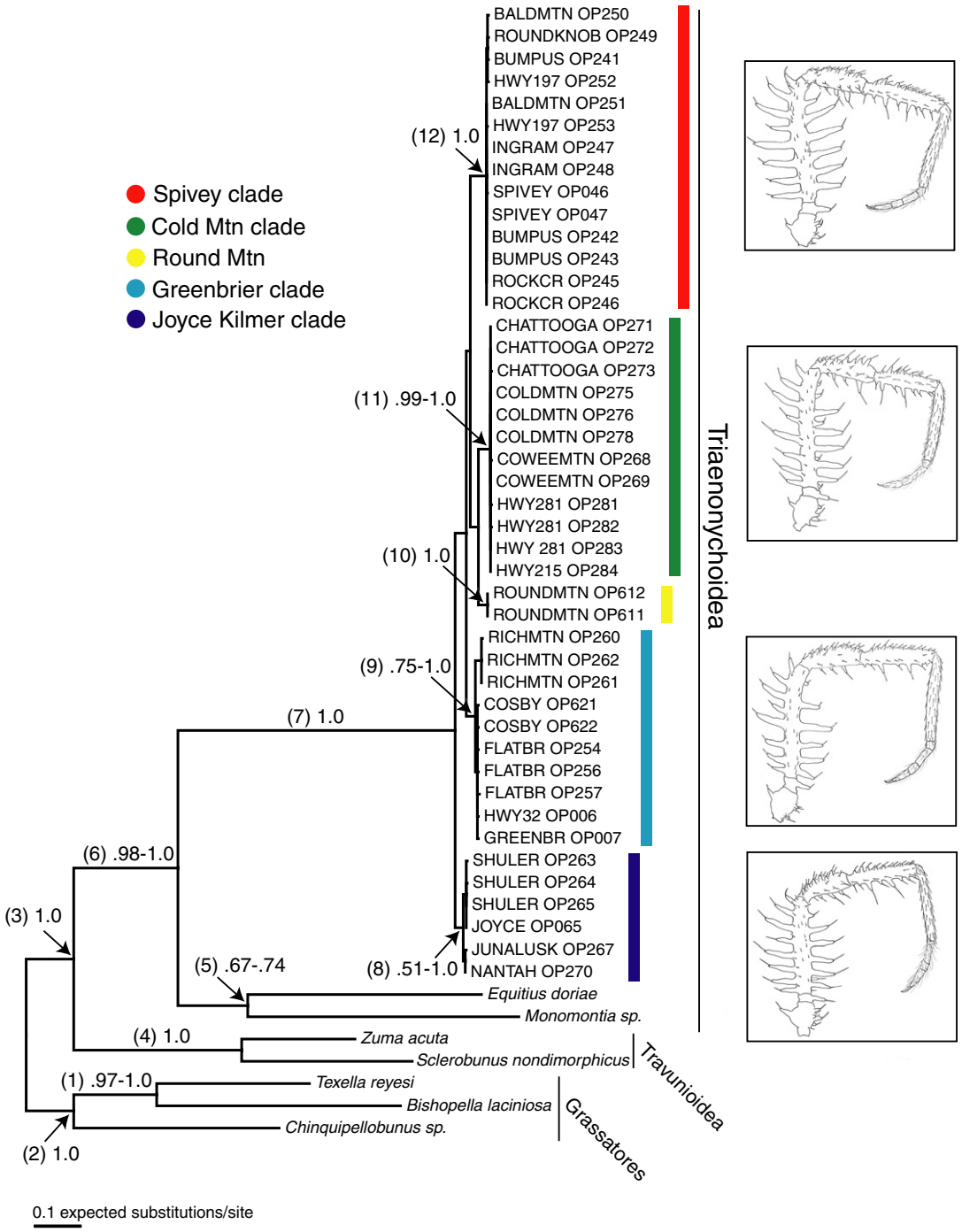


Fig. 2. Bayesian phylogram resulting from all analyses that included *F. deprehendor* and outgroups (analyses 1–4). Important nodes are numbered with the range of recovered Bayesian posterior probabilities (in parentheses) from various analyses (see Table 3). Well-supported clades are named as in Fig. 1. The illustrations are of a representative male's first leg from the corresponding genetic clade (see Thomas and Hedin, 2006).

"Spivey" clade, comprising seven sampled sites northeast of the French Broad River and the Asheville Basin; (2) the "Greenbrier" clade, comprising five sites in or near the Great Smoky Mountains National Park, north and northeast of the Little Tennessee and Tuckasegee Rivers, and west of the Pigeon River; (3) the "Joyce Kilmer" clade, comprising four sampled sites in the vicinity of the Joyce Kilmer Memorial Forest, southwest of the Little Tennessee River; (4) the "Round Mountain" clade, comprising only one sampled site, between the Pigeon and French Broad Rivers; and (5) the "Cold Mountain" clade, comprising five sites east of the Little Tennessee River, south of the Great Smoky Mountains, and west of the Asheville Basin (Figs. 1–3). These five clades were recovered in all nine Bayesian analyses (see Tables 2 and 3). We note that although the Cold Mountain clade is potentially paraphyletic with respect to the Round Mountain population in ITS-only analyses (Fig. 3), the 95% credible set of trees included 3677 trees (of 11388 total trees from both runs) consistent with reciprocal monophyly of these groupings.

Posterior probability support values for the *Fumontana* genetic clades are generally high (node numbers 8–12, see Table 3), with all clades receiving the maximum value of 100% in most analyses. However, support values for the Greenbrier and Joyce Kilmer clades vary widely across analyses, and receive only modest support in the "all-taxa" analyses (see Table 3). However, it was the case that all analyses that excluded outgroups exhibit significantly higher nodal support for clades within *Fumontana*. The observation of reduced support when including extremely divergent outgroup sequences is consistent with previous findings in both simulation (Holland et al., 2003) and empirical studies (Buckley et al., 2006). Nodal support for the branches representing inter-relationships between *Fumontana* clades was always consistently lower, indicating that the relationships among these clades are less clear. This lack of resolution is further supported by the observation that clade interrelationships differ in COI-only versus ITS-only trees (Fig. 3).

### 3.3. Haplotype networks

Five unconnected (i.e., distinct at the 95% connection limit) mtDNA haplotype networks were resolved using TCS statistical parsimony (Fig. 4), corresponding to the five *Fumontana* clades recovered in Bayesian analyses. A conspicuous pattern seen in these unconnected networks is the relative genetic homogeneity (few, closely-related haplotypes; many haplotypes shared across multiple sites), contrasting with relatively high divergence implied by unconnected networks. However, two of the networks (clades) reveal greater internal divergence, suggesting either sampling gaps or population structure within these geographic clades.

## 4. Discussion

The discovery of divergent, genealogically concordant genetic clades contrasts with the monotypic taxonomy and general morphological uniformity observed in *Fumontana*. In the discussion below, we address three questions that arise from this phylogenetic discovery: What are the spatial and temporal patterns of divergence in this group of independent evolutionary lineages? Should morphologically indistinguishable genetic clades be considered divergent phylogeographic units, or separate cryptic species? How does this phylogenetic research impact conservation interest and actions directed at these relatively rare, cryophilic arachnids?

### 4.1. Patterns of spatial and temporal divergence

The southern Blue Ridge province includes the highest mountains in eastern North America (1500–2000 m). These forested uplands are separated by major drainage systems, some lying within lower-elevation, relatively xeric intermontane basins. The distributional limits of *Fumontana* clades *appear to* correspond very closely to these large riverine barriers and intermontane basins (see Figs. 1 and 3). For example, the northeastern Spivey clade is isolated from all other clades by the French Broad River, which flows north through the Asheville Basin, and is by far the largest intermontane basin in the region (Hack, 1989). The southwestern Joyce Kilmer clade appears separated from the Greenbrier and Cold Mountain clades by the route of the Little Tennessee River, and the latter two clades are themselves apparently separated by low elevations associated with the Tuckaseegee River (and tributaries). Finally, the Round Mountain population is geographically nestled between the Pigeon and French Broad Rivers, which appear to separate this genetically unique population from the neighboring Greenbrier and Spivey clades, respectively.

The general spatial positioning of genealogical breaks observed in *Fumontana*, although preliminary (see below), have also been observed in phylogeographic or phylogenetic studies of other regional upland taxa. The Asheville Basin divergence is most conspicuous, and is seen in *Trechus* beetles (Kane et al., 1990), and spider species of the *Nesticus nasicus* species group (Hedin, 1997). The Asheville Basin is also both a phylogeographic (*Desmosgnathus wrighti* (Crespi et al., 2003) and *Plethodon montanus* (Kozak and Wiens, 2006)) and species barrier (e.g., *P. teyahalee* versus *P. cylindraceus*; Kozak and Wiens, 2006) for many salamander taxa. Breaks corresponding to the Little Tennessee, Tuckaseegee, and Pigeon Rivers are also seen in *Trechus* (Kane et al., 1990), and in the *Plethodon jordoni* complex (Weisrock and Larson, 2006; although this group may not be monophyletic, see Wiens et al., 2006). Weaker evidence for an influence of the Little Tennessee and Tuckaseegee is seen in phylogeographic data for *D. wrighti* (Crespi et al., 2003). Interestingly, phylogeographic

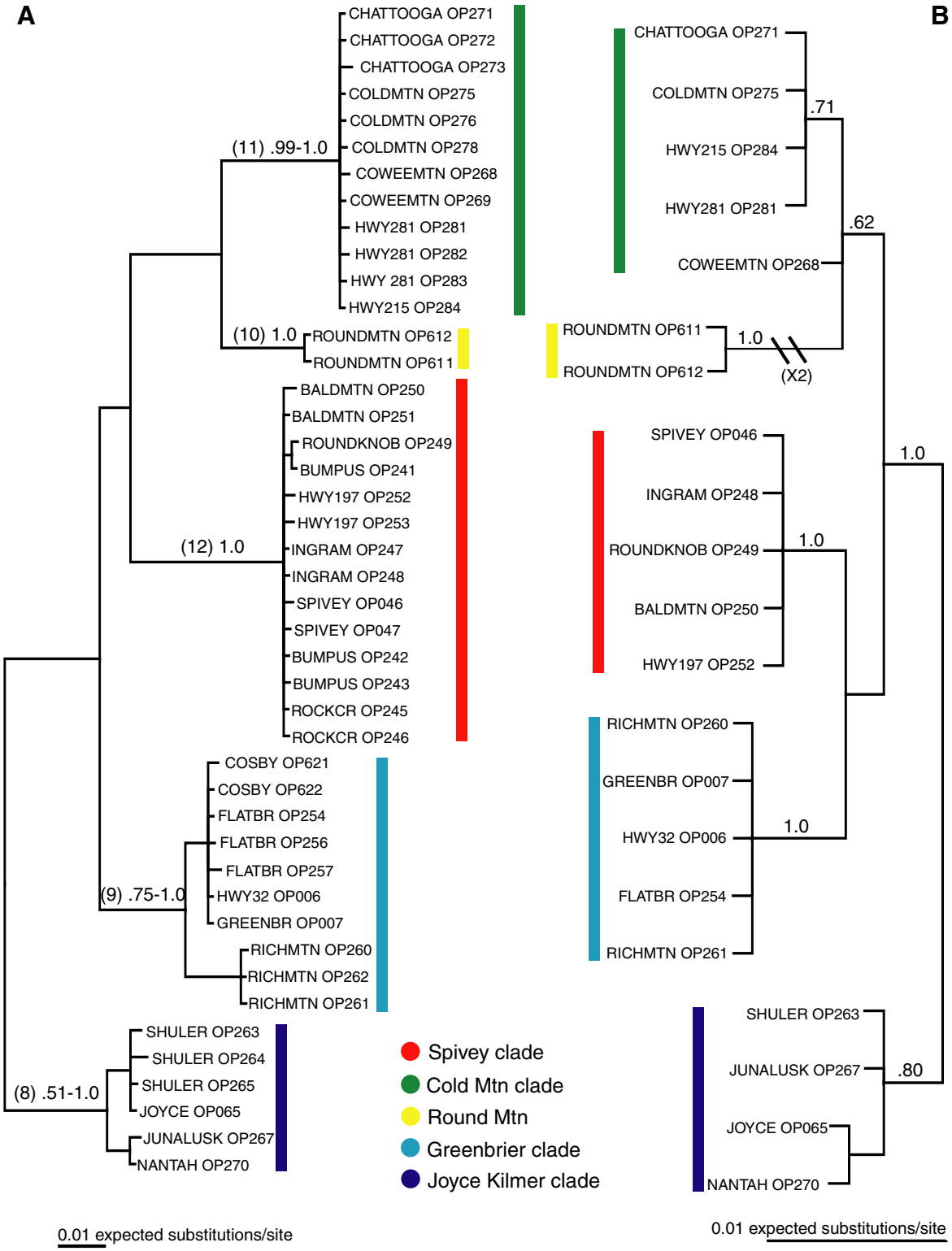


Fig. 3. (A) Bayesian phylogram of *F. deprehendor* genetic clade relationships recovered in all analyses that included COI alone (and in combination with ITS). Although these ingroup relationships were consistently recovered, support was never significant ($P << 0.95$). (B) Bayesian phylogram recovered in the single ITS-only analysis (analysis 7, Table 2). Nodal values correspond to Bayesian posterior probabilities. Since ITS data was not collected for outgroups, the phylogeny was rooted at the branch leading to the Joyce Kilmer clade, following topologies recovered in all other analyses.

data for wingless *Cryptocercus* wood roaches, which are ecologically similar to *Fumontana* (decaying wood specialists), do not indicate spatial congruence (see Nalepa et al., 2002). In roaches, different chromosomal races meet

Table 3
Bayesian posterior probabilities

| Analysis No.: | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Taxa: | All-taxa | | | | Ingroup only | | | | |
| Node No. | COI | | COI and ITS | | COI | | ITS | COI and ITS | |
| 1 | 1.0 | .98 | 1.0 | .97 | — | — | — | — | — |
| 2 | 1.0 | 1.0 | 1.0 | 1.0 | — | — | — | — | — |
| 3 | 1.0 | 1.0 | 1.0 | 1.0 | — | — | — | — | — |
| 4 | 1.0 | 1.0 | 1.0 | 1.0 | — | — | — | — | — |
| 5 | .74 | .71 | .74 | .67 | — | — | — | — | — |
| 6 | .98 | 1.0 | .98 | 1.0 | — | — | — | — | — |
| 7 | 1.0 | 1.0 | 1.0 | 1.0 | — | — | — | — | — |
| 8 | .78 | .51 | .76 | .57 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| 9 | .75 | .93 | .77 | .87 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| 10 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| 11 | .99 | .99 | 1.0 | 1.0 | 1.0 | 1.0 | ** | 1.0 | 1.0 |
| 12 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |

*Notes:* Values for the 12 nodes labeled in Figs. 2 and 3 recovered in nine individual analyses (Table 2). Node 11 for analysis 7 is not given a support value as this group was paraphyletic in the ITS-only analysis.

in parapatry at high elevations, and distributional breaks do not coincide with any of the aforementioned riverine barriers (e.g., the same chromosome form spans the French Broad River).

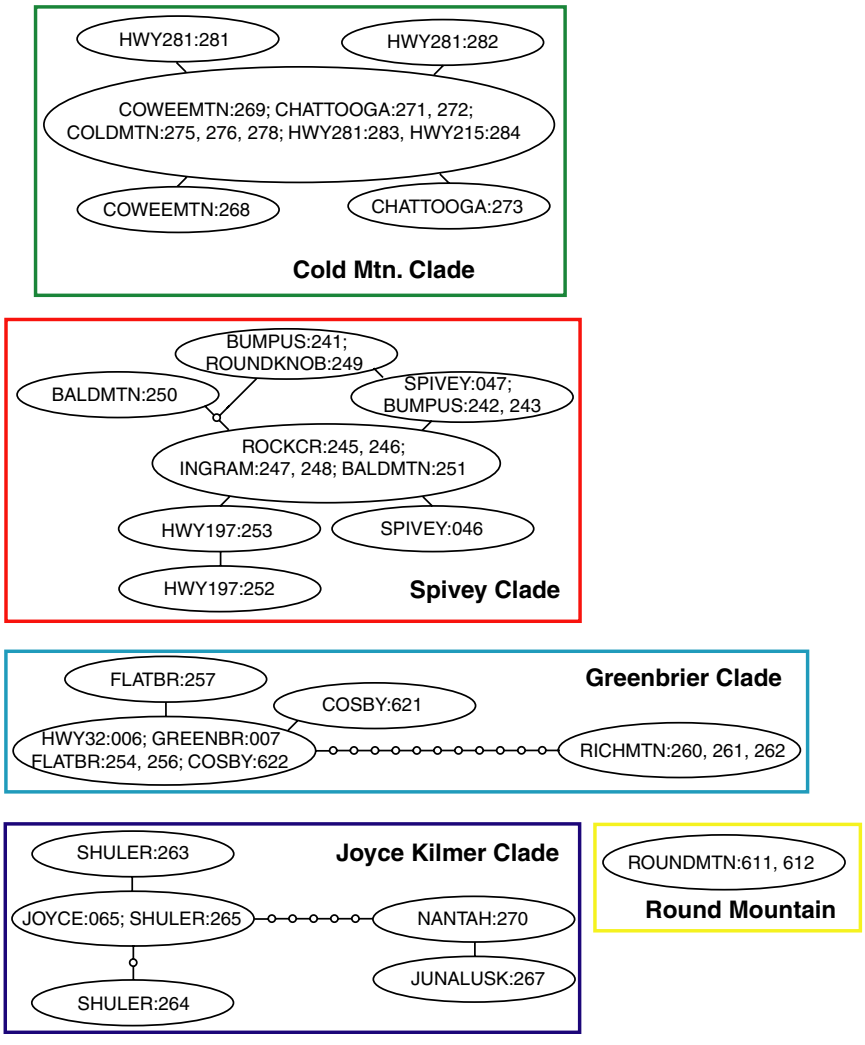


Fig. 4. COI TCS networks, corresponding to five geographic clades. Connections between genetic clades are beyond the 95% connection criterion (i.e., the networks are ''unconnected'').

Given our current sample, it seems most-parsimonious to infer clade distributional limits in *Fumontana* that correspond to riverine barriers (as indicated in Fig. 1), but these need to be tested and strengthened by further sampling in the region. We view the Asheville Basin and Little Tennessee barrier hypotheses as the best-supported; conversely, much denser sampling is needed to understand where the Cold Mountain and Greenbrier clades meet (likely near the junction of the Great Smoky Mountains and the Plott Balsam Mountains), and how the Round Mountain population relates (both genetically and geographically) to the Cold Mountain clade.

We lack fossil data for *Fumontana*, and are generally unsure about absolute rates of molecular evolution in Opiliones—as such, inferences regarding divergence timing in *Fumontana* must be considered preliminary. If we assume a strict molecular clock, and further assume that absolute rates of COI evolution in *Fumontana* correspond to a "standard" arthropod rate of ~2.3% pairwise sequence divergence per million years (Brower, 1994; Pons et al., 2006), then it is possible to convert observed genetic divergence values into estimates of divergence time. Average K2P-corrected inter-clade divergences range from 6.56% to 3.66% (Table 4), which corresponds to clade separations occurring in a mid-Pliocene to the early-Pleistocene time interval, approximately 2.9–1.6 Ma.

Several lines of evidence provide independent corroboration for hypothesized mid-Pliocene to the early-Pleistocene divergence times. First, there is ample evidence for global climatic fluctuations since the mid-Pliocene (Webb and Bartlein, 1992; Jansson and Dynesius, 2002; Molnar, 2004), and it is well-known that recent climatic variation has dramatically influenced plant communities in the Blue Ridge (e.g., Delcourt and Delcourt, 1998). Given the modern-day cryophilic ecology of *Fumontana*, we hypothesize that populations would have experienced relatively more continuous habitats during cool, moist periods, and that these habitats would have been more fragmented during warmer, drier periods. Also, paleodrainage maps suggest that major north-flowing Blue Ridge rivers (e.g., Little Tennessee, French Broad) were in place in the Pliocene (e.g., fig. 6.1 of Hocutt et al., 1986), so even if woodlands were continuous across low-lying basins, the rivers themselves could still have acted as barriers. Finally, molecular clock-based time estimates for many of the upland taxa referenced above are consistent with mid-Pliocene to the early-Pleistocene divergences. For example, Crespi et al. (2003) estimate the Asheville Basin break within *D. wrighti* at approximately 4.3 Ma, and discuss other phylogeographic divergences that are generally pre-Pleistocene. Kozak and Wiens (2006) cite a 2–5 Ma timeframe for divergence of Blue Ridge salamander sister-lineages (both within and between species), and Wiens et al. (2006), using relaxed clock methods for four genes, estimated divergence times for Blue Ridge species of the *Plethodon* "*glutinosus* group" (their Clade B) consistent with mid-Pliocene divergence.

A surprising result of this study is the almost complete lack of genetic structuring observed within major genetic clades. Most clades are characterized by a common, geographically widespread haplotype, with a few minimally divergent mutational derivatives (Fig. 4). Although much larger sample sizes are needed to discriminate among alternative population genetic scenarios, these data are at least consistent with historical reductions in population size, and more recent population and geographic expansion (Slatkin and Hudson, 1991). This type of population genetic dynamic would be consistent with the known Pleistocene climatic fluctuations in Appalachia (Delcourt and Delcourt, 1998), with warm interglacials resulting in elevational retreat and population bottleneck, and glacial maximum periods allowing for population increase and range expansion.

### 4.2. Cryptic speciation?

The process of species delimitation, which has seen a long history of both empirical and conceptual argumentation, is a difficult, contentious, and vitally important problem (Agapow et al., 2004; Sites and Marshall, 2004). Although we favor a single species hypothesis at this time, there are many arguments that could be made for a multiple species hypothesis, briefly outlined here. Independently segregating mitochondrial and nuclear genes recover the same genetic clades, consistent with the genealogical congruence criterion (Avise and Ball, 1990; Baum and Shaw, 1995). Genealogical congruence is expected only when lineages have been separated for long periods of time (relative to population size), and have maintained this evolutionary separation (i.e., no gene flow after divergence). As such, the observation of congruent genealogical structuring in separate genes is perhaps the strongest evidence for species

Table 4
Average K2P-corrected (Kimura, 1980) COI pairwise divergences within and between *Fumontana* genetic clades

| | Spivey (%) | ColdMtn. (%) | RoundMtn. (%) | Greenbrier (%) | JoyceKilmer (%) |
|---|---|---|---|---|---|
| Spivey | 0.14 | | | | |
| ColdMtn. | 6.02 | 0.07 | | | |
| RoundMtn. | 5.15 | 3.66 | 0.00 | | |
| Greenbrier | 5.10 | 6.43 | 5.33 | 0.69 | |
| JoyceKilmer | 4.78 | 6.56 | 5.24 | 4.53 | 0.49 |

status, although such congruence has also been shown for isolated populations *within* species (see examples in Avise, 2000).

Focusing only on the more rapidly-evolving COI data, these data reveal the presence of geographically contiguous (cohesive), reciprocally-monophyletic clades, consistent with the tree-based criterion of Wiens and Penkrot (2002). Also, COI divergence values among clades are generally an order of magnitude greater than divergences within clades (see Table 4), a pattern used in species discovery in so-called "DNA barcoding" efforts (e.g., see Barrett and Hebert, 2005; Pons et al., 2006). Fragmentation of the COI data into five separate TCS networks is a fourth criterion (Cardoso and Vogler, 2005), but is very similar to the "low within, high between" argument. Finally, we note that although Thomas and Hedin (2006) were unable to show obvious morphological divergence among populations of *Fumontana*, it is also true that studies demonstrating cryptic divergence in arachnids are becoming fairly common (e.g., Wilcox et al., 1997; Bond et al., 2001; Hendrixson and Bond, 2005; Starrett and Hedin, 2007). It seems that morphology is sometimes conservative with respect to species limits in cryophilic arachnids.

Given these multiple arguments for multiple *Fumontana* species, it is valid to ask why we continue to favor a monotypic taxonomy? Put simply, we argue that more data are needed before formal taxonomic changes are recommended. The collection of many more populations is needed to understand the geographic limits of clades, if and how clades interact in parapatry, and population genetic structure within clades. Collection of data from more genes would allow further tests for genealogical congruence, but also, perhaps provide more resolution of interclade relationships. Revealing clade inter-relationships consistent with *secondary* contact would greatly increase evidence for species status. Collection of more individuals would allow for a better understanding of morphological variation, and more detailed studies of population genetic structuring. Finally, more careful studies of population ecology are needed. Although our studies have greatly increased our understanding of *Fumontana* diversity, we are still somewhat limited by the available sample, which includes relatively few individuals from few sites.

### 4.3. Conservation implications

Prior to 2006 (Thomas and Hedin, 2006), *F. deprehendor* was known from only four individuals from two old-growth forest sites (Shear, 1977, 1978). Despite this apparent rarity, we are unaware of any regional conservation efforts that considered this taxon. However, this perceived rarity was shown to be a collecting artifact, and over twenty new populations were discovered after gaining a better understanding of *Fumontana* microhabitat preferences (Thomas and Hedin, 2006), and many more populations are likely to be discovered with continued sampling efforts. The observed morphological homogeneity and larger distribution of *Fumontana* would thus seem to indicate that conservation attention is not warranted. However, our genetic data suggest the presence of five independent evolutionary units within the species, which may possibly represent cryptic species, and are minimally "evolutionarily significant units" (e.g., Moritz, 1994). These independent evolutionary units inhabit relatively small geographic distributions, as exemplified by the Round Mountain population. Thus, *Fumontana* has gone from an initially hyperrare taxon of very limited distribution (Shear, 1977, 1978), to a more widespread taxon (Thomas and Hedin, 2006), with phylogeographic data now revealing subdivision into five independent groups, each of which we feel should be recognized in regional conservation decisions.

Validating previous morphological hypotheses, our molecular data demonstrate that *F. deprehendor* is a phylogenetic relict within the Triaenonychidae, and is clearly one of the most distinctive laniatoreans in North America. Given this phylogenetic distinctiveness, *Fumontana* represents a key component of regional "phylogenetic diversity", often a criterion in conservation planning (Rodrigues and Gaston, 2002). *Fumontana* populations also appear very sensitive to habitat alteration (Thomas and Hedin, 2006), and both anthropogenic (e.g., deforestation, fragmentation, etc.) and non-anthropogenic impacts (e.g., the invasive Hemlock Woolly adelgid currently causing extreme damage to natural hemlock populations) continue to threaten *Fumontana* populations in the southern Appalachians. Lastly, we emphasize that despite our increased population sample, *Fumontana* is still a relatively uncommon opilionid, often difficult to find, and rarely collected in large numbers. We argue that this combination of phylogenetic distinctiveness, habitat specialization, rarity, and phylogeographic diversification warrants conservation attention and action, and hope that this contribution provokes further studies of this elusive opilion taxon.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2007.10.013.

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