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Prodigious polyphyly in Pleuroceridae (Gastropoda: Cerithioidea)

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Abstract

Phylogenomic studies with hundreds or thousands of loci are rare for most invertebrate groups, including freshwater gastropods. This can prevent understanding of phylogeny, which hinders many areas of research. Pleuroceridae is a family of freshwater snails that is highly imperiled and plays an essential role in the ecology of many freshwater systems of the eastern United States. However, the evolutionary history of the family is not understood, and the systematics of the family has not been revised in a modern framework. Pleurocerids display a variety of egg-deposition behaviors and shell shapes, making the family an ideal system for studying evolution of invertebrate life history and morphology. However, past mitochondrial-based phylogenetic analyses have failed to produce meaningful phylogenetic hypotheses, preventing conclusions about pleurocerid systematics and evolution. Here, we generated a novel anchored hybrid enrichment probe set with phylogenetic utility for Pleuroceridae. We sampled pleurocerids from across their range to test the probe set and generated a backbone phylogeny. Our analyses uncovered striking levels of polyphyly among currently accepted genera. Numerous species were also polyphyletic, indicative of unrecognized diversity. Phylogenetic patterns also revealed considerable convergence of shell morphologies. In contrast, anatomical and life history features appeared to be much less homoplastic. Despite generic paraphyly, high support for most major clades and phylogenetic cohesiveness of non-shell characters indicate utility of the AHE probe set for studying pleurocerid evolution.

Keywords: Anchored hybrid enrichment; probe set; freshwater; biodiversity; snails; evolution

1 INTRODUCTION

Phylogenetics forms the cornerstone of modern

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evolutionary biology and taxonomy. Understanding evolutionary relationships is essential for studying processes that gave rise to biodiversity (Maddison 1994; Soltis and Soltis 2003) and for constructing a natural classification (Sites Jr. and Marshall 2003; Sites Jr. and Marshall 2004; Wiley and Lieberman 2011). Yet, many groups lack a well-resolved phylogeny, which hinders studies of life history, toxicology, biogeography, and ecology because results cannot be placed in a robust comparative context. Furthermore, a classification that does not accurately reflect diversity can hamper effective conservation efforts (Mace 2004; Wilson 2017). In recent years, development of genomic tools for inferring phylogenetic relationships has greatly advanced our ability to infer robust evolutionary frameworks. Specifically, ultraconserved elements (UCEs) and anchored hybrid enrichment (AHE) loci have shown considerable utility in metazoan phylogenetics (McCormack et al. 2013; Stout et al. 2016; Esselstyn et al. 2017; Homiziak et al. 2019; Buenaventura et al. 2020). However, a disproportionate focus on vertebrates and arthropods has left many invertebrates lacking necessary genomic resources (but see Teasdale et al. 2016; Abdelkrim et al. 2018; Quattrini et al. 2018; Pfeiffer et al. 2019; Moles and Giribet 2021).

The freshwater gastropod family Pleuroceridae (Cerithioidea) (Figs. 1, 2) is one such understudied group of high conservation concern that lacks genomic resources. Pleurocerids live in habitats ranging from springs to big rivers in North America east of the Rocky Mountains (Burch and Tottenham 1980; Strong and Köhler 2009; Johnson et al. 2013). Pleurocerids play important nutrient cycling roles in rivers of the eastern United States (Richardson et al. 1988; Rosemond et al. 1993; Huryn et al. 1995), and they can make up over 90% of macroinvertebrate biomass in some southeastern United States streams (Newbold et al. 1983). The family's diversity is centered in the Mobile and Tennessee River basins of the southeastern United States, both of which are under considerable anthropogenic stress (Lydeard and Mayden 1995; Lydeard et al. 1997). Recent estimates indicate that over 79% of pleurocerid species are imperiled, and 33 are considered extinct (Johnson et al. 2013). The latter includes all six species of the genus *Gyrotoma* Shuttleworth, 1845, which was endemic to the mainstem Coosa River, Alabama, USA, prior to extensive modifications for hydropower. At least 24 other pleurocerids went extinct in the 20th century, most from the mainstem Coosa River but also from the Tennessee and Ohio River drainages (Johnson et al. 2013). Remaining pleurocerid diversity is largely fragmented, particularly in the Mobile and Tennessee River drainages, with many species restricted to lower sections of large tributaries. One notable exception where species do not suffer considerable fragmentation is in the Cahaba River system, which remains one of the least modified rivers in the southeastern United States (Ward et al. 2005). Despite being less speciose than in the Tennessee and Mobile River drainages, pleurocerids are also found throughout central and eastern North America in Gulf Coast drainages, the eastern Atlantic slope, the Ohio River drainage, the Mississippi River drainage, and the Great Lakes drainages where they serve as dominant grazers that have an essential role in nutrient cycling (Dazo 1965; Power et al. 1988; Miller-Way and Way 1989; Johnson and Brown 1997).

Like other freshwater gastropods, pleurocerids are understudied relative to their ecological importance, and the systematics is woefully out of date (Lysne et al. 2008; Perez and Minton 2008; Johnson et al. 2013). Pleuroceridae was traditionally

subdivided into two subfamilies: the Pleurocerinae with species from North America and the Semisulcospirinae with those from Asia (Strong and Köhler 2009). In light of molecular phylogenetic analyses, Strong and Köhler (2009) transferred western North American *Juga* to the Semisulcospirinae and elevated it to family rank while restricting the Pleuroceridae to species east of the Rocky Mountains. Over 850 available pleurocerid species group names have been established, but only ~166 species are currently considered valid (Tryon 1873; Graf 2001; Johnson et al. 2013). Of the roughly 35 available genus-group names, only seven are considered valid for extant species (Fig. 1).

The largely shell-based classification of pleurocerids has remained essentially unchanged since the seminal work of Burch and Tottenham (1980) and has not been revised within a robust molecular framework. Several attempts have been taken to apply molecular data to pleurocerid systematics, and four genera, *Elimia* Adams and Adams, 1854; *Lithasia* Haldeman, 1840; *Pleurocera* Rafinesque, 1818; and *Leptoxis* Rafinesque, 1819, were shown to be polyphyletic. Yet, no widely accepted taxonomic revisions have been made. Furthermore, past molecular phylogenetic studies relied almost exclusively on mitochondrial genes and were significantly under-sampled relative to the diversity of Pleuroceridae (Lydeard et al. 1997; Lydeard et al. 1998; Holznagel and Lydeard 2000; Minton and Lydeard 2003; Lee et al. 2006).

The exclusive use of mitochondrial genes for species tree inference can be problematic for any organismal group (Funk and Omland 2003). However, evolutionary analyses with mitochondrial genes are particularly problematic for Pleuroceridae. Mitochondrial loci in pleurocerids can display intraspecific variation of over 20%, including among morphologically identical individuals occurring in syntopy (Dillon and Robinson 2009; Whelan and Strong 2016). Although the precise cause of extreme mitochondrial heterogeneity has yet to be determined, morphological data and limited nuclear sequencing indicate that mitochondrial gene trees do not reflect species relationships (Whelan and Strong 2016; Whelan et al. 2019). The majority of phylogenetic studies in this group took place before mitochondrial heterogeneity was well documented (Lydeard et al. 1997; Lydeard et al. 1998; Holznagel and Lydeard 2000; Minton and Lydeard 2003; Lee et al. 2006), so the true extent of generic polyphyly is unknown. Furthermore, traditional nuclear markers used in molluscan systematics do not appear to be sufficiently variable to resolve pleurocerid species relationships (Lee et al. 2006; Whelan and Strong 2016). Overall, past molecular phylogenetic studies suggest that the current, shell-based classification does not accurately reflect evolutionary relationships at the genus and species level, but limited taxon sampling and reliance on mitochondrial genes precludes meaningful conclusions.

Phylogenomics offers the most promising method for resolving pleurocerid phylogeny. Anchored hybrid enrichment is a cost-effective method for generating data for hundreds of nuclear loci with baits, or probes, to enrich DNA sequencing libraries with target genome regions (Lemmon et al. 2012; Lemmon and Lemmon 2013). For phylogenomics, this method has advantages over other cost-effective methods for sequencing nuclear loci (e.g., RAD-seq) as probes can be designed to be effective across broad taxonomic scales. However, unlike RAD-seq, AHE requires *a priori* genomic information to inform marker selection and development of baits. Although this can be

an initial barrier to using an AHE approach, a well-designed probe-set is a valuable tool for systematics, allowing for a repeatable sequencing strategy so future studies can leverage previously generated datasets (e.g., Pfeiffer et al. 2019; Smith et al. 2020). Here, we develop an AHE probe set for the Pleuroceridae and test the efficacy of the novel probe set by inferring a backbone phylogeny for the family. In doing so, we examine insights that can be gained from the use of this genomic resource. We also outline areas of research on pleurocerids that will benefit from a well-tested AHE probe set.

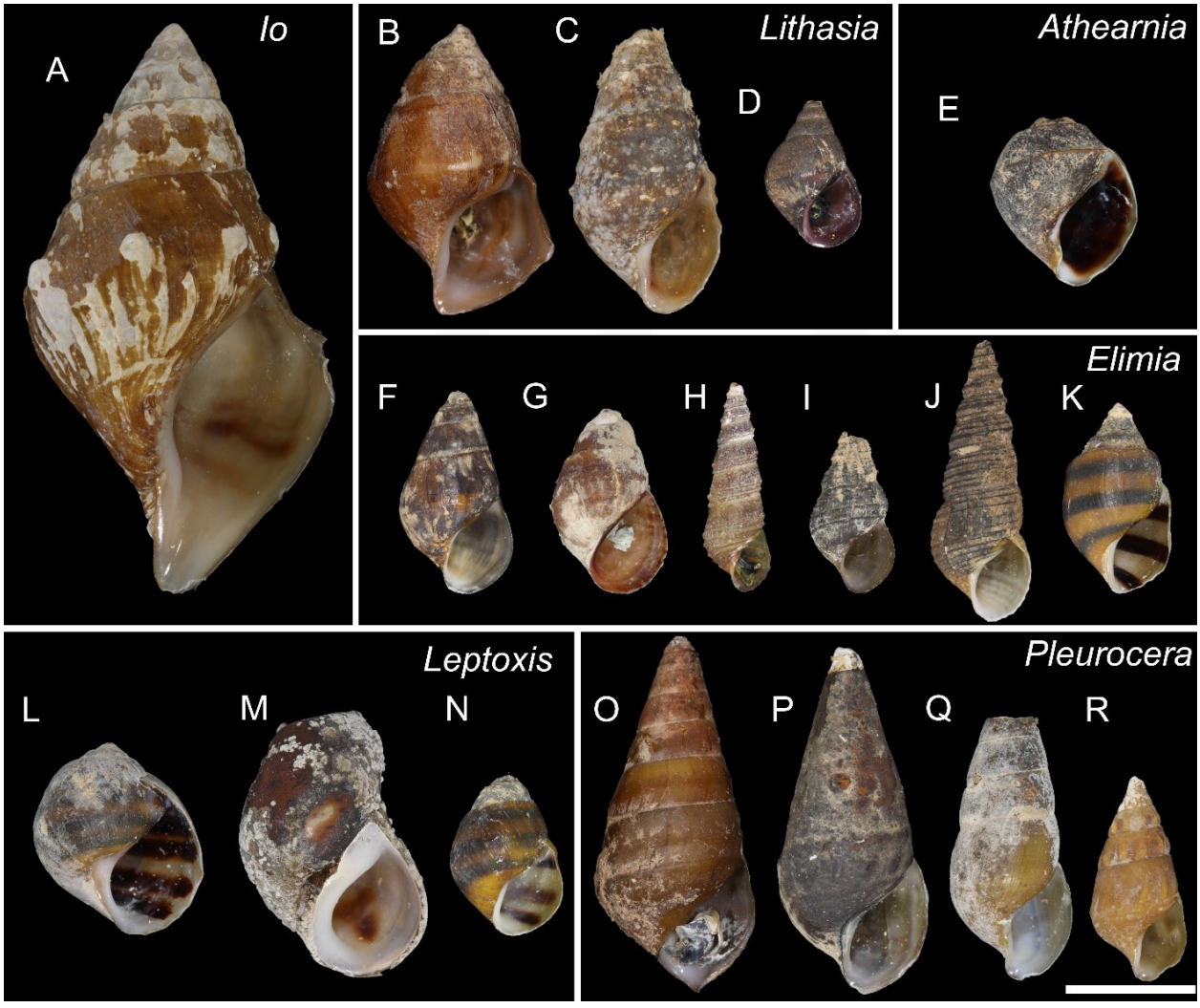

Figure 1: Shell morphology of currently recognized genera. See Figure 3 for clade designations of each individual. A) *Io fluvialis*, USNM 1597739, clade M; B) *Lithasia jayana*, USNM 1638600, clade M; C) *Lithasia verrucosa*, USNM 1597791, clade M; D) *Lithasia pinguis*, USNM 1597787, clade J; E) *Athearnia anthonyi*, USNM 1597544, clade E; F) *Elimia flava*, USNM 1597623, clade N; G) *Elimia potosiensis*, USNM 1597658, clade F; H) *Elimia cochliaris*, USNM 1597596, clade N; I) *Elimia interveniens*, USNM 1597717, clade H; J) *Elimia arachnoidea*, USNM 1597560, clade L; K) *Elimia showalteri*, USNM 1597595, clade N; L) *Leptoxis praerosa*, USNM 1597846, clade E; M) *Leptoxis picta*, USNM 1597796, clade A; N) *Leptoxis plicata*, USNM 1597799, clade N; O) *Pleurocera prasinata*, USNM 1638609, clade G; P) *Pleurocera foremani*, USNM 1597873, clade N; Q) *Pleurocera walkeri*, USNM 1597903, clade G; R) *Pleurocera alveare*, USNM 1597855, clade I. Scale bar = 1 cm. See Table S1 for collection details.

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2 METHODS

2.1 Taxon Sampling

Five species were sampled for transcriptome sequencing: *Leptoxis ampla* (Anthony, 1855), *Pleurocera prasinata* (Conrad, 1834), *Elimia "clavaeformis"* (Adams and Adams, 1854) (see results), *Elimia crenatella* (Lea, 1860), and *Lithasia geniculata* (Haldeman, 1840). These species were chosen to maximize phylogenetic diversity (Lydeard et al. 1997; Lydeard et al. 1998; Holznagel and Lydeard 2000; Minton and Lydeard 2003) for the purposes of designing AHE baits for use across Pleuroceridae. Tissue clips were harvested from the mantle (*Le. ampla* only) or foot, placed in RNAlater, and stored at -80°C.

Specimens for phylogenomic analyses were collected from across most of the geographic range of Pleuroceridae, focusing on centers of species diversity such as the Mobile, Tennessee, and Cumberland River drainages (Fig. 2; Table S1). We collected 192 individuals from 92 putative species representing all pleurocerid genera except the Mexican endemic *Lithasiopsis* Pilsbry, 1910 and the extinct *Gyrotoma*. Two specimens of *Juga plicifera* (Lea, 1838) (Semisulcospiridae; Table S1) were used as outgroups to root the tree. Species- and genus-level taxonomy follow the authoritative list of Johnson et al. (2013), except for species described since 2013 (e.g., Minton 2013) and in some cases where junior synonyms could be morphologically distinguished and were hypothesized to represent distinct species (see Table S1). Species were identified based on morphological comparisons to type material, original species descriptions, and other published accounts (Tryon 1873; Burch and Tottenham 1980). However, species-level dichotomous keys do not exist for Pleuroceridae, and poorly understood conchological variation makes identification difficult in some situations (Ó Foighil et al. 2009). Most species identifications were unambiguous, but we include notes for a few particularly difficult species in the Supplementary Material. Broadly, species identified as "*Genus* sp." fit the current concept of any given genus, but we could not confidently assign a currently recognized name to them. In a small number of cases where relationships among closely related individuals were unresolved and it was unclear whether all individuals should be a single species, we erred on the side of splitting individuals into different species if their morphology was distinct. For many taxa, more extensive geographical sampling will be necessary to determine if a name is available or if the taxon is undescribed. However, such species-level taxonomy was outside the scope of the current study. Individuals that could not be confidently assigned to a described species were labelled "sp.". We used the qualifier "aff." (see Bengtson 1988) to denote individuals that were morphologically identified as described species but that were distinct lineages based on phylogenetic analyses (Wiley 1978). When multiple lineages were identified within the same nominal species, lineages were distinguished by collection site (Table S1).

For almost all species, at least two individuals were sampled (Table S1). For species with comparatively large ranges, or for those with variable shell morphologies (Fig. 3; Supplementary Material), we sampled more than two individuals so that shell shape variation could be placed in a phylogenetic context. This also allowed us to examine the potential for cryptic diversity. All sequenced individuals were photographed, and shell vouchers for all sequenced specimens have been deposited in the collections of the National Museum of Natural History (Table S1). Some species were photographed alive in aquaria to document anatomical features,

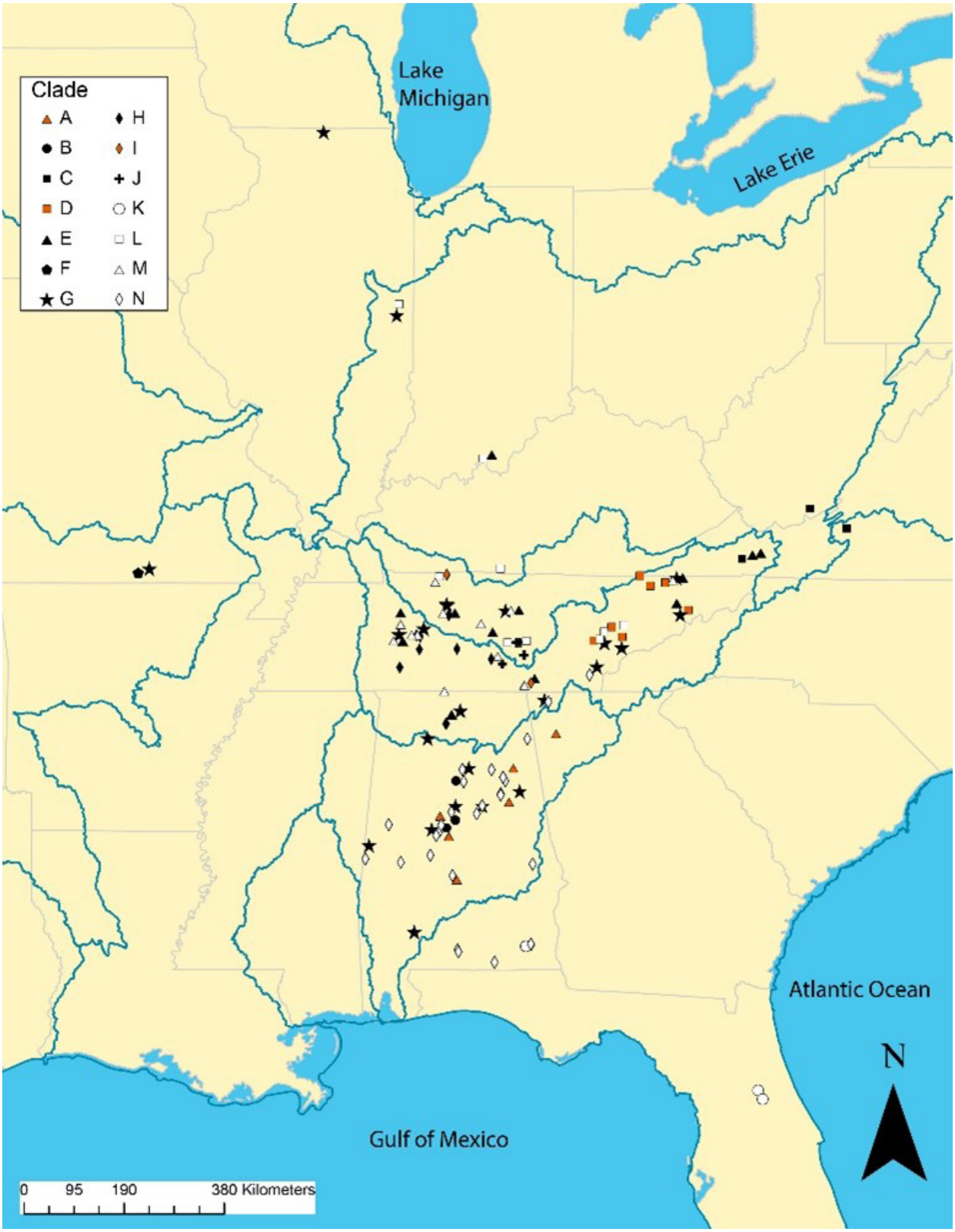

Figure 2: Map of sampled individuals. Standard hydrologic unit 2 watersheds are displayed, plus the Mobile River and Cumberland River drainages. In some cases, representatives from multiple clades were sampled from the same location, and points were artificially spread apart for visualization purposes. No symbol was moved across demarcated drainages. See Table S1 for precise collection localities.

but these individuals were not sequenced.

2.2 AHE marker design

Tissues in RNAlater were sent to Macrogen (Rockville, Maryland) for RNA extraction, transcriptome sequencing library prep using the Illumina TruSeq RNA library preparation kit V2, and 100bp paired-end sequencing on an Illumina HiSeq 2500. Raw-reads were assembled *de novo* with Trinity v2.40 (Haas et al. 2013). Adaptor sequences and low-quality reads were trimmed with Trimmomatic (Bolger et al. 2014), and all Trinity parameters were set to defaults.

Marker design utilized the five transcriptomes generated here and the *Biomphalaria glabrata* (Say, 1818) genome (Adema et al. 2017), which, at the time of marker design, was the species most closely related to Pleuroceridae with a published genome. We also used a draft genome of *Le. ampla* that was generated with Illumina paired-end and mate-pair sequencing (see Supplementary Material). Loci discovery and choice for the AHE probe set followed Breinholt et al. (2018; see Supplementary Material for more information). Briefly, potential loci useful for pleurocerid phylogenomics were screened from the *B. glabrata* genome with single copy and orthology criteria from Breinholt et al. (2018). This approach implicitly assumed genes had two alleles as *B. glabrata* and Pleuroceridae are diploid (Thiriot-Quiévreux 2003). These potential loci were extracted from pleurocerid transcriptomes and the *Le. ampla* genome with the genome_getprobe_BLAST.py script from Espeland et al. (2018); mitochondrial genes were excluded. Following Breinholt et al. (2018), loci were screened for orthology with s_hit_checker.py and ortholog_filter.py. To be chosen for downstream analyses, loci had to be present in at least 4 of 5 pleurocerid transcriptomes and larger than 120 bp in length. For the 742 loci that passed all screens, probes of 120bp were tiled across target regions of each reference taxon at 2X coverage. SureSelect Custom DNA Target Enrichment probes were ordered from Agilent Technologies for use in AHE sequencing. We performed a megablast search (Zhang et al. 2000) of the *B. glabrata* reference sequence for each locus against the NCBI non-redundant nucleotide database to annotate loci.

2.3 Phylogenomic data generation

DNA was extracted from foot tissue using the Qiagen DNeasy Plant Kit following Whelan et al. (2019). A plant kit was used as pleurocerids produce large amounts of mucopolysaccharides that are not handled well by extraction kits designed for animals. DNA was quantified with a Qubit Fluorometer, and each extraction was normalized to 50 ng/µL and sent to RAPID Genomics (Gainesville, Florida) for AHE library prep and sequencing. Library prep, sequencing, and dataset assembly followed Breinholt et al. (2018) and Pfeiffer et al. (2019) with slight modifications (see Supplementary Material for more details).

After assembly, orthology filtering, and alignment, we generated three datasets. The first dataset, pleurocerid_full, was not modified after the final alignment. An alignment-masked dataset, pleurocerid_masked, was generated by assessing alignment accuracy with Aliscore (Misof and Misof 2009) and by trimming ambiguously aligned sites with Alicut (Kück 2009). A third dataset, pleurocerid_probe, was generated by extracting probe regions (see Supplementary Material). The probe-regions-only dataset was generated to minimize missing data as flanking regions were not evenly sequenced owing to the non-specificity of AHE sequencing for regions that flank

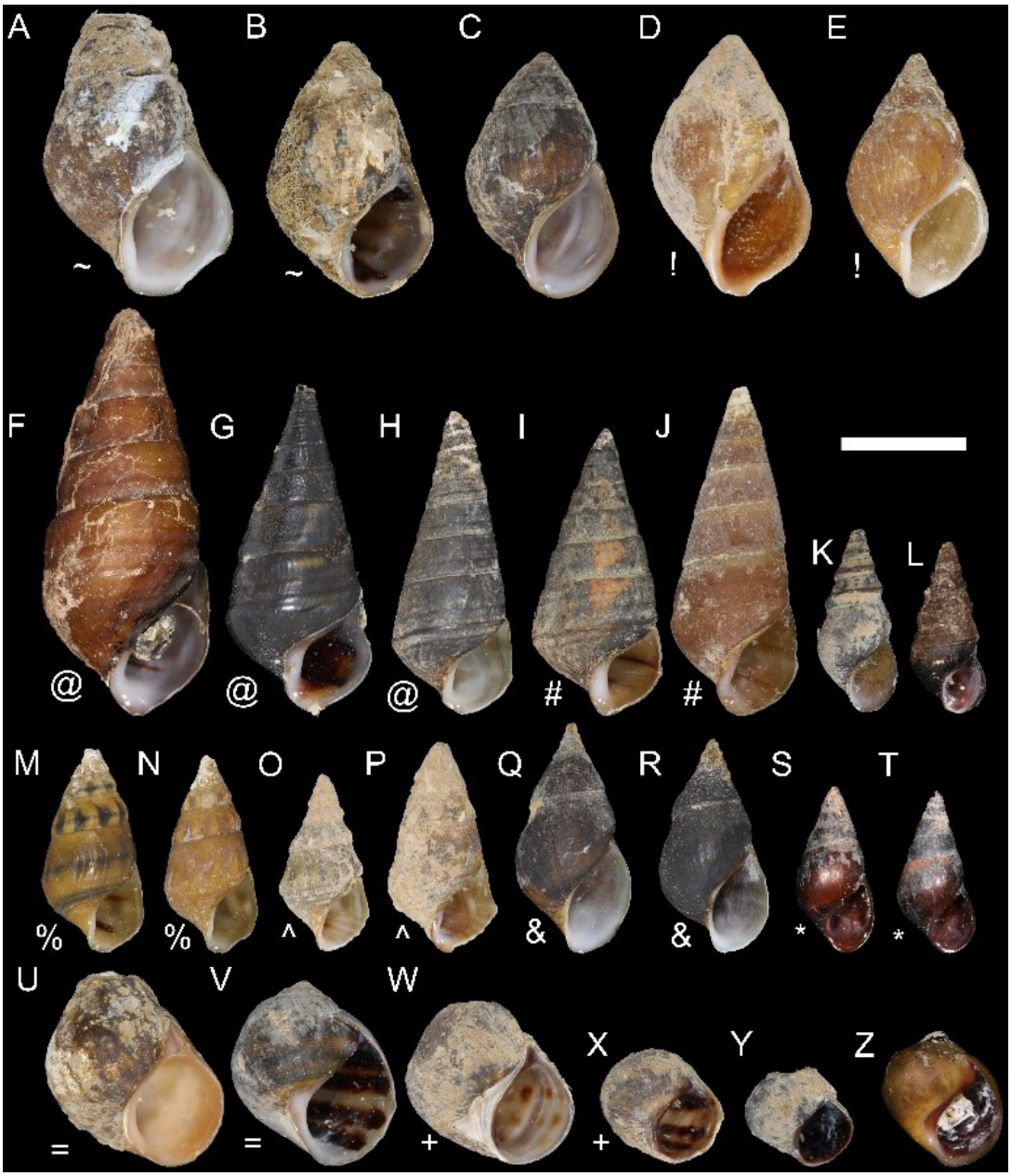

Figure 3: Intraspecific variation and interspecific convergence in shell morphology. Symbols indicate individuals are from the same evolutionary lineage (see Fig. 4). A) *Lithasia fuliginosa*, USNM 1597783, clade M; B) *Lithasia fuliginosa*, USNM 1597765, clade M; C) *Lithasia* aff. *fuliginosa* East Fork Stones River, USNM 1597766, clade M; D) *Lithasia* aff. *fuliginosa* Red and Harpeth Rivers, USNM 1597771, clade M; E) *Lithasia* aff. *fuliginosa* Red and Harpeth Rivers, USNM 1597769, clade M; F) *Pleurocera prasinata*, USNM 1638608, clade G; G) *Pleurocera prasinata*, USNM 1597950, clade G; H) *Pleurocera prasinata*, USNM 1597908, clade G; I) *Pleurocera* aff. *prasinata*, USNM 1597875, clade G; J) *Pleurocera* aff. *prasinata*, USNM 1597876, clade G; K) *Elimia carinifera*, USNM 1597581. Clade N; L) *Elimia* aff. *carinifera*, USNM 1638569, clade B; M) *Pleurocera alveare*, USNSM 1597855, clade I; N) *Pleurocera alveare*, USNM 1597856, clade I; O) *Pleurocera* aff. *alveare*, USNM 1597859, clade I; P) *Pleurocera* aff. *alveare*, USNM 1597858, clade I; Q) *Elimia simplex*, USNM 1597711, clade D; R) *Elimia simplex*, USNM 1597710, clade D; S) *Elimia* aff. *simplex*, USNM 1638580, clade D; T) *Elimia* aff. *simplex*, USNM 1638579, clade D; U) *Leptoxis virgata*, USNM 1597837, clade E; V) *Leptoxis virgata*, 1597846, clade E; W) *Leptoxis* aff. *praerosa*, Duck and Harpeth Rivers, USNM 1597821, clade E; X) *Leptoxis* aff. *praerosa*, Duck and Harpeth Rivers, USNM 1597805, clade E; Y) *Leptoxis* aff. *praerosa*, Limestone Creek USNM 1597807, clade E; X) *Leptoxis praerosa*, USNM 1638604, clade E. Scale bar = 1 cm. See Table S1 for collection details.

the AHE probes. The pleurocerid_probe alignment was not masked with Aliscore and Alicut as there were few gaps in the alignment. Moreover, we chose to analyze the pleurocerid_full dataset because automated alignment masking approaches may result in worse trees, at least in some situations (Tan et al. 2015). Loci from each dataset were concatenated with FASconCAT-G (Kück and Longo 2014) for some downstream analyses. See Supplementary Material for more details on dataset characteristics.

Once we generated the three initial datasets, two taxa that were represented by less than 10% of loci in each dataset were removed with AMAS (Borowiec 2016; see Table S1). Further filtering was done with TreSpEx (Struck 2014) following Struck (2014) and Whelan et al. (2015a; see Supplementary Material for more details), resulting in three datasets. Individual loci for each dataset were concatenated with FASconCAT-G (Kück and Longo 2014) to create concatenated datasets (Table 1).

2.4 Phylogenetic Analyses

Model testing and maximum likelihood (ML) phylogenetic inference for each concatenated dataset was done with IQTREE 1.6.12 (Nguyen et al. 2015). Best-fit partitions and models were inferred using ModelFinder (Kalyaanamoorthy et al. 2017), as implemented in IQTREE, using 20% fast relaxed hierarchical clustering and limiting the maximum number of partition pairs in each merging phase to 1000 to limit computational demands (Lanfear et al. 2014; Lanfear et al. 2017; Supplementary Material). Starting partition blocks were limited to individual loci. Codon positions were not used as starting partition blocks as some loci appeared to span introns, and we decided that trying to determine open reading frames for our target capture data would likely introduce error, rather than facilitate accurate phylogenetic inference. All available GTR-nested nucleotide models in IQTREE were tested, including those with the FreeRates model for rate heterogeneity across sites (Yang 1995; Soubrier et al. 2012). Bayesian information criteria were used to identify best-fit partitions and models. Model testing was followed by tree inference using best-fit partitions and models, allowing each partition to have its own evolutionary rate (Chernomor et al. 2016). Tree inference was done with 10 independent runs, reporting only the best-scoring tree. Nodal support was measured with 1000 ultrafast bootstrap replicates (BS; Hoang et al. 2018).

Dataset	Loci	Total Alignment Sites	Parsimony informative Sites	Singleton Sites	Invariant Sites	Taxon Occupancy	Total Missing Data
pleurocerid_probe	553	123,820	17,532	6,921	99,367	97%	3.15%
pleurocerid_masked	503	226,694	55,797	23,198	147,699	97.30%	8.66%
pleurocerid_full	441	241,990	64,529	28,139	149,322	97%	17.14%

Table 1: Dataset statistics

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As phylogenetic inference on concatenated datasets can result in positively misleading trees (Kubatko 2007), ASTRAL-III was used for species tree inference (Zhang et al. 2018). ASTRAL uses the multispecies coalescent to resolve individual gene tree conflict, and differences between trees inferred with concatenated datasets and ASTRAL can identify areas of the phylogeny under incomplete lineage sorting. Input single gene trees were inferred with IQTREE as above, except perturbation strength was set to 0.2 and 500 iterations had to be unsuccessful to stop tree inference; these parameters were chosen for ASTRAL-input tree inference because most loci were short (< 1,000 bp), and many individuals were closely related. Single-gene tree inference for ASTRAL was done with 10 independent runs for each locus, and best-scoring trees for each locus were placed into a single file. We collapsed nodes with BS equal to 10 or less with Newick Utilities (Junier and Zdobnov 2010) as collapsing poorly supported nodes has been shown to increase accuracy of species tree inference with ASTRAL (Zhang et al. 2018). We inferred an ASTRAL tree using a taxon map that enforced monophyly on individuals grouped into the same "species" (Table S1). Individuals were grouped into species based on shell morphology, collection locality, and results of the ML inference on concatenated datasets (Table S1; see above and Supplementary Material for more details). However, species boundaries for some taxa were unclear, and cryptic species are clearly present in our dataset (see below). When species boundaries were unclear, we erred on the side of splitting individuals rather than incorrectly lumping individuals into the same species. We also inferred an ASTRAL tree for each dataset without using a taxon map, as *a priori* species groupings may result in error if individuals are incorrectly grouped together. Support for relationships inferred by ASTRAL was measured with local posterior probability (LPP; Sayyari and Mirarab 2016).

Trees were visualized with FigTree (http://tree.bio.ed.ac.uk/software/figtree). ASTRAL does not infer branch lengths for tips with only one individual (i.e., all tips from analyses without a taxon map) so we used the R (R Core Development Team 2020) package APE (Paradis et al. 2004) to arbitrarily set undefined branch lengths to 0.15 coalescent units for visualization purposes of ASTRAL analyses without a taxon map. Trees were rooted with *Juga plicifera* and rendered in Adobe Illustrator. For discussion purposes, the ASTRAL species tree inferred for the probe-regions-only dataset (pleurocerid_probe) with a taxon map is used as the standard reference (Fig. 4); significant differences from the results obtained with ML on the concatenated pleurocerid_probe dataset, or with other datasets, are noted. Major clades are indicated with uppercase letters as referenced throughout the text.

3 RESULTS

3.1 Molecular data generation

Illumina sequencing of AHE libraries resulted in 65,119-3,829,792 raw paired-end reads per individual (average = 1,476,608; Table S1). Assembly was successful for 629 loci. The two individuals with the lowest number of reads, with only 14 and 24 loci respectively (Table S1), were discarded. For the other individuals, between 292-627 loci were assembled (average = 608; Table S1). After BLAST-based filtering of paralogs and removal of outlier genes that could cause systematic error, the final datasets had 441-553 loci and 123,820-241,990 characters (Table 1; more details in Supplementary Material).

3.2 Phylogenetic inference

Phylogenetic inference with different datasets and methodology resulted in trees that were largely congruent, especially at deeper nodes. All genera, except *Athearnia* Morrison, 1971 and *Io* Lea, 1831, were resolved as non-monophyletic (Figs. 4, S4-S11), but *Athearnia* and *Io* only have one extant representative. The sister clade to all other pleurocerids, clade A, comprises extant Mobile River drainage *Leptoxis sensu lato* (s.l.), minus *Leptoxis compacta* (Anthony, 1864) and *Leptoxis plicata* (Conrad, 1834) (Figs. 4, S4-S11). The majority of other *Leptoxis* s.l. species, including the type species of *Leptoxis*, *Leptoxis praerosa* (Say,

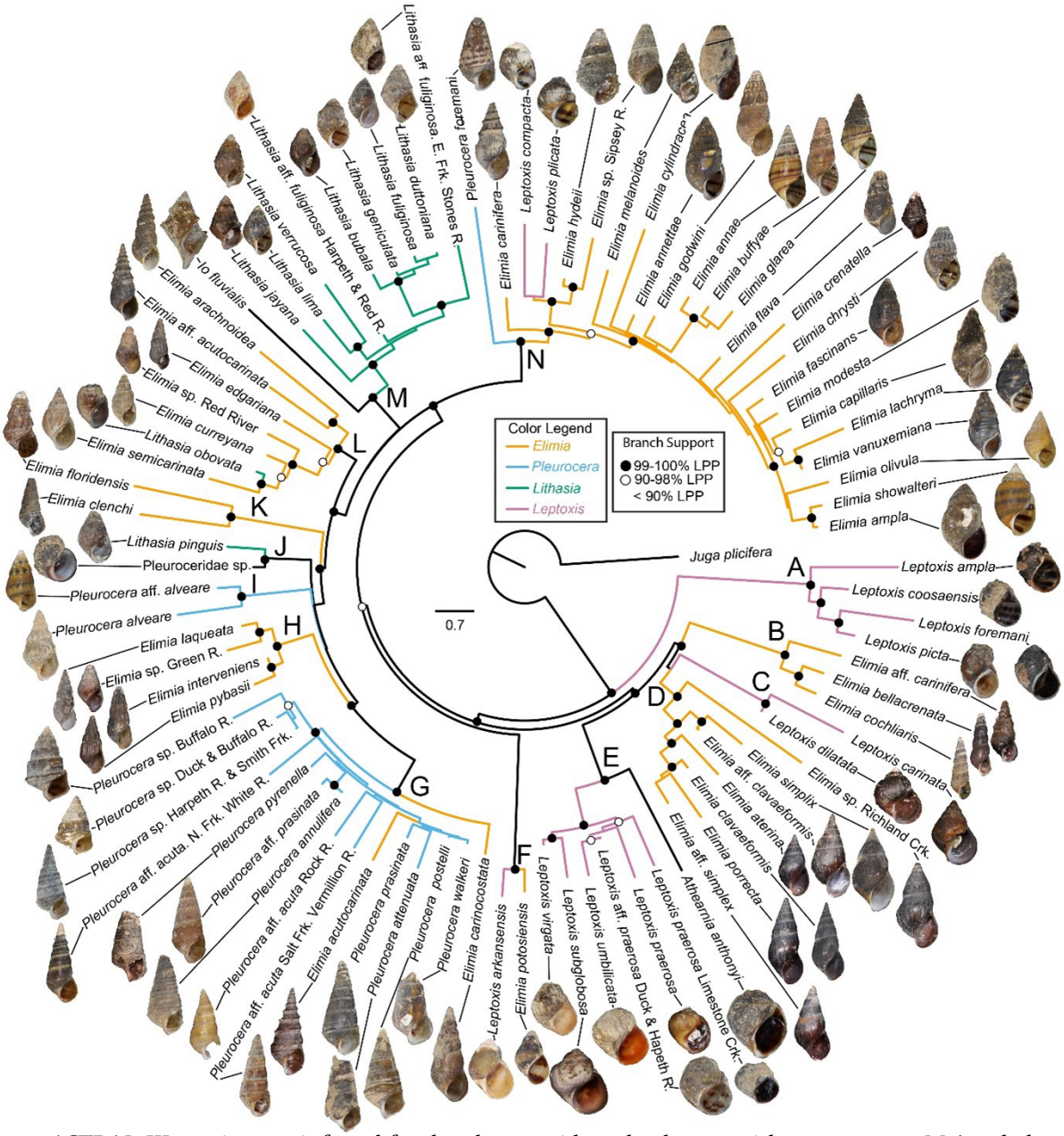

Figure 4: ASTRAL-III species tree inferred for the pleurocerid_probe dataset with a taxon map. Major clades are indicated with uppercase letters as referenced throughout the text. Figured specimens are sequenced vouchers; shells are not to scale. Branch support is measured by LPP; branch lengths and scale bar are in coalescent units. Branch colors correspond to current generic placement.

1821), were recovered sister to *Athearnia anthonyi* (Redfield, 1854) and together comprise clade E. Within clade E, *Le. praerosa* is paraphyletic with respect to *Leptoxis umbilicata* (Wetherby, 1876). Clade E was recovered sister to clade (C + B + D). Clade C contains two *Leptoxis* s.l. species, *Leptoxis dilatata* (Conrad, 1835) and *Leptoxis carinata* (Bruguière, 1789), which is notable as *Le. dilatata* is found in both the upper Ohio River drainage and upper Tennessee River drainage. Furthermore, *Le. carinata* is found in Atlantic Coast drainages (Fig. 2; Table S1). Clade D contains smooth-shelled *Elimia* s.l. species from the upper Tennessee River drainage, and clade B contains three spring-associated species from north-central Alabama. Placement of clade B was poorly supported as either sister to clade (C + D) (Figs. 4, S4, S6-S9) or as sister to clade D (Figs. S5, S10, S11) with no clear correlation to analysis type or dataset. *Leptoxis arkansensis* (Hinkley, 1915), the only *Leptoxis* s.l. from west of the Mississippi River, was recovered in clade F with *Elimia potosiensis* (Lea, 1841), a species that also occurs west of the Mississippi River. Clade F was sister to clades G-N in every analysis (Figs. 4, S4-S11). The two other *Leptoxis* species, *Le. compacta* and *Le. plicata,* were nested within clade N, which mostly includes Mobile River basin *Elimia* s.l.

We recovered two distinct lineages of *E. clavaeformis* and of its putative synonym, *E. acutocarinata* Adams and Adams, 1854 (Figs. 4, 5, S4-S11). Both *E. clavaeformis* lineages were recovered in clade C, with the individual most resembling the type specimen and sampled from the type locality, "Ocoee District, Tennessee", representing true *E. clavaeformis* (Figs. 4, 5; Table S1). We were unable to locate an extant population of *E. acutocarinata* from its exact type locality, Holston River, but sampled three sites in geographic proximity (Table S1); these individuals were recovered in both clades L and G. The *E. acutocarinata* individual most closely resembling the type specimen was recovered in clade G (Figs. 4, 5, S4-S11), which mostly consists of *Pleurocera* species. *Elimia* aff. *acutocarinata* was placed in clade L with *Elimia* s.l. species from the Tennessee, Cumberland, and Ohio River drainages plus *Lithasia obovata* (Say, 1821). Given phylogenetic results and collection locality, we determined that the "*E. clavaeformis*" individual used to design the probe set was actually *E.* aff. *acutocarinata* (Table S1).

Clades K, H, and N also include *Elimia* s.l. species (Figs. 4, S4-S11). Clade K consists of two *Elimia* species: *Elimia clenchi* (Goodrich, 1924) from the Gulf of Mexico coastal plain in Alabama and *Elimia floridensis* (Reeve, 1860) from two springs in central Florida. These two species were recovered sister to clades G-J (Figs. 4, S4-S11). The majority of *Elimia* s.l. were recovered in clade N, which consists of species from the Mobile River basin, one taxon from an adjacent Tennessee River drainage, and coastal drainages in southern Alabama and the Florida panhandle. Notably, individuals identified according to the current concept of *Elimia carinifera* (Lamarck, 1822) were recovered in two different clades, N and D (Figs. 4, S4-S11). Three non-*Elimia* species were recovered in clade N: *Le. plicata*, *Le. compacta*, and *Pleurocera foremani* (Lea, 1843). Many relationships within clade N were poorly resolved, particularly among *Elimia* s.l. species from the Cahaba River and Coosa River systems (Figs. 4, S1-S8).

Clades G-J include *Lithasia pinguis* (Lea, 1852), six *Elimia* s.l. species, and all *Pleurocera*, except *P. foremani*. Two distinct lineages, one from the Tennessee River drainage and one from the Cumberland River drainage, that would both be classified

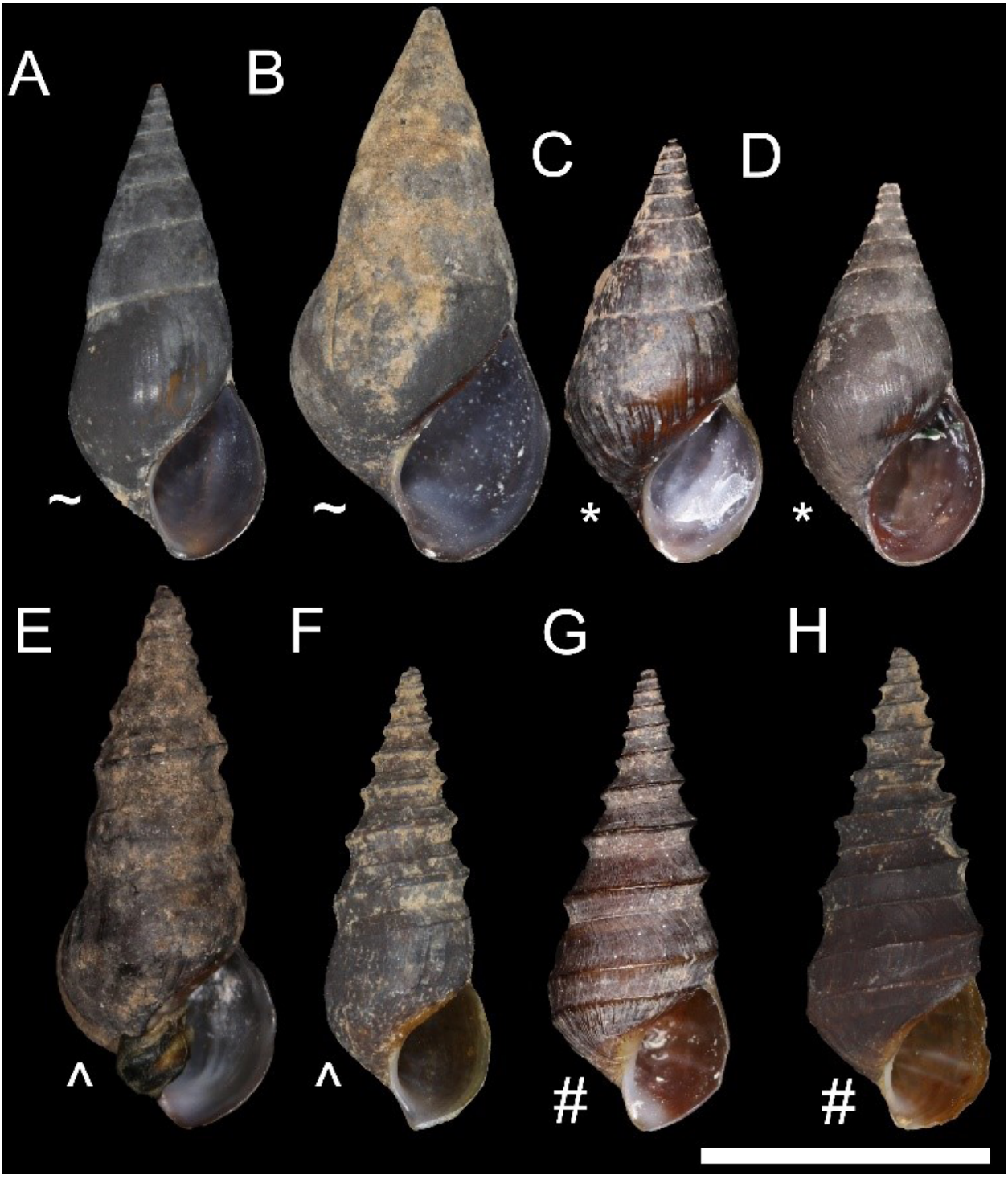

Figure 5: Shell morphology of *Elimia clavaeformis* s.l. Symbols indicate individuals are from the same evolutionary lineage (see Fig. 4). A) *Elimia clavaeformis*, USNM 1597591, clade D; B) *Elimia clavaeformis*, USNM 1597594, clade D; C) *Elimia* aff. *clavaeformis,* USNM 1638570, clade D; D) *Elimia* aff. *clavaeformis*, USNM 1638571, clade D; E) *Elimia* aff. *acutocarinata*, USNM 1638566, clade L; F) *Elimia* aff. *acutocarinata*, USNM 1597548, clade L; G) *Elimia acutocarinata*, USNM 1638565, clade G; H) *Elimia acutocarinata*, USNM 1597551 (not sequenced). A and E were collected from the same location, and C and G were collected from the same location. Scale bar = 1 cm. See Table S1 for details.

as *Pleurocera alveare* (Conrad, 1834) based on shell morphology (Figs. 3, 4) are the sole members of clade J. *Lithasia pinguis* and a sympatric, undescribed pleurocerid comprise clade I and were recovered sister to clades G-H (Figs. 4, S4-S11). Clade G comprises *Pleurocera sensu stricto* (s.s.) as it includes the type species *Pleurocera acuta* Rafinesque in Blainville, 1824. We were unable to locate a population of *P. acuta* from its type locality, Lake Erie, but most species with the typical apertural canal and high, narrow spire of *Pleurocera* were recovered within clade G. Thus, we are confident that this clade represents true *Pleurocera* s.s. Relationships within clade G were mostly unresolved. ASTRAL analyses and the ML analysis on the concatenated pleurocerid_probe dataset placed *Elimia carinocostata* (Lea, 1845) as sister to all other taxa in clade G (Figs. 4, S4, S5, S7, S8, S10, S11), but ML of the concatenated pleurocerid_all and pleurocerid_masked datasets supported the placement of *E. carinocostata* nested within clade G (Figs. S6, S9). This conflict between ML on concatenated datasets and ASTRAL may indicate some degree of incomplete lineage sorting among species in clade G, but conflict could also be a result of gene tree inference error, at least in part.

Most *Lithasia* species, including the type species *Li. geniculata*, were recovered in clade M, and *Io fluvialis* (Say, 1825) was recovered as sister to all other species in clade M (Figs. 4, S4-S1). We recovered clade M either sister to clades G-L with ASTRAL (Figs. 4, S5, S7, S8, S10, S11) or sister to clade N with ML inference on concatenated datasets (Figs. S4, S6, S9). However, support for placement of clade M was moderate to low in all analyses. *Lithasia jayana* (Lea, 1841) was recovered sister to all other *Lithasia* s.s. in ASTRAL analyses. *Lithasia* aff. *fuliginosa* from East Fork of the Stones River was inferred sister to *Li. jayana* in ML inference of concatenated datasets (Figs., S4, S7, S9) but more closely related to other *Lithasia* species in clade M in ASTRAL analyses (Figs. 4, S5, S7, S8, S10, S11). *Lithasia fuliginosa* (Lea, 1841), considered a subspecies of *Li. geniculata* by Burch and Tottenham (1980), was not recovered monophyletic with *Li. geniculata*, nor were all *Li. fuliginosa* s.l. individuals recovered monophyletic (Figs. 4, S4-S11). Relationships among *Lithasia* from the Duck River were poorly resolved. Maximum likelihood inference on all three concatenated datasets resulted in *Li. fuliginosa* from the Duck River drainage being polyphyletic with *Lithasia duttoniana* (Lea, 1841) and *Li. geniculata* from the Duck River drainage. However, these three species from the Duck River were reciprocally monophyletic in the ASTRAL analysis with dataset pleurocerid_probe. *Lithasia geniculata* and *Li. fuliginosa* from the Duck River were also reciprocally monophyletic in ASTRAL analyses without a taxon map on datasets pleurocerid_full and pleurocerid_masked, but *Li. duttoniana* was paraphyletic. These conflicting results between dataset and analysis type are likely caused by the presence of incomplete lineage sorting among *Lithasia* from the Duck River drainage, noise from sequence regions that flank the probe region, or, more likely, both.

4 DISCUSSION

The AHE probe set developed here has demonstrated utility for studying pleurocerid evolution, and our phylogenetic hypotheses represent a critical leap forward in understanding the evolutionary history of the Pleuroceridae. For the first time, we robustly resolved pleurocerid relationships, unbiased by the high levels of intraspecific mitochondrial heterogeneity that have plagued previous inferences. One of the

most striking results was the ubiquity of polyphyly at the genus level, with all traditionally accepted genera recovered as polyphyletic except *Io* and *Athearnia*, which each only have one extant representative. Numerous species were also polyphyletic, sometimes highly so, with members of traditionally recognized species dispersed across distantly related clades. Polyphyly of genera and species are the result of high levels of conchological convergence, which has also been seen in other gastropod groups (Emberton 1995; Webster et al. 2012; Stelbrink et al. 2020). Thus, our results demonstrate that the shell characters traditionally used to classify pleurocerids are unsuitable for the task. Conversely, our analyses resolved numerous major clades that will merit formal taxonomic recognition. Importantly, our results allow for the first robust insights into morphological evolution, biogeography, life history, and taxonomy of pleurocerids. In doing so, this study highlights promising areas of future research enabled by our AHE probe set.

4.1 Utility of the Pleuroceridae probe set compared to traditional markers

Our probe set represents a considerable advance over markers previously used for pleurocerid phylogenetics. As noted above, most past phylogenetic studies relied on mitochondrial markers, which can have high levels of intraspecific variation. Such variation has rendered species and genera as polyphyletic in many past analyses. We also recovered considerable polyphyly in our study, but the patterns recovered here differ in notable ways and allow us to conclude that our probe set allows for inferring pleurocerid evolutionary history better than traditional markers. For example, no individuals with identical shell morphology and collected from the same location were polyphyletic in our analyses (Figs. S4-S7, S9, S10), which is a yet unexplained phenomenon on pleurocerid mitochondrial gene trees (Whelan and Strong 2016). Furthermore, despite widespread generic polyphyly, clade membership generally follows geographic patterns and species appear united by non-shell characters (see below). This indicates that species and genus non-monophyly revealed with our AHE probe set is not an artifact.

Although not all relationships were resolved with high support (e.g., the placement of clades B and E), all major clades were supported by LPP and BS of 99-100% (Figs. 4, S4-S11). Uncertainty surrounding the placement of clades B and E may be the result of rapid divergence as indicated by short internal branch lengths. At the species level, resolution of relationships varied somewhat among clades, with limited resolution and poor support in the two most speciose clades (clades G and N). We anticipate that areas of poor resolution and low support will improve with increased taxon sampling.

4.2 Morphological convergence revealed by AHE phylogenomic inference

Perhaps unsurprisingly given the pervasive para- and polyphyly of traditionally accepted taxa, phylogenetic results indicate high levels of convergence in shell morphology (Figs. 1, 3-5), with ostensibly diagnostic features resolved as variable among or within species and genera. For example, a thickening of the parietal wall (i.e., a callus on the interior wall of the aperture) has been cited as one of the distinguishing features of *Lithasia*. However, it is also present in *Le. compacta* (clade N), contributing to the complicated and unstable taxonomic history of this species (e.g., Tryon 1873; Goodrich 1922). This feature is supported as having arisen independently in clades M and N. Similarly, the genus *Leptoxis* has been tradi-

tionally diagnosed by its subglobose, broadly conic, or ovate shell, but this shape can be found in six different clades (A, C, E, F, J, N; Figs. 1, 4). Likewise, the genus *Pleurocera* has been recognized by the presence of an anterior canal forming an auger-shaped base of the shell. However, this feature is found in at least three clades (G, I, N).

Morphological convergence, and the potential taxonomic consequences, are perhaps best exemplified by *E. clavaeformis.* Conchological variation in *E. clavaeformis,* as presently conceived, spans two morphotypes: 1) smooth, medium-spired shells indicative of *E. clavaeformis* s.s. (Fig. 5A-D) and 2) medium-spired, heavily carinate shells indicative of *E. acutocarinata* s.s (Fig. 5E-G). Goodrich (1940) was the first to consider *E. acutocarinata* to be a morphological variant and synonym of *E. clavaeformis*. Dillon (2011) postulated that limited variation at allozyme loci corroborated this view. Instead, *E. clavaeformis* and *E. acutocarinata* were not supported here as closely related and, hence, are not synonyms. Surprisingly, even the *clavaeformis* and *acutocarinata* morphotypes were not monophyletic. The two shells most similar to the lectotype of *Melania clavaeformis* Lea, 1841 (Graf 2001) have a less acute spire (Fig. 5A, B) than *E.* aff. *clavaeformis* (Fig. 5C, D). Furthermore, the individual most like the holotype of *Melania acutocarinata* Lea, 1841 was nested within clade G (Figs. 4, S4-S11). The two other *E. acutocarinata* s.l. individuals, denoted here as *E.* aff. *acutocarinata*, were recovered in clade L (Figs. 4, S4-S11).

The phylogenetic placement of *E. acutocarinata* s.l. and *E. clavaeformis* s.l. illustrates the need to critically reevaluate the utility of pleurocerid shell characters for identifying species and circumscribing genera. However, it may not be possible to generalize about what types of conchological differences are useful. For instance, the *acutocarinata* and *clavaeformis* morphotypes differ by the presence or absence of carinae. However, the presence of carinae is not always a useful guide and has been found to be variable among populations of *Le. ampla* (Whelan et al. 2012a; Whelan et al. 2019). Similar examples can be found throughout the tree. Shells of *E. carinifera* s.l. are characterized by a narrow spire with carinae and beaded striae (Fig. 3k, l). Yet, this morphology is found in both clades B and N (Figs. 3, 4). Furthermore, paraphyly of *Le. praerosa* and closely related lineages with similar shell shapes indicates that conchological differentiation may not accompany speciation (Clade E; Figs. 3u-z, 4). A similar pattern was documented for *Li. fuliginosa* (Clade M; Figs. 3a-e, 4). Widespread homoplasy of conchological characters also complicates the use of museum records for inferring clade membership and historical species ranges and should be interpreted with caution. Dense population and geographic sampling in a phylogenomic context will be required to accurately circumscribe species and understand the significance of geographical and population morphological variation.

Exploring the processes driving morphological differentiation and convergence in Pleuroceridae should be a priority for future research. More work is also needed to understand the influence of phenotypic plasticity on pleurocerids. Only one past study has experimentally shown that pleurocerid shell shape is influenced by phenotypic plasticity, but the study only focused on *Elimia livescens* (Menke, 1830) and the differences between treatment and control groups were also small (< 1 mm) (Krist 2002). In contrast, a study on *Le. ampla* indicated that the presence or absence of carinae was not the result of phenotypic plasticity, but the

generality of this finding is unclear (Whelan et al. 2012a). Nevertheless, available data suggest that presence of discrete characters (e.g., carinae, costae, striae, nodules) are not influenced by phenotypic plasticity (see also Whelan 2021). The drivers of pleurocerid shell shape evolution are undoubtedly complex, and other characters will likely be more useful for systematics.

4.3 Phylogenetic patterns of non-shell morphology and life history

Our analyses revealed that other characters not traditionally used in pleurocerid taxonomy have potential utility in recognizing species and diagnosing major clades. In particular, distinct external anatomical features of the head-foot appear to be associated with specific clades. For example, all species in clade M have a body fold on the back of the neck, and all *Lithasia* in clade M possess a glandular lobe on the back of the foot (Fig. 5G, H). Although the precise functions of these morphologies are unknown, photographs of living animals suggest they play a role in controlling water flow similar to the nuchal lobes of Viviparidae, which are also derived from the foot (Ponder et al. 2020). At least one *Lithasia* s.l. outside clade M, *Li. obovata*, lacks the body fold and glandular lobe features seen in *Lithasia* of clade M (Fig. 5I). Both species in clade C possess a flap-like ocular peduncle (Fig. 5C), and a prominent, globose ocular peduncle is found in all species of clade E (Fig. 5A, B). Neither feature has been documented in other clades, suggesting that anatomical convergence is far less prevalent than conchological convergence.

Some soft-body pigmentation patterns may also prove to be conserved within certain clades. For example, all species in clade M possess uniformly dark pigmented tentacles, which is a feature not documented in any other clade (Fig. 5G, H). However, intrapopulation color variation in *Le. ampla* was previously documented (Whelan and Strong 2016). Furthermore, at least three species have a black pigmented head and snout with a bright yellow horizontal band below the eyes: *Le. plicata* (clade N), *Le. compacta* (clade N), and most individuals of the distantly related *Le. ampla* (clade N) (Fig. 5D, E). These pigmentation patterns are retained in captively reared offspring (P. Johnson, *unpublished data*), suggesting that soft-body pigmentation is genetically controlled, rather than influenced by environmental factors such as diet. As with external anatomical features, pigmentation patterns have not been documented in most species, but placing them in a robust phylogenomic framework will allow an assessment of their utility and facilitate study on their possible function and evolution.

The phylogenetic framework inferred here is also consistent with observations of radular morphology, which was previously at odds with the historical shell-based classification and with mitochondrial gene trees. For instance, *Le. ampla* and *Le. coosaensis* (clade A) have nearly identical radular morphologies, but they differ from *Le. compacta* (clade N) and from *Le. praerosa* (clade E) (Minton 2002; Whelan et al. 2012b; Whelan and Strong 2016), which are now confirmed to represent three different clades. Moreover, the radular morphology of *Le. compacta* is more like that of *Elimia christyi* (Lea, 1841) (both clade N) than to that of *Le. praerosa* and *Le. ampla* (Minton et al. 2004; Whelan et al. 2012b). Patterns in radular morphology were also inconsistent with the results obtained in past analyses of mitochondrial genes. Whelan and Strong (2016) found that *Pleurocera pyrenella* (Conrad, 1834) and *P. prasinata* (clade G) had radular morphologies more similar to each other than to *Le.*

ampla, regardless of mitochondrial lineage. They hypothesized that phylogenomic analyses would reveal *P. pyrenella* and *P. prasinata* were more closely related to each other than to *Le. ampla*, which was corroborated by our analyses.

Few, if any, freshwater gastropod groups display a comparable diversity of egg-laying patterns as that seen in pleurocerids. Within *Leptoxis* s.l. alone, three different patterns have been identified: eggs deposited singly, in a line, or in circular clutches. However, all *Leptoxis* spp. with circular clutches occur in clade A, the two *Leptoxis* species that comprise clade C deposit eggs in lines, and all *Leptoxis* in clade E deposit single eggs (Whelan et al. 2015b). No other pleurocerid group has received similar attention in documenting spawn type, but scattered reports in the literature suggest some convergence may exist as *P. acuta* and *E. floridensis* lay circular clutches (Dazo 1965;

Figure 6: Live pleurocerids. A) *Athearnia anthonyi*, clade E; arrows: ocular peduncles. B) *Leptoxis praerosa*, clade E; arrows: ocular peduncles. C) *Leptoxis dilatata*, clade C; arrow: flap-like ocular peduncle. D) *Leptoxis plicata*, clade N. E) *Leptoxis ampla*, clade A. F) *Leptoxis picta*, clade A. G) *Lithasia verrucosa*, clade M; left arrow: dorsal body fold; right arrow: glandular lobe. H) *Lithasia fuliginosa*, clade M; top arrow: dorsal body fold; bottom arrow: glandular lobe. I) *Lithasia obovata*, clade L. J) *Elimia* aff. *carinocostata*, clade G. K) *Pleurocera alveare*, clade G. J) *Elimia melanoides*, clade N.

Chambers 1980) similar to clade A. Egg-laying patterns are more easily categorized than highly variable shell morphology, and there is no evidence of intraspecific variation in egg-laying patterns. Therefore, documenting egg patterns in a phylogenetic context, particularly in concert with other characters, may be useful for characterizing genera and should be a focus of future studies.

4.4 Biogeographical patterns revealed with AHE phylogenetics

Most pleurocerid species are narrow-range endemics, and polyphyly of wide-ranging species like *Le. praerosa* and *E. carinifera* indicates that at least some pleurocerids previously considered to have wide distributions may comprise multiple small-range endemics. Low dispersal ability and small ranges facilitate reconstructing biogeographic patterns as there are fewer possible dispersal routes. At the clade level, we found that most clades are dominated by species from a single drainage (Fig. 2). For example, the sister clade to all other pleurocerids comprises species from the Mobile River basin. Furthermore, repeated patterns of relationships among species from different drainages suggest historical or ongoing connectivity that we can begin to reconstruct within this novel phylogenetic framework. Specifically, clades E, G, H, I, J, L, and M all contain closely related species or individual species distributed in both the lower Tennessee and Cumberland River drainages (Fig. 2), suggesting historical connectivity. Similarly, close or sister relationships among species from the Cahaba River and Black Warrior drainages were inferred in clades G (*P.* aff. *prasinata* + *Pleurocera annulifera* (Conrad, 1834)), N (*Le. compacta* + *Le. plicata*), and B (*E.* aff. *carinifera* + (*Elimia bellacrenata* (Haldeman, 1842) + *Elimia cochliaris* (Lea, 1868))) (Figs. 2, 3). This repeated pattern supports historical connectivity between the Cahaba and Black Warrior Rivers, possibly in the form of headwater capture given that these species occur above the fall line in Alabama. The pattern is similar to that seen in several localized darters (Percidae) in a complex of interconnected spring systems surrounding Birmingham, Alabama that straddles both the Cahaba River and Black Warrior River drainages (Boschung and Mayden 2004).

Likewise, the distribution of species in clade N suggests historical connectivity between the Mobile River basin and the upper Tennessee River drainage and between Gulf Coast drainages and the Mobile River basin. For example, we sampled *E. carinifera* from the upper Coosa River drainage and from the adjacent Tennessee River drainage in Georgia east of Walden's Ridge, which forms the eastern escarpment of the Cumberland Plateau (Wilson Jr. and Stearns 1958). The presence of *E. carinifera* in both drainages suggests recent headwater capture or ongoing groundwater-facilitated dispersal in this spring-associated species (Fig. 2). Whereas the distribution of *E. carinifera* points to recent or ongoing connectivity, historical connectivity is indicated by the placement of *E. christyi* from the Hiwassee River, a Tennessee River tributary in eastern Tennessee, north Georgia, and North Carolina, in clade N (Figs. 2, 4), which consists mostly of Mobile River basin species. Three closely related species from Gulf Coast drainages are also nested in clade N (*Elimia glarea* Mihalcik and Thompson, 2002; *Elimia buffyae* Mihalcik and Thompson, 2002; and *Elimia annae* Mihalcik and Thompson, 2002; Figs. 2, 4, S4-S11), which is indicative of historical connectivity with the Mobile River basin.

Clade G contains representatives from every major drainage we sampled (Fig.

2). Despite limited resolution for most relationships in clade G, some relationships are notable for their biogeographic implications. *Pleurocera walkeri* Goodrich, 1928, *Pleurocera postellii* (Lea, 1862), and *P. attenuata* from Tennessee River drainages are closely related to *P. prasinata* from the Coosa River drainage, which is a part of the Mobile River basin, suggesting historical connectivity. Notably, *P. walkeri* was collected from the Hiwassee River and shows a similarly close relationship to Coosa River species as *E. christyi*. The close relationship between species from the Coosa River drainage and the Hiwassee River may support the hypothesis of Ross (1971) that drainage capture occurred between the Oostanaula River, an upper Coosa River tributary, and the Hiwassee River. Such an event would explain observed patterns without having to invoke the presence of a historical Appalachian River that connected the present-day Tennessee River east of Walden's Ridge and the Coosa River, for which there is little geological evidence (Adams 1928; Milici 1968). These preliminary patterns indicate that greater taxon sampling, combined with the genomic resources developed here, could aid understanding historical river connectivity of the eastern United States.

4.5 Pleurocerid taxonomy

A major goal of this study was to establish a framework and toolkit for future revisionary systematics. Our analyses show that accepted genera are not natural groups, and some currently recognized, wide-ranging species conceal unrecognized diversity. However, taxonomic revision with current taxon sampling would be premature and would leave many unsampled species in taxonomic limbo, especially given limited ability to resolve systematic affinities based on shells alone (see above). Nevertheless, we can explore the available genus-group names in the context of this new phylogenomic framework and point to areas of needed work.

The two largest genera, *Elimia* and *Pleurocera*, were both robustly resolved as polyphyletic. Past results indicating that *Elimia* and *Pleurocera* were monophyletic and sister were apparently the consequence of limited taxon sampling (Lydeard et al. 1997; Holznagel and Lydeard 2000), and increased resolution of relationships compared to those obtained by Lee et al. (2006) were enabled by our probe set. *Elimia* may be a junior synonym of *Pleurocera*, given the placement of the type species of *Elimia, E. acutocarinata* s.s., in clade G with the type species of *Pleurocera, P. acuta*. However, other clades containing *Elimia* s.l. likely merit recognition at the rank of genus, and synonymization of *Elimia* with *Pleurocera* would not render a monophyletic *Pleurocera*. Thus, the broad synonymy of *Elimia* with *Pleurocera*, as advocated by Dillon (2011), is ill conceived.

Our findings corroborate the results of Minton and Lydeard (2003) that *Lithasia* is polyphyletic but with greater resolution. The type species of *Lithasia, Li. geniculata,* was recovered in clade M, and *Li. pinguis* and *Li. obovata* were recovered in clade J and L, respectively. The genus-group name *Melasma* Adams and Adams, 1854 may be available for clade L. The type species of *Melasma*, *Melania crebricostata* Lea, 1841, is considered a synonym of *Elimia edgariana* (Lea, 1841), which was also recovered in clade L. However, we have not been able to test this synonymy with specimens identical to the types from the type localities. We also anticipate that greater taxon sampling of the lower Ohio River and lower Cumberland River drainages will reveal additional species in clade L. Thus, future sampling efforts should emphasize type localities of *E. edgariana* and

its synonyms.

Two potential genus-group names are available for clade B: *Anaplocamus* Dall, 1896 and *Alleghenya* Clench and Boss, 1967. *Anaplocamus borealis* Dall, 1896, the type species of *Anaplocamus*, is morphologically identical to *Le. dilatata*. The range of *Le. dilatata* overlaps with the inferred type locality of *A. borealis,* rendering *Alleghenya* a junior synonym of *Anaplocamus*.

The genus-group name *Mudalia* Haldeman, 1840 may be available for clade N owing to the placement of *Elimia melanoides* (Conrad, 1834) in this clade. The type species of *Mudalia* is *Mudalia turgida* Haldeman, 1840, described from "Alabama", and is a subjective junior synonym of *E. melanoides* (Tryon 1873). Another genus-group name formerly used for many species in clade N is *Goniobasis* Lea, 1862. The type species of *Goniobasis* is *G. osculata* Lea, 1862, which is a subjective junior synonym of *Elimia alabamensis* (Lea, 1861). Although we were unable to sample *E. alabamensis*, we predict that the species is a member of clade N based on its geographical distribution in the Coosa River drainage and morphological similarity to other species in the clade. Sequencing *E. alabamensis* with our AHE probe set will allow testing the hypothesis that *Goniobasis* is a junior synonym of *Mudalia*.

At the species level, our results revealed the presence of a number of apparently unrecognized species complexes that will have taxonomic and conservation implications. In clade E, the placement of *Leptoxis umbilicata* rendered *Le. praerosa* s.l. paraphyletic. We hypothesize that at least three species are represented by *Le. praerosa* s.l., but greater geographical sampling is needed. *Leptoxis virgata* is morphologically similar to *Le. praerosa*, which is petitioned for listing under the U.S. Endangered Species Act. Phylogenetic results indicate that *Le. virgata* is a valid species, but the extent of its range, which is important for conservation decisions, requires clarification in light of morphological similarity to *Le. praerosa* s.l. According to the current classification, *Leptoxis subglobosa* (Say, 1825) is considered an upstream variant and synonym of *Le. praerosa* (Goodrich 1938; Burch and Tottenham 1980; Burch 1982). However, we sampled *Le. subglobosa* from its type locality, North Fork of the Holston, and our results support recognition of *Le. subglobosa* as distinct from *Le. praerosa* (Figs. 4, S4-S11). In this case, we reject the oft-repeated hypothesis that many pleurocerid species have upstream, conspecific variants that differ in shell sculpture (Goodrich 1938; Burch and Tottenham 1980; Minton et al. 2008; Dillon 2011; Dillon and Robinson 2011; Dillon 2014).

Similarly, in clade M, *Li. fuliginosa* was recovered as comprising at least three distinct lineages (see taxa labelled as *Li. fuliginosa* and *Li.* aff. *fuliginosa* in Figs. 4, S4-S11). At present, *Li. fuliginosa* is considered a midriver subspecies of *Li. geniculata,* along with the nominotypical subspecies found in the main stem and *Lithasia geniculata pinguis* (Lea, 1852) in the headwaters (Burch and Tottenham 1980). However, our data reject this hypothesis, with *Li. geniculata* and its traditionally recognized subspecies recovered as highly polyphyletic (Figs. 4, S4-S11). Our results also suggest that *Li. geniculata, Li. fuliginosa, and Li. duttoniana* from the Duck River drainage are all distinct species but are likely undergoing some degree of incomplete lineage sorting.

5 CONCLUSIONS

The AHE probe set developed here is a much-needed tool for resolving recalcitrant relationships among pleurocerids spanning multiple taxonomic levels within the family.

As evidence of its utility, our study provides the first robustly resolved hypothesis of pleurocerid relationships and serves as a foundation for resolving the systematics of the family. Our findings indicate that many pleurocerid shell morphologies are convergent, and subtle morphological differences among taxa obscure recognition of species-level pleurocerid diversity. External soft tissue morphology and life history traits, features that traditionally have been overlooked, appear to be useful for recognizing and identifying some natural groups.

Despite advances made here, additional work is required before a comprehensive systematic revision will be possible. Morphological convergence is common, and we recommend caution when interpreting conchological characters and historical distribution records based on museum specimens alone. We anticipate that use of the AHE probe set in concert with improved sampling of *Elimia* s.l. and *Pleurocera* s.l., in particular, will be fruitful for improving support, determining clade membership, and informing taxonomic revisions. The pleurocerid probe set is also likely to be useful for degraded material (Blaimer et al. 2016), which could enable testing the phylogenetic position of extirpated populations and extinct species from museum records to yield a more comprehensive picture of pleurocerid evolution. Biogeographic patterns and historical river connectivity in the eastern United States are also likely to be revealed with greater taxon sampling. In the meantime, researchers should carefully reevaluate their assumptions about pleurocerids, particularly those concerning the causal mechanisms of shell shape variation across the family. Finally, putatively distinct, cryptic lineages revealed here may require conservation attention, further emphasizing the importance of additional systematics research.

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Data Availability

Photographs of all individuals included in phylogenetic analyses, probe sequences, sequence alignments, gene trees, concatenated matrices, tree files, commands used for tree inference, *Le. ampla* genome assembly, transcriptome assemblies, and AHE locus annotations are available from Data Dryad: https://doi.org/10.5061/dryad.pnvxok6qd. Supplementary Text, Table and Figures are available on Zenodo: https://doi.org/10.5281/zenodo.6564938. Data assembly and alignment pipeline and scripts for data wrangling are av ailable from https://github.com/nathanwhelan/AHE-data-processing. Raw sequence data are available from NCBI Short Read Archive under BioProject PRJNA781144. Supplemental materials associated with this manuscript are available from the Dryad repository: https://doi.org/10.5061/dryad.pnvxok6qd.

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