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SHORT COMMUNICATION

Cryptic diversity in the southern Appalachian Mountains: genetic data reveal that the red centipede, *Scolopocryptops sexspinosus*, is a species complex

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Abstract
Invertebrates can often persist in habitat patches too small to support larger, more mobile taxa, and as such, they may be strong predictors of conservation priorities for vertebrates. In the southeastern United States, the southern Appalachian Mountains are a well-known center of endemism for amphibians, and recent work has also uncovered extensive cryptic diversity in dispersal-limited forest invertebrate species. Here, intensive geographic and genetic sampling of a widely distributed centipede, *Scolopocryptops sexspinosus* [84 individuals from 42 sites across seven states, assayed for DNA sequence variation at a mitochondrial (632-bp) and nuclear (879-bp) locus] revealed two deeply divergent well-supported major clades (lineages A and B). Notably, the spatial distribution of each genetic lineage was largely geographically cohesive and broadly parapatric but with large areas of allopatry, and levels of mitochondrial divergence between lineages are comparable to that seen between named species within the genus. Indeed, no nuclear alleles are shared despite opportunities for interbreeding at locations where these lineages occur in close proximity. This study provides a foundation for follow-up work focusing on the description of species within the *S. sexspinosus* complex, and importantly, contributes to a growing body of research that identifies a high incidence of cryptic diversity in the southern Appalachian Mountains.

Keywords Appalachian Mountains · Conservation priorities · Cryptic diversity · DNA sequences · Forest invertebrate · Species complex

Introduction

The spatial distribution of community- and population-level biodiversity in dispersal-limited species has the potential to offer fine-scale information for the identification of biogeographically unique areas that warrant protection (Moritz 2002). Compared to more mobile vertebrate taxa, flightless invertebrates can persist in small habitat patches, and may therefore more readily retain signatures of past habitat fragmentation and/or long-term in situ isolation (Sunnucks et al. 2006). Furthermore, unlike vascular plants with wind-mediated pollen or seed dispersal, colonization of new areas by flightless invertebrates may be strongly limited by local landscape features (e.g., deep ravines and exposed ridgelines, or river and stream networks that subdivide catchments; Harvey 2002; Garrick et al. 2004). In montane forest regions, several studies have highlighted the value of invertebrate-based biodiversity metrics for efficiently capturing spatial patterning that is evident from co-distributed taxa (Hodkinson and Jackson 2005, and references therein). For example, in the Southern Hemisphere, community-level analyses of vertebrate and vascular plant biodiversity in the Australian Wet Tropics were found to be relatively poor surrogates for that of invertebrates, but conversely, invertebrates were strong predictors of conservation priorities for other biota (Moritz et al. 2001). Furthermore, in the same study region, population genetic data showed that the phylogeographic patterns exhibited by an ecologically-specialized low-mobility land snail represented a composite of patterns seen in lizards and frogs (Hugall et al. 2002). These findings also extend to temperate forests in southeastern Australia,

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where similar community- and population-level conclusions have been reached for ground-dwelling arthropods (Ferrier et al. 1999) and dead-wood-dependent invertebrates (Garrick et al. 2012a and references therein; Bull et al. 2013). To date, however, very few analogous comparative studies have been conducted in montane temperate forests in North America.

In the southeastern United States, the southern Appalachian Mountains are a well-known center of endemism for Plethodontid salamanders (Petranka 1998) and other amphibians (Rissler and Smith 2010), as well as vascular plants (Estill and Cruzan 2001). Emerging work is also revealing short-range endemism in other groups such as crayfish (Crandall and Buhay 2008), arachnids (Hedin and McCormack 2017 and references therein) and millipedes (Marek 2010). Indeed, owing to their topographic complexity, steep environmental gradients, and paleoclimatic history as unglaciated refuge area (Garrick 2011 and references therein), the southern Appalachian Mountains are expected to harbor high levels of as-yet unknown biodiversity in other invertebrate taxa (Carlton and Bayless 2007). As part of an on-going research program focusing on population- and community-level biodiversity in the region, we have sampled dead-wood-associated invertebrates (Garrick 2016, 2017; Garrick et al. 2015, 2017). One species of particular interest for understanding organismal responses to past environmental change is the red centipede, *Scolopocryptops sexspinosus* Say, and accordingly, our genetic and geographic sampling of this taxon has been intensive.

Briefly, *S. sexspinosus* is a large (up to ~7 cm long), venomous centipede that is broadly distributed across the eastern United States (i.e., longitudinally from eastern Texas to the Atlantic Coast, and latitudinally from the Gulf of Mexico up to the lower reaches of the Great Lakes), and its range also extends into the Niagara Region of Ontario, Canada (Shelley 2002). Members of the species are blind (i.e., lack ocelli) generalist predators that often feed on insects, spiders and worms, as well as other centipedes (Auerbach 1951). Individuals are usually found within the decomposing heartwood and under the bark of rotting logs, but during the breeding season and after periods of prolonged rainfall they exhibit high levels of surface activity (Auerbach 1951). They can rapidly move through leaf litter while maintaining crypsis (RC Garrick pers. obs.), so dispersal over moderate distances seems possible. However, densities are considerably reduced at forest edges (Hickerson et al. 2005), such that movement among fragmented habitat patches may be limited.

In this short communication, we present evidence that strongly indicates that the taxon *S. sexspinosus* contains cryptic species. Although many alternative species concepts exist (e.g., De Queiroz 2007, and references therein), we apply the phylogenetic species concept because these evolutionary units are spatially and temporally discrete and directly comparable across different organismal groups (Cracraft 1987). Also, in the context of conservation, it is the integrity of distinct lineages with unique historical evolutionarily trajectories that are the primary targets for protection (Garrick et al. 2012b). Our inference about the existence of cryptic species is underpinned by DNA sequence data from a maternally-inherited haploid mitochondrial locus and a biparentally-inherited diploid nuclear autosomal locus, obtained from large sample of individuals that were unambiguously identified as *S. sexspinosus* based on diagnostic morphological characters. Our goals are to (1) highlight the existence of cryptic diversity in this taxon and provide a foundation for follow-up work that focuses on formally describing species within the complex, and (2) place the present discovery within the broader context of a growing body of research that suggests invertebrate biodiversity in the southern Appalachian Mountains may be exceptionally high.

Methods

Sampling

From 2012 to 2017, centipedes were sampled from intermediate- to late-stage rotting logs along a northeast-southwest oriented ~970 km-long transect, covering all structurally continuous forest in the montane regions (and surrounding areas) of the southern Appalachians. Sampling was conducted under scientific collecting permits issued by the Alabama Department of Conservation and Natural Resources, Georgia Department of Natural Resources (permit # 29-WBH-12-16), United States Department of Agriculture Forest Service, and United States National Park Service (permit # GRSM-2012-SCI-2242, SHEN-2012-SCI-0015, and CUGA-2012-SCI-0008). All specimens were geo-referenced, and subsequently keyed to species following Shelley (1987, 2002).

Molecular data

Genomic DNA was extracted from 4 to 8 *S. sexspinosus* legs using a DNeasy Blood and Tissue Kit (Qiagen, Valencia CA, USA), following the manufacturer's recommendations. For each individual, a fragment of the mitochondrial (mtDNA) cytochrome oxidase subunit I (COI) gene was amplified via polymerase chain reaction (PCR) using primers LCO-1490 and HCO-2198 (Folmer et al. 1994), and a fragment of the nuclear RNA Polymerase II (RNP2) gene was amplified using primers developed here (SsRNP2-F2: 5′-CACATC AACACTCGAATCC-3′, SsRNP2-R2: 5′-TTGCGGTCA AGTTCGATACGC-3′). Amplifications were performed in 10 µL volumes (or multiples thereof) comprised of 2.0

µL 5 × PCR buffer (Promega, Madison WI, USA), 0.8 µL $MgCl_2$ (25 mM, Promega), 1.6 µL dNTPs (1.25 mM, Promega), 0.5 µL Bovine Serum Albumin (10 mg/mL, New England BioLabs, Ipswich, MA, USA), 3.0 µL dH_2O, 0.5 µL of each primer, 0.1 µL Go-Taq (5 U/µL, Promega), and 1.0 µL of genomic DNA. The following PCR profile was used for both genes: 95 °C for 2 min (1 cycle), 95 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min (35 cycles), and a final extension of 72 °C for 2 min (1 cycle). Amplified products were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA, USA), then sequenced on an Applied Biosystems 3730x Genetic Analyzer at Yale University's DNA Analysis Facility on Science Hill. Sequences were edited and aligned in MEGA v6.06 (Tamura et al. 2013). RNP2 sequences with heterozygous sites were coded using IUPAC ambiguity characters; following Gifford and Larson (2008), a site was considered heterozygous if the secondary peak was > 25% of the amplitude of the primary peak. Gametic phase of segregating sites in multi-site heterozygotes was then inferred using DNASP v5.10 (Librado and Rozas 2009), using search settings described by Garrick et al. (2010). DNA sequences and alignments are available from GenBank under the following Accession Numbers: MH680589–MH680622 (COI), and MH680623–MH680669 (RNP2).

Analyses

Phylogenetic relationships among non-redundant DNA sequence haplotypes sampled from *S. sexspinosus* individuals were estimated separately for each gene, using maximum-likelihood, implemented in MEGA. For each locus, we used the best-fit model of nucleotide substitution identified using jModelTest 2 (Darriba et al. 2012), empirical base frequencies, a maximum parsimony starting tree, extensive subtree-pruning-regrafting branch swapping, with node support assessed using 1000 bootstrap replicates. Next, the geographic distributions of deeply divergent well-supported clades were mapped, and DNA sequence-based differences between them were characterized using several summary statistics, calculated in DNASP. These included F_{ST} (Hudson et al. 1992), the number fixed nucleotide differences between groups, and a compound genetic diversity-based statistic, Nei and Li's (1979) net nucleotide differences between groups (π_{net}). Given two groups, A and B, π_{net} is calculated as $\pi_{AB} - ((\pi_A + \pi_B)/2)$, where π is the average number of nucleotide differences between two randomly chosen sequences. Finally, to provide context for interpreting the magnitude of genetic differences detected between genetically divergent lineages of *S. sexspinosus* sampled from the southern Appalachian Mountains, we quantified typical levels of intra- and interspecific sequence divergence for *Scolopocryptops* species. To do this, all non-redundant mitochondrial COI sequences from named members of the genus were retrieved from GenBank (http://www.ncbi.nlm.nih.gov; accessed 10 July 2018), aligned, trimmed, and then pairwise uncorrected *p*-distances calculated in MEGA were summarized as a frequency distribution. Corresponding mean *p*-distances between genetic groups identified in the present study were also calculated and plotted on the same set of axes.

Results and discussion

Eighty-four individuals were collected from 51 rotting logs across 42 sampling sites spanning seven states (Fig. 1; Online Resource 1), and adult specimens were clearly identified as *S. sexspinosus* based on diagnostic morphological characters described by Shelley (1987, 2002). The mitochondrial COI sequences generated here (632-bp alignment, 81 individuals) had characteristics of "true" arthropod mtDNA (cf. nuclear pseudogenes), such as open reading frames when translated to amino acids, and A + T-biased nucleotide composition (Sunnucks and Hales 1996). As a group, *S. sexspinosus* samples had 34 unique mtDNA haplotypes, with 146 variable sites. Nuclear RNP2 sequences (879-bp alignment, 74 individuals) spanned coding and non-coding (intron) regions, and this dataset contained 47 unique allele haplotypes and 122 variable sites excluding alignment gaps. The best models of nucleotide substitution for COI and RNP2 were HKY + I + G and GTR + I + G, respectively.

Molecular phylogenetic analyses of each gene region consistently identified two deeply divergent well-supported major clades (lineages A and B herein; Fig. 2), each containing individuals that were unambiguously morphologically identified as *S. sexspinosus* according to Shelley's (1987, 2002) keys. Notwithstanding some missing data for 13 individuals (i.e., three individuals without COI sequences, and ten individuals without RNP2 data; Online Resource 1) due to some PCR amplifications that failed repeatedly despite good quality DNA, the maximum-likelihood trees for each gene showed full concordance with respect to the membership of individuals in each group. That is, of the 72 individuals with a complete two-locus sequence dataset, there was 100% agreement between loci regarding lineage assignment (however, we do acknowledge the possibility that instances of discordance may exist, but have not yet been sampled). Overall, lineage A contained 64 individuals from 27 sites and lineage B had 20 members from 15 sites (21 vs. 13 COI haplotypes respectively; 29 vs. 18 RNP2 haplotypes respectively; Fig. 1; Online Resource 1). Furthermore, the spatial distribution of each genetic lineage was largely geographically cohesive; relative to one another, they appear to be broadly parapatric. However, there are clearly large zones of allopatry, with spatial subdivision between the two lineages coinciding with the orientation of major ridgelines (i.e.,

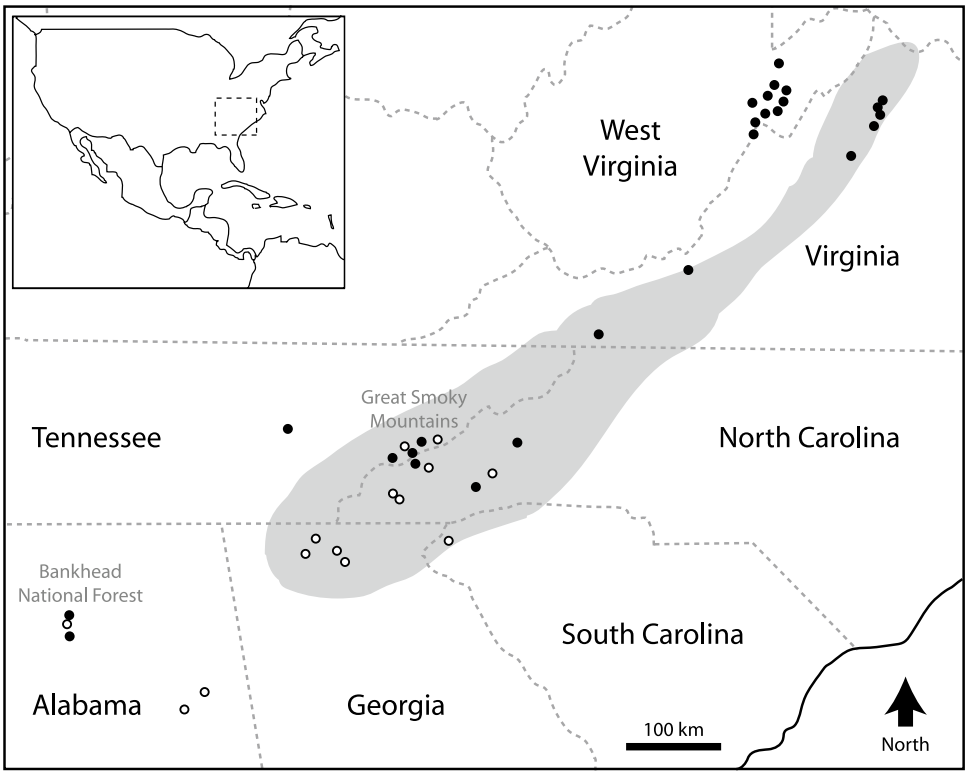

Fig. 1 Map showing the spatial distribution of 42 *Scolopocryptops sexspinosus* sampling sites in the southern Appalachian Mountains. Different symbols for each distinct genetic lineage are used to denote their spatial distributions (lineage A, solid circles; lineage B, open circles; also see Online Resource 1). Grey shading indicates the location of the central mountainous Blue Ridge physiographic subregion, with many high-elevation peaks > 1800 m. Inset: map showing location of study area within the continental United States (dashed black box)

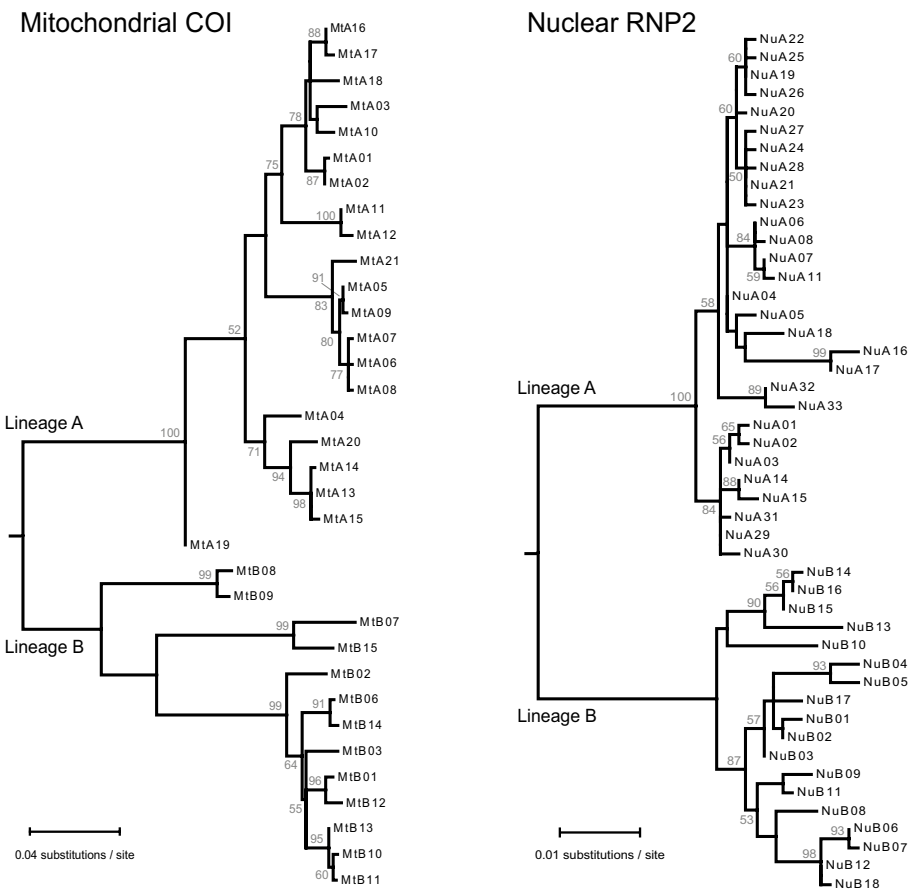

Fig. 2 Midpoint-rooted Maximum likelihood phylogenetic trees showing relationships among non-redundant mitochondrial COI (left) and nuclear RNP2 (right) haplotypes identified from southern Appalachian *Scolopocryptops sexspinosus* samples. Numbers on nodes are bootstrap values (only those ≥ 50% are shown). Names of unique haplotypes correspond with those in Online Resource 1 (but note that the numbering system used for haplotype names is arbitrary)

running approximately northeast-southwest) in the southern Appalachian Mountains (Fig. 1). The narrow zones of apparent parapatry occur in both Bankhead National Forest, Alabama, and in the Great Smoky Mountains National Park on the Tennessee/North Carolina border. Although our sampling indicates microallopary at the local scale given that members of the two lineages did not co-occur in the same rotting logs, they were nonetheless sampled just 200 m and 4.5 km apart in the two aforementioned regions, respectively. In the Great Smoky Mountains National Park, similar spatial-genetic patterns have been reported for the *Cryptocercus punctulatus* wood-feeding cockroach species complex, which is syntopic with *S. sexspinosus*. In the wood roach, different chromosomal races/divergent mtDNA clades have interdigitated distributions in the Great Smoky Mountains, but elsewhere, they are broadly allopatric (Everaerts et al. 2008; Garrick et al. 2017; Nalepa et al. 2017). Furthermore, Jones and Weisrock (2018) recently reported that genome-wide sequence data had uncovered a pair of cryptic lineages within a clade of *Desmognathus* spp. salamanders—each containing the same two morphotypes—with an abrupt geographic transition between them in close proximity to the Great Smoky Mountains. Indeed, the possibility that this geographic area represents a suture zone demands further investigation.

In the present study, based on a 632-bp alignment, mitochondrial COI sequences showed seven fixed nucleotide differences (five transitions and two transversions, all 3rd position synonymous substitutions) between the two lineages (mean uncorrected *p*-distance = 0.11, range 0.08–0.13,). Although Burnside et al. (1999) described and named four lineages of the southern Appalachian *C. punctulatus* wood roach complex as separate species based on seven mtDNA nucleotide characters from non-coding 12S and 16S rRNA genes (subsequently reduced to four characters by Steinmiller et al. 2001), we prefer an approach based on cross-validation, using multiple independent datasets. Indeed, the phylogenetic species concept (see Introduction) is underpinned by the notion that an estimated phylogeny represents organismal (cf. gene-specific) evolutionary history. Other measures of mtDNA genetic differentiation also indicated a deep divergence ($F_{ST} = 0.61$, $\pi_{net} = 31.41$). A coarse understanding of typical levels of mitochondrial COI divergence for *Scolopocryptops* species was based on 25 intraspecific and 54 interspecific comparisons, using COI data from GenBank (Online Resource 2), aligned and trimmed down to 588-bp to minimize missing data. Notwithstanding the limitations associated with using sequence similarity to recognize species boundaries (Cognato 2006), the level of mtDNA divergence between the two *S. sexspinosus* lineages is comparable to that seen between named species within the genus (albeit on the lower end of the frequency distribution; Fig. 3). However, the most compelling evidence

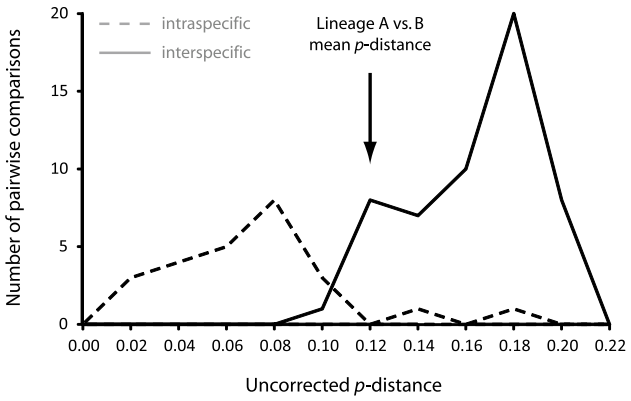

Fig. 3 Frequency distributions of mitochondrial COI divergence showing typical intra- and interspecific differences for *Scolopocryptops* species based on GenBank accessions (see Online Resource 2). For comparison, corresponding COI divergence between *S. sexspinosus* lineages A versus B (mean uncorrected *p*-distance = 0.11) based on empirical data generated in this study is also plotted

that the taxon is a species complex comes from the nuclear RNP2 sequences (22 fixed differences, 16 transitions and six transversions; $F_{ST} = 0.62$, $\pi_{net} = 37.46$), given the biparental mode of inheritance and larger genetically effective population size (and thus longer coalescence times) of this locus relative to mtDNA (Palumbi et al. 2001). Indeed, despite the potential opportunities for interbreeding at locations where the two mtDNA clades occur in close proximity, no RNP2 allele haplotypes are shared by their members. Our inference that *S. sexspinosus* is a species complex is not without precedent—the southern Appalachian Mountains harbor many morphologically cryptic species complexes of forest arthropods. For example, genetic data have revealed divergent mtDNA-based phylogeographic lineages (i.e., with as-yet undermined taxonomic status) within the *Narceus americanus* millipede species complex (Walker et al. 2009), the opilion *Fumontana deprehendor* (Thomas and Hedin 2008), and several *Hypochilus* spp. spiders (Hedin and Wood 2002; Keith and Hedin 2012). In other taxa, the combination of mitochondrial and nuclear genetic data confirmed the existence of multiple species nested within a single named taxon [e.g., the opilion *Sabacon cavicolens* (Hedin and McCormack 2017), and the spider *Antrodiaetus unicolor* (Hendrixson and Bond 2005)].

Overall, our study shows that the taxonomy of *S. sexspinosus* warrants re-evaluation, and that the integration of multiple data types (e.g., distributions, genetics and ecology; Ulyshen et al. 2017) will likely be necessary for formal description of species within the complex. High-resolution imaging such as microCT scanning of whole-organism phenotypes or specific anatomical features (sometimes coupled with scanning electron microscopy of genitalia or other reproductive structures) is an emerging tool that can help

resolve the taxonomy of complexes that contain cryptic species (Akkari et al. 2015; Sarnat et al. 2016), and may be helpful in the present case. Other information that could be used to uncover a suite of characters for diagnosis includes cuticular hydrocarbon profiles (e.g., Everaerts et al. 2008), quantitative comparisons of environmental niche models (e.g., Edwards et al. 2014) and DNA sequence data from additional loci (e.g., Jarman et al. 2002). More broadly, the present paper contributes to a growing body of research that identifies a high incidence of cryptic diversity in the southern Appalachian Mountains—particularly in ecologically-specialized dispersal limited invertebrates. This suggests that the predominant focus on vertebrates and vascular plants in conservation research and planning is likely to result in management strategies that fail to cater to a large proportion of biodiversity (Hugall et al. 2002).

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Compliance with ethical standards

Conflict of interest There are no conflicts of interest between the authors, the institution and fund donors.

Ethical approval The paper has been prepared and submitted in accordance with the ethical standards of the Committee on Publication Ethics. Specimens were obtained under scientific collecting permits, and no ethical permits were required.

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