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ARTICLE

An integrative phylogenetic analysis of eastern Nearctic *Leuctra* (Plecoptera: Leuctridae), with an emphasis on the fauna of a southern Appalachian Highlands landscape

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Abstract

Leuctra Stephens, 1836 is the fourth most speciose genus of Plecoptera (Leuctridae) east of the Rocky Mountains with 31 recognised species, trailing only *Isoperla* Banks, 1906 (58), *Allocapnia* Claassen, 1928 (47), and *Perlesta* Banks, 1906 (34). Although *Leuctra* females are described in taxonomic literature, they are difficult to morphologically distinguish among regional congeners, and identifications are often made through inference only (*i.e.,* presence of males). This is particularly problematic in the Appalachian Mountains of the United States of America, which host numerous *Leuctra* species. We sampled the stonefly fauna of Mount Mitchell, western North Carolina, United States of America, from April to October 2019. The mitochondrial cytochrome C oxidase subunit 1 gene was sequenced for 39 adult males and 239 adult females of *Leuctra.* This allowed us to confidently place species names on all of the latter individuals. Phylogenetic tree- and genetic distance–based methods consistently grouped females with males for nine recognised species. Two separate *L. ferruginea* (Walker, 1852) operational taxonomic units were recognised, albeit with low divergence values, and an additional undetermined *Leuctra* was identified based solely on females. Digital stereomicroscope images were taken from females of each species unit to identify variation among and between species. This approach allowed for a more robust assessment of regional biogeographic patterns.

Introduction

The ability to correctly identify individual specimens to a species level is important for water quality and biodiversity evaluations of aquatic systems (Jones 2008). Separating closely related species is typically completed through a comparison of diagnostic characteristics, yet these comparisons are often limited by the amount of morphological divergence visible by eye (Bickford *et al.* 2007). Most descriptions of stoneflies are based primarily on adult males because their genitalia are distinctive enough among species to be diagnostic (Szczytko and Kondratieff 2015). Accurate morphological descriptions of females and larvae remain difficult for many species and, in some instances, are impossible with standard light microscopy (Zhou *et al.* 2010). Moreover, there are few stonefly taxonomists, and at times they are unable to identify specimens due to the lack of objective diagnostic traits (Beaty 2015).

Modern molecular techniques, coupled with open access data (*e.g.,* GenBank), can help overcome taxonomic issues such as insufficient knowledge of morphological variability for traditional species descriptions (Hausmann *et al.* 2016). Molecular data can provide a less labour-intensive and faster method for making male–female and adult–larvae associations of

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Plecoptera where only subtle morphological differences exist (Miller *et al.* 2005; Mynott *et al.* 2011; Hausmann *et al.* 2013). Molecular data of species for any combination of male, female, and larvae remain highly conserved within species (Calleja *et al.* 1993). Therefore, gene sequences from authoritatively identified adult males can be used as a reference for associating females and larvae. Integrative molecular and morphological approaches can delineate minor morphological differences that conform to diagnostic characters (McDaniel and Shaw 2003). Several recent studies have used molecular data for the purposes of evolutionary systematics of stoneflies (Mynott *et al.* 2011; Gill *et al.* 2015a, 2015b; Vitecek *et al.* 2017; South *et al.* 2021). Studies have also used DNA to associate different stonefly life stages or to associate males with females (Miller *et al.* 2005; Mynott *et al.* 2011; Grubbs *et al.* 2020). The increasing use of gene sequence data for stoneflies has allowed for an integrated approach that combines morphology and molecular information (Vitecek *et al.* 2017). Much of the previously completed work focusing on DNA-based life-stage association relies on the mitochondrial cytochrome c oxidase subunit 1 (mt*CO1*) gene (Caterino and Tishechkin 2006).

Despite decades of technological advances with light and electron microscopy, describing the adult female stage and associating females with males remain difficult with some stonefly genera. One example is with Nearctic *Leuctra* Stephens, 1836 (Plecoptera: Leuctridae). Thirty-one *Leuctra* species are currently recognised from the eastern Nearctic region, with most found in the Appalachian Mountains and unglaciated regions of the southeastern United States of America (DeWalt *et al.* 2020). Harper and Harper (1997) placed most of the species known at that time ($n = 26$) into five proposed species groups: *L. biloba* Claassen, 1923 group, *L. duplicata* Claassen, 1923 group, *L. ferruginea* (Walker, 1852) group, *L. grandis* Banks, 1906 group, and *L. tenuis* (Pictet, 1841) group. Two species were left unassigned, but the five species described since 1997 have each been placed into a group (Grubbs and Sheldon 2008; Grubbs 2010; Harrison and Stark 2010). The most recent Nearctic taxonomic key (Hitchcock 1974) for this genus includes only 17 species, with descriptions of larva and females provided for only seven of these species. Taxonomic information in general on females is absent or minimal at best (*e.g.,* Hitchcock 1974; Harper and Harper 1997) due to conservative morphological characters of females and larvae characteristics. Although the females of some *Leuctra* species are described in taxonomic literature (*e.g.,* Claassen 1923; Poulton and Stewart 1991; Grubbs 2010; Harrison and Stark 2010), they are often difficult to distinguish due to conservative morphology of the subgenital plate, setal patterns, and shape of the internal spermathecae (Harper and Harper 1997). This is especially problematic in the more species-rich Appalachian Mountains, where as many as 7–10 species may inhabit the same drainage. Despite this, confident identification of females and larvae is critical for understanding the diversity, ecology, and conservation of species in the region.

Only one phylogenetic treatment of Nearctic *Leuctra* has been undertaken (Grubbs *et al.* 2020), and it is small in scope, with only 12 of the 31 known species. The two partial phylogenetic treatments of Palearctic *Leuctra* are comparatively larger in scope. Gattolliat *et al.* (2016) analysed an mt*CO1* sequence library for 90 species from Switzerland, including 37 *Leuctra* species. Vitecek *et al.* (2017) used mitochondrial and nuclear genes to assess the phylogenetic relationships of 18 species of the *L. inermis* Kempny, 1899 species group.

This study had two main objectives. The first objective was to incorporate mt*CO1* sequence data with tree- and genetic distance–based phylogenetic approaches to associate putative females with males collected in 2019 for several species of *Leuctra* known from a southern Appalachian Highlands landscape, western North Carolina. The second objective was to build upon the phylogenetic framework established in Grubbs *et al.* (2020) to a more robust assessment of phylogenetic relationships amongst eastern Nearctic *Leuctra* species and the proposed species groups.

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Materials and methods

Study region

Fieldwork occurred in Mount Mitchell State Park and in the adjacent Appalachian Ranger District of Pisgah National Forest (Fig. 1) in North Carolina. Mount Mitchell is the high point in North America east of Hudson Bay and the Mississippi River Basin. The mountain is located within the ancient Blue Ridge Physiographic region that extends from Pennsylvania to northern Georgia, United States of America, and it varies from narrow ridges to high peaks in mountainous areas. Both landscapes are nested within the United States Environmental Protection Agency Level III Blue Ridge Ecoregion 65. A total of 44 sites were selected to represent a broad range of stream sizes, from small seeps to the South Toe River, and along an elevation gradient that spanned from 831 to 1983 m (from 2726 to 6506 ft) above sea level (Supplementary material, Table S1). Most sites were accessed along backcountry trails and United States Forest Service roads (Fig. 1). Coordinates for each site were taken with handheld geographic positioning system units and were subsequently projected to check for accuracy using Acme Mapper 2.2 (WGS-84; Acme Laboratories, Vancouver, British Columbia, Canada; http://mapper.acme.com). All geographic coordinate data are represented in decimal degrees. Dot distribution maps were prepared using ArcMap 10.7 (Esri, Redlands, California, United States of America).

Field sampling

Fresh adult specimens of *Leuctra* were collected in April, June, July, August, and October 2019. We used beating sheets to dislodge specimens from riparian vegetation during cool weather, sweep nets on warmer days, hand-picking from emergent leaf packs, rocks, trees, and bridges, and light trapping with a standard ultraviolet light. *Leuctra* specimens were preserved in 95% ethanol for molecular analyses.

Microscopy and digital imaging

Males were identified using an Olympus SZ61 stereomicroscope (Olympus, Shinjuku City, Tokyo, Japan) and several resources (Claassen 1923; Hanson 1941; Hitchcock 1974; Harper and Harper 1997; Grubbs 2015). Subgenital plates for all females sequenced in this study ($n = 272$) were digitally imaged using a Leica MZ16 stereomicroscope (Leica, Wetzler, Germany) equipped with a JVC KY-F75U camera (JVC, Yokohama, Japan). Images were layered using the Auto-Montage Pro programme (Syncroscopy, Cambridge, United Kingdom).

Molecular methods

Total DNA was extracted from two adult legs and associated thoracic tissue per individual from nine regional species collected in 2019 (*L. ferruginea* (Walker, 1852), *L. grandis* Banks, 1906, *L. mitchellensis* Hanson, 1941, *L. sibleyi* Claassen, 1923, *L. tenella* Provancher, 1878, *L. tenuis* (Pictet, 1841), *L. triloba* Claassen, 1923, *L. truncata* Claassen, 1923, and *L. variabilis* Hanson, 1941) (Supplementary material, Table S2). We extracted DNA using a DNeasy Blood and Tissue Kit (Qiagen, Inc., Hilden, Germany) following the manufacturer's instructions. We amplified the mt*CO1* gene through a polymerase chain reaction using the primers HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) and LCO1490 (5′-GGTCAACAAATCATAAA GATATTGG-3′; Folmer *et al.* 1994). The polymerase chain reaction was performed with 10 µL total (5 µL GoTaq® Colorless Master Mix (Promega Corporation, Madison, Wisconsin, United States of America), 0.5 µL of each of 10-µM solutions of HCO2198 and LCO1490, and 4 µL DNA). The polymerase chain reaction thermal regime consisted of an initial denaturation at 95 °C (1 minute), 35 cycles at 95 °C (20 seconds each), annealing at 50 °C (20 seconds), and an extension period at 72 °C (1 minute), followed by a final extension period at 72 °C (5 minutes).

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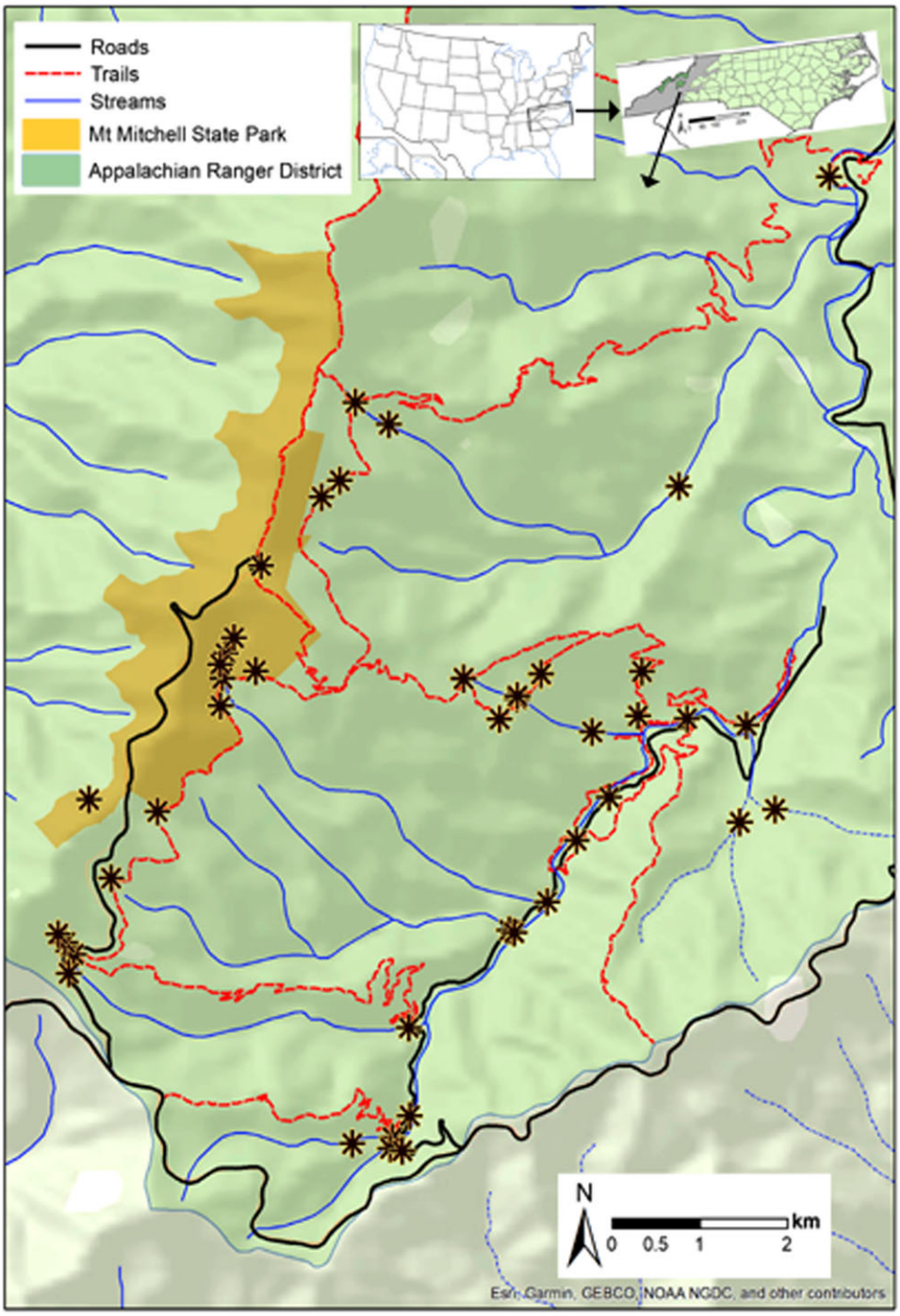

Fig. 1. Collection sites during April–October 2019 from Mount Mitchell State Park and Pisgah National Forest, North Carolina, United States of America. Map inset of North Carolina in upper right shows the Appalachian Ranger District, Pisgah National Forest in dark green. Sites are represented by black stars.

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Polymerase chain reaction products were verified on 1% agarose gel (0.35 g agarose and 35 mL 1 × Tris-acetate-EDTA buffer; Thermo Fisher Scientific, Waltham, Massachusetts, United States of America) using a FisherBiotech FB-SB-710 Electrophoresis System (Thermo Fisher Scientific) and subsequently visualised compared to a 1-kB DNA ladder with an IO Rodeo Large Blue LED transilluminator (IO Rodeo Inc., Pasadena, California, United States of America). Polymerase chain reaction products were sequenced at the North Carolina State University Genomics Lab (Raleigh, North Carolina). Full collection data, photographs, sequencing primers, and all sequences have been added to a private data set on the Barcode of Life Database (BOLD; University of Guelph, Guelph, Ontario, Canada). Barcode of Life Database IDs can be seen in Supplementary material, Table S1.

To add more information for phylogenetic analyses, six species were also collected in 2019 (*L. alta* James, 1974, *L. carolinensis* Claassen, 1923, *L. moha* Ricker, 1952, *L. rickeri* James, 1976, *L. schusteri* Grubbs, 2015, and *L. tenuis*). Cytochrome c oxidase subunit 1 sequences were treated as above, and high-quality mt*CO1* sequences for *L. alexanderi* Hanson, 1941, *L. duplicata* Claassen, 1923, *L. ferruginea*, *L. rickeri*, *L. sibleyi*, and *L. tenuis* were downloaded from BOLD (Supplementary material, Table S2). Phred quality scores indicated the quality of each base in the sequences. All sequences were trimmed to a score of 30 using 4peaks, version 1.8 (Nucleobytes, Aalsmeer, The Netherlands). Edited sequences were assembled and aligned using Muscle in MEGA X (Kumar *et al.* 2016).

Molecular analyses

Molecular data were analysed with tree- and genetic distance–based phylogenetic methods. Overall tree topology and a preliminary assessment of putative female–male associations were visualised with a neighbour-joining tree constructed in MEGA X. Nodal support was assessed using 1000 bootstrap replicates (Felsenstein 1985). With one exception, males of each species clustered as monophyletic units. The neighbour-joining tree was used to determine the species identity of each female. To confirm the placement of females, two more robust phylogenetic approaches were used. A maximum-likelihood tree was generated using RAxML with the autoMRE setting. Although nodal support was again assessed using 1000 bootstrap replicates, the autoMRE setting allowed this analysis to finish earlier if 1000 replicates were unnecessary. A Bayesian-inference tree was also constructed using MrBayes MPI to further assess species relationships (Huelsenbeck *et al.* 2002) with the following settings: sequence data, GTR_I_G model, default Markov chain Monte Carlo commands (four chains, three heated), default number of runs ($n = 2$), stationary nucleotide frequency, set burn-in of 25%, random starting tree, sampling frequency 1000 generation, and a stop value between 0.01 and 0.05. Approximately 65 000 000 generations were required. A consensus tree was generated following the stop rule value.

Pairwise divergence distances were calculated in MEGA X using the Kimura two-parameter model of nucleotide substitution with the complete codon position deletion option. We compared pairwise divergence values among males and putative females of the same species (intraspecific) and between all species (interspecific). Minimum and maximum intraspecific and interspecific values were generated as more reliable indicators of genetic distinctiveness than referring only to mean values (Meier *et al.* 2006; Mynott *et al.* 2011). Automatic barcode gap discovery (Puillandre *et al.* 2012) was performed on the pairwise-distance data generated in MEGA X to complement the phylogenetic approach. Automatic barcode gap discovery creates operational taxonomic units based on comparisons of pairwise distances. Relative gap width was set at 1.5, the Kimura two-parameter model was applied, and all other settings were default (Puillandre *et al.* 2012). This method has been used successfully for stoneflies with new species descriptions (Teslenko *et al.* 2019) and for supporting operational taxonomic unit delineations based on mt*CO1* sequences (Morinière *et al.* 2017; Grubbs *et al.* 2020).

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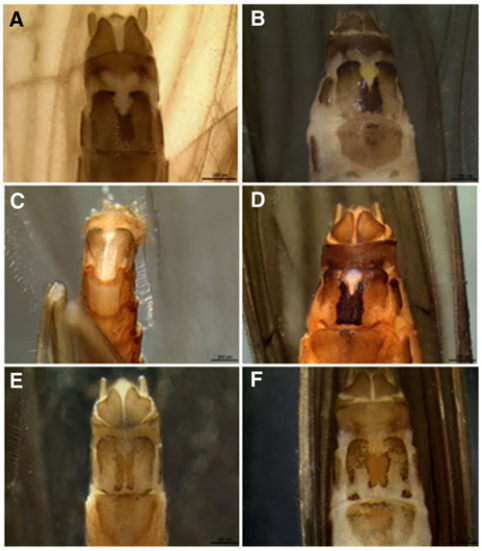

Fig. 2. *Leuctra ferruginea* group females, abdominal terminalia, ventral view: **A**, *L. ferruginea* OTU 1, Setrock Creek; **B**, *L. ferruginea* OTU 1, Setrock Creek; **C**, *L. ferruginea* OTU 2, Lower Creek; **D**, *L. ferruginea* OTU 2, tributary Lower Creek; **E**, *L. truncata*, Big Lost Cove Creek; and **F**, *L. truncata*, Neals Creek. OTU, operational taxonomic unit.

Results

In total, 360 *Leuctra* males were collected from Mount Mitchell streams in 2019, and all could be readily identified to species level using traditional morphological characteristics (*i.e.,* dorsal processes and paraprocts). We collected 477 females, and most could not be identified to the species level with any confidence because subgenital plate features were similar across several species (Figs. 2–4).

We sequenced or gathered from BOLD a total of 401 sequences. These consisted of 162 determined males and *L. grandis* females collected in 2019, as well as BOLD downloads, and 239 undetermined females collected in 2019 (Supplementary material, Table S1). With two exceptions (see below), all valid species were supported by high bootstrap values (80–100%) and posterior probabilities (0.80–1.00). The maximum-likelihood and Bayesian-inference phylogenies displayed similar topologies, but none strongly supported the species group hypotheses of Harper and Harper (1997).

Morphology-based *L. grandis* female determinations were confirmed through molecular methods with successful pairing with males of the same species. The maximum-likelihood and Bayesian-inference trees associated females with males with very high nodal support for at least 16 species, including two *L. ferruginea* operational taxonomic units and one undetermined *Leuctra* operational taxonomic unit. The two *L. ferruginea* operational taxonomic units (*L. ferruginea* OTU 1 and *L. ferruginea* OTU 2) overlapped in space (Fig. 5). In addition, six of the seven other species that were included to provide additional information also had well-supported clades.

Of particular importance was the clade for *L. carolinensis*. This is a regional species with a "North Carolina, Black Mountains" type locality (Claassen 1923) that was collected in east Tennessee, United States of America, only approximately 40 km from Mount Mitchell but not during this study. No Mount Mitchell females grouped with the *L. carolinensis* individuals (Figs. 6–7).

Genetic divergence and automatic barcode gap discovery

There was overlap between maximum intraspecific and minimum interspecific divergence values for several species. Intraspecific divergence values ranged from 0% to 18.6% (Table 1),

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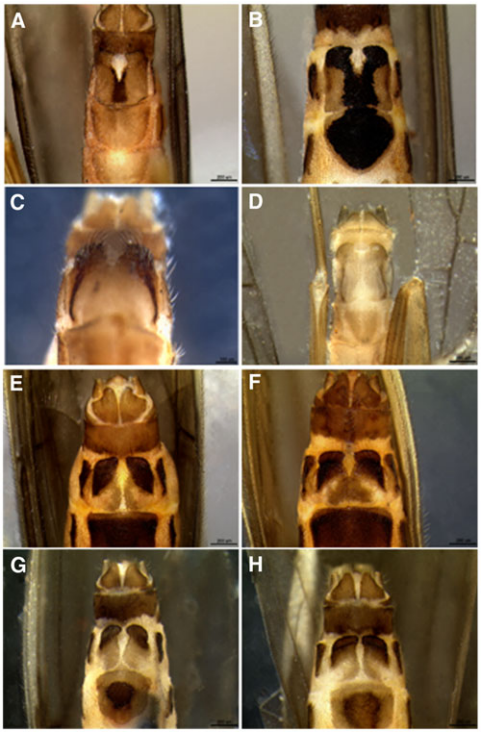

Fig. 3. *Leuctra tenuis* group females, abdominal terminalia, ventral view: **A**, *L. tenella*, Balsam Spring; **B**, *L. tenella*; seep; **C**, *L. tenuis*, South Toe River; **D**, *L. tenuis*; South Toe River; **E**, *L. triloba*, tributary Setrock Creek; **F**, *L. triloba*, seep; **G**, *L. variabilis*, Lower Creek; and **H**, *L. variabilis*, seep.

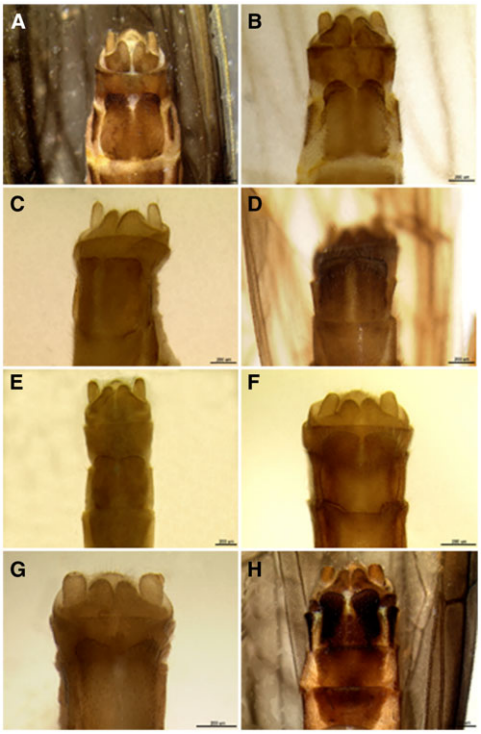

Fig. 4. *Leuctra biloba* group (**A–B**), *L. grandis* group (**C–F**), and undetermined *Leuctra* (**G–H**) females, abdominal terminalia, ventral view: **A**, *L. mitchellensis*, seep; **B**, *L. mitchellensis*, seep; **C**, *L. grandis*, Big Lost Cove Creek; **D**, *L. grandis*, Lower Creek; **E**, *L. sibleyi*, Left Prong South Toe River; **F**, *L. sibleyi*, Big Lost Cove Creek; **G**, Undetermined *Leuctra* operational taxonomic unit, Lower Creek; and **H**, Undetermined *Leuctra* operational taxonomic unit, Lower Creek.

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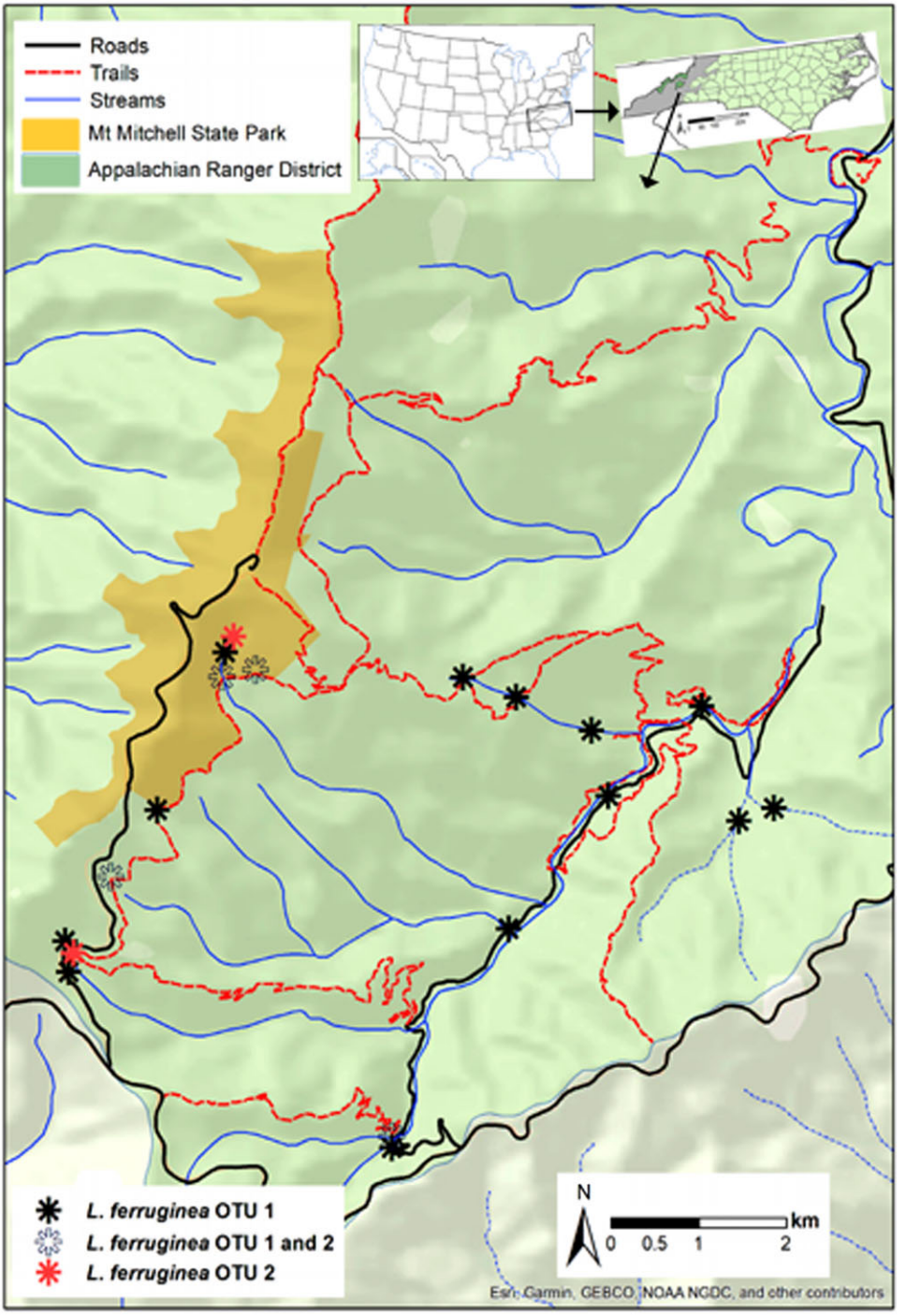

Fig. 5. Distribution of the two *Leuctra ferruginea* operational taxonomic units from Mount Mitchell State Park and Pisgah National Forest, North Carolina, United States of America. Map inset of North Carolina in upper right shows the Blue Ridge Ecoregion in grey and the Appalachian Ranger District, Pisgah National Forest in dark green.

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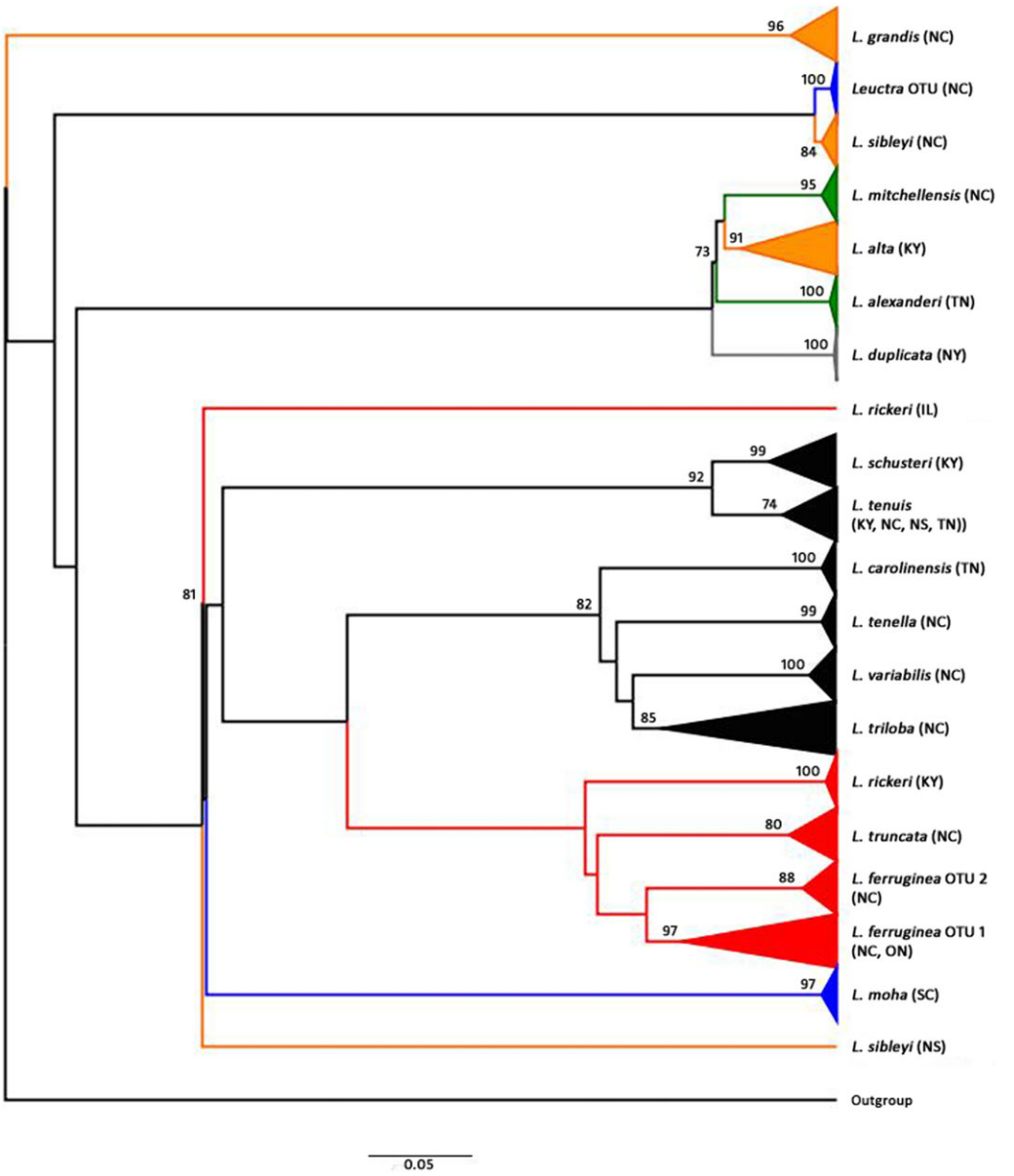

Fig. 6. Maximum likelihood tree for 18 eastern Nearctic *Leuctra* species made in RAxML. Support greater than 70% is represented on nodes. Horizontal triangle width is a function of the number of sequences per species. Scale bar represents substitution rate. Canadian and United States of America postal codes: Canada, ON = Ontario, NS = Nova Scotia; United States of America, IL = Illinois, KY = Kentucky, NC = North Carolina, NY = New York, SC = South Carolina, and TN = Tennessee. Proposed *Leuctra* species groups are represented by colour: orange = *L. grandis* group, grey = *L. duplicata* group, green = *L. biloba* group, black = *L. tenuis* group, red = *L. ferruginea* group, and blue = ungrouped species.

whereas minimum interspecific values ranged from 2.2% to 15.5% (Table 2). Although intraspecific values of at most 3% occurred only with a combination of six species and operational taxonomic units (Table 1), there was a general, albeit variable, relationship with the number of individuals sequenced (Fig. 8). A larger number of individuals sequenced (*e.g.*, *L. alta*, *L. triloba*; Fig. 8) were associated with a broader range of intraspecific divergence values. The notable exception was for

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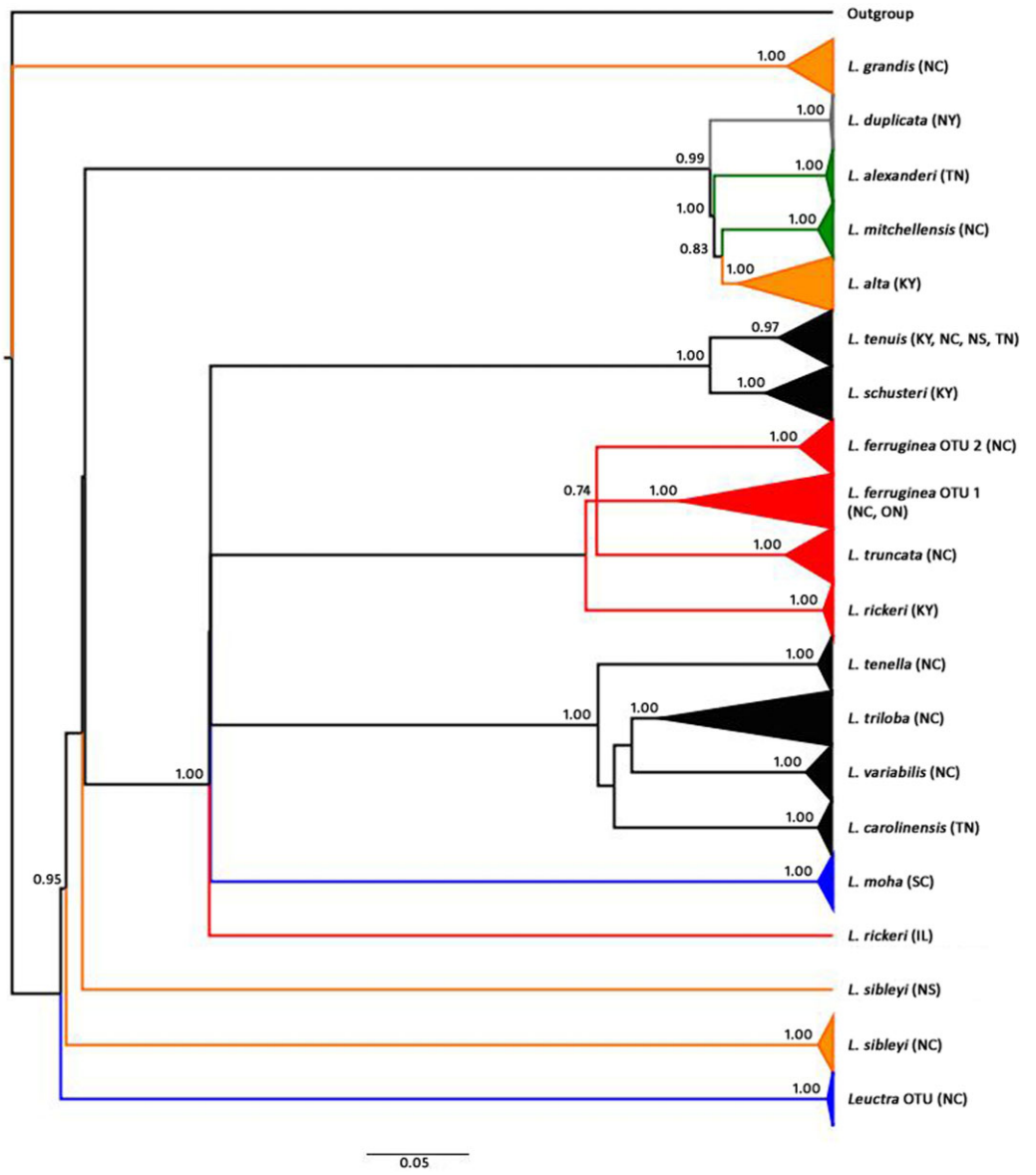

Fig. 7. Bayesian-inference tree for 18 eastern Nearctic *Leuctra* species made in MrBayes. Support greater than 0.7 is represented on nodes. Horizontal triangle width is a function of the number of sequences per species. Scale bar represents substitution rate. Canadian and United States of America postal codes: Canada, ON = Ontario, NS = Nova Scotia; United States of America, IL = Illinois, KY = Kentucky, NC = North Carolina, NY = New York, SC = South Carolina, and TN = Tennessee. Scale bar represents estimated substitution rate. Proposed *Leuctra* species groups are represented by colour: orange = *L. grandis* group, grey = *L. duplicata* group, green = *L. biloba* group, black = *L. tenuis* group, red = *L. ferruginea* group, and blue = ungrouped species.

L. sibleyi, with a high intraspecific divergence value (18.6%; Table 1) that was attributed to one individual (Fig. 8). Interestingly, the haplotype outlier was from Mount Mitchell and not the BOLD sequence of a Nova Scotia, Canada male.

Higher intraspecific divergence values were also associated with a higher number of automatic barcode gap discoveries (Table 1). Automatic barcode gap discovery groupings were concordant

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Table 1. Mean and range of intraspecific divergence values of mt*CO1* sequences for 16 eastern Nearctic *Leuctra* species, including two *L. ferruginea* operational taxonomic units, plus three undetermined females (*L.* operational taxonomic unit). *Leuctra* species are organised by groups as proposed in Harper and Harper (1997). "F" and "M" refer to numbers of female and male mt*CO1* sequences included in this study, respectively. Species in bold type were collected from Mount Mitchell, North Carolina, United States of America in 2019. Standard Canadian and United States postal codes were used in the table. OTU, operational taxonomic unit

Species group and species	No. F	No. M	Range	ABGD clusters
L. biloba Group				
L. alexanderi[*]	1	3	0.0–0.0	1
L. mitchellensis	6	2	0.0–1.4	1
L. duplicata Group				
L. duplicata[†]	1	1	0.3–0.3	1
L. ferruginea Group				
***L. ferruginea* OTU 1**[‡]	68	7	0.0–10.0	6
***L. ferruginea* OTU 2**	14	1	0.0–1.9	1
L. rickeri (KY – 2019)[§]	5	1	0.0–0.8	2
L. truncata	16	8	0.0–3.0	1
L. grandis Group				
L. alta (KY – 2019)	20	27	0.0–7.4	3
L. grandis	20	4	0.0–10.0	5
L. sibleyi[⊠]	5	4	0.0–18.6	3
L. tenuis Group				
L. carolinensis (TN – 2019)	7	1	0.0–1.6	1
L. schusteri (KY – 2019)	17	17	0.0–2.2	1
L. tenella	6	2	0.0–1.6	1
L. tenuis[#] (plus, KY – 2019)	13	16	0.0–4.8	6
L. trilobal	79	5	0.0–6.3	3
L. variabilis	13	1	0.0–1.6	1
Unplaced species				
L. moha (SC – 2019)	4	4	0.0–3.0	1
***Leuctra* OTU**	3	0	0.8–1.4	1

[*]All from BOLD sequences from the United States of America – Tennessee.
[†]All from BOLD sequences from the United States of America – New York.
[‡]Includes one BOLD sequence from Canada – Ontario.
[§]Includes one BOLD sequence from the United States of America – Illinois.
[⊠]Includes one BOLD sequence from Canada – Nova Scotia.
[#]Includes one BOLD sequence each from Canada – Nova Scotia and the United States of America – Tennessee. ABGD, automatic barcode gap discovery.

with species clades. There were 41 sequence operational taxonomic units (31 clusters and 10 singletons) recognised with automatic barcode gap discovery. All operational taxonomic units were generated by the initial partition with a prior intraspecific divergence of $Pmax = 0.00774$ (Table 1). Most groups represented species clades (24%), including the two *L. ferruginea* operational taxonomic units, and clades within species clades (71%). The remaining 5% were the undetermined *Leuctra* operational taxonomic unit and the outgroup.

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Table 2. Minimum interspecific divergence values of mt*CO1* sequences for at least 16 eastern Nearctic *Leuctra* species, including two *L. ferruginea* operational taxonomic units and three undetermined females (*L.* operational taxonomic unit), collected from Mount Mitchell, North Carolina, United States of America in 2019. Species are organised alphabetically by groups as proposed in Harper and Harper (1997). Species names are abbreviated: alex (*L. alexanderi*), mitc (*L. mitchellensis*), dupl (*L. duplicata*), ferr (*L. ferruginea* OTU 1 and OTU 2), rick (*L. rickeri*), trun (*L. truncata*), alta (*L. alta*), gran (*L. grandis*), sibl (*L. sibleyi*), caro (*L. carolinensis*), schu (*L. schusteri*), tene (*L. tenella*), tenu (*L. tenuis*), tril (*L. triloba*), vari (*L. variabilis*), and moha (*L. moha*). OTU, operational taxonomic unit

Species group and species	*L. biloba* group		*L. duplicata* group	*L. ferruginea* group				*L. grandis* group			*L. tenuis* group						Unplaced species	
	alex	mitc	dupl	ferr 1	ferr 2	rick	trun	alta	gran	sibl	caro	schu	tene	tenu	tril	vari	moha	*L.* OTU
alex																		
mitc	13.5																	
dupl	15.1	13.4																
ferr 1	11.9	15.2	14.7															
ferr 2	14.5	15.5	15.1	3.3														
rick	15.6	15.5	16.1	5.4	4.5													
trun	13.2	14.8	13.7	2.2	2.8	5.4												
alta	15.3	9.6	12.4	12.1	11.8	11.2	10.5											
gran	13.8	13.4	16.4	11.2	12.4	13.8	11.2	14.4										
sibl	12.7	15.1	13.4	11.5	12.8	12.1	12.4	11.7	13.7									
caro	15.2	15.8	15.8	9.3	10.3	9.6	9.9	13.1	13.4	12.8								
schu	15.9	15.8	16.9	7.2	7.2	8.8	6.6	13.1	12.7	11.5	10.6							
tene	14.5	13.4	14.7	7.8	9.0	10.3	8.1	13.8	13.2	12.2	8.1	9.4						
tenu	11.9	15.8	14.4	7.2	6.3	8.4	6.0	12.1	12.4	11.5	11.0	5.4	8.4					
tril	14.1	13.4	14.1	7.8	7.1	7.4	6.8	11.5	12.1	9.8	6.9	7.8	5.7	7.8				
vari	15.2	15.1	15.1	5.4	7.8	8.7	7.1	11.5	12.8	11.8	6.6	8.1	6.9	7.8	4.5			
moha	15.6	16.1	15.8	4.8	5.9	8.4	3.6	13.4	12.1	12.5	11.2	6.6	9.1	7.4	7.8	9.3		
Leuctra OTU	17.6	14.1	16.4	12.1	13.1	13.8	13.1	15.7	12.7	10	13.8	11.8	12.8	13.1	10.8	12.8	13.5	

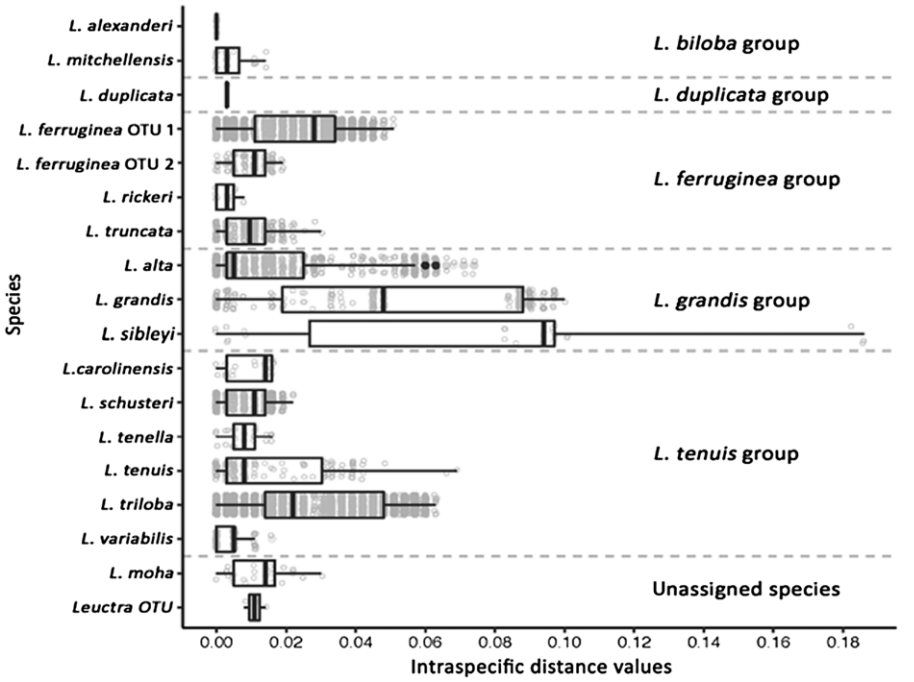

Fig. 8. Box and whisker jitter plot of intraspecific genetic distance values for each individual species unit. Species are arranged alphabetically within each proposed species group. Each dot represents a distance value between two unique sequences. The vertical line, rectangle, and horizontal line represent the median, interquartile range, and entire range of data, respectively.

There were low (≤ *ca.* 3%) minimum interspecific divergence values for regional members of the *L. ferruginea* group, and these were only most notably between *L. truncata* and the two *L. ferruginea* operational taxonomic units. The low-divergence value between the two *L. ferruginea* operational taxonomic units is concordant with the maximum-likelihood tree (Fig. 6). These two operational taxonomic units were nested as a single clade, albeit without strong nodal support (< 80%). The Bayesian-inference tree grouped both *L. ferruginea* operational taxonomic units with *L. truncata* as a three-taxon polytomy, also without strong nodal support (< 0.80). Overall, the general lack of resolution depicted within and between species-group relationships was a common theme with both the maximum-likelihood and Bayesian-inference analyses. Most notable was paraphyly shown for the *L. tenuis* group (Figs. 5–6). Whereas *L. schusteri* and *L. tenuis* appear to form a sister-species group with strong nodal support (maximum-likelihood: 92%; Bayesian-inference: 1.00), the remaining species (*L. carolinensis*, *L. tenella*, *L. triloba*, and *L. variabilis*) form their own monophyletic group (maximum-likelihood: 82%; Bayesian-inference: 1.00). Despite the possible paraphyletic nature of the *L. tenuis* group (Figs. 5–6), this group as a whole was most closely grouped to members of the *L. ferruginea* group. Minimum interspecific divergence values ranged from 5.4% to 9.0% (Table 1).

Discussion

General patterns

For both the Gattolliat *et al.* (2016) and Vitecek *et al.* (2017) phylogenetic treatments of Palearctic *Leuctra*, the molecular approach generally supported morphology-based species

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groups (*i.e.,* Harper and Harper 2003). In this study, mt*CO1* phylogenetic analyses showed strong nodal support for 18 monophyletic groups that represented at least 16 of the 31 Nearctic species. Males were grouped into distinct clades with high to very high nodal support. These values likewise provided strong support for inclusion of sequenced females, thus permitting confidence with mt*CO1*-based species determinations in lieu of subjective, subtle morphological differences.

Females of most Nearctic *Leuctra* species are difficult to identify to species with confidence. Notable exceptions include *L. duplicata* and *L. maria* Hanson, 1941 (Grubbs and Wei 2017), *L. hicksi* Harrison and Stark, 2010 (Grubbs *et al.* 2020), and *L. grandis*. We found that the shape of the subgenital plate lobes and medial cleft for females studied here appear different with small changes in the viewing angle (*e.g.,* Fig. 3E–F). Plates can appear different with age (*e.g.,* teneral *versus* mature; Fig. 3A–B), and the space between lobes also varies between gravid and nongravid females. Using morphological features alone to identify females would have resulted in many incorrect determinations for all species except *L. grandis*. An integrative taxonomic approach has provided a more accurate and robust data set.

Many *Leuctra* species in our study showed a high amount of maximum intraspecific genetic variation. Broad ranges have been reported for several species of Plecoptera (Zhou *et al.* 2009; Mynott *et al.* 2011; Sweeney *et al.* 2011; Boumans and Baumann 2012; Avelino-Capistrano *et al.* 2014; Gill *et al.* 2015a). For example, Avelino-Capistrano *et al.* (2014) reported maximum intraspecific values ranging from 0% to 15% for Brazilian *Kempnyia* Klapálek, 1914. Mynott *et al.* (2011) reported maximum intraspecific values ranging from 0% to 5.8% for Australian *Riekoperla* McLellan, 1971. Sweeney *et al.* (2011) reported high intraspecific variation for Nearctic *Perlesta* Banks, 1906 but with no gaps between intraspecific and interspecific pairwise values.

The bi- and tri-model distributions reported here for intraspecific data are not due solely to a large proportion of individuals. *Leuctra grandis*, *L. ferruginea* OTU 1, *L. sibleyi*, and *L. triloba* each had maximum intraspecific values that were greater than 5%. High values for *L. ferruginea* OTU 1 and *L. triloba*, however, were both due to only one divergent individual. *Leuctra sibleyi* exhibited a trimodal divergence distribution, with one individual yielding the highest intraspecific variation value (18.6%) and three individuals representing an intermediate haplotype (10%). Males of *L. sibleyi* were morphologically inseparable even though the intraspecific variation was high. Considering the small geographic scope of this study, there are essentially no barriers separating these groups, and intermediate haplotypes may not have been collected. *Leuctra grandis* exhibited a bimodal divergence distribution, with 16 individuals showing maximum intraspecific divergence values that were greater than 5% (Fig. 8). Again, intermediate haplotypes may not have been collected.

There was overlap in distance values between individuals of species with high maximum intraspecific and low minimum interspecific values. Most overlaps were due to low numbers of divergent individuals, as noted above. For example, if those few individuals were removed, the following species or operational taxonomic unit pairs would no longer overlap – *L. ferruginea* OTU 1 and *L. ferruginea* OTU 2, and *L. variabilis* and *L. triloba*. The only species pairs that would still have overlapping values would be *L. ferruginea* OTU 2 and *L. truncata*, and *L. ferruginea* OTU 1 and *L. truncata*. These latter two species have been placed in the *L. ferruginea* group (Harper and Harper 1997).

The overlap between intraspecific and interspecific genetic distance values suggests that other methods may better explain the data. The automatic barcode gap discovery algorithm separated all species into singletons or clusters. There were no shared clusters between morphologically defined species. In many cases, automatic barcode gap discovery overdivided species into multiple clusters or singletons due to variations in intraspecific values (*e.g., L. sibleyi*). The variation could be due to uncommon variant haplotypes,

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diverse population genetic structure, incomplete geographic sampling (*i.e.,* intermediate elevation sites were most difficult to access), or a combination of the three.

Species groups

Members of all five Nearctic species groups (Harper and Harper 1997) were included in this study, although none were fully represented. Group membership, however, was only partially supported by the mt*CO1* phylogenetic analyses. Including *L. moha*, which remains ungrouped (Harper and Harper 1997; Grubbs *et al.* 2020), the maximum-likelihood and Bayesian-inference phylogenetic trees had similar topology. The maximum-likelihood analyses recognised all individuals from Mount Mitchell as monophyletic units, including nine recognised species. The Bayesian-inference tree similarly showed similar patterns of monophyly, except for the polytomy of *L. ferruginea* OTU 1, *L. ferruginea* OTU 2, and *L. truncata*. The deeper relationships (*e.g.,* species groups), however, were not well resolved in either the Bayesian-inference or maximum-likelihood analysis.

Leuctra alexanderi and *L. mitchellensis* (*L. biloba* group) consistently grouped together but only with *L. atla* (*L. grandis* group). Harper and Harper (1997) noted an apparent close relationship between the *L. biloba* and *L. grandis* groups. Interestingly, in the present study, *L. duplicata* always grouped with the three aforementioned species with strong nodal support. The inclusion of more sequences of *L. duplicata*, together with *L. maria* – the two species of the *L. duplicata* group – in subsequent analyses is needed to better confirm this relationship. Although the original inclusion of *L. maria* was tentative (Harper and Harper 1997), morphological similarities between these two species in paraproct, vesicle, and subgenital plate features (Grubbs and Wei 2017) suggest a sister-species relationship.

Leuctra alta was placed tentatively in the *L. grandis* group (Harper and Harper 1997) due to common characteristics with other members (*i.e*., bilobed dorsal process, narrow subanal lobes). In the present study, the *L. grandis* group was represented by three of five species, but none grouped together. Despite the superficial similarities in the shape of the dorsal process of *L. alta* with that of *L. sibleyi* and in the paraproct characteristics of *L. grandis* with those of *L. sibleyi* (Grubbs, unpublished data), from a phylogenetic perspective, these three species were always recovered as completely independent of each other. Moreover, minimum interspecific divergence values ranged from 11.7% to 14.4%.

The *L. tenuis* group was paraphyletic. Harper and Harper (1997) split this group into two subgroups, based on emergence timing and subanal lobe characteristics. They placed *L. carolinensis* and *L. tenella* into one subgroup and the remaining species into a second. Although two monophyletic units were recovered in the present study, they do not support Harper and Harper (1997). Monophyly of *L. carolinensis* and *L. tenella* was not recovered in either analysis. In the present study, *L. schusteri* and *L. tenuis* were clustered as a sister-species pair with high nodal values. This was further supported by relatively low interspecific divergence (5.4%). This agrees with Grubbs (2015), who noted that these two species vary from the other members of the group by dorsal-process characteristics. Of additional importance was that the sequences of *L. tenuis* from Kentucky and Tennessee, United States of America, and from Nova Scotia, Canada, grouped together with the North Carolina specimens with good to strong nodal support. The remaining four group species (*L. carolinensis*, *L. tenella*, *L. triloba*, and *L. variabilis*) grouped together with good to strong nodal support. Similarly, interspecific divergence only ranged from 4.5% to 8.1%.

Although two subgroups were proposed for the *L. ferruginea* group (Harper and Harper 1997), this taxon is an enigma. For example, Harrison and Stark (2010) provided good evidence using scanning electron microscopy that *L. rickeri* is a junior synonym of *L. alabama* James, 1974. This is particularly relevant because these two species were placed into different subgroups. Although the Kentucky specimens of *L. rickeri* that were used in the present study grouped with

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L. ferruginea and *L. truncata*, the single sequence of *L. rickeri* from Illinois, United States of America, was consistently basal to the clade of *L. ferruginea* group + *L. tenuis* group + *L. moha*.

Unresolved species

There were two separate *L. ferruginea* operational taxonomic units and the undetermined *Leuctra* operational taxonomic unit. The low interspecific divergence value (3.3%) between the *L. ferruginea* operational taxonomic units and the close grouping on both the Bayesian-inference and maximum-likelihood trees suggest that they represent one distinct, albeit genetically variable, species. An important item to note is that the single BOLD sequence for a male from Ontario, Canada consistently grouped with *L. ferruginea* OTU 1 in all phylogenetic approaches.

Although there were only three undetermined *Leuctra* operational taxonomic unit females, the high minimum interspecific divergence values suggest that this may represent a distinct species. What are the options based on currently recognised species? No Mount Mitchell females grouped with the *L. carolinensis* individuals (Figs. 5–6). This is a regional species with a "North Carolina, Black Mountains" type locality (Claassen 1923) that was collected in east Tennessee, only approximately 40 km from Mount Mitchell but not during the present study. *Leuctra biloba* Claassen, 1923, *L. nephophila* Hanson, 1941, and *L. monticola* Hanson, 1941 were the only three remaining regional Nearctic *Leuctra* species not included in this analysis. *Leuctra biloba* is endemic to the southern Appalachian Highland region but was not collected in our study. *Leuctra nephophila* is also endemic to the southern Appalachian Highland region. Although not included here, we included BOLD sequences (SMSTO172, SMSTO173) of *L. nephophila* in preliminary analyses. This species did not group with the *Leuctra* operational taxonomic unit females, and interspecific divergence values exceeded 10%. *Leuctra monticola* is arguably the rarest Nearctic *Leuctra*, known only from the type series collected from Cades Cove (Great Smoky Mountain National Park, Tennessee) in 1938. Overall, males and subsequent molecular analyses are needed before a species name can be confidently applied to the *Leuctra* operational taxonomic unit individuals.

Conclusions

An integrative morphological and molecular approach added robust taxonomic information to the *Leuctra* faunal diversity of Mount Mitchell, North Carolina. Species names were assigned with confidence to an additional 235 females. The additional information included from other 2019 localities and from BOLD sequences served to better contextualise tree- and genetic distanced–based analyses. Morphological characteristics and gene sequence analyses may not align the same species into individual groups. Evidence presented in the present study suggests revisions to group memberships are plausible with future phylogenetic work. No changes, however, are proposed at this time to the Nearctic *Leuctra* species groups (Harper and Harper 1997) due to the use of only one gene. Previous studies have shown that the use of a single mitochondrial gene is often insufficient to understand relationships amongst different species (Mynott *et al.* 2011; Gill *et al.* 2015a). Additional genes (*e.g.,* nuclear *28S*) or single nucleotide polymorphisms of whole genomes could help resolve these older relationships. Vitecek *et al.* (2017) used a concatenated data set of two mitochondrial and two nuclear genes to assess the *L. inermis* group, but even a combination of faster- and slower-evolving genes did not resolve relationships between some species. The authors suggested that diversification within the species group was recent. A similar phenomenon could be operating on Nearctic *Leuctra*. Molecular characterisation of the entire genus *Leuctra* from both Nearctic and Palearctic realms should be considered as more DNA sequence data become available.

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Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.4039/tce.2022.5.

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Competing interests. The authors declare no competing interests.

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