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Patterns of intra- and interspecific diversity in araneomorph spiders of southern high Appalachian leaf litter

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Abstract. The intraspecific diversity of the few mygalomorph spiders in southern Appalachia has received considerable attention, revealing highly localized and deeply divergent populations and in some cases cryptic species. The araneomorph fauna of the region has received comparatively little study, due to its higher diversity, more tentative taxonomy, and perhaps perception that short-range endemicity might be lower. In this study, we examined this last assumption, surveying patterns of intraspecific diversity across Araneomorphae occurring in the leaf litter of high elevation spruce-fir sky islands of southern Appalachia, as revealed by population level metabarcoding. In most species, intraspecific haplotypic diversity, while high, revealed little geographic pattern, generally supporting assumptions of low regional endemicity. Of 50 spider species found in spruce-fir forest litter, more than 30 occur more widely, and showed little sign of subdivision even over major biogeographic features in the region, such as the French Broad River valley. By analyzing 10 of these well-sampled species with population genetic and phylogeographic methods, we found that only a few showed significant population level subdivisions. We suggest that the ballooning behavior of most immature araneomorph spiders is sufficiently frequent to overcome divergence of otherwise isolated populations, and that in those few exceptions the species are more specialized to particular microhabitats in the spruce-fir forest and such unpredictable dispersal may have been selected against.

Keywords: Cytochrome c oxidase I, endemism, metabarcoding, sky islands

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The high elevation spruce-fir sky islands of southern Appalachia host a rich and still underdocumented arthropod fauna (Caterino & Recuero 2024). These coniferous peaks present a distinct set of microhabitats from the surrounding sea of deciduous forest, and consequently harbor numerous distinct lineages. Furthermore, their isolation from one another has driven among-peak differentiation, resulting in considerable peak or range level endemism (Caterino & Recuero 2024), and numerous cryptic species complexes, such as *Trechus* Clairville, 1806 ground beetles (Barr 1979); myriapods (Garrick et al. 2018); various salamanders (Niemiller et al. 2022; Pierson et al. 2023); *Dasycerus* Brongniart, 1800 rove beetles (Caterino & Harden 2024), and many others.

Spiders have drawn considerable attention in the region, owing in part to the presence of the federally listed mygalomorph *Microhexura montivaga* Crosby & Bishop, 1925 (Microhexuridae) which shows deep phylogeographic structure across the high peaks of the region (Hedin et al. 2015). Recent studies of the more widespread mygalomorph genus *Antrodiaetus* Ausserer, 1871 (Antrodiaetidae) in this region have also revealed considerable cryptic diversity, some of it in sympatry, with *A. microunicolor* Hendrixson & Bond, 2005 recognized within the past 20 years as a species distinct from *A. unicolor* (Hentz, 1842) (Hendrixson & Bond 2005a), and more recent analyses uncovering several distinct lineages within what remains of *A. unicolor* (Newton et al. 2020). Most of the arachnological work in the region, however, has focused on lineages more common to lower elevations or subterranean environments. *Nesticus* Thorell, 1869 (Nesticidae) spiders, most frequently associated with cave habitats, show deep divergence and high regional diversity in southern Appalachia (Hedin 1997; Hedin & Milne 2023). The lampshade spiders *Hypochilus* Marx, 1888 (Hypochilidae), with populations limited to rock outcrops in southern Appalachia, show high local endemism and deep regional structure (Keith & Hedin 2012), though a few *Hypochilus* distributions extend to the highest elevations of these mountains. More widely distributed harvestmen have also received considerable attention. But lineages occurring in, or restricted to, the spruce-fir islands have not been the focus of much arachnological work.

Spider phylogeography more generally suggests some general expectations. In particular, mygalomorph spiders have typically shown high levels of local phylogeographic structure (Hendrixson & Bond 2005b; Hamilton et al. 2011; Hedin et al. 2015; Rix et al. 2017; Newton et al. 2020; Korba et al. 2022), whereas araneomorph taxa tend to exhibit lower degrees of structure, presumably due to ballooning behavior, a dispersal process in which spiders use silk threads to become airborne, exhibited by many araneomorph spiderlings (Dean & Sterling 1985; Hamilton et al. 2011; Agnarsson et al. 2016). In some cases, apparently wide biogeographic distributions have been shown to result from inadequately resolved cryptic taxonomy (Abel et al. 2020). In other sky island systems, the results have been intermediate, with jumping spiders in western sky islands showing high local but lower regional structure, which in that case was attributed to incomplete lineage sorting (Masta 2000). Perhaps consistent with the latter observation, some work has shown ballooning to be less common in habitat specialist species, presumably due to risks of landing in unsuitable habitat patches (Bonte et al. 2003; Bonte & Lens 2007), comparable to losses of wings in insular insect taxa (Darwin 1859; Darlington 1943; Wagner & Liebherr 1992).

Given these ambiguous previous findings, it is difficult to predict what sorts of intraspecific patterns might be exhibited by the majority of high Appalachian spiders. On the one hand, most

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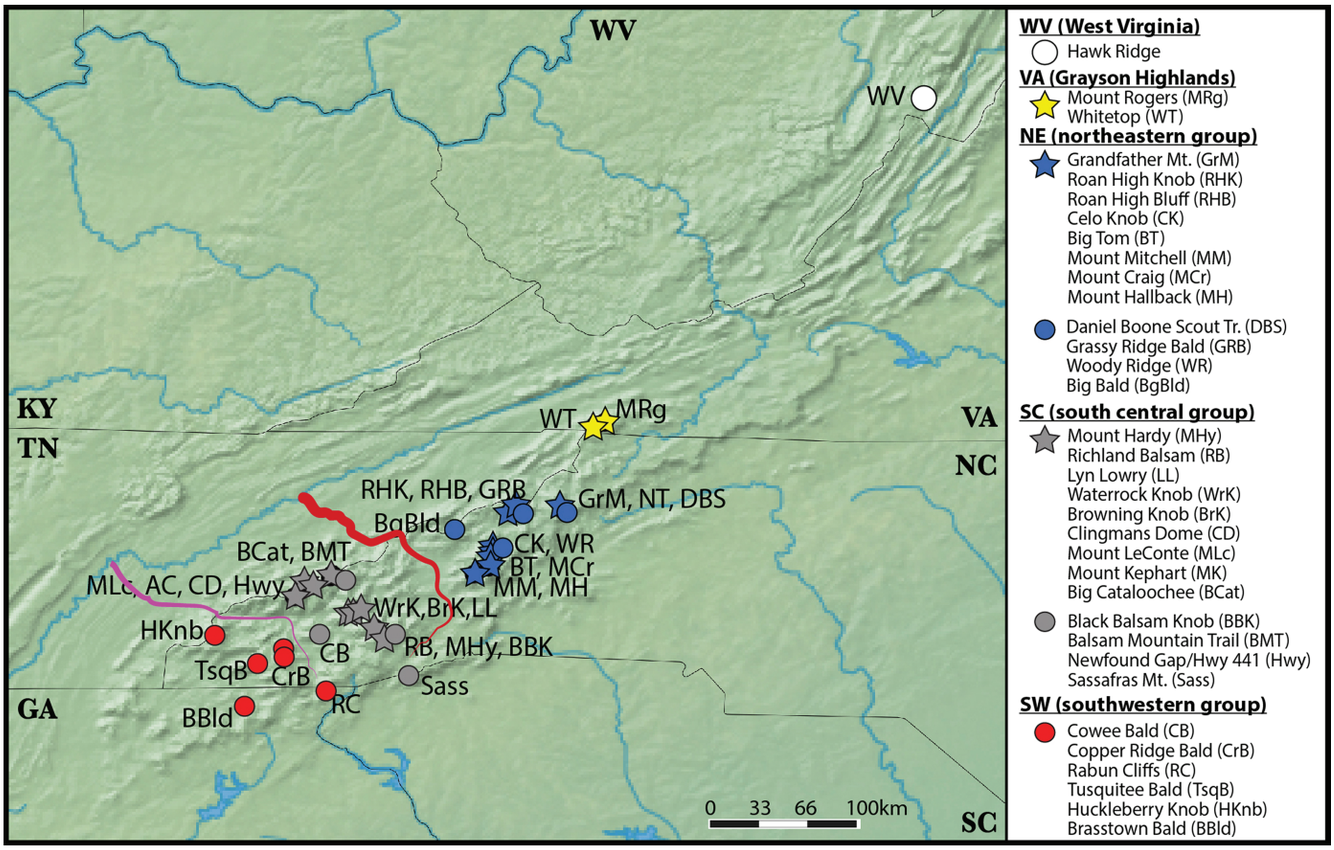

Figure 1.—All sampling sites, colors used to distinguish by population genetic 'group' (SW – red; SC – gray; NE – blue; Grayson Highlands – yellow; West Virginia – white). Stars indicate high elevation sites with spruce-fir cover. Circles represent lower elevation sites and/or those lacking in spruce-fir forest. Red line marks the course of the French Broad River, while the purple line marks the Little Tennessee River.

araneomorph species might be expected to show low phylogeographic structure due to a presumed proclivity to balloon, and therefore able to readily disperse among sky islands. On the other hand, these sky islands might represent sufficiently specialized and distinctive habitats that natal dispersal will have been selected against, leading to greater philopatry and intraspecific structure.

Among litter-associated spiders in the region, most that have been identified to date seem to represent relatively widespread species, not obviously restricted to the region, let alone to higher elevation peaks. However, a few species have been recognized as having narrower and more limited distributions, such as the erigonine linyphiid *Sisicottus montigena* Bishop & Crosby, 1938, described from Mt. Mitchell in the Black Mts., but reported from a few other spruce-fir peaks in the region (Miller 1999).

Our purpose in this paper is to assess levels of phylogeographic structure across numerous species of spiders occurring in the leaf litter of high elevation spruce-fir forests in the southern Appalachians. We employ Cytochrome c oxidase I (COI) barcoding data to reveal intra- and interpopulation diversity patterns and explore the possibility that any of the lineages in the region represent cryptic species complexes, with narrowly distributed endemics restricted to particular peaks or ranges. Lastly, given insights into these questions, can we hypothesize any biological reasons to explain the observed patterns, in particular aspects of behavior and natural history that might be associated with more or less geographic structuring?

METHODS

Sampling.—We sampled spiders from numerous medium- to high-elevation sites in southern Appalachia, including several spruce-fir peaks within 7 more or less well-defined ranges, from (southwest to northeast): Clingmans Dome, Mount Kephart, Mount LeConte, and Big Cataloochee (Great Smoky Mountains), Browning Knob and Mt. Lyn Lowry (Plott Balsams), Richland Balsam and Mt. Hardy (Great Balsams), Mount Mitchell and Celo Knob (Black mountains), Roan High Knob and Roan High Bluff (Roan Highlands), Grandfather Mountain (basically a one-peak range), and Whitetop and Mount Rogers (Grayson Highlands of Virginia). To assess endemism to higher, spruce-fir dominated areas, we also sampled numerous intervening sites. All spider sampling sites are shown in Fig. 1, with additional details on each site in Table 1. Samples from Hawk Ridge, a disjunct spruce-only site (~250km NE of Grayson Highlands) in West Virginia, were included to see if genetic distances in shared species are disproportionately low to the north, consistent with post-glacial expansion. Most sites were visited twice, once in late spring/early summer, and once in fall. Each visit we sifted three leaf litter samples through an 8mm mesh and extracted all arthropods using Berlese funnels. Spiders were separated out and stored cold in 100% ethanol. These were sorted to morphospecies, considering adult males, adult females, and immatures as distinct OTUs. One individual of each morphospecies from each

Table 1.—Sampling sites, abbreviations as in Figs. 1 & 2. Bold, underlined locality abbreviations represent high elevation spruce-fir forested sites. 'Region' pertains to coarse distributional ranges as shown in Fig. 3.

Abbrev.	Locality	Region	Mountain range	Elev. (m)	Coordinates
AC	TN: Sevier Co., Great Smoky Mts. NP, Alum Cave Tr.	SC	Great Smoky Mts.	1317–1581	35.64, −83.44
BBK	NC: Haywood Co., Pisgah NF, Black Balsam Knob	SC	Great Balsams	1796–1851	35.32, −82.87
BBld	GA: Towns Co., Chattahoochee NF, Brasstown Bald	SW		1346–1408	34.87, −83.81
BCat	NC: Haywood Co., Great Smoky Mts. NP, Big Cataloochee Mt.	SC	Great Smoky Mts.	1703–1876	35.67, −83.17
BgBld	TN: Unicoi Co., Cherokee NF, Big Bald	NE		1596–1665	35.99, −82.49
BMT	NC: Haywood Co., Great Smoky Mts. NP, Balsam Mt. Tr.	SC	Great Smoky Mts.	1448–1575	35.64, −83.20
BrK	NC: Jackson Co., Blue Ridge Pkwy NP, Browning Knob	SC	Plott Balsams	1871–1898	35.46, −83.13
BT	NC: Yancey Co., Mt. Mitchell SP, Big Tom near summit	NE	Black Mts.	1991–2007	35.77, −82.26
CB	NC: Macon Co., Nantahala NF, Cowee Bald	SC		1475–1506	35.33, −83.34
CD	NC: Swain Co., Great Smoky Mts. NP, Clingmans Dome	SC	Great Smoky Mts.	1900–1981	35.56, −83.5
CK	NC: Yancey, Pisgah NF, Celo Knob	NE	Black Mts.	1908–1920	35.85, −82.25
CrB	NC: Macon Co., Nantahala NF, Copper Ridge Bald	SW		1534–1597	35.23, −83.56
NT/DBS	NC: Caldwell Co., Grandfather Mt. SP, Boone Scout Tr.	NE	Grandfather Mt.	1218–1558	36.11, −81.79
GRB	NC: Mitchell Co., Pisgah NF, Grassy Ridge Bald	NE	Roan Highlands	1846–1874	36.1, −82.1
GrM	NC: Caldwell Co., Grandfather Mt. SP, Calloway Peak	NE	Grandfather Mt.	1760–1803	36.11, −81.81
HKnb	NC: Graham Co., Nantahala NF, Huckleberry Knob	SW		1662–1683	35.32, −83.99
Hwy	TN: Sevier Co., Great Smoky Mts. NP, Off Hwy 441	SC	Great Smoky Mts.	1394–1405	35.6, −83.4
LL	NC: Jackson Co., Blue Ridge Pkwy NP, Mt. Lyn Lowry	SC	Plott Balsams	1858–1893	35.46, −83.11
MCr	NC: Yancey Co., Mt. Mitchell SP, Mt. Craig near summit	NE	Black Mts.	1984–2016	35.78, −82.26
MH	NC: Yancey Co., Mt. Mitchell SP, Mt. Hallback near summit	NE	Black Mts.	1927	35.75, −82.28
MHy	NC: Haywood Co., Blue Ridge Pkwy NP, Mt. Hardy	SC	Great Balsams	1857–1865	35.3, −82.93
MK	TN: Sevier Co., Great Smoky Mts. NP, Mt. Kephart	SC	Great Smoky Mts.	1883–1894	35.63, −83.39
MLc	TN: Sevier Co., Great Smoky Mts. NP, Mt. LeConte	SC	Great Smoky Mts.	1971–2008	35.65, −83.44
MM	NC: Yancey Co., Mt. Mitchell SP, Mt. Mitchell	NE	Black Mts.	1985–2023	35.76, −82.26
MRg	VA: Smyth Co., Mt. Rogers NRA, Mt. Rogers	VA	Grayson Highlands	1731–1743	36.66, −81.54
RB	NC: Haywood Co., Blue Ridge Pkwy NP, Richland Balsam	SC	Great Balsams	1881–1950	35.36, −82.99
RC	GA: Rabun Co., Chattahoochee NF, Rabun Cliffs	SW		1207–1320	34.97, −83.3
RHB	NC: Mitchell Co., Pisgah NF, Roan High Bluff	NE	Roan Highlands	1873–1905	36.09, −82.14
RHK	NC: Mitchell Co., Pisgah NF, Roan High Knob	NE	Roan Highlands	1754–1916	36.1, −82.12
Sass	SC: Pickens Co., Sassafras Mt.	SC		1029–1047	35.06, −82.78
TsqB	NC: Clay Co., Nantahala NF, Tusquitee Bald	SW		1262–1606	35.14, −83.73
WR	NC: Yancey, Pisgah NF, Woody Ridge Tr.	NE	Black Mts.	1259–1642	35.84, −82.24
WT	VA: Smyth Co., Whitetop Mt.	VA	Grayson Highlands	1644–1678	36.64, −81.6
WV	WV: Randolph Co., Monongahela NF, Cheat Mt.	WV		1330–1350	38.56, −79.94

sampling site was photographed and prepared for DNA extraction. Images of morphospecies are archived on our lab Flickr page, named by morphospecies code (online at https://www.flickr.com/photos/183480085@N02/albums/72157710331079771). Each specimen was subdivided (if large) or punctured to permit tissue digestion and placed in a separate well in a 96-well plate, where they were digested with lysis buffer and proteinase K (Omega BioTek, Norcross, GA). The voucher was saved for archiving in the Clemson University Arthropod Collection (CUAC), and the liquid fraction was removed to a new plate. Genomic DNA was purified using Omega BioTek's MagBind HDQ Blood and Tissue kit on a Hamilton Microlab Star automated liquid handling system, eluting with 150 µL elution buffer.

We amplified a 421 bp fragment of the mitochondrial COI gene using the primers BF2-BR2 (5′-GCHCCHGAYATRGCHTTY CC-3′ & 5′-TCDGGRTGNCCRAARAAYCA-3′, respectively; Elbrecht & Leese 2017), corresponding to the downstream two-thirds of the standard barcoding region (e.g., Hebert et al. 2003). Each reaction was tagged with a unique combination of forward and reverse 9bp indices (from Meier et al. 2016), synthesized as part of the primer by Eurofins Genomics (Louisville, KY). All PCRs were conducted in 12.5µL volumes (5.6 µL water, 1.25 µL Platinum Taq buffer, 1.25 µL dNTP mix [2.5 mM each], 0.4 µL $MgCl_2$ [50 mM], 1.5 µL each primer [5 µM], 0.05 µL Platinum Taq polymerase, 1 µL DNA template), with a 95°C initial denaturation for 5 minutes, followed by 35 cycles of 94°C (30 sec), 50°C (30 sec), 72°C (30 sec), and a 5-minute 72°C final extension on an Eppendorf Gradient Mastercycler.

The amplicon was sequenced using either an Illumina MiSeq or an Oxford Nanopore MinION. For Illumina library preparation, PCR products were combined and purified using Omega Bio-Tek's Mag-Bind Total Pure NGS Kit, in a ratio of 0.7:1 (v:v, bead mix: sample volume), (enriching for fragments >300bp). Illumina adapters and sequencing primers were ligated to PCR products using New England BioLab's Blunt/TA Ligase Master Mix. The amplicon+adapter library was again purified using Mag-Bind Total Pure NGS and subsequently quantified using a Qubit fluorometer (Thermo-Fisher Scientific). Nanopore libraries were prepared using the ligation sequencing kit LSK-112 (Oxford Nanopore Technologies, Oxford, UK), then loaded onto a MinION running a v10.4 flow cell.

Data analysis.—Illumina reads were processed with bbtools software package (online at https://jgi.doe.gov/data-and-tools/bbtools/ v38.87, Bushnell et al. 2017) to merge paired read ends,

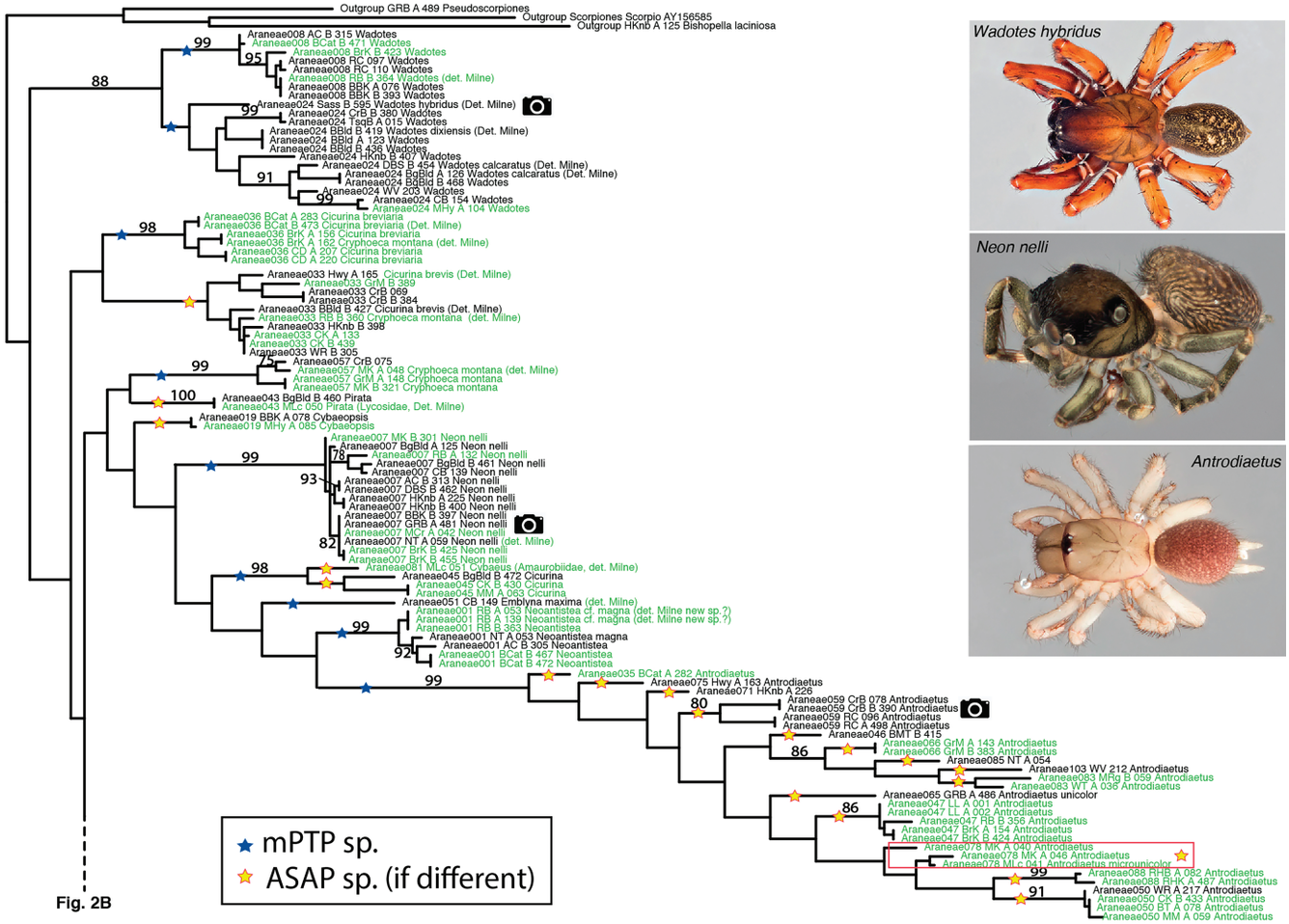

Figure 2A.—Top quarter of RaxML phylogeny of all species occurring in spruce-fir forest. Taxon designations in green represent those individuals from spruce-fir sites. Specimens directly examined by M. Milne are designated 'Det. Milne'. Blue stars on branches represent mPTP suggested species. Yellow stars indicate ASAP delimited species if they differ from mPTP (red box around some *Antrodiaetus* specimens in 2A enclose an ASAP-delimited species not found to be monophyletic). Ultrafast bootstrap values >75% are shown. Camera icons indicate specimens or populations figured at right.

remove PhiX reads, trim Illumina adapters, filter reads for the correct size, remove reads with quality score<30, cluster sequences by similarity allowing 5 mismatches (~1%), and generate a final matrix in FASTA format. Nanopore reads were basecalled using the 'super-accurate' algorithm of Guppy v6.1.2, then demultiplexed using ONTbarcoder v0.1.9 (Srivathsan et al. 2021), with minimum coverage set at 5. All sequences were combined and aligned with the online version of Mafft v7 (Katoh et al. 2017) using the auto strategy. All barcode sequences have been deposited in GenBank; accession numbers are provided in Supplemental Table S1 (available online at https://doi.org/10.1636/JoA-S-24-007.s1).

Though morphological identifications of many adult specimens were carried out (by MAM), we primarily assessed endemicity by distributions of OTUs hypothesized by automated species delimitation under the presumption that some morphological species might contain cryptic diversity, as do many other flightless arthropods in the region. We used ASAP (Assemble Species by Automatic Partitioning; Puillandre et al. 2021), which seeks a barcoding gap in the data provided, a discontinuity between presumably intraspecific and interspecific genetic distances. These were assessed using Kimura 2-parameter distances, and the top three delimitations were saved, though only the best (lowest ASAP) score results are reported here. We also explored species limits as hypothesized by a coalescence-based method, mPTP, which seeks a discontinuity in branching patterns distinguishing intra- and interspecific phylogeny (Kapli et al. 2017), using the web service online at https://mptp.h-its.org/#/tree. As an input tree we used a topology estimated by RaxML (Stamatakis 2014) on the Cipres server (Miller et al. 2010) that included all spider sequences from all sampling sites, including low elevation sites, running 1000 ultrafast bootstrap replicates to measure branch support. Below we only discuss those results where at least one occurrence of the delimited species was in the spruce-fir biome. Determinations not designated as 'Det. Milne' in Fig. 2a-d were assigned by BOLD or GenBank matches (see Recuero et al. 2024 for details).

For each species found to occur in the spruce-fir zone and represented by more than 10 individuals, we assessed broad-scale geographic structure of genetic diversity quantitatively using θ_{st}.

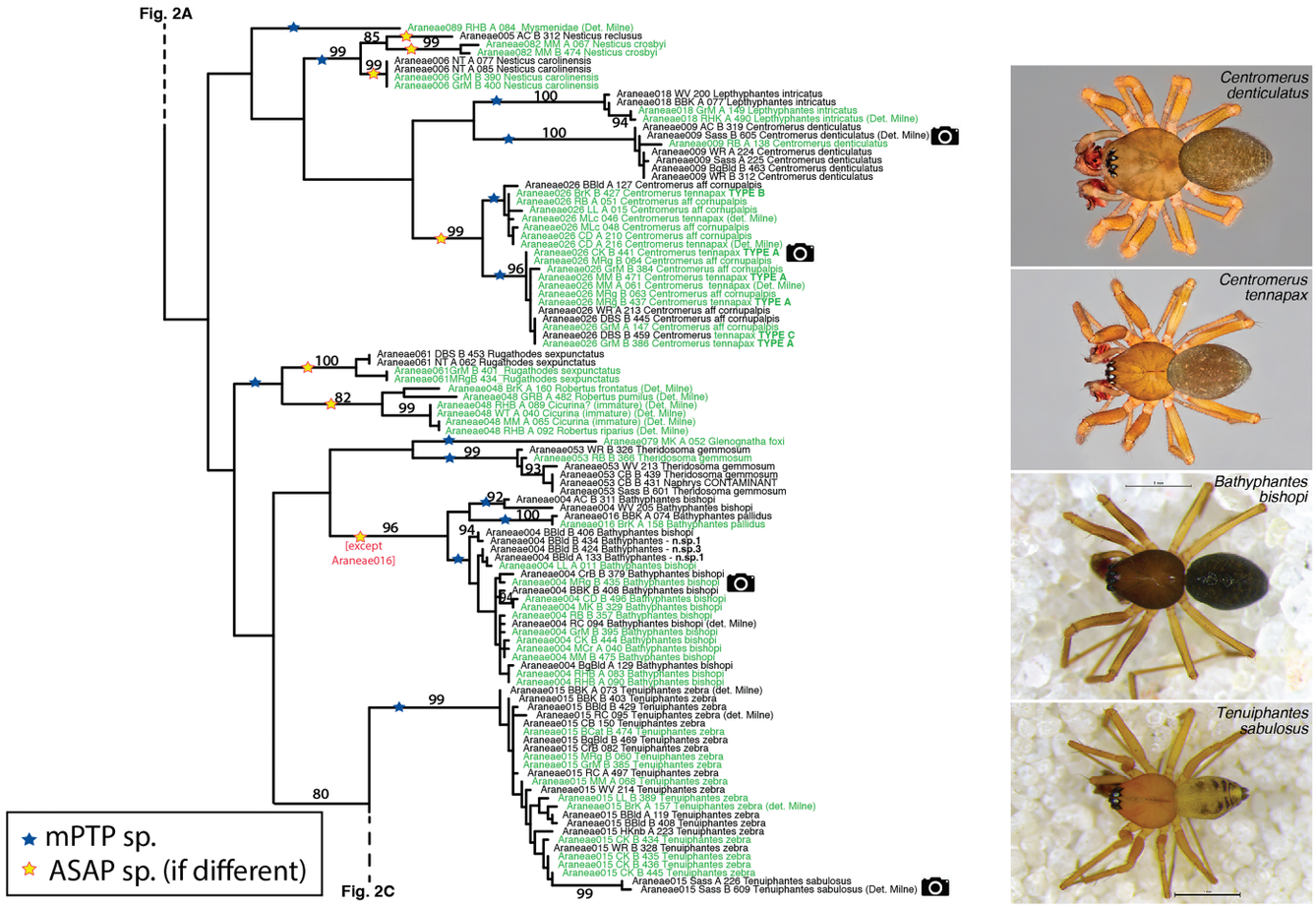

Figure 2B.—Second quarter of RaxML phylogeny of all species occurring in spruce-fir forest., Taxon designations in green represent those individuals from spruce-fir sites. Specimens directly examined by M. Milne are designated 'Det. Milne'. Blue stars on branches represent mPTP suggested species. Yellow stars indicate ASAP delimited species if they differ from mPTP. Ultrafast bootstrap values >75% are shown. Camera icons indicate specimens or populations figured at right.

Populations were assigned to 5 coarse groups: southwestern ('SW', all populations southwest of the Little Tennessee River [LTR] drainage); south-central ('SC', all populations between LTR and the French Broad River [FBR]; northeast ('NE', ranges north of FBR within North Carolina); VA (Grayson Highlands); and WV (for species sampled at our far northern West Virginia site). For these groups, we performed a 2-group AMOVA (east and west of FBR, lumping the first two and the remaining three listed above, to obtain sufficient sample sizes), and report overall θ_{st} and its significance. We also examined intraspecific network structure qualitatively, to explore distribution and relatedness of haplotypes within delimited groups. AMOVAs were conducted in Arlequin (ver. 3.5.2.2) and TCS networks (Clement et al. 2000) were constructed in PopART (Leigh & Bryant 2015).

To explore the temporal depth of diversity within each delimited species (those with at least one spruce-fir occurrence), we constructed dated intraspecific topologies in BEAST. v1.10.4 (Drummond et al. 2012). Each species dataset was analyzed with linked tree and clock models and partitioned by codon position and best-fitting nucleotide substitution models as determined with PartitionFinder 2 (Lanfear et al. 2017). We implemented a coalescent Bayesian Skyline tree prior and a strict molecular clock using a substitution rate with normal distribution, a mean value of 0.015, and a standard deviation of 0.01, based on empirical estimates for different spider families (Bidegaray-Batista & Arnedo 2011; Bidegaray-Batista et al. 2014; Arnedo & Hormiga 2021). We used Tracer v1.7.2 (Rambaut et al. 2018) to check effective sample sizes (> 200) considering a 25% burn-in, and to generate Bayesian Skyline Plots for each species, examining trends in apparent effective population sizes over time. Estimated ages were obtained from maximum credibility trees constructed with TreeAnnotator v.1.10.4, also considering a 25% burn-in.

Vouchering.—Following digestions, remains of extracted specimens were recombined with any non-extracted body parts, labelled, assigned unique CUAC (Clemson University Arthropod Collection) identifiers, and curated into the CUAC. Unextracted representatives of morphospecies, if any, remain in bulk order-level samples, and are also permanently vouchered in the CUAC, as are unsorted residues (containing additional representatives of more abundant morphospecies). A complete list of all specimens extracted, with collecting data, DNA extraction codes, GenBank accession numbers, and voucher codes is available in Supplemental Table S1.

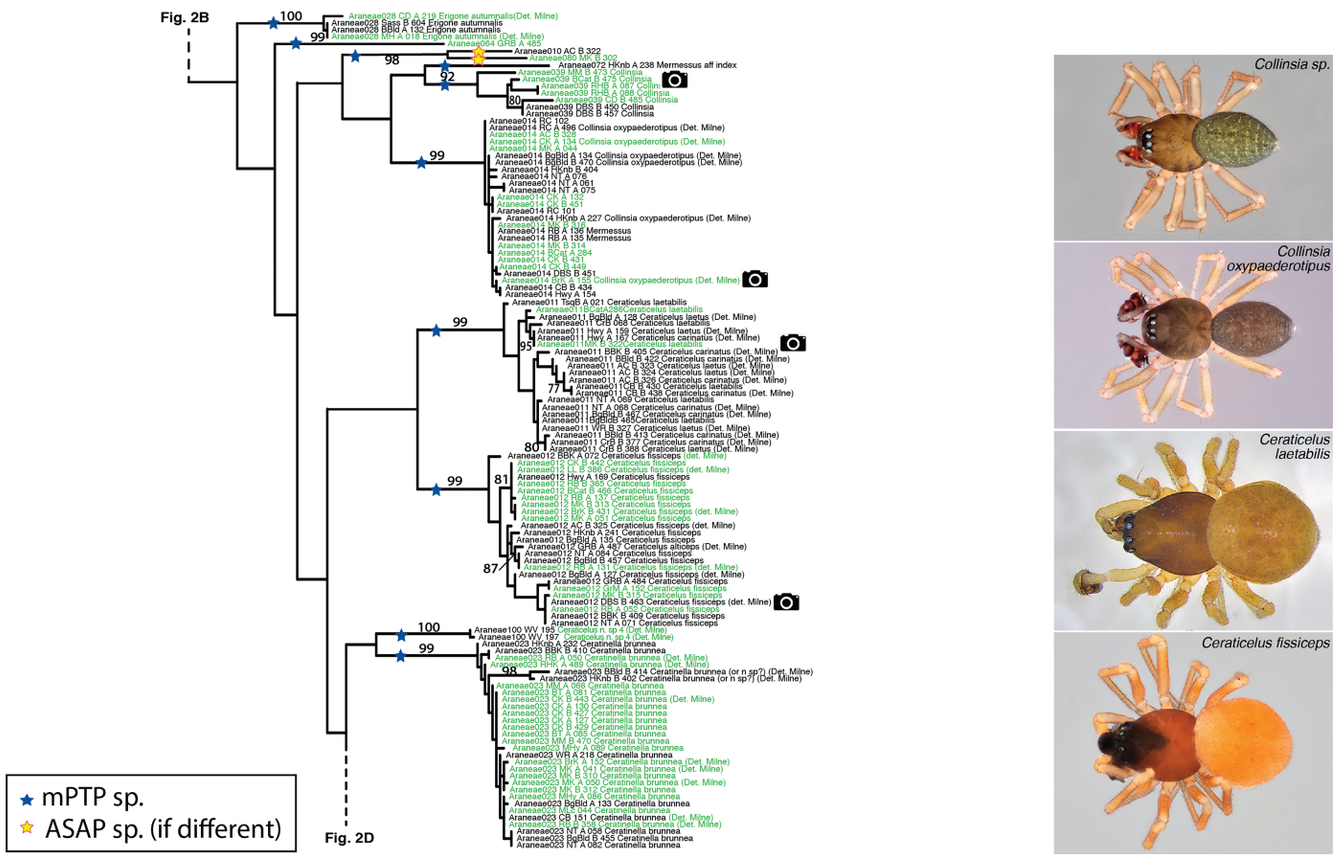

Figure 2C.—Third quarter of phylogeny RaxML phylogeny of all species occurring in spruce-fir forest. Taxon designations in green represent those individuals from spruce-fir sites. Specimens directly examined by M. Milne are designated 'Det. Milne'. Blue stars on branches represent mPTP suggested species. Yellow stars indicate ASAP delimited species if they differ from mPTP. Ultrafast bootstrap values >75% are shown. Camera icons indicate specimens or populations figured at right.

RESULTS

Our barcoding resulted in a total of 572 spider barcodes from all sites. ASAP analyses partitioned these into 103 putative species. Of these, 50 had at least one spruce-fir occurrence. These were represented by 430 sequences, which were retained for subsequent analyses. mPTP analysis partitioned the 'total' data set into 74 putative species, and those occurring in spruce-fir into 41, most of the differences resulting from lumping of lineages among divergent *Antrodiaetus* (Mygalomorphae) trapdoor spider populations. These delimitations are indicated on the tree in Fig. 2. Below, all references to 'species' refer to those as inferred by ASAP, for consistency's sake, unless otherwise indicated. Relationships among COI lineages aren't expected to reveal meaningful phylogenetic structure at the deeper levels of the ML tree, as indicated by low bootstrap supports on most supraspecific branches (Fig. 2), so we don't explore those further here. But relationships among populations and closely related species may reveal meaningful patterns.

Of the 50 species occurring at least partially in spruce-fir forest, 18 are known only from this forest type (see Supplemental Table S2, available online at https://doi.org/10.1636/JoA-S-24-007.s2). Eight of these are represented only by singletons, and little can therefore be said about their preferences and/or endemicity. Seven of the remainder are known from only a single range, whereas four are known from multiple ranges. Those from single ranges include four ASAP lineages of *Antrodiaetus*, and only three aranaeomorphs. The most widely distributed putative spruce-fir endemic is a yet-undescribed linyphid species, *Sisicus* n. sp. 1 (Araneae049_Sisicus in Table 2); it is known from seven sites, in five different ranges, maximally separated by 214 km (from Clingmans Dome in the Smokies to Mt. Rogers in SW Virginia). Only this species and two others with predominantly spruce-fir occurrences have ranges that span the French Broad River (FBR). Sampling for many of these species was probably insufficient to reveal their complete ranges, so these numbers likely overestimate apparent endemicity.

Of the better sampled species (10 or more individuals), those occurring at fewer sites have generally higher θ_{ST} values (Table 2), indicating reduced gene flow among their populations. The highest θ_{ST} values in species that nominally spanned the FBR were in the linyphiid *Centromerus tennapax* (Barrows, 1940) ($\theta_{ST} = 0.94$), with completely isolated north-south clades (Fig. 3). This species in our sampling is furthermore largely (16 of 20 individuals) restricted to spruce-fir sites, and its prevalence in these higher elevations may be associated with its higher degree of isolation. The next highest apparent population subdivision is observed in *Cicurina brevis* (Emerton, 1890) (Cicurinidae) ($\theta_{ST} = 0.97$), though with only ~40% of occurrences in the spruce-fir sites, any dispersal limitation would not be limited by

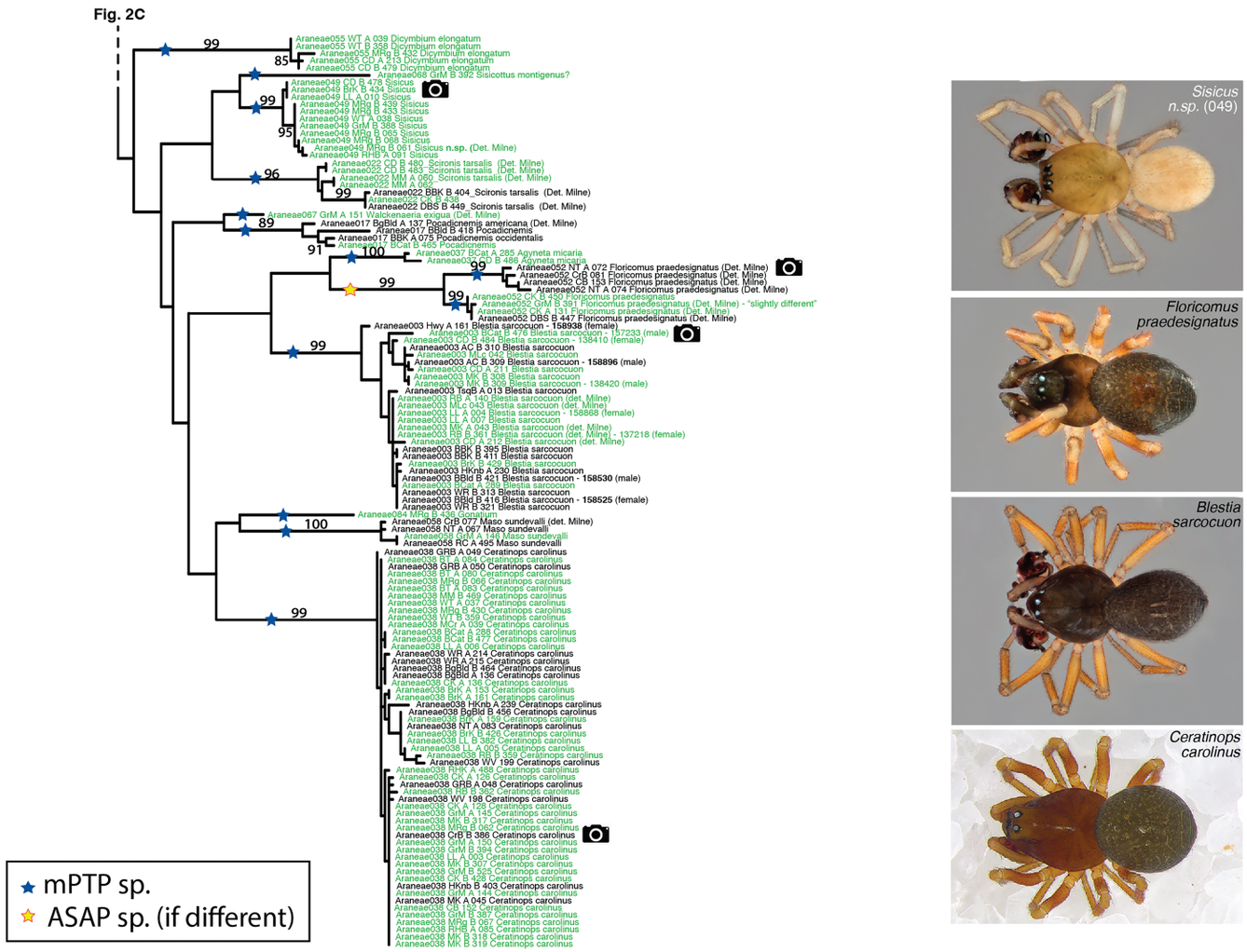

Figure 2D.—Bottom quarter of phylogeny RaxML phylogeny of all species occurring in spruce-fir forest. Taxon designations in green represent those individuals from spruce-fir sites. Specimens directly examined by M. Milne are designated 'Det. Milne'. Blue stars on branches represent mPTP suggested species. Yellow stars indicate ASAP delimited species if they differ from mPTP. Ultrafast bootstrap values >75% are shown. Camera icons indicate specimens or populations figured at right.

the same ecological factors as others here. Its north-south haplotypes are intermingled with each other phylogenetically (Fig. 3), but also highly divergent from each other.

More widespread species, correspondingly, tended to exhibit low population structure. The most abundantly and widely distributed species in our sampling was *Ceratinops carolinus* (Banks, 1911), a distinctive linyphiid. With a θ_{ST} of 0.22, it was found at 12 spruce-fir sites, accounting for two-thirds of its records, but it was found at seven other sites, lower in elevation and/or predominantly open or deciduous habitats, indicating considerable ecological latitude. Indeed, its most common haplotype was found across nearly all of its sampled range, from Huckleberry Knob in southwestern North Carolina to Mount Rogers in Virginia. A similar pattern was observed in the linyphiid *Collinsia oxypaederotipus* (Crosby, 1905), with relatively low structure ($\theta_{ST} = 0.19$), considerable haplotype diversity, and yet with multiple individual haplotypes spanning the FBR. The linyphiids *Ceraticelus fissiceps* (O. Pickard-Cambridge, 1874) and *Blestia sarcocuon* (Crosby & Bishop, 1927) fit this category as well, with θ_{ST} values of 0.1 and 0.13, respectively. *Ceraticelus fissiceps* has three haplotypes that span the FBR, and limited phylogeographic structure among populations. *Blestia sarcocuon* includes two rather divergent clades, one with a couple of widespread haplotypes, the other comprising a number of distinctive haplotypes limited to the Great Smoky Mountains.

A number of species appear intermediate in degree of structure, being both widespread but revealing moderately high θ_{ST} values. *Tenuiphantes zebra* (Emerton, 1882), (Linyphiidae) for example, is known from 17 sites, including several high elevation spruce-fir forest patches, with a θ_{ST} of 0.57. Yet one haplotype is common to six of those sites, ranging from Cowee Bald to Mt. Rogers. The linyphiids *Ceratinella brunnea* Emerton, 1882 ($\theta_{ST} = 0.54$) and *Bathyphantes bishopi* Ivie, 1969 ($\theta_{ST} = 0.38$) exhibit similar patterns. The first of these shared haplotypes is mostly restricted to smaller geographic areas, none spanning the FBR. But at a deeper phylogenetic level, major lineages mostly comprise mixed northern

Table 2.—Table of ASAP-delimited species used in intraspecific analyses. N = total specimens sampled. '# Peaks' indicates how many high elevation spruce-fir localities the species was collected from; '# Ranges' indicates how many separate ranges (see methods) species were found in; '# spruce-fir' and '% spruce-fir' indicates how many total individuals and what percentage of total individuals were found at high elevation spruce-fir sites; 'spruce-fir only?' indicates whether the species was only found at spruce-fir sites; 'sites span FBR' indicates whether species records span the French Broad River valley; 'θ_{st}' is provided for two-group AMOVA; 'max range (km)' indicates maximum linear distance between collecting sites; 'Min. age' is the age of the deepest split in the Bayesian tree as estimated by BEAST; and 'Haps' and 'Haps/Ind.' indicate the total number of haplotypes and the relative to total N.

ASAP delimited sp.	Family	Genus	Species	N	# Peaks	# Ranges	# spruce-fir	% spruce-fir	spruce-fir only?	sites span FBR	θ_{st}	max range (km)	Min. age	Haps	Haps/Ind.
Araneae038	Linyphiidae	*Ceratinops*	*carolinus*	55	12	12	36	65%	N	Y	0.22	510	0.77	17	0.31
Araneae023	Linyphiidae	*Ceratinella*	*brunnea*	30	8	10	18	60%	N	Y	0.54	230	1.65	15	0.50
N/A		*Antrodiaetus*	(lumped)	29	14	10	18	62%	N	Y			13.61	24	0.83
Araneae003	Linyphiidae	*Blestia*	*sarcocuon*	26	7	7	15	58%	N	Y	0.13	181	1.29	15	0.58
Araneae014	Linyphiidae	*Collinsia*	*oxypaederotipus*?	26	5	9	12	46%	N	Y	0.19	217	0.29	12	0.46
Araneae012	Linyphiidae	*Ceraticelus*	*fissiceps*	25	7	8	12	48%	N	Y	0.10	217	1.07	13	0.52
Araneae015	Linyphiidae	*Tenuiphantes*	*zebra*/*sabulosus*	24	7	13	10	42%	N	Y	0.57	535	2.99	19	0.79
Araneae011	Linyphiidae	*Ceraticelus*	*laetus*/*laetabilis*?	22	2	10	2	9%	N	Y	0.02	230	1.14	14	0.64
Araneae004	Linyphiidae	*Bathyphantes*	*bishopi*	21	9	11	11	52%	N	Y	0.38	535	3.26	20	0.95
Araneae026	Linyphiidae	*Centromerus*	*tennapax*?/*cornuipalpis*	20	9	8	16	80%	N	Y	0.94	287	1.96	10	0.50
Araneae007	Salticidae	*Neon*	*nelli*	15	3	9	4	27%	N	Y	0.21	217	0.86	9	0.60
Araneae024	Agelenidae	*Wadotes*	sp.	13	1	9	1	8%	N	Y	0.65	535	5.42	10	0.77
Araneae049	Linyphiidae	*Sisicus*	n. sp. 1	11	7	5	11	100%	Y	Y	0.87	214	0.35	5	0.45
Araneae033	Dictynidae	*Cicurina*	*brevis*	10	3	7	4	40%	N	Y	0.97	230	1.36	8	0.80

and southern representatives. In *B. bishopi*, there is no haplotype sharing among areas, but deeper branches of its tree loosely reflect geographic proximity.

Species exhibit a broad range of divergence times in the region, as estimated by Bayesian divergence-rate calibrated trees (Table 2, Supplemental Fig. S1, online at https://doi.org/10.1636/JoA-S-24-007.s3;). At the oldest extreme, the haplotypes present in regional *Antrodiaetus* spp. would represent a minimum of approximately 13MY (similar to estimates in Hendrixson & Bond 2007). Even restricting this to lineages of either *A. unicolor* or *A. microunicolor* lowers it only to 11.6MY or 8.5MY, respectively. Divergences in araneomorph lineages would appear considerably younger than this, the oldest splits there being found in the theridiid *Robertus* sp. (which morphology suggests are actually multiple species) and the agelenid *Wadotes* sp. (#024), both dated at a little over 5MY. Most remaining intraspecific diversities are dated no older than 3MY, with many under 1MY. Even some linyphiid species exhibiting high haplotype diversities may have generated this diversity over surprisingly short timeframes. The 17 haplotypes of *Ceratinops carolinus* are dated to have arisen over 0.77MY, while the 12 haplotypes of *Collinsia oxypaederotipus* are dated to only 0.29MY.

Historical demographic analyses with Bayesian Skyline Plots show no common clear trend among the analyzed species (Supplemental Fig. S1), although many showed signs of stable population sizes or moderate demographic expansions during the late Pleistocene. Several among them suffered weak to moderate demographic contractions in relatively recent times, since the last glacial maximum [e.g., *Ceraticelus fissiceps, Ceraticelus laetus* (O. Pickard-Cambridge, 1874)*, Collinsia oxypaederotipus*]. A few showed signs of moderate (*Bathyphantes bishopi*) to strong demographic expansion since the late Pleistocene (*Ceratinops carolinus*, *Tenuiphantes zebra*).

DISCUSSION

The spruce-fir forests of southern Appalachia are generally associated with high levels of endemism in arthropods (Caterino & Recuero 2024), including mygalomorph spiders like *Microhexura montivaga* (Hedin et al. 2015) and *Antrodiaetus* (Hendrixson & Bond 2005a, b). However, this is not the case in most araneomorph spiders found in this restricted, high-elevation habitat. Most araneomorphs that are found in spruce-fir show lower phylogeographic restriction, with lower population subdivision and frequent sharing of haplotypes among peaks and ranges. This suggests a few possible explanations. One, it is possible that the species we examined utilize a broader range of habitat types and elevations and thus are less limited to particular sky islands. It is also possible that, despite some preference for high-elevation sites, they may be capable of dispersing among mountaintops and thereby preventing divergence into localized endemic species. Lastly, it is possible that the lack of subdivision we observed is indicative of insufficient time to sort ancestral diversity into more exclusive haplotype clades.

Some of the most common species in the region provide some context for weighing these alternatives. *Ceratinops carolinus* was one of the most abundant and widespread species we encountered in the spruce-fir environment. Yet it exhibited little population level structuring or phylogeographic pattern. Similar distributions and lack of structure were found in numerous other minute linyphiid spiders whose dispersal abilities might have been questionable, like

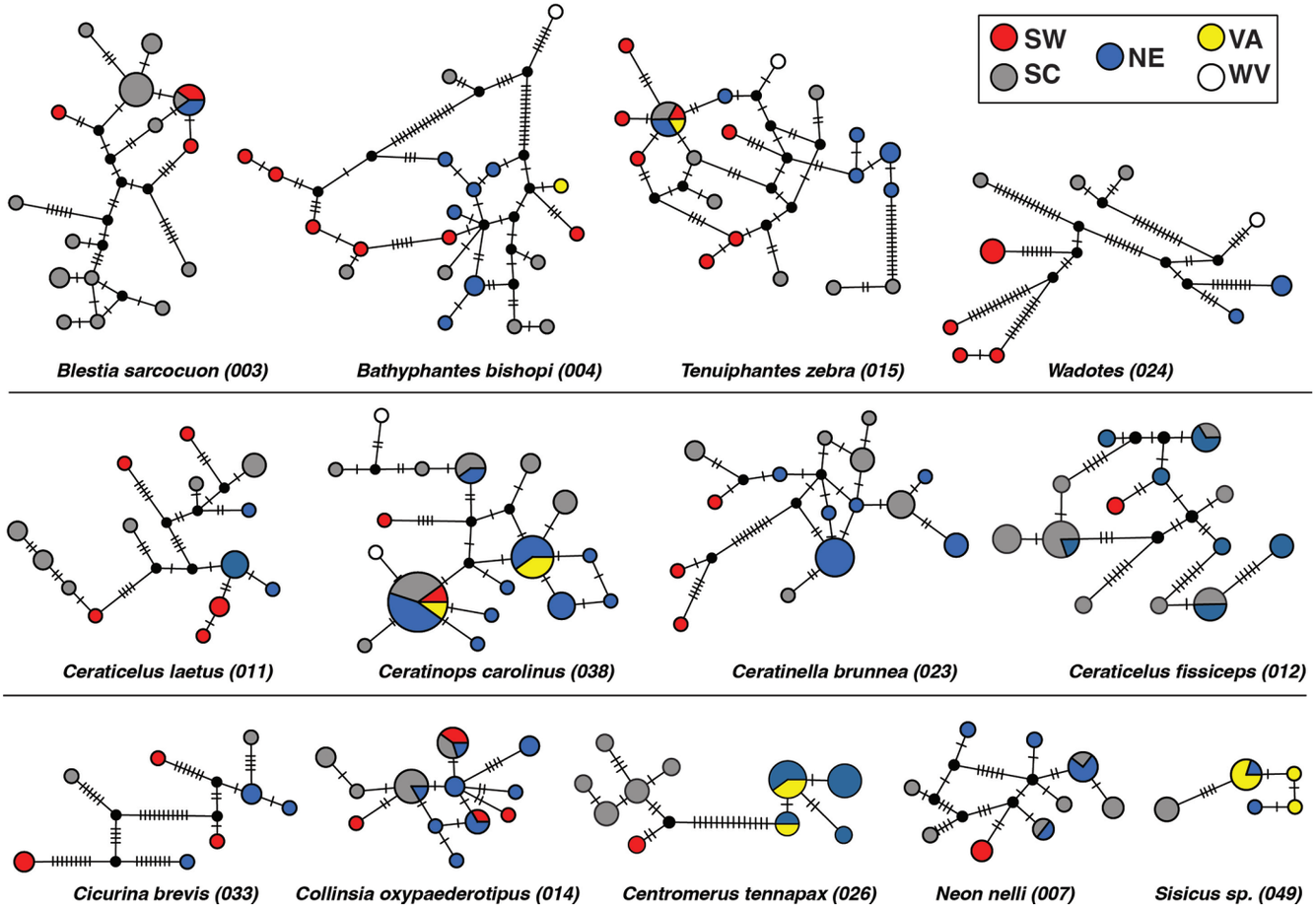

Figure 3.—TCS networks for all species occurring in spruce-fir forest that are represented by more than 10 individuals (see Table 2 for details).

Bathyphantes bishopi, *Ceraticelus fissiceps*, *Ceraticelus laetus*, and *Collinsia oxypaederotipus*. The salticid *Neon nelli* G. W. Peckham & E. G. Peckham, 1888 was not quite as abundant or widespread but showed a similar lack of structuring, with some haplotypes shared over rather broad areas.

Only a few species of araneomorphs studied here pose exceptions to the prevailing pattern of limited isolation, appearing not only restricted to spruce-fir habitats, but to have distinctly isolated populations. The clearest of these is *Sisicus* n. sp. 1 (049), which while found at high elevation sites on both sides of the FBR, does not share any haplotypes across it. One other perhaps similar example is found in *Centromerus 'tennapax'*, with distinct clades to either side of the FBR and a deep phylogenetic separation between them. However, even in these species, some haplotype sharing is observed among northeastern localities as distant as 120 km (Mount Mitchell, NC to Mount Rogers, VA). A few other species may represent spruce-fir endemics though sample sizes remain too small to have much certainty; the linyphiid *Dicymbium elongatum* (Emerton, 1882) is represented by five specimens from three sites. But these sites are quite disjunct, found in the Smokies in the west and the Grayson Highlands of southern Virginia, suggesting it was missed at intervening localities. *Cicurina breviaria* Bishop & Crosby, 1926 (Cicurinidae) was found at three sites in two neighboring ranges (Smokies and Plott Balsams), but again, with only five individuals sampled it reveals relatively little. All other putative species so far found only in spruce-fir are known from only one or two specimens, many of them singletons. Further focused sampling will be needed to determine their broader distributions and associations.

During Pleistocene climatic fluctuations, when populations were presumably being pushed up and down in elevation, and north and south in latitude, one might have expected repeated bottlenecks to have pruned intraspecific diversity over these periods. It may be that the dispersal abilities of these species were sufficient to have overcome some of these pressures as well, moving readily rather than suffering high levels of local extinction. Most (if not all, pending further taxonomic work) of the observed intraspecific genetic diversity among the studied species likely originated during the Pleistocene in the last million years, which could indicate that major colonization events occurred once global temperatures had become colder, allowing the expansion of ranges and population sizes of cool-temperate species. In general, historical demography indicates stable populations for most taxa, which is indicative of homogenous ecological conditions through time. This, together with the potential for relatively long-distance dispersal through ballooning, might have allowed for the maintenance of high levels of genetic diversity. Even if Pleistocene glacial cycles may have triggered range and demographic

shifts, the overall lack of phylogeographic structure suggests that those changes must have been shallow, leaving only the faintest traces in the extant diversity, with only a few exceptions as discussed further below. In cases where the observed diversity predates the Pleistocene, as in the case of *Antrodiaetus* or *Wadotes* Chamberlin, 1925 (Agelenidae) ('sp #024'), it is possible that old speciation processes and competitive exclusion processes may have played a role in the observed diversity patterns. A thorough taxonomic revision would be necessary to determine the real number of species in these groups, to assess their precise distributions, and to permit further examination of these scenarios.

Despite predominantly low phylogeographic structure across araneomorphs, results from several species did reveal subdivisions sufficiently deep or geographically restricted to hypothesize that they have remained largely in place and undergone cryptic speciation over this time frame. In *Blestia sarcocuon*, a basal split separates a precinctive lineage in the Smokies from a more widespread one (including sympatric representatives in parts of the Smokies). In *Bathyphantes bishopi*, a deeply divergent lineage of two disjunct individuals (one from the Smokies, one from West Virginia) appears isolated from a more abundantly represented lineage that covers much of the intervening area. In *Ceratinella brunnea*, we observed a similarly divergent lineage from western North Carolina and northern Georgia as sister to a more widespread (and partly sympatric) one. *Sisicus* n. sp. 1 (049) shows a deep split across the FBR, with one lineage (a single haplotype) in the Smokies and Plott Balsams, and another ranging from the Roan Highlands and Grandfather Mt. to the Grayson Highlands of Virginia. The northernmost of these in Grayson Highlands appears to be a new species, but it remains unclear if the southwestern lineage might represent yet another.

There are very few comparable studies of araneomorph spiders in sky-island systems with which to contrast our results. Masta's (2000) work on the salticid *Habronattus pugillis* Griswold, 1987 in the Madrean sky islands provides perhaps the best, coming from similar latitudes in North America, and involving similar historical environmental regimes. She also found somewhat complex patterns, with some indication of isolation, and even morphological differentiation over post-Pleistocene time frames (last 10,000 years). Most divergences, however, were estimated to be much older. Many mountain ranges (topographically connected clusters of proximate peaks) did not exhibit predicted patterns of monophyly, comparable to patterns in our more widespread species. While she considered dispersal a potentially responsible factor, the results in that system pointed more strongly to retention of ancestral polymorphism as the most important explanation, in part, however, because *H. pugillis* was not suspected to balloon.

Our results offer an opportunity to examine the accuracy of ASAP (or mPTP) species delimitations relative to 'true' morphological species. In the linyphiid *Tenuiphantes* Saaristo & Tanasevitch, 1996 two morphological species, *T. zebra* (widespread) and *T. sabulosus* (Keyserling, 1886) (only represented by individuals from Sassafras Mt., SC), appear to have been lumped as one, by both ASAP and mPTP delimitation methods. The two have been considered closely related but with consistent differences in male palpal morphology (Zorsch 1937). A deep basal split in *Centromerus tennapax* probably corresponds to a split between *C. tennapax* (described from and in our sampling largely restricted to the Great Smoky Mountains) and *C. cornupalpis* (O. Pickard-Cambridge, 1875), which is reported mainly from northern areas, and in our sampling is restricted to areas east of the FBR. Existing literature (e.g., van Helsdingen 1973) has these species overlapping in western North Carolina, so the clean split we observed may be due only to insufficient sampling. However, we have also detected a third morphotype among the northeastern examples, and there may be still more species represented. The '*Robertus* sp.' (048) reported here apparently includes three named theridiid species – *R. frontatus* (Banks, 1892) from Browning Knob (Plott Balsams), *R. pumilus* (Emerton, 1909) from Roan Highlands, and *R. riparius* (Keyserling, 1886) from the Roan Highlands, Black Mountains, and Grayson Highlands. A potential fourth, undescribed species near *R. riparius* was also seen from Roan High Bluff (though not sequenced). A closer look at their morphology may reveal them to have been over split by morphology, or, alternatively, that COI is not a suitable marker to separate them.

Species level splits in other noticeably structured ASAP species may indeed correspond to multiple species, to varying degrees of crypticity. The species *Blestia sarcocuon* exhibits some morphological diversity within the species as delimited by ASAP; considerable variation in the clypeal protuberance and tibial apophyses of mature males has been observed by previous authors (Crosby & Bishop 1927; Millidge 1993), as well as by us. Although the morphological variation does not correspond well to patterns of COI variation discovered here, it should be explored further in the context of additional geographic sampling and molecular markers. A lot of taxonomic work regarding Appalachian spiders remains to be done, including many undescribed yet morphologically divergent species, and likely a number of cryptic taxa as well, as has been increasingly observed across various invertebrate organisms (e.g., Nalepa et al. 2017; Garrick et al. 2018; Newton et al. 2020; Dukes et al. 2022).

At the most general level, spruce-fir sites host slightly lower total spider diversity than non-spruce fir sites. Of the 10 most species-rich sites (of 33 total), only one, Grandfather Mountain, is a spruce-fir dominated peak. The rest are either slightly lower (with the exception of Black Balsam Knob, which approaches 2000 m, but hosts an open shrubby vegetation) or are dominated by deciduous vegetation. These richest sites are represented by 15–19 litter-dwelling spider species in our sampling. On the other end of the richness spectrum, 8 of the 10 least species-rich sites (9 or fewer litter-dwelling spider species) are spruce-fir dominated peaks. Examining whether this relates to environmental factors at these sites, or a measure of prey availability, or some other factors would be a worthwhile future study. The diversity of leaf litter sources (trees and shrubs) in these forest types, and niche diversity generally, would seem to be lower at the highest elevations. Our collections were made mostly in predominantly dense mats of coniferous needles, and there was relatively little riparian or wet habitat, limiting the diversity of habitat specialists that might be present.

In conclusion, our results here reveal a complex picture. The majority of araneomorph species that live in the higher elevations of southern Appalachia do not show great fidelity to the spruce-fir habitat and exhibit relatively low degrees of phylogeographic structuring across their ranges. This is most likely attributable to some combination of large and stable populations being slow to sort ancestral polymorphism, and the ballooning behavior of immatures of most of these species. Examination of phylogeographic patterns in light of explicit data on population sizes (current and historical) may help determine whether connectedness or haplotype retention is more likely responsible. But the sharing

of identical, not merely related, haplotypes seems to be reasonably strong evidence that at least some dispersal is involved.

What is less clear is what accounts for those few species or lineages (e.g., *Sisicus* Bishop & Crosby, 1938, *Wadotes*, *Blestia* Millidge, 1993) that do show significant patterns of isolation and subdivision. Are they less prone to ballooning, and was such a behavior perhaps selected against by more particular microhabitat requirements? The dense needle litter in these forests is itself unusual in the region and the moist cool climatic conditions found on these peaks are otherwise rare. Such questions remain to be addressed. The rather high degree of intraspecific mitochondrial variation in many of these species, structured or not, is also noteworthy. Haplotype diversity is quite high in several lineages that may have diverged from conspecifics only a few hundred thousand years ago. High and stable effective population sizes without significant bottlenecks through Pleistocene fluctuations may also account for some of this, as inferred for many of the species examined here. Such a hypothesis bodes well for these species' adaptability to anthropogenic climatic shifts. As such, they would be well worth closer attention as we monitor the status of these vulnerable habitats into the future.

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SUPPLEMENTAL MATERIALS

Supplemental Table S1.—All specimens sequenced for this study, with identifications, identifiers, morphospecies codes, DNA extraction numbers, voucher codes, GenBank accession numbers, localities, and dates. Morphospecies codes (column B) in green text indicate those specimens collected in spruce-fir dominated sites. Names in red text (columns D, E) indicate identifications inconsistent with the ASAP species delineation purporting to delineate one species. Yellow highlighting indicates those that we consider to represent likely contaminants (i.e., morphological identifications are very inconsistent with the prevailing species delimitation). Available online at https://doi.org/10.1636/JoA-S-24-007.s1

Supplemental Table S2.—All ASAP-delimited species with at least one occurrence in spruce-fir forest. N=total specimens sampled. '# Peaks' indicates how many high elevation spruce-fir localities the species was collected from; '# Ranges' indicates how many separate mountain ranges (see methods) species were found in; '# spruce-fir' and '% spruce-fir' indicate how many total individuals and what percentage of total individuals were found at high elevation spruce-fir sites; 'spruce-fir only?' indicates whether the species was only found at spruce-fir sites; 'sites span FBR' indicates whether species records span the French Broad River valley; 'θ_{st}' is provided for two-group AMOVA; 'max range (km)' indicates maximum linear distance between collecting sites; 'Min. age' is the age of the deepest split in the Bayesian tree as estimated by BEAST; and 'Haps' and 'Haps/Ind.' indicate the total number of haplotypes and the number relative to total N. Available online at https://doi.org/10.1636/JoA-S-24-007.s2

Supplemental Figure S1.—Bayesian divergence-rate calibrated trees (with 95% HPD error bars in light blue) and Bayesian skyline plots for all species. Bayesian trees: colored taxon names refer to geographic regions (light red = SW, gray = SC, blue = NE, golden yellow = VA, uncolored = WV). Bayesian skyline plots represent the estimated effective population (y-axis) size through time (x-axis) (shaded area corresponds to the 95% HPD) for all species. Available online at https://doi.org/10.1636/JoA-S-24-007.s3.

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