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A phylogeny of Vetigastropoda and other "archaeogastropods": re-organizing old gastropod clades

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Abstract. The phylogenetic relationships among the "archaeogastropod" clades Patellogastropoda, Vetigastropoda, Neritimorpha, and Neomphalina are uncertain; the phylogenetic placement of these clades varies across different analyses, and particularly among those using morphological characteristics and those relying on molecular data. This study explores the relationships among these groups using a combined analysis with seven molecular loci (18S rRNA, 28S rRNA, histone H3, 16S rRNA, cytochrome *c* oxidase subunit I [COI], myosin heavy-chain type II, and elongation factor-1α [EF-1α]) sequenced for 31 ingroup taxa and eight outgroup taxa. The deep evolutionary splits among these groups have made resolution of stable relationships difficult, and so EF-1α and myosin are used in an attempt to re-examine these ancient radiation events. Three phylogenetic analyses were performed utilizing all seven genes: a single-step direct optimization analysis using parsimony, and two-step approaches using parsimony and maximum likelihood. A single-step direct optimization parsimony analysis was also performed using only five molecular loci (18S rRNA, 28S rRNA, histone H3, 16S rRNA, and COI) in order to determine the utility of EF-1α and myosin in resolving deep relationships. In the likelihood and POY optimal phylogenetic analyses, Gastropoda, Caenogastropoda, Neritimorpha, Neomphalina, and Patellogastropoda were monophyletic. Additionally, Neomphalina and Pleurotomariidae fell outside the remaining vetigastropods, indicating the need for further investigation into the relationship of these groups with other gastropods.

Additional key words: Gastropoda, Mollusca, phylogenetics, parsimony, maximum likelihood

Vetigastropoda Salvini-Plawen 1980 is currently considered to include the extant gastropod clades Fissurelloidea Fleming 1822, Haliotioidea Rafinesque 1815, Lepetelloidea Dall 1882, Lepetodriloidea McLean 1988, Neomphaloidea McLean 1981, Pleurotomarioidea Swainson 1840, Scissurelloidea Gray 1847, Seguenzioidea Verrill 1884, Trochoidea Rafinesque 1815, and Turbinoidea Rafinesque 1815, as well as many extinct gastropod lineages (e.g., Anomphaloidea Wenz 1938, Cirroidea Bandel 1993, Euomphaloidea de Koninick 1881) (Bouchet et al. 2005; Geiger et al. 2008). Gastropods from the modern groups Vetigastropoda, Patellogastropoda Lindberg 1986, Neritimorpha Golikov & Starobogatov 1975, and Cocculinoidea Dall 1882 were combined in the clade Archaeogastropoda by Thiele (1929). Archaeogastropoda, however, is not monophyletic and is no longer recognized as an independent clade (see Bieler 1992; Ponder & Lindberg 1997; Aktipis et al. 2008; Lindberg 2008). Additionally, the placement of Neomphaloidea within Vetigastropoda is controversial, with some authors identifying Neomphalina Warén & Bouchet 1993 as an independent clade outside of Vetigastropoda (Heß et al. 2008; Kano 2008). The former "archaeogastropod" clades, Vetigastropoda, Neritimorpha, Cocculinoidea, and Neomphalina (the "hot-vent" taxa), therefore, are recognized in this study as independent groups with uncertain affinities to one another and other "higher" gastropod clades.

There are significant morphological, ecological, and behavioral variations among vetigastropods (see Geiger et al. 2008). Accounts of characteristic morphological traits for vetigastropods vary, but the presence of bursicles—a type of sensory organ found on the gills—and epipodial tentacles with epipodial sense organs (ESO) at their base are currently considered two well-documented synapomorphies of the

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clade (Salvini-Plawen 1980; Haszprunar 1987, 1988; Salvini-Plawen & Haszprunar 1987; Salvini-Plawen & Steiner 1996; Sasaki 1998; Geiger & Thacker 2005; Geiger et al. 2008). These characteristics, however, are absent or reduced in some vetigastropod taxa, namely some fissurellids (ESO) and lepetodrilids (bursicles). The presence of nacre and the presence of a shell slit or hole have been considered by some to be additional synapomorphies even though they are also secondarily lost in many species (Geiger et al. 2008). Likewise, there are significant differences among other archaeogastropod clades (see Lindberg 2008), and these morphological discrepancies have made determining the relationships between these groups difficult.

The taxonomic classification of Vetigastropoda, Patellogastropoda, Neritimorpha, Cocculinoidea, and Neomphalina has varied significantly over time. Furthermore, determination of evolutionary relationships among these groups has been problematic as their phylogenetic placement has varied across different analyses. This variation has been particularly drastic among analyses using morphological characteristics and those relying on molecular data (e.g., Ponder & Lindberg 1996, 1997; Sasaki 1998; Colgan et al. 2000, 2003; Geiger & Thacker 2005; Giribet et al. 2006; Williams et al. 2008). Studies incorporating only morphological characteristics resolve many relationships among these clades with high support values (Ponder & Lindberg 1997; Sasaki 1998; Aktipis et al. 2008), but these results often conflict with those obtained in molecular analyses (Harasewych et al. 1997; Colgan et al. 2000, 2003, 2007; McArthur & Harasewych 2003; Remigio & Hebert 2003; Giribet et al. 2006). In the only analysis to date of all gastropod clades incorporating both morphological and molecular data (Aktipis et al. 2008), the placement of Neritimorpha as a sister clade to the "higher" gastropod clade Apogastropoda Salvini-Plawen & Haszprunar 1987 stabilized when multiple sets of alignment parameters were studied, but the placement of Patellogastropoda, Vetigastropoda, Cocculinoidea, and Neomphalina remained uncertain. Furthermore, nodal support for the placement of these clades within Gastropoda remained low. Thus, additional sources of data are necessary to recover stable and well-supported relationships among these archaeogastropod clades.

Our ability to develop a clear understanding of the placement of Vetigastropoda among other gastropods is limited by their ancient and varied times of origin, as well as the high rate of extinction and subsequent rapid radiations occurring across these gastropod clades (see Frýda et al. 2008). The deeper the evolutionary relationships between clades, the more difficult it is to obtain a clear phylogenetic signal due to the increased frequency of character convergence in molecular data (Rokas et al. 2005; Rokas & Carroll 2006). Phylogenetic reconstruction is also made more difficult when many taxa in an extant group are extinct (Donoghue et al. 1989), such as in Patellogastropoda (Lindberg 2008). Furthermore, although gastropods have a rich fossil history dating back to the Cambrian, the limited number of well-preserved morphological characteristics for many of these fossils has contributed to their uncertain phylogenetic placement. This lack of phylogenetically informative morphological data makes the determination of the origin of archaeogastropod (and other gastropod) taxa problematic (Frýda et al. 2008).

The existence of these deep-level phylogenetic relationships necessitates the use of multi-gene analyses. The inclusion of different loci allows for the resolution of evolutionary relationships across a broad geologic time framework and improves phylogenetic inference (Cummings et al. 1995; Giribet 2002a; Rokas & Carroll 2005). Recent multi-locus analyses of taxa representing Patellogastropoda, Vetigastropoda, Neomphalina, Neritimorpha, and Cocculinoidea are based on molecular data from as few as two different genes (854 bp) to as many as five different genes ($\leq$6.5 kb) (Colgan et al. 2000; Giribet et al. 2006; Aktipis et al. 2008). All of these analyses fail to recover clear, well-supported relationships among the clades. One putative cause of this problem may be limited taxon sampling (Graybeal 1998; Pollock et al. 2002). Another possible explanation for the lack of supported nodes may be that the genes used in existing analyses do not provide sufficient resolution for the deep evolutionary splits between the different clades.

This study increases both the number of taxa sampled and the molecular loci analyzed in order to address the deep evolutionary splits between Patellogastropoda, Vetigastropoda, Neritimorpha, and Neomphalina. Seven genes representing nearly 7 kb of sequence data were sequenced for 31 ingroup taxa from all archaeogastropod clades, except Cocculinoidea, and eight outgroup taxa representing Caenogastropoda Cox 1960 as well as three additional molluscan classes. Five of these loci, including three nuclear genes (complete ribosomal gene 18S rRNA, partial 28S ribosomal rRNA, and histone H3) and two mitochondrial genes (partial ribosomal 16S rRNA and cytochrome *c* oxidase subunit I [COI]), have been used individually or in different combinations in other phylogenetic studies of gastropods (e.g., Harasewych et al. 1997, 1998; Colgan

et al. 2000, 2003; McArthur & Harasewych 2003; Nakano & Ozawa 2004; Vonnemann et al. 2005). In addition to these five genes, sequences of two additional nuclear protein-encoding genes were analyzed: fragments of the head portion of myosin heavy chain type II and elongation factor-1α (EF-1α), referred to as myosin and EF-1α, respectively. EF-1α has been used in conjunction with other genes in a molecular phylogeny of Caenogastropoda (Colgan et al. 2007), but myosin has yet to be incorporated into a gastropod phylogeny. Both of these genes have been used successfully in other contexts to resolve deep splits in metazoan phylogenies (Ruiz-Trillo et al. 2002; Peterson et al. 2004).

Methods

Taxon selection and identification

The 31 ingroup taxa represent the gastropod clades Patellogastropoda (six species), Vetigastropoda (18 species), Neomphalina (two species), and Neritimorpha (five species). Vetigastropoda is represented by taxa from the families Fissurellidae Fleming 1822, Haliotidae Rafinesque 1815, Pyropeltidae McLean & Haszprunar 1987, Lepetodrilidae McLean 1988, Pleurotomariidae Swainson 1840, Trochidae Rafinesque 1815, and Turbinidae Rafinesque 1815. Eight outgroup taxa representing the gastropod clade Caenogastropoda and the molluscan classes Scaphopoda, Polyplacophora, and Bivalvia were also included. Ingroup and outgroup species representation was limited to taxa that could be collected fresh for RNA extraction and subsequent sequencing of myosin and EF-1α. Seven sequences for COI and 28S rRNA were downloaded from GenBank. All the remaining sequences were generated from the material available to the authors, although some gene sequences had been generated for previous studies (Giribet et al. 2006). In total, 205 novel sequences were generated for this study. The species included in the phylogenetic analysis along with GenBank accession numbers for all molecular loci are given in Table 1. Specimen identification was conducted by the authors or with the assistance of Anders Warén (Sweden). The higher classification of the species follows Bouchet et al. (2005) for the most part; as the placement of Neomphalina among Vetigastropoda is explored in this study, however, it is identified as an independent clade and not part of Vetigastropoda *s.s.* Therefore, Vetigastropoda is represented by specimens from the families Fissurellidae, Haliotidae, Pyropeltidae, Lepetodrilidae, Pleurotomariidae, Trochidae, and Turbinidae. Appendix SA in the supporting information presents locality information, collection details, and museum voucher numbers for the specimens used in this analysis.

Nucleic acid extraction and PCR

Two different protocols were used to isolate genomic DNA and total RNA. Genomic DNA was extracted from specimens preserved in 96% ethanol (EtOH), RNA*later*® (Ambion, Austin, TX), or frozen at −80°C, using the DNeasy™ tissue kit from Qiagen (Valencia, CA, USA). Total RNA was isolated from tissues preserved in RNA*later*® (Ambion) or frozen at −80°C, using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA synthesis was performed with 1–2 μg of total RNA using the RETROscript™ kit (Ambion).

Data collection

Five loci were PCR amplified from genomic DNA: 18S rRNA, 28S rRNA, histone H3, 16S rRNA, and COI. See supporting information (Table S1) for the nucleotide sequences and original references of all primers used for amplification and sequencing in this analysis. Alternative primers and techniques were used when the initial amplification attempts were unsuccessful. The details of these methods are also explained in the caption for supporting information Table S1.

Standard PCR reactions occurred in a final volume of 50 μL (2 μL genomic DNA template, 41 μL ddH_2O, 5.0 μL AmpliTaq™ 10 × PCR buffer, 1 μL dNTPs [10 μmol L^{-1}], 0.5 μL of each primer [100 μmol L^{-1}], and 1.25 U AmpliTaq™ enzyme, Applied Biosystems, Carlsbad, CA, USA). PCR amplification was optimized for the ribosomal 18S, 28S, and 16S rRNA genes by increasing the $MgCl_2$ levels and using DMSO to restrict secondary structures in the template DNA. These less stringent reactions were carried out in a final volume of 25 μL (1 μL genomic DNA template, 15.15 μL ddH_2O, 2.5 μL AmpliTaq™ 10 × PCR buffer, 2.5 μmol L^{-1} of $MgCl_2$, 0.25 μL dNTPs [100 μmol L^{-1} each], 1.2 μL of each primer [10 μmol L^{-1}], and 0.625 U AmpliTaq™ enzyme). After an initial denaturation step at 95°C for 5 min, reactions were cycled 35 × with the following parameters: 30 s of denaturation at 95°C, 30 s of annealing at gene-dependent temperatures, and 60 s of extension at 72°C, with a final 6-min extension at 72°C. Annealing temperatures for 18S rRNA, 16S rRNA, and 28S rRNA ranged between 46°C and 48°C for standard reactions and 54°C for the DMSO reactions. Annealing temperatures for COI and H3 ranged between 42°C and 44°C.

Table 1. List of family, terminal, and genes sequenced with GenBank accession numbers and specimen voucher numbers.

Family	Taxa	DNA#	18S	28S	16S	H3	COI	EF-1α	MYO
Lottiidae	*Tectura testudinalis*	MCZ DNA101952	FJ977630	FJ977660	FJ977694	FJ977723	FJ977748	FJ977770	FJ977803
Lottiidae	*Tectura fenestrata*	MCZ DNA102022	FJ977631	FJ977661	FJ977695	FJ977724	FJ977749	FJ977771	FJ977804
Lottiidae	*Lottia gigantea*[a]	MCZ DNA101968 and (MCZ DNA102019)	FJ977632	FJ977662	FJ977696	FJ977725	FJ977750		(FJ977805)
Lottiidae	*Lottia jamaicensis*	MCZ DNA102130	FJ977633	FJ977663	FJ977697	FJ977726	FJ977751	FJ977772	FJ977806
Lottiidae	*Lottia asmi*	MCZ DNA102020	FJ977634	FJ977664	FJ977698	FJ977727		FJ977773	FJ977807
Neolepetopsidae	*Paralepetopsis* sp.	MCZ DNA102471	FJ977635	FJ977665	FJ977699	FJ977728	FJ977752	FJ977774	FJ977808
Lepetelloidea	*Pyropelta* sp.	MCZ DNA102472	FJ977636	FJ977666	FJ977700	FJ977729	FJ977753	FJ977775	FJ977809
Pleurotomariidae	*Entemnotrochus adansonianus*[a]	MCZ DNA100665 and (MCZ DNA102481)	AF120509	FJ977667	AY377621	AY377774	L78910[b]	(FJ977776)	(FJ977810)
Pleurotomariidae	*Bayerotrochus midas*	MCZ DNA100666 and (MCZ DNA102482)	(FJ977637)	FJ977668	DQ093474	DQ093500	AY296820[b]		(FJ977811)
Fissurellidae	*Diodora dysoni*	MCZ DNA102140	FJ977638	FJ977669	FJ977701	FJ977730	FJ977754	FJ977777	FJ977812
Fissurellidae	*Fissurella barbadensis*	MCZ DNA102128	FJ977639	FJ977670	FJ977702	FJ977731	FJ977755	FJ977778	FJ977813
Fissurellidae	*Fissurella nodosa*	MCZ DNA102153	FJ977640	FJ977671	FJ977703	FJ977732	FJ977756	FJ977779	
Fissurellidae	*Puncturella* sp.	MCZ DNA102473	FJ977641	FJ977672	FJ977704	FJ977733	FJ977757	FJ977780	FJ977814
Fissurellidae	*Lucapina suffusa*	MCZ DNA102017	FJ977642	FJ977673	FJ977705	FJ977734	FJ977758		FJ977815
Fissurellidae	*Hemitoma octoradiata*	MCZ DNA102469	FJ977643	FJ977674	FJ977706	FJ977735	FJ977759	FJ977781	
Haliotidae	*Haliotis corrugata*	MCZ DNA102585	FJ977644	FJ977675	FJ977707	FJ977736	FJ977760	FJ977782	FJ977816
Trochidae	*Gibbula cineraria*[a]	MCZ DNA102440 and (MCZ DNA103760)	FJ977645	FJ977676	FJ977708	FJ977737	AM049339[b]	(FJ977783)	(FJ977817)
Trochidae	*Cittarium pica*	MCZ DNA102127	FJ977646	FJ977677	FJ977709	FJ977738	FJ977761	FJ977784	FJ977818
Trochidae	*Pseudostomatella erythrocoma*	MCZ DNA102148	FJ977647	FJ977678	FJ977710	FJ977739		FJ977785	FJ977819
Turbinidae	*Cantrainea macleani*	MCZ DNA102474	FJ977648	FJ977679	FJ977711	FJ977740			FJ977820
Turbinidae	*Astralium phoebium*	MCZ DNA102144	FJ977649	FJ977680	FJ977712	FJ977741	FJ977762	FJ977786	FJ977821
Turbinidae	*Turbo castanea*	MCZ DNA102131	FJ977650	FJ977681	FJ977713	FJ977742	FJ977763	FJ977787	
Liotiidae	*Marevalvata* sp.	MCZ DNA102467	FJ977651	FJ977682	FJ977714	FJ977743			FJ977822
Lepetodrilidae	*Lepetodrilus pustulosus*	MCZ DNA101606	FJ977652	FJ977683	FJ977715	FJ977744		FJ977788	FJ977823
Peltospiridae	*Peltospira delicata*	MCZ DNA101609	FJ977653	FJ977684	FJ977716	FJ977745	FJ977764	FJ977789	FJ977824
Neomphalidae	*Cyathermia naticoides*[a]	MCZ DNA100855 and (MCZ DNA101607)	DQ093430	FJ977685	DQ093472	DQ093498	DQ093518	(FJ977790)	(FJ977825)
Neritidae	*Nerita tessellata*	MCZ DNA102135	FJ977654	FJ977686	FJ977717		FJ977765	FJ977791	FJ977826
Neritidae	*Neritina viriginea*	MCZ DNA102465	FJ977655	FJ977687	FJ977718		FJ977766	FJ977792	
Neritidae	*Puperita pupa*	MCZ DNA102136	FJ977656	FJ977688	FJ977719		FJ977767	FJ977793	FJ977827
Neritidae	*Smaragdia viridis*	MCZ DNA102162	FJ977657	FJ977689	FJ977720	FJ977746		FJ977794	
Neritidae	*Bathynerita naticoidea*	MCZ DNA102209	FJ977658	FJ977690	FJ977721	FJ977747	FJ977768	FJ977795	FJ977828
Outgroups									
Turritellidae	*Turritella communis*	MCZ DNA103761	FJ977659	FJ977691	FJ977722				FJ977829
Littorinidae	*Littorina littorea*	MCZ DNA101389 and (MCZ DNA101221)	DQ093437	FJ977692	DQ093481	DQ093507	DQ093525	(FJ977796)	(FJ977830)
Calyptraeidae	*Crepidula fornicata*	MCZ DNA102572	AY377660		AY377625	AY377778	AF353154[b]	FJ977797	FJ977831
Ampullariidae	*Pomacea bridgesi*[a]	MCZ DNA101450 and (MCZ DNA102528)	DQ093436	FJ977693	DQ093480	DQ093506	DQ093524	(FJ977798)	
Scaphopoda	*Antalis entalis*	AToL000061	DQ279936	AY145388[b]	DQ280027	DQ280000	DQ280016	FJ977799	FJ977832
Polyplacophora	*Leptochiton asellus*[a]	AToL000071/ (AToL000316)	AY377631	AY145414[b]	AY377586	AY377734		(FJ977800)	(FJ977833)
Bivalvia	*Neotrigonia margaritacea*	AToL000073	AF411690	DQ279963	DQ280034	AY070155	FJ977769	FJ977801	FJ977834
Bivalvia	*Mya arenaria*	AToL000002	AF120560	AB126332[b]	AY377618	AY377770	AY070140	FJ977802	FJ977835

[a]Multiple specimens were used for the terminal taxon, and sequences and voucher numbers in parentheses are the alternative specimens.
[b]Obtained from GenBank.

Fragments from the head portion of myosin heavy-chain type II (myosin hereinafter) and EF-1α were amplified from cDNA using nested PCR reactions. For the initial reaction, myosin was amplified using the external primers Ext F and Ext R, or MIO 1 and MIO 4, in a 25 μL reaction (1 μL genomic cDNA template, 20.5 μL ddH_2O, 2.5 μL AmpliTaq™ 10 × PCR buffer, 0.5 μL dNTPs [10 μmol L^{-1}], 0.25 μL of each primer [100 μmol L^{-1}], and 0.625 U AmpliTaq™ enzyme). After an initial denaturation at 94°C for 1 min, reactions were cycled 35 × with the following parameters: 35 s of denaturation at 94°C, 45 s of annealing at 48°C, and a 2-min extension at 72°C, with a final 7-min extension at 72°C. In the second 50 μL PCR reaction (see the description of standard PCR reactions for volumes), this PCR product was amplified using the internal primers Mio 7F and Mio 6R and the same thermocycler parameters to obtain the final myosin fragment. These internal primers were also used to generate the 624-bp myosin sequence. EF-1α was amplified in a 25 μL reaction using the same volumes as in the first myosin reaction and the external primers RS1F and RS7R. After an initial 2-min denaturation at 94°C, 25 touchdown cycles were carried out at the following parameters: 30 s of denaturation at 94°C, 30 s of annealing at 55°–45°C, and a 1-min extension at 72°C. Immediately following the touchdown cycles, 14 cycles were carried out using the following parameters: 30 s of denaturation at 94°C, 30 s of annealing at 45°C, and a 1-min extension at 72°C, with a final 7-min extension at 72°C. This initial PCR product was amplified again in a 50 μL PCR reaction (see the description of standard PCR reactions for volumes) using the internal primers RS2F and RS4R with the following thermocycler parameters: initial 2-min denaturation at 94°C, then 25 cycles of 35 s of denaturation at 94°C, 45 s of annealing at 45°C, and a 2-min extension at 72°C, with a final 7-min extension at 72°C. The EF-1α fragment was sequenced using the internal primers and ranged in size between 540 and 588 bp.

All amplified samples were purified using an Eppendorf vacuum (Hamburg, Germany) and Millipore Multiscreen® $PCR_{\mu 96}$ cleanup filter plates (Billerica, MA, USA) following the manufacturers' instructions. Sequencing was performed in a GeneAmp® PCR system 9700 (Perkin Elmer, Waltham, MA, USA) using ABI PRISM™ BigDye™ v. 3 Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) and following the manufacturer's protocol. The BigDye-labeled PCR products were cleaned with Performa® DTR v3 96-well short plates (Edge BioSystems, Gaithersburg, MD, USA) and directly sequenced using an automated ABI Prism® 3730 Genetic Analyzer.

Chromatograms obtained from the automatic sequencer were viewed and contigs were assembled using the sequence editing software Sequencer™4.8 (Gene Codes Corporation, Ann Arbor, MI, USA). The concatenated sequences were then edited in MacGDE: Genetic Data Environment for MacOSX (Central Michigan University, Mt. Pleasant, MI, USA) (Linton 2005). External primer regions were removed from these edited sequences, protein coding genes were checked to ensure the absence of stop codons and indels, and long sequences were separated according to the internal primer regions and secondary structure features following Giribet (2001).

Phylogenetic reconstruction: direct optimization

Sequence data were analyzed in a single-step phylogenetic approach using parsimony under the direct optimization method (Wheeler 1996) implemented in the computer program POY v. 4.0.2885 and v. 4.0.2911 (American Museum of Natural History, New York, NY, USA) (Varón et al. 2010). To increase analysis efficiency, some gene sequences were divided into fragments as follows: 18S rRNA (23 fragments), 28S rRNA (eight fragments), 16S rRNA (seven fragments), COI (five fragments), and myosin (four fragments). EF-1α and histone H3 were not partitioned and, due to the lack of sequence length variability, histone H3 sequences were used in the phylogenetic analysis as pre-aligned data. Length variations in the myosin and EF-1α sequences were due to amino acid insertion or deletion events among different gastropod clades. In contrast, incomplete sequencing of the COI for some specimens resulted in length variation for the gene as all fully sequenced genes were of equal length. Nine data sets were analyzed independently, including each of the seven individual molecular loci, all of the sequence data combined (totaling seven genes), and all sequence data excluding EF-1α and myosin (totaling five genes). Although four loci are protein-encoding (myosin, EF-1α, COI, and H3), all sequence data were examined on a DNA level. The POY analyses were run in a Linux cluster using 14–20 processors at Harvard University (http://www.portal.cgr.harvard.edu). Processes were executed in parallel and the preliminary tree space was searched (search [hits: 200, max_time: 0:04:00]) with random addition replicates. Subtree pruning and regrafting, (SPR) and tree bisection and reconnection (TBR) branch swapping, followed by tree fusing (Goloboff 1999, 2002), were used in all replicates.

A parameter space of two variables was explored (Wheeler 1995; Giribet 2003) for each of the parti-

tions. Specifically, these variables were an indel/change ratio (change refers to the highest value for a base transformation) and a transversion/transition ratio. A total of ten parameter sets were analyzed per partition: gap/change ration values of 1, 2, 3, and 4 as well as transversion/transition ratios of 1 (transversions and transitions of equal weight), 2 (transversions twice the weight of transitions), and 4 (transversions 4 × the weight of transitions). To summarize, the ten parameter sets used in this analysis were 111, 121, 141, 211, 221, 241, 411, 421, 441, and 3221, with 111 signifying a weighting scheme where all changes were equal, 121 signifying one where the indel/change ration was 1:1 and the transversion/transition ratio was 2:1, 221 signifying one where the indel/change ration was 2:1 and the transversion/transition ratio was 2:1, etc. Under the 3221 parameter, gap extensions were down-weighed in comparison with the gap opening costs (gap opening 3 × the weight of extensions), with transversion and transitions assigned an equal cost of two (Varón & Wheeler 2008). In a direct optimization framework, this transformation is considered by some to be comparable to unweighted parsimony (De Laet 2005).

The parameter set that maximized the overall congruence among molecular partitions as indicated by a modified version of the incongruence length difference (ILD) metric (Mickevich & Farris 1981; Farris et al. 1995) was used as the "optimal" set (Wheeler 1995). The ILD value was determined for the five-gene and seven-gene combined analyses by subtracting the sum of the length of each individual tree for the genes used in that analysis from the length of the combined tree and dividing that result by the length of the combined tree (see Table 2). The parameter with the lowest ILD score is the one minimizing incongruence among all the partitions.

Following this preliminary search and the identification of the most congruent parameters, the shortest trees from all the initial searches were pooled and used in a sensitivity analysis tree-fusing (SATF) search in order to search tree space more thoroughly. In this method, trees retained from earlier analyses are used as the starting trees for a new round of TBR branch swapping, and tree fusing to further improve tree length (Giribet 2007). SATF has been used successfully to obtain shorter trees in other analyses (D'Haese 2003; Boyer et al. 2005). The shortest trees from all parameter sets were used as the starting trees in each consecutive round of SATF. New SATF searches were repeated for each single gene partition under the most congruent parameter set and for combined partitions (seven genes and five genes) using all the parameters until the reported tree length stabilized and did not improve in subsequent analyses. Tree lengths for all analyses are summarized in Table 2. Dynamic bootstrap support values under the optimal parameters for both the five- and the seven-gene data set were calculated using the partitioned data and an automated procedure for partitioning the data for 100 replicates (commands: transform (auto_sequence_partition); calculate_support (bootstrap:100)) (see Giribet et al. 2009). Nodal stability (Giribet 2003) under the ten different parameter sets was also explored (Figs. 1A, 2A).

Phylogenetic reconstruction: a two-step approach

In addition to the parsimony direct optimization analyses, we explored a two-step approach using both maximum likelihood and parsimony optimality criteria. In this approach, each gene was first independently aligned with MUSCLE 3.7 using the default setting in the EMBL-EBI online interface (http://www.ebi.ac.uk/Tools/muscle/index.html). This program uses sum-of-pairs gap costs with affine gap penalties and multiple iterative refinements of each subtree to generate the final alignment (Edgar 2004). Additionally, variable regions of this aligned data set were removed using the program Gblocks (Castresana 2000) at Phylogeny.fr (Dereeper et al. 2008). The reduced data set (53% of the original data) was then analyzed under both parsimony and maximum likelihood using TNT and RAxML. The MUSCLE aligned gene files as well as implied alignments from the optimal tree and the resulting tree files are available at Treebase (study accession number S2598, matrix accession numbers M4962, M4963, and M4964).

For the parsimony analyses, the complete and partial MUSCLE alignments were analyzed in the program TNT (Goloboff et al. 2005). Data were analyzed using 100 random addition replicates, followed by sectorial searches, parsimony ratchet, tree drifting, and tree fusing. All nucleotide changes were equally weighted, corresponding to the preferred parameter set in POY, 111. Nodal support was calculated using 100 bootstrap replicates.

For the maximum likelihood analyses, both MUSCLE alignments were first concatenated using Phyutility (Smith & Dunn 2008) and then analyzed in combination using the phylogenetic analysis program RAxML version 7.0.4 (Stamatakis et al. 2005) on the web-server CIPRES portal (http://www.phylo.org/portal2). This program utilizes GTR, the "best-fit" model for all genes, and the combined data set as selected by the Akaike information criterion in Modeltest 3.7 (Posada 2005). In the RAxML

Table 2. Tree lengths for the individual and combined data sets at different parameter values, with incongruence length difference (ILD) values. Individual data sets: 18S, 18S rRNA; 28S, 28S rRNA; 16S, 16S rRNA; H3, histone H3; COI, cytochrome *c* oxidase subunit I; MYO, myosin heavy-chain type II; EF-1α, elongation factor-1α. Combined data set five-gene: 5-gene = (18S+28S+16S+H3+COI). Combined data seven-gene: ALL = (18S+28S+16S+H3+COI+MYO+EF-1α). Bold ILD and rows reflect the parameter set that minimizes incongruence among data sets.

	18S	28S	16S	H3	COI	MYO	EF-1α	5-gene	ILD 5-gene	ALL	ILD ALL
111	**3550**	**6772**	**3433**	**733**	**2986**	**2274**	**2329**	17,981	0.0282	**23,137**	**0.0458**
121	5716	11,302	5367	1035	4482	3405	3549	28,760	0.0298	36,626	0.0483
141	9813	19,908	9015	1619	7323	5549	5875	49,296	0.0328	62,332	0.0518
211	4576	9249	4168	734	3046	2383	2428	22,622	0.0375	28,049	0.0522
221	7652	15,862	6700	1035	4568	3583	3720	37,399	0.0423	45,729	0.0571
241	13,586	28,682	11,575	1619	7500	5906	6240	66,059	0.0469	80,108	0.0624
411	6311	13,158	5279	734	3085	2507	2567	30,212	0.0544	36,134	0.0690
421	11,024	23,580	8817	1035	4626	3799	3995	52,251	0.0606	61,417	0.0739
441	20,185	43,892	15,763	1619	7621	6328	6789	95,448	0.0667	111,307	0.0818
3221	**7057**	**13,103**	**6978**	**1468**	**6009**	4612	4716	**35,589**	**0.0274**	46,077	0.0463

analysis, the data were partitioned according to the seven genes used to incorporate rate heterogeneity among the multiple loci and a gamma distribution (*G*) was used to estimate the rate of variation among sites. The proportion of invariable sites (*I*), however, was not estimated in the analysis as there is a high correlation between the two parameters (*G* and *I*) that can negatively affect the accuracy of the likelihood estimation (Sullivan et al. 1999). Nodal support was measured in RAxML using 1000 bootstrap replicates (Stamatakis et al. 2008).

Results

Table 2 shows the tree lengths for the individual and combined data sets at different parameter values, with ILD values. The congruence among molecular partitions in the five-gene analyses (excluding only myosin and EF1α) was maximized under the parameter set 3221, with an ILD score of 0.0274 (Table 2). The congruence among molecular partitions in the seven-gene (18S, 28S, H3, 16S, COI, EF-1α, and myosin) analyses was maximized under parameter set 111, where the ratio between all data changes (indels/transformations and transversions/transitions) was equally weighted. The ILD score for the seven-gene data set was 0.0458 (Table 2).

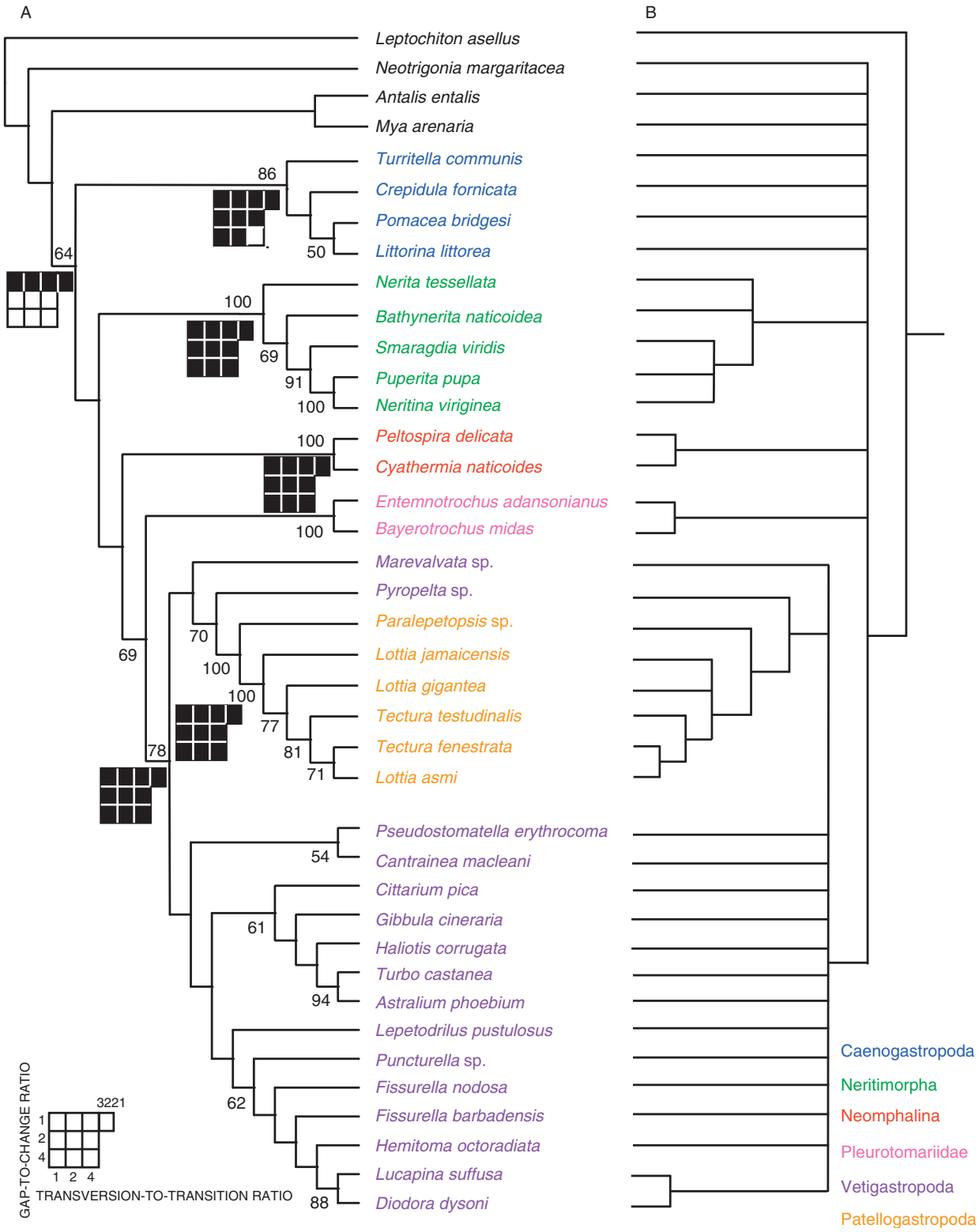
Five-gene data set: direct optimization parsimony

Combined analysis of the five "standard" genes (18S, 28S, H3, 16S, and COI) under the optimal parameter set (3221) yielded a single shortest tree of 35,589 steps. When rooted with Polyplacophora, the monophyly of the Gastropoda was recovered, and the clade received 64% bootstrap support (Fig. 1A). Gastropoda was, however, not recovered as monophyletic under all the explored parameters sets and, therefore, collapsed into the strict consensus tree of all the parameter sets explored (Fig. 1B). The gastropod clades Caenogastropoda, Neritimorpha, Patellogastropoda, and Neomphalina were monophyletic in this analysis, with bootstrap support values of 86% for Caenogastropoda and 100% for all remaining clades. Although caenogastropods were not monophyletic under all weighting schemes, the neritimorph, patellogastropod, and neomphaline clades remained stable to parameter set variation and were recovered in all weighting schemes.

Under the optimal parameter set, Caenogastropoda was sister to all the remaining gastropods—the clade Archaeogastropoda—albeit with low nodal support and stability. Neritimopha formed a clade with Caenogastropoda under the majority of the remaining parameter sets (excluding 411, 441). The

Fig. 1. Cladogram based on the parsimony analyses of the five-gene combined molecular data using POY. **A.** Cladogram is the single shortest tree (35,589 weighted steps) under the optimal parameter set (3221). See text for further details and Table 1 for family designations. Numbers on branches indicate bootstrap support values >50%. Graphic plots of sensitivity analyses (Navajo Rugs) indicate monophyly of nodes under the different parameter sets. Black squares indicate monophyly for a given parameter set, while white squares indicate non-monophyly. Colored terminal taxa indicate clade designations: blue for Caenogastropoda, green for Neritimorpha, red for Neomphalina, pink for Pleurotomariidae, purple for Vetigastropoda, and orange for Patellogastropoda. **B.** Cladogram is a strict consensus of all trees obtained under all the ten parameters explored for the five-gene molecular analysis.

placement of Neomphalina sister to a clade of vetigastropods and patellogastropods was not supported in bootstrap analyses and is unstable to parameter set variation. Alternative placements of the Neomphalina were sister to Pleurotomariidae or sister to the Caenogastropoda+Neritimorpha clade. In the optimal parameter set, Pleurotomariidae was recovered as sister to a clade where Patellogastro-



poda was placed within all the remaining vetigastropods, and this sister group of pleurotomariids to Vetigastropoda/Patellogastropoda was supported in 69% of bootstrap replicates. This placement of Pleurotomariidae occurred only under parameter sets 111, 121, and 3221; under all other parameter sets, Pleurotomariidae was sister to Neomphalina. The placement of Patellogastropoda within a clade of all non-pleurotomariid vetigastropods was supported with 78% bootstrap support (Fig. 1A) and recovered under every weighting scheme (Fig. 1B).

The internal relationships of neritimorph and patellogastropod taxa were more stable to parameter set variation than those of many vetigastropod taxa. Within Neritimorpha, the clade comprised of *Puperita pupa* (Linné, 1767), *Smaragdia viridis* (Linné, 1758), and *Neritina viriginea* (Linné, 1758) was recovered in all parameter sets and received high bootstrap support (91%). *Nerita tessellata* Gmelin, 1791 and *Bathynerita naticoidea* Clarke, 1989 alternated placement outside the (*P. pupa*+*S. viridis*+*N. viriginea*) clade as the parameter sets varied. Patellogastropod families Lottiidae Gray 1840 and Neolepetopsidae were represented in this analysis and in all parameter sets the single neolepetopsid *Paralepetopsis* sp. was sister to Lottiidae (100% bootstrap support). Although the genus *Lottia* was not monophyletic, *Tectura testudinalis* (Müller, 1776), *Tectura fenestrata,* (Reeve, 1855), and *Lottia asmi* (Middendorff, 1849) formed a clade under the optimal parameter set and received 81% bootstrap support. The sister relationship between *T. fenestrata* and *L. asmi* was recovered under all parameter sets and received 71% bootstrap support. The vetigastropod *Pyropelta* sp. fell sister to the patellogastropods (70% bootstrap support) in all parameter sets. Another vetigastropod, the fissurellid *Hemitoma octoradiata* (Gmelin, 1791), fell sister to the *Pyropelta*+Patellogastropoda clade in all parameter sets except for the most congruent, 3221, but this topology did not receive significant bootstrap support. Under the most congruent parameter set, *Marevalvata* sp. fell outside the *Pyropelta*+Patellogastropoda clade, again without bootstrap support.

Many vetigastropod families were not monophyletic and were also unstable to parameter set variation in this analysis. Pleurotomariidae was the only vetigastropod family stable to parameter set variation and recovered with high bootstrap support in the optimal parameter set. Fissurellidae was only recovered as a monophyletic taxon under the optimal parameter scheme (62% bootstrap support) due to the placement of *H. octoradiata* as a sister to Patellogastropoda+*Pyropelta* in all parameter sets except 3221. Within Fissurellidae, *Lucapina suffusa* Reeve, 1850+*Diodora dysoni* (Reeve, 1850) was the only clade recovered under all parameter sets and that received significant bootstrap support (88%). The internal relationships among the other fissurellids were not stable to the variation of all ten parameter sets. Representatives of the vetigastropod groups Haliotidae, Lepetodrilidae, and Trochoidea formed a clade in all parameter sets, except for 441 and 3221 (Fig. 1). The internal relationships among these taxa, however, varied under different parameter sets. Trochoidea, Trochidae, and Turbinidae were never monophyletic, but *Astralium phoebium* (Röding, 1798)+*Turbo castanea* (Gmelin, 1791) formed a clade under the optimal parameter set (94% bootstrap support). The placement of *Haliotis corrugata* Wood, 1828 and *Lepetodrilus pustulosus* McLean, 1988 changed as the parameter sets varied, with *H. corrugata* falling sister to *Gibbula cineraria* (Linnaeus, 1758) under the 111, 121, and 211 parameter sets and *L. pustulosus* falling within Turbinidae, sister to *Pseudostomatella erythrocoma* (Dall, 1889), or sister to Fissurellidae under different parameter sets.

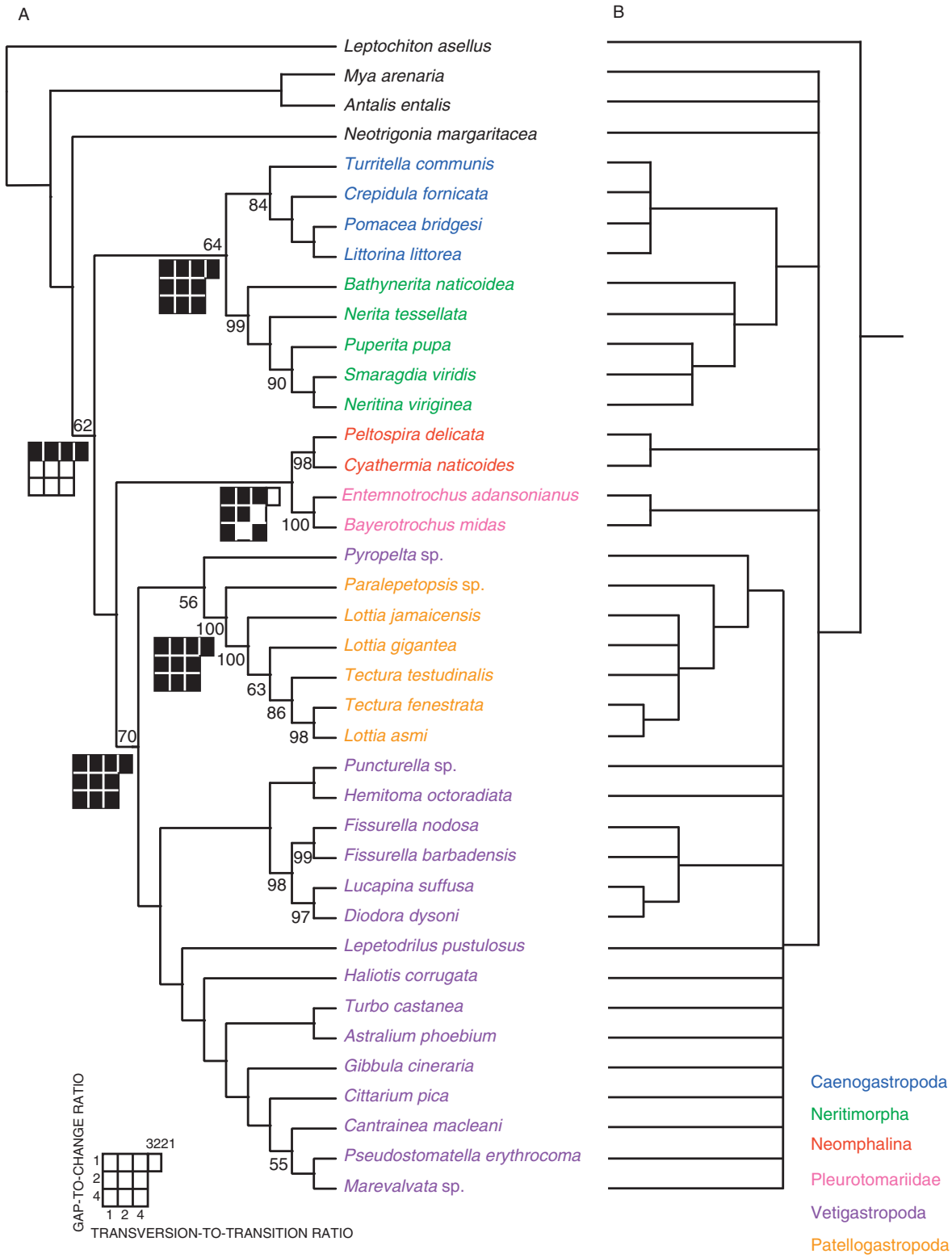
Seven-gene data set: direct optimization parsimony

The optimal parameter set used in the seven-gene analysis (18S, 28S, H3, 16S, COI, EF1α, and myosin) was 111 and yielded a single shortest tree of 23,137 steps. When rooted with *Leptochiton asellus* (Gmelin, 1791), Gastropoda was monophyletic under the 111, 121, 141, and 3221 weighting schemes, and received a bootstrap support of 62% under the optimal parameter set (Fig. 2). All major gastropod clades, except for Vetigastropoda, were monophyletic with high bootstrap support and were stable to parameter set variation. Under the optimal parameter set,

Fig. 2. Cladogram based on the parsimony analyses of the seven-gene combined molecular data using POY. **A.** Cladogram is the single shortest tree (23,137 weighted steps) under the optimal parameter set (111). See text for further details and Table 1 for family designations. Numbers on branches indicate bootstrap support values >50%. Graphic plots of sensitivity analyses (Navajo Rugs) indicate monophyly of nodes under the different parameter sets. Black squares indicate monophyly for a given parameter set, while white squares indicate non-monophyly. Colors correspond to those assigned to the clades in Fig. 1. **B.** Cladogram is a strict consensus of all trees obtained under all the ten parameters explored for the seven-gene molecular analysis.

Caenogastropoda received 84% bootstrap support, Neritimorpha 99%, Neomphalina 98%, and Patellogastropoda 100%.

Caenogastropoda was sister to Neritimorpha with 64% bootstrap support under the optimal parameter set and this relationship was stable to parameter



variation, contradicting the monophyly of Archaeogastropoda found under some parameter sets in the five-gene analysis. The sister relationship between the clades Pleurotomariidae and Neomphalina did not receive bootstrap support above 50%, but was recovered in seven of the ten parameter sets. A clade composed of Patellogastropoda and all vetigastropods, except for Pleurotomariidae, was recovered under all parameter sets and received 70% bootstrap support in the optimal parameter set.

Within Neritimorpha, (*P. pupa* (*S. viridis*+*N. viriginea*)) received 90% bootstrap support under the 111 parameter set and was monophyletic under all parameter sets except 3221. The specific placement of *Bathynerita naticoidea* and *N. tessellata* outside this clade was variable depending on the weighting schemes. Among the patellogastropods, the sister relationship between *T. fenestrata* and *L. asmi* (98% bootstrap) was stable to parameter set variation. The placement of *T. testudinalis, Lottia gigantea* Sowerby, 1834 and *L. jamaicensis* (Gmelin 1791) in relation to *T. fenestrata*+*L. asmi* varied as the parameters changed. *Paralepetopsis* sp. fell sister to all the remaining patellogastropods (100% bootstrap) under all parameter sets. The placement of the vetigastropod *Pyropelta* sp. as a sister to Patellogastropoda (bootstrap support of 56%) was consistent under all parameter sets. A second vetigastropod, *H. octoradiata,* fell sister to the *Pyropelta* sp.+Patellogastropoda clade in all weighting schemes, except for 3221 and the optimal parameter set, 111.

The placement of vetigastropod species among the other ingroup taxa was unstable to parameter set variation. Pleurotomariidae was monophyletic under all parameter sets but was never recovered in a clade with the other vetigastropod taxa. Fissurellidae was only monophyletic under 3221 and the optimal parameter set, 111, but did not receive significant bootstrap support. Under the eight other parameter sets, *H. octoradiata* was not recovered with the other fissurellids. The placement of *Puncturella* sp. also varied across parameter sets; sometimes, it was sister to the other four fissurellid taxa, but in the 141, 211, and 221 weighting schemes, it was placed within non-fissurellid vetigastropod taxa. The clade ([*Fissurella nodosa* (Born 1778)+*Fissurella barbadensis* (Gmelin, 1791)]+[*L. suffusa*+*D. dysoni*]) was stable to parameter set variation and received 98% bootstrap support in the optimal parameter set. Furthermore, the internal *Fissurella* and (*L. suffusa*+*D. dysoni*) clades received 99% and 97% bootstrap support, respectively. Although the *Fissurella* clade was not stable to parameter variation, (*L. suffusa*+*D. dysoni*) was recovered in all parameter sets. The remaining vetigastropods, representing Haliotidae, Lepetodrilidae, Trochidae, and Turbinidae, form a monophyletic group in seven parameter sets (excluding 411, 421, and 441) but do not receive significant bootstrap support under the optimal parameter set. Furthermore, the internal relationships among these taxa vary depending on the parameter set used. The only internal clade recovered in $>50\%$ of bootstrap replicates under the optimal parameter set was (*Cantrainea macleani* Warén and Bouchet, 1993 (*P. erythrocoma*+*Marevalvata* sp.)).

Seven-gene data set: TNT

The single shortest tree found in the TNT parsimony analysis had 28,721 unweighted steps. When rooted with *L. asellus,* Gastropoda was monophyletic with a bootstrap support of 96% (Fig. 3, supporting information Fig. S1). Patellogastropoda was the only monophyletic major gastropod clade (100% bootstrap support); all the other major groups were not monophyletic. Additionally, few deep nodes received bootstrap support $>50\%$. Support was recovered for some higher groups; Pleurotomariidae, (*C. macleani*+*P. erythrocoma*) and ((*Fissurella nodosa*+*F. barbadensis*)+(*L. suffusa*+*D. dysoni*)) were monophyletic in all bootstrap replicates. As in some POY analyses, *Pyropelta* sp. and *H. octoradiata* fell sister to Patellogastropoda. The vetigastropods *L. pustulosus* and *Marevalvata* sp. were also not found within Vetigastropoda; they were instead recovered within a clade of caenogastropods and *Peltospira delicata* McLean, 1989. All the remaining non-pleurotomariid vetigastropods formed a clade without significant bootstrap support. Within this group, the fissurellids excluding *H. octoradiata* (58% bootstrap support) were sister to a clade representing Haliotidae, Lepetodrilidae, Trochidae, and Turbinidae (98% bootstrap support).

In contrast, the optimal tree generated in TNT using data from the Gblocks reduced data set (53% of the original data) was 13,098 steps (Fig. S3). Gastropoda was not monophyletic but all major gastropod clades, except Vetigastropoda, received bootstrap support varying from 94% (Caenogastropoda) to 100% (Patellogastropoda, Neritimorpha, Neomphalina, Pleurotomariidae). Deep nodes connecting these clades, however, did not receive bootstrap support $>50\%$, and many of the internal relationships among vetigastropods also lacked significant bootstrap support.

Seven-gene data set: maximum likelihood

A maximum likelihood tree based on the data from all seven genes had a $-\log L = 95{,}447.379113$

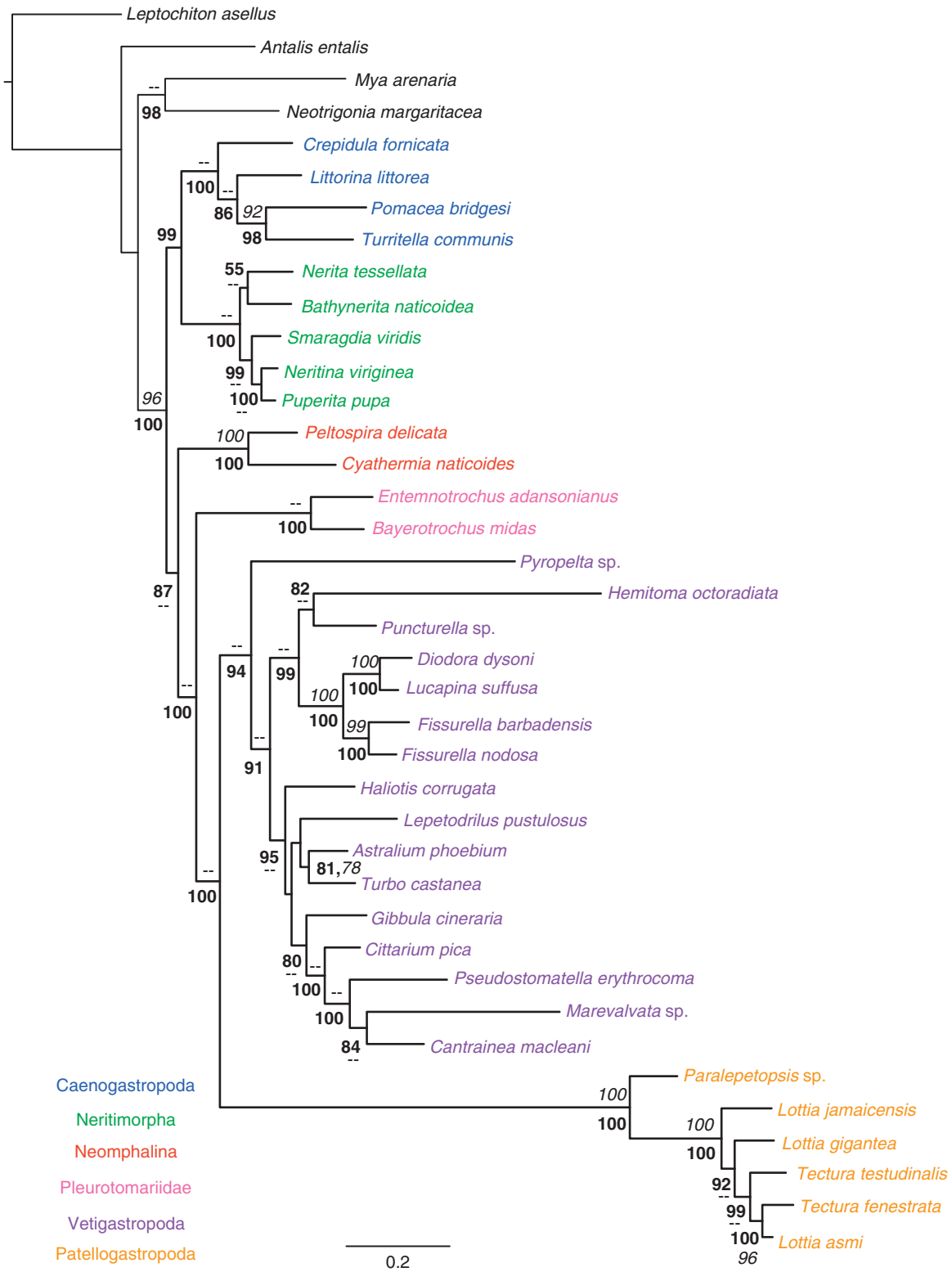


Fig. 3. Maximum likelihood tree based on seven-gene combined molecular data ($-\log L = 95{,}447.379113$). Bold numbers on branches indicate bootstrap support values >50% recovered in the RaxML analysis. Italicized numbers indicate support for nodes recovered in the complete TNT parsimony analysis and dashes indicate that the node was not recovered in the TNT analysis (Fig. S1). Bold branches indicate gastropod taxa and colors correspond to those assigned to the clades in Fig. 1. See Table 1 for the familial classification for each species.

(Fig. 3) and a topology very similar to that recovered under the seven-gene direct optimization parsimony analysis (Fig. 2). Furthermore, bootstrap support for recovered clades was, in general, higher than that found in the seven-gene parsimony analysis, as expected for a static alignment. Gastropoda was monophyletic with 100% bootstrap support, and a Caenogastropoda+Neritimorpha clade (99% bootstrap support) was sister to the remaining gastropods. The internal relationships within Caenogastropoda and Neritimorpha were the same as those recovered in the seven-gene parsimony analysis, except that a *N. tessellata*+*B. naticoidea* clade was recovered within Neritimorpha. Neomphalina was sister to a clade of vetigastropods and patellogastropods in 87% of bootstrap replicates.

Among the vetigastropods, Pleurotomariidae fell sister to a clade of the remaining vetigastropods plus patellogastropods in all the bootstrap replicates. Patellogastropoda was monophyletic (100% bootstrap support), with the same internal relationships as in the seven-gene parsimony analysis. In contrast to the seven-gene analysis, *Pyropelta* sp. and *H. octoradiata* were not sister to the patellogastropods and, instead, were placed within the non-pleurotomariid vetigastropods (94% bootstrap support). *Pyropelta* sp. was sister to all non-pleurotomariid vetigastropods and Fissurellidae was monophyletic (99% bootstrap support), with an internal topology of (*H. octoradiata*+*Puncturella* sp.) sister to ((*D. dysoni*+*L. suffusa*)+(*F. barbadensis*+*F. nodosa*)). Fissurellidae was sister to a clade composed of taxa representing Haliotidae, Lepetodrilidae, Liotiidae, Trochidae, and Turbinidae (91% bootstrap support). The latter non-fissurellid vetigastropod clade was recovered in 95% of bootstrap replicates. Although Trochoidea, Trochidae, and Turbinidae were not monophyletic, the turbinid species *A. phoebium* and *T. castanea* were sister taxa with 81% bootstrap support. A clade comprised of *G. cineraria, Cittarium pica, P. erythrocoma, Marevalvata* sp., and *C. macleani* was recovered with a bootstrap support value of 80%.

The maximum likelihood tree based on the data from the Gblocks reduced data set (53% of the original data) had a $-\log L = 57{,}247.227828$ (Fig. S2) and a topology very similar to that recovered under the seven-gene likelihood analysis using the complete data set (Fig. 3). Bootstrap support was also similar, except for the support values for a few deep nodes. Specifically, the (Pleurotomariidae (Vetigastropoda+Patellogastropoda)) clade was supported in only 56% bootstrap replicates in the Gblocks data set compared with all bootstrap replicates in the complete analysis. Additionally, support for Patellogastropoda and for the non-pleurotomariid vetigastropods was higher for the complete analysis than it was in the Gblocks data set (100% vs. 86% bootstrap support).

Discussion

Gastropoda

A monophyletic Gastropoda—despite admittedly spare outgroup representation and low bootstrap support in the POY analyses—was recovered in all optimal phylogenetic analyses utilizing molecular characteristics, except one (Figs. 1–3 and Figs. S1, S2). While gastropods are recognized as a well-supported clade in cladistic analyses utilizing morphological characteristics individually or in conjunction with molecular data (Salvini-Plawen & Steiner 1996; Ponder & Lindberg 1997; Aktipis et al. 2008), previous molecular analyses failed to recover the monophyly of Gastropoda (Colgan et al. 2000, 2003; Giribet et al. 2006). Although bootstrap support for Gastropoda did not change significantly with the addition of the protein-coding genes, the supported clades recovered within Gastropoda in the seven-gene analyses correspond more closely to those found in other studies, reinforcing the argument that increasing the data sources in an analysis improves phylogenetic reconstruction (Giribet 2002a; Rokas & Carroll 2005; Lindgren & Daly 2007; Dunn et al. 2008). Despite the greater phylogenetic congruence between this and previous studies, some of the internal relationship results of this study contradict those identified in other gastropod phylogenies.

One such important difference is the lack of support for the Eogastropoda and Orthogastropoda classification of gastropods where Patellogastropoda is a separate clade sister to all the remaining gastropods. Formally proposed by Ponder & Lindberg (1997), this topology has been recovered in other morphological analyses (Golikov & Starobogatov 1975; Salvini-Plawen & Haszprunar 1987; Haszprunar 1988; Aktipis et al. 2008) and a few molecular analyses (Tillier et al. 1992, 1994; Harasewych & McArthur 2000; McArthur & Harasewych 2003). In other molecular analyses, the Eogastropoda/Orthogastropoda split was not recovered and the placement of Patellogastropoda varied (Harasewych et al. 1997; Rosenberg et al. 1997; Colgan et al. 2000, 2003; Giribet et al. 2006; Aktipis et al. 2008). Eogastropoda and Orthogastropoda were inconsistently recovered when both morphological and molecular characteristics were used in a phylogenetic analysis; the two clades were recovered in five of the nine different parameter sets explored (Aktipis et al. 2008). In this study, Patellogastropoda was only separate from all the remaining gastropods in the Gblocks

reduced data set representing only 53% of the original data, and this placement did not receive bootstrap support >50% (Fig. S3).

Another interesting result was shown under only one of the parameter sets of the five-gene analysis in this study: the 3221 optimal parameter set. Although Archaeogastropoda has long been recognized as a paraphyletic grade (Haszprunar 1993), it was monophyletic (although with low bootstrap support) in the optimal parameter set for the five-gene analysis. This is likely due to the movement of Neritimorpha, as neritimorphs were not sister to Caenogastropoda, although this was found only in three parameter sets in the whole study.

Although some results in the TNT analyses do not correspond to those found in the POY and maximum likelihood analyses, none of the deep gastropod nodes received bootstrap support >50% and therefore the results of the TNT analyses do not contradict the stable and supported results from the other analyses. In all analyses performed with the complete data set, Patellogastropoda was nested within or sister to the non-pleurotomariid vetigastropods, and this result received high support and was stable to parameter set variation. In addition, some direct optimization parsimony analyses and the maximum likelihood analyses place Pleurotomariidae as the sister group to the former clade with bootstrap support >50%, indicating that Pleurotomariidae may be a separate clade from Vetigastropoda. Neritimorpha+Caenogastropoda was also recovered as a separate clade under all parameter sets in the seven-gene POY analysis, the ML analyses, eight of the ten parameter sets explored in the five gene POY analysis, and the TNT parsimony analysis of the Gblocks reduced data set. These results lend support to the hypothesis that Neritimorpha is more closely related to Caenogastropoda (used here as a proxy for Apogastropoda) than to Vetigastropoda. Contradicting some current phylogenetic classifications, Neomphalina fell outside Vetigastropoda in all analyses, except for the TNT parsimony analysis of the Gblocks reduced data set. In this analysis, however, the placement of Neomphalina among Gastropoda did not receive bootstrap support >50%. Furthermore, gastropods are split between two major clades, Vetigastropoda+Patellogastropoda and Neritimorpha+Caenogastropoda, with the placement of Pleurotomariidae and Neomphalina varying according to the parameter set used.

Neritimorpha

Neritimorpha was monophyletic in all analyses, except the TNT parsimony analysis; this relationship has been consistently revealed in other phylogenetic analyses using both molecular and morphological characters (Salvini-Plawen & Haszprunar 1987; Haszprunar 1988; Ponder & Lindberg 1997; Harasewych et al. 1998; Sasaki 1998; McArthur & Harasewych 2003; Aktipis et al. 2008). While the relative placement of Neritimorpha to other gastropods has been inconsistent (see Lindberg 2008 for a historical overview), the results of molecular, morphological and combined analyses are beginning to converge upon the placement of Neritimorpha as sister to Apogastropoda, the clade composed of Caenogastropoda+Heterobranchia (Harasewych et al. 1998; McArthur & Harasewych 2003; Aktipis et al. 2008). Although heterobranchs are not included in this study, the monophyly of Apogastropoda has broad support in previous analyses (e.g., Harasewych et al. 1998; Aktipis et al. 2008) and, therefore, Caenogastropoda is used as a proxy for Apogastropoda. A large majority of analyses in this study reveal a Caenogastropoda+Neritimorpha clade. Yonge (1947) initially proposed a close relationship between Neritimorpha and Caenogastropoda based on pallial features and especial similarities in the ctenidial structure. More recent embryological studies focusing on the timing of the division of the 3D macromere and subsequent production of the mesentoblast also provide support for the sister relationship between nerites and apogastropods (van den Biggelaar 1996; Lindberg & Guralnick 2003).

Neomphalina

Neomphalina, represented by *P. delicata* and *Cyathermia naticoides* Warén and Bouchet, 1989, was monophyletic in all analyses except one and independent from all non-pleurotomariid vetigastropods in all analyses (Figs. 1–3 and Figs. S1–S3), but the placement of this clade within gastropods was unstable to parameter set variation. While the position of Neomphalina has varied in morphological analyses (Ponder & Lindberg 1997), its placement outside Vetigastropoda has been obtained in other molecular and combined analyses (McArthur & Koop 1999; McArthur & Harasewych 2003; Aktipis et al. 2008). Although this analysis only includes representatives from two major families in Neomphalina (Peltospiridae McLean 1989 and Neomphalidae McLean 1981), the consistent monophyly of *P. delicata*+*C. naticoides* and the recovery of Neomphalina outside of Vetigastropoda suggests the recognition of this clade as independent from Vetigastropoda.

The results in this study contradict the expansion of Vetigastropoda to include Neomphalina (Bouchet

et al. 2005; Geiger et al. 2008). This re-classification was based on a molecular phylogenetic analysis and the shared presence of bursicles in these groups. The only member of Neomphalina with documented ''bursicle-like'' structures, however, is the peltospirid *Melanodrymia* (Geiger et al. 2008), and no molecular data are available for any members of this genus. While these structures have been treated as homologous to the bursicles found in vetigastropods (Ponder & Lindberg 1997; Geiger & Thacker 2005; Geiger et al. 2008), bursicles have not yet been described on the gills of other Neomphalina (Haszprunar 1993; Ponder & Lindberg 1997), and so there is some uncertainty regarding the existence of true bursicles in this group. Some have suggested, however, that bursicles are lost or difficult to observe in adult specimens and are easily visible only on juveniles (A. Warén, pers. comm.). Further research regarding the presence of this morphological characteristic in Neomphalina is therefore warranted. ESO, another characteristic trait for the Vetigastropoda (Geiger et al. 2008), are also absent in members of Neomphalina (Fretter 1989; Warén & Bouchet 1989; Haszprunar 1993; Ponder & Lindberg 1997). Although some researchers attribute this absence of bursicles and epipodial tentacles as a secondary reduction, as observed in some vetigastropods (Geiger & Thacker 2005; Geiger et al. 2008), others note that Neomphalina have more shared morphological features with cocculinids, neritimorphs, and other rhipidoglossate clades than with vetigastropods (see table 3 in Heß et al. 2008). The absence of key traits such as ESO along with differences in other key morphological features as well the results from this molecular study suggest that Neomphalina should be recognized as an independent gastropod clade (Fretter et al. 1981; Heß et al. 2008). The formal position of Neomphalina within Gastropoda, however, remains ambiguous, with some analyses placing them as sister to (Pleurotomariidae (Vetigastropoda, Patellogastropoda)) while other analyses place them as sister to Pleurotomariidae, and may require additional phylogenetic research.

Pleurotomarioidea

Pleurotomarioidea, represented in this analysis by two species from the genera *Entemnotrochus* and *Bayerotrochus,* was recovered as a clade separate from all remaining vetigastropods in every multigene analysis. This result contradicts standard phylogenetic classifications identifying Pleurotomariidae within vetigastropods (Haszprunar 1988; Ponder & Lindberg 1997; Sasaki 1998; Harasewych 2002; Bouchet et al. 2005; Geiger & Thacker 2005), but confirms results from other molecular analyses (McArthur & Harasewych 2003). The placement of pleurotomariids, however, varies in the individual gene trees analyzed in POY under the seven-gene optimal parameter set 111. They fall outside vetigastropods in the 18S rRNA, 28S rRNA, and EF-1α analyses, but group with vetigastropods in the other single-gene analyses. Specifically, pleurotomariids fall sister to fissurellids in the H3, 16S rRNA, and myosin analyses, and sister to *H. corrugata* based on the COI data. It is possible that the phylogenetic signal from the 18S rRNA and 28S rRNA is overwhelming that of the other genes because pleurotomariid 18S rRNA genes and 28S rRNA genes tend to be $\sim$200–300 bp longer than other vetigastropod sequences. To confirm that the inserted regions are transcribed regions, the 18S rRNA gene was sequenced directly from total RNA and compared with the 18S rRNA gene sequence from genomic DNA. The sequences were identical, indicating that the extended gene regions are transcribed regions and therefore may have phylogenetic significance. 18S and 28S rRNAs are frequently used in conjunction with other molecular loci to elucidate deep evolutionary splits among molluscs (Giribet & Carranza 1999; Giribet et al. 2006) and may in fact be providing an informative phylogenetic signal (Giribet 2002a,b; Okusu et al. 2003; Lindgren et al. 2004).

The placement of Pleurotomariidae as an independent clade outside the remaining Vetigastropoda in this analysis concurs with the results of other molecular and combined phylogenetic analyses (although, admittedly, all of these analyses incorporate data from the phylogenetically influential nuclear ribosomal genes). In the molecular analysis of Aktipis et al. (2008), Pleurotomariidae was recovered outside a clade of vetigastropods and patellogastropods. Furthermore, Giribet et al. (2006) recovered Pleurotomariidae outside a clade of vetigastropods, cocculinids, and hot vent taxa. Some published analyses revealed a sister relationship between Pleurotomariidae and all remaining vetigastropods, but this relationship never received high nodal support (Harasewych et al. 1997; Geiger & Thacker 2005; Aktipis et al. 2008). Furthermore, the presence of bursicles has yet to be confirmed for Pleurotomariidae. Haszprunar (1987) identified the structure in *Mikadotrochus caledonicus* Warén and Bouchet, 1982, but other researchers failed to identify bursicles on other pleurotomariid species (Sasaki 1998; Harasewych 2002; Geiger & Thacker 2005). The presence of another suggested vetigastropod synapomorphy, ESO, is also controversial in the literature. Sasaki (1998)

documented small tuberculate papillae as ESO in pleurotomariids and Geiger et al. (2008) described ESO as reduced in pleurotomariids, but others describe pleurotomariids as lacking ESO entirely (Woodward 1901; Fretter 1964; Hickman 1996; Harasewych 2002). Although these characteristics could have been secondarily lost, their absence, in addition to the frequent phylogenetic placement of Pleurotomariidae outside all the remaining vetigastropods, indicates that the taxonomic placement of Pleurotomariidae within Vetigastropoda should be reconsidered. Further research should be performed to reinvestigate the anatomy of this enigmatic group of gastropods.

Patellogastropoda

Patellogastropoda was monophyletic in all analyses (100% bootstrap support) and fell sister to or within a clade of non-pleurotomariid vetigastropods in the optimal parameter sets of the POY parsimony analyses, TNT analysis using the complete data set and both maximum likelihood analyses (Figs. 1–3 and Figs. S1–S3). Patellogastropoda was only separate from all the remaining gastropods in the TNT parsimony analysis of the reduced data set. As this placement did not receive significant bootstrap support, it does not contradict results from the other analyses. While Patellogastropoda appears on a long branch in the maximum likelihood analyses, long-branch attraction is generally thought to be a problem with parsimony analysis, not so much with maximum likelihood, and so it is not considered to be a likely reason for the placement of Patellogastropoda in this study. Additionally, Patellogastropoda+Vetigastropoda was stable to parameter set variation in POY and recovered in all ML analyses, and long-branch problems have been shown by Giribet (2003) to be related to instability to parameter set variation. As mentioned in Aktipis et al. (2008), clades with high stability but low support may be united by a low (but uncontradicted) number of supporting characteristics. It is acknowledged, however, the nested position of Patellogastropoda within Vetigastropoda must be subjected to further testing.

Although these results contradict those recovered in morphological cladistic analyses (Golikov & Starobogatov 1975; Haszprunar 1988; Ponder & Lindberg 1997; Sasaki 1998), the close relationship between Vetigastropoda and Patellogastropoda has been recovered in some molecular studies (Tillier et al. 1994; Colgan et al. 2003) and in analyses using both molecular and morphological data (Aktipis et al. 2008). Furthermore, the radula of juvenile patellogastropods is very different from that of adult patellogastropods and instead resembles that of some adult vetigastropods (A. Warén, pers. comm.). The homology between juvenile patellogastropod radulas and adult vetigastropod radulas, therefore, should be investigated as it may represent morphological evidence for the close relationship between patellogastropods and vetigastropods.

Although the gill configuration varies among Patellogastropoda, all but one patellogastropod sampled in the study have a single left ctenidium without a skeletal rod. The five taxa representing Lottiidae formed a distinct clade in all analyses. The remaining patellogastropod, *Paralepetopsis* sp., a hydrothermal vent limpet with an uncertain relationship with Acmaeidae Carpenter 1857 and Lottiidae (Lindberg 1998, 2008; Sasaki 1998; Harasewych & McArthur 2000), lacks gills entirely (Fretter 1990) and was sister to Lottiidae in every analysis. The relationship of *Paralepetopsis* and other members of the Neolepetopsidae with other patellogastropods will remain uncertain until the family is better represented in molecular phylogenetic analyses. Lindberg (2008) has suggested, however, that there are two distinct lineages of patellogastropods, Lottiidae+Acmaeidae and Patelloidea Rafinesque 1815+Nacelloidea Thiele 1891, and that Patellogastropoda may actually represent a paraphyletic grade defined by plesiomorphic morphological characteristics. Pallial characteristics support this split in two clades. Most members of Acmaeidae and Lottiidae have a single left ctenidium, while members of Patelloidea and Nacelloidea have secondary gill leaflets located around the edge of the pallial cavity (Sasaki 1998; Lindberg 2008).

Further molecular studies testing Lindberg's hypothesis regarding a polyphyletic Patellogastropoda may also resolve the conflicting hypotheses regarding its placement within gastropods. The recovery of a polyphyletic Patellogastropoda with the Acmaeidae+Lottiidae lineage located sister to the Vetigastropoda, and the Patelloidea+Nacelloidea placed outside the remaining gastropods, would rectify the conflict among the different phylogenetic results. Only one molecular study to date incorporates taxa from Lottiidae, Acmaeidae, Patelloidea, and Nacelloidea with higher gastropod taxa. A monophyletic Patellogastropoda was recovered sister to Cocculiniformia, but the Lottiidae+Acmaeidae and Patelloidea+Nacelloidea split was not recovered, with Nacellidae sister to all other patellogastropods (Harasewych & McArthur 2000). These results may, however, be affected by the limited taxa and genes sampled in the analysis. Additionally, we were not

able to specifically test the results of Harasewych & McArthur (2000) as our taxon sampling design focused primarily on vetigastropods.

Vetigastropoda

Representatives of the proposed vetigastropod clades Fissurellidae, Haliotidae, Lepetelloidea, Lepetodrilidae, Neomphalina, Peltospiridae, Pleurotomariidae, Trochidae, and Turbinidae were not monophyletic in any analyses in this study. As addressed previously, Pleurotomariidae and Neomphalina were recovered consistently outside a clade comprised of patellogastropods and the vetigastropod families/superfamilies Fissurellidae, Haliotidae, Lepetelloidea, Lepetodrilidae, and Trochoidea. Vetigastropod taxa recovered as a clade are members of Haliotidae, Fissurellidae, Lepetodrilidae, and Trochoidea. These families all display the characteristic vetigastropod bursicles and ESO, and have regularly been recovered in other phylogenetic analyses (e.g., Geiger et al. 2008). The phylogenetic relationships among these families varied depending on the optimality criteria and parameter sets used, something that is likely to stabilize as taxon sampling increases. The significance of these relationships, therefore, will be better addressed in future studies with increased vetigastropod representation.

Because of the instability of *Pyropelta* and *Hemitoma,* Patellogastropoda was often recovered within the remaining vetigastropods in the parsimony analyses, but these specimens nested with the non-pleurotomariid vetigastropods in the maximum likelihood analyses. Although a previous molecular analysis based on 18S rRNA recovered a sister relationship between the Patellogastropoda and Cocculiniformia (Cocculinioidea+Lepetelloidea) (Harasewych & McArthur 2000), the placement of *Pyropelta* sp. and *H. octoradiata* in our study may be caused by some sort of systematic error. *Pyropelta* sp. and *H. octoradiata* have aberrant 18S and 28S rRNA sequences with some long insertions, molecular patterns that are also seen in Patellogastropoda. Furthermore, the placement of *Pyropelta* sp. and *H. octoradiata* stabilized in the maximum likelihood analysis. *Hemitoma octoradiata* was also recovered within Fissurellidae under the 3221 parameter set, a weighting scheme that accounts for long insertions by minimizing the cost of indel extensions. In the maximum likelihood analysis, *Pyropelta* sp. was sister to all non-pleurotomariid vetigastropods and *H. octoradiata* was sister to *Puncturella* sp. within Fissurellidae, positions that correspond to those described in current taxonomic classifications (McLean 1984).

Summary and conclusions

Although bootstrap support for Gastropoda and other main groups did not change significantly with the addition of the nuclear protein-coding genes, the supported clades recovered in the seven-gene parsimony and maximum likelihood analyses correspond more closely to those found in other molecular and combined molecular and morphological analyses than those recovered under the most optimal five-gene tree. Congruence between the deep gastropod relationships recovered in the seven-gene analyses and those using only morphological characteristics, however, only occurs with the monophyly of Caenogastropoda (proxy for Apogastropoda) and Gastropoda. In contrast, the results of the seven-gene POY and maximum likelihood analyses reveal similar relationships with increased bootstrap support among the ''archaeogastropod'' groups to those recovered in analyses utilizing both morphological and molecular data. This convergence between the results of analyses using ''combined'' data sources and analyses with new molecular loci indicates that the utilization of additional data sources may be useful in resolving the deep splits among gastropods.

Recovered clades were also more stable to parameter set variation in the POY analysis of the seven-gene complete data set, supporting the argument that increasing the data sources in an analysis improves phylogenetic reconstruction (Cummings et al. 1995; Giribet 2002a; Rokas & Carroll 2005). Some relationships recovered in this analysis indicate a need to reconsider high-level gastropod systematics. In particular, Neritimorpha should be considered to be more closely related to Apogastropoda (represented in this analysis by Caenogastropoda) than to Vetigastropoda. The independence of Neomphalina and Pleurotomariidae from Vetigastropoda should be further investigated in analyses utilizing increased data sampling. The sister relationship between Patellogastropoda and Vetigastropoda *s.str.* also deserves further exploration with increased taxon and character sampling, although this pattern has been recovered in most of the analyses presented here as well as in some recent analyses considering a large number of taxa, genes, and morphology (e.g., Aktipis et al. 2008). This study did not elucidate the internal relationships among the vetigastropod families due to the sampling design, which had to include fresh tissues preserved for RNA work. Future exploration of Vetigastropoda *sensu stricto* with increased taxon sampling is necessary in order to better understand the relationships within vetigastropods and inform upon a new definition of Vetigastropoda. This study is only a beginning

step toward understanding the origin of major gastropod lineages; increased taxon sampling, genomic sequencing, and analysis will provide more understanding about the ancient splits existing between gastropod clades.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Cladogram based on the parsimony analyses of the full 7-gene MUSCLE-aligned molecular data using TNT. The cladogram shown is the single shortest tree (28,721 steps) found under the 7-gene optimal parameter set (111). Numbers on branches indicate bootstrap support above 50%. Bold branches indicate gastropod taxa while colored terminal taxa indicate clade designations: blue for Caenogastropoda, green for Neritimorpha, red for Neomphalina, pink for Pleurotomariidae, purple for Vetigastropoda and orange for Patellogastropoda.

Figure S2. Maximum likelihood tree based on 7-gene MUSCLE-aligned molecular data with variable regions removed using Gblocks ($-\log L = 57247.227828$). Numbers on branches indicate bootstrap support above 50%. Bold branches indicate gastropod taxa and colors correspond to those assigned to the clades in Figure S1.

Figure S3. Cladogram based on the parsimony analyses of the Gblocks reduced 7- gene molecular data using TNT. The cladogram shown was the single shortest tree (13,098 steps) found under the 7-gene optimal parameter set (111). Support was calculated using 100 bootstrap replicates and numbers on branches indicate bootstrap support above 50%. Bold branches indicate gastropod taxa and colors correspond to those assigned to the clades in Figure S1.

Table S1. Primers used in the study. Primers identified with an asterisk were used as alternative primers when initial amplification attempts were unsuccessful. The internal primers 4R and 18Sbi were used to amplify challenging 18S rRNA sequences. Due to the difficulty of amplifying the first portion of 18S rRNA for Patellogastropoda species, novel primers Pat1F and Pat1R were designed and successfully used to amplify and sequence the starting region of 18S rRNA. When the first fragment of 28S rRNA was not easily amplified, the external primer rd1a was used in place of D1F. For COI, more challenging specimens were amplified using alternative amplification methods described by Kano (2008) using the degenerate primer pairs LCOmod and HCOmod, or a nested PCR reaction with the COI-NERa and HCO primers for the first reaction.

Appendix SA. Voucher data for specimens used in this study. Specimens with sequence data obtained from GenBank are not listed. See Table 1 for family assignments. Abbreviations: Museum of Comparative Zoology, Harvard University (MCZ); Penn State University (PSU); U.S. National Museum, Smithsonian Institution (UNM).