Received: 21 September 2023 | Revised: 20 March 2024 | Accepted: 20 April 2024

DOI: 10.1111/zsc.12661

Zoologica Scripta | WILEY

ORIGINAL ARTICLE

Hidden diversity in eastern North America: The genus *Ligidium* (Oniscidea, Ligiidae) in the southern Appalachian Mountains

Ernesto Recuero | **Michael S. Caterino**

Department of Plant & Environmental Sciences, Clemson University, Clemson, South Carolina, USA

Correspondence
Ernesto Recuero, Department of Plant & Environmental Sciences, Clemson University, 277 Poole Agricultural Center, Clemson, SC 29634-0310, USA.
Email: ernestorecuerogil@gmail.com

Funding information
Clemson University Experiment Station, Grant/Award Number: SC-1700596; National Science Foundation, Grant/Award Number: DEB-1916263

Abstract

The terrestrial isopod genus *Ligidium* includes 58 species from Europe, Asia, and North America. In Eastern North America four species are recognized: *L. floridanum* and *L. mucronatum*, known just from their type localities in Florida and Louisiana respectively, *L. blueridgensis*, endemic to the southern Appalachians, and *L. elrodii*, widespread from Georgia to Ontario. The genus shows a marked morphological conservatism, and species are differentiated mostly by small morphological differences; it is not always easy to determine if such variability represents inter- or intraspecific variation. Here, we explore the diversity of *Ligidium* from the southern Appalachian Mountains, exploring the congruence of morphologically defined groups with multilocus phylogenetic reconstructions and molecular species delimitation methods. We have studied a total of 130 specimens from 37 localities, mostly from the southern Appalachians, and analysed mtDNA (Cox1) and nuclear (28S, NaK) sequences. Morphologically, we recognized eight morphotypes, most of them assignable to current concepts of *L. elrodii* and *L. blueridgensis*. Phylogenetic analyses supported the evolutionary independence of all morphotypes, and suggest the existence of 8–9 species, including limited cryptic diversity. Single-locus delimitation analyses based on mtDNA data suggest the existence of a much higher number of species than the multilocus analyses. The estimated age of the ancestors of sampled lineages indicates a long presence of the genus in eastern North America and old speciation events through the Miocene. Our results indicate a higher diversity than previously thought among the *Ligidium* populations present in the southern Appalachian Mountains, with several species to be described.

KEYWORDS

Crustacea, cryptic species, endemism, native, phylogeography, species delimitation

1 | INTRODUCTION

Terrestrial isopods are one of the main detritivore animal groups in the soils of all continents except Antarctica; they represent, according to our current taxonomic knowledge, the most speciose of Isopod groups, with around 3700 named species living in all kinds of terrestrial habitats. However, this diversity is not uniformly distributed, with the highest diversity centered in some tropical regions and Palearctic areas such as the Mediterranean (Sfenthourakis & Taiti, 2015).

The diversity of Oniscidea in the Nearctic is relatively low when compared with other climatically similar regions as the western Palearctic. For instance, in North America, 109 species have been reported from the United States and Canada, and more than one-third of them are considered exotic taxa coming mostly from Europe (Jass & Klausmeier, 2000), while in Mexico 86 species are known, with around 11% being exotic forms (Jass & Klausmeier, 2006; Segura-Zarzosa et al., 2020). In contrast, in regions such as the Western Palearctic, diversity is much higher. For instance, Alexiou and Sfenthourakis (2013) listed 238 species just from Greece, of which 161 are endemic, while in Italy there are 367 species recorded, with a 60% endemicity (Taiti, 2017). Such differences are likely a consequence of the evolutionary history of the group, but perhaps also multiplied by geographic biases in the taxonomic work done throughout history.

Among the native North American species, 8 of them belong to the genus *Ligidium* Brandt, 1833 (Jass & Klausmeier, 2000). This genus has a patchy Holarctic distribution and includes 58 species from Europe, Asia, and North America (Schmalfuss, 2003; Wang et al., 2022). In western North America, the genus is present along the Pacific coast, with one widespread species and 3 Californian endemics. In eastern North America, it is represented by locally endemic species from Florida, *Ligidium floridanum* Schultz & Johnson, 1984, and Louisiana, *Ligidium mucronatum* Mulaik & Mulaik, 1942; one endemic to the southern Appalachians, *Ligidium blueridgensis* Schultz, 1964, and one widespread species, *Ligidium elrodii* (Packard, 1873), ranging from northern Georgia to Ontario. According to the current information only two of them, *L. elrodii* and *L. blueridgensis*, overlap in their distributional ranges, specifically in the southern Appalachian Mountains (Schultz, 1982).

Ligidium species present a marked morphological conservatism, and species are differentiated mostly by small differences in the apex of the elongated male pleopod 2 endopodite, the shape of male pleopod 1 and 2 exopodites, or the length of uropod exopodites and endopodites (Klossa-Kilia et al., 2006; Schultz, 1970; Sfenthourakis, 1993; Wang et al., 2022). Within the widespread *Ligidium elrodii*, some morphological variability has been described, which has led to the definition of several subspecies known only from their type localities: *L. e. chatoogaensis* Schultz, 1970, from Chattooga Co. in northwestern Georgia, *L. e. scottensis* Schultz, 1970 and *L. e. leensis* Schultz, 1970, from Scott Co. and Lee Co. respectively in southwestern Virginia, and *L. e. hancockensis* Schultz, 1970 from Hancock Co. in Northern Tennessee.

Molecular studies in different regions across the genus's range have shown unexpected levels of diversity, suggestive of the existence of species complexes and even cryptic species. In Greece, works using mtDNA markers indicated high interspecific and interpopulation genetic distances and, in some cases such as in *Ligidium beieri* Strouhal, 1928, a strong discordance between mtDNA lineages and morphological identification (Klossa-Kilia et al., 2005, 2006). The authors suggest that the observed high genetic differentiation may result from strict ecological specialization to very humid soil habitats, causing strong geographic isolation in a relatively dry region such as the Eastern Mediterranean. However similar patterns have been found among temperate, subtropical and montane climates in regions of Japan (Harigai et al., 2020, 2023; Yoshino & Kubota, 2022) and China, where several new species have been delimited with the help of molecular data (Li, 2017; Wang et al., 2022). Overall, this suggests that *Ligidium* species have limited dispersal capacities, favouring genetic lineage differentiation and eventually speciation, most likely by allopatric isolation coupled with ecological niche conservatism (Wiens, 2004).

Thus, widespread species such as *Ligidium elrodii* are excellent candidates to explore the distribution of hidden diversity, including local endemics and the existence of species complexes. Here, we explore the diversity of *Ligidium* from the southern Appalachian Mountains, exploring the congruence of morphologically defined groups with multilocus phylogenetic reconstructions and automated species delimitation using molecular data. Previous studies indicate that the diversity of many groups of small, flightless Arthropoda is underestimated in this region (e.g., Caterino & Recuero, 2024; Derkarabetian et al., 2022; Dukes et al., 2022; Hedin & Milne, 2023). Our main hypothesis is that the diversity of southern Appalachian *Ligidium* is higher than the two species currently reported, and that more than one species are hidden under a single name.

2 | MATERIALS AND METHODS

2.1 | Sampling

We have studied a total of 130 *Ligidium* specimens from 37 localities, mostly from the southern Appalachians in the

states of North Carolina, Tennessee, Georgia, and South Carolina, with two localities located in the Allegheny Mountains in West Virginia (Table 1). Sampling was performed mostly by leaf litter sifting and subsequent Berlese extraction. Litter samples were collected down to the soil surface at points where humidity was high, sifted through an 8 mm mesh, and taken to the lab in cloth bags. Berlese-extracted specimens were directly collected into absolute ethanol, and subsequently sorted and stored in absolute ethanol at −20°C. A few specimens were also collected by direct search in leaf litter or under logs and rocks. Studied material is currently deposited at the Clemson University Arthropod Collection (CUAC).

Samples were examined using an Olympus SZX7 stereomicroscope, and the general habitus of several specimens was photographed using a Nikon EOS 6D with a Tamron AF 1.4× teleconverter and a Canon MP-E 65 mm macro lens. For each sample 10 to 20 pictures were taken and stacked with a Visionary Digital Passport system and Helicon Focus software v.8.1.1 (HeliconSoft, Ukraine). Most male samples could be morphologically assigned to species following the character descriptions in Schultz (1964, 1970, 1982). Some specimens did not match any described species. Specifically, we revised the shape of male pleopod 2 endopodite for species identification, and considered it as a discrete character to define morphospecies and compare them with genetic lineages. Females and juveniles were identified based on males from the same locality or on their position in the phylogeny. All samples were georeferenced and mapped using QGIS v3.28 (available at http://www.qgis.org) (Figure 1).

2.2 | DNA extraction and sequencing

We extracted genomic DNA from one or two pereiopods from each specimen, digested with lysis buffer and proteinase K (Omega BioTek, Norcross, GA, USA). Most samples were extracted using Omega BioTek's MagBind HDQ Blood and Tissue kit, eluting with 150 µL elution buffer, on a Hamilton Microlab Star automated liquid handling system, which allows processing up to 96 samples in a single run. Some samples were extracted using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA), following standard protocol and eluting in 150 µL of molecular grade water. Both methods yielded high quality DNA extractions with similar concentrations.

Amplification with the polymerase chain reaction (PCR) of fragments of the mitochondrial gene cytochrome c oxidase subunit I (Cox1) was made using primers LCO-1490 and HCO-2198 (Folmer et al., 1994) or BF2 and BR2 (Elbrecht & Leese, 2017); the latter pair was indexed with 9 base pair (bp) tags (Meier et al., 2016) to allow multiplexed high-throughput sequencing as part of a larger megabarcoding project (Caterino & Recuero, 2024). For a subset of samples representing all main mtDNA lineages, we amplified fragments of the nuclear genes 28S ribosomal RNA (28S), using the primers 28Sa and 28Sb (Whiting et al., 1997), and the sodium-potassium ATPase α-subunit (NaK), using the primers NaK for-b and NaK Rev2 (Tsang et al., 2008). The PCR conditions were employed as described by Dukes et al. (2022), with annealing temperatures of 50°C for Cox1 and 28S and 62°C for NaK. PCR products were visualized using electrophoresis on 1% agarose gels to assess amplification success. Clean-up and Sanger sequencing were performed by Psomagen (Rockville, MD, USA). Amplicons obtained with primers BF2-BR2 were sequenced using a Nanopore MinION (Oxford Nanopore Technologies, Oxford, UK); we prepared the library using the ligation sequencing kit LSK-112 and sequenced it with a R10.4 flow cell. Demultiplexing was performed using ONTbarcoder (Srivathsan et al., 2021).

2.3 | Phylogenetic analyses

Sequences were revised using Sequencher v.5.4.1 (Gene Codes Corporation), and aligned either manually (Cox1, NaK) or using MAFFT v7 (28S) (Katoh et al., 2019; available online at https://mafft.cbrc.jp/alignment/server/) with the FFT-NS-2 method.

For the coding Cox1 and NaK matrices, we used PartitionFinder (Lanfear et al., 2012) to establish the best partition scheme for the different codon positions, resulting in three different partitions for each of these two genes. This partition scheme was implemented in all phylogenetic analyses.

We performed phylogenetic analyses using the whole Cox1 matrix, as well as for each nuclear gene, a concatenated nuclear genes matrix, and a matrix concatenating all three genes. Analyses were performed under maximum likelihood (ML) and Bayesian approaches.

ML trees were obtained with W-IQ-TREE (Trifinopoulos et al., 2016; available online at http://iqtree.cibiv.univie.ac.at), using the Auto option to estimate the best fitting substitution model, and measuring branch support with 1000 ultrafast bootstrap replicates.

Bayesian phylogenetic inference was performed with MrBayes v3.2.6 (Ronquist & Huelsenbeck, 2003) and BEAST2.7.5 (Bouckaert et al., 2014), the latter including estimates of time to the most recent common ancestor (TMRCA). MrBayes analyses were run four independent times to assess consistency of results; runs used one cold and three heated chains for 10 million generations sampling every 1000. Convergence was assessed checking the

TABLE 1 *Ligidium* specimens included in this study with GenBank accession numbers for the analysed sequences.

Species	DNA_code	CUAC#	Locality	Latitude	Longitude	Cox1	28S	NaK
L. blueridgensis	RC.A.505	CUAC000138049	USA: GA: Rabun Co.: Chattahoochee N. F.: Rabun Cliffs	34.9713°N	83.2978°W	OR172582	–	–
L. blueridgensis	RC.080	CUAC000138078	USA: GA: Rabun Co.: Chattahoochee N. F.: Rabun Cliffs	34.9709°N	83.3004°W	OR172609	–	–
L. blueridgensis	Lig018	CUAC000171280	USA: GA: Rabun Co.: Chattahoochee N. F.: Rabun Cliffs	34.9709°N	83.3004°W	OR169848	–	–
L. blueridgensis	Lig016	CUAC000180796	USA: GA: Rabun Co.: Chattahoochee N. F.: Rabun Cliffs	34.9713°N	83.2978°W	OR169933	PP741487	PP739563
L. blueridgensis	Lig017	CUAC000180797	USA: GA: Rabun Co.: Chattahoochee N. F.: Rabun Cliffs	34.9713°N	83.2978°W	OR169934	–	–
L. blueridgensis	Lig058a	CUAC000171346	USA: NC: Avery Co.: Grandfather Mountain	36.0893°N	81.8388°W	OR169912	–	–
L. blueridgensis	Lig058b	CUAC000171347	USA: NC: Avery Co.: Grandfather Mountain	36.0893°N	81.8388°W	OR169913	–	–
L. blueridgensis	Lig058c	CUAC000171348	USA: NC: Avery Co.: Grandfather Mountain	36.0893°N	81.8388°W	OR169914	–	–
L. blueridgensis	Lig050	CUAC000171339	USA: NC: Avery Co.: Grandfather Mountain: Bridge Trail	36.0948°N	81.8311°W	OR169905	–	–
L. blueridgensis	Lig051	CUAC000171340	USA: NC: Avery Co.: Grandfather Mountain: Bridge Trail	36.0948°N	81.8311°W	OR169906	–	–
L. blueridgensis	Lig045	CUAC000171334	USA: NC: Avery Co.: Grandfather Mountain: Grandfather Trail	36.0978°N	81.8293°W	OR169900	–	–
L. blueridgensis	Lig030	CUAC000171307	USA: NC: Caldwell Co.: Grandfather Mountain S P: Boone Scout Trail	36.1164°N	81.7844°W	OR169874	PP741473	PP739567
L. blueridgensis	Lig031	CUAC000171308	USA: NC: Caldwell Co.: Grandfather Mountain S P: Boone Scout Trail	36.1164°N	81.7844°W	OR169875	–	–
L. blueridgensis	Lig032	CUAC000171309	USA: NC: Caldwell Co.: Grandfather Mountain S P: Boone Scout Trail	36.1164°N	81.7844°W	OR169876	–	–
L. blueridgensis	DBS.B.465	CUAC000138059	USA: NC: Caldwell Co.: Grandfather Mountain S. P.: Boone Scout Trail	36.1164°N	81.7844°W	OR172588	–	–
L. blueridgensis	Lig011	CUAC000180792	USA: NC: Graham Co.: Nantahala N. F.: Huckleberrry Knob	35.3210°N	83.9934°W	OR169929	PP741484	PP739580
L. blueridgensis	BBK.B.415	CUAC000138045	USA: NC: Haywood Co.: Black Balsam Knob	35.3289°N	82.8745°W	OR172578	PP741468	PP739579

TABLE 1 (Continued)

Species	DNA_code	CUAC#	Locality	Latitude	Longitude	Cox1	28S	NaK
L. blueridgensis	Lig062	CUAC000171352	USA: NC: Madison Co.: Pisgah N. F.: Camp Creek Bald	36.0220°N	82.7167°W	OR169918	–	–
L. blueridgensis	Lig063	CUAC000171353	USA: NC: Madison Co.: Pisgah N. F.: Camp Creek Bald	36.0220°N	82.7167°W	OR169919	–	–
L. blueridgensis	Lig064	CUAC000171354	USA: NC: Madison Co.: Pisgah N. F.: Camp Creek Bald	36.0220°N	82.7167°W	OR169920	–	–
L. blueridgensis	Lig065	CUAC000171355	USA: NC: Madison Co.: Pisgah N. F.: Camp Creek Bald	36.0220°N	82.7167°W	OR169921	–	–
L. blueridgensis	Lig066	CUAC000171356	USA: NC: Madison Co.: Pisgah N. F.: Camp Creek Bald	36.0220°N	82.7167°W	OR169922	–	–
L. blueridgensis	Lig067	CUAC000171357	USA: NC: Madison Co.: Pisgah N. F.: Camp Creek Bald	36.0220°N	82.7167°W	OR169923	PP741481	PP739577
L. blueridgensis	Lig055	CUAC000171344	USA: NC: Polk Co.: North Pacolet River	35.2221°N	82.3059°W	OR169910	–	–
L. blueridgensis	Lig046	CUAC000171335	USA: NC: Polk Co.: Tryon: Melrose Falls	35.2199°N	82.2987°W	OR169901	PP741477	PP739573
L. blueridgensis	Lig047	CUAC000171336	USA: NC: Polk Co.: Tryon: Melrose Falls	35.2199°N	82.2987°W	OR169902	–	–
L. blueridgensis	Lig048	CUAC000171337	USA: NC: Polk Co.: Tryon: Melrose Falls	35.2199°N	82.2987°W	OR169903	–	–
L. blueridgensis	Lig073	CUAC001169835	USA: NC: Rutherford Co.: Chimney Rock S.P.	35.4372°N	82.2506°W	PP737143	–	–
L. blueridgensis	Lig074	CUAC001169838	USA: NC: Rutherford Co.: Chimney Rock S.P.	35.4372°N	82.2506°W	PP737144	–	–
L. blueridgensis	Lig044a	CUAC000171326	USA: NC: Yancey Co.: Pisgah NF: Woody Ridge Trail	35.8455°N	82.2279°W	OR169892	–	–
L. blueridgensis	Lig044b	CUAC000171327	USA: NC: Yancey Co.: Pisgah NF: Woody Ridge Trail	35.8455°N	82.2279°W	OR169893	PP741476	PP739572
L. blueridgensis	Lig044c	CUAC000171328	USA: NC: Yancey Co.: Pisgah NF: Woody Ridge Trail	35.8455°N	82.2279°W	OR169894	–	–
L. blueridgensis	Lig044d	CUAC000171329	USA: NC: Yancey Co.: Pisgah NF: Woody Ridge Trail	35.8455°N	82.2279°W	OR169895	–	–
L. blueridgensis	Lig044e	CUAC000171330	USA: NC: Yancey Co.: Pisgah NF: Woody Ridge Trail	35.8455°N	82.2279°W	OR169896	–	–
L. blueridgensis	Lig044	CUAC000171331	USA: NC: Yancey Co.: Pisgah NF: Woody Ridge Trail	35.8455°N	82.2279°W	OR169897	–	–
L. blueridgensis	Lig044g	CUAC000171332	USA: NC: Yancey Co.: Pisgah NF: Woody Ridge Trail	35.8455°N	82.2279°W	OR169898	–	–

(Continues)

TABLE 1 (Continued)

Species	DNA_code	CUAC#	Locality	Latitude	Longitude	Cox1	28S	NaK
L. blueridgensis	Lig044h	CUAC000171333	USA: NC: Yancey Co.: Pisgah NF: Woody Ridge Trail	35.8455°N	82.2279°W	OR169899	–	–
L. blueridgensis	Lig006	CUAC000180795	USA: NC: Yancey Co.: Pisgah NF: Woody Ridge Trail	35.8455°N	82.2279°W	OR169932	–	–
L. blueridgensis	Lig060	CUAC000171350	USA: SC: Greenville Co.: Chesnut Ridge H. P.: South Pacolet River	35.1503°N	82.2804°W	OR169916	PP741480	PP739576
L. blueridgensis	Sass.A.344	CUAC000138055	USA: SC: Pickens Co.: Sassafras Mountain	35.0647°N	82.7774°W	OR172587	–	–
L. blueridgensis	Lig012	CUAC000180798	USA: SC: Pickens Co.: Sassafras Mountain	35.0647°N	82.7774°W	OR169935	PP741485	PP739581
L. blueridgensis	Lig013	CUAC000180799	USA: SC: Pickens Co.: Sassafras Mountain	35.0647°N	82.7774°W	OR169936	–	–
L. blueridgensis	Lig061	CUAC000171351	USA: TN: Greene Co.: Cherokee N. F.: Firescald Knob	36.0337°N	82.7024°W	OR169917	–	–
L. blueridgensis	Lig043a	CUAC000171323	USA: TN: Sevier Co.: Great Smoky Mountains N. P.: Alum Cave Tr.	35.6382°N	83.4387°W	OR169890	–	–
L. blueridgensis	Lig043c	CUAC000171325	USA: TN: Sevier Co.: Great Smoky Mountains N. P.: Alum Cave Tr.	35.6382°N	83.4387°W	OR169891	–	–
L. blueridgensis	Lig008	CUAC000171276	USA: TN: Sevier Co.: Great Smoky Mountains N. P.: Mount Le Conte	35.6427°N	83.4426°W	OR169844	PP741471	PP739560
L. blueridgensis	Lig049	CUAC000171338	USA: TN: Sevier Co.: Great Smoky Mountains N. P.: Mount LeConte	35.6427°N	83.4426°W	OR169904	–	–
L. blueridgensis	Lig007	CUAC000171275	USA: TN: Sevier Co.: Great Smoky Mountains N. P.: Off Highway 441	35.6237°N	83.4163°W	OR169843	PP741470	PP739559
L. blueridgensis	Lig024d	CUAC000171299	USA: TN: Sevier Co.: Great Smoky Mountains N. P.: Off Highway 441	35.6240°N	83.4163°W	OR169866	–	–
L. blueridgensis	Lig033	CUAC000171310	USA: TN: Unicoi Co.: Cherokee NF: Big Bald	35.9938°N	82.4573°W	OR169877	–	–
L. blueridgensis	Lig034	CUAC000171311	USA: TN: Unicoi Co.: Cherokee NF: Big Bald	35.9938°N	82.4573°W	OR169878	–	–
L. elrodii	WV.235	CUAC000174439	USA: WV: Pocahontas Co.: Pocahontas Campground	38.1026°N	79.9666°W	PP737149	PP741492	PP739578
L. elrodii	WV.236	CUAC000174440	USA: WV: Randolph Co.: Monongahela National Forest	38.5595°N	79.9262°W	PP737150	–	–

TABLE 1 (Continued)

Species	DNA_code	CUAC#	Locality	Latitude	Longitude	Cox1	28S	NaK
L. elrodii	WV.237	CUAC000174441	USA: WV: Randolph Co.: Monongahela National Forest	38.5595°N	79.9262°W	PP737151	–	–
L. elrodii	WV.238	CUAC000174442	USA: WV: Randolph Co.: Monongahela National Forest	38.5595°N	79.9262°W	PP737152	–	–
L. elrodii	WV.239	CUAC000174443	USA: WV: Randolph Co.: Monongahela National Forest	38.5595°N	79.9262°W	PP737153	–	–
Ligidium sp. 1	BBld.A.223	CUAC000138051	USA: GA: Towns Co.: Brasstown Bald	34.8763°N	83.8107°W	OR172584	–	–
Ligidium sp. 1	Lig035	CUAC000171312	USA: GA: Towns Co.: Chattahoochee N. F.: Brasstown Bald	34.8766°N	83.8109°W	OR169879	PP741474	PP739568
Ligidium sp. 1	Lig036	CUAC000171313	USA: GA: Towns Co.: Chattahoochee N. F.: Brasstown Bald	34.8766°N	83.8109°W	OR169880	PP741490	PP739569
Ligidium sp. 1	Lig037	CUAC000171314	USA: GA: Towns Co.: Chattahoochee N. F.: Brasstown Bald	34.8766°N	83.8109°W	OR169881	–	–
Ligidium sp. 2	Lig019b	CUAC000171282	USA: NC: Clay Co.: Nantahala N. F.:Chunky Gal Trail	35.1471°N	83.7144°W	OR169849	–	–
Ligidium sp. 2	Lig020	CUAC000180793	USA: NC: Clay Co.: Nantahala N. F.:Chunky Gal Trail	35.1471°N	83.7144°W	OR169930	PP741488	PP739565
Ligidium sp. 2	Lig021	CUAC000180794	USA: NC: Clay Co.: Nantahala N. F.:Chunky Gal Trail	35.1471°N	83.7144°W	OR169931	–	–
Ligidium sp. 3	TsqB.B.464	CUAC000138058	USA: NC: Clay Co.: Nantahala N. F.: Tusquitee Bald	35.1467°N	83.7146°W	OR172645	–	–
Ligidium sp. 3	Lig039a	CUAC000171316	USA: NC: Macon Co.: Nantahala N. F.: Van Hook Glade Campground	35.0783°N	83.245°W	OR169883	PP741475	PP739570
Ligidium sp. 3	Lig039b	CUAC000171317	USA: NC: Macon Co.: Nantahala N. F.: Van Hook Glade Campground	35.0783°N	83.245°W	OR169884	–	–
Ligidium sp. 3	Lig059	CUAC000171349	USA: NC: Swain Co.: Great Smoky Mountains N. P.: Payne Ck at Lakeshore Trail	35.4855°N	83.8028°W	OR169915	PP741479	PP739575
Ligidium sp. 3	Lig075	CUAC000177088	USA: SC: Pickens Co.: Chimney Top Gap	35.0644°N	82.7953°W	PP737145	–	–
Ligidium sp. 3	Lig076	CUAC000177089	USA: SC: Pickens Co.: Chimney Top Gap	35.0644°N	82.7953°W	PP737146	–	–
Ligidium sp. 3	Lig077	CUAC000177090	USA: SC: Pickens Co.: Chimney Top Gap	35.0644°N	82.7953°W	PP737147	–	–

(Continues)

TABLE 1 (Continued)

Species	DNA_code	CUAC#	Locality	Latitude	Longitude	Cox1	28S	NaK
Ligidium sp. 4	Lig019a	CUAC000171281	USA: NC: Clay Co.: Nantahala N. F.:Chunky Gal Trail	35.1471°N	83.7144°W	PP737141	PP741489	PP739564
Ligidium sp. 4	BCat.A.294	CUAC000138053	USA: NC: Haywood Co.: Big Cataloochee Mt.	35.6675°N	83.1805°W	PP737148	–	–
Ligidium sp. 4	Lig043b	CUAC000171324	USA: TN: Sevier Co.: Great Smoky Mountains N. P.: Alum Cave Tr.	35.6382°N	83.4387°W	PP737142	PP741491	PP739571
Ligidium sp. 5	Lig052	CUAC000171341	USA: NC: Polk Co.: North Pacolet River	35.2221°N	82.3059°W	OR169907	PP741478	PP739574
Ligidium sp. 5	Lig053	CUAC000171342	USA: NC: Polk Co.: North Pacolet River	35.2221°N	82.3059°W	OR169908	–	–
Ligidium sp. 5	Lig054	CUAC000171343	USA: NC: Polk Co.: North Pacolet River	35.2221°N	82.3059°W	OR169909	–	–
Ligidium sp. 5	Lig056	CUAC000171345	USA: NC: Polk Co.: North Pacolet River	35.2221°N	82.3059°W	OR169911	–	–
Ligidium sp. 5	Lig005	CUAC000171274	USA: TN: Blount Co.: Great Smoky Mountains N. P.: Rich Mountain Gap	35.6455°N	83.8058°W	OR169842	PP741469	PP739558
Ligidium sp. 5	Lig002a	CUAC000171267	USA: TN: Blount Co.: Great Smoky Mountains N. P.: White Oak Sink	35.6362°N	83.7412°W	OR169835	–	–
Ligidium sp. 5	Lig002b	CUAC000171268	USA: TN: Blount Co.: Great Smoky Mountains N. P.: White Oak Sink	35.6362°N	83.7412°W	OR169836	–	–
Ligidium sp. 5	Lig002c	CUAC000171269	USA: TN: Blount Co.: Great Smoky Mountains N. P.: White Oak Sink	35.6362°N	83.7412°W	OR169837	–	–
Ligidium sp. 5	Lig002d	CUAC000171270	USA: TN: Blount Co.: Great Smoky Mountains N. P.: White Oak Sink	35.6362°N	83.7412°W	OR169838	–	–
Ligidium sp. 5	Lig002e	CUAC000171271	USA: TN: Blount Co.: Great Smoky Mountains N. P.: White Oak Sink	35.6362°N	83.7412°W	OR169839	–	–
Ligidium sp. 5	Lig002f	CUAC000171272	USA: TN: Blount Co.: Great Smoky Mountains N. P.: White Oak Sink	35.6362°N	83.7412°W	OR169840	–	–
Ligidium sp. 5	Lig002g	CUAC000171273	USA: TN: Blount Co.: Great Smoky Mountains N. P.: White Oak Sink	35.6362°N	83.7412°W	OR169841	–	–
Ligidium sp. 5	Lig003	CUAC000180800	USA: TN: Blount Co.: Great Smoky Mountains N. P.: White Oak Sink	35.6362°N	83.7412°W	OR169937	–	–
Ligidium sp. 5	Lig004	CUAC000180801	USA: TN: Blount Co.: Great Smoky Mountains N. P.: White Oak Sink	35.6362°N	83.7412°W	OR169938	–	–
Ligidium sp. 6	BBld.B.558	CUAC000138052	USA: GA: Towns Co.: Brasstown Bald	34.8782°N	83.8108°W	OR172585	–	–
Ligidium sp. 6	Lig015	CUAC000171279	USA: GA: Towns Co.: Chattahoochee N. F.: Brasstown Bald	34.8772°N	83.8108°W	OR169847	–	–

TABLE 1 (Continued)

Species	DNA_code	CUAC#	Locality	Latitude	Longitude	Cox1	28S	NaK
Ligidium sp. 6	Lig014	CUAC000180791	USA: GA: Towns Co.: Chattahoochee N. F.: Brasstown Bald	34.8772°N	83.8108°W	OR169928	PP741486	PP739562
Ligidium sp. 6	HKnb.A.128	CUAC000138050	USA: NC: Graham Co.: Nantahala N. F.: Huckleberry Knob	35.3210°N	83.9934°W	OR172583	–	–
Ligidium sp. 6	BMT.B.432	CUAC000138054	USA: NC: Haywood Co.: Balsam Mountain Trail	35.6425°N	83.2007°W	OR172586	–	–
Ligidium sp. 6	Lig038	CUAC000171315	USA: NC: Macon Co.: Highland Biological Station: Coker Rhododendron Tr.	35.0558°N	83.1840°W	OR169882	–	–
Ligidium sp. 6	Lig040a	CUAC000171319	USA: NC: Macon Co.: Highland Biological Station: Coker Rhododendron Tr.	35.0558°N	83.1840°W	OR169886	–	–
Ligidium sp. 6	Lig040b	CUAC000171320	USA: NC: Macon Co.: Highland Biological Station: Coker Rhododendron Tr.	35.0558°N	83.1840°W	OR169887	–	–
Ligidium sp. 6	CrB.B.433	CUAC000138046	USA: NC: Macon Co.: Nantahala N. F.: Copper Ridge Bald	35.2376°N	83.5594°W	OR172579	–	–
Ligidium sp. 6	Lig009	CUAC000171277	USA: NC: Macon Co.: Nantahala N. F.: Copper Ridge Bald	35.2348°N	83.5596°W	OR169845	PP741483	PP739561
Ligidium sp. 6	Lig010	CUAC000171278	USA: NC: Macon Co.: Nantahala N. F.: Copper Ridge Bald	35.2357°N	83.5610°W	OR169846	–	–
Ligidium sp. 6	CB.B.543	CUAC000138047	USA: NC: Macon Co.: Nantahala N. F.: Cowee Bald	35.3270°N	83.3359°W	OR172580	–	–
Ligidium sp. 6	Lig022a	CUAC000171283	USA: NC: Macon Co.: Nantahala N. F.: Cowee Bald	35.3270°N	83.3359°W	OR169850	PP741472	PP739566
Ligidium sp. 6	Lig022b	CUAC000171284	USA: NC: Macon Co.: Nantahala N. F.: Cowee Bald	35.3270°N	83.3359°W	OR169851	–	–
Ligidium sp. 6	Lig022c	CUAC000171285	USA: NC: Macon Co.: Nantahala N. F.: Cowee Bald	35.3270°N	83.3359°W	OR169852	–	–
Ligidium sp. 6	Lig022d	CUAC000171286	USA: NC: Macon Co.: Nantahala N. F.: Cowee Bald	35.3270°N	83.3359°W	OR169853	–	–
Ligidium sp. 6	Lig022e	CUAC000171287	USA: NC: Macon Co.: Nantahala N. F.: Cowee Bald	35.3270°N	83.3359°W	OR169854	–	–
Ligidium sp. 6	Lig022f	CUAC000171288	USA: NC: Macon Co.: Nantahala N. F.: Cowee Bald	35.3270°N	83.3359°W	OR169855	–	–

(Continues)

TABLE 1 (Continued)

Species	DNA_code	CUAC#	Locality	Latitude	Longitude	Cox1	28S	NaK
Ligidium sp. 6	Lig022g	CUAC000171289	USA: NC: Macon Co.: Nantahala N. F.: Cowee Bald	35.3270°N	83.3359°W	OR169856	–	–
Ligidium sp. 6	Lig039c	CUAC000171318	USA: NC: Macon Co.: Nantahala N. F.: Van Hook Glade Campground	35.0783°N	83.245°W	OR169885	–	–
Ligidium sp. 6	Lig041	CUAC000171321	USA: NC: Macon Co.: Nantahala N. F.: Van Hook Glade Campground	35.0776°N	83.2452°W	OR169888	–	–
Ligidium sp. 6	Lig042	CUAC000171322	USA: NC: Macon Co.: Nantahala N. F.: Van Hook Glade Campground	35.0786°N	83.2442°W	OR169889	–	–
Ligidium sp. 6	Lig023a	CUAC000171290	USA: NC: Swain Co.: Great Smoky Mountains N. P.: Off Highway 441	35.5824°N	83.3979°W	OR169857	–	–
Ligidium sp. 6	Lig023b	CUAC000171291	USA: NC: Swain Co.: Great Smoky Mountains N. P.: Off Highway 441	35.5824°N	83.3979°W	OR169858	–	–
Ligidium sp. 6	Lig023c	CUAC000171292	USA: NC: Swain Co.: Great Smoky Mountains N. P.: Off Highway 441	35.5824°N	83.3979°W	OR169859	–	–
Ligidium sp. 6	Lig023d	CUAC000171293	USA: NC: Swain Co.: Great Smoky Mountains N. P.: Off Highway 441	35.5824°N	83.3979°W	OR169860	–	–
Ligidium sp. 6	Lig023e	CUAC000171294	USA: NC: Swain Co.: Great Smoky Mountains N. P.: Off Highway 441	35.5824°N	83.3979°W	OR169861	–	–
Ligidium sp. 6	Lig023f	CUAC000171295	USA: NC: Swain Co.: Great Smoky Mountains N. P.: Off Highway 441	35.5824°N	83.3979°W	OR169862	–	–
Ligidium sp. 6	Lig025	CUAC000171302	USA: NC: Transilvania Co.: Brevard, Pisgah Forest	35.3291°N	82.7890°W	OR169869	–	–
Ligidium sp. 6	Lig026	CUAC000171303	USA: NC: Transilvania Co.: Brevard, Pisgah Forest	35.3291°N	82.7890°W	OR169870	–	–
Ligidium sp. 6	Lig027	CUAC000171304	USA: NC: Transilvania Co.: Pisgah National Forest, Hwy 215	35.2910°N	82.9133°W	OR169871	–	–
Ligidium sp. 6	Lig028	CUAC000171305	USA: NC: Transilvania Co.: Pisgah National Forest, Hwy 215	35.2910°N	82.9133°W	OR169872	–	–
Ligidium sp. 6	Lig029	CUAC000171306	USA: NC: Transilvania Co.: Pisgah National Forest, Hwy 215	35.2910°N	82.9133°W	OR169873	–	–
Ligidium sp. 6	Lig001a	CUAC000171264	USA: TN: Blount Co.: Great Smoky Mountains N. P.: Rich Mountain Gap	35.6450°N	83.8061°W	OR169832	PP741482	PP739557

TABLE 1 (Continued)

Species	DNA_code	CUAC#	Locality	Latitude	Longitude	Cox1	28S	NaK
Ligidium sp. 6	Lig001b	CUAC000171265	USA: TN: Blount Co.: Great Smoky Mountains N. P.: Rich Mountain Gap	35.6450°N	83.8061°W	OR169833	–	–
Ligidium sp. 6	Lig001c	CUAC000171266	USA: TN: Blount Co.: Great Smoky Mountains N. P.: Rich Mountain Gap	35.6450°N	83.8061°W	OR169834	–	–
Ligidium sp. 6	Hwy.A.147	CUAC000138081	USA: TN: Sevier Co.: Great Smoky Mountains N. P.: Off Highway 441	35.6240°N	83.4163°W	OR172613	–	–
Ligidium sp. 6	Lig024a	CUAC000171296	USA: TN: Sevier Co.: Great Smoky Mountains N. P.: Off Highway 441	35.6240°N	83.4163°W	OR169863	–	–
Ligidium sp. 6	Lig024b	CUAC000171297	USA: TN: Sevier Co.: Great Smoky Mountains N. P.: Off Highway 441	35.6240°N	83.4163°W	OR169864	–	–
Ligidium sp. 6	Lig024c	CUAC000171298	USA: TN: Sevier Co.: Great Smoky Mountains N. P.: Off Highway 441	35.6240°N	83.4163°W	OR169865	–	–
Ligidium sp. 6	Lig024e	CUAC000171300	USA: TN: Sevier Co.: Great Smoky Mountains N. P.: Off Highway 441	35.6240°N	83.4163°W	OR169867	–	–
Ligidium sp. 6	Lig024f	CUAC000171301	USA: TN: Sevier Co.: Great Smoky Mountains N. P.: Off Highway 441	35.6240°N	83.4163°W	OR169868	–	–

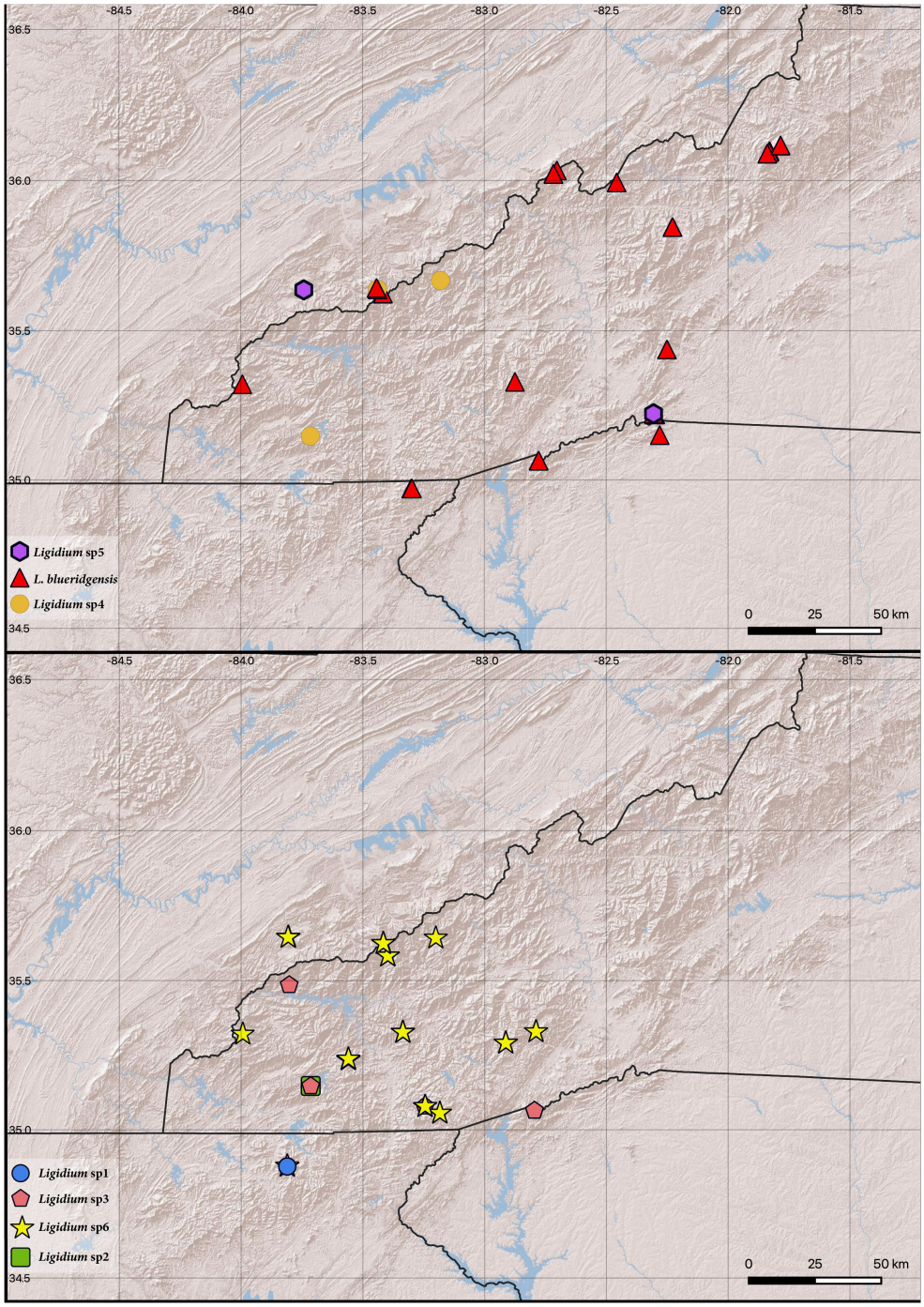

FIGURE 1 Map showing the studied populations and the distribution of southern Appalachian *Ligidium* main lineages. The two studied populations from West Virginia are not shown.

ending average standard deviation of split frequencies values, which were always below 0.01, and effective sample sizes (ESS), always above 100; consensus trees were obtained after applying a 25% burn-in. BEAST2 analyses were also independently repeated four times to assess consistency, using the package bModelTest (Bouckaert & Drummond, 2017) to estimate the best-fitting substitution models for the different partitions, running for 100 million generations sampled every 10,000 and implementing an optimized lognormal relaxed clock. We used Birth and Death as the tree prior to analyse all concatenated matrices, while for the Cox1 matrix, considering the high number of haplotypes per species, we chose a Bayesian Skyline coalescent tree prior, in both cases starting with default values. Having no available fossil records or any other adequate information to calibrate the molecular clock, we chose to use a substitution rate with a mean value of 0.017 (±0.007) following previous works on Peracarid crustaceans as Amphipoda and Isopoda

(Lee et al., 2014; Mamos et al., 2021). No other priors were modified. ESS for all parameters were checked with Tracer v.1.7.2 (Rambaut et al., 2018) and higher than 200. We used TreeAnnotator v.1.10.4 to build maximum clade credibility trees considering a 25% burn-in.

2.4 | Species delimitation analyses

We performed single-locus species delimitation analyses on each gene alignment. To compare different approaches, we used a distance-based method, ASAP (Assemble Species by Automatic Partitioning, Puillandre et al., 2021), based on the detection of barcoding gaps from observed genetic pairwise distances, and a phylogenetic-based method, mPTP (multirate Poisson Tree Process, Kapli et al., 2017), that compares branching rate transitions using speciation and coalescent models. ASAP analyses were run on the web server (available at https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html), with a split probability of 0.01 and using simple p-distances. For mPTP analyses we used the web server (available at https://mptp.h-its.org/#/tree) and the trees generated with BEAST2 as input.

For multilocus matrices, we used BPP v4.6.2 (Bayesian Phylogenetics and Phylogeography; Yang & Rannala, 2010). This is considered a validation method that will compare posterior probabilities (pp) of different delimitation models that consider different numbers of units among a priori specified species (Carstens et al., 2013). For these analyses, we considered both a dataset including only nuclear genes and a dataset including nuclear and Cox1 sequences. In the second case we tried using the complete Cox1 alignment and a reduced alignment including only those represented also in the nuclear alignments. This analysis generates the delimitation models to be tested by lumping a priori species, but it can't split them further, so we tested different hypotheses considering 15 a priori species (considering shallower clades as potential species), or 25 species (considering each specimen a potential species). We ran 'A11' analyses for 200,000 generations with 20,000 generations as burnin, sampling every five generations, using a guide tree based on our previous multilocus phylogenetic analyses, and mean values of 0.02 for theta and tau, with inverse gamma distributions.

3 | RESULTS

3.1 | Morphology

The morphological examination of the studied samples allowed us to differentiate three main morphotypes based on the general shape of male pleopod 2 endopodite (Figure 2a), despite the generally conserved general habitus (Figure 3a). Two of these main morphotypes present some degree of variation that is congruent with the identified genetic lineages, which allowed us to differentiate at least eight different, discrete morphological units among the studied samples.

The first main morphotype (morphotype I) shows a largely acuminate tip, and it was found only in a single adult male, corresponding with the most divergent genetic lineage, referred to here as *Ligidium* sp. 4.

The second one (morphotype II) shows a broadly squarish or rounded tip with an elongated excrescence, as typically observed in *Ligidium blueridgensis*. Within morphotype II we could distinguish three different variations of male pleopod 2 endopodite. One of them corresponds with *Ligidium blueridgensis* sensu stricto (s. str.), in which an elongated excrescence is located in the inner corner of the squarish or rounded pleopod tip and directed inwards or backwards. The other two correspond to yet undescribed species, denoted hereafter *Ligidium* sp. 5, characterized by a more rounded tip and centered excrescence, and *Ligidium* sp. 2, with a robust excrescence located in the inner corner but directed outwards.

The third (morphotype III) includes broadly rounded or squarish tip with no elongated excrescences, as has been described for the different subspecies of *Ligidium elrodii*. Among morphotype III, we could identify up to four more or less different variations among all the collected samples. One of them, found in the northernmost studied population, corresponds with *Ligidium elrodii* s. str. as currently defined. *Ligidium* sp. 1 presents a broad, squarish tip of male pleopod 2 endopodite with a short row of small denticles in the inner corner. *Ligidium* sp. 3 also presents a row of small denticles in the inner corner, but has a rounded tip with a short, squarish terminal projection. *Ligidium* sp. 6 has an uneven, rounded profile of male pleopod 2 endopodite, with the inner corner truncated.

3.2 | Molecular data and phylogenetic analyses

The final alignments for phylogenetic and species delimitation analyses include 130 sequences of Cox1 with a total of 658 bp, although for samples amplified with BF2-BR2 primers, the fragment size is 421 bp, and the missing part was treated as missing data. For both NaK and 28S the alignments include 25 sequences of 664 and 430 bp respectively, representing the morphologically defined groups and all major mtDNA lineages.

MtDNA shows very high genetic distances between and within the mentioned morphological groups. Mean

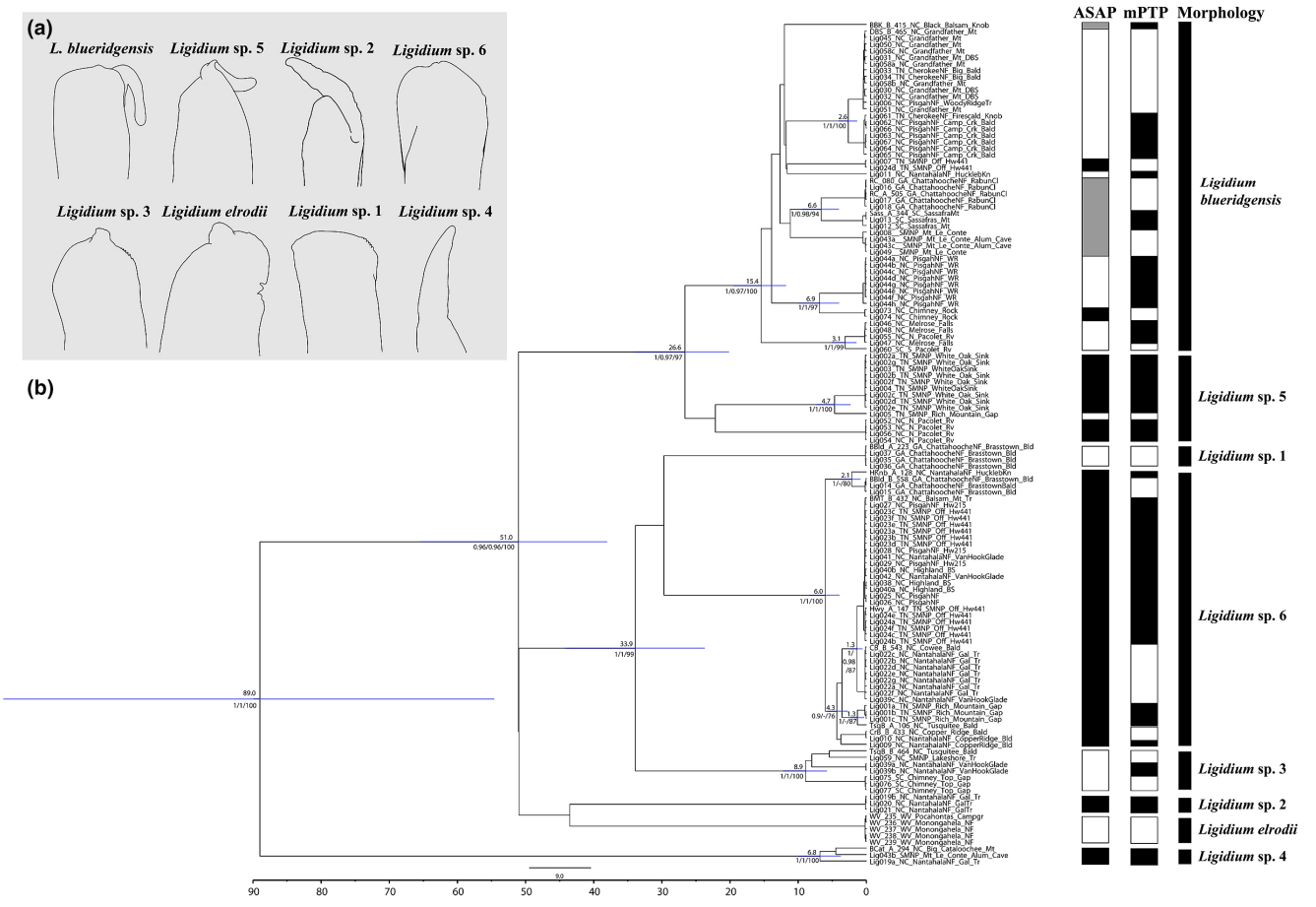

FIGURE 2 (a) Male pleopod 2 endopodites of the eight observed morphotypes (non-scaled). (b) Chronogram based on Cox1 sequences of all studied specimens. Information by the nodes represent mean values of TMRCAs (above) and support values of phylogenetic analyses (below; BEATS2/MrBayes/ML); blue bars on nodes represent TMRCA's 95% HDP. The bottom bar indicates time in millions of years. On the right we show the morphologically defined units and the results on single-locus species delimitation analyses.

uncorrected p-distances between morphological groups range from 15.6% to 26.9%, while within them mean distances range from 0% to up to 10% (Table S1). The 28S data revealed mean uncorrected p-distances between groups of 0.98%–9.5% and very low within those groups, 0%–0.1% (Table S1). For NaK, the observed mean uncorrected p-distances between groups range from 0.46% to 5.3%, and within group distances also 0%–0.1% (Table S1).

Most specimens with morphotype II were initially assigned to *Ligidium blueridgensis*, including several deep mitochondrial lineages grouped in two main clades (Figure 2b), although one of them is not supported in any single locus phylogenetic analyses. Both groups are strongly supported as monophyletic when analysing the nuclear genes together (Figure S1) and, when combining nuclear and mtDNA genes, they are recovered as sister clades with high support (Figure 3). One of them, corresponding to *L. blueridgensis* s. str., is widespread across the southern Appalachian Mountains, being present on both sides of the French Broad River Basin, a major biogeographic barrier in the region (Figure 1). The second one, corresponding to *Ligidium* sp. 5, shows a more restricted distribution and presents two genetically divergent but morphologically similar subgroups; one is found in the western Great Smoky Mountains in Tennessee, and the other close to the headwaters of the French Broad River, in south-central North Carolina (Figure 1). The third clade, representing *Ligidium* sp. 2, is phylogenetically unrelated to the other two lineages with distal excrescences, and shows a pleopod 2 endopodite different from the typical *L. blueridgensis*. It has been found exclusively at the Chunky Gal Trail in the Nantahala National Forest, SW North Carolina (Figure 1).

In the case of samples with morphotype III, initially assigned to *Ligidium elrodii* sensu lato (s. l.), we found several divergent lineages in all our analyses. The northernmost one, found in West Virginia, is assigned to *L. elrodii* s. str. (type locality, southern Indiana; Packard, 1873) and, according to our phylogenetic reconstructions, is not closely related to the other lineages (Figures 2b–3b). The

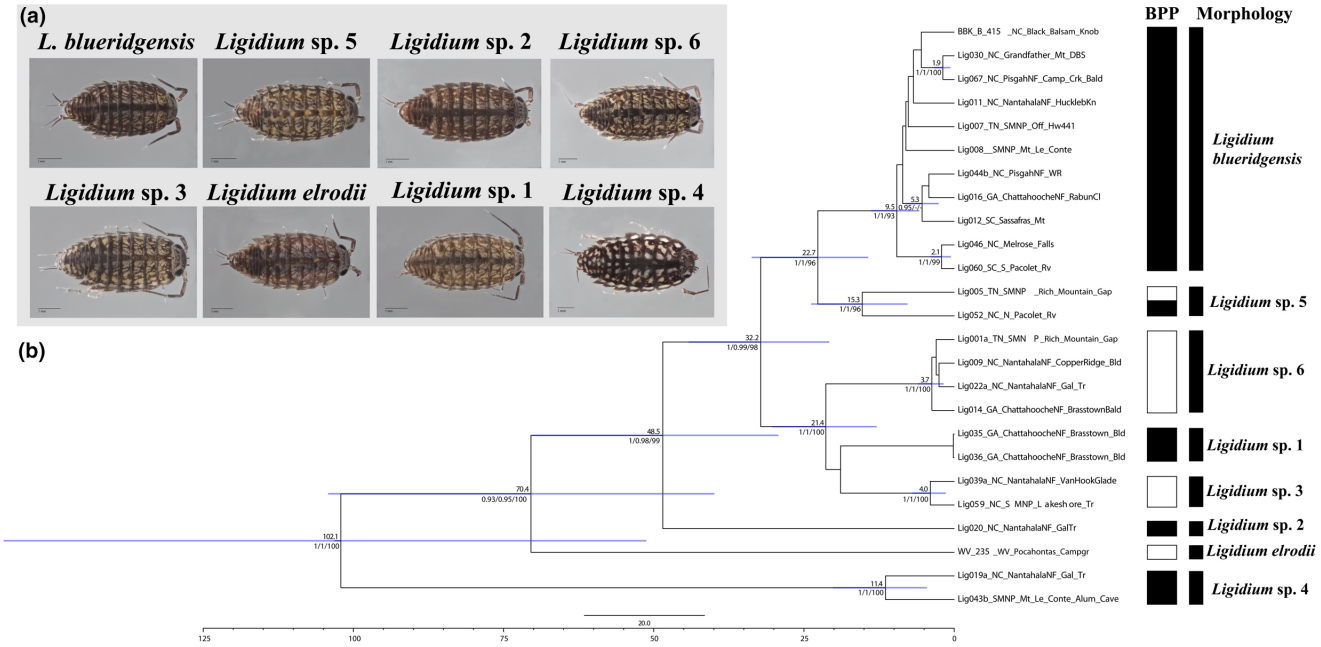

FIGURE 3 (a) External habitus of the eight observed morphotypes. (b) Chronogram based on mtDNA and nDNA sequences of selected specimens. Information by the nodes represent mean values of TMRCAs (above) and support values of phylogenetic analyses (below; BEATS2/MrBayes/ML); blue bars on nodes represent TMRCA's 95% HDP. The bottom bar indicates time in millions of years. On the right we show the morphologically defined units and the results on BPP species delimitation analyses.

other three form a monophyletic clade both using mtDNA and concatenated nuclear genes (Figure 3b). One of these lineages, *Ligidium* sp. 6, is widespread and frequent in the southern Appalachians west of the French Broad River Basin, while the other two appear to be much less frequent, one restricted to Brasstown Bald (*Ligidium* sp. 1), the highest mountain in Georgia, and the other (*Ligidium* sp. 3) showing a scattered range in southwestern North Carolina (Figure 1).

Finally, for *Ligidium* sp. 4 (including the male with morphotype I), only three specimens have been found in three rather widely scattered localities (Figure 1). Since only one of them is an adult male, assignment to this morphotype of the other specimens is based on their position in the phylogenetic trees.

The estimates of TMRCAs indicate a very old age of *Ligidium* species inhabiting the southern Appalachians (Figures 2b–3b). Estimates on deeper nodes were slightly older when analysing the multilocus dataset. The studied lineages share a common ancestor dated in the Upper-Lower Cretaceous boundary, with subsequent cladogenic events occurring likely during the Paleogene. Most of the eight main lineages are estimated as old groups, with TMRCAs dating back to the Miocene. The only exceptions are *L. elrodii* s. str., *Ligidium* sp. 1 and *Ligidium* sp. 2, characterized by very shallow genetic diversity, perhaps due to insufficient sampling, as they have been scarcely found and only in one or two localities each.

3.3 | Species delimitation analyses

Single-locus automated species delimitation analyses using the Cox1 matrix suggested the existence of several more species than morphotypes. The best proposed model by ASAP ($p = 0.00019$, ASAP-score = 3.0) indicated the existence of 16 potential species, as it suggested splitting *Ligidium blueridgensis* into eight species and *Ligidium* sp. 5 into three (Figure 2b). The result of mPTP analysis suggests even more potential species, by splitting *L. blueridgensis* into 12 species, *Ligidium* sp. 5 into three, *Ligidium* sp. 6 into seven, and *Ligidium* sp. 3 into three (Figure 2b). Contrarily, these analyses on the nuclear datasets suggested fewer species than the observed morphotypes. Neither 28S nor NaK performed well for ASAP analyses, as no significant models were proposed. The results of mPTP both with 28S and NaK delimit the same four units corresponding with morphotypes *Ligidium* sp. 4, *L. elrodii* s. str., and *Ligidium* sp. 2, and another group including all other morphotypes. BPP results, including analyses with all three genes (15 a priori species, $pp = 0.36$) or only with nuclear ones (15 a priori species, $pp = 0.47$), indicate that the best-supported model includes 9 units, suggesting the split of *Ligidium* sp. 5 in two potential species (Figure 3b).

We have found no clear allopatric distribution patterns among the sampled species and populations (Figure 1). *Ligidium elrodii* s. str. has been found only outside the southern Appalachians, but our sampling to the north is

limited. Within the southern Appalachians we found several localities where two or even three lineages are found together just a few meters apart. At the sampling points along the Chunky Gal Trail in the Nantahala National Forest, SW North Carolina, we have collected three lineages, *Ligidium* sp. 2, *Ligidium* sp. 4, and *Ligidium* sp. 3. The widespread *L. blueridgensis* shares some localities with *Ligidium* sp. 5 (northern part of the Pacolet River, North Carolina), with *Ligidium* sp. 6 (in the Smoky Mountains near Newfound Gap, Tennessee), and with *Ligidium* sp. 2 (Alum Cave trail at Mount LeConte, Tennessee). It has been found also near *Ligidium* sp. 3 at Sassafras Mountain, South Carolina. The second most widespread lineage, *Ligidium* sp. 6, has also been found close to *Ligidium* sp. 1 (Brasstown Bald, Georgia) and *Ligidium* sp. 3 (Van Hook Glade Campground, Nantahala National Forest, North Carolina). Since at each place we collected several litter samples it is hard to say if in these localities they live in microsympatry, but they were mostly collected in the same kind of habitat.

4 | DISCUSSION

The genus *Ligidium* has proven to be more diverse than initially expected in several parts of the Holarctic, after the integration of dense sampling and molecular data (Harigai et al., 2023; Klossa-Kilia et al., 2006; Wang et al., 2022). Integrative taxonomic approaches have found the existence of truly cryptic *Ligidium* species, hardly diagnosable based on morphological characters (Wang et al., 2022). In other cases, it has been shown that morphological variability, once considered intraspecific variation, can reflect the existence of independent evolutionary lineages and potentially different species (Li, 2017; Wang et al., 2022). Similarly, our results indicate a higher diversity than previously thought among the *Ligidium* populations present in the southern Appalachian Mountains.

Ligidium elrodii has been considered a widespread species present over a large portion of eastern North America. Within its range, a considerable morphological variability has been described as intraspecific variation, particularly in the southern half of its range. Some of this variability has been considered taxonomically significant, and up to 5 different subspecies are traditionally recognized (Schultz, 1970). These subspecies are largely based on differences in the male pleonite 2 endopodite, a main source of characters for species recognition in this and other groups of Oniscidea (Schultz, 1970; Vandel, 1960). Besides that, at least another morphotype has been described with no taxonomic recognition (Schultz, 1982). Without further evidence, evaluating the relevance of such variability within what is traditionally considered a widespread, variable species can be challenging (e.g., Montesanto et al., 2007; Rodriguez-Flores et al., 2021; Sánchez-Vialas et al., 2020; Vasquez-Valverde & Marek, 2022). This is particularly true when such variability is not geographically or ecologically structured, and we find some of the different morphological variants even in the same locality. In this case, it seems reasonable to assume that the observed differences, sometimes relatively subtle, are indeed part of a single species variability. The use of phylogeographic methods can help us to increase our understanding of such patterns, and an integrative taxonomic approach is necessary to delimit species in these complexes (Padial et al., 2010).

We have observed that the morphological variants that could be initially attributed to *Ligidium elrodii s. l.* indeed represent old, independent evolutionary lineages that can be postulated as different species. Within the southern Appalachians we have discovered three of them and, given the complex geography of this region, there could be more to be discovered yet. Based on our results, we anticipate that the four morphotypes described as different subspecies within *L. elrodii* will also represent deep, independent evolutionary lineages that will be eventually considered full species. However, a more complete sampling including representatives of these groups will be needed before their taxonomic status is revised. Even if somewhat modest, the differences observed in the male pleopode 2 endopodite seem to be constant and seem to be of diagnostic value, so these aren't necessarily 'cryptic species' in the strictest sense within this complex.

In the case of *Ligidium blueridgensis s. l.*, only two different morphotypes were clearly separated, representing the two main lineages within this group. In this case, the existence of deeper sublineages indicates the existence of cryptic diversity, perhaps even some cryptic species. Certainly, automated species delimitation methods based on Cox1 sequences split both main lineages into several units. However, our nuclear and morphological evidence is not enough to recognize most of them. It will be necessary to generate more data to study in more detail the evolutionary history of this species and explore its real species diversity. In the case of *Ligidium* sp. 5, we found two genetically distinct and apparently geographically segregated lineages. Pending a deeper morphological assessment, they may represent two true cryptic species as suggested by multilocus species delimitation.

Besides the above semi-cryptic diversity, we still have been able to locate new morphotypes unrelated to previously described taxa. This is indicative of insufficient exploration of the diversity of *Ligidium*, and suggests that still more species might be discovered not only in this area but across North America. Indeed, not many zoologists have focused their attention on terrestrial isopods in the

Nearctic region, and the lack of taxonomic expertise and of functional keys makes it difficult to identify current diversity (Shultz, 2018). Thus, it would not be surprising that species with restricted distribution have not been found and described yet. In many parts of North America, the dominant terrestrial isopod species are just a bunch of exotic species, some of them with presence in the continent already for hundreds of years (Jass & Klausmeier, 2000; Van Name, 1936). We do not know whether this is caused by native species being displaced by invasive ones, or by an originally limited native fauna that allowed exotic species to easily colonize available ecological niches. But this may have reduced the general interest for this group, particularly for local taxonomists. In the southern Appalachians we have found indeed poor communities of terrestrial isopods, with native representatives of the genera *Ligidium* and *Miktoniscus* Kesselyak, 1930; in the studied litter samples we have found *Ligidium* as the dominant taxon, frequently living in sympatry with *Trichoniscus pusillus* Brandt, 1833, and more rarely with *Oniscus asellus* Linnaeus, 1758 or *Hyloniscus riparius* (C. Koch, 1838), all three exotic species of European origin (Schmalfuss, 2003).

Most of the diversity found in the southern Appalachians lives west of the French Broad River Basin, considered one of the major geographical barriers in the area acting as such at least since the Pliocene (Caterino & Langton-Myers, 2019; Crespi et al., 2003; Hedin et al., 2015), while other commonly recognized riverine barriers, such as the Little Tennessee and Tuckasegee River basins (Hedin & McCormack, 2017; Thomas & Hedin, 2008), seem not to be influencing the observed distributions. In fact, among species in the region, only *Ligidium blueridgensis* seems to have crossed the French Broad River Basin, showing a distinct mitochondrial sublineage exclusively present in those parts. In our multilocus chronogram, time estimates are compatible with a Mio-Pliocene origin of this clade, around 5.5 mya. However, all main lineages are estimated to be much older than the Pliocene. Among terrestrial Isopoda, as in any other terrestrial animal group, allopatric speciation is the most frequently considered process for the formation of species (Hernández-Hernández et al., 2021). Even if we have found several cases of sympatry in southern Appalachia, their ages would allow current species to have originated by allopatric speciation in southern Appalachia or in other regions with subsequent range shifts generating currently sympatric populations.

The estimated ages in our chronograms suggest an ancient presence of the genus in eastern North America. *Ligidium* has a broad distribution encompassing all the Holartic and, considering the dispersal limitations of these organisms, it is possible that the ancestors of current diversity were already living in Laurasia at least when this supercontinent started to split between North America and Eurasia. The Appalachian Mountains are very old (Rast, 1989), so indeed they could have been in these lands for the last hundred million years. The only published fossil record of *Ligidium* is a Baltic amber inclusion dated in the Paleogene Eocene (Broly et al., 2013), which tells us little regarding the age of Appalachian *Ligidium* but that is compatible with the TMRCAs estimated in our analyses. However, we lacked adequate calibration points for a molecular clock, and the substitution rate we used could be either over- or underestimated. It is relatively frequent among terrestrial isopods to exhibit very high mtDNA genetic distances, inter- and intraspecific (e.g., Raupach et al., 2022; Recuero et al., 2022; Zimmermann et al., 2015). The reasons for this have never been clear and could be explained under different hypotheses (Raupach et al., 2022). First, they could indeed be old organisms with an evolutionary history of millions of years. In this case, old mtDNA lineages could be indicative of the existence of cryptic species, although mtDNA alone is often not enough to establish the current evolutionary independence of such lineages (Després, 2019). Second, a potential effect of *Wolbachia* bacteria has been hypothesized to have shaped the mitochondrial diversity of infected populations, generating a strong selective pressure that could rapidly induce differentiation of mtDNA lineages (Hurst & Jiggins, 2005; Kodandaramaiah et al., 2013). In this case it is expected to find deeply differentiated lineages, but extremely homogeneous within-lineage haplotype diversity. A third alternative is that mitochondrial substitution rates are higher than normal in these organisms. However, the few studies trying to calibrate a molecular clock for terrestrial isopods do not support this (Ketmaier et al., 2003; Poulakakis & Sfenthourakis, 2008; Wysocka et al., 2008). Lastly, the presence of nuclear copies of the mtDNA genes (NUMTs) can yield deceptive results including extremely divergent lineages (Bensasson et al., 2001). However, in the case of coding genes, these divergent NUMTs should be plagued with stop codons and would be easy to detect.

Regardless of our estimates, it is clear that the *Ligidium* fauna in the southern Appalachian Mountains is more diverse than previously thought, mostly due to misinterpretation of the observed morphological variation, but also by the existence of unexplored and cryptic diversity. We are working to describe the species presented here in the near future, while continuing to explore the southern Appalachians in search of yet undiscovered species.

ACKNOWLEDGEMENTS

Thanks to P. Wooden, A. Haberski, C. Harden, M. Ferro, L. Cushman, R. Kucuk, P. Marek, S. Payne, L. Kairy, C. McGehee, C. MacCheyne and F. Etzler for their help in

the field or collecting samples. Also, to the North Carolina State Parks, Monongahela National Forest, Great Smoky Mountains National Park, and Blue Ridge Parkway National Park for collecting permit. We acknowledge the support of the John and Suzanne Morse Endowment for Arthropod Biodiversity. This study represents Technical Contribution Number 7240 of the Clemson University Experiment Station.

FUNDING INFORMATION

This study was funded by the U.S. National Science Foundation (Award DEB-1916263 to MSC) and the Clemson University Experiment Station (SC-1700596 to MSC).

ORCID

Ernesto Recuero https://orcid.org/0000-0001-8086-8667
Michael S. Caterino https://orcid.org/0000-0002-2597-5707

REFERENCES

Alexiou, S., & Sfenthourakis, S. (2013). The terrestrial isopods (Isopoda: Oniscidea) of Greece. *Parnassiana Archives*, *1*, 3–50.

Bensasson, D., Zhang, D. X., Hartl, D. L., & Hewitt, G. M. (2001). Mitochondrial pseudogenes: evolution's misplaced witnesses. *Trends in Ecology & Evolution*, *16*(6), 314–321. https://doi.org/10.1016/S0169-5347(01)02151-6

Bouckaert, R., Heled, J., Kühnert, D., Vaughan, T., Wu, C. H., Xie, D., Suchard, M. A., Rambaut, A., & Drummond, A. J. (2014). BEAST 2: A software platform for Bayesian evolutionary analysis. *PLoS Computational Biology*, *10*, e1003537. https://doi.org/10.1371/journal.pcbi.1003537

Bouckaert, R. R., & Drummond, A. J. (2017). bModelTest: Bayesian phylogenetic site model averaging and model comparison. *BMC Evolutionary Biology*, *17*, 1–11. https://doi.org/10.1186/s12862-017-0890-6

Broly, P., Deville, P., & Maillet, S. (2013). The origin of terrestrial isopods (Crustacea: Isopoda: Oniscidea). *Evolutionary Ecology*, *27*, 461–476. https://doi.org/10.1007/s10682-012-9625-8

Carstens, B. C., Pelletier, T. A., Reid, N. M., & Satler, J. D. (2013). How to fail at species delimitation. *Molecular Ecology*, *22*(17), 4369–4383. https://doi.org/10.1111/mec.12413

Caterino, M. S., & Langton-Myers, S. S. (2019). Intraspecific diversity and phylogeography in southern Appalachian *Dasycerus carolinensis* (Coleoptera: Staphylinidae: Dasycerinae). *Insect Systematics and Diversity*, *3*(6), 8. https://doi.org/10.1093/isd/ixz022

Caterino, M. S., & Recuero, E. (2024). Shedding light on dark taxa in sky-Island Appalachian leaf litter: Assessing patterns of endemicity using large-scale, voucher-based barcoding. *Insect Conservation and Diversity*, *17*(1), 16–30. https://doi.org/10.1111/icad.12697

Crespi, E. J., Rissler, L. J., & Browne, R. A. (2003). Testing Pleistocene refugia theory: Phylogeographical analysis of *Desmognathus wrighti*, a high-elevation salamander in the southern Appalachians. *Molecular Ecology*, *12*(4), 969–984. https://doi.org/10.1046/j.1365-294x.2003.01797.x

Derkarabetian, S., Starrett, J., & Hedin, M. (2022). Using natural history to guide supervised machine learning for cryptic species delimitation with genetic data. *Frontiers in Zoology*, *19*(1), 8. https://doi.org/10.1186/s12983-022-00453-0

Després, L. (2019). One, two or more species? Mitonuclear discordance and species delimitation. *Molecular Ecology*, *28*, 3845–3847. https://doi.org/10.1111/mec.15211

Dukes, C. D., Janssens, F., Recuero, E., & Caterino, M. S. (2022). Specific and intraspecific diversity of Symphypleona and Neelipleona (Hexapoda: Collembola) in southern high Appalachia (USA). *Diversity*, *14*, 847. https://doi.org/10.3390/d14100847

Elbrecht, V., & Leese, F. (2017). Validation and development of COI metabarcoding primers for freshwater macroinvertebrate bioassessment. *Frontiers in Environmental Science*, *5*, 11. https://doi.org/10.3389/fenvs.2017.00011

Folmer, O., Hoeh, W. R., Black, M. B., & Vrijenhoek, R. C. (1994). Conserved primers for PCR amplification of mitochondrial DNA from different invertebrate phyla. *Molecular Marine Biology and Biotechnology*, *3*(5), 294–299.

Harigai, W., Saito, A., Suzuki, H., & Yamamoto, M. (2020). Genetic diversity of *Ligidium* isopods in Hokkaido and Niigata, northern Japan, based on mitochondrial DNA analysis. *Zoological Science*, *37*(5), 417–428. https://doi.org/10.2108/zs200017

Harigai, W., Saito, A., Zemmoto, C., Karasawa, S., Yokoi, T., Nagano, A. J., Suzuki, H., & Yamamoto, M. (2023). History of the terrestrial isopod genus *Ligidium* in Japan based on phylogeographic analysis. *BMC Ecology and Evolution*, *23*(1), 1–25. https://doi.org/10.1186/s12862-023-02144-8

Hedin, M., Carlson, D., & Coyle, F. (2015). Sky Island diversification meets the multispecies coalescent–divergence in the spruce-fir moss spider (*Microhexura montivaga*, Araneae, Mygalomorphae) on the highest peaks of southern Appalachia. *Molecular Ecology*, *24*(13), 3467–3484. https://doi.org/10.1111/mec.13248

Hedin, M., & McCormack, M. (2017). Biogeographical evidence for common vicariance and rare dispersal in a southern Appalachian harvestman (Sabaconidae, *Sabacon cavicolens*). *Journal of Biogeography*, *44*(7), 1665–1678. https://doi.org/10.1111/jbi.12973

Hedin, M., & Milne, M. A. (2023). New species in old mountains: Integrative taxonomy reveals ten new species and extensive short-range endemism in *Nesticus* spiders (Araneae, Nesticidae) from the southern Appalachian Mountains. *ZooKeys*, *1145*, 1–130. https://doi.org/10.3897/zookeys.1145.96724

Hernández-Hernández, T., Miller, E. C., Román-Palacios, C., & Wiens, J. J. (2021). Speciation across the tree of life. *Biological Reviews*, *96*(4), 1205–1242. https://doi.org/10.1111/brv.12698

Hurst, G. D., & Jiggins, F. M. (2005). Problems with mitochondrial DNA as a marker in population, phylogeographic and phylogenetic studies: The effects of inherited symbionts. *Proceedings of the Royal Society B: Biological Sciences*, *272*(1572), 1525–1534. https://doi.org/10.1098/rspb.2005.3056

Jass, J., & Klausmeier, B. (2000). Endemics and immigrants: North American terrestrial isopods (Isopoda, Oniscidea) north of Mexico. *Crustaceana*, *73*, 771–799. https://doi.org/10.1163/156854000504804

Jass, J. P., & Klausmeier, B. R. (2006). Transborder associations of terrestrial isopods (Crustacea, Isopoda, Oniscidea) of Mexico and the United States. *Western North American Naturalist*, *66*, 132–134. https://doi.org/10.3398/1527-0904(2006)66[132:TAOTIC]2.0.CO;2

Kapli, P., Lutteropp, S., Zhang, J., Kobert, K., Pavlidis, P., Stamatakis, A., & Flouri, T. (2017). Multi-rate Poisson tree processes for single-locus species delimitation under maximum likelihood and Markov chain Monte Carlo. *Bioinformatics*, *33*(11), 1630–1638. https://doi.org/10.1093/bioinformatics/btx025

Katoh, K., Rozewicki, J., & Yamada, K. D. (2019). MAFFT online service: Multiple sequence alignment, interactive sequence choice and visualization. *Briefings in Bioinformatics*, *20*(4), 1160–1166. https://doi.org/10.1093/bib/bbx108

Ketmaier, V., Argano, R., & Caccone, A. (2003). Phylogeography and molecular rates of subterranean aquatic stenasellid isopods with a peri-Tyrrhenian distribution. *Molecular Ecology*, *12*(2), 547–555. https://doi.org/10.1046/j.1365-294X.2003.01734.x

Klossa-Kilia, E., Kilias, G., & Sfenthourakis, S. (2005). Increased genetic diversity in Greek populations of the genus *Ligidium* (Crustacea: Isopoda: Oniscidea) revealed by RFLP analysis of mtDNA segments. *Contributions to Zoology*, *74*(3–4), 255–264. https://doi.org/10.1163/18759866-0740304003

Klossa-Kilia, E., Kilias, G., Tryfonopoulos, G., Koukou, K., Sfenthourakis, S., & Parmakelis, A. (2006). Molecular phylogeny of the Greek populations of the genus *Ligidium* (Isopoda, Oniscidea) using three mtDNA gene segments. *Zoologica Scripta*, *35*, 459–472. https://doi.org/10.1111/j.1463-6409.2006.00243.x

Kodandaramaiah, U., Simonsen, T. J., Bromilow, S., Wahlberg, N., & Sperling, F. (2013). Deceptive single-locus taxonomy and phylogeography: Wolbachia-associated divergence in mitochondrial DNA is not reflected in morphology and nuclear markers in a butterfly species. *Ecology and Evolution*, *3*(16), 5167–5176. https://doi.org/10.1002/ece3.886

Lanfear, R., Calcott, B., Ho, S. Y., & Guindon, S. (2012). PartitionFinder: Combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Molecular Biology and Evolution*, *29*, 1695–1701. https://doi.org/10.1093/molbev/mss020

Lee, T. R., Ho, S. Y., Wilson, G. D., & Lo, N. (2014). Phylogeography and diversity of the terrestrial isopod *Spherillo grossus* (Oniscidea: Armadillidae) on the Australian East Coast. *Zoological Journal of the Linnean Society*, *170*(2), 297–309. https://doi.org/10.1111/zoj.12105

Li, W. C. (2017). Morphology and molecules reveal high species diversity of *Ligidium* (Crustacea: Oniscidea: Ligiidae) in Jiangxi, China. *Zoological Journal of the Linnean Society*, *179*(3), 627–641. https://doi.org/10.1111/zoj.12464

Mamos, T., Grabowski, M., Rewicz, T., Bojko, J., Strapagiel, D., & Burzyński, A. (2021). Mitochondrial genomes, phylogenetic associations, and SNP recovery for the key invasive Ponto-Caspian amphipods in Europe. *International Journal of Molecular Sciences*, *22*(19), 10300. https://doi.org/10.3390/ijms221910300

Meier, R., Wong, W., Srivathsan, A., & Foo, M. (2016). $1 DNA barcodes for reconstructing complex phenomes and finding rare species in specimen-rich samples. *Cladistics*, *32*, 100–110. https://doi.org/10.1111/cla.12115

Montesanto, G., Caruso, D., & Lombardo, B. M. (2007). Taxonomic status of the Mediterranean terrestrial isopod, *Porcellio lamellatus* Budde-Lund, 1885 as inferred from genetic and morphological differentiation (Isopoda, Oniscidea). *Crustaceana*, *80*, 917–938. https://doi.org/10.1163/156854007781681229

Padial, J. M., Miralles, A., De la Riva, I., & Vences, M. (2010). The integrative future of taxonomy. *Frontiers in Zoology*, *7*(1), 1–14. https://doi.org/10.1186/1742-9994-7-16

Poulakakis, N., & Sfenthourakis, S. (2008). Molecular phylogeny and phylogeography of the Greek populations of the genus *Orthometopon* (Isopoda, Oniscidea) based on mitochondrial DNA sequences. *Zoological Journal of the Linnean Society*, *152*(4), 707–715. https://doi.org/10.1111/j.1096-3642.2007.00378.x

Puillandre, N., Brouillet, S., & Achaz, G. (2021). ASAP: Assemble species by automatic partitioning. *Molecular Ecology Resources*, *21*(2), 609–620. https://doi.org/10.1111/1755-0998.13281

Rambaut, A., Drummond, A. J., Xie, D., Baele, G., & Suchard, M. A. (2018). Posterior summarization in Bayesian phylogenetics using tracer 1.7. *Systematic Biology*, *67*(5), 901–904. https://doi.org/10.1093/sysbio/syy032

Rast, N. (1989). The evolution of the Appalachian chain. In A. W. Bally & A. R. Palmer (Eds.), *The geology of North America-an overview* (pp. 323–348). Geological Society of America. https://doi.org/10.1130/DNAG-GNA-A.323

Raupach, M. J., Rulik, B., & Spelda, J. (2022). Surprisingly high genetic divergence of the mitochondrial DNA barcode fragment (COI) within central European woodlice species (Crustacea, Isopoda, Oniscidea). *ZooKeys*, *1082*, 103–125. https://doi.org/10.3897/zookeys.1082.69851

Recuero, E., Rodríguez-Flores, P. C., & García-París, M. (2022). Homoplasy and morphological stasis revealed through multilocus phylogeny of new myrmecophilous species in Armadillidiidae (Isopoda: Oniscidea). *Zoological Journal of the Linnean Society*, *194*(4), 1312–1340. https://doi.org/10.1093/zoolinnean/zlab066

Rodriguez-Flores, P. C., Macpherson, E., & Machordom, A. (2021). Revision of the squat lobsters of the genus *Phylladiorhynchus* Baba, 1969 (Crustacea, Decapoda, Galatheidae) with the description of 41 new species. *Zootaxa*, *5008*(1), 1–159. https://doi.org/10.11646/ZOOTAXA.5008.1.1

Ronquist, F., & Huelsenbeck, J. P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, *19*, 1572–1574. https://doi.org/10.1093/bioinformatics/btg180

Sánchez-Vialas, A., García-París, M., Ruiz, J. L., & Recuero, E. (2020). Patterns of morphological diversification in giant *Berberomeloe* blister beetles (Coleoptera: Meloidae) reveal an unexpected taxonomic diversity concordant with mtDNA phylogenetic structure. *Zoological Journal of the Linnean Society*, *189*(4), 1249–1312. https://doi.org/10.1093/zoolinnean/zlz164

Schmalfuss, H. (2003). World catalog of terrestrial isopods (Crustacea: Isopoda). *Stuttgarter Beiträge Zur Naturkunde, A: Biologie*, *654*, 1–341.

Schultz, G. A. (1964). Two additional data on terrestrial isopod crustaceans: *Ligidium blueridgensis*, sp. nov., from Georgia and a north Carolina cave location for *Miktoniscus linearis* (patience, 1908). *Journal of the Elisha Mitchell Scientific Society*, *80*, 90–94.

Schultz, G. A. (1970). Descriptions of new subspecies of *Ligidium elrodii* (Packard) comb. nov. with notes on other isopod crustaceans from caves in North America (Oniscoidea). *American Midland Naturalist*, *84*, 36–45. https://doi.org/10.2307/2423724

Schultz, G. A. (1982). Terrestrial isopods (Crustacea: Isopoda: Oniscoidea) from North Carolina. *Brimleyana*, *8*, 1–26.

Segura-Zarzosa, I. E., Rodríguez-Almaraz, G. A., Obregón-Barboza, H., Murugan, G., Treviño-Flores, J. A., & Maeda-Martínez, A. M. (2020). New records of exotic species of Oniscidea (Crustacea: Isopoda) from northern Mexico. *Revista Mexicana de Biodiversidad*, *91*, e913098. https://doi.org/10.22201/ib.20078706e.2020.91.3098

Sfenthourakis, S., & Taiti, S. (2015). Patterns of taxonomic diversity among terrestrial isopods. *ZooKeys*, *515*, 13–25. https://doi.org/10.3897/zookeys.515.9332

Sfenthourakis, S. (1993). The genus *Ligidium* Brandt (Isopoda, Oniscidea) in Greece. Taxonomy and distribution. *Biologia Gallo-Hellenica*, *20*, 45–53.

Shultz, J. W. (2018). A guide to the identification of the terrestrial isopoda of Maryland, USA (crustacea). *ZooKeys*, *801*, 207–228. https://doi.org/10.3897/zookeys.801.24146

Srivathsan, A., Lee, L., Katoh, K., Hartop, E., Kutty, S. N., Wong, J., Yeo, D., & Meier, R. (2021). ONTbarcoder and MinION barcodes aid biodiversity discovery and identification by everyone, for everyone. *BMC Biology*, *19*, 1–21. https://doi.org/10.1186/s12915-021-01141-x

Taiti, S. (2017). Biologia e biogeografia degli isopodi terrestri (Crustacea, Isopoda, Oniscidea). *Atti Della Accademia Nazionale Italiana di Entomologia, Rendiconti*, *65*, 83–90.

Thomas, S. M., & Hedin, M. (2008). Multigenic phylogeographic divergence in the paleoendemic southern Appalachian opilionid *Fumontana deprehendor* shear (Opiliones, Laniatores, Triaenonychidae). *Molecular Phylogenetics and Evolution*, *46*(2), 645–658. https://doi.org/10.1016/j.ympev.2007.10.013

Trifinopoulos, J., Nguyen, L.-T., von Haeseler, A., & Minh, B. Q. (2016). W-IQ-TREE: A fast online phylogenetic tool for maximum likelihood analysis. *Nucleic Acids Research*, *44*(W1), W232–W235. https://doi.org/10.1093/nar/gkw256

Tsang, L. M., Ma, K. Y., Ahyong, S. T., Chan, T. Y., & Chu, K. H. (2008). Phylogeny of Decapoda using two nuclear protein-coding genes: Origin and evolution of the Reptantia. *Molecular Phylogenetics and Evolution*, *48*, 359–368. https://doi.org/10.1016/j.ympev.2008.04.009

Van Name, W. G. (1936). The American land and fresh-water isopod crustacea. *Bulletin of the American Museum of Natural History*, *71*, 1–535.

Vandel, A. (1960). *Faune de France. Isopodes Terrestres (Première Partie)*. P. Lechevalier.

Vasquez-Valverde, L. F., & Marek, P. E. (2022). Phylogenetic review of the millipede genus *Cherokia* Chamberlin, 1949 (Polydesmida, Xystodesmidae). *ZooKeys*, *1106*, 141–163. https://doi.org/10.3897/zookeys.1106.81386

Wang, J., Yang, J., Zeng, X., & Li, W. (2022). Integrative taxonomy on the rare sky-Island *Ligidium* species from southwest China (isopoda, Oniscidea, Ligiidae). *BMC Zoology*, *7*, 1–21. https://doi.org/10.1186/s40850-022-00120-1

Whiting, M. F., Carpenter, J. C., Wheeler, Q. D., & Wheeler, W. C. (1997). The Strepsiptera problem: Phylogeny of the holometabolous insect orders inferred from 18S and 28S ribosomal DNA sequences and morphology. *Systematic Biology*, *46*, 1–68. https://doi.org/10.1093/sysbio/46.1.1

Wiens, J. J. (2004). Speciation and ecology revisited: Phylogenetic niche conservatism and the origin of species. *Evolution*, *58*(1), 193–197. https://doi.org/10.1111/j.0014-3820.2004.tb01586.x

Wysocka, A., Kostoski, G., Kilikowska, A., Wróbel, B., & Sell, J. (2008). The *Proasellus* (crustacea, isopoda) species group, endemic to the Balkan Lake Ohrid: A case of ecological diversification? *Fundamental and Applied Limnology*, *172*(4), 301–313. https://doi.org/10.1127/1863-9135/2008/0172-0301

Yang, Z., & Rannala, B. (2010). Bayesian species delimitation using multilocus sequence data. *Proceedings of the National Academy of Sciences*, *107*(20), 9264–9269. https://doi.org/10.1073/pnas.0913022107

Yoshino, H., & Kubota, K. (2022). Phylogeographic analysis of *Ligidium japonicum* (isopoda: Ligiidae) and its allied species reveals high biodiversity and genetic differentiation in the Kanto region, Japan. *Entomological Science*, *25*, e12501. https://doi.org/10.1111/ens.12501

Zimmermann, B. L., Campos-Filho, I. S., Depra, M., & Araujo, P. B. (2015). Taxonomy and molecular phylogeny of the neotropical genus *Atlantoscia* (Oniscidea, Philosciidae): DNA barcoding and description of two new species. *Zoological Journal of the Linnean Society*, *174*(4), 702–717. https://doi.org/10.1111/zoj.12256

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Recuero, E., & Caterino, M. S. (2024). Hidden diversity in eastern North America: The genus *Ligidium* (Oniscidea, Ligiidae) in the southern Appalachian Mountains. *Zoologica Scripta*, *00*, 1–20. https://doi.org/10.1111/zsc.12661