Molecular Phylogenetics and Evolution xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Molecular phylogenetic analysis supports a Gondwanan origin of the Hyriidae (Mollusca: Bivalvia: Unionida) and the paraphyly of Australasian taxa

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ARTICLE INFO

Article history:
Received 22 August 2014
Revised 22 January 2015
Accepted 28 January 2015
Available online xxxx

Keywords:
Freshwater mussel
Hyriidae
Phylogeny
Australasia
Gondwana

ABSTRACT

The freshwater mussel family Hyriidae (Mollusca: Bivalvia: Unionida) has a disjunct trans-Pacific distribution in Australasia and South America. Previous phylogenetic analyses have estimated the evolutionary relationships of the family and the major infra-familial taxa (Velesunioninae and Hyriinae: Hyridellini in Australia; Hyriinae: Hyriini, Castaliini, and Rhipidodontini in South America), but taxon and character sampling have been too incomplete to support a predictive classification or allow testing of biogeographical hypotheses. We sampled 30 freshwater mussel individuals representing the aforementioned hyriid taxa, as well as outgroup species representing the five other freshwater mussel families and their marine sister group (order Trigoniida). Our ingroup included representatives of all Australian genera. Phylogenetic relationships were estimated from three gene fragments (nuclear 28S, COI and 16S mtDNA) using maximum parsimony, maximum likelihood, and Bayesian inference, and we applied a Bayesian relaxed clock model calibrated with fossil dates to estimate node ages. Our analyses found good support for monophyly of the Hyriidae and the subfamilies and tribes, as well as the paraphyly of the Australasian taxa (Velesunioninae, (Hyridellini, (Rhipidodontini, (Castaliini, Hyriini)))). The Hyriidae was recovered as sister to a clade comprised of all other Recent freshwater mussel families. Our molecular date estimation supported Cretaceous origins of the major hyriid clades, pre-dating the Tertiary isolation of South America from Antarctica/Australia. We hypothesize that early diversification of the Hyriidae was driven by terrestrial barriers on Gondwana rather than marine barriers following disintegration of the super-continent.

1. Introduction

The phylogenetic relationships among the freshwater mussels of the family Hyriidae (Mollusca: Bivalvia: Unionida) have received considerable attention but little resolution (Walker et al., 2014). Representative species have been included in morphological (Graf, 2000), molecular (Bogan and Hoeh, 2000; Graf and Ó Foighil, 2000a,b; Graf, 2002; Hoeh et al., 2002; Baker et al., 2003, 2004; Walker et al., 2006; Whelan et al., 2011; Pfeiffer and Graf, 2013; Marshall et al., 2014), and combined (Hoeh et al., 2001, 2009; Roe and Hoeh, 2003; Graf and Cummings, 2006) phylogenetic analyses, but sampling has been sparse and repetitive (Graf, 2013). Moreover, the analytical methods employed by the most comprehensive analyses are, in many cases, dated. Three outstanding problems in need of clarification are (1) the reported paraphyly of the Australasian taxa relative to those of South America, (2) the position of the Hyriidae among the other freshwater mussels of the order Unionida, and (3) molecular clock estimates of clade ages. The objectives of this study are to test the family-level relationships of the Australasian freshwater mussels using broader taxon and molecular character sampling than has previously been brought to bear and to do so in a rigorous molecular phylogenetic context.

The Hyriidae is composed of around 90 Recent species in 16 genera (Graf and Cummings, 2007; Pereira et al., 2014; Walker et al., 2014). The family has a disjunct distribution, occurring in

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http://dx.doi.org/10.1016/j.ympev.2015.01.012
1055-7903/

South America and Australasia (including Australia, New Guinea, the Solomon Islands, and New Zealand). Like almost all freshwater mussels of the order Unionida, hyriids are obligate parasites of freshwater fishes during their larval stages (Wächtler et al., 2001; Graf and Cummings, 2006), and this period of encystment on the gills or fins of their hosts is the primary dispersal phase of their life cycle (Graf, 1997, 2013). As adults, freshwater mussels are sedentary filter feeders. Females exhibit parental care, brooding their larvae in the interlamellar spaces of their ctenidia. Such behavior provides not only osmotic protection in nutrient-poor waters but also inertia in lotic habitats (Needham, 1930; Pennak, 1985; Gray, 1988). This peculiar life history makes freshwater mussels, including hyriids, poor dispersers across terrestrial and marine barriers. Consequently, disjunctions among freshwater mussels are strong evidence for vicariance, and the recent literature has attributed the current distribution pattern of the Hyriidae to the Mesozoic disintegration of Gondwana (Graf and Ó Foighil, 2000b; Walker et al., 2014).

Historically, the family-group level classifications of the Neotropical and Australasian hyriids were studied in isolation and without the benefit of modern cladistic theory, continental drift theory, or molecular sequence data. The current taxonomy of South American hyriids dates largely from Parodiz and Bonetto (1963), with three endemic taxa: Rhipidontini (= Diplodontini), Castaliini, and Hyriini. Largely contemporaneously, McMichael and Hiscock (1958) divided the Australasian hyriids among four subfamilies: Hyridellinae, Cucumerunioninae, Velesunioninae, and Lortiellinae. Both arrangements were based mostly on shell characters (e.g., external sculpture, shell outline). Each of these faunas on opposite sides of the Pacific Ocean were regarded as endemic radiations, though waif dispersal and hypothetical "land bridges" had been invoked to explain their disjunction (Ortmann, 1921; Modell, 1942; McMichael and Hiscock, 1958; Parodiz and Bonetto, 1963; Walker et al., 2001).

Molecular phylogenetic studies since 2000 have shed new light on the traditional classification of the Australasian hyriids and their relationships to those of South America. Graf and Ó Foighil (2000b) recovered the Australasian fauna as paraphyletic relative to the South American taxa, and this result has been confirmed by subsequent re-analysis (Graf and Cummings, 2006). Based on these results, the family-group level classification of the Hyriidae was revised to delimit two subfamilies: Velesunioninae in Australasia and Hyriinae in Australasia and South America. The latter subfamily is composed of four tribes: Hyridellini in Australasia and the three Neotropical tribes listed above (Bieler et al., 2010). Ponder and Bayer (2004) synonymized the Lortiellinae with the Velesunioninae on anatomical grounds, and this result was confirmed by Graf and Cummings (2006). The Cucumerunioninae is currently regarded as a synonym of the Hyridellini (Graf and Cummings, 2006; Marshall et al., 2014; Walker et al., 2014), although no phylogenetic analysis has tested this hypothesis. The current classification of the Hyriidae is summarized in Table 1.

While the classification of the Hyriidae has reached a consensus (Bieler et al., 2010; Carter et al., 2011), the phylogenetic position of the family among freshwater mussels has remained contentious. The monophyly of the Hyriidae is well supported (Graf and Cummings, 2006; Whelan et al., 2011), but analyses emphasizing different character sets have supported various sister groups for the family. Traditionally, the six Recent families of the Unionida were divided into two superfamilies based solely on larval morphology (Parodiz and Bonetto, 1963). The Unionoidea (= "Unionacea") was composed of the three families with glochidium-type larvae: Unionidae, Margaritiferidae, and Hyriidae. The families Etheriidae, Mycetopodidae, and Iridinidae comprised the Etherioidea (= "Mutelacea"), diagnosed by the presence of lasidium-type larvae (Wächtler et al., 2001; Graf and Cummings, 2006). However, cladistic analyses of larval and adult morphology supported a sister relationship between the Hyriidae and the lasidium-bearing mussels based on synapomorphies of the adult ctenidia and posterior mantle apertures (Graf, 2000; Hoeh et al., 2001; Roe and Hoeh, 2003; Graf and Cummings, 2006). Graf (2000) and Graf and Cummings (2006) advocated reclassifying the Hyriidae among the Etherioidea.

Contrary to morphological analyses, molecular phylogenetic studies of cytochrome oxidase subunit I (COI) have generally recovered the Hyriidae as sister to the five other extant families of the order (Bogan and Hoeh, 2000; Hoeh et al., 2001, 2002; Walker et al., 2006). Graf and Cummings (2006) reanalyzed the published COI data in combination with morphology and available 28S (large nuclear ribosomal subunit) sequences, and they recovered the Hyriidae as sister to the lasidium-bearing mussels – albeit with weak support. More recently, Whelan et al. (2011) analyzed COI and 28S sequences for representatives of five freshwater mussel families with mixed results. Whereas parsimony resolved a (Hyriidae + Margaritiferidae + Unionidae) clade, likelihood-based methods weakly supported Hyriidae as sister to the remainder of the Unionida. The compromise solution has been to classify the Hyriidae in a separate superfamily, Hyrioidea (Hoeh et al., 2009; Bieler et al., 2010; Carter et al., 2011).

A common feature of the analyses reviewed above is the inadequacy of character and taxon sampling to rigorously test either the interfamilial relationships of the Hyriidae or the sister group of the family. Too much emphasis has been placed on single character sets and serendipitous representation of hyriid lineages. Moreover, many of these studies relied strictly upon maximum parsimony, demonstrated to produce unreliable results with deep divergences and fast-evolving characters like COI (Graf and Cummings, 2010). While previous results have been sufficient to reject the traditional taxonomic arrangement, they provide little basis for a stable and predictive classification. We set out to test the inter-generic relationships of the Australian Hyriidae and the position of the family with (1) broader, targeted taxon sampling, (2) a larger character set including both nuclear and mitochondrial DNA, and (3) methods of analysis that extend beyond maximum parsimony.

Table 1
Classification, diversity, and biogeography of the Hyriidae. Summarized from Graf and Cummings (2007), Walker et al. (2014) and Pereira et al. (2014).

Taxon	Generic richness	Species richness	Geographical distribution
HYRIIDAE Swainson, 1840	16	91	
VELESUNIONINAE Iredale, 1934	5	13	Australia, New Guinea
HYRIINAE s.s.			
HYRIDELLINI McMichael, 1956 (1934)	4	14	Australia, New Guinea, Solomon Islands, New Zealand
HYRIINI s.s.	2	4	South America
CASTALIINI Morretes, 1949	3	17	South America
RHIPIDODONTINI Starobogatov, 1970	2	43	South America

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2. Materials & methods

2.1. Taxon sampling

Sampled taxa are listed in Table 2. Ingroup taxa were chosen to represent both of the subfamilies and all of the tribes of the Hyriidae from Australasia and South America (Table 1). The outgroup was composed of taxa representing the five other extant families as well as *Neotrigonia* (Trigoniida), the sister group to freshwater mussels (Hoeh et al., 1998; Giribet and Wheeler, 2002). All Australian freshwater mussel genera were represented (Walker et al., 2014), and all ingroup specimens were identified by the authors.

2.2. Character sampling

Characters for phylogenetic analyses were drawn from three genes: mitochondrial protein-coding cytochrome oxidase subunit I (COI), the large mitochondrial ribosomal subunit (16S), and the large nuclear ribosomal subunit (28S). All novel sequences were obtained using standard PCR and dye-terminator sequencing methods (Graf and Ó Foighil, 2000a,b). Primer sequences used for amplification and sequencing are listed in Table 3. Several outgroup DNA sequences and five ingroup sequences were published previously and obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/). References and accession numbers are cited in Table 2.

Ribosomal 16S and 28S sequences were aligned using CLUSTAL X (Larkin et al., 2007) and refined by eye using Mesquite version 2.75 (Maddison and Maddison, 2011). Protein-coding COI was translated into amino acids and nucleotides were aligned manually by codon position.

2.3. Phylogenetic analyses

The three character partitions (COI, 16S and 28S) were analyzed separately and in combination using maximum parsimony (MP) and maximum likelihood (ML). Bayesian inference (BI) was applied only to the combined dataset. An incongruence length difference test (ILD = partition homogeneity test) was implemented in PAUP* version 4b10 (Swofford, 2002) to confirm the congruence of phylogenetic signals among the three character sets (Farris et al., 1995).

PAUP* (Swofford, 2002) was used for all MP analyses. Tree searches were performed first as heuristic searches with 100 random sequence additions and default settings. The resultant trees were then used as the starting trees for another bout of tree searching to work around a known PAUP* bug (http://paup.csit.fsu.edu/problems.html). Bootstrap analyses (2000 replicates, heuristic search with 10 random sequence additions) were employed to measure clade support.

For likelihood-based analyses (ML and BI), COI was analyzed both as a single partition and as three partitions (i.e., one for each codon position). Thus, there were two different combined datasets: 3 partitions (28S + 16S + COI) and 5 partitions (28S + 16S + 3 COI codon positions). jModelTest version 2.1.1 (Darriba et al., 2012) was used to determine the optimal substitution model for each partition under the Akaike information criterion. All ML analyses

Table 2
Specimens analyzed. Voucher specimens have been (or will be) deposited at the Academy of Natural Sciences of Drexel University (ANSP), Philadelphia, Field Museum of Natural History (FMNH), Chicago, Illinois Natural History Survey (INHS), Champaign, and the University of Michigan Museum of Zoology (UMMZ), Ann Arbor, USA as listed. A dagger (†) indicates chimeric taxa.

Taxon	Locality	Genbank Accession #			Source
		28S	16S	COI	
TRIGONIOIDA: TRIGONIDAE					
Neotrigonia margaritacea †	Australia (marine)	DQ279963	DQ280034	U56850	Hoeh et al. (1998), Giribet et al. (2006)
UNIONIDA: UNIONIDAE					
Unio pictorum †	Europe	AF305383	DQ060163	AF156499	Graf and Ó Foighil (2000a,b) and Källersjö et al. (2005)
MARGARITIFERIDAE					
Margaritifera margaritifera †	Europe	JN243869	AF303281	JN243891	Machordom et al. (2003), Whelan et al. (2011)
IRIDINIDAE					
Chambardia wahlbergi †	Zambia	JN243864	KP184845	JN243886	FMNH 343927-343928, Whelan et al. (2011)
MYCETOPODIDAE					
Anodontites elongata	Peru	KP184872	KP184846	KP184896	FMNH 343931 ex ANSP 416349
ETHERIIDAE					
Etheria elliptica	Zambia	KP184873	KP184847	KP184897	FMNH 343930 ex ANSP 419710
HYRIIDAE					
Triplodon corrugatus (*n* = 2)	Peru	JN243868	KP184851	JN243890	FMNH 343929 ex ANSP 416338, Whelan et al. (2011)
		KP184876	KP184852	KP184900	FMNH 343925
Castalia ambigua	Peru	JN243867	KP184848	JN243889	ANSP 416341
Diplodon demeraraensis (*n* = 2)	Guyana	KP184874	KP184849	KP184898	INHS 27889
		KP184875	KP184850	KP184899	
Cucumerunio novaehollandiae (*n* = 2)	New South Wales	KP184877	KP184853	KP184901	UMMZ 304501
		KP184878	KP184854	KP184902	UMMZ 304502
Hyridella australis (*n* = 2)	New South Wales	KP184883	KP184859	KP184907	FMNH 343926
		KP184884	KP184860	KP184908	UMMZ 304507
Hyridella depressa (*n* = 2)	New South Wales	KP184879	KP184855	KP184903	UMMZ 304503
		KP184880	KP184856	KP184904	UMMZ 304504
Hyridella drapeta (*n* = 2)	New South Wales	KP184881	KP184857	KP184905	UMMZ 304505
		KP184882	KP184858	KP184906	UMMZ 304506
Microdontia anodontaeformis †	New Guinea	KP184885	KP184861	KP184909	UMMZ 304508-304509
Alathyria jacksoni	New South Wales	KP184888	KP184864	KP184912	UMMZ 304512
Alathyria pertexta (*n* = 2)	Queensland	KP184886	KP184862	KP184910	UMMZ 304510
	New South Wales	KP184887	KP184863	KP184911	UMMZ 304511
Alathyria profuga (*n* = 2)	New South Wales	KP184889	KP184865	KP184913	UMMZ 304513
	New South Wales	KP184890	KP184866	KP184914	UMMZ 304514
Lortiella froggatti †	Western Australia	KP184891	KP184867	AF231746	UMMZ 304515, Bogan and Hoeh (2000)
Velesunio ambiguus (*n* = 2)	New South Wales	KP184892	KP184868	KP184915	FMNH 337195
		KP184893	KP184869	KP184916	
Westralunio carteri (*n* = 2)	Western Australia	KP184894	KP184870	KP184917	UMMZ 304516
		KP184895	KP184871	KP184918	UMMZ 304517

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Table 3
Primer sequences for PCR amplification and sequencing.

Gene	Name	Direction	Sequence (5′–3′)	References
COI	LCO1490	Forward	ggtcaacaaatcataaagatattgg	Folmer et al. (1994)
	HCO2198	Reverse	taaacttcagggtgaccaaaaaatca	
	HCO2198-RH	Reverse	tcagggtgaccaaaaaatca	Graf and Ó Foighil (2000a)
	LCO22ME2	Forward	ggtcaacaaaycataargatattgg	Walker et al. (2006)
	HCO700dy2	Reverse	tcagggtgaccaaaaaayca	
16S	16Sar-L-myt	Forward	cgactgtttaacaaaaacat	Lydeard et al. (1996)
	16Sbr-H-myt	Reverse	ccgttctgaactcagctcatgt	
28S	D23F	Forward	gagagttcaagagtacgtg	Park and Ó Foighil (2000)
	D4RB	Reverse	tgttagactccttggtccgtgt	

were performed using RAxML version 7.0.3 under the GTR + Γ model, as recommended in the manual (Stamatakis, 2006). The ML topology was estimated using 1000 separate inferences from each partition and combination, and clade support was determined from 2000 standard (i.e., not rapid) bootstrap replicates. MrBayes version 3.2.1 (Ronquist et al., 2012) was used for BI analyses of the combined partitions (4 runs, 4 chains each, 24×10^6 MCMC generations). The substitution models applied to each partition are listed in Table 4. Trees were sampled every 1000 generations and the first 25% were discarded as burn-in. Sufficient mixing of the chains was monitored using the average of the standard deviations of the splits frequencies (<0.01), and stationarity was verified using TRACER version 1.5 (Rambaut and Drummond, 2009).

2.4. Comparison of alternative topologies

Conflicting clade analysis (CCA), following the methods of Whelan et al. (2011), was used to summarize topological differences among the MP, ML, and BI results. CCA identifies those clades that have high MP or ML bootstrap support (⩾70%) or BI posterior probabilities (⩾95%) in the various analyses but that have low support in the preferred topology. This practice eliminates the need to illustrate multiple similar trees and focuses discussion on conflicting clades that are well supported (ignoring those that are only resolved with low support). The analysis was performed using a custom perl script (available from the corresponding author).

Templeton, Winning Sites, Shimodaira–Hasegawa (S–H), and Bayes Factor analyses were undertaken to statistically compare optimal topologies against three different constraint topologies: (1) *Alathyria* monophyletic, (2) *Hyridella* monophyletic, and (3) Australasia monophyletic. Templeton and Winning Sites tests (Felsenstein, 2003) were implemented in PAUP* (Swofford, 2002). The S–H tests (Shimodaira and Hasegawa, 1999) were done using RAxML (Stamatakis, 2006), and Bayes Factors were calculated and interpretted following the methods of Kass and Raftery (1995) and Nylander et al. (2004).

2.5. Estimation of node ages

The topology and timing of freshwater mussel diversification were simultaneously estimated by conducting a Bayesian uncorrelated relaxed clock analysis using BEAST version 1.7.5 (Drummond et al., 2012). For this analysis only the Combined (5 partition) dataset described above was used, employing the same substitution models used in the MrBayes analysis (Table 4) and a Birth–Death speciation topology-prior. A complete summary of the priors used for each parameter can be found in the Supplementary materials. The phylogeny was calibrated at three nodes: the Hyridellini, the core Velesunioninae (= *Velesunio* + *Alathyria* + *Lortiella* + *Microdontia*), and Unionoidea (= *Unio* + *Margaritifera*). The minimum ages of the first two nodes were both calibrated at 99.6 My based on the fossil ages of *Hyridella* and *Alathyria* in the upper-most Albian-Cenomanian (Hocknull, 2000; Walker and Geissman, 2009) and the minimum for the divergence of Unionoidea was based on the oldest unionid in the Morrison Formation of western North America. That fossil freshwater mussel assemblage is well regarded as belonging to the stem or crown Unionidae (Watters, 2001). The oldest exemplar, *Hadrodon jurassicus*, was described from the lower portion of the formation, spanning the Tidwell and Salt Wash members (Yen, 1952; Evanoff et al., 1998). Kowallis et al. (1998) provided dates bracketing these members from 150 to 155 My, and we calibrated the minimum age of the Unionidae at 152 My. Each calibration was modeled with the date representing the youngest possible age and an exponential distribution for the probability of an age older than the calibration data. A lambda rate parameter of 30 was used for the 99.6 My calibration dates resulting in a 95% credibility interval of 100–210 My and a lambda of 20 for the 152 My calibration date (95% CI 153–226 My). The MrBayes consensus tree inferred from the Combined (5 partition) dataset was used to create a starting tree. Branch lengths of this tree were converted to be roughly time-calibrated via penalized likelihood using the chronos function in the R package APE (Paradis et al., 2004). Three independent analyses were performed for 24×10^6 generations each, sampling trees every 1000 generations. TRACER (Rambaut and Drummond, 2009) was used to identify plateaus in likelihood scores and the posterior estimates of model parameters. All runs reached a stationary distribution prior to 2.4 million generations, and these were discarded as burn-in.

AWTY (Nylander et al., 2008) was used to evaluate concordance among the three independent runs, the cumulative posterior probabilities of tree bipartitions were examined using the "cumulative" utility, and bipartition posterior probabilities between independent analyses were compared with the "compare" utility. After confirming concordance, the output of independent runs was combined and the maximum clade credibility chronogram calculated for the posterior sample (64,803 trees) using the LOGCOMBINER and TREEANNOTATOR utilities included in BEAST (Drummond et al., 2012).

Table 4
Substitution models applied for BI analyses.

DNA Sequence Partition	Substitution Model
28S	GTR + G
16S	GTR + G
COI	GTR + I + G
COI codon position 1	GTR + I + G
COI codon position 2	GTR
COI codon position 3	HKY + G

3. Results

The combined datasets (28S + 16S + COI) comprised a matrix of 30 individuals (21 species in 16 genera, *a priori*) by 1639 aligned

nucleotides (nt). All terminals were represented by all genes, and nearly all were non-chimeric (i.e., all three gene fragments were obtained from the same individual). The following terminals were chimeric: *Neotrigonia margaritacea*, *Unio pictorum*, *Margaritifera margaritifera, Chambardia wahlbergi* (all outgroup), *Microdontia anodontaeformis*, and *Lortiella froggatti* (both ingroup) (Table 2). Preliminary analyses employing other alignment algorithms recovered similar results to those presented below based on CLUSTAL X. All novel sequences have been deposited in GenBank (KP184845-KP184918).

An ILD test found no significant phylogenetic conflict among 28S, 16S and COI ($p = 0.204$). Table 5 reports the tree statistics from the MP analysis of the combined dataset, showing the relative contribution of each partition to the tree score. See the Supplementary materials for more details of the MP, ML, and BI analysis results, including topologies, branch support, and model parameters. Fig. 1 depicts a time-calibrated phylogram recovered from the BI Combined (5 partitions) analysis, including clade bootstraps and posterior probabilities from the other analyses of the combined matrix. The four ML and BI combined analyses (3 and 5 partition) recovered the same topology, although they differed slightly in their degree of support for some clades. The MP combined analysis recovered a conflicting topology (see Supplementary materials). The CCA analysis among all topologies is summarized in Table 6.

Only two conflict clades (8 & 9) in the CCA (Table 6) are relevant to the results of this study. Clades 1–5 were resolved in the BI Combined (5 partitions) tree shown in Fig. 1. These clades merely indicate where alternative analyses found higher support for clades than were recovered in the preferred topology. Clades 6–7 represent interspecific relationships supported by ML analyses of single genes. Clades 8–9 indicate important conflicting clades that were recovered with high support (i.e., bootstrap $\geqslant$70%) in only the MP combined analysis. Clade 8, a sister relationship between the Hyriidae and the (Unionidae + Margaritiferidae) clade, was resolved in <50% of all of bootstrap or MCMC trees for all other MP, ML, and BI analyses. Clade 9 supports the monophyly of the Australian mussels (Velesunioninae + Hyridellini). It also had low support in all other analyses.

BI and ML analyses resolved the Hyriidae as monophyletic and sister to all other freshwater mussel families, although support for the latter clade was ambiguous (Fig. 1). The two subfamilies, Velesunioninae and Hyriinae were monophyletic. Support for the Hyriinae was robust (except MP). Support for the core velesunionine clade (*Velesunio* + *Alathyria* + *Lortiella* + *Microdontia*) was also robust, but the placement of *Westralunio* as sister to the core clade was generally weak. Both 16S and COI individually resolved *Westralunio* with the Hyriinae (not shown, see Supplementary material). A clade of the genera representing the three South American tribes was well supported. Only two genera, (*Alathyria* and *Hyridella*) included multiple congeners, and neither was recovered as monophyletic.

Statistical analyses comparing the optimal MP, ML, and BI trees to topologies constraining the monophyly of *Alathyria, Hyridella* and Australian mussels are summarized in Tables 7 and 8. MP Templeton and Winning Sites tests (Table 7), ML S–H test, and BI Bayes Factor analysis (Table 8) robustly rejected *Alathyria* monophyly. Both BI and ML also rejected *Hyridella* monophyly, but MP was ambiguous. The best MP topology was identical to the Australasian monophyly constraint topology (Table 6: clade 9). However, that MP topology did not differ significantly from the best BI/ML topology (Table 7). Neither the S–H test nor Bayes Factor analysis found a significant topological difference between the best topology (Fig. 1) and the Australia monophyly constraint topology (Table 8).

Age estimates for key clades are reported in Table 9. The mean age derived from Bayesian MCMC analysis for the Hyriidae was 194 My, placing its origin in the Early Jurassic. Both the Velesunioninae (mean age = 172 My) and Hyriinae (167 My) arose in the Middle Jurassic (Walker and Geissman, 2009).

Table 5
Tree statistics for MP analysis of the combined matrix. Data for the contributions of the individual partitions of the MP are also provided.

	Combined	28S	16S	COI
Taxa	30	30	30	30
Characters	1639	481	518	640
Informative characters	641	156	212	273
# of trees	1			
Length	2637	481	966	1190
RC	0.3282	0.5797	0.3255	0.2518

RC = Rescaled Consistency Index.

4. Discussion

Although many studies have touched on the topic of Australian hyriid phylogeny (listed above), ours has the broadest taxon sampling and deepest character sampling to date. Our likelihood-based analyses recovered good support for the monophyly of the Hyriidae, Velesunioninae, Hyriinae (i.e., the paraphyly of the Australasian freshwater mussels), and Hyridellini (Table 1, Fig. 1). Though the paraphyly of the Australasian freshwater mussels was not resolved by maximum parsimony, this is hardly a cause for concern. The shortcomings of parsimony in molecular analyses with deep divergence situations (i.e., long branches) are well known (Felsenstein, 1978, 1981). While likelihood-based methods are not a panacea, the topology in Fig. 1 and our clade age estimates in Table 9 agree well with the morphological, biogeographical, and fossil evidence. Moreover, our results are in accordance with the modern classification of the Hyriidae and freshwater mussels generally.

4.1. Monophyly & sister-group of the Hyriidae

The Hyriidae was supported as monophyletic, confirming the results of nearly all previous analyses. The aforementioned phylogenetic studies had recovered the Hyriidae as either (1) sister to the other freshwater mussel families (Bogan and Hoeh, 2000; Hoeh et al., 2001, 2002; Walker et al., 2006), or (2) sister to the lasidium-bearing mussels (Graf, 2000; Roe and Hoeh, 2003; Graf and Cummings, 2006). Most of our analyses resolved the former hypothesis (Fig. 1), although the Bayesian inference (BI) combined (5 partition: 28S + 16S + 3 COI codon positions) analysis was the only one to provide strong support. The maximum parsimony (MP) combined analysis topology provided a well-supported (>70% bootstrap) alternative: (Hyriidae + Unionidae + Margaritiferidae) sister to (Etheriidae + Iridinidae + Mycetopodidae) (tree not shown, see Supplementary materials) (Table 6: clade 8). The MP result is consistent with the traditional classification proposed by Parodiz and Bonetto (1963), with the Hyriidae grouped with other mussels with glochidium-type larvae, and the mussels with lasidium-type larvae in a separate clade. Our preferred topology (Fig. 1) supports the hypothesis that possession of glochidium-type larvae (as observed in the Hyriidae, Unionidae, and Margaritiferidae) was the ancestral condition among freshwater mussels, and the lasidium-type larvae were derived from glochidia on the branch leading to the Etheriidae, Iridinidae, and Mycetopodidae (Graf and Cummings, 2006). Our preferred topology also supports classifying the Hyriidae in their own superfamily, Hyrioidea, distinct from the Unionoidea and Etherioidea (Hoeh et al., 2009; Bieler et al., 2010; Carter et al., 2011). The family (and superfamily) is readily diagnosed by the presence of hooked-type glochidia (lacking

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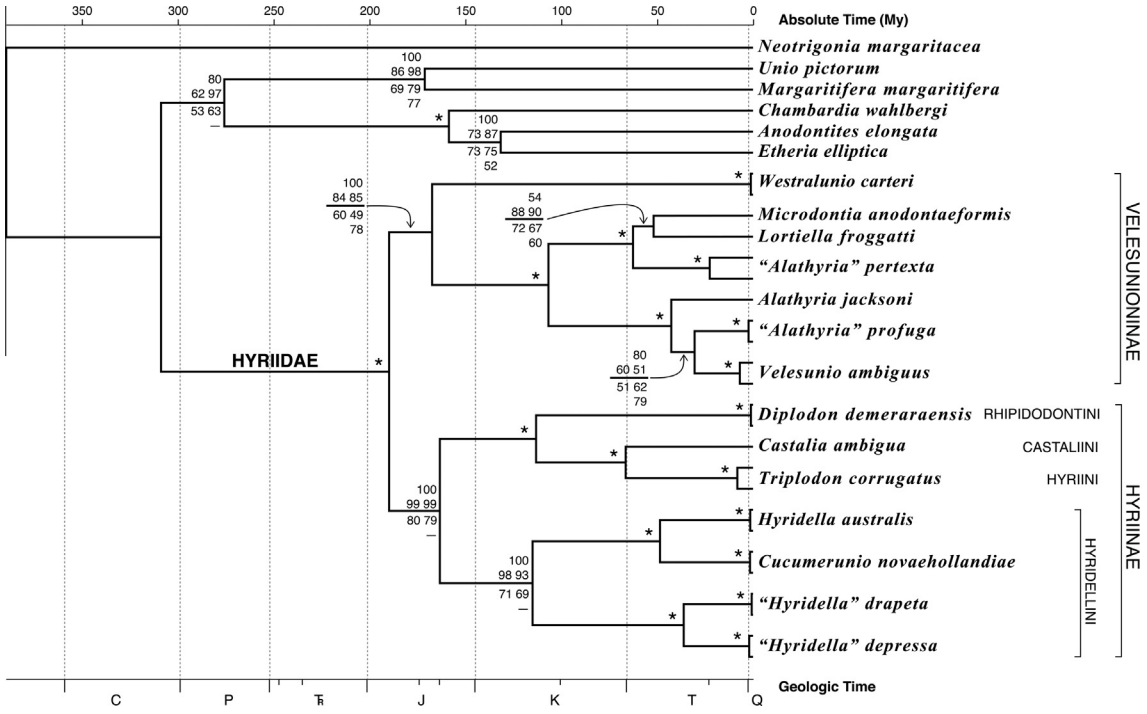

Fig. 1. BI/ML topology with branch lengths determined by the molecular clock. All BI and ML combined analyses (3 and 5 partition) returned the same topology. Node ages are the mean value returned from Bayesian molecular clock analysis. An asterisk (*) indicates nodes with ⩾98% bootstrap or posterior probability support in all analyses of the combined matrix. For other clades, branch support values above the branches are posterior probability percentages from BEAST and the two combined BI analyses (3 and 5 partition). Bootstrap percentages are given below the branches for the two combined ML analyses (3 and 5 partition) and MP.

Table 6
Conflict Clade Analysis (CCA) relative to the preferred topology (BI Combined, 5 partitions). Clades listed are those with low support in the BI Combined (5 partition) (<95%; underlined) but high support in the other analyses (BI posterior probability ⩾95%, MP/ML bootstrap ⩾70%; **bold**). † indicates clades resolved in Fig. 1.

#	Clade partitions	BI		MP				ML					
		Combo 5	Combo 3	Combo 1	28S 1	16S 1	COI 1	Combo 5	Combo 3	28S 1	16S 1	COI 3	COI 1
1†	(*Anodontites* + *Etheria*)	87	73	52	38	28	35	**75**	**74**	55	18	**76**	67
2†	HYRIDELLINI	93	**98**	47	46	57	9	69	**71**	54	45	26	27
3†	VELESUNIONINAE	85	84	**78**	39	6	19	49	60	48	5	0	0
4†	(*Microdontia* + *Lortiella*)	90	88	60	68	19	48	67	**72**	**73**	25	23	40
5†	(*"Alathyria" profuga* + *Velesunio ambiguus*)	51	60	**80**	0	**92**	36	62	51	0	**71**	22	12
6	(*Chambardia* + *Anodontites*)	0	11	6	0	60	14	13	12	0	**77**	10	18
7	(*Alathyria jacksoni* + *"A." profuga*)	47	39	20	0	7	62	38	49	48	23	**76**	**87**
8	(UNIONOIDEA + HYRIIDAE)	0	17	**75**	45	38	38	15	15	0	21	40	30
9	(VELESUNIONINAE + HYRIDELLINI)	0	0	**72**	16	0	38	13	12	0	7	14	15

HYRIDELLINI = (*Cucumerunio* + *Hyridella*); VELESUNIONINAE = (*Alathyria* + *Lortiella* + *Microdontia* + *Velesunio* + *Westralunio*); HYRIIDAE = (HYRIDELLINI + VELESUNIONINI + *Diplodon* + *Castalia* + *Triplodon*); UNIONOIDEA = (*Unio* + *Margaritifera*).

Table 7
Statistical comparison of MP constraint topologies. The MP tree is compared to the trees recovered from three different constraint topologies: *Alathyria* monophyletic, *Hyridella* monophyletic, and the preferred BI/ML topology (Fig. 1). The BEST MP tree resolves the Australasian taxa as monophyletic. * indicates statistically significant *p*-values ($\alpha = 0.05$).

Tree	Length	Length difference	Templeton (*p*-value)	Winning sites (*p*-value)
BEST	2637	–	–	–
Alathyria-1	2687	50	0.0001*	0.0001*
Alathyria-2	2687	50	<0.0001*	<0.0001*
Australasia	2637	0	–	–
Hyridella-1	2652	15	0.12	0.1334
Hyridella-2	2652	15	0.0287*	0.0411*
BI/ML (Fig. 1)	2652	15	0.1654	0.211

marginal spines) brooded in a marsupium composed of the females' inner demibranchs, as well as the presence of a complete excurrent siphon. The incurrent aperture generally lacks ventral mantle fusion (Graf and Cummings, 2006).

4.2. Hyriid subfamilies, tribes & genera

Our results further support the division of the Hyriidae into two subfamilies: Velesunioninae and Hyriinae (Fig. 1, Table 1). The

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Table 8
Statistical comparison of BI/ML constraint topologies. The BI/ML topology (BEST) is compared with three constraint topologies: *Alathyria* monophyletic, Australasia monophyletic, and *Hyridella* monophyletic. ∗ indicates statistically significant *p*-values ($\alpha = 0.05$).

Tree	−ln*L*	−ln*L* Difference	S–H (*p*-value)	Arithmetic Mean −ln*L*	−ln*L* Difference	Harmonic Mean −ln*L*	−ln*L* Difference	2 ln (B_{10})	Decision
BEST	−12503.59289	–	–	−12512.83	–	−12556.09			
Alathyria	−12631.44270	−127.84981	<0.05*	−12640.52	−127.69	−12686.04	−129.95	−259.89	Reject
Australasia	−12508.77216	−5.17927	>0.05	−12517.10	−4.27	−12560.81	−4.72	−9.43	Reject?
Hyridella	−12535.14477	−31.55188	<0.05*	−12543.22	−30.39	−12587.83	−31.74	−63.47	Reject

Table 9
Estimation of clade ages. Three minimum clade ages were used to calibrate ages (see text for explanation). Ages are given in millions of years before present.

Clades	Calibration	Mean	Median	95% range
Root	–	403	389	241–589
UNIONIDA	–	315	308	212–424
UNIONOIDEA	152	177	171	152–222
(*Anodontites* + *Etheria*)	–	136	131	73–209
HYRIIDAE	–	194	190	143–251
VELESUNIONINAE	–	172	168	127–223
(*Alathyria* + *Lortiella* + *Microdontia* + *Velesunio*)	99.6	110	107	99.6–129
HYRIINAE	–	167	163	119–219
HYRIDELLINI	99.6	119	115	99.6–150
(*Castalia* + *Diplodon* + *Triplodon*)	–	115	113	72–164

Velesunioninae is strictly Australasian whereas the Hyriinae has a disjunct distribution, occurring in both Australasia and South America. The paraphyly of the Australasian hyriids has been previously proposed (Graf and Ó Foighil, 2000b; Graf and Cummings, 2006) and has been generally accepted despite insufficient taxon and character sampling (Bieler et al., 2010; Carter et al., 2011; Walker et al., 2014). The phylogeny in Fig. 1 includes representatives of all but two Australasian freshwater mussel genera (i.e., *Virgus* and *Echyridella*, both traditionally Hyriinae). The two subfamilies are well supported by our likelihood-based analyses except for the placement of *Westralunio* as sister to the core Velesunioninae (= *Velesunio* + *Alathyria* + *Lortiella* + *Microdontia*). No well-supported alternative position for that genus was recovered by any of our analyses (Table 6), and the other core velesunionines are well supported as monophyletic. Only the MP combined analysis supported a clade composed of Australasian mussels sister to those from South America (tree not shown, see Supplementary materials) (Table 6: clade 9). None of our constraint analyses were able to find significant support to distinguish Australasian monophyly from paraphyly (Tables 7 and 8). Nevertheless, likelihood-based analyses of the combined dataset consistently returned high bootstrap and posterior probabilities supporting the monophyly of the Hyriinae, a clade composed of both Australasian and Neotropical mussels (Fig. 1). The two subfamilies can be diagnosed by the degree of development of umbo sculpture as well as larval characteristics (Walker et al., 2014). Species of the Hyriinae tend to have well-developed "radial" or V-shaped umbo sculpture, whereas members of the Velesunioninae generally have weakly developed or no umbo sculpture (Graf and Cummings, 2006; Zieritz et al., 2013). These shell characters are useful for distinguishing fossil as well as extant taxa (Hocknull, 2000).

The subfamily Hyriinae is split between two clades in our analyses (Fig. 1). One is equivalent to the Australian tribe Hyridellini, and the other clade is comprised of the three Neotropical tribes: Hyriini, Castaliini, and Rhipidodontini. The latter clade lacks formal nomenclature. The genus *Echyridella* from New Zealand was not available for inclusion, but in previous studies, it has been recovered in various weakly supported positions relative to these two clades (Graf and Cummings, 2006; Walker et al., 2006; Marshall et al., 2014). If *Echyridella* is found to be sister to either (Hyriini + Castaliini + Rhipidodontini) or (Hyridellini, ((Hyriini + Castaliini + Rhipidodontini)), then it may represent a 5th tribe within the Hyriinae. The clades within the Hyriinae are best diagnosed by molecular characters and geography. McMichael and Hiscock (1958) distinguished Australian hyriines from Neotropical species by the presence of a perforated septum dividing the infrabranchial from the suprabranchial chambers of the mantle cavity, but this has been questioned by Walker et al. (2014).

Only two genera in our analyses, *Hyridella* and *Alathyria*, were represented by multiple species (Table 2), and neither was recovered as monophyletic (Fig. 1). *Hyridella* (Hyriinae: Hyridellini) was represented by three species that were resolved in two well supported clades: (*H. depressa* + *H. drapeta*) and (*H. australis* + *Cucumerunio novaehollandiae*). *Alathyria* (Velesunioninae) was also recovered as paraphyletic, with none of the three included species forming an *Alathyria*-exclusive clade. The problem of non-monophyly of the Australian freshwater mussel genera has been reported previously (Baker et al., 2003, 2004). However, sorting out genus-level nomenclature is beyond the scope of this paper. It should be sufficient to point out that the names *Hyridella* Swainson, 1840 and *Alathyria* Iredale, 1934 will remain with their respective type species, *H. australis* (Lamarck, 1819) and *A. jacksoni* Iredale, 1934, and that other genus-group level names are already available for the other lineages (McMichael and Hiscock, 1958).

4.3. Origin & diversification of the Hyriidae

The clade ages of the Australian and South American hyriid lineages as well as the dispersal capabilities of freshwater mussels are entirely consistent with Mesozoic Gondwanan origins of the major ingroup clades depicted in Fig. 1: Hyriidae, Velesunioninae, Hyriinae, Hyridellini, and the clade of Neotropical tribes (Table 1). With only the spotty fossil record available to estimate clade ages, dispersalist hypotheses were considered untestable under the paradigm of the Cladistic Revolution (Briggs, 2003; de Queiroz, 2005; McGlone, 2005). Nevertheless, over the last 10–15 years, molecular clock analyses have repeatedly discovered that the geographical distributions of a variety of traditional Gondwanan taxa are better explained by subsequent transoceanic dispersal than by ancient vicariance – e.g., southern beeches (Knapp et al., 2005), ratite birds (Haddrath and Baker, 2001), and galaxiid fishes (Burridge et al., 2012). This is not the case with the disjunct distribution of the

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Australasian and South American hyriids. Even our minimum clade age calibrations for the core Velesunioninae and Hyridellini from the earliest Late Cretaceous (99.6 Mya) (Hocknull, 2000) substantially pre-date the latest hypothesized continental connection between South America and Australia/Antarctica during the Eocene (52 Mya) (Sanmartín and Ronquist, 2004). The mean molecular age estimates for the origins of core velesunionines and Hyridellini as well as the Neotropical clade are Early Cretaceous (110–119 Mya), and the mean age estimates for the Velesunioninae (including *Westralunio*) and Hyriinae fall in the latest Middle Jurassic (167–172 Mya) (Table 9). This suggests that vicariance resulting from the disintegration of Gondwana was not the driver of cladogenesis among the major hyriid lineages. Rather, these clades (or their stem groups) had diversified before to the breakup of the southern continents.

Although the major hyriid clades originated prior to the isolation of South America from Australia by marine dispersal barriers, we hypothesize that terrestrial barriers isolated these clades even while these continental elements of Gondwana remained intact. That is, it was the evolution of Mesozoic river basins on Gondwana that precipitated cladogenesis in the Hyriidae, localizing the Velesunioninae, Hyridellini and Neotropical tribes to specific catchments, and subsequent tectonic rifting reinforced this isolation. Alternatively these clades were widespread on Gondwana, and the distributions observed today represent relictual lineages and the products of differential extinction. The former hypothesis is supported by the Mesozoic fossil record of the Hyriidae, which is largely (see below) restricted to South America and Australasia, and the fossil taxa in those areas are assigned to their respective modern taxa (Martínez and Figueiras, 1991; Hocknull, 2000; Perea et al., 2009; Thompson and Stilwell, 2010; Parras and Griffin, 2013).

We estimate an Early Jurassic (mean age = 194 My) origin of the crown-group Hyriidae (Table 9). That is younger than the Late Triassic (Carnian, 217–229 Mya) alleged hyriid records from Australasia (McMichael, 1957; Hocknull, 2000) and North America (Wanner, 1921; Good, 1998). Those fossils are not assigned to modern genera (e.g., *Antediplodon*, *Prohyria*, *Megalovirgus*, *Mesohyridella*), and only the Australasian taxa have been assigned to the modern family-group level taxa described above. Skawina and Dzik (2011) regarded those pre-Jurassic fossils as the stem-groups of the modern unionoid clades, and that hypothesis is consistent with our results. The relationships of the North American Triassic fossils to the extinct Trigonioidoidea remains to be determined (Gray, 1988). We hypothesize that the most recent common ancestor of the modern Hyriidae arose on post-Triassic Gondwana, and the descendants of that freshwater mussel species gave rise to the ancestors of the Velesunioninae, Hyridellini and the (Hyriini + Castaliini + Rhipidodontini) clade before South America, Antarctica, and Australia were isolated by marine barriers in the Tertiary.

4.4. Areas for future study

Our work provides a sound basis for continued phylogenetic studies of the global Hyriidae. The phylogenetic positions of two additional Australasian genera, *Virgus* from New Guinea and *Echyridella* from New Zealand, could be added in future studies to test their positions relative to the well supported clades we have recovered. Both genera have traditionally been placed in the Hyridellini, although work to-date has demonstrated that *Echyridella*, geographically isolated from the other Australasian clades by 80 Mya (Sanmartín and Ronquist, 2004), may represent a distinct lineage (Graf and Cummings, 2006; Walker et al., 2006; Marshall et al., 2014). Moreover, the monophyly of the Neotropical tribes remains to be examined. We hope that this research stimulates further interest in this ancient family of freshwater mussels.

Acknowledgments

This research was funded by grants from the National Science Foundation of the USA to DLG (DEB-0542575, DEB-AToL-0732903). Mark Sabaj-Pérez collected most of the South American taxa. Maria Byrne, David Cale, Michael Caley, David Morgan, Adrian Pinder, and Andrew Storey provided specimens or facilitated collecting of Australasian taxa. Laboratory assistance was provided by Sergei Bombin, Nathan Whelan, and Sarah Wofford at the University of Alabama.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2015.01.012.

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Please cite this article in press as: Graf, D.L., et al. Molecular phylogenetic analysis supports a Gondwanan origin of the Hyriidae (Mollusca: Bivalvia: Unionida) and the paraphyly of Australasian taxa. Mol. Phylogenet. Evol. (2015), http://dx.doi.org/10.1016/j.ympev.2015.01.012