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Unioverse: A phylogenomic resource for reconstructing the evolution of freshwater mussels (Bivalvia, Unionoida)

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ABSTRACT

Freshwater mussels (order Unionoida) are a diverse radiation of parasitic bivalves that require temporary larval encystment on vertebrate hosts to complete metamorphosis to free-living juveniles. The freshwater mussel-fish symbiosis represents a useful relationship for understanding eco-evolutionary dynamics in freshwater ecosystems but the practicality of this promising model system is undermined by the absence of a stable freshwater mussel phylogeny. Inadequate character sampling is the primary analytical impediment obfuscating a coherent phylogeny of freshwater mussels, specifically the lack of nuclear molecular markers appropriate for reconstructing supraspecific relationships and testing macroevolutionary hypotheses. The objective of this study is to develop a phylogenomic resource, specifically an anchored hybrid enrichment probe set, capable of capturing hundreds of molecular markers from taxa distributed across the entirety of freshwater mussel biodiversity. Our freshwater mussel specific anchored hybrid enrichment probe set, called Unioverse, successfully captures hundreds of nuclear protein-coding loci from all major lineages of the Unionoida and will facilitate more data-rich and taxonomically inclusive reconstructions of freshwater mussel evolution. We demonstrate the utility of this resource at three disparate evolutionary scales by estimating a backbone phylogeny of the Bivalvia with a focus on the Unionoida, reconstructing the subfamily-level relationships of the Unionidae, and recovering the systematic position of the phylogenetically unstable genus *Plectomerus*.

1. Introduction

Freshwater mussels (order Unionoida) are an ancient, globally distributed, and species-rich radiation distinguished from all other bivalves by their restriction to freshwater ecosystems and having an obligate parasitic life history (Graf and Cummings, 2006, 2007). These parasitic bivalves require temporary larval encystment on vertebrate hosts, primarily freshwater fishes, to complete metamorphosis from parasitic larvae to free-living juveniles. This parasitic relationship is thought to be Triassic in origin (> 200MY; Watters, 2001) and ubiquitous in permanent freshwater habitats globally, making it a consequential selective force shaping the evolution and ecology of both symbionts. The freshwater mussel-fish symbiosis represents an interesting and useful relationship for better understanding eco-evolutionary dynamics of two of the most diverse, ecologically important, and imperiled animals in freshwater ecosystems. However, the practicality of this promising evolutionary and ecological model system is undermined by the absence of a stable freshwater mussel phylogeny. This systematic shortcoming not only limits our understanding of freshwater mussel biology, but also our appreciation of freshwater ecology and evolution more broadly (e.g., comparative biogeography, coevolution, community assembly).

Beyond the macroevolutionary insights extended from a robust phylogeny of the Unionoida are its varied uses to the freshwater mussel conservation community (e.g., identifying and preserving phylogenetic diversity, utilizing synapomorphy to infer ecological interactions and population-level processes) – an enterprise aimed at stewardship of one of the world's most threatened animal groups (Lopes-Lima et al., 2018). The objective of the present study is to develop an accessible phylogenomic resource, specifically an anchored hybrid enrichment probe set, that facilitates more data-rich and taxonomically inclusive reconstructions of freshwater mussel evolution.

The application of molecular phylogenetics has transformed freshwater mussel systematics but limited phylogenetic consensus has emerged in regard to the early evolution of the Unionoida. This is especially true of the relationships of the families and the suprageneric clades of its most diverse radiation, the Unionidae (Graf, 2013). Inadequate character sampling appears to be the primary analytical

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impediment obfuscating a coherent phylogeny of the Unionoida, specifically the lack of nuclear molecular markers appropriate for estimating supraspecific relationships and testing macroevolutionary hypotheses (Graf and Cummings, 2006, 2010; Hoeh et al., 2009). The inadequacies of the available molecular markers in describing the higher-level relationships of freshwater mussels is apparent in the numerous conflicting hypotheses generated using the conventional two- or three-locus reconstructions (Fig. 1). Fortunately, approaches for generating genome-scale datasets designed to estimate the evolutionary relationships of phylogenetically divergent groups is becoming much more accessible for non-model organisms (McCormack et al., 2013).

Two increasingly common methods of generating phylogenomic datasets aimed at estimating higher-level evolutionary relationships are transcriptome sequencing (a.k.a. RNA-seq) and sequence capture (e.g., ultraconserved elements and anchored hybrid enrichment). Transcriptomic approaches to reconstructing phylogeny typically involve extracting high quality RNA from fresh or properly preserved tissues, next-generation sequencing of cDNA libraries, orthology detection, and the assembly of hundreds of multiple sequence alignments. Sequence capture, and specifically anchored hybrid enrichment (AHE: Lemmon et al., 2012), uses genetic resources (genomes and transcriptomes) to identify target regions to which DNA probes are designed to hybridize with and selectively enrich to facilitate capture during next-generation sequencing, resulting in hundreds of preselected molecular markers sequenced from taxa distributed across the desired phylogenetic scope.

To date, systematic malacology has, with great success, relied largely on transcriptomic approaches to generate phylogenomic datasets and estimate the evolutionary history of diverse molluscan groups, including bivalves (Gonzalez et al., 2015; Lemer et al,. 2019), gastropods (Zapata et al., 2014), cephalopods (Lindgren and Anderson, 2018), and the phylum as a whole (Smith et al., 2011; Kocot et al., 2011). Although sequence capture has been used to reconstruct the evolution of many species-rich and phylogenetically diverse clades (e.g., vertebrates–Lemon et al., 2012; brittle stars–Hugall et al., 2015; minnows and relatives–Stout et al., 2016; butterflies and moths–Breinholt et al., 2018; mayflies–Miller et al., 2018), it has only begun to revolutionize molluscan phylogenomics (Gastropoda, Conidae: Abdelkrim et al., 2018; Phuong and Mahardika, 2018). One logistical advantage of AHE over phylotranscriptomics is that it has less demanding sample requirements. Rather than relying on fresh tissue or properly preserved RNA, as is the case with phylotranscriptomics, AHE can be used to sequence the genomic DNA contained in a wide variety of historical samples, including alcohol-preserved specimens, dried tissues, and shells (Yeates et al., 2016; Sproul and Maddison, 2017; Der Sarkissian, 2017). The capacity to more completely leverage the genomic resources of historical specimens would be an important development in freshwater mussel systematics, especially given the high proportion of extinct and rare taxa.

The objective of this study is to develop an AHE probe set designed to capture hundreds of molecular markers from taxa distributed across the entirety of freshwater mussel biodiversity and demonstrate the phylogenetic utility of these loci at multiple evolutionary scales. We designed our taxon sampling to 1) estimate a backbone phylogeny for the Bivalvia with a focus on the Unionoida, 2) re-evaluate the subfamily-level relationships of the most species-rich unionoid family, the Unionidae, and 3) determine the tribe-level position of the phylogenetically unstable North American genus *Plectomerus*.

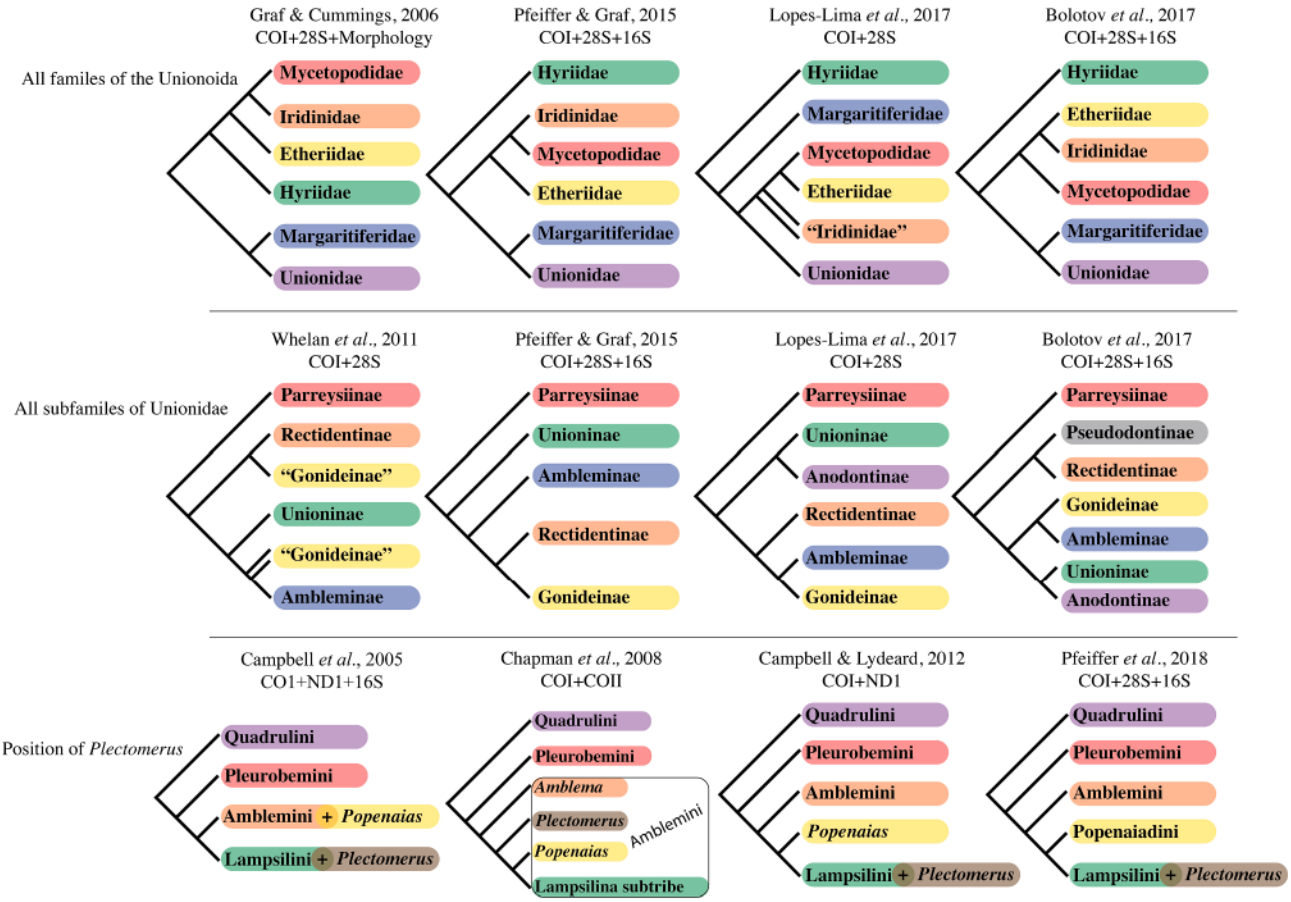

Fig. 1. Summary of recent multi-locus phylogenetic hypotheses including all the families of the Unionoida, all subfamilies of the Unionidae, and the position of *Plectomerus* among the subfamily Ambleminae.

Table 1
Taxa, vouchers, and source information utilized in phylogenomic analysis of the Bivalvia.

Taxon	Voucher	Accession	Source	Type
PROTOBRANCHIA				
Nuculidae				
Ennucula tenuis (Montagu, 1808)	NA	SRR331123	Smith et al. (2011)	Transcriptome
Solemyidae				
Solemya velum Say 1822	NA	SRR330465	Smith et al. (2011)	Transcriptome
PTERIOMORPHA				
Mytillidae				
Bathymodiolus platifrons Hashimoto & Okutani, 1994	Jiaolong_Div055_RL01	MJUT00000000.1	Sun et al. (2017)	Ref. Genome
Mytilus edulis Linneaus, 1758	MCZ 381397	SRR1560431	Gonzalez et al. (2015)	Transcriptome
HETERODONTA				
Archiheterodonta				
Astarte sulcata (da Costa, 1778)	MCZ 378853	SRR1559270	Gonzalez et al. (2015)	Transcriptome
Cardites antiquatus (Linnaeus, 1758)	MCZ Spain	SRR1560458	Gonzalez et al. (2015)	Transcriptome
Anomalodesmata				
Lyonsia floridana Conrad, 1849	BivATOL 248.1a	SRR1560310	Gonzalez et al. (2015)	Transcriptome
Myochama anomioides Stutchbury, 1830	BivAToL 84.1a	SRR1560429	Gonzalez et al. (2015)	Transcriptome
Imparidentia				
Hiatella arctica (Linnaeus, 1767)	BivAToL 195.1a	SRR1560281	Gonzalez et al. (2015)	Transcriptome
Corbicula fluminea (O.F. Müller, 1774)	BivATOL 242.1a	SRR1559272	Gonzalez et al. (2015)	Transcriptome
PALEOHETERODONTA				
Trigoniidae				
Neotrigonia margaritacea (Lamarck, 1804)	MCZ 379092	SRR1560432	Gonzalez et al. (2015)	Transcriptome
Mycetopodidae				
Anodontites elongatus (Swainson, 1823)	UA 20859	SRR8473049	This study	AHE
Etheriidae				
Etheria elliptica Lamarck, 1807	ANSP419710	SRR8473065	This study	AHE
Etheria elliptica	UNI_Eell	SRX5036374	Lemer et al. (2019)	Transcriptome
Iridinidae				
Aspatharia pfeifferiana (Bernardi, 1860)	UNI_Apfe	SRX5036375	Lemer et al. (2019)	Transcriptome
Hyriidae				
Hyridella australis (Lamarck, 1819)	UA 20825	SRR8473062	This study	AHE
Margaritiferidae				
Margaritifera margaritifera (Linnaeus, 1758)	BivATOL 299.2d	SRR1560312	Gonzalez et al. (2015)	Transcriptome
Margaritifera hembeli (Conrad, 1838)	UF 521837	SRR8473036	This study	AHE
Unionidae				
Ambleminae				
Amblema plicata (Say, 1817)	UF 438572	SRR8473047	This study	AHE
Cyrtonaias tampicoensis (Lea, 1838)	UF 438559	SRR8473040	This study	AHE
Elliptio complanata (Lightfoot, 1786)	NA	SRR5136467	Cornman et al. (2014)	Ref. Transcriptome
Elliptoideus sloatianus (Lea, 1840)	UF 440850	SRR8473067	This study	AHE
Lampsilis cardium Rafinesque, 1820	BivATOL 421.5a	SRR1560282	Gonzalez et al. (2015)	Ref. Transcriptome
Lampsilis cardium	UMMZ 304654	SRR8473035	This study	AHE
Leaunio lienosus (Conrad, 1834)	NA	SRR354206	Wang et al. (2012)	Transcriptome
Obliquaria reflexa Rafinesque, 1820	UF 438940	SRR8473034	This study	AHE
Pachynaias spheniopsis (Morelet, 1849)	UF 507900	SRR8473029	This study	AHE
Plectomerus dombeyanus (Valenciennes, 1827)	UF 438655	SRR8473056	This study	AHE
Pleurobema strodeanum (B.H. Wright, 1898)	UF 441317	SRR8473051	This study	AHE
Popenaias popeii (Lea, 1857)	UF 438742	SRR8473050	This study	AHE
Psoronaias semigranosa (Philippi, 1843)	UF 507899	SRR8473037	This study	AHE
Quadrula apiculata (Say, 1829)	UF 441088	SRR8473075	This study	AHE
Reginaia ebena (Lea, 1831)	UF 438113	SRR8473078	This study	AHE
Uniomerus tetralasmus (Say, 1831)	NA	SRR910418	Luo et al. (2014)	Ref. Transcriptome
Gonideinae				
Bineurus mouhotii (Lea, 1863)	UF 507896	SRR8473054	This study	AHE
Chamberlainia hainesiana (Lea, 1856)	UF 507722	SRR8473043	This study	AHE
Contradens contradens (Lea, 1838)	UF 507562	SRR8476280	This study	Ref. Transcriptome
Contradens contradens	UF 507874	SRR8473045	This study	AHE
Ensidens ingallsianus (Lea, 1852)	UF 507686	SRR8473066	This study	AHE
Gonidea angulata (Lea, 1838)	NCSM41055.202	SRR8473064	This study	AHE
Hyriopsis bialata Simpson, 1900	UF 507433	SRR8473061	This study	AHE
Lamprotula cornuumlunae (Heude, 1883)	UMMZ 304345	SRR8473058	This study	AHE
Monodontina vondembuschiana (Lea, 1840)	UF 507593	SRR8473068	This study	AHE
Physunio superbus (Lea, 1843)	UF 507729	SRR8473039	This study	AHE
Pilsbryoconcha compressa (Martens, 1860)	UF 507540	SRR8473057	This study	AHE
Pseudodon inoscularis (Gould, 1844)	UF 507661	SRR8473055	This study	AHE
Rectidens sumatrensis (Dunker, 1852)	BIV-1665	SRR8473077	This study	AHE
Sinohyriopsis cumingii (Lea, 1852)	NA	SRR3499637	Zhang et al. (2016)	Ref. Transcriptome
Solenaia khwaenoiensis Deein et al., 2004	UF 521836	SRR8473074	This study	AHE
Trapezoideus foliaceus (Gould, 1843)	UF 507865	SRR8473069	This study	AHE
"*Trigonodon*" *crebristruatus* (Anthony, 1865)	UF 507703	SRR8473052	This study	AHE
Yaukthwa paiensis Konopleva et al., 2019	UF 507709	SRR8473071	This study	AHE

(*continued on next page*)

Table 1 (*continued*)

Taxon	Voucher	Accession	Source	Type
Parreysiinae				
Coelatura choziensis (Preston, 1910)	ANSP 416276	SRR8473042	This study	AHE
Harmandia somboriensis Rochebrune, 1881	UF 507831	SRR8473063	This study	AHE
Indochinella pugio (Benson, 1862)	UMMZ 304644	SRR8473032	This study	AHE
Lamellidens corrianus (Lea, 1834)	UA 20729	SRR8473060	This study	AHE
Lamellidens generosus (Gould, 1847)	UA 20727	SRR8473059	This study	AHE
Leoparreysia bhamoensis (Theobald, 1873)	UA 20723	SRR8473030	This study	AHE
Leoparreysia olivacea (Prashad, 1930)	UMMZ 304641	SRR8473038	This study	AHE
Prisodontopsis aviculaeformis F.R. Woodward, 1991	ANSP 416269	SRR8473053	This study	AHE
Radiatula caerulea (Lea, 1831)	UF 507572	SRR8473076	This study	AHE
Scabies phaselus (Lea, 1856)	UF 507552	SRR8476281	This study	Ref. Transcriptome
Scabies phaselus	UF 507472	SRR8473072	This study	AHE
Unioninae				
Anodonta cygnea (Linnaeus, 1758)	AC6	SRR8473046	This study	AHE
Cristaria plicata (Leach, 1814)	NA	SRR3095781	Wang et al. (2017)	Ref. Transcriptome
Cristaria plicata	UA 20691	SRR8473044	This study	AHE
Cuneopsis pisciculus (Heude, 1874)	NCSM 26903	SRR8473041	This study	AHE
Lanceolaria lanceolata (Lea, 1856)	UA 20692	SRR8473049	This study	AHE
Nodularia douglasiae (Griffith & Pidgeon, 1833)	UA 20694	SRR8473033	This study	AHE
Nodularia jourdyi (Morlet, 1886)	UF 507885	SRR8473031	This study	AHE
Pyganodon grandis (Say, 1829)	NA	SRR910339	Luo et al. (2014)	Ref. Transcriptome
Sinanodonta woodiana (Lea, 1834)	UF 507884	SRR8473073	This study	AHE
Unio gibbus Spengler, 1793	BIV738	SRR8473070	This study	AHE

2. Materials and methods

2.1. Probe design

Our anchored hybrid enrichment probe set was designed to capture exonic regions present in taxa distributed across the family Unionidae. The *Bathymodiolus platifrons* (Bivalvia, Mytillidae) genome (Sun et al., 2017) and eight unionid transcriptomes, representing five of the six subfamilies of the family (*sans* Modellnaiinae), were used as genomic references to identify target loci and develop the probes. Raw reads from six previously published transcriptomes were downloaded from GenBank SRA (https://www.ncbi.nlm.nih.gov/sra), representing the subfamilies Ambleminae (3 taxa), Unioninae (2 taxa), and Gonideinae (1 taxon) (Table 1). Novel transcriptomic data were generated for representatives of the subfamilies Parreysiinae and Rectidentinae (*Scabies* and *Contradens*, respectively) (Table 1). Novel transcriptomic data were generated from mantle tissue preserved in RNALater® (Ambion, ThermoFisher). Tissue was rinsed with alcohol, freeze dried, and ground for mRNA extraction with the Dynabeads mRNA DIRECT Purification Kit (Life Technologies). The mRNA was heat fragmented and converted to cDNA. Sequencing libraries were built by end-repairing the cDNA, A-tailing, adapter ligation, bar-coding, and PCR amplification. The libraries were sequenced on an Illumina HiSeq 3000 for 100-bp, paired-end reads. Transcriptomes were assembled using Bridger (Chang et al., 2015) using a pair gap length of 200 and a kmer length of 25.

The *Bathymodiolus platifrons* genome was used to screen for exons that passed the single copy and orthology mapping criteria described in Breinholt et al. (2018). These single copy exons were extracted from the eight unionid transcriptomes using the genome_getprobe_BLAST.py from Espeland et al. (2018) and screened for orthology with s_hit_checker.py and ortholog_filter.py from Breinholt et al. (2018). Target loci were required to be present in at least six of the eight transcriptomes and be over 120 nt in length. Probes of 120 nt were tiled across target regions of each reference taxon at 2× coverage. The resulting freshwater mussel specific AHE probe set, called Unioverse, consists of 11,131 individual probes (Unioverse_probes.txt available on Dryad) designed to capture 811 nuclear protein-coding loci with a total probe region length of 173,707 nt (Unioverse_reference_probe_regions.zip available on Dryad). The Unioverse probes were synthesized as Custom SureSelect probes from AgilentTechnologies (Santa Clara, CA).

2.2. Taxon sampling and sequencing

We designed our ingroup taxon sampling to include representatives of each of the seven extant families of the subclass Palaeoheterodonta, with a focus on the most species-rich family, the Unionidae. Representatives of the bivalve subclasses Protobranchia, Pteriomorphia, and Heterodonta were sampled to include an outgroup. Fifty samples were selected for anchored hybrid enrichment using the Unioverse probe set (Table 1). Genomic resources from an additional twenty-three taxa were screened for the Unioverse loci to include an appropriate outgroup (10 taxa: 1 genome, 9 transcriptomes) and to augment ingroup sampling (13 taxa: 2 novel and 11 previously published transcriptomes) (Table 1) following the methods described in our probe design and Breinholt et al. (2018).

DNA was extracted from mantle tissue using a QIAamp® DNA Mini Kit (Qiagen, Inc.), and each isolation was quantified using PicoGreen®. Next-generation sequencing libraries, enrichment, and Illumina sequencing were done at RAPiD GENOMICS (Gainesville, FL). Libraries were constructed by shearing DNA to an average length of 300 bp followed by an end-repair reaction and ligation of an adenine residue to the 3′-end of the blunt-end fragments. Barcoded adapters were ligated to the libraries followed by PCR amplification. Libraries were pooled into groups of up to 16 samples and the SureSelect^{xt} Target Enrichment System for Illumina Paired-End Multiplexed Sequencing Library protocol (AgilentTechnologies Santa Clara, CA) was followed for solution-based target enrichment of the Unioverse loci. An Illumina HiSeq 3000 was used to generate 100 bp, paired-end reads.

2.3. Anchored hybrid enrichment data processing

The AHE data were processed and assembled following the pipeline developed by Breinholt et al. (2018) and is described in brief below. TRIM GALORE! v 0.4.0 (www.bioinformatics.babraham.ac.uk/projects/trim_galore/) was used to clean Illumina data with a minimum read size of 30 nt and quality trim of Phred score < 20. Loci were assembled using the iterative bait assembly script (IBA.py) of Breinholt et al. (2018) using the Unioverse reference probe regions as baits and modifying IBA.py userach target coverage parameters for the "blast_command_final" and "blast_filter" commands to 0.60 and 0.50 respectively. Loci were screened with single hit and orthology location mapping to the *B. platifrons* genome following Breinholt et al. (2018).

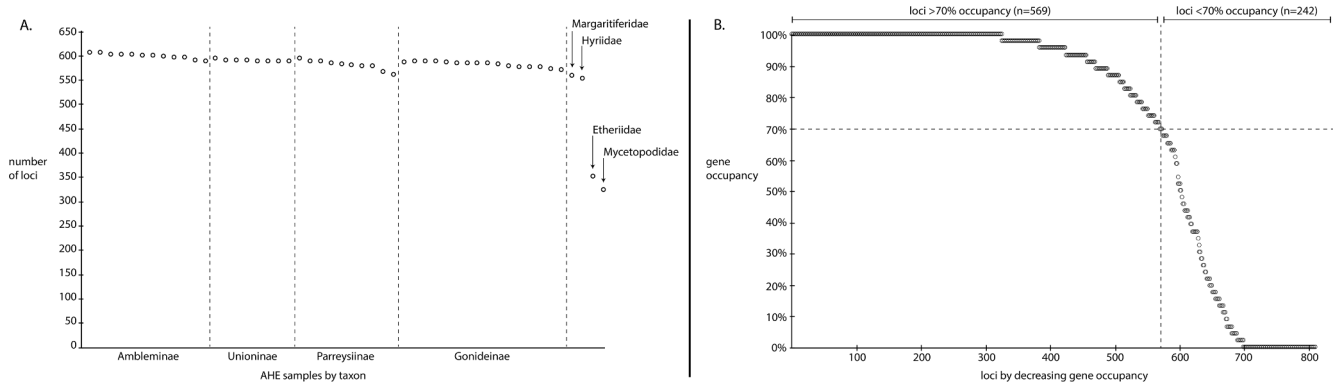

Fig. 2. Number of loci captured for each of the AHE unionoid samples (A) and percent gene occupancy of the 811 targeted loci (B).

The orthologs were further screened for contamination to remove nearly identical sequences from different bivalve families (Breinholt et al., 2018 – contamination_filter.py).

To reduce the amount of missing data in our datasets we included loci that had a minimum of 70% AHE gene occupancy across the Unionidae. Loci meeting this requirement were aligned using MAFFT v 7.294 (Katoh and Standley, 2013) and trimmed using Trimal v 1.2 (Capella-Gutiérrez et al., 2009) using a 50 percent gap threshold (i.e., columns represented by less than half of the taxa in the alignment were removed). Each trimmed alignment was translated to amino acids to ensure an open reading frame and incomplete terminal codons were deleted.

2.4. Phylogenetic analyses

PartitionFinder v 2.1.1 (Lanfear et al., 2016) was used to find the best codon partitioning scheme and GTR model of nucleotide substitution by means of the AICc selection criterion and the rcluster search method (rcluster 10 percent, rcluster-max 1000). Maximum likelihood (ML) analyses were implemented in RAxML v 8.2.8 (Stamatakis, 2014) using the best codon partitioning scheme and model of nucleotide evolution with 1000 rapid bootstraps. Bayesian inference (BI) analysis was performed using MrBayes v 3.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) using the best codon partitioning scheme and model of nucleotide evolution with 5×10^6 generations sampling a total of 20,000 trees with a burnin of 5000. Convergence of the two runs was monitored by the average standard deviation of split frequencies, the potential scale reduction factor (PSRF) and effective sample size (ESS) of the estimated parameters.

We also employed ASTRAL v 5.6.1 (Zhang et al., 2018), a multi-species coalescent-based approach to phylogenetic reconstruction that uses individual gene trees to estimate a species tree. Unpartitioned gene trees for each locus were estimated following the ML methods described above. Bipartitions in each gene tree with < 10 BS were removed prior to species tree estimation, as this has been shown to improve species tree accuracy (Zhang et al., 2017). Nodal support and gene tree conflict in the ASTRAL reconstruction were estimated using local posterior probabilities and the proportion of individual gene trees recovering that node.

To examine the distribution of the percentage of variable sites per locus (a proxy for evolutionary rate) and its potential impact on phylogenetic inference, we quantified the percentage of variable sites (% VS) for each locus, sorted the loci by increasing %VS (i.e., conserved to variable), grouped the 569 loci into ten 57-loci supermatrices (i.e., % VS_1 – %VS_10; %VS_10 consisted of 56 loci, not 57 as the total number of loci was not evenly divisible by ten), and generated unpartitioned ML phylogenies for each subset with 1000 rapid bootstraps. We compared topological differences between the %VS topologies and the ML NT_Supermatrix topology using Robinson-Foulds distances and the percent of unique bipartitions as calculated in RAxML v 8.2.8 (Stamatakis, 2014).

We calculated a phylogenetic distance matrix from the ML NT_Supermatrix tree using the cophenetic function in the R package ape v 5.1 (Paradis et al., 2004) to determine how capture efficiency varied as a function of phylogenetic distance to the nearest reference taxon used in probe design.

3. Results

The Unioverse probe set was very effective at capturing loci from individuals distributed across the entirety of freshwater mussel diversity but especially among representatives of the Unionidae, Margaritiferidae, and Hyriidae (Fig. 2a). Within the Unionidae (46 taxa), the number of loci captured per individual ranged from 560 to 606 (*Lamellidens* and *Amblema*, respectively), with an average of 586 loci. Outside the Unionidae (4 taxa), the number of captured loci per individual ranged from 324 to 558 (*Anodontites* and *Margaritifera*, respectively), with an average of 446 loci. The number of loci captured per individual decreased with increasing phylogenetic distance to the nearest reference taxon used in probe design (adjusted $R^2 = 0.953$, $p < 0.001$) (Fig. 3a). The number of loci captured per individual was not negatively affected by the number of years of ETOH preservation (adjusted $R^2 = 0.005$, $p = 0.27$) (Fig. 3b).

Of the 811 targeted loci, 569 loci had a gene occupancy of at least 70% across the Unionidae (Fig. 2b). After trimming and the deletion of incomplete terminal codons, the 569 loci had a total length of 98,817 nt. The average loci length was 174 nt, with a minimum and maximum length of 84 nt and 849 nt. Two concatenated supermatrices were constructed using the 569 loci with > 70% gene occupancy: all nucleotides included (NT_Supermatrix–98,817 nt, 39.6% parsimony informative sites, 15.1% missing data) and a protein translation of the previous matrix (AA_Supermatrix–32,939 AA, 18.5% parsimony informative sites, 15.3% missing data).

Maximum likelihood and BI analysis of the NT_Supermatrix recovered identical topologies with strong support for nearly all clades – five nodes had less than 100 posterior probability (PP)/100 bootstrap support (BS) (Figs. 4 and 5). Maximum likelihood and BI analysis of the AA_Supermatrix recovered identical topologies and were very similar to the NT_Supermatrix topology – seven nodes differed between the AA_Supermatrix topology and the NT_Supermatrix topology (Table 2). The ASTRAL topology (Fig. 6) was also very similar to the supermatrix topologies – six nodes differed between the ASTRAL topology and the NT_Supermatrix topology, two of which were also recovered in AA_Supermatrix topology (Table 2). Convergence of the BI runs was supported by the average deviation of split frequencies (0.000015), average PSRF values (1.000), and high average ESS values for each parameter (> 1008.14).

The average proportion of variable sites per locus was 48.2%, with a

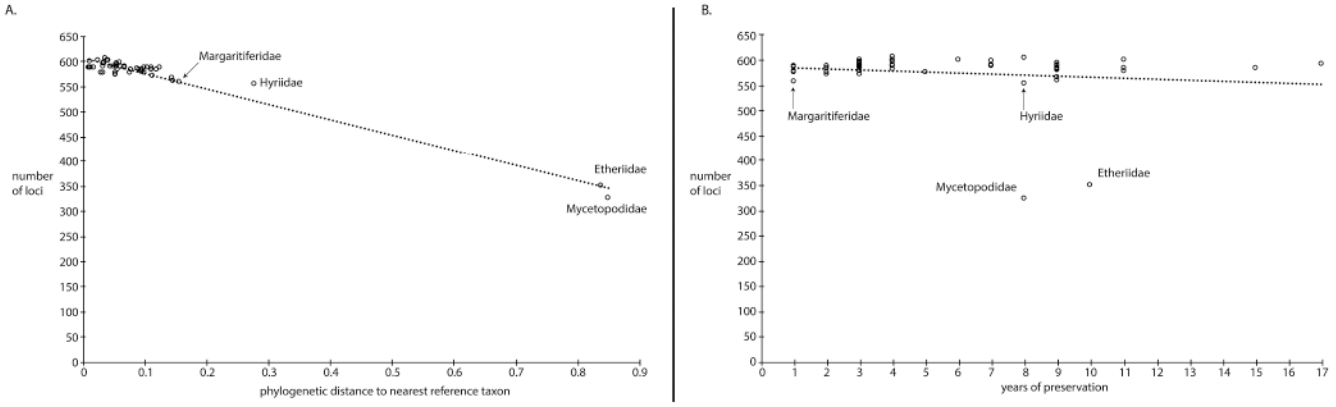

Fig. 3. Number of loci captured per individual as a function of (A) phylogenetic distance to nearest reference taxon used in probe design and (B) number of years of ETOH preservation.

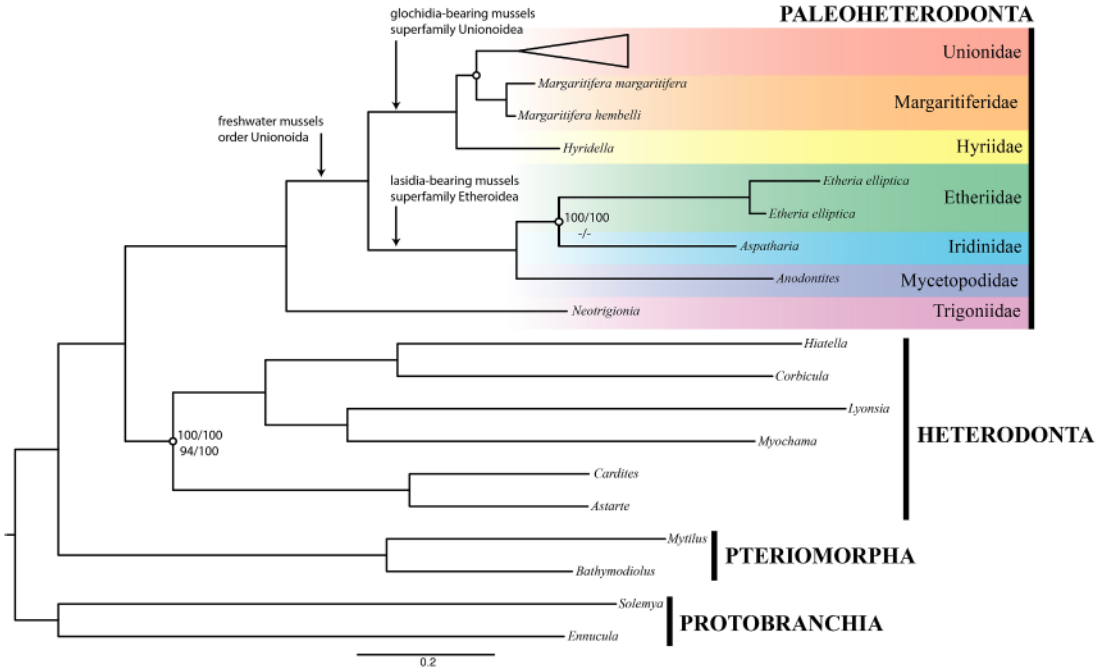

Fig. 4. Maximum likelihood reconstruction of the NT_Supermatrix with the Unionidae collapsed. Support values are listed as ML BS/BI PP: Top – NT_Supermatrix; Bottom – AA_Supermatrix. Nodes without support values are 100 PP/BS across all four analyses. Nodes with circles highlight topological incongruences recovered in the AA_Supermatrix and ASTRAL analyses.

minimum and maximum of 30.7% and 74.3% (Fig. 7a). Table 3 describes the support values for nodes of interest recovered in each of the ten %VS reconstructions. Robinson-Foulds distances and the percentage of unique bipartitions between the %VS trees and the ML NT_Supermatrix tree were small and tended to decrease with increasing variability (Fig. 7b).

4. Discussion

Our novel AHE probe set successfully captured hundreds of nuclear markers from all major branches of the freshwater mussel tree of life. The Unioverse probe set was designed specifically to capture loci from taxa distributed across the Unionidae, and capture efficiency was consistently high in individuals from that family (Fig. 2a). Expectedly, the number of loci captured per individual decreased as a function of increasing phylogenetic distance to the nearest reference taxon utilized in probe design (Fig. 3a). There was no apparent decrease in capture efficiency associated with the number of years a specimen had been preserved in ETOH – up to at least 17 years (Fig. 3b). The ability to capture hundreds of nuclear protein-coding loci from individuals distributed across the entirety of freshwater mussel biodiversity, and from museum specimens, dramatically improves the practicality of generating data-rich and taxonomically inclusive phylogenies of freshwater mussels.

Phylogenetic analysis of the loci captured via the Unioverse probe set recovered strongly supported and largely congruent topologies regardless of the dataset (NT_Supermatrix/AA_Supermatrix) or reconstruction method (ML/BI/ASTRAL). The higher-level relationships of the Bivalvia recovered here are congruent with recent phylotranscriptomic reconstructions of the Bivalvia (Gonzalez et al., 2015; Lemer et al., 2019), suggesting there is a consistent phylogenetic signal in the concatenated Unioverse loci and the previously analyzed bivalve transcriptomes. The level of conservatism in the loci varied substantially from strongly conserved (~30% of the sites varied across the Bivalvia) to highly variable (~75% of the sites varied across the Bivalvia), but phylogenetic reconstructions deliberately biased towards these extremes recovered largely congruent topologies (Table 3 and Fig. 7), further suggesting consistent phylogenetic signal among the Unioverse

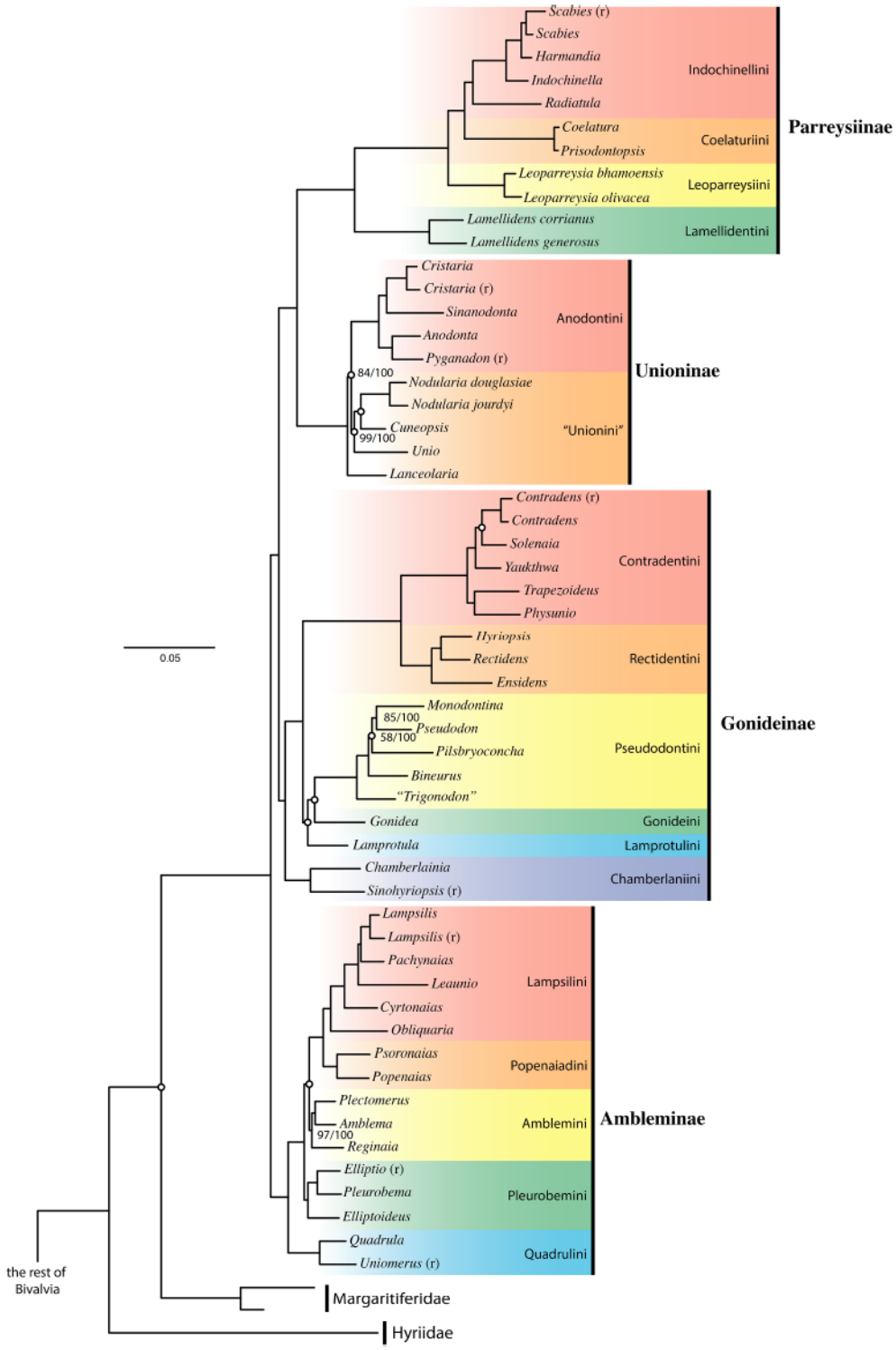

Fig. 5. Maximum likelihood reconstruction of the NT_Supermatrix with Unionidae expanded. Support values are listed as ML BS/BI PP. Nodes without support values are 100 BS/PP across all four analyses. Nodes with circles highlight topological incongruences recovered in the AA_Supermatrix and ASTRAL analyses. Reference taxa used in probe design are indicated with "(r)".

loci under concatenation.

The few topological differences between the NT_Supermatrix, AA_Supermatrix, and ASTRAL reconstructions are described in Table 2, and their relevance to freshwater mussel evolution and classification are discussed below. Although all approaches recovered similar topologies, coalescent methods revealed considerable gene tree conflict; of the 71 clades recovered in the ASTRAL reconstruction 23 were found in less than half of the 569 individual gene trees (Fig. 6). This result underscores the importance of sampling many loci as gene tree conflict appears to be quite common across the sampled mussel genomes.

We demonstrate that these loci are useful for reconstructing freshwater mussel evolution at three disparate phylogenetic scales and discuss our results in the context of fundamental aspects of freshwater mussel morphology and its importance in classification.

Table 2
Nodal support for the clades recovered in the ML/BI AA_Supermatrix topology and the ASTRAL species tree not present in the ML/BI NT_Supermatrix topology.

Clade	AA_Supermatrix ML/BI	ASTRAL
Lanceolaria + *Cuneopsis*	52/97	–
Lanceolaria + *Cuneopsis* + *Unio*	40/62	–
Lanceolaria + *Cuneopsis* + *Unio* + *Nodularia*	71/100	93
Contradens + *Yaukthwa*	60/100	–
Bineurus + *Pseudodon* + *Monodontina*	53/100	100
(Pseudontini + *Gonidea*) + (Rectidentini + Contradentini)	48/95	–
Etheriidae + Mycetopodidae	58/91	–
Gonidea + *Lamprotula*	–/–	100
Amblemini + Pleurobemini	–/–	82
Margaritiferidae + Hyriidae	–/–	100
(*Cardites* + *Astarte*) + Paleoheterodonta	–/–	53

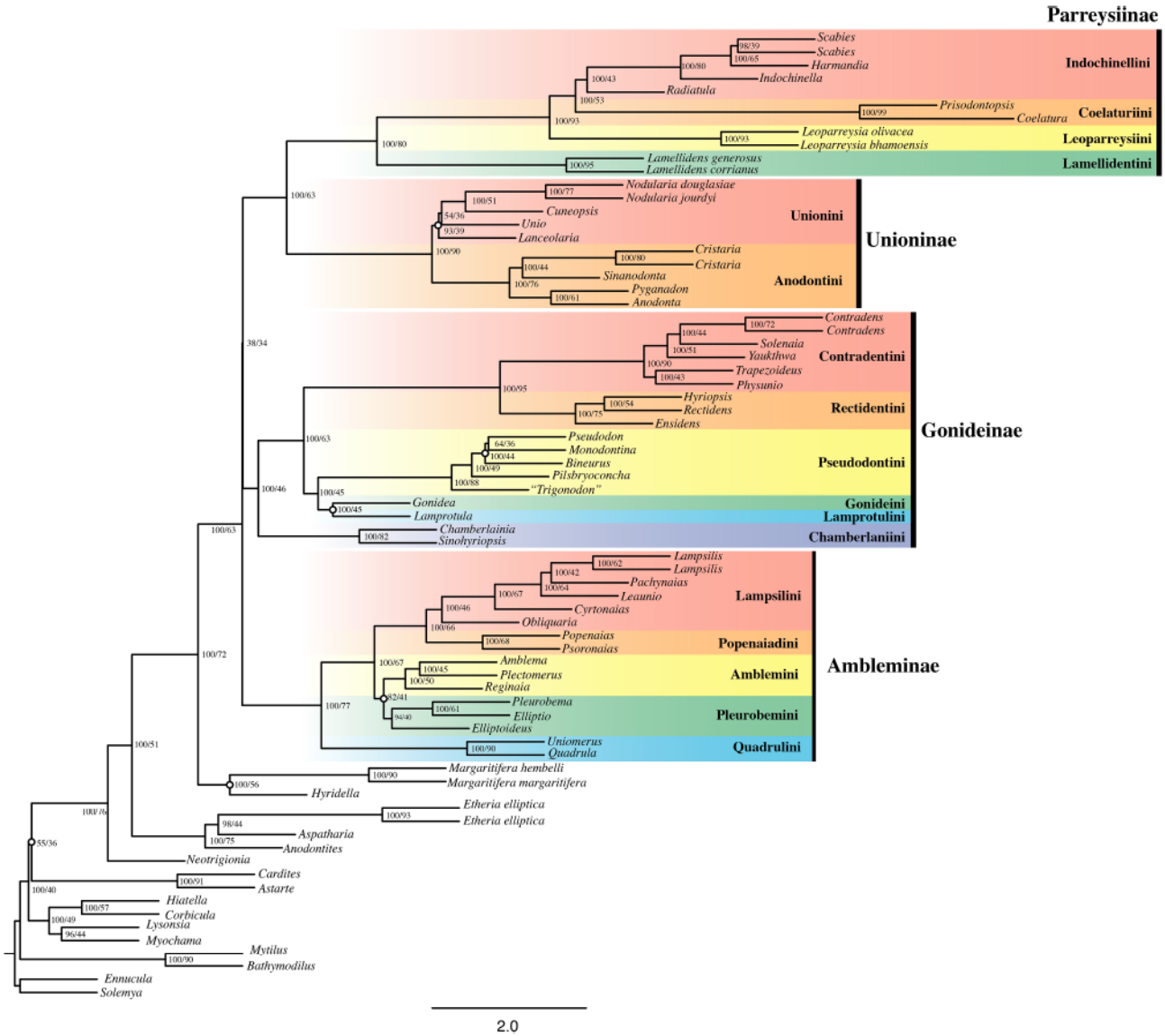

Fig. 6. Coalescent-based ASTRAL species tree of the Unionoida. Values at nodes are local posterior probabilities and the percentage of the gene trees recovering that node. Nodes with circles highlight topological incongruences recovered in the NT_Supermatrix and the AA_Supermatrix.

4.1. Monophyly of the glochidia- and lasidia-bearing mussels

Among the Unionoida are two distinct types of parasitic larvae: glochidia – calcified bivalved larvae with a single adductor muscle, and lasidia – uncalcified trilobed larvae with a long larval thread (Graf and Cummings, 2006). Molecular and morphological phylogenetic analyses sampling representatives of each unionoid family have consistently recovered the glochidia-bearing mussels as paraphyletic; either the

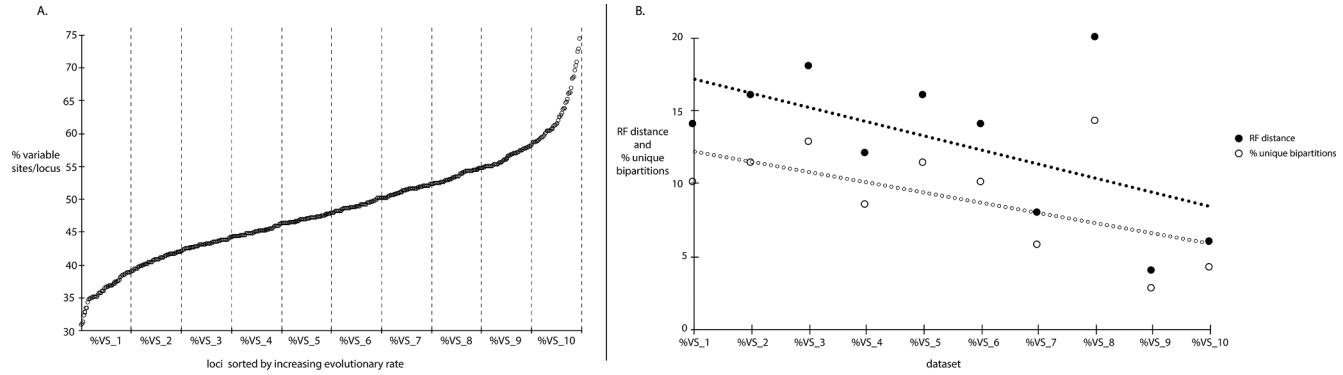

Fig. 7. Percent variable sites of each locus and visualization of the ten %VS subsets (A). Robinson-Foulds distances and % unique bipartitions of each of the %VS subsets in comparisons to the ML NT_Supermatrix topology (B).

Table 3
Bootstrap support for the clades of interest recovered in each of the percentage variable site subsets (%VS_1 – %VS_10) not recovered in the ML/BI NT_Supermatrix topology.

	%VS_1	%VS_2	%VS_3	%VS_4	%VS_5	%VS_6	%VS_7	%VS_8	%VS_9	%VS_10
Average % variable sites	36.0	40.5	43.0	44.9	46.8	48.8	51.1	53.3	56.1	62.7
Clade										
Unionoid monophyly	–	100	68	100	99	96	95	100	95	100
Neotrigonia + Unionoidea	77	–	–	–	–	–	–	–	–	–
Iridinidae + Etheriidae	95	100	92	69	–	76	54	–	97	85
Mycetopodidae + Iridinidae	–	–	–	–	84	–	–	45	–	–
Margaritiferidae + Unionidae	97	98	99	79	100	100	–	98	98	81
Margaritiferidae + Hyriidae	–	–	–	–	–	–	42	–	–	–
Ambleminae sister to rest of Unionidae	67	92	100	66	93	99	99	78	92	64
Unioninae + Parreysiinae	98	100	100	100	100	100	100	98	100	100
Pseudodontini + *Gonidea* + *Lamprotula*	58	–	57	64	69	91	71	91	94	64
Gonideinae	67	96	94	95	74	100	78	99	72	96
Amblema + *Plectomerus*	94	–	35	79	51	–	43	80	55	70
Amblema + *Plectomerus* + *Reginaia*	85	–	26	–	51	–	56	56	82	–

Table 4
Key to the subfamilies of the Unionidae.

1	adult shell with anterior hook	Modellnainae (Southeast Asia)
–	adult shell without anterior hook	2
2	hooked glochidia	Unioninae (Holarctic + Afrotropical)
–	unhooked glochidia	3
3	complete (imperforate) septa	Ambleminae (Nearctic + Mesoamerica)
–	incomplete (perforate) septa	4
4	Ascending lamella (inner demibranch) completely fused to visceral mass	Parreysiinae (Afrotropical + Indotropical)*
–	Ascending lamella (inner demibranch) not completely fused to visceral mass	Gonideinae (Palearctic + Indotropical + Western Nearctic)

* The Chamberlainiini (Paleacrtic + Indotropical) also has the ascending lamella of the inner demibranch completely fused to the visceral mass but is part of the Gonideinae – representatives of the Chamberlainiini are distinguished from the Parreysiinae by their large size and more obvious ligamental fossette.

Hyriidae is recovered as sister to the remaining five families (the most common topology – Bogan and Hoeh, 2000; Hoeh et al., 2001, 2002; Whelan et al., 2011; Pfeiffer and Graf, 2015; Graf et al., 2015; Lopes-Lima et al., 2017; Bolotov et al., 2017a; Huang et al., 2018, 2019) or the Hyriidae is recovered as sister to the lasidia-bearing mussels (Graf, 2000; Roe and Hoeh, 2003; Graf and Cummings, 2006) (Fig. 1). These two alternative topologies both suggest that glochidia are the plesiomorphic parasitic larval condition of freshwater mussels, and the ancestral state from which lasidia was derived. However, some recent mitogenomic reconstructions have recovered the glochidia-bearing mussel as monophyletic (Guerra et al., 2017; Huang et al., 2018, 2019).

Our phylogenomic reconstruction strongly supports the glochidia-bearing and lasidia-bearing mussels as reciprocally monophyletic. This early cladogenesis in freshwater mussel phylogeny bisects the order into two phylogenetically divergent clades diagnosed by their parasitic larval types. However, the biological importance of the monophyly of the glochidia- and lasidia-bearing mussels has yet to be recapitulated into the classification of freshwater mussels. Despite recovering the glochidia- and lasidia-bearing mussels as deeply divergent and reciprocally monophyletic, Guerra et al., (2017) advocated for a three superfamily classification – recognizing the lasidia-bearing mussels as the superfamily Etherioidea (=Etheriidae + Mycetopodidae + Iridinidae) but splitting the glochidia-bearing mussels into the superfamily Hyrioidea (=family Hyriidae) and the superfamily Unionoidea (=Unionidae + Margaritiferidae). However, they provided no basis for recognizing the Hyrioidea as distinct from the Unionoidea – the only "evolutionary event" characterizing the Hyrioidea was the loss of an open reading frame in the male mitochondrial genome, although this trait is also shared by the Unionidae (Guerra et al., 2017: their Fig. 4). More importantly, the three superfamily classification fails to capture the monophyly of the glochidia-bearing mussels and the shared similarities between the three families (several of which Guerra et al., 2017 accurately illustrate). We prefer an alternative superfamily classification, initially proposed by Parodiz and Bonneto (1963) which

Table 5
Classification of the genera of the Unionidae.

Ambleminae Rafinesque, 1820
Amblemini Rafinesque, 1820
Amblema Rafinesque, 1820
Plectomerus Conrad, 1853
Reginaia Campbell & Lydeard, 2012
Quadrulini von Ihering, 1901
Quadrula Rafinesque, 1820
Cyclonaias Pilsbry in Ortmann & Walker, 1922
Megalonaias Utterback, 1915
Theliderma Swainson, 1840
Tritogonia Agassiz, 1852
Uniomerus Conrad, 1853
Pleurobemini Hannibal, 1912
Pleurobema Rafinesque, 1819
Elliptio Rafinesque, 1819
Elliptoideus Frierson, 1927
Eurynia Rafinesque, 1820
Fusconaia Simpson, 1900
Hemistena Rafinesque, 1820
Parvaspina Perkins, Johnson & Gangloff, 2017
Plethobasus Simpson, 1900
Pleuronaia Frierson, 1927
Popenaiadini Heard & Guckert, 1970
Popenaias Frierson, 1927
Barynaias Fischer & Crosse, 1894
Disconaias Fischer & Crosse, 1894
Martensnaias Frierson, 1927
Micronaias Simpson, 1900
Nephritica Frierson, 1927
Nephronaias Fischer & Crosse, 1894
Psoronaias Fischer & Crosse, 1894
Sphenonaias Fischer & Crosse, 1894
Lampsilini von Ihering, 1901
Lampsilis Rafinesque, 1820
Actinonaias Fischer & Crosse, 1894
Arotonaias Martens, 1900
Cambarunio Watters, 2018
Cyprogenia Agassiz, 1852
Cyrtonaias Fischer & Crosse, 1894
Delphinonaias Fischer & Crosse, 1894
Dromus Simpson, 1900
Ellipsaria Rafinesque, 1820
Epioblasma Rafinesque, 1831
Friersonia Ortmann, 1912
Glebula Conrad, 1853
Hamiota Roe & Hartfield, 2005
Leaunio Watters, 2018
Lemiox Rafinesque, 1831
Leptodea Rafinesque, 1820
Ligumia Swainson, 1840
Medionidus Simpson, 1900
Obliquaria Rafinesque, 1820
Obovaria Rafinesque, 1819
Ortmanniana Frierson, 1927
Pachynaias Fischer & Crosse, 1894
Paetulunio Watters, 2018
Potamilus Rafinesque, 1818
Ptychobranchus Simpson, 1900
Reticulatus Frierson, 1927
Sagittunio Watters, 2018
Toxolasma Rafinesque, 1831
Truncilla Rafinesque, 1819
Venustaconcha Frierson, 1927
Villosa Frierson, 1927
Gonideinae Ortmann, 1916
Gonideini Ortmann, 1916
Gonidea Conrad, 1857
Leguminaia Conrad, 1865
Microcondylaea Vest, 1866
Paravasolenaia Huang & Wu in Huang *et al.*, 2018
Pseudodontopsis Kobelt, 1913
Ptychorhynchus Simpson, 1900
Rectidentini Modell, 1942
Rectidens Simpson, 1900
Ctenodesma Simpson, 1900
Elongaria Haas, 1911

Table 5 (*continued*)

Ensidens Frierson, 1911
Hyriopsis Conrad, 1853
Prohyriopsis Haas, 1914
Contradentini Modell, 1942
Contradens Haas, 1911
Physunio Simpson, 1900
Pressidens Haas, 1910
Solenaia Conrad, 1869
Trapezoideus Simpson, 1900
Yaukthwa Konopleva *et al.*, 2019
Chamberlainiini Bogan, Froufe & Lopes-Lima in Lopes-Lima *et al.*, 2017
Chamberlainia Simpson, 1900
Caudiculatus Simpson, 1900
Sinohyriopsis Starobogatov, 1970
Pseudodontini Frierson, 1927
Pseudodon Gould, 1844
Bineurus Simpson, 1900
Monodontina Conrad, 1853
Pilsbryoconcha Simpson, 1900
"*Trigonodon*" Conrad, 1865
Lamprotulini Modell, 1942
Lamprotula Simpson, 1900
? Discomya Simpson, 1900
Inversidens Haas, 1911
Potomida Swainson, 1840
Pronodularia Starobogatov, 1970
? Schepmania Haas, 1910
Modellnaiinae Brandt, 1974
Modellnaia Brandt, 1974
Parreysiinae Henderson, 1935
Parreysiini Henderson, 1935
Parreysia Conrad, 1853
Leoparreysiini Vikhrev, Bolotov & Kondakov in Bolotov *et al.*, 2018
Leoparreysia Vikhrev, Bolotov & Kondakov in Bolotov *et al.* 2018
Indochinellini Bolotov, Pfeiffer, Vikhrev, & Konopleva in Bolotov *et al.*, 2018
Indochinella Bolotov, Pfeiffer, Vikhrev, & Konopleva in Bolotov *et al.*, 2018
Harmandia Rochebrune, 1881
Radiatula Simpson, 1900
Scabies Haas, 1911
Unionetta Haas, 1955
Lamellidentini Modell, 1942
Lamellidens Simpson, 1900
Arcidopsis Simpson, 1900
Trapezidens Bolotov, Vikhrev & Konopleva in Bolotov *et al.*, 2017
Coelaturini Modell, 1942
Coelatura Conrad, 1853
Brazzaea Bourguignat, 1885
Grandidieria Bourguignat, 1885
Nitia Pallary, 1924
Nyassunio Haas, 1936
Prisodontopsis Tomlin, 1928
Pseudospatha Simpson, 1900
Unioninae Rafinesque, 1820
Unionini Rafinesque, 1820
Unio Philipsson in Retzius, 1788
Aculamprotula Wu, Liang, Wang & Ouyang, 1999
Acuticosta Simpson, 1900
Cuneopsis Simpson, 1900
Diaurora Cockerell, 1903
Inversiunio Habe, 1991
Lanceolaria Conrad, 1853
Lepidodesma Simpson, 1896
Nodularia Conrad, 1853
Protunio Haas, 1912
Pseudobaphia Simpson, 1900
Rhombuniopsis Haas, 1920
Schistodesmus Simpson, 1900
Anodontini Rafinesque, 1820
Anodonta Lamarck, 1799
Alasmidonta Say, 1818
Anemina Haas, 1969
Anodontoides Simpson in F.C. Baker, 1898
Arcidens Simpson, 1900
Cristaria Schumacher, 1817
Lasmigona Rafinesque, 1831
Pegias Simpson, 1900
Pletholophus Simpson, 1900

(*continued on next page*)

Table 5 (*continued*)

Pseudanodonta Bourguignat, 1876
Pseudodontoides Frierson, 1927
Pyganodon Fischer & Crosse, 1894
Simpsonaias Frierson, 1914
Simpsonella Cockerell, 1903
Sinanodonta Modell, 1945
Strophitus Rafinesque, 1820
Utterbackia F.C. Baker, 1927
Utterbackiana F.C. Baker, 1927
Unionoidea *incertae sedis* (previously considered Unionidae)
Germainaia Germain, 1911
Haasodonta McMichael, 1956

recognizes the glochidia-bearing mussels (Unionoidea = Hyriidae + Margaritiferidae + Unionidae) and lasidia-bearing mussels (Etherioidea = Etheriidae + Mycetopodidae + Iridinidae) as the two unionoid superfamilies. This classification more accurately reflects the ancient divergence of these two freshwater mussel clades – each of which is diagnosed by their parasitic larval semaphorants and supported by the analysis of our AHE data (Fig. 4).

Moreover, this classification is not contingent on the sister relationship of the Unionidae and the Margaritiferidae, which remains tenuous. Our ML and BI reconstructions of both the NT_Supermatrix and AA_Supermatrix strongly support the monophyly of the Margaritiferidae + Unionidae (Fig. 5), while the coalescent-based ASTRAL analysis has 100 PP for a sister relationship of the Margaritiferidae + Hyriidae (Fig. 6). Clearly there is some conflicting phylogenetic signal surrounding the relationships of these families – only 56% of the individual gene trees recovered the Margaritiferidae + Hyriidae clade. Admittedly, the taxon sampling in this phylogeny does little to resolve the family-level relationships within the glochidia- or lasidia-bearing mussels. Greater sampling of the non-unionid freshwater mussel families is necessary to resolve the relationships within the two superfamilies, as well as test the monophyly of the families themselves (e.g., Iridinidae, Etheriidae).

4.2. Subfamily-level relationships and early evolution of the Unionidae

The family Unionidae represents over 75 percent of the total species richness of the subclass Palaeoheterodonta and with over 680 species it is by far the most species-rich freshwater mussel family (Graf and Cummings, 2007). Our appreciation for the mode and tempo of this diversification is limited (in part) by uncertainties surrounding the early evolution of the group and the genesis of its major lineages.

The subfamily Parreysiinae has been consistently recovered as the sister group to the rest of the Unionidae in multi-locus reconstructions (Fig. 1) and many hypotheses of unionid biogeography, morphological evolution, and classification are at least partially predicated on this hypothesized early unionid bifurcation (Pfeiffer and Graf, 2015; Lopes-Lima et al., 2017; Bolotov et al., 2017a). In no analysis did we recover the Parreysiinae as sister to the rest of the Unionidae – the subfamily Ambleminae is the recovered sister group to the remainder of the Unionidae. This relationship was recovered with 100 BS/PP in all concatenated and coalescent-based analyses (Figs. 5 and 6). However, there is significant gene tree conflict surrounding this node and others in the early evolution of the Unionidae (Fig. 6). The short internal branch lengths "uniting" the major clades of the Unionidae may suggest a rapid cladogenesis in the early evolution of the group, perhaps revealing why the backbone of the Unionidae has been (and remains) so difficult to reconstruct with any certainty.

There have been several recent revisions to the higher-level classification of the Unionidae, including the resurrection of several available subfamily-level nomina and the description of new tribes (e.g., Anodontinae, Lanceolariini – Lopes-Lima et al., 2017; Pseudodontinae, Pilsbryoconchini – Bolotov et al., 2017a, 2017b; Lepidodesmini, Acuticostini – Huang et al., 2019). However, many of these recently recognized taxa have limited molecular support, uncertain sister relationship, no known diagnostic morphological characters, and/or contain a limited number of taxa – raising doubts about the usefulness of recognizing these taxa. This practice has destabilized aspects of freshwater mussel classification by undermining the capacity for utilizing morphological characters to recognize higher-level taxa (e.g., if the Anodontinae is considered a subfamily, hooked glochidia is no longer a synapomorphy of any named clade). The five unionid subfamilies recognized here are consistently recovered and strongly supported in our reconstructions and are morphologically recognizable using four simple morphological characters; shell outline, glochidia morphology, completeness of the interlamellar septa, and fusion of the ascending lamella of the inner demibranch to the visceral mass (Table 4). This dichotomous key does not represent explicit hypotheses of character evolution, rather it is a simple and useful method for recognizing these higher-level taxa and identifying their constituents. We utilize this key, inferences from other morphological traits, and previous treatments of the family to generate an updated hypothesis of unionid classification (Table 5). We recognize that our classification of the Unioninae is especially coarse and that several recently recognized tribe-level names may indeed be valid (e.g., Lanceolariini), but without useful morphological circumscriptions these names cannot be practically applied. We look forward to improvements to this working hypothesis.

4.3. Systematic position of Plectomerus among the Ambleminae

The subfamily Ambleminae is the most diverse freshwater mussel subfamily and is endemic to the Nearctic and Mesoamerica realms. This radiation has been the primary focus of freshwater mussel molecular systematics (Graf, 2013), and has resulted in a robust classification of the group (Lopes-Lima et al., 2017; Williams et al., 2017; Pfeiffer et al., 2018). The Ambleminae is divided into five more or less morphologically cohesive tribes; Amblemini, Lampsilini, Pleurobemini, Popenaiadini, and Quadrulini (Pfeiffer et al., 2018). The tribe Lampsilini is morphologically one of the most recognizable and diverse clades of the Ambleminae, characterized by a suite of morphological characters, including (1) restriction of the marsupium to only a portion of the outer demibranch, (2) sexually dimorphic shells, (3) female mantle modifications, (4) ascending lamellae of the inner demibranchs completely attached to the visceral mass, and (5) the unambiguous synapomorphy of the clade, ventral expansion of the marsupial water tubes relative to the rest of the demibranch (Pfeiffer et al., 2018).

Notwithstanding the apparent morphological cohesiveness of the Lampsilini, multi-locus phylogenies often fail to recover that taxon as monophyletic (Fig. 1). The Lampsilini is most commonly rendered paraphyletic with respect to the North American genus *Plectomerus,* which does not possess the traits typical of most lampsilines (Campbell et al., 2005; Campbell and Lydeard, 2012; Pfeiffer et al., 2018; but see Chapman et al., 2008). Despite the unstable position of *Plectomerus* in amblemine phylogeny and its morphological disparity in comparison to the Lampsilini, many systematists (including the lead author here) have recently considered *Plectomerus* to be a lampsiline (Lopes-Lima et al., 2017; Williams et al., 2017). With the understanding that higher-level taxonomic names are a means of communicating synapomorphy and evidence of common ancestry (i.e., the names are not ends in themselves), the classification of *Plectomerus* as a lampsiline suggests that the traits thought to characterize the Lampsilini need to be re-evaluated or simply that the tribe-level classification of the genus is flawed.

Our phylogeny strongly supports *Plectomerus* as sister to the genus *Amblema* – a genus with which it shares several morphological characteristics, including tetragenous brooding, entire demibranch is marsupial, short term brooding, ascending lamella of inner demibranch fused to visceral mass only anteriorly, and overall shell shape (Williams et al., 2008 and citations therein). The morphological traits of the tribe

Amblemini have been poorly characterized and the tribe is not yet diagnosed by any known morphological synapomorphy but robustly recovering the relationships of *Plectomerus* and *Amblema* (and *Reginaia*) provides a useful perspective in which to better characterize the morphology of the Amblemini and the other clades of the Ambleminae.

5. Conclusion

The lack of nuclear markers available for phylogenetic inference has stymied freshwater mussel systematics and its broader applications in freshwater ecology, evolution, and conservation. The Unioverse probe set rectifies this shortcoming, equipping systematists with genomic resources aimed at advancing hypotheses of freshwater mussel evolution, ecology, and conservation. We have demonstrated the utility of this resource across the freshwater mussel tree of life – but have done so at a very coarse scale. We hope the malacological community will leverage the Unioverse probe set to more densely sample freshwater mussel biodiversity and generate more taxonomically complete, data rich, and useful phylogenetic hypotheses.

Data accessibility

Raw transcriptomic and AHE reads are available on Genbank SRA database via BioProject PRJNA515912. The novel transcriptomic assemblies, genomic reference matrix used for probe design, probe sequences, processed AHE data, assembled matrices, and all tree files are available on Dryad (https://doi.org/10.5061/dryad.8575jr5).

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