Phylogenetic revision of the genus *Cherokia* (Chamberlin, 1949) (Polydesmida: Xystodesmidae)

Luisa Fernanda Vasquez-Valverde

Thesis submitted to the faculty of Virginia Polytechnic Institute and State University

in partial fulfillment of the requirements for the degree of

Master of Science

in

Entomology

Paul Marek

Robin Andrews

William Shear

May 7, 2021

Blacksburg, VA

Keywords: *Cherokia*, phylogenetics, subspecies, morphology.

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Academic Abstract

The family Xystodesmidae (Polydesmida) includes 521 species with a center of diversity concentrated in the Appalachian Mountains. Within this family, the genus *Cherokia*, a monotypic taxon with the type species *Cherokia georgiana*, is divided into three subspecies. The last revision of this genus was made by Richard Hoffman in 1960. Here, I used morphological and molecular data sets to review the genus, and evaluate whether it is a monophyletic group. I included material from literature records and three natural history collections. Newly collected samples were obtained through a citizen science project. Morphological characters such as the shape of the paranota, body size, and coloration were evaluated. Seven gene loci were used to estimate a molecular phylogeny of the genus, and a species delimitation analysis was used to evaluate the status of the subspecies. The geographical range of *Cherokia* was expanded to include a newly reported state (Virginia) and ca. 160 new localities compared to the previously known range. Morphological characters such as the shape of the paranota and body size that were historically used to establish subspecies, showed a direct relation with geographical distribution and elevation (clinal variation), but not with the phylogeny. Coloration was variable and did not accord with geography or phylogeny. The phylogeny recovered a monophyletic lineage, and the species delimitation test supports a single species. The molecular and morphological evidence showed that *Cherokia* is a monotypic genus with the sole species *Cherokia georgiana* being geographically widespread and highly variable in its morphology.

General Audience Abstract

Millipedes are a mega-diverse group of soil dwelling animals that feed on leaf litter. The Appalachian Mountains has a huge diversity of millipedes, in particular those in the family Xystodesmidae. Within this family, I studied the genus *Cherokia*, commonly known as "Georgia flat-backed millipedes". The single species in this group, *Cherokia georgiana*, is divided into three subspecies. The last thorough study of this genus was done by Richard Hoffman in 1960, so a modern analysis with DNA sequencing was needed to test subspecies boundaries. Here, I used hundreds of specimens from three natural history museums, and fresh specimens obtained for DNA sequencing with the help of citizen scientists. I measured the shape and size of the body and coloration patterns to determine if they were related to the geographical distribution of *Cherokia*. I used DNA sequencing to make an evolutionary tree of the genus. I found *Cherokia* individuals in Virginia for the first time and found ca. 160 new sites or locations not reported previously. The shape and size of the body was related to millipede location and elevation. Coloration was not related to geography or phylogeny, and in some localities, multiple color patterns co-existed. The genetic information from DNA sequencing indicated that all *Cherokia* were more closely related to each other than to any other millipede genus. In conclusion, I found that the genus *Cherokia* is a single species, *Cherokia georgiana*, that has a wide geographical distribution and a considerable diversity of body shape and color. Diversity of shape and color does not reflect subspecies boundaries but instead reflects intra-population and geographic variation.

Acknowledgments

I thank Dr. Marek, whom I met in 2016 while doing my first ever field work on millipedes outside my home country. Paul, I appreciate all your help and guidance during the past two years, in my research, and in all the other aspects since I came to the United States to start my Master's program. I am grateful for all your patience and guidance in stressful times, and for trusting me and giving me the chance to grow as a researcher in the fascinating field of myriapodology.

I thank my committee, Dr. Robin Andrews and Dr. William Shear, for their guidance, advice and support during the development of this research. I look forward to learn more in the future from both of you. The Marek lab has been my home during my research. I want to specially thank Dr. Derek Hennen for his patience and help in the lab, and all the laughs, memes and funny stories we have shared while running PCR's and figuring out Mesquite. I also want to thank Maddie Hellier and Dr. Jackson Means for their help and support with this project.

I would like to especially thank my mom who, despite the distance, has been my support in the harder times, and who motivated me to be better every day. Thanks to the rest of my family for always checking in and taking care of me, with a call, a text, a cat picture or a meme. I will always be grateful with Dr. Eduardo Florez and Ms. Daniela Martinez, who introduced me to the fascinating world of millipedes around 5 years ago, and still support me to this day. To all my friends here and back home (whom I cannot list here or this paragraph would be way too long) for their support and encouragement when I felt like giving up.

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CHAPTER 1

1. Introduction

The subphylum Myriapoda includes the classes Pauropoda, Symphyla, Chilopoda, and Diplopoda. The Diplopoda, commonly known as millipedes, is the most diverse and abundant of the group. This class has 14,336 described species, and an estimated undescribed diversity of between 20,000 and 80,000 species (Brewer *et al.,* 2012; Sierwald & Spelda, 2021). In contrast, there are about 3,500 species in the class Chilopoda (centipedes), 800 in the class Pauropoda, and 200 in the class Symphyla (Means *et al.,* 2021a). Millipedes are detritivores, feeding mainly on decaying logs and leaves, with some fungivorous species (Marek *et al.,* 2012). Consequently, millipedes play a key role in the cycling of nutrients in the soil. By initiating the decomposition process via mechanical fragmentation of organic matter, millipedes increase the surface area available for subsequent colonization by bacteria and fungi (Pitz & Sierwald, 2010). This process liberates nutrients and frees up carbon, nitrogen, and simple sugars for utilization by plants, microorganisms and other animals. Millipedes consume and process nearly 30.6% of the leaf litter produced annually in tropical forests (Dangerfield & Telford, 1991) and 36% in conifer forests (Cárcamo *et al.,* 2000).

Millipedes are present on all the continents except Antarctica. Nevertheless, despite their geographical ubiquity, due to their small size and low dispersion capacity, most millipede species have restricted distributions and high levels of endemicity (Enghoff, 2015; Means & Marek, 2017). The Appalachian Mountains ecoregion is a hotspot of biodiversity, especially for low-mobility animals such as millipedes, other invertebrates, and salamanders. The millipede family Xystodesmidae has its greatest species diversity in this region, (Marek *et al.,* 2014; Means

& Marek, 2017; Means *et al.,* 2021a). This family, has 521 species and exhibits a considerable diversity of body sizes, aposematic colors, mimicry assemblies, and gonopodal variation (Marek *et al.,* 2014; Means *et al.,* 2021b). Xystodesmid millipedes have been traditionally differentiated at the species level by their male genitalia, specifically their gonopods, the first pair of legs of the seventh body ring that are modified as sperm transfer organs (Means & Marek, 2017). Currently, species delimitation relies on molecular phylogenetics in combination with morphological (genitalic) features (Marek *et al.,* 2018).

The monotypic Appalachian genus *Cherokia* (Xystodesmidae) was described by Ralph Vary Chamberlin with *Fontaria georgiana* (Bollman, 1888) as its generotype (Chamberlin, 1949). After its description, several authors proposed multiple synonymies of the type species *Cherokia georgiana* based on gonopod morphology (Causey, 1950; Hoffman, 1950; Chamberlin & Hoffman, 1958). These authors, however, pointed out considerable color and size variation of individuals of *Cherokia*.

When Richard Hoffman's (1960) revised *Cherokia,* he proposed it as a monotypic genus, with three subspecies, *Cherokia georgiana georgiana*, *Cherokia georgiana ducilla,* and *Cherokia georgiana latassa.* He differentiated the three subspecies from each other based on morphological features including the position of the scapulora (see definition in next sentence), and the ratio of the body length versus its width. The scapulora is a term defined by Hoffman as *'from the Latin "scapula," a shoulder, and "ora," the rim of a shield'* (Hoffman, 1960: 231). The scapulora in *C. g. latassa* is found in a marginal position, which separates it from *C. g. georgiana* and *C. g. ducilla* that have a submarginal scapulora (Fig. 1, a - b). The subspecies, *Cherokia georgiana georgiana* and *C. g. ducilla,* are differentiated from each other based on the ratio of the body length versus its width (Hoffman, 1960).

Hoffman confronted several problems during his revision of the genus *Cherokia*. The first one was that *"despite the diversity of body form, color pattern, and morphological details which occurs in the genus, the male gonopods remain essentially similar"* (Hoffman, 1960: 227). He also struggled to assign all individuals to one of the subspecies. For this reason, Hoffman proposed an intermediate form, termed an "intergrade" between *C. g. georgiana* and *C. g. ducilla.* These intergrades made up a wide geographical band (~30 km) between the distributions of *C. g. georgiana* and *C. g. ducilla*.

Cherokia is mentioned in tribal revisions (Hoffman, 1978) and checklists (Shelley, 1980 and 2000; Marek *et al.,* 2014) after 1960. Recent syntheses of morphological and molecular characteristics, placed the genus *Cherokia* within the family Xystodesmidae as sister to the genus *Pleuroloma* (Means & Marek, 2017; Means *et al.* 2021a).

For my research, I used natural history collections in combination with material sampled since 2016 to now from nearly 200 locations within the range of *Cherokia*. These samples, prepared for preservation of DNA, provided the basis to infer an evolutionary history using molecular phylogenetics and to determine the status of the three subspecies within *Cherokia*. I found that *Cherokia* is a monophyletic lineage that is geographically widespread and morphologically variable. Although morphological features such as the scapulora, width-to-length ratio, color, and gonopods vary as a function of geography, and the molecular phylogeny uncovered discrete and statistically well-supported clades, these characters are not consistent with one another and do not support multiple intraspecific taxa. Using a distance-based species delimitation method, divergence was not detected, thereby supporting a single and yet highly morphologically diverse species.

1.1. Objectives

The genus *Cherokia* was described more than 70 years ago, and no modern revision of the genus has been conducted. In this work, I used material from natural history collections and literature records to infer a comprehensive geographical distribution of *Cherokia*. I used molecular phylogenetics for 106 specimens of *Cherokia* sampled from throughout its range to infer a phylogeny of the genus. This phylogeny was used to evaluate morphological characters that I measured on each specimen to produce a comprehensive understanding of the genus while clarifying the status of the three subspecies of *Cherokia georgiana*.

2. Materials and Methods

To describe the geographical range of the genus *Cherokia,* I used records in the literature, natural history collections, and new collections from the field. All the localities of specimens of *Cherokia* documented in Hoffman (1960) and from the Virginia Tech Insect Collection, Virginia Museum of Natural History (VMNH), and Florida State Collection of Arthropods (FSCA) were digitized. Digitization involved transcribing the label data of specimens in a spreadsheet using the Darwin Core data standard (Wieczorek, 2012). I entered the text-based details of the label including state, county, and any other locality information in the spreadsheet. In cases where precise geographical coordinates (e.g. latitude and longitude) were not provided, the text of the localities from the labels was georeferenced and geographical coordinates automatically extracted using the software GEOLocate (Rios & Bart, 2010) to retrospectively obtain decimal degree coordinates. To supplement this data set, localities from *Cherokia* specimens from the Virginia Tech Insect Collection (VTEC) that were already digitized with geographical coordinates recorded at the time of collection were downloaded from the online database SCAN (Barkworth *et al.,* 2019). I used this data set of coordinates (from collections and literature), to produce a comprehensive map of the geographical range of *Cherokia*.

To document molecular and morphological evolution of *Cherokia*, I selected specimens in the Virginia Tech Insect Collection. These specimens were selected because both morphological and molecular characters could be scored. Individual millipede specimens or their tissues were fixed in either ≥ 95% ethanol or Qiagen RNALater to preserve DNA and other genetic material. Whole body specimens (not including tissue preserved for DNA) were preserved in 70% isopropanol.

New samples were needed from some localities that had not previously been sampled; these localities were in the periphery of the distribution of *Cherokia* or in areas where DNA-grade specimens were unavailable. A season of fieldwork was planned for the Summer 2020, however, due to the SARS-CoV-2/COVID-19 pandemic, and state and university restrictions, travel was not feasible. In response, and with the objective of obtaining these required samples, I developed a citizen science project. This enabled the general public to become involved in the collection of millipedes of the genus *Cherokia*. For the Citizen Science project, I designed collection kits and information pamphlets, with step-by-step instructions and other information for the public to obtain samples in an accurate and legal way (Fig. 3). Citizen scientists were recruited with social media through Facebook and Twitter, and the kits were shipped to interested participants. A small plastic keychain with a picture of *Cherokia* was included in the kit as a gift for participants. Once the participants received the kit and collected millipedes, they were instructed to ship the millipedes back to the lab at Virginia Tech, so I could identify, process, and preserve them.

I included specimens of *Cherokia* obtained from the citizen science initiative for the molecular and morphological analyses. For each of the newly obtained samples, I removed the legs from the left side of the body from segments 8 - 20 and preserved them in 1.5 mL microcentrifuge tubes with 500 µL of 100% ethanol. Afterward, I labeled the tubes with a unique specimen number with the prefix "MPE-" and stored them in -80ºC ultracold freezer. The rest of the specimen's body was preserved in 70% isopropanol, labeled with the same specimen code, and registered in the SCAN database with all the collection information provided from the citizen science participant (e.g. state, county, date, time, geographical coordinates, collector) (Means *et al.*, 2015).

For the specimens selected for the molecular analysis, I took four legs from each individual for DNA extractions with a Qiagen DNeasy kit. The DNA obtained from the extraction was amplified via polymerase chain reaction (PCR) for seven gene regions: cytochrome oxidase subunit I (COI), small subunit RNA (12S), tRNA-Valine (tRNA-Val), large subunit RNA (16S), elongation factor-alpha (EF1α), RNA polymerase II largest subunit (RNAPol2) and F-box (fBox). Amplification of DNA was carried out according to Means *et al* (2021b). These PCR amplicons were cleaned, quantified, normalized, and sequenced on an Applied Biosystems ABI 3730 capillary sequencer at the University of Arizona Genetics Core.

The sequences were analyzed in Mesquite (Version 3.61) (Maddison & Maddison, 2019) using the sequence analysis module Chromaseq (Version 1.52) and phred and phrap (Maddison & Maddison, 2020, Ewing *et al.,* 1998), for chromatogram base calling, trimming, quality control and generation of matrices. The outgroups were selected based on the analysis conducted by Means et al. (2021a) and included: *Pleuroloma flavipes*, *Pleuroloma plana* and *Pleuroloma cala*. I then aligned *Cherokia* sequences with the progressive sequence alignment program MAFFT (Version 7) using the model L-INS-I, that is reported to be probably a more accurate model from the alternatives available in MAFFT (Katoh & Standley, 2013). With the sequences aligned for each of the genes, I ran a nucleotide base composition chi-square test in IQ-TREE 2 (Version 2.0.4, Minh *et al.,* 2020) to test the heterogeneity of the sequences ($H_{alternative}$ = homogeneity), and excluding the sequences of the outgroup taxa. The sequences that failed the heterogeneity test were excluded from the phylogeny. Afterwards, I partitioned each locus by gene, intron/exon location, and codon position, and concatenated the seven loci in a single matrix. The partitioned matrix was analyzed using ModelFinder to test alternative nucleotide evolution models and to infer the best-fitting model (Kalyaanamoorthy *et al.,* 2017). The selected

model was then used to estimate a phylogenetic tree for the genus, in the maximum likelihood based software IQ-TREE 2 (Version 2.0.4, Minh *et al.,* 2020).

A species delimitation analysis was used to determine whether or not the subspecies of *Cherokia georgiana* represent distinct groups. To test this, I used Automatic Barcode Gap Discovery (ABGD). This method uses an alignment of sequences of a single locus (COI) to make a pairwise distance matrix and determine if a barcode gap exists. A barcode gap is observed when the intraspecific distance among unique sequences is smaller than the interspecific distance (Puillandre *et al.*, 2012). I ran this analysis in the ABGD online server using the alignment of *Cherokia* sequences for the locus COI, excluding the outgroup the sequences.

For the analysis of morphological features, I revisited the morphological traits described in Hoffman (1960): width-to-length ratio, color, gonopods, and the position of the scapulora (Fig. 1, A- B). Hoffman (1960) measured the entire length of the trunk of the millipedes; however, due to the flexibility of the trunk and the rings that make up the trunk—causing concertinaing compression and extension—these overall length measurements typically have a high amount of error. To better evaluate size variation, I dissected the 12^{th} body ring and measured the (1) width (Fig. 1, C) and (2) length (Fig. 1, D) of the metazonite in dorsal view, and (3) the paranota extension from a posterior view (Fig. 1, E). Measurement of a single ring reduces error because a single diplosegmental ring is rigid and inflexible, and presumably correlated with overall length. To control for an irregular body size distribution, I used a natural logarithm to transform the raw measurements. Linear regressions were used to evaluate the relationship between the measurements and elevation.

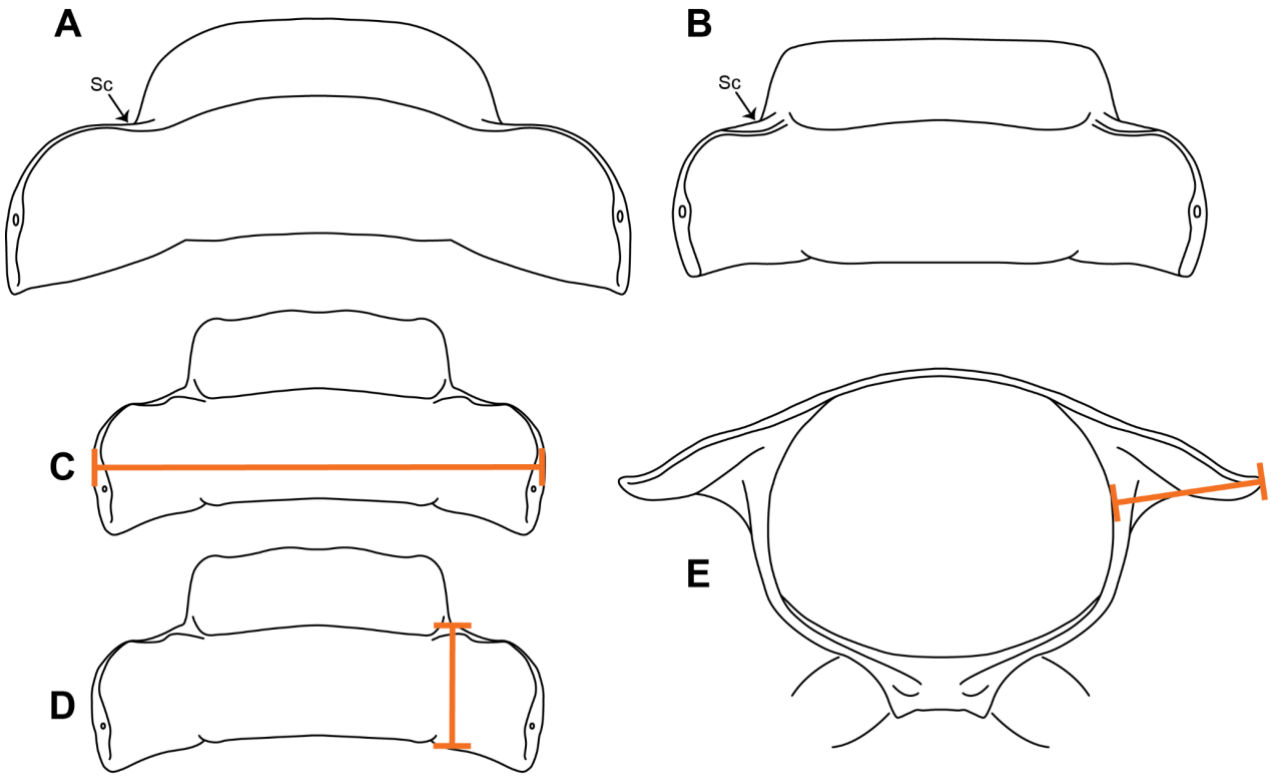

Figure 1. Position of the scapulorae. A) Strictly marginal and **B)** Submarginal; **Measurements of the 12th body ring. C)** Metazonite width, **D)** Metazonite length and **E)** Paranota extension. Adapted from: Hoffman, 1960

Cherokia georgiana exhibits a considerable diversity in coloration patterns throughout its geographical distribution. To evaluate this variation I examined, coded, and scored pictures of *Cherokia* from the specimens selected for the analysis, and those observed on iNaturalist (Available from https://www.inaturalist.org. Accessed May, 2020). These pattern codes were then used to score for coloration patterns and were mapped onto the distribution of *Cherokia* to test if there is any correspondence with geographical areas.

I used the concept that species are separately evolving metapopulation lineages, meaning that there is limited gene flow between lineages and species are on their own trajectories and do not exchange substantial genetic information (De Queiroz, 2007). I used a phylogenetic based

species delimitation criterion that species are a monophyletic and genetically distinct group of organisms that are diagnosable from others by a combination of unique characteristics (Cracraft, 1983 and 1992). A subspecies is defined as multiple populations of the same species, with sufficient morphological characters to differentiate them from another; the populations are located in subdivisions of the geographical range of the species (Mayr & Ashlock 1991, in Jorgensen *et al.* 2013). Subspecies are not a natural category, but more a useful tool for the taxonomist and natural history collections to consider geographical, ecological and behavioral differences inside a species (Mayr, 1982). The delimitation of subspecies most of the time will rely on a specialist on the group (Jorgensen *et al.* 2013), as is the case of *Cherokia* subspecies described by Hoffman (1960). I used the phylogeny, the ABGD analysis, and the distribution of morphological and molecular characters to test the hypothesis that *Cherokia* is a monotypic genus, and to determine the status of the subspecies.

3. Results

3.1. Geographical distribution

A total 201 reports were digitized and georeferenced from Hoffman (1960) (n = 103), the VMNH (n = 31), and FSCA (n = 67) natural history collections. Localities from the VTEC were already databased and thereby added 222 *Cherokia* records to my database. Table 1 shows the number of individuals, total number of states, counties and localities obtained from each data source. The total of states and counties in the table do not sum to localities because multiple localities can occur in a single county. Duplicated data was excluded.

Source		Number of individuals				Number of localities		
		Males	**Females**	**Juveniles**	**Total**	**States**	**Counties**	**Localities**
Literature records	Hoffman, 1960	224	136	1	361	6	43	93
Collections	VMNH	39	25	1	65	7	25	30
	FSCA	107	62	21	190	4	24	45
	VTEC	143	78	11	232	7	47	85
Total		513	301	34	848	7	96	253

Table 1. Total number of individuals and localities used to determine the geographical range of *Cherokia*.

A map of the geographical distribution of the genus *Cherokia* (Fig. 2) was constructed using 253 coordinates from localities representing 848 individuals. The geographical distribution includes seven states: Kentucky, Virginia, Tennessee, North Carolina, South Carolina, Alabama and Georgia. Overall, ninety-six counties have records of *Cherokia* individuals. The geographical range of *Cherokia* described by Hoffman (1960), included six states, 43 counties and 93 localities (Table 1). Here I report a seventh state (Virginia) and 53 new counties, for a total of 160 new localities where specimens of the genus have been collected.

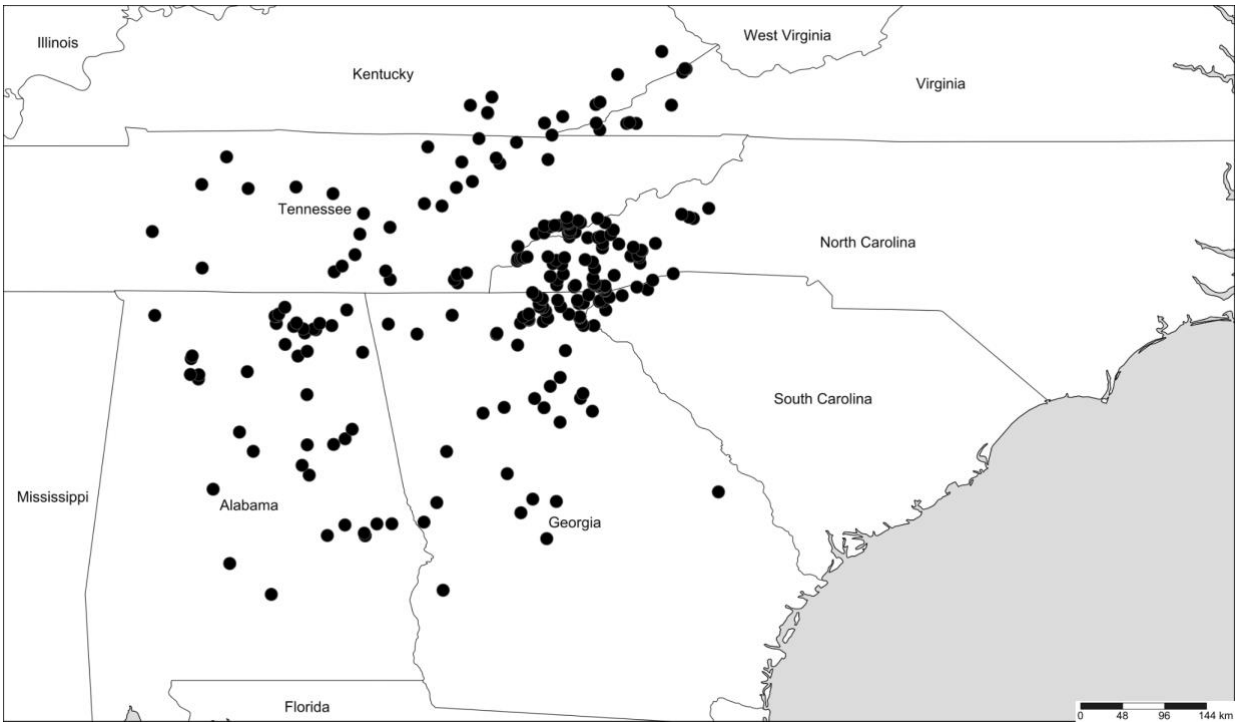

Figure 2. Geographical distribution of the genus *Cherokia.*

3.2. Citizen Science project

The Citizen Science initiative received a positive response on social media, with more than 100 responses from both the Facebook post and the tweet in Twitter. This resulted in 68 people completing a Google form to express their interest in participating in the project. Fifty people were selected based on their location in proximity to areas previously not surveyed. Due the limited number of kits available, our efforts focused on the collection of millipedes in localities needed in Georgia, Alabama and Tennessee. A total of 41 kits (Fig. 3) were shipped to participants during the months of July and August of 2020. From October 2020 to March 2021, a total of 23 live millipedes were received as a result of this project, and 13 of them were identified as *Cherokia* and included in the morphological and molecular analysis.

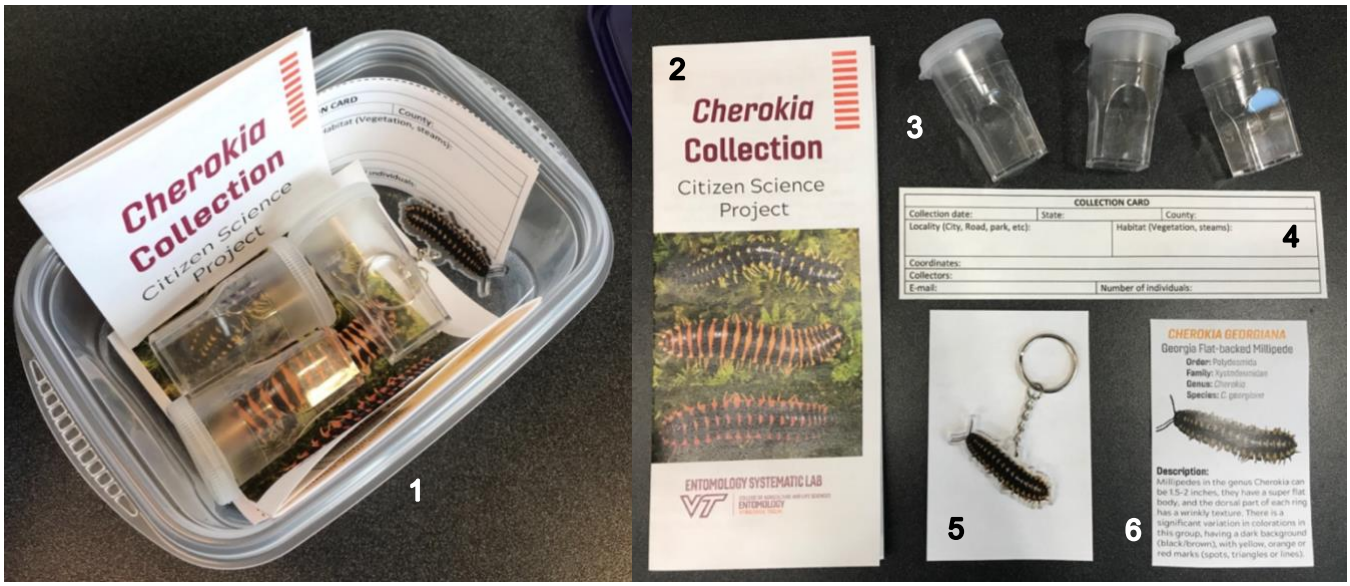

Figure 3. Citizen Science collection kit. Each collection kit contained: **1)** A plastic food container (32 FL OZ), **2)** Instruction flyer: with step-by-step instructions of collecting and shipping, **3)** Clear plastic collection vials, **4)** Collection card, **5)** gift for the participant: millipede keychain, and **6)** *Cherokia* identification card.

3.2. Molecular and phylogenetic analysis

A total of 106 individual *Cherokia* were included in the molecular phylogenetic analysis: 74 males, 31 females and one juvenile. The amplification and sequencing of DNA for the loci COI, 12S, tRNA-Val and 16S, had a high rate of success, and only one specimen did not amplify (Table 2). For the locus fBox, the rate of success in amplification and sequencing was 96%, and for the loci EF1α and RNAPol2 that rate was considerably lower with 75% and 55% of the total sequences obtained. When amplifications and/or sequencing failed, amplifications were repeated up to three times using the same DNA extraction before discontinuing attempts.

Table 2. List of specimens used in the phylogenetic reconstruction and the loci that were successfully sequenced for each one.
The column "16S" includes the loci 12S, tRNA-VAL and 16S

Specimen information				High quality sequence				
Specimen code	**State, County**	**Latitude**	**Longitude**	**COI**	**EF1a**	**16S**	**RNAPol2**	**fBox**
MMC0264	Virginia, Lee	36.73846	-82.87839	Yes	Yes	Yes	Yes	-
MMC0265	Virginia, Lee	36.73846	-82.87839	Yes	Yes	Yes	-	Yes
MMC0269	Virginia, Lee	36.73846	-82.87839	Yes	Yes	Yes	Yes	Yes
MPE00490	North Carolina, Macon	35.07875	-83.24715	Yes	-	Yes	-	Yes
MPE00493	North Carolina, Macon	35.08157	-83.23599	Yes	-	Yes	-	Yes
MPE00499	North Carolina, Macon	35.08157	-83.23599	Yes	Yes	Yes	-	Yes
MPE00501	North Carolina, Macon	35.08157	-83.23599	Yes	Yes	Yes	-	Yes
MPE00506	North Carolina, Macon	35.06815	-83.23860	Yes	-	Yes	-	Yes
MPE00512	North Carolina, Macon	35.05170	-83.19330	Yes	Yes	Yes	Yes	Yes
MPE00514	South Carolina, Oconee	34.90753	-83.18076	Yes	Yes	Yes	Yes	Yes
MPE00517	South Carolina, Walhalla	34.81014	-83.12329	Yes	-	Yes	Yes	Yes
MPE00523	North Carolina, Jackson	35.16706	-83.03644	Yes	-	Yes	-	Yes
MPE00535	Tennessee, Pioneer	36.37317	-84.25250	Yes	Yes	Yes	Yes	Yes
MPE00540	Tennessee, Campbell	36.31748	-84.21465	Yes	Yes	Yes	Yes	-
MPE01258	Tennessee, Van Buren	35.66126	-85.34640	Yes	Yes	Yes	Yes	-
MPE01263	Alabama, Winston	34.09967	-87.31973	Yes	Yes	Yes	-	Yes
MPE01271	Georgia, Dawson	34.57043	-84.24454	Yes	Yes	Yes	-	Yes
MPE01272	Alabama, Madison	34.74383	-86.51136	Yes	Yes	Yes	-	Yes
MPE01308	Georgia, Dawson	34.55937	-84.24968	Yes	Yes	Yes	Yes	Yes
MPE01336	Alabama, Winston	34.10174	-87.32034	Yes	Yes	Yes	-	Yes
MPE01473	North Carolina, Jackson	35.49380	-83.15572	Yes	Yes	Yes	-	Yes
MPE01508	Tennessee, Sevier	35.63609	-83.49378	Yes	-	Yes	Yes	Yes
MPE01512	North Carolina, Macon	35.07812	-83.24668	Yes	Yes	Yes	-	Yes

MPE01532	South Carolina, Greenville	35.18396	-82.42769	Yes	Yes	Yes	Yes	Yes
MPE01547	North Carolina, Haywood	35.58614	-83.07365	Yes	Yes	Yes	-	Yes
MPE01585	Georgia, Rabun	34.74020	-83.39213	Yes	Yes	Yes	-	Yes
MPE01822	Georgia, Rabun	34.84465	-83.58818	Yes	Yes	Yes	Yes	Yes
MPE02181	Tennessee, Morgan	36.06981	-84.66313	Yes	Yes	Yes	Yes	Yes
MPE02360	Georgia, Floyd	34.56287	-85.06811	Yes	Yes	Yes	-	Yes
MPE02683	Tennessee, Dickson	36.10172	-87.28539	Yes	Yes	Yes	-	Yes
MPE02700	Tennessee, Dickson	36.10172	-87.28539	Yes	Yes	Yes	-	Yes
MPE02702	Tennessee, Dickson	36.10172	-87.28539	Yes	Yes	Yes	Yes	Yes
MPE02823	Tennessee, Morgan	36.13206	-84.49780	Yes	Yes	Yes	Yes	Yes
MPE03167	Kentucky, Pulaski	36.91564	-84.51827	Yes	Yes	Yes	Yes	Yes
MPE03215	Kentucky, Pulaski	36.91564	-84.51827	Yes	Yes	Yes	Yes	Yes
MPE03216	Kentucky, Pulaski	36.91564	-84.51827	Yes	Yes	Yes	Yes	Yes
MPE03234	Kentucky, Pike	37.46902	-82.54621	Yes	Yes	Yes	Yes	Yes
MPE03252	Kentucky, Knott	37.23148	-83.00081	Yes	Yes	Yes	Yes	Yes
MPE03316	Georgia, Towns	34.83647	-83.77109	-	Yes	-	Yes	Yes
MPE03318	Georgia, Towns	34.83326	-83.77644	Yes	-	Yes	Yes	Yes
MPE03331	Georgia, Union	34.84084	-83.80223	Yes	Yes	Yes	-	Yes
MPE03692	Georgia, Tift	34.39251	-83.53917	Yes	Yes	Yes	Yes	Yes
MPE03902	Georgia, Barrow	34.02540	-83.69500	Yes	-	Yes	-	Yes
MPE03992	Virginia, Wise	36.91761	-82.44585	Yes	Yes	Yes	Yes	Yes
MPE04252	Georgia, Jackson	34.11667	-83.59278	Yes	Yes	Yes	-	Yes
MPE04345	Georgia, Lumpkin	34.67500	-84.00150	Yes	-	Yes	-	Yes
MPE04356	Georgia, Towns	34.95380	-83.83590	Yes	-	Yes	-	Yes
MPE04365	Georgia, Lumpkin	34.70780	-83.91500	Yes	Yes	Yes	Yes	Yes
MPE04376	Georgia, Rabun	34.73910	-83.38870	Yes	-	Yes	-	Yes
MPE04383	Georgia, Rabun	34.88130	-83.35390	Yes	Yes	Yes	-	Yes
MPE04408	Georgia, Towns	34.92560	-83.77760	Yes	-	Yes	Yes	Yes
MPE04422	Georgia, Union	34.77010	-83.91630	Yes	Yes	Yes	-	Yes

MPE04515	South Carolina, Oconee	34.89680	-83.18960	Yes	-	Yes	Yes	Yes
MPE04539	Georgia, White	34.72620	-83.72160	Yes	Yes	Yes	-	Yes
MPE04550	South Carolina, Oconee	35.94270	-83.08660	Yes	Yes	Yes	-	Yes
MPE04551	South Carolina, Oconee	34.94270	-83.08660	Yes	-	Yes	Yes	Yes
MPE04552	South Carolina, Oconee	34.94270	-83.08660	Yes	-	Yes	Yes	Yes
MPE04558	Georgia, White	34.69200	-83.76740	Yes	-	Yes	Yes	Yes
MPE04662	Alabama, Madison	34.74770	-86.53050	Yes	Yes	Yes	-	Yes
MPE04808	Tennessee, Sevier	35.70450	-83.52470	Yes	-	Yes	-	Yes
MPE04820	Tennessee, Sevier	35.72890	-83.40640	Yes	Yes	Yes	Yes	Yes
MPE05011	North Carolina, Monroe	35.46320	-84.02790	Yes	-	Yes	Yes	Yes
MPE05012	North Carolina, Graham	35.34880	-83.97680	Yes	Yes	Yes	Yes	Yes
MPE05013	North Carolina, Graham	36.35790	-83.71850	Yes	Yes	Yes	-	Yes
MPE05014	North Carolina, Yancey	35.76480	-82.26510	Yes	-	Yes	Yes	Yes
MPE05052	Alabama, Macon	32.51455	-85.61167	Yes	-	Yes	Yes	Yes
MPE05057	Georgia, Lamar	33.12607	-84.13859	Yes	-	Yes	-	Yes
MPE05058	Georgia, Lamar	33.12607	-84.13859	Yes	Yes	Yes	Yes	Yes
MPE05059	Georgia, Lamar	33.12607	-84.13859	Yes	Yes	Yes	-	-
MPE05064	Alabama, Macon	32.48710	-85.60289	Yes	Yes	Yes	Yes	Yes
MPE05071	Alabama, Macon	32.48710	-85.60289	Yes	Yes	Yes	Yes	Yes
MPE05087	Alabama, Macon	32.49060	-85.99154	Yes	Yes	Yes	Yes	Yes
MPE05088	Alabama, Macon	32.48949	-85.60178	Yes	Yes	Yes	Yes	Yes
MPE05090	Georgia, Harris	32.62842	-84.99641	Yes	-	Yes	Yes	Yes
MPE05093	Alabama, Tallapoosa	32.59872	-85.81039	Yes	-	Yes	Yes	Yes
MPE05094	Alabama, Tallapoosa	32.59872	-85.81039	Yes	Yes	Yes	-	Yes
MPE05095	Alabama, Tallapoosa	32.59872	-85.81039	Yes	Yes	Yes	Yes	Yes
SPC000009	Tennessee, Cumberland	35.90401	-84.99195	Yes	Yes	Yes	Yes	Yes
SPC000035	North Carolina, Graham	35.34870	-83.97660	Yes	-	Yes	-	Yes
SPC000045	South Carolina, Greenville	35.11707	-82.63942	Yes	-	Yes	Yes	Yes
SPC000050	Georgia, Lumpkin	34.70749	-83.91617	Yes	Yes	Yes	Yes	Yes

SPC000053	Georgia, Murray	34.75646	-84.70615	Yes	Yes	Yes	Yes	Yes
SPC000054	Georgia, Murray	34.75646	-84.70615	Yes	Yes	Yes	Yes	Yes
SPC000056	Georgia, Murray	34.75646	-84.70615	Yes	Yes	Yes	-	Yes
SPC000060	Alabama, Marshall	34.38539	-86.19906	Yes	Yes	Yes	-	Yes
SPC000061	Alabama, Marshall	34.38539	-86.19906	Yes	Yes	Yes	-	Yes
SPC000062	Alabama, Marshall	34.38539	-86.19906	Yes	Yes	Yes	-	Yes
SPC000064	Alabama, Jackson	34.64966	-85.94576	Yes	Yes	Yes	-	Yes
SPC000073	Alabama, Jackson	34.60658	-86.11101	Yes	Yes	Yes	-	Yes
SPC000174	Kentucky, Harlan	36.92358	-83.22477	Yes	Yes	Yes	Yes	Yes
SPC000307	Virginia, Dickenson	37.29292	-82.30893	Yes	Yes	Yes	Yes	Yes
SPC000354	North Carolina, Macon	35.06337	-83.43687	Yes	-	Yes	Yes	Yes
SPC000469	Alabama, Jackson	34.64966	-85.94576	Yes	Yes	Yes	-	Yes
SPC000477	Tennessee, Wilson	36.07506	-86.31454	Yes	Yes	Yes	Yes	Yes
SPC000490	Tennessee, Davidson	36.05945	-86.80724	Yes	Yes	Yes	Yes	Yes
SPC000597	Kentucky, McCreary	36.83420	-84.33970	Yes	Yes	Yes	Yes	Yes
SPC000604	Kentucky, Whitley	36.84121	-84.34126	Yes	Yes	Yes	Yes	Yes
SPC000790	Kentucky, Harlan	36.73285	-83.22161	Yes	Yes	Yes	Yes	Yes
SPC000892	North Carolina, Graham	35.34608	-83.96907	Yes	Yes	Yes	-	Yes
SPC000897	North Carolina, Macon	35.18037	-83.56044	Yes	Yes	Yes	Yes	Yes
SPC000903	North Carolina, Macon	35.26787	-83.57239	Yes	Yes	Yes	-	Yes
SPC000911	Tennessee, Monroe	35.46322	-84.02760	Yes	Yes	Yes	Yes	Yes
SPC000917	North Carolina, Graham	35.35760	-83.71830	Yes	Yes	Yes	-	Yes
SPC000975	Alabama, Lawrence	34.33760	-87.38400	Yes	Yes	Yes	-	Yes
SPC001108	Tennessee, Cocke	35.75170	-83.20680	Yes	Yes	Yes	-	Yes
SPC001124	Alabama, Calhoun	33.58338	-85.73607	Yes	-	Yes	Yes	Yes
			Total	**105**	**79**	**105**	**59**	**102**

The multiple sequence alignment in MAFFT and inference of nucleotide evolution models in ModelFinder resulted in a 3,865 bp concatenated matrix divided into six partitions and composed of 142 bp (12S, TIM+F+G4 nucleotide evolution model), 82 bp (tRNA-Val, TIM+F+G4), 1081 bp (16S, TIM+F+G4), 600 bp (COI, pos1 TN+I+G4, pos 2 TIM3+F+R2 and pos 3 TIM3+F+G4), 585 bp (EF1α, pos 1 & 2 TN+I+G4, pos 3 TIM3+F+R2 and intron GTR+F+I+G4), 978 bp (RNAPol2, pos 1, 2, 3 & intron 1 TN+F+R2 and intron 2 TIM+F+G4) and 397 bp (fBox, pos 1 & 2 TN+I+G4 and pos 3 TIM3+F+R2). Out of the 3,865 nucleotide characters, 2,726 correspond to constant sites, 738 were parsimony-informative, and 401 were singleton sites. The average uncorrected pairwise distance for COI sequences between individuals from the same locality was 0.00470 (max. = 0.01644, min. = 0, s = 0.005). The estimated phylogeny for *Cherokia* using the seven loci and the above-mentioned partitions and models is shown in figure 5.

The ABGD analysis included high-quality COI sequences for 105 specimens of *Cherokia*. The analysis was carried out on the ABGD web server using the Jukes-Cantor (JC69) substitution model and a relative gap width of 1.5X. The results of this analysis showed that the COI sequences of *Cherokia* do not have a barcode gap (Fig. 4a), and supports the idea that all individuals belong to the same species. The simulated histogram expected for two species and that has a barcode gap is shown in figure 4b, where the dotted line marks the separation between two species.

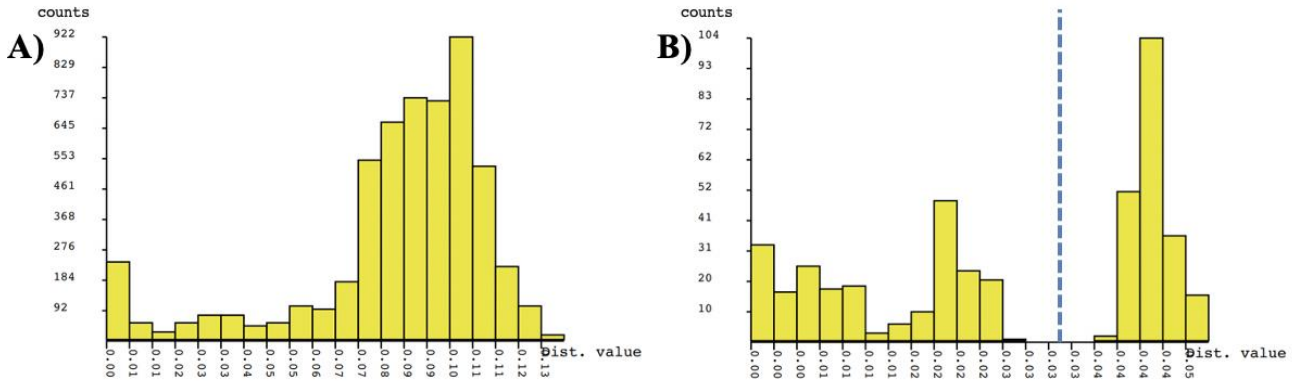

Figure 4. ABGD results A) *Cherokia* sequences, no barcode gap observed. **B)** Simulated sequences, barcode gap marked by the dotted line.

3.3. Morphological analysis

Genus diagnosis. Adult *Cherokia* have a total body length ranging from 30 to 40 mm. The paranota are horizontal and wide, with little curvature downwards making the body appear flat. The dorsal surface of the metazonites have a noticeable wrinkly texture; not smooth as in apheloriine millipedes. Coloration can be variable, with yellow to red hues. The yellow trimaculate is the most frequent color morph, although bimaculate and striped color patterns occur.

All the adult individuals used for the phylogeny reconstruction were included in the morphological analysis. The juvenile (Fig. 5, GA-TIF-MPE03692^{+}) was excluded due to lack of development in its morphological characters. Measurements of metazonite width had the greatest variation (range = 6.0 - 9.3 mm, $\bar{x}$ = 7.62, 95% CI = 7.47 - 7.76, n = 105), followed by the paranotal extension (range = 1.25 - 2.17 mm, $\bar{x}$ = 1.57, 95% CI = 1.52 - 1.62, n = 105), and lastly by the metazonal length (range = 1.54 - 2.60 mm, $\bar{x}$ = 1.90, 95% CI = 1.86 - 1.94, n = 105).

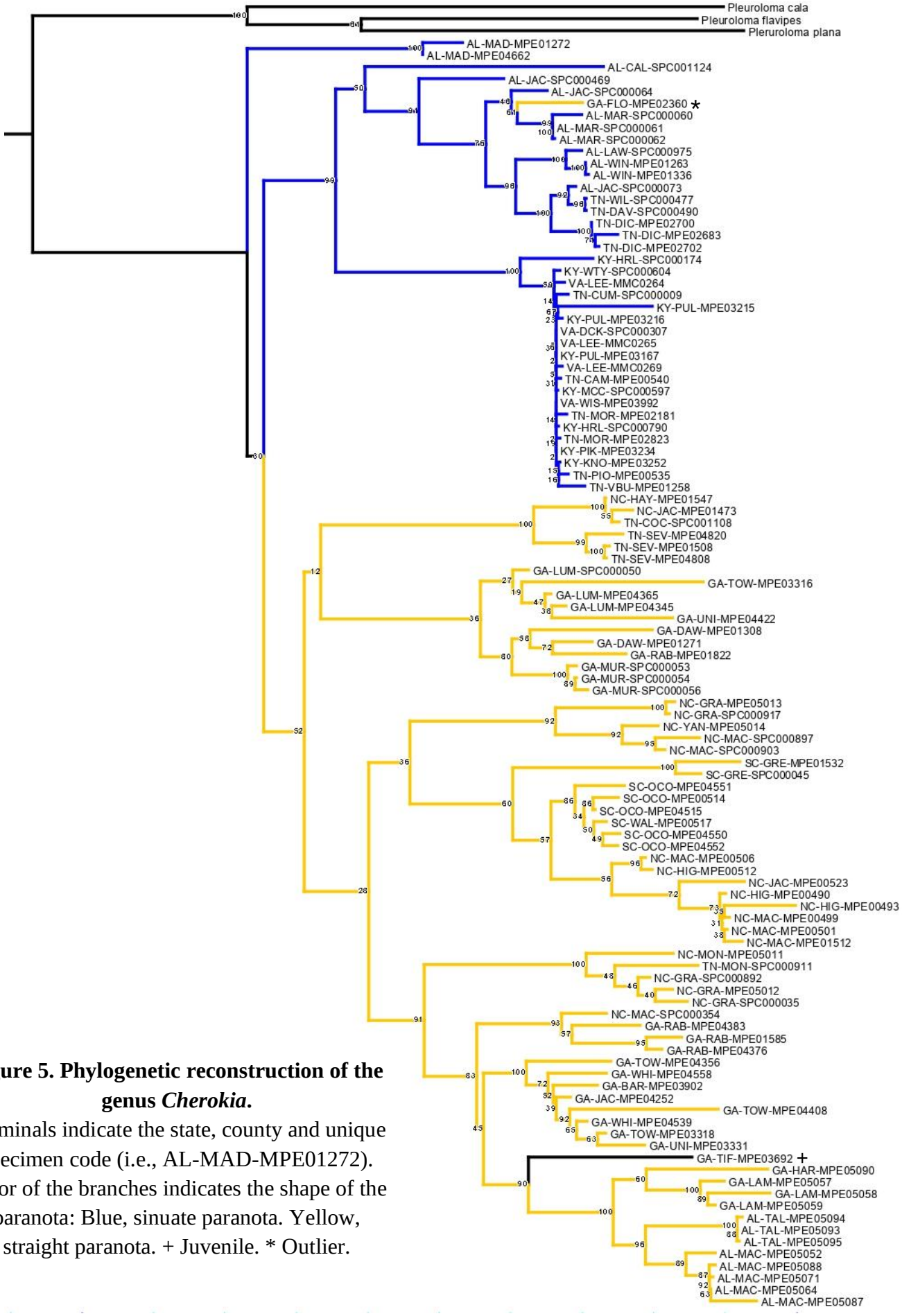

Figure 5. Phylogenetic reconstruction of the genus *Cherokia*.

Terminals indicate the state, county and unique specimen code (i.e., AL-MAD-MPE01272). Color of the branches indicates the shape of the paranota: Blue, sinuate paranota. Yellow, straight paranota. + Juvenile. * Outlier.

Linear regression was used to determine the relationship between elevation and the natural log (Ln) of the three morphological measurements (Fig. 6). These analyses indicate that Ln body measurements are negatively related to elevation; millipedes with smaller body sizes tend to be present at higher elevation than those with larger sizes.

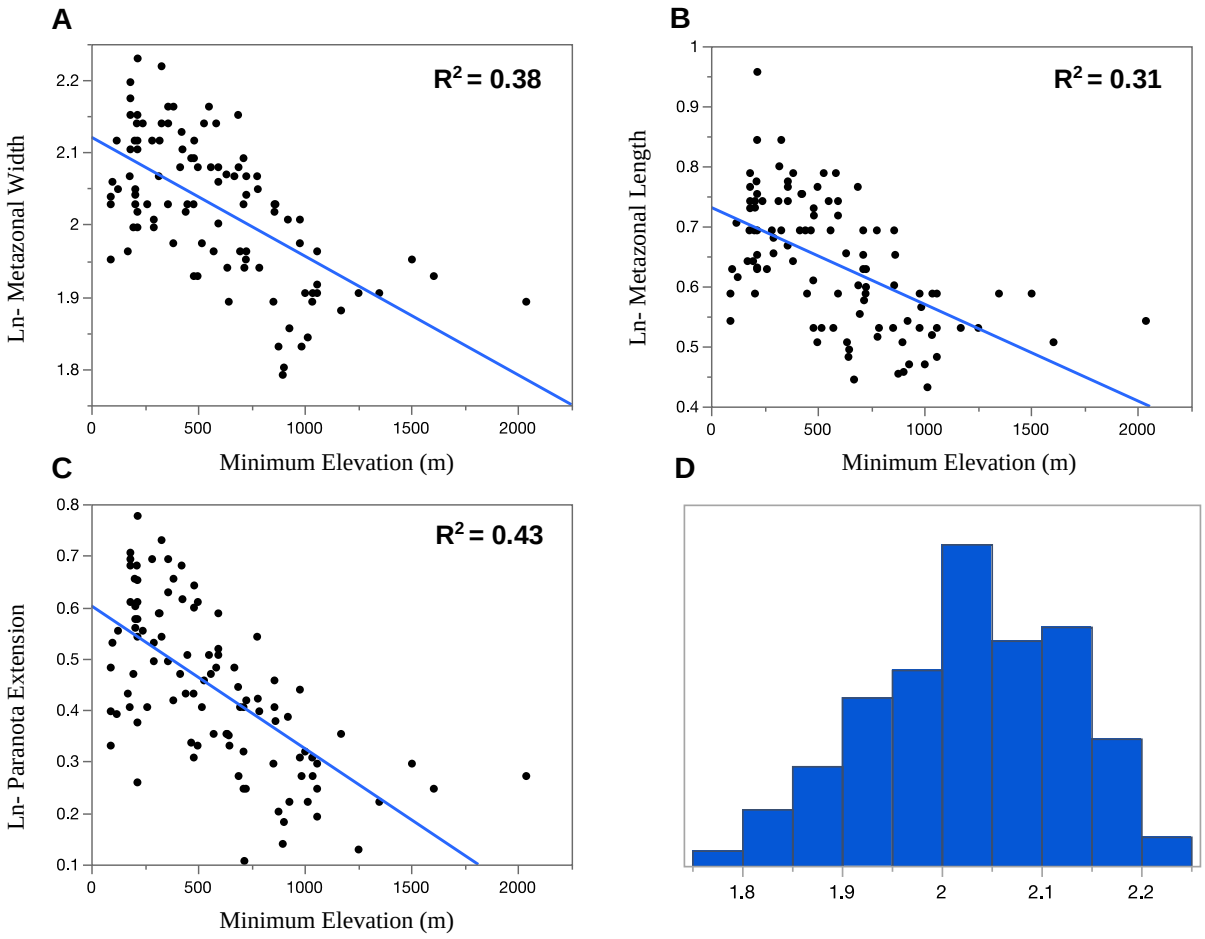

Figure 6. Linear regression of elevation and body measurements.
A. Metazonal width, **B.** Metazonal length, **C.** Paranota extension and **D.** Ln-transformed metazonal width distribution.

The position of the scapulora as described in Hoffman (1960) (Fig. 1) could not be consistently scored and was not included in analyses. Nevertheless, I observed a consistent dichotomy in the shape of the anterior border of the paranota. One phenotype has a distinct sinuous curvature on the anterior border of the paranota, while the posterior corner protrudes

backward beyond the margin of the posteromedial margin of the metazonite (Fig. 7A, blue lines). The second phenotype has an almost straight anterior border, and the posterior corner is mostly aligned with the posterior margin of the metazonite (Fig. 7B, yellow lines).

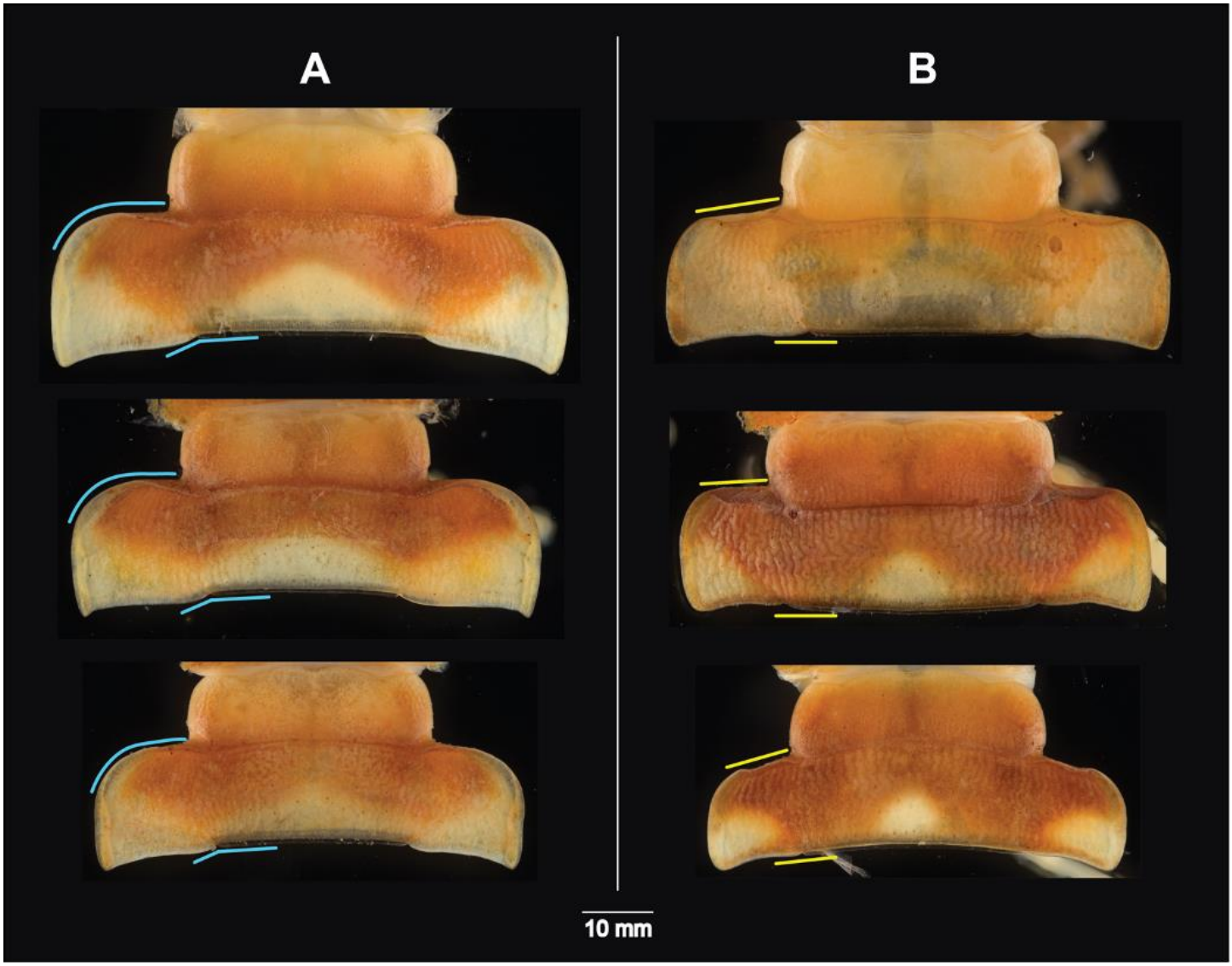

Figure 7. Variation in the paranota shape in *Cherokia*.
A) Sinuate paranota, **B)** Straight paranota.
Blue and Yellow lines denote the differences between the paranota shape.

The final morphological character that I evaluated was coloration. A total of 124 images of individuals that I identified as *Cherokia* on iNaturalist were used for this analysis. Pictures were downloaded and then coded using three colors (red, orange and yellow) and three coloration patterns (bimaculate, trimaculate, and striped) based on the color descriptions of Hoffman (1960). Most individuals exhibited only one of the colors, and a small proportion, two. White was only present in combination with another color (i.e. white and orange), while the other colors were present by themselves or with another color.

Individuals with a bimaculate pattern, had a colored spot on each paranota (there are two paranota per ring) with the center lacking coloration (Fig. 8A). Individuals with a trimaculate pattern, had a colored spot on each paranota with a spot on the middorsal part of the ring. The spots (middorsal or paranotal) were in different sizes and could be one of three shapes: a circle, oval, or a triangle (Fig. 8B). Individuals with a striped pattern had a colored band on the posterior margin of the ring that runs from one paranota to the other. The band could have various thicknesses, and in some cases a superposition with the trimaculate pattern was evident atop the banded pattern (Fig. 8C). Geographical distribution and colors or patterns were not associated; in some cases, *Cherokia* individuals from the same locality exhibited different color patterns.

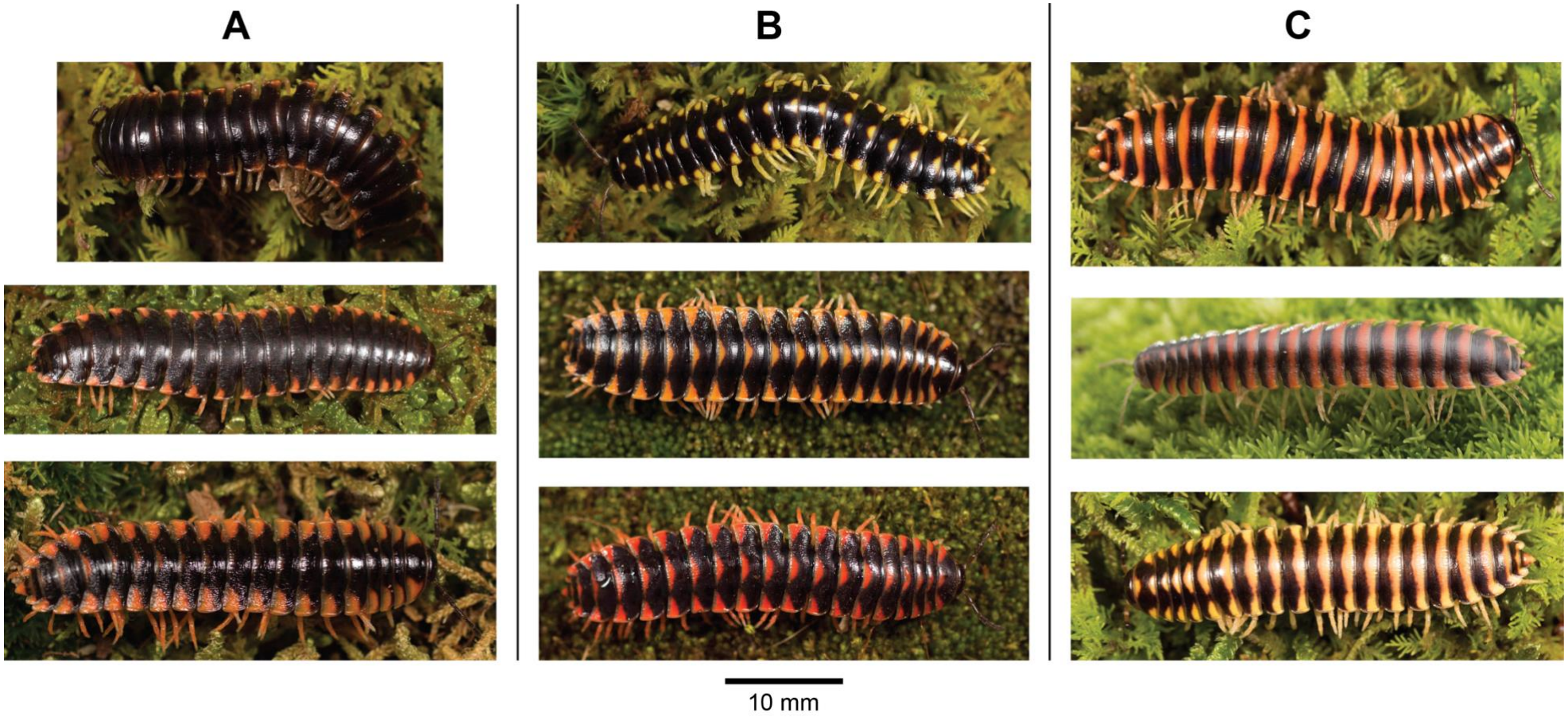

Figure 8. Color patterns of *Cherokia*.
A. Bimaculate, **B.** Trimaculate and **C.** Striped.

4. Discussion

Cherokia is a monophyletic group (Fig. 5). A clade formed by two individuals from Monte Sano State Park, Madison Co., Alabama is sister to the remaining species. Three statistically well-supported clades are subtended by long branches; however, the other individuals in the genus are paraphyletic with respect to these clades and are not reciprocally monophyletic with them. In general, individuals from the same locality or nearby localities are grouped together. Individuals from Kentucky and Virginia group together with some individuals from Tennessee in a clade with very short branches. This block of individuals corresponds with the northeast limit of the geographical range of the genus, and to the Cumberland Mountain Thrust Block region, a mountainous and complex region lying between the dissected Appalachian Plateau to the west and the Valley and Ridges to the east. The millipede genus *Brachoria*, with similarly shallow genetic divergences, is endemic to this region as well also (Marek, 2010). These shallow branches in *Cherokia*, as in *Brachoria*, may represent relatively recent diversification in this area, due to common drivers of regional diversity or to evolution associated with mimicry complexes (Marek & Bond, 2009).

The morphological characters I examined in my work were compared to elevation and phylogeny. The measurements taken from the 12th body ring and its inverse linear relation with elevation showed that body size and paranota extension of *Cherokia* individuals decrease with elevation (Fig. 6). Although my new measurements generally had the same distributions as Hoffman's (1960), variation appears to be clinal, and not discordant variation that corresponds to species boundaries. Although they are qualitative, the scapulora exhibit a similar pattern of variation. The ABGD analysis indicates that there are no clear genetic clusters indicative of subspecies or more than one species(Fig. 4A).

The position of the scapulora (sensu Hoffman, 1960) was not a useful character because of the difficulty of distinguishing its two states from each other (strictly marginal and submarginal) (Fig. 7). Nonetheless, I discovered that the shape of the anterior margin of the paranota was dichotomous; individuals exhibited either sinuate or straight margins. I mapped the geographical distribution of this character (Fig. 9), and traced it on the phylogenetic tree (Fig. 5). Individuals with sinuate paranota tend to be mainly located in the western part of the Appalachian region, while the individuals with a straight paranota are located in the east part. This separation appears to correspond to the Tennessee River Valley and the geological barrier that it represents for the genus, and other co-distributed taxa. However, in the southern part of the geographical distribution of *Cherokia*, especially in the state of Alabama, the paranota overlap with no clear geographical separation (Fig. 9).

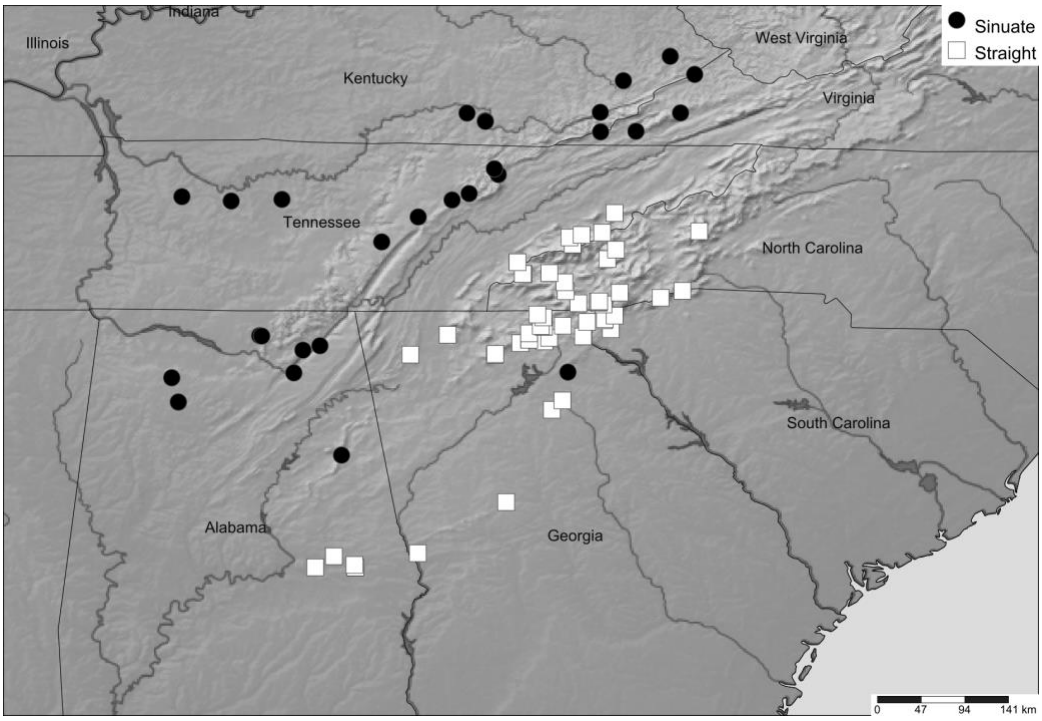

Figure 9. Geographical distribution of *Cherokia* showing the two paranota shapes.

When the shape of the paranotal margin was traced on the phylogeny, most individuals in one clade exhibited straight paranota (Fig. 5, blue), while the other clade (and two individuals from Monte Sano State Park, Alabama) possessed sinuate paranota (Fig. 5, Yellow). One individual in the whole phylogeny and geographical distribution appears as an outlier for the general trend of this character (Fig. 5, GA-FLO-MPE03260*). Although the qualitative character of the shape of the paranota is correlated with metazonite width ($p = 0.0001$), in some cases it is a bit difficult to distinguish straight versus sinuate, and the variation appears to be clinal in some regions. In contrast with the scapulora and color characteristics, the shape of the paranotal margin is largely concordant with the phylogeny, but in itself as a single character, insufficient for species or subspecies delimitation.

The coloration patterns were plotted on a map to assess if there was concordance with the geographical distribution. Figure 10 shows the distribution of the pattern (bimaculate, trimaculate, or striped), and the colors (red, orange or yellow). Some localities have all three types of patterns and/or colors, in contrast with Hoffman's (1960) supposition that each coloration is geographically isolated. Nearly all possible combinations of colors and patterns were observed, but the trimaculate yellow color morph was the most common (both in frequency of individuals and geographical area). The bimaculate pattern was only presented in orange (the bimaculate orange color morph, Fig. 8A). Figure 10 shows that neither the pattern (bimaculate, trimaculate, striped) nor the colors (red, orange, yellow), have any clear geographical concordance. The number of geographical data points that I used for these maps (Fig. 10) were greater ($n = 124$) than the one used for the phylogenetic analysis ($n = 106$). Here I included iNaturalist reports for *Cherokia*, because the number of images available for the specimens used in the phylogeny was relatively small ($n = 26$), and limited the scope of inference. Perception of

color can be affected by the observer, lighting conditions, and distance thereby adding error to the evaluation of this character (Endler, 1990). More sophisticated techniques that are less error-prone and a non-human-centric technique should be implemented such as using a spectrophotometer and incorporating the visual systems of the predators of *Cherokia* (likely avian) to evaluate the coloration according to the perceivers' eyes to have more accurate results.

The use of citizen science as a tool for obtaining and analyzing data has been successfully demonstrated by various research groups. The Cornell Lab of Ornithology, for example, has developed multiple projects involving amateur ornithologists and the general public for around two decades. Data obtained from those initiatives has been published in several peer-reviewed research papers in various journals (Bonney *et al.*, 2009). The small-scale citizen science project that I made as part of this research demonstrated that it is an effective method to obtain samples from remote and inaccessible localities, or in special situations such as the SARS-CoV-2 pandemic. Although the first response to the initiative was highly positive, follow-up contact with the interested participants was more difficult and less successful. The number of samples shipped back to us (n = 12) corresponds to around the 30% of the kits shipped to selected participants (n = 41). Improved communication with the participants, and a more structured timeline will be needed in the future to increase the overall success of this initiative in future projects.

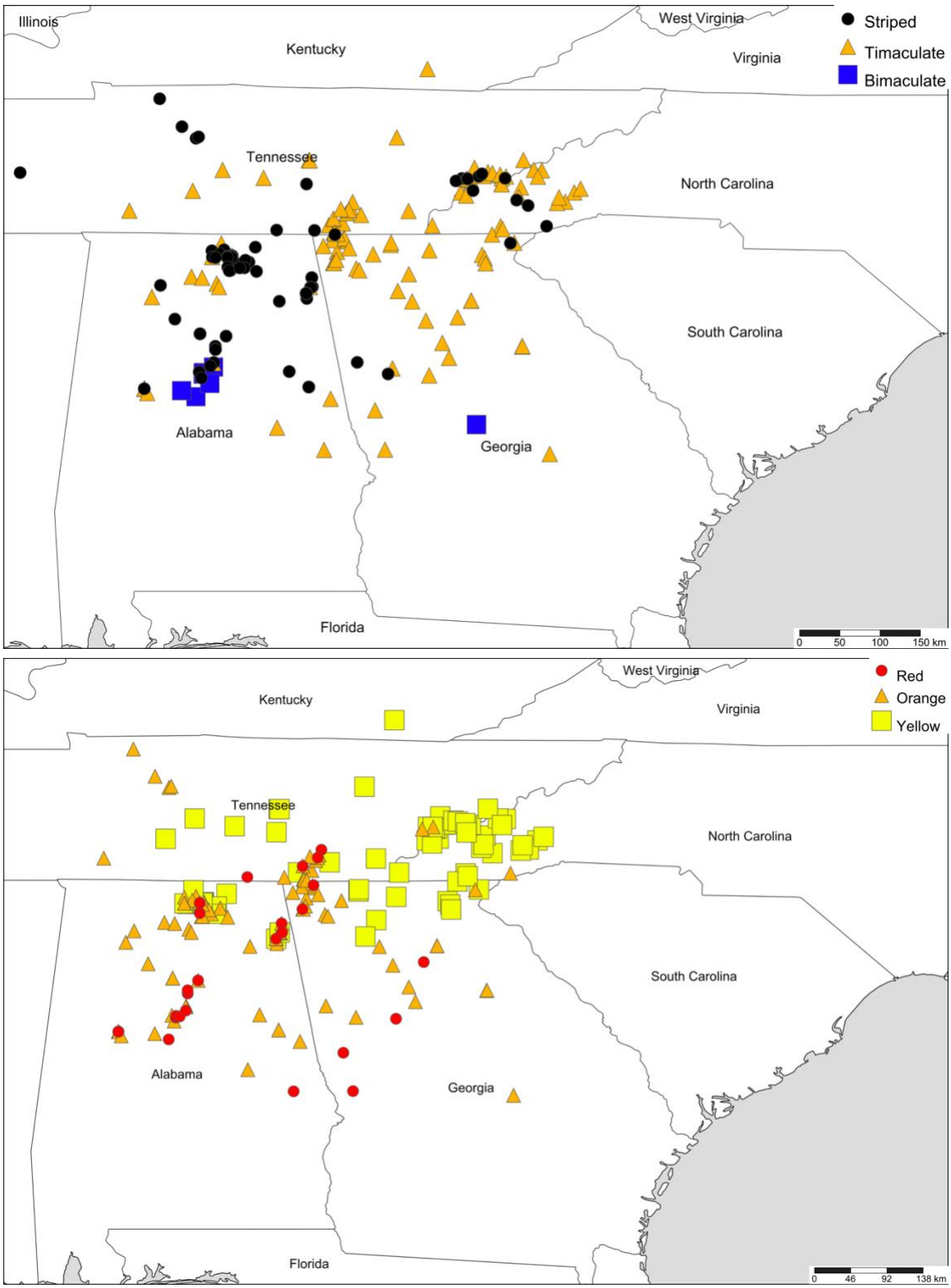

Figure 10. Geographical distribution of *Cherokia* vs. coloration patterns.
Top: Patterns**. Bottom:** Colors**.**

Despite at least three well supported clades in the phylogeny, and the relationship of the two paranota shapes with them and the geographical distribution, I conclude that the genus *Cherokia* is not divided in multiple well-defined groups that correspond to species or subspecies. The absence of a barcode gap in the sequences of *Cherokia* and the phylogeny support a grouping of all *Cherokia* individuals into a single morphologically diverse clade closely related to the sister genus, *Pleuroloma* (Means *et al.*, 2021a). Evidence presented here therefore supports the hypothesis that *Cherokia* is a monotypic genus.

5. Annotated literature review

5.1. Literature pertaining to the genus *Cherokia* and some species synonymized with *Cherokia georgiana*

Bollman, C. H. (1888). Notes upon some myriapods belonging to the U.S. National Museum. *Proceedings of the US National Museum.* This paper described the species *Fontaria georgiana* from an adult male and gives a brief description of its gonopod. In addition, *Fontaria tallulah* was described from an adult female. The two species were only separated by the coloration pattern and were proposed to be related to *F. virginiensis.*

Loomis, H. F. (1943). New cave and epigean millipedes of the United States, with notes on established species. *Bulletin of the Museum of Comparative Zoology at Harvard College 92: 373- 410.* In this paper, the author proposed the synonymy of *Mimuloria georgiana* (Chamberlin, 1939) with *Mimuloria ducilla* (Bollman, 1888), based on the examination of the types and the original descriptions of the species.

Loomis, H. F. & Hoffman, R. L. (1948). Synonymy of various diplopods. *Proceedings of The Biological Society of Washington 61:51- 54.* Here the authors made the species *F. tallulah* and *M. ducilla* synonyms of *M. georgiana.* They also pointed out that based on the drawings and its morphological description, *Dynoria parvior* should be synonymized with *M. georgiana,* too.

Chamberlin, R. V. (1949). A new genus and four new species in the diplopod family Xystodesmidae. *Proceedings of the Biological Society of Washington 62: 3-6.* This paper includes the original description of the genus *Cherokia.* The description is a brief paragraph and a simple illustration of the male gonopod. The author also designates *Fontaria georgiana* (Bollman, 1888) as the type species for the genus, changing it to *Cherokia georgiana.*

Causey, N. B. (1950). A collection of Xystodesmid millipedes from Kentucky and Tennessee. *Entomological News, 61:5-7, figs. 1-3.* Here the author mentions 16 males of *Mimuloria georgiana* collected in the Great Smoky Mountains National Park. *M. georgiana* was previously synonymized with *Fontaria georgiana* now *Cherokia georgiana*. Causey describes as well the color variation in the specimens.

Hoffman, R. L. (1950). Records and descriptions of diplopoda from the southern Appalachians. *Journal of the Elisha Mitchell Scientific Society (Chapel Hill NC) 66(1): 11-33.* This is the first paper after the description of the genus *Cherokia* that places some preexisting species in it. The author synonymized *Fontaria georgiana, F. tallulah, Mimuloria ducilla, M. furcifer, M. georgiana* and *Dynoria parvior* with *Cherokia georgiana.* Additionally, the author described the known geographical range and reported the northernmost locality in North Carolina. This work also includes a brief description of the life cycle of *C. georgiana*, and mentions its color variation. In this paper Hoffman explains that *Cherokia* is a highly variable monotypic genus.

Chamberlin, R. V. & Hoffman, R. L. (1958). Checklist of the millipedes of North America. *United States National Museum Bulletin 212.* In this checklist, the genus *Cherokia* is listed together with the species that have been previously synonymized with *C. georgiana*. The author also included an explanation of the genus distribution and a brief description of its phenotypic variation.

Hoffman, R. L. (1960). Revision of the millipede genus *Cherokia* (Polydesmida: Xystodesmidae). *Proceedings of the United States National Museum 112: 227-264.* This is the most current revision of the genus *Cherokia* to date. Here the author reviewed most of the museum material from different repositories and listed it in detail with the locality, sex,

collection date, collector, and museum acronym where the specimen was deposited. The paper includes a detailed description of the morphology of the genitalia including drawings of the gonopods and cyphopods (female openings of the oviducts). Here, *Cherokia* was proposed as being a monotypic genus, with three subspecies: *Cherokia georgiana georgiana, Cherokia georgiana ducilla* (new status), and *Cherokia georgiana latassa* (new subspecies). The author also provided maps showing the geographical distribution of each subspecies and proposed morphological characters to separate them.

Hoffman, R. L. (1978). A new genus and species of Rhysodesmine millipede from Southern Georgia (Polydesmida: Xystodesmidae). ***Proceedings of the Biological Society of Washington, 91(2): 365- 373.*** In this paper, eight genera in the Tribe Rhysodesmini are mentioned. The author suggests an affinity between the genera *Caralinda* and *Cherokia* based on the structures of the gonopods. The author also included an identification key for the genera in the tribe Rhysodesmini.

Shelley, R. M. (1980). Revision of the millipede genus *Pleuroloma* (Polydesmida: Xystodesmidae). ***Canadian Journal of Zoology, 58*****(2), 129-168.** While the main focus of this paper was not the genus *Cherokia,* the author highlighted the problem of overly brief descriptions of new species as well as the over-splitting of groups based purely on geographical distributions and nonsexual characters such as coloration and size. Shelley mentioned that *Cherokia* and *Pleuroloma* have similar characteristics, including a wide geographical distribution, substantial variation in coloration and body size, and uniform gonopods across their generic distributions.

Shelley, R. M. (2000). Annotated checklist of the millipedes of North Carolina (Arthropoda: Diplopoda), with remarks on the genus *Sigmoria* Chamberlin (Polydesmida:

Xystodesmidae). ***Journal of the Elisha Mitchell Scientific Society, 177-205.*** In this paper, the author presented a complete list of the occurrences of millipedes in North Carolina, including individuals of the genus *Cherokia*. However, the author presented a number of intergrades of the genus that could not be assigned to one of the subspecies.

Marek, P.; Tanabe, T. & Sierwald, P. (2014). A species catalog of the millipede family Xystodesmidae (Diplopoda: Polydesmida). ***Virginia Museum of Natural History Publications.*** This checklist provided a detailed summary of the species of the family Xystodesmidae. The authors included the family's morphological characteristics, geographical distribution, and a literature review of all the taxa in the family. For the genus *Cherokia*, the paper summarized its species and a list of synonymies. It also outlines the general distribution of the subspecies.

Means, J. C., & Marek, P. E. (2017). Is geography an accurate predictor of evolutionary history in the millipede family Xystodesmidae?. ***PeerJ, 5, e3854.*** In this paper, the authors compared phylogenetic hypotheses estimated with morphological and geographical characters against an updated molecular phylogeny for the family Xystodesmidae. They concluded that morphological and geographical phylogenies do not give a good hypothesis by itself and should be used in conjunction with molecular phylogeny. Regarding the genus *Cherokia*, this paper shows in the molecular phylogeny that two of the subspecies of the genus assemble into one clade, and they are well differentiated. The article provides an analysis of one of the morphological diagnostic characters of the genus, the males gonopods, and its high percentage of homoplasy indicating convergent evolution and poor choice as a diagnostic character.

6. References

Barkworth, M., Brandt, B., Dyreson, C., Cobb, N., & Pearse, W. (2019). Symbiota2: Enabling greater collaboration and flexibility in mobilizing biodiversity data. Biodiversity *Information Science and Standards.*

Bollman, C. H. (1888). Notes upon some myriapods belonging to the US National Museum. *Proceedings of the United States National Museum.*

Bonney, R., Cooper, C. B., Dickinson, J., Kelling, S., Phillips, T., Rosenberg, K. V., & Shirk, J. (2009). Citizen science: a developing tool for expanding science knowledge and scientific literacy. *BioScience, 59(11), 977-984.*

Brewer, M. S., Sierwald, P., & Bond, J. (2012). Millipede Taxonomy after 250 Years: Classification and Taxonomic Practices in a Mega-Diverse yet Understudied Arthropod Group. *PLoS ONE, 7(5): 37240.*

Cárcamo, H. A., Abe, T. A., Prescott, C. E., Holl, F. B., & Chanway, C. P. (2000). Influence of millipedes on litter decomposition, N mineralization, and microbial communities in a coastal forest in British Columbia, Canada. *Canadian Journal of Forest Research, 30(5), 817-826.*

Causey, N. B. (1950). A collection of Xystodesmid millipeds from Kentucky and Tennessee. *Entomological News, 61:5-7, figs. 1-3.*

Chamberlin, R. V. (1949). A new genus and four new species in the diplopod family Xystodesmidae. *Proceedings of the Biological Society of Washington 62: 3-6*

Chamberlin, R. V., & Hoffman, R. L. (1958). Checklist of the millipeds of North America. *United States National Museum Bulletin 212.*

Cracraft, J. (1983). Species concepts and speciation analysis. In Current ornithology (pp. 159-187). *Springer, New York, NY.*

Cracraft, J. (1992). The species of the birds-of-paradise (Paradisaeidae): applying the phylogenetic species concept to a complex pattern of diversification. *Cladistics, 8(1), 1-43.*

Dangerfield, J. M., & Telford, S. R. (1991). Seasonal activity patterns of julid millipedes in Zimbabwe. *Journal of tropical ecology, 281-285.*

De Queiroz, K. (2007). Species concepts and species delimitation. *Systematic biology, 56(6), 879-886.*

Endler, J. A. (1990). On the measurement and classification of colour in studies of animal colour patterns. *Biological Journal of the Linnean Society, 41(4), 315-352.*

Enghoff, H. (2015). 13 Diplopoda - Geographical Distribution. In: Minelli, A. (Editor). The Myriapoda Volume 2. *Leiden and Boston: Brill. p. 329-336*

Ewing, B., Hillier, L., Wendl, M. C., & Green, P. (1998). Base-calling of automated sequencer traces usingPhred. I. Accuracy assessment. *Genome research, 8(3), 175-185.*

Hoffman, R. L. (1950). Records and descriptions of Diplopoda from the southern Appalachians. *Journal of the Elisha Mitchell Scientific Society (Chapel Hill NC) 66(1): 11- 33.*

Hoffman, R. L. (1960). Revision of the milliped genus *Cherokia* (Polydesmida: Xystodesmidae). *Proceedings of the United States National Museum 112: 227-264. https://www.biodiversitylibrary.org/page/7717047*

Hoffman, R. L. (1978). A new genus and species of Rhysodesmine milliped from Southern Georgia (Polydesmida: Xystodesmidae). *Proceedings of the Biological Society of Washington, 91(2): 365- 373.*

Jorgensen, M. C., Sierwald, P., & Mason-Gamer, R. J. (2013). A review of subspecies recognition in polydesmidan millipedes (Diplopoda) with a revision of the subspecies of Euryuridae (Xystodesmoidea). *Zoologica Scripta, 42(3), 317-326.*

Kalyaanamoorthy, S., Minh, B. Q., Wong, T. K., Von Haeseler, A., & Jermiin, L. S. (2017). ModelFinder: fast model selection for accurate phylogenetic estimates. *Nature methods, 14(6), 587-589.*

Katoh, K., & Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular biology and evolution, 30(4), 772-780.*

Maddison, W. P., & D.R. Maddison. (2019). Mesquite: a modular system for evolutionary analysis. Version 3.61. *http://www.mesquiteproject.org*

Maddison, D.R., & W.P. Maddison. (2020). Chromaseq: a Mesquite package for analyzing sequence chromatograms. Version 1.52. *http://chromaseq.mesquiteproject.org*

Marek, P. E., & Bond, J. E. (2009). A Müllerian mimicry ring in Appalachian millipedes. *Proceedings of the National Academy of Sciences, 106(24), 9755-9760.*

Marek, P. E. (2010). A revision of the Appalachian millipede genus *Brachoria* Chamberlin, 1939 (Polydesmida: Xystodesmidae: Apheloriini). *Zoological Journal of the Linnean Society, 159(4), 817-889.*

Marek, P. E., Shear, W. A., & Bond J. (2012). A redescription of the leggiest animal, the millipede *Illacme plenipes*, with notes on its natural history and biogeography (Diplopoda, Siphonophorida, Siphonorhinidae). *ZooKeys. 241: 77–112.*

Marek, P., Tanabe, T., & Sierwald, P. (2014). A species catalog of the millipede family Xystodesmidae (Diplopoda: Polydesmida). Virginia Museum of Natural History Publications

Marek, P. E., Means, J. C., & Hennen, D. A. (2018). *Apheloria polychroma*, a new species of millipede from the Cumberland Mountains (Polydesmida: Xystodesmidae). *Zootaxa 4375 (3): 409–425.*

Mayr, E. (1982). Of what use are subspecies?. *The Auk, 99(3), 593-595.*

Means, J. C., Francis, E. A., Lane, A. A., & Marek, P. E. (2015). A general methodology for collecting and preserving xystodesmid and other large millipedes for biodiversity research. *Biodiversity data journal, (3).*

Means, J. C., & Marek, P. E. (2017). Is geography an accurate predictor of evolutionary history in the millipede family Xystodesmidae?. *PeerJ, 5, e3854.*

Means, J. C., Hennen, D. A., Tanabe, T., & Marek, P. E. (2021a). Phylogenetic Systematics of the Millipede Family Xystodesmidae. *Insect Systematics and Diversity, 5(2), 1.*

Means, J. C., Hennen, D. A., & Marek, P. E. (2021b). A revision of the minor species group in the millipede genus *Nannaria* Chamberlin, 1918 (Diplopoda, Polydesmida, Xystodesmidae). *ZooKeys 1030: 1-180.*

Minh BQ, Schmidt HA, Chernomor O, Schrempf D, Woodhams MD, von Haeseler A, Lanfear R. (2020). IQ-TREE 2: New models and efficient methods for phylogenetic inference in the genomic era. *Molecular biology and evolution 37(5): 1530–1534.*

Pitz, K., & Sierwald, P. (2010). Phylogeny of the millipede order Spirobolida (Arthropoda: Diplopoda: Helminthomorpha). *Cladistics; 26: 497–525.*

Puillandre N., Lambert A., Brouillet S., & Achaz G. (2012). ABGD, Automatic Barcode Gap Discovery for primary species delimitation. *Molecular ecology, 21(8), 1864-1877.*

Rios, N. E., & Bart, H. L. (2010). GEOLocate (Version 3.22) [computer software]. *Belle Chasse, LA: Tulane University Museum of Natural History.*

Shelley, R. M. (1980). Revision of the milliped genus *Pleuroloma* (Polydesmida: Xystodesmidae). *Canadian Journal of Zoology, 58(2), 129-168.*

Shelley, R. M. (2000). Annotated checklist of the millipeds of North Carolina (Arthropoda: Diplopoda), with remarks on the genus *Sigmoria* Chamberlin (Polydesmida: Xystodesmidae). *Journal of the Elisha Mitchell Scientific Society, 177-205.*

Sierwald, P.; Spelda, J. (2021). MilliBase. *Accessed at http://www.millibase.org on 2021-05-20. doi:10.14284/370*

Wieczorek, J., Bloom, D., Guralnick, R., Blum, S., Döring, M., Giovanni, R., Robertson, T. & Vieglais, D. (2012). Darwin Core: an evolving community-developed biodiversity data standard. *PloS one, 7(1), e29715.*