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Towards a phylogeny of chitons (Mollusca, Polyplacophora) based on combined analysis of five molecular loci

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Abstract

This study represents the first phylogenetic analysis of the molluscan class Polyplacophora using DNA sequence data. We employed DNA from a nuclear protein-coding gene (histone H3), two nuclear ribosomal genes (18S rRNA and the D3 expansion fragment of 28S rRNA), one mitochondrial protein-coding gene (cytochrome *c* oxidase subunit I), and one mitochondrial ribosomal gene (16S rRNA). A series of analyses were performed on independent and combined data sets. All these analyses were executed using direct optimization with parsimony as the optimality criterion, and analyses were repeated for nine combinations of parameters affecting indel and transversion/transition cost ratios. Maximum likelihood was also explored for the combined molecular data set, also using the direct optimization method, with a model equivalent to GTR + I + Γ that accommodates gaps. The results of all nine parameter sets for the combined parsimony analysis of all molecular data (as well as ribosomal data) and the maximum-likelihood analysis of all molecular data support monophyly of Polyplacophora. The resulting topologies mostly agree with a division of Polyplacophora into two major lineages: Lepidopleuridae and Chitonida (sensu Sirenko 1993). In our analyses the genus *Callochiton* is positioned as the sister group to Lepidopleuridae, and not as sister group to the remaining Chitonida (sensu Buckland-Nicks & Hodgson 2000), nor as the sister group to the remaining Chitonina (sensu Buckland-Nicks 1995). Chitonida (excluding *Callochiton*) is monophyletic, but conventional subgroupings of Chitonida are not supported. Acanthochitonina (sensu Sirenko 1993) is paraphyletic, or alternatively monophyletic, and is split into two clades, both with abanal gills only and cupules in the egg hull, but one has simple cupules whereas the other has more strongly hexagonal cupules. Sister to the Acanthochitonina clades is Chitonina, including taxa with adanal gills and a spiny egg hull. *Schizochiton*, the only genus with adanal gills that has an egg hull with cupules, is the sister-taxon to one of the Acanthochitonina clades plus Chitonina, or alternatively basal to Chitonina. Support values for either position are low, leaving this relationship unsettled. Our results refute several aspects of conventional classifications of chitons that are based primarily on shell characters, reinforcing the idea that chiton classification should be revised using additional characters.

Key words: evolution, molecular phylogeny, POY, parsimony, maximum likelihood, direct optimization

Introduction

The members of Polyplacophora, commonly referred to as chitons, are dorso-ventrally flattened, bilaterally symmetrical molluscs that are characterized by eight dorsal calcium carbonate shell plates, or valves, and a broad ventral ciliated foot. Surrounding the valves – or even nearly completely engulfing them in some species – there is a thick marginal girdle (perinotum) embedded with calcium carbonate spines and spicules. The approximately 900 living species of chitons worldwide are exclusively marine animals, with the majority found from the intertidal to the sublittoral, but some deep-sea species are also known (Beesley et al. 1998). Chitons are thought to have diverged relatively early from other molluscan lineages, and their known fossil record ex-

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tends from as early as the Upper Cambrian (Yochelson et al. 1965, Runnegar et al. 1979, Yates et al. 1992, Stinchcomb & Darrough 1995, Slieker 2000).

Since chitons were first described by Linnaeus (1758) there have been extensive taxonomic studies at the species level (Pilsbry 1892–1894; Thiele 1909; Kaas & Van Belle 1985a, 1985b, 1987, 1990, 1994). However, the taxonomic classification at higher levels in the group has remained somewhat unsettled (Thiele 1909; Bergenhayn 1930, 1955; Smith 1960; Van Belle 1983; Eernisse 1984; Sirenko 1993, 1997; Buckland-Nicks 1995; Okusu 2003). Most traditional classifications of major groups of chitons have been based primarily on shell (valve) characters. For example, Van Belle (1983, 1985; see also Smith 1960, Kaas & Van Belle 1985–1994, Kaas et al. 1998) mostly followed Bergenhayn (1930, 1955) in dividing all extant species (order Neoloricata) into three suborders: Lepidopleurina, Acanthochitonina (including Acanthochitonidae and Cryptoplacidae), and Ischnochitonina (the most speciose group, including Ischnochitonidae, Mopaliidae, Chitonidae, and Schizochitonidae). Gowlett-Holmes (1987) additionally assigned the monotypic genus *Choriplax* to its own suborder, Choriplacina. A historical review of chiton taxonomy can be found in Van Belle (1983) and Kaas & Van Belle (1985a: 26–28). For a detailed historical perspective of the early study of chitons, with special emphasis on Australian species, consult Kaas et al. (1998: 161–163).

Most of the early work on chiton systematics relies on characters of the shell plates, and even modern taxonomic treatises follow a system based mostly on shell characters (e.g. Kaas et al. 1998). More recent efforts to test chiton classification and phylogeny have utilized egg hull morphology, gill placement and morphology, and sperm ultrastructure in addition to shell valve morphology (Eernisse 1984; Sirenko 1993, 1997; Buckland-Nicks 1995). The prevalent near-exclusive use of valve characters, despite being the only character set available for fossil chitons, has been criticised as not sufficient to reveal phylogenetic relationships. In contrast to the situation in gastropods, radular morphology in chitons is generally too conservative, even at deep levels, and characters such as radular tooth details, girdle morphology, and shell spicular processes are mostly valuable at lower taxonomic levels (Eernisse 1984; Sirenko 1993, 1997; Eernisse & Reynolds 1994; Buckland-Nicks 1995).

Sirenko (e.g. 1993) recognized the taxon Chitonida, which includes most extant chitons with elaborate extracellular hull processes surrounding their eggs (Pearse 1979, Eernisse 1984, Eernisse & Reynolds 1994). These hull processes typically can be either cup-like or spiny. They are primarily secreted by the egg (Richter 1986), thus are not strictly a chorion. The hulls might have diverse functions. Parachuting and chain forming have been suggested to allow slow sinking or to deter predators; spacing eggs apart may have an effect for oxygenation; adhesion has also been suggested (Eernisse 1988, Buckland-Nicks 1993). Most importantly, they seem to direct sperm to localized areas during fertilization (Buckland-Nicks 1993). All chitons with elaborate egg hulls also have sperm with asymmetrically arranged mitochondria and a long filamentous anterior extension of the nucleus (Hodgson et al. 1988), which has at its tip a reduced acrosomal vesicle (Buckland-Nicks et al. 1990, Buckland-Nicks 1995).

Chitons also have serially repeated posterior or lateral gills that run along each side of the foot in the pallial cavity (e.g see Kaas & Van Belle 1985a: Fig. 3). The gills are variable in number and arrangement. Their number ranges from 6 to 88 pairs but is not constant within a species and may differ between body sides (Hyman 1967). It also increases with growth in size of the animal. Developmentally, the first gill pair to appear is post-renal (right behind the nephridiopore) (Pelseneer 1899). Most chitons (adanal type) add gills both anterior and posterior to this pair, but some (abanal type) only add gills to the anterior (Eernisse 1984, Eernisse & Reynolds 1994, Sirenko 1993).

A correlation among egg hull type, sperm morphology and gill placement has been recognized in the recent literature (e.g. Eernisse 1984, Sirenko 1993, Buckland-Nicks 1995). Based on these characters the members of extant chitons were proposed to constitute two major lineages: (1) Lepidopleurida, chitons with presumed primitive features of valves without slitted insertion plates, adanal gills restricted to the posterior region, ectaquasperm, and smooth eggs; and (2) Chitonida, chitons with presumed derived features of valves with slitted insertion plates extending laterally into the girdle, lateral gills with separation between left and right rows (of adanal or abanal type), elaborate egg hull processes, and sperm with a filamentous extension of the nucleus and reduced acrosome (Sirenko 1993, Buckland-Nicks 1995). In the Results and Discussion sections below we will often refer to these groups as Lepidopleurida sensu Sirenko and Chitonida sensu Sirenko. The Chitonida were further divided into taxa with a spiny egg hull and adanal gill placement (Chitonina), and taxa with an egg hull with cupules and abanal gill placement (Tonicellina and Acanthochitonina). Unlike those who have emphasized the importance of the distinctive valve sculpturing in *Acanthochitona* and its relatives as revealing their early divergence within Chitonida (Bergenhayn 1930, Smith 1960, Van Belle 1983), Sirenko (1993) interpreted these differences as more recently derived traits, and expanded Acanthochitonina to also include his previously proposed Tonicellina grouping, on the basis of similarity of egg hull morphology and gill placement. Sirenko (1997) recently added another set of characters related to the articulamentum shell layer. Based on these he reor-

ganized subclades within Acanthochitonina, separating it into Mopalioidea and Cryptoplacoidea. He also divided the other suborder of Chitonida, Chitonina, into Chitonoidea and Schizochitonoidea.

Sirenko's (1997) classification system is generally corroborated by the only available cladistic analysis of morphology, based on 25 characters scored from valve, egg hull, sperm, and gill morphology of 10 polyplacophoran families (25 species examined in total) and two aplacophorans as outgroups (Buckland-Nicks 1995). This analysis supported two major lineages (summarized in Fig. 1): Lepidopleurida and Chitonida (including Acanthochitonina and Chitonina).

The phylogenetic position of the polyplacophorans within Mollusca is still a contentious issue. Polyplacophorans have been suggested to be the sister-taxon to aplacophorans, forming the Aculifera (= Amphineura), by having similar girdle spicules and a generally elongate body form (Scheltema 1996). The recent discovery of an aplacophoran larva with seven transverse dorsal imbricating spaces devoid of spicules by Scheltema & Ivanov (2002) may support the earlier idea that aplacophorans may have shared plate-like dorsal structures similar to those of polyplacophorans. This is further supperted by the discovery (Sutton et al. 2001a) of the Silurian "aplacophoran" mollusc, *Acaenoplax hayae*, with seven dorsal valves and one ventral valve, interpreted to be homologous to valves 1–6 and 8 of chitons, would appear to strengthen the Aculifera hypothesis. However, the molluscan affinities of *A. hayae* are still disputed (see Steiner & Salvini-Plawen 2002, Sutton et al. 2001b). Other hypotheses view aplacophorans as paraphyletic with respect to a clade comprising the remaining molluscs, Testaria, within which polyplacophorans are sister taxon to conchiferans (e.g. Wingstrand 1985, Haszprunar 2000). This position of the Polyplacophora is often assumed in studies of conchiferan relationships (i.e. Giribet & Wheeler 2002). It is beyond the scope of this study to assess the position of Polyplacophora within Mollusca. Thus, we have included sequence data for all classes of molluscs as outgroups (with the exception of the unavailable monoplacophorans), with the aims to examine the relationships within Polyplacophora and to perform the strictest test of the monophyly of the class short of also including non-molluscan outgroups (i.e., we must assume that Mollusca is not rooted within Polyplacophora).

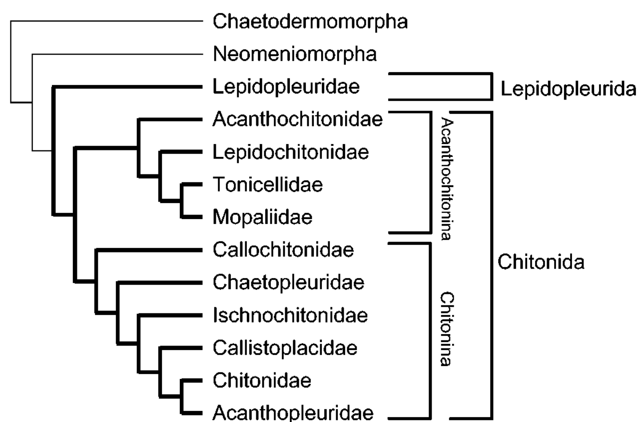

Fig. 1. Tree of chiton families based on 25 morphological characters including shell, spicule, girdle, egg hull, sperm, and gill morphology and placement (redrawn from Buckland-Nicks 1995). The topology indicates two major lineages, Lepidopleurida and Chitonida, the latter including the Acanthochitonina, Tonicellina and Chitonina sensu Sirenko (1993, 1997).

Here, we present a phylogenetic analysis of chiton relationships, including representatives of 28 species belonging to 13 families (Table 1). This study represents the first molecular analysis of polyplacophoran relationships (after the unpublished thesis by Okusu 2003) and uses up to 5 Kb of sequence data including nuclear protein-coding (histone H3) and ribosomal (18S rRNA and 28S rRNA) genes, as well as mitochondrial protein-coding (cytochrome *c* oxidase subunit I) and ribosomal (16S rRNA) genes.

Material and methods

Taxon sampling

A total of 28 polyplacophoran species have been chosen and collected for the study to represent 13 families from all three orders (see Table 1 and Appendix 1 for voucher information). Species from 15 other families could not be obtained for the present study. All the material was collected alive and either frozen or fixed in 70–96% EtOH and kept at –80 °C. Outgroup taxa representing each of the conventional molluscan classes, Aplacophora, Gastropoda, Bivalvia, Scaphopoda, and Cephalopoda, have been obtained and preserved in a similar fashion.

DNA isolation, amplification, and sequencing

Total DNA was extracted from a small tissue sample of each individual, from the body wall, foot or gonads (see Okusu & Giribet 2003), using the DNeasy Tissue Kit from QIAGEN© and the protocol provided by the manufacturer. The purified total DNA was used as template for amplification of a portion for each of the 18S rRNA, 28S rRNA, 16S rRNA, cytochrome *c* oxidase subunit I (COI hereafter), and histone H3 loci, using the polymerase chain reaction (PCR). The complete 18S rRNA (ca. 1.8 kb) was amplified in three overlapping fragments of about 950, 900, and 850 bp each, using primer pairs 1F–5R, 3F–18Sbi, and 18Sa2.0–9R (primer se-

Table 1. Taxon sampling and GenBank accession numbers for each sequenced locus. Chiton classification based on Sirenko (1997).

		18S rRNA	28S rRNA	16S rRNA	H3	COI
Chaetodermomorpha	*Chaetoderma nitidulum*	AY377658	AY377692	AY377612	AY377763	AY377726
	Scutopus ventrolineatus	X91977				
	Prochaetoderma sp.				AY377762	
Neomeniomorpha	*Helicoradomenia* sp.	AY21210	AY377688	AY377613	AY377764	AY377725
	Epimenia n. sp.	AY377657	AY377691	AY377615	AY377765	AY377723
	Epimenia babai		AY377690	AY377616	AY377766	AY377724
	Epimenia australis		AY377689	AY377614	AY377767	AY377722
Polyplacophora						
Lepidopleurida						
Lepidopleuridae	*Lepidopleurus cajetanus*	AF120502	AF120565	AY377585	AY377735	AF120626
	Leptochiton asellus	AY377631	AY377662	AY377586	AY377734	
Chitonida						
Chitonina						
Callochitonidae	*Callochiton septemvalvis*	AY377632	AY377663		AY377736	AY377700
Chitonidae	*Tonicia lamellosa*	AY377634	AY377666	AY377589	AY377738	
Chaetopluridae	*Chaetopleura apiculata*	AY377636	AY377667	AY377590	AY377741	AY377704
	Chaetopleura angulata	AY377637	AY377668	AY377591	AY377740	AY377703
Ischnochitonidae	*Ischnochiton comptus*	AY377639	AY377673	AY377593	AY377744	AY377709
	Ischnochiton australis	AY377641	AY377670	AY377596	AY377746	AY377707
	Ischnochiton elongatus	AY377642	AY377672	AY377595	AY377743	AY377708
	Ischnochiton rissoi	AY377640	AY377671	AY377594	AY377745	AY377706
	Lepidozona mertensii	AY377643	AY377674	AY377597	AY377747	AY377710
	Stenoplax alata	AY377644	AY377675	AY377598	AY377748	AY377711
Callistoplacidae	*Callistochiton antiquus*	AY377645	AY377676	AY377599	AY377749	AY377712
Schizochitonidae	*Schizochiton incisus*	AY377646	AY377677	AY377600	AY377750	
Loricidae	*Lorica volvox*	AY377647	AY377678	AY377601	AY377751	
Chitonidae	*Chiton olivaceus*	AY377651	AY377682	AY377605	AY377755	AY377716
	*Liolophura japonica**	AY377652	AY377683	AY377606	AY377756	AY377717
	*Sypharochiton pelliserpentis**	AY377653	AY377684	AY377607	AY377757	AY377718
	Acanthopleura granulata	AY377654	AY377685	AY377608	AY377758	AY377719
Acanthochitonina						
Mopalliidae	*Mopalia muscosa*	AY377648	AY377679	AY377602	AY377752	AY377713
	Nuttallochiton mirandus	AY377638	AY377669	AY377592	AY377742	AY377705
	Plaxiphora albida	AY377649	AY377680	AY377603	AY377753	AY377714
	Katharina tunicata	AY377650	AY377681	AY377604	AY377754	AY377715
Tonicellidae	*Tonicella lineata*	AY377635	AY377665	AY377588	AY377739	AY377702
	Lepidochitona cinerea	AY377633	AY377664	AY377587	AY377737	AY377701
Acanthochitonidae	*Acanthochitona crinita*	AF120503	AF120566	AY377609	AY377759	AF120627
	Cryptochiton stelleri	AY377655	AY377686	AY377610	AY377760	AY377720
Cryptoplacidae	*Cryptoplax japonica*	AY377656	AY377687	AY377611	AY377761	AY377721
Gastropoda						
Cocculinidae	*Cocculina messingi*	AF120508	AY377696	AY377624	AY377777	AY377731
Vetigastropoda	*Entemnotrochus adansonianus*	AF120509	AY377694	AY377621	AY377774	
	Diodora cayenensis	AY377659	AY377695	AY377623	AY377776	AY377730
	Haliotis tuberculata	AF120511	AF120570	AY377622	AY377775	AY377729
	Sinezona confusa	AF120512	AF120571	AY377620	AY377773	AF120631
Caenogastropoda	*Viviparus georgianus*	AF120516	AF120574	AY377626	AY377779	AF120634
	Crepidula fornicata	AY377660	AY377697	AY377625	AY377778	AF353154
Pulmonata	*Siphonaria pectinata*	X91973	AF120578	AY377627	AY377780	AF120638
Bivalvia						
Solemyoidea	*Solemya velum*	AF120524	AF120581		AY070146	U56852**
Nuculoidea	*Nucula proxima (N. sulcata)*	AF120526	AF120583	AY377617	(AY070147)	AF120641

Table 1. Continued.

			18S rRNA	28S rRNA	16S rRNA	H3	COI
	Nuculanoidea	*Nuculana pella*	AY070111	AY070124		AY070148	AY070138
		Yoldia limatula	AF120528	AF120585		AY377768	AF120642
	Pteriomorpha	*Mytilus edulis*	L33448**	AF120587	AJ293738**	AY377769	AY377727
		Limaria hians	AF120534	AF120595		AY070152	AF120650
		Pecten jacobaeus	AY070112	AY070125		AY070153	AY377728
	Palaeoheterodonta	*Neotrigonia margaritacea*	AF411690	AF411689		AY070155	U56850**
	Heterodonta	*Cardita calyculata*	AF120549	AF120610		AY070156	AF120660
		Corbicula fluminea	AF120557	AF131009		AY070161	AF120666
		Dreissena polymorpha	AF120552	AF120613		AY070165	AF120663
		Mya arenaria	AF120560	AF120621	AY377618	AY377770	AY070140
Scaphopoda		*Rhabdus rectius*	AF120523	AF120580	AY377619	AY377772	AF120640
		Antalis pilsbryi	AF120522	AF120579			AF120639
		Dentalium vulgare		AY377693		AY377771	
Cephalopoda							
	Nautiloidea	*Nautilus pompilius*	AF207641	AF411688	AY377628	AF033704	AF120628
	Sepioidea	*Sepia elegans*	AF120506-7	AF120569	AY377630	AY377784	
	Teuthoidea	*Loligo pealei*	AF120505	AF120568	AF421958	AY377782	AF120629
		Architeuthis dux	AY377661	AY377699	AY377629	AY377783	AY377733
	Octopoda	*Octopus joubini*		AY377698		AY377781	AY377732

* Genus not listed in Sirenko (1997)
** Sequences not generated by the authors.
Since histone H3 data for *Nucula proxima* are lacking, those for *N. sulcata* are given.

quences are listed in Table 2). An additional primer pair internal to 1F–5R was used for sequencing: 1F–4R. The D3 fragment of the 28S rRNA locus was amplified and sequenced using primer pair 28Sa–28Sb. The16S rRNA gene was amplified and sequenced using primer pair 16Sa–16Sb. The COI was amplified and sequenced using a primer pair LCO1490–HCO 2198. The complete coding region of histone H3 was amplified and sequenced using primer pair H3aF–H3aR.

PCR reactions (50 µL) included 2 µL of the template DNA, 1 µM of each primer, 200 µm of dNTP's (Invitrogen), 1× PCR buffer containing 1.5 mM $MgCl_2$ (Perkin Elmer), and 1.25 units of AmpliTaq DNA polymerase (Perkin Elmer). The PCR reactions were carried out using a GeneAmp PCR System 9700 thermal cycler, and involved an initial denaturation step (5 min at 95 °C) followed by 35 cycles including denaturation at 95 °C for 30 s, annealing (ranging from 45 to 49 °C) for 30 s, and extension at 72 °C for 1 min, with a final extension step at 72 °C for 1 min.

The double-stranded PCR products were verified by agarose gel electrophoresis (1% agarose), and purified using GENECLEAN II Kit (BIO 101). The purified PCR products were sequenced directly; each sequence reaction of a total volume of 10 µL included 2 µL of the PCR product, irrespective of PCR yield, 1 µM of one of

Table 2. Sequences of primers used for the analyses. References for primers: 18S rRNA (Giribet et al. 1996, Whiting et al. 1997); 28S rRNA (Whiting et al. 1997); 16S rRNA (Xiong & Kocher 1991); COI (Folmer et al. 1994); histone H3 (Colgan et al. 1998).

18S rRNA	
1F	5′-TAC CTG GTT GAT CCT GCC AGT AG-3′
3F	5′-GTT CGA TTC CGG AGA GGG A-3′
3R	5′-AGG CTC CCT CTC CGG AAT CGA AC-3′
4R	5′-GAA TTA CCG CGG CTG CTG G-3′
5R	5′-CTT GGC AAA TGC TTT CGC-3′
18Sa2.0	5′-ATG GTT GCA AAG CTG AAA C-3′
18Sbi	5′-GAG TCT CGT TCG TTA TCG GA-3′
9R	5′-GAT CCT TCC GCA GGT TCA CCT AC-3′
28S rRNA	
28Sa	5′-GAC CCG TCT TGA AAC ACG GA-3′
28Sb	5′-TCG GAA GGA ACC AGC TAC-3′
16S rRNA	
16Sa	5′-CGC CTG TTT ATC AAA AAC AT-3′
16Sb	5′-CTC CGG TTT GAA CTC AGA TCA-3′
COI	
LCO1490	5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′
HCO2198	5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′
Histone H3	
H3aF	5′-ATG GCT CGT ACC AAG CAG AC(ACG) GC-3′
H3aR	5′-ATA TCC TT(AG) GGC AT(AG) AT(AG) GTG AC-3′

the PCR primer pairs, 2 μL of halfTERM Dye Terminator Reagent (Genpak), and 2 μL of ABI BigDye™ Terminator v3.0 (Applied Biosystems). The sequence reactions, performed using the thermal cycler described above, involved an initial denaturation step for 3 min at 95 °C, and 25 cycles (95 °C for 10 s, 50 °C for 5 s, 60 °C for 4 min). The BigDye-labelled PCR products were cleaned with AGTC® Gel Filtration Cartridges (Edge BioSystems). The sequence reaction products were then analyzed using an ABI Prism 3100 Genetic Analyzer.

Sequence editing

Chromatograms obtained from the automated sequencer were read and "contig sequences" (assembled sequences) were made using the sequence editing software Sequencher™ 4.0. For the non-coding genes (18S, 28S, 16S), complete sequences were edited and aligned against secondary structure models and then split into accordant fragments using internal primers and the visualized secondary structure features (Giribet & Wheeler 2001, Giribet 2002b). These fragments were subsequently used as the input files for the phylogenetic analyses. Protein coding genes (H3 and COI) were taken as 'prealigned' when no insertion/deletion (indel hereafter) event needed to be postulated among taxa. All these sequences were visualized and manipulated in GDE (Smith et al. 1994). The external primer regions 1F and 9R (for 18S), 28Sa and 28Sb (for the 28S fragment), 16Sa and 16Sb (for 16S), LCO and HCO (for COI), and H3aF and H3aR (for H3) were removed and hence excluded from the analyses. All the new sequences have been deposited in GenBank under accession numbers AY377585-AY377784 (Table 1).

In most cases we have included a total of up to 5 kb of sequence data from a wide representation of polyplacophoran and other molluscan taxa. A small number of extremely hypervariable regions of the ribosomal genes (16S rRNA, 18S rRNA and 28S rRNA) were excluded from the analyses. Such hypervariable regions are difficult to align and are not only uninformative, but can also cause conflict, as demonstrated in previous analyses (e.g. Giribet et al. 2000). The regions excluded are: fragments 0, 1 and 8 of 16S rRNA sequence file (16s.seq); fragment 25 of 18S rRNA sequence file; fragments 4 & 5 of 18S rRNA and fragments 2 & 3 of 28S rRNA of all cephalopod species; and fragment 5 of 18S rRNA and fragment 3 of 28S rRNA of *Entemnotrochus adansonianus*.

Phylogenetic analysis

The data were analyzed in the computer program POY (Wheeler et al. 2002), using the direct optimization method (Wheeler 1996) and parsimony as the optimality criterion. Independent sets of five partitions were analyzed: each of the 16S, COI and H3 data, and the combined data sets ribosomal genes (18S and 28S) and all genes (18S, 28S, 16S, COI, and H3). This method allows analyzing sequences of unequal length without the necessity of providing aligned matrices, via a dynamic optimization process that generates phylogenetic trees that minimize the number of transformations by specifying certain parameters for those transformations. The same criterion and model is thus employed through the phylogenetic construction procedure (Wheeler 1996). The method allows the data to be analyzed in a sensitivity analysis framework (Wheeler 1995) using multiple parameter sets with different transversion/transition cost ratios and gap costs (see Giribet 2001 for a review of POY).

Parallel tree searches were conducted using pvm (parallel virtual machine) on a cluster of 14 dual-processor nodes assembled at Harvard University (darwin.oeb.harvard.edu). Commands for load balancing of spawned jobs were in effect to optimize parallelization procedures (-parallel –dpm –jobspernode 2). Trees were built through a random addition sequence procedure (usually 100 replicates) followed by a combination of branch-swapping steps (SPR 'subtree pruning and regrafting' and TBR 'tree bisection and reconnection') and tree fusing (Goloboff 1999, 2002) in order to further improve tree length. Discrepancies between heuristic and actual tree length calculations were addressed by adjusting slop values (-slop 5 – checkslop 10).

Each of the five partitions was analyzed under nine parameter sets, for a variation of indel costs and transversion/transition ratios, where indel cost refers to the highest nucleotide transformation (in this case always transversions). Gap/transversion ratios of 1, 2 and 4, and transversion/transition ratios of 1, 2 and 4 were explored (see the specific step matrices in Table 3). In total we performed 64 analyses taking ca. 60 days of computation time in the 28-processor cluster. Implied alignments, a sort of alignment based on a synapomorphy scheme (Wheeler 2003), can be easily generated for each tree.

Due to the fact that several "models" were explored, in order to select the optimal one among the 9 parameter sets studied, we employed a character-congruence technique, a modification of the ILD (incongruence length difference) metric developed by Mickevich & Farris (1981, see also Farris et al. 1995), as proposed by Wheeler (1995) (Table 4). The value is calculated for each parameter set by subtracting the sum of the scores of all partitions from the score of the combined analysis of all partitions, and normalizing it for the score of the combined length. This has been interpreted as a meta-optimality criterion for choosing the parameter set that best explains all partitions in combination, the one that maximizes overall congruence and minimizes character

Table 3. Symmetrical step matrices used in the analyses (a series of three numbers assigned to each parameter – 111, 121, 141, 211, 221, 241, 411, 421, and 441 – corresponds to the ratios of gap/transversion, transversion/transition, and transition values (always set as 1)).

111

	A	C	G	T	–
A	0	1	1	1	1
C	1	0	1	1	1
G	1	1	0	1	1
T	1	1	1	0	1
–	1	1	1	1	0

211

	A	C	G	T	–
A	0	1	1	1	2
C	1	0	1	1	2
G	1	1	0	1	2
T	1	1	1	0	2
–	2	2	2	2	0

411

	A	C	G	T	–
A	0	1	1	1	4
C	1	0	1	1	4
G	1	1	0	1	4
T	1	1	1	0	4
–	4	4	4	4	0

121

	A	C	G	T	–
A	0	2	1	2	2
C	2	0	2	1	2
G	1	2	0	2	2
T	2	1	1	0	2
–	2	2	2	2	0

221

	A	C	G	T	–
A	0	2	1	2	4
C	2	0	2	1	4
G	1	2	0	2	4
T	2	1	2	0	4
–	4	4	4	4	0

421

	A	C	G	T	–
A	0	2	1	2	8
C	2	0	2	1	8
G	1	2	0	2	8
T	2	1	2	0	8
–	8	8	8	8	0

141

	A	C	G	T	–
A	0	4	1	4	4
C	4	0	4	1	4
G	1	4	0	4	4
T	4	1	4	0	4
–	4	4	4	4	0

241

	A	C	G	T	–
A	0	4	1	4	8
C	4	0	4	1	8
G	1	4	0	4	8
T	4	1	4	0	8
–	8	8	8	8	0

441

	A	C	G	T	–
A	0	4	1	4	16
C	4	0	4	1	16
G	1	4	0	4	16
T	4	1	4	0	16
–	16	16	16	16	0

Table 4. Tree length and calculated ILD (incongruence length difference) values for the partitions including each of 16S, H3, COI, ribosomal (18S + 28S) and molecular (18S + 28S + 16S + COI + H3) data sets at different parameter set values (111, 121, 141, 211, 221, 241, 411, 421, 441). Optimal (121) and immediate suboptimal (111) parameter sets are indicated in boldface.

Set	16S	COI	H3	Ribosomal	Molecular	ILD
111	2708	4913	1276	4773	14132	**0.0327**
121	4311	7565	1813	7200	21579	**0.0320**
141	7337	12601	2843	11842	35882	0.0351
211	3148	4998	1276	5579	15527	0.0339
221	5098	7671	1814	8622	24117	0.0378
241	8837	12866	2843	14614	40860	0.0416
411	3708	5056	1277	6782	17630	0.0458
421	6164	7780	1815	10965	28202	0.0524
441	10956	13053	2843	19198	48790	0.0562

conflict among all the data. Alternatively, we present the strict consensus of all parameter sets explored, which has been interpreted as a measure of stability to model choice, as applied in statistical sensitivity analyses (Wheeler 1995, Giribet 2003). Nodal supports for all the topologies were measured by parsimony jackknifing (Farris et al. 1995, Farris 1997).

Furthermore, we explored an alternative tree construction method by using the maximum-likelihood criterion (Felsenstein 1981) as implemented in POY using direct optimization for the combined molecular data set. This incorporates values for gaps as proposed in earlier maximum-likelihood alignment based methods (Thorne et al. 1991) and parameters are allowed to vary and re-estimated for each pair of sequences as they are encountered (Wheeler et al. 2002). Parallel tree searches with random addition sequence followed by branch swapping and tree fusing were executed as described above. A model with parameter estimation comparable to the General Time Reversible (GTR) + I + Γ model was used; all base and indel frequencies, transition probabilities, and likelihoods for theta fraction of invariant sites and discrete gamma distribution (rate class four) were allowed to vary and were re-estimated for each pair of sequences as they were encountered. Finally, the likelihood of an optimization alignment was determined by summing all optimization alignments, i.e. the paths taken for each possible optimization alignment with their corresponding likelihoods.

All input files, data files, analysis batch files and output files are available online at the following url: http://www.mcz.harvard.edu/Departments/InvertZoo/ giribet_data.htm. These files will allow easy generation of implied alignments for each output tree in a matter of seconds, without need for supercomputers.

Results

After analyzing our data under the nine specified sets of parameters, the one that minimized overall incongruence is that with a gap/change ratio of 1:1 and a transversion/transition ratio of 2:1 (parameter set 121, ILD = 0.0320). The results presented here and referred to as "optimal parameter set" are therefore mostly based on the analyses performed under these analytical conditions, irrespective of the marker or combined matrix discussed. The results under equal weights will also be discussed; equal weights (parameter set 111) constitutes the immediate suboptimal parameter set, at an ILD = 0.0327 (Table 4). For the combined analysis of all data, both trees are fundamentally similar. Given the minor difference in the ILD of parameter sets 121 and 111, which could be

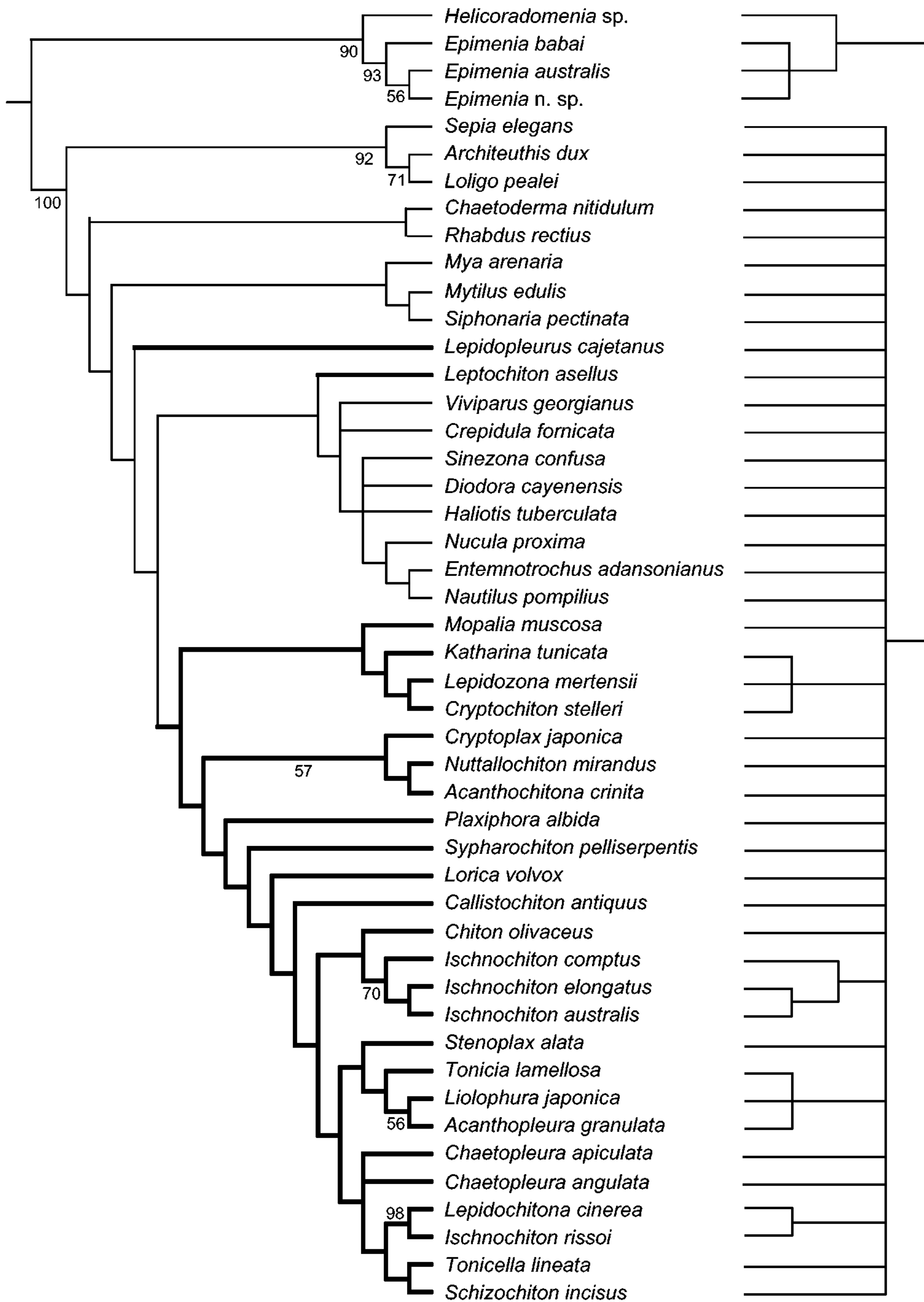

Fig. 2. Left: Strict consensus of 3 shortest trees at length 4,311 for the 16S rRNA data at parameter set 121 (optimal tree based on ILD value). A single tree at length 4,311 was found a single time after 100 replicates of SPR + TBR; two extra trees of minimum length were found after tree fusing. Branches in bold represent polyplacophoran clades; numbers on branches represent jackknife proportions above 50%. Right: Strict consensus of 16 trees found for all nine explored parameter sets.

given by the heuristics of the tree searches, we decided to discuss results of both parameter sets, named “optimal” and “immediate suboptimal”, hereafter.

Mitochondrial genes (Figs. 2, 3)

There was little agreement among all the parameter sets for the mitochondrial genes, 16S rRNA (Fig. 2, right tree) and COI (Fig. 3, right). The strict consensus of 16 optimal trees obtained after analysis of all parameter sets for 16S rRNA had little resolution (Fig. 2, right), with only seven nodes being completely stable to parameter variation. The optimal parameter set (121) yielded three shortest trees of length 4,311 after SPR + TBR. Tree fusing did not improve the length of the trees. A clade consisting of some gastropods, bivalves and *Nautilus* rendered Polyplacophora paraphyletic in the strict consensus of the three shortest trees (Fig. 2, left tree), with *Lep-*

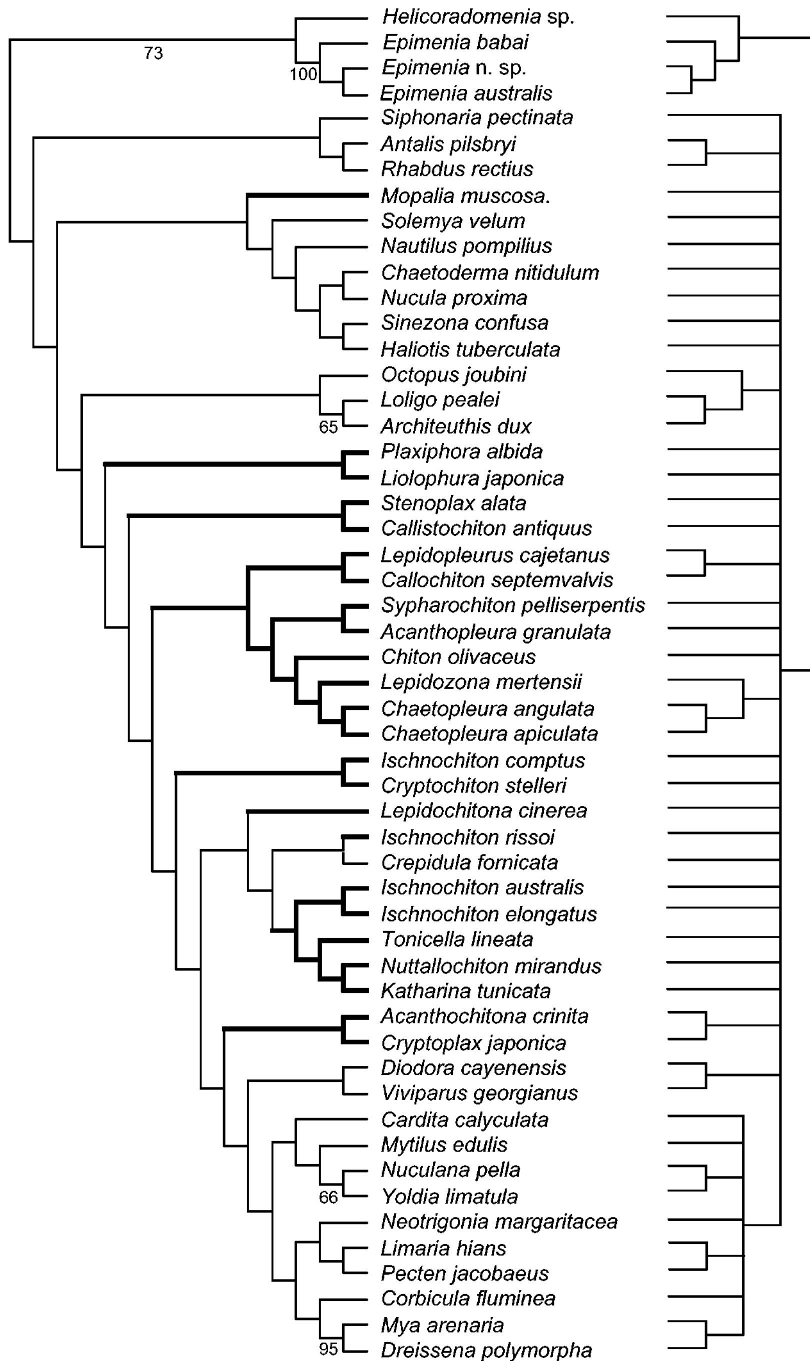

Fig. 3. Left: Shortest tree at length 7,565 after 100 replicates of SPR + TBR + TF for the COI data at parameter set 121 (optimal tree based on ILD value). This tree was found in 36 replicates. Right: Strict consensus of 14 trees found for all nine explored parameter sets. Branches in bold represent polyplacophoran clades; numbers on branches represent jackknife proportions above 50%.

idopleurus and *Leptochiton* branching off before the other chitons. Chitonida sensu Sirenko is monophyletic, although 16S rRNA data for *Callochiton* is not available (see discussion on the position of *Callochiton* below). On the contrary, equal weights supported monophyly of Polyplacophora (tree not shown), with Lepidopleuridae as sister group to Chitonida. The optimal tree also recovered Neomeniomorpha, and within Polyplacophora the following clades were obtained under all analytical conditions: (*Katharina, Lepidozona, Cryptochiton*), *Ischnochiton* species (except for *I. rissoi*), (*Tonicia, Liolophura, Acanthopleura*), and (*Lepidochitona, I. rissoi*). In addition to these clades, the topology of the optimal tree supports monophyly of Coleoidea and a clade formed by (*Cryptoplax* (*Nuttallochiton*, *Acanthochitona*)) with a jackknife value above 50%. Other interesting clades place *Mopalia* as sister taxon to the *Katharina* – *Cryptochiton* complex; *Chiton* as the sister taxon to *Ischnochi-*

ton; *Stenoplax* as the sister taxon to the *Tonicia – Acanthopleura* complex; the clade (*Tonicella, Schizochiton*) as the sister taxon to (*Lepidochitona, I. rissoi*); and the *Chaetopleura* species forming a clade with the *Tonicella – I. rissoi* complex. All these clades receive jackknife support values below 50%.

The strict consensus of the 14 optimal trees of all parameter sets for COI also had little resolution with 16 nodes resolved (Fig. 3, right tree). The optimal parameter set yielded a single shortest tree of length 7,565 after SPR + TBR; tree fusing did not improve the length of the tree. None of the parameter sets analyzed supported polyplacophoran monophyly, the optimal parameter set nesting a bivalve-gastropod clade and a second lineage, the gastropod *Crepidula*, within Polyplacophora (Fig. 3, left). For the clades outside Polyplacophora, the strict consensus of all parameter sets supports Scaphopoda, Coleoidea, (*Diodora*, *Viviparus*), and a bivalve clade excluding Nuculoidea and Solemyoidea. Within Polyplacophora, the strict consensus of all parameter sets analyzed supports the following clades: (*Lepidopleurus, Callochiton*); (*Chaetopleura, Lepidozona*); and (*Acanthochitona, Cryptoplax*). Of these clades, (*Loligo, Architeuthis*), (*Nuculana, Yoldia*), and (*Mya, Dreissena*) receive jackknife values above 50% for the optimal parameter set. Interestingly, all parameter sets for the COI data support a sister group relationship of *Lepidopleurus* and *Callochiton*, but no COI data are available for other lepidopleurids.Therefore, as it would be expected, the mitochondrial genes are adding little information to deep splits as judged by the instability of such results to parameter variation.

Nuclear protein-coding gene (Fig. 4)

The strict consensus of the 236 optimal trees obtained for all the parameter sets for histone H3 resolved more deep nodes than the mitochondrial genes did (Fig. 4, right tree). The strict consensus tree nests the two gastropod species of the genera *Viviparus* and *Siphonaria* within Polyplacophora, whereas it does not support monophyly of *Leptochiton* with respect to the remaining members of the chiton clade. For the putative outgroup taxa, all parameter sets support the monophyly of *Epimenia*, Coleoidea, and a clade including certain bivalves + Coleoidea.

Within Polyplacophora, the strict consensus tree of all parameter sets supports monophyly of two large chiton clades, one containing the genera *Tonicia*, *Acanthopleura*, *Lorica*, *Chiton, Sypharochiton*, *Liolophura*, and *Ischnochiton* (excluding *I. rissoi*), and another containing the members of the genera *Katharina*, *Mopalia*, *Tonicella*, *Cryptochiton*, *Acanthochitona*, *Nuttallochiton*, *Plaxiphora*, *Lepidochitona*, *Callistochiton*, *Chaetopleura*, *Stenoplax*, *Lepidozona*, *Callochiton*, *Cryptoplax*, and the species *I. rissoi*. The latter clade receives a jackknife support value of 77%. Other subclades of chitons found across the entire parameter space include *Chiton* + *Sypharochiton*; (*Liolophura* (*Ischnochiton australis* (*I. elongatus* + *I. comptus*))), as well as the genus *Chaetopleura*, or a clade containing the genera *Lepidozona*, *Callochiton*, and *Cryptoplax*. The optimal parameter set yielded a single shortest tree of length 1,813 after SPR + TBR (Fig. 4, left tree). Tree fusing did not improve the length of the tree. In addition to the clades mentioned above, the optimal parameter set supports Nuculanoidea, *Crepidula* as sister taxon to *Nautilus*-Neomeniomorpha, and *Haliotis* + *Diodora* with jackknife frequencies above 50%. Within Polyplacophora, the optimal parameter set supports, in addition to the clades mentioned above, (*Lorica (Chiton, Sypharochiton*)); *Acanthopleura* as sister taxon to *Lorica – I. comptus* complex; and (*Katharina, Mopalia, Tonicella, Cryptochiton*) as sister taxon to (*Acanthochitona, Nuttallochiton, Plaxiphora, Lepidochitona, Callistochiton, Chaetopleura, I. rissoi, Stenoplax, Lepidozona, Callochiton, Cryptoplax*).

Nuclear ribosomal genes (Fig. 5)

The combined analysis of the ribosomal genes 18S rRNA and the D3 region of 28S rRNA resulted in a topology mostly in agreement with the combined molecular tree. The strict consensus of 65 trees for all parameter sets of the combined analyses of the nuclear ribosomal loci (Fig. 5, right tree) supports the monophyly of Polyplacophora under all analytical conditions. This tree also supports monophyly of Scaphopoda, Cephalopoda, *Epimenia*, Chaetodermomorpha, Vetigastropoda, (*Limaria, Pecten*), Euheterodonta, and Nuculanoidea. Within Polyplacophora, all trees agree in the monophyly of clades such as Lepidopleuridae (*Lepidopleurus, Leptochiton*); Tonicellina (*Lepidochitona, (Tonicella, Katharina, Cryptochiton, Mopalia*)); (*Chiton, Sypharochiton*); (*Tonicia* (*Liolophura, Acanthopleura*)); ((*Chaetopleura apiculata, C. angulata), Ischnochiton rissoi*); ((*Callistochiton, Stenoplax), Lepidozona*); and ((*Ischnochiton comptus, I. elongatus), I. australis*). The optimal parameter set yielded a single shortest tree of length 7,200 after SPR + TBR (Fig. 5, left tree), and tree fusing did not improve tree length or find additional trees. In this tree Polyplacophora is split into two major lineages, one includes most of the species within Chitonina sensu Sirenko (except for *Tonicia, Liolophura,* and *Acanthopleura*), and the other includes most Acanthochitona sensu Sirenko with *Callochiton* and Lepidopleuridae as their successive sister taxa. Support for Polyplacophora as measured by jackknife values is 100%, and several nodes within and outside Polyplacophora receive values above 90% (Fig. 5), including those of *Chaetopleura*, *Ischnochiton* (excluding *I. rissoi*), *Chiton* + *Sypharochiton*, *Liolophura* + *Acantho-*

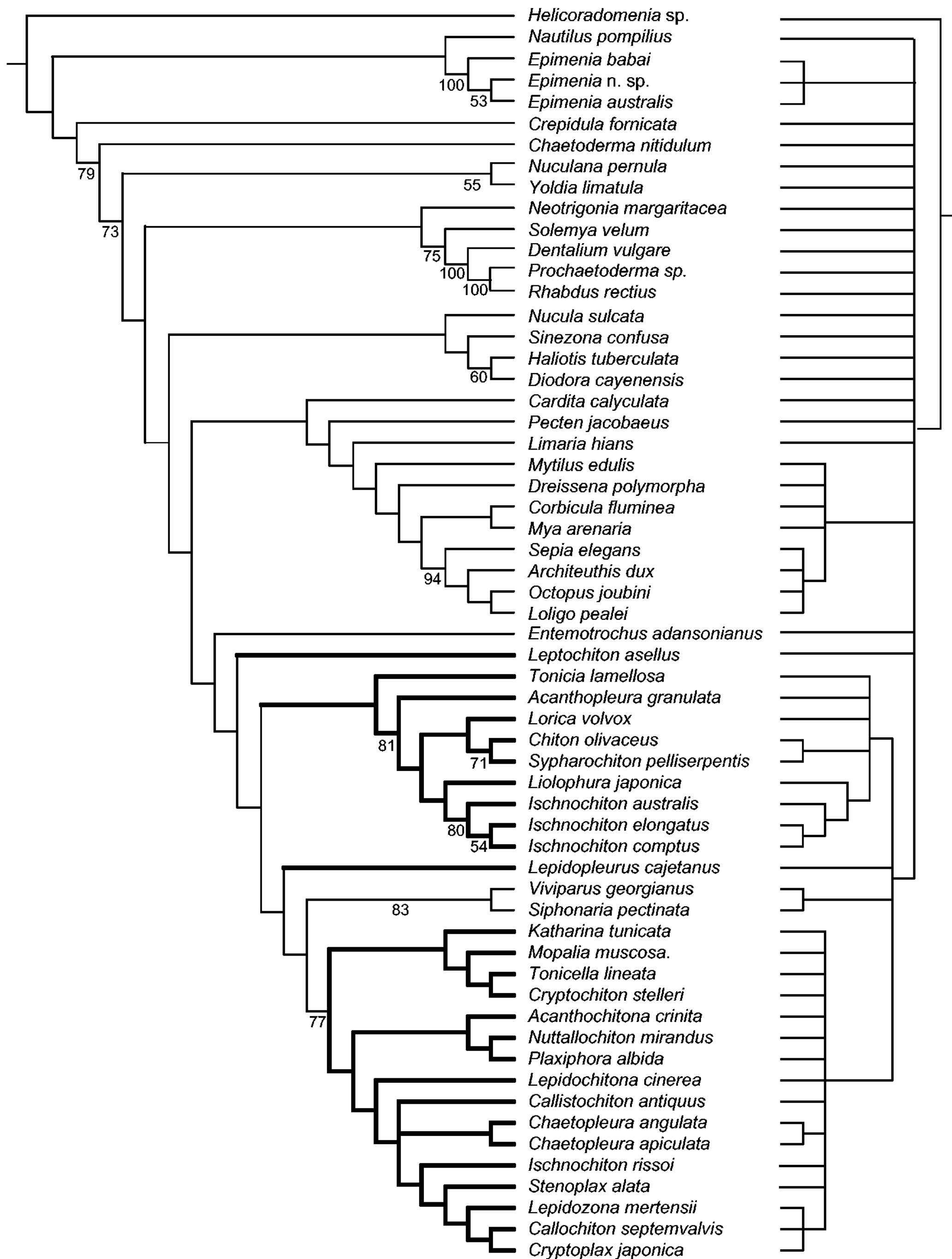

Fig. 4. Left: Shortest tree at length 1,813 after 100 replicates of SPR + TBR branch swapping and tree fusing for the histone H3 data at parameter set 121 (optimal tree based on ILD value). This tree was found in 11 replicates. Right: Strict consensus of 236 trees found for all nine explored parameter sets. Branches in bold represent polyplacophoran terminals; numbers on branches represent jackknife proportions above 50%.

pleura, (*Tonicia* (*Liolophura* + *Acanthopleura*), Lepidopleuridae, or a clade formed by Lepidopleuridae, Acanthochitonina, Callochitoninae, *Tonicia*, *Acanthopleura* and *Liolophura*.

Combined molecular data (Figs. 6, 7)

The strict consensus of all parameter sets for the combined analysis of all molecular data (Fig. 6, right tree) shows monophyly of several lineages, including Chaetodermomorpha, Polyplacophora, Neomeniomorpha, *Epimenia*, Cephalopoda, Coleoidea, Nuculanoidea, a bivalve-heterodont clade containing *Corbicula*, *Mya*, and *Dreissena*, and a vetigastropod clade containing *Diodora*, *Sinezona*, and *Haliotis*. Within Polyplacophora, the following clades appear monophyletic under all examined parameter sets: (*Acanthochitona* + *Nuttallochiton* + *Cryptoplax*); (*Katharina* + *Cryptochiton*); *Chaetop-*

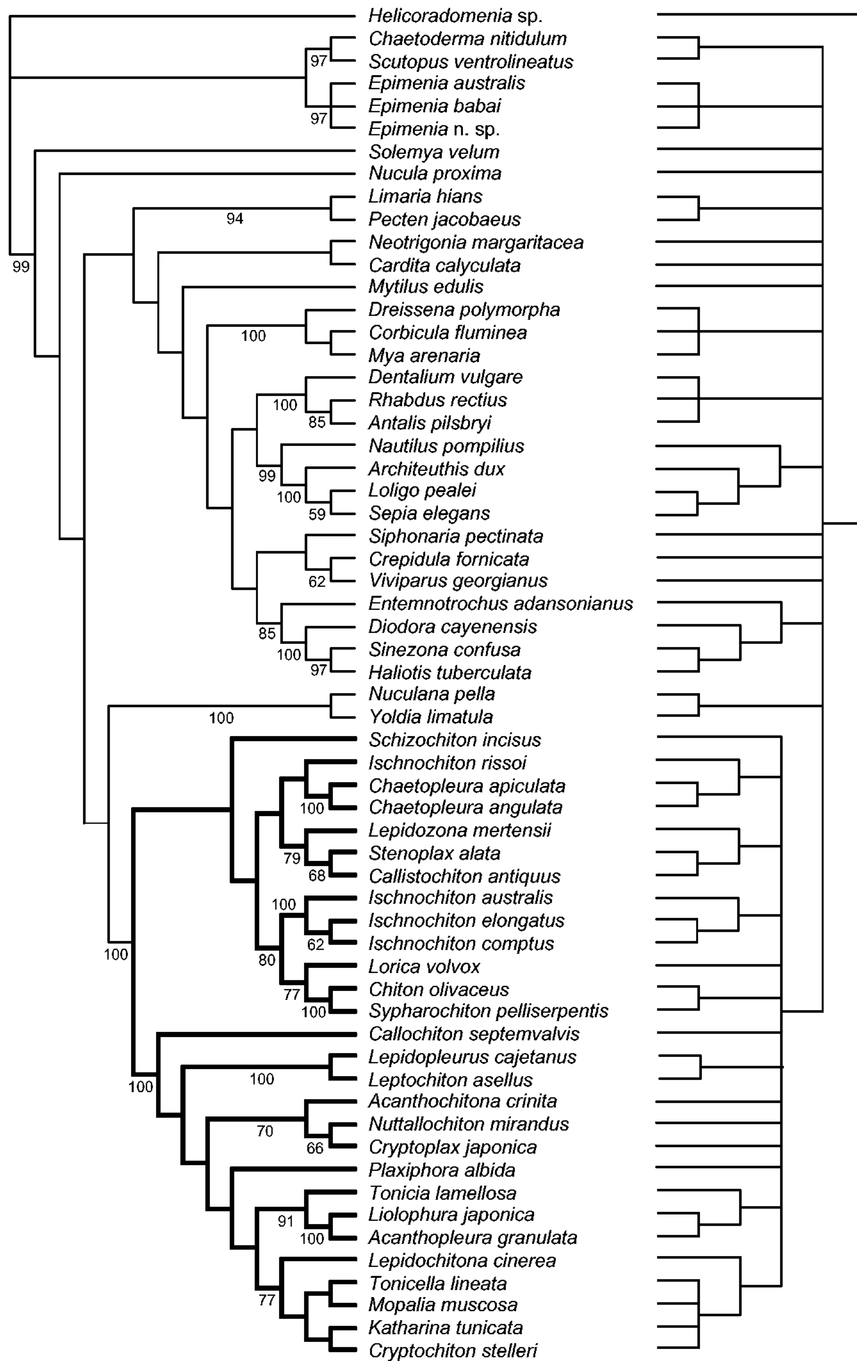

Fig. 5. Left: Shortest tree at length 7,200 after 100 replicates of SPR + TBR branch swapping and tree fusing for the combined ribosomal data (28S rRNA + 18S rRNA) at parameter set 121 (optimal tree based on ILD value). This tree was found on a single replicate and no more trees were found after tree fusing. Right: Strict consensus of 65 trees found for all nine explored parameter sets. Branches in bold represent polyplacophoran clades; numbers on branches represent jackknife proportions above 50%.

leura; (*Lepidozona* + *Stenoplax* + *Callistochiton*); *Ischnochiton* excluding *I. rissoi*; (*Tonicia* + *Liolophura* + *Acanthopleura*); and (*Chiton* + *Sypharochiton*).

The analysis under the optimal parameter set yielded one shortest tree of length 21,579 (Fig. 6, left tree) after SPR + TBR. Tree fusing did not improve the length of the tree. During the searches of the molecular combined data under the optimal parameter set, a total of 22,928,162 trees were examined and 252,903,002 alignments were processed in a little more than 11 hours in the 28-processor cluster, although the optimal tree was hit only once. The suboptimal parameter set (111) yielded one shortest tree of length 14,132 (Fig. 7A), and the maximum-likelihood analysis (GTR + I + Γ) yielded a tree of likelihood –ln L = 55,451.88 (Fig. 7B). Topologies of the three trees (optimal, equal-weights, and maximum-likelihood) are topologically congruent and all show monophyly of Polyplacophora, Neomeniomorpha,

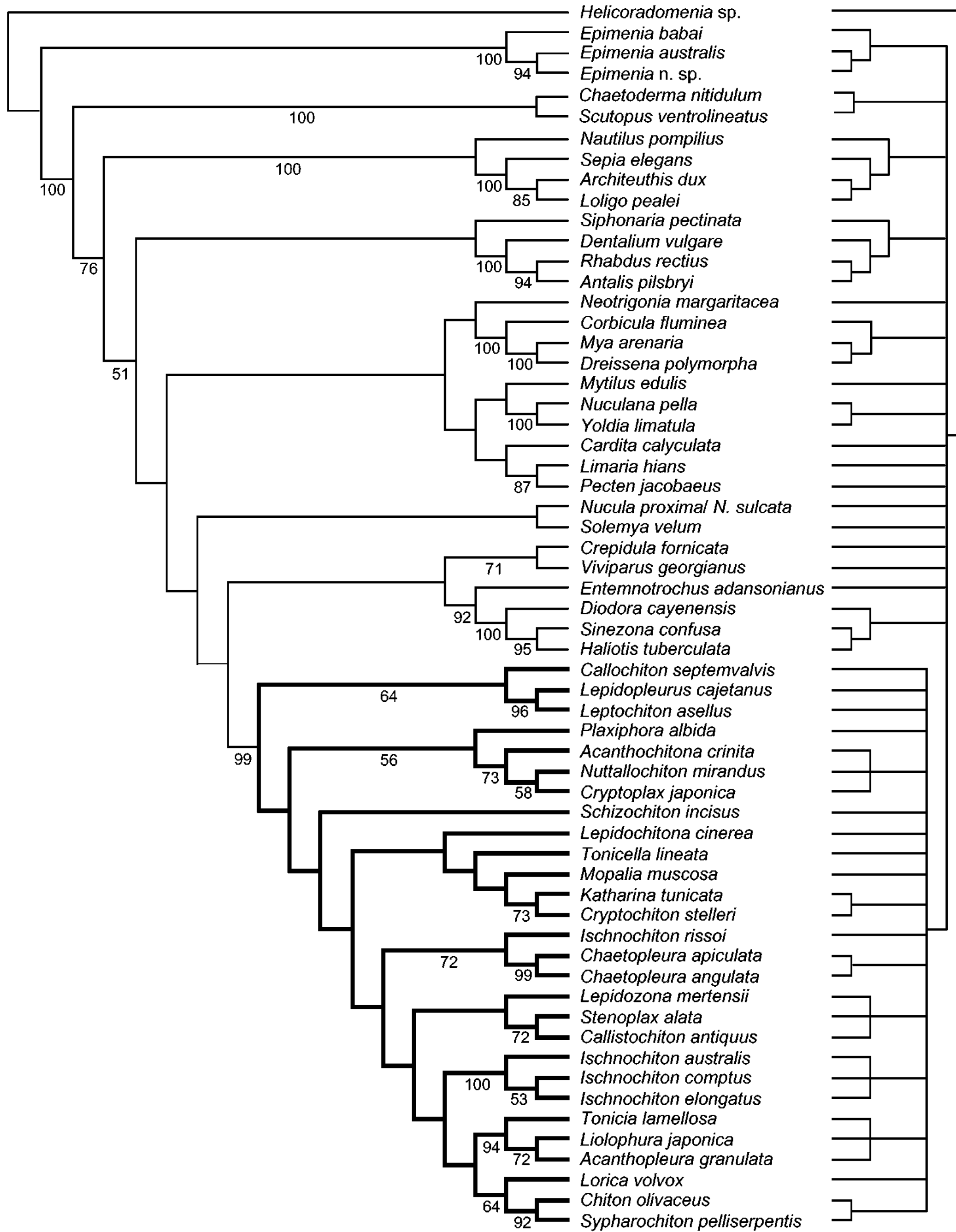

Fig. 6. Left: Shortest tree at 21,579 steps found after 100 replicates of SPR + TBR branch swapping and tree fusing for all molecular data combined (18S + 28S + 16S + COI + H3) at parameter set 121 (optimal tree based on ILD value). This tree was found on a single replicate and no additional trees were obtained after tree fusing. Branches in bold represent polyplacophoran clades; numbers on branches represent jackknife proportions above 50%. Right: Strict consensus of 16 trees found for all nine explored parameter sets.

Cephalopoda, and Scaphopoda. Under maximum likelihood, gastropod monophyly was also supported.

For the internal relationships within polyplacophorans all three topologies agree in the presence of two lineages: Lepidopleuridae (*Lepidopleurus cajetanus* and *Leptochiton asellus*), and Chitonida sensu Sirenko, which includes the rest of the chitons here represented, except *Callochiton*. All three topologies also support the chitonid species *Callochiton septemvalvis* as the sister group to Lepidopleuridae. In order to facilitate further

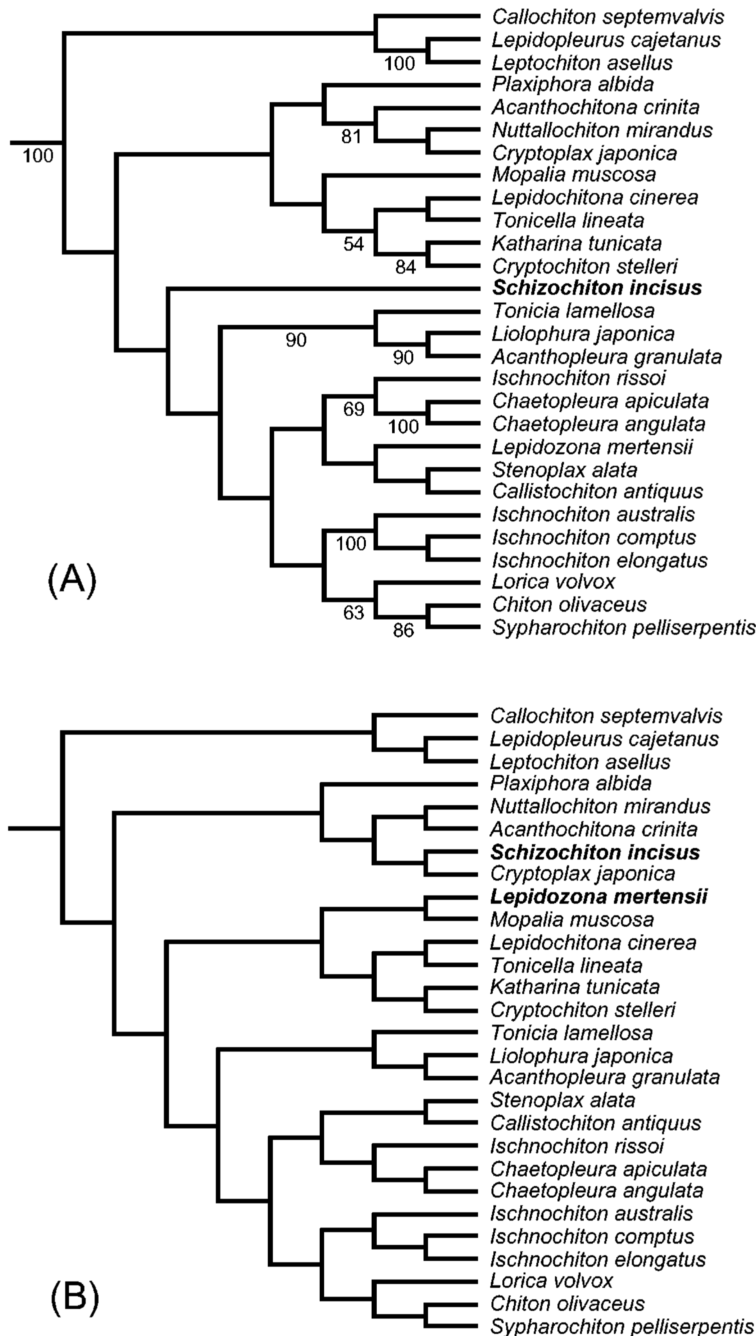

Fig. 7. Alternative topologies for the combined molecular data (18S + 28S + 16S + COI + H3): (A) Shortest tree at 14,132 steps after 100 replicates of SPR + TBR branch swapping and tree fusing at parameter set 111 (equal weights); and (B) Tree that maximized the likelihood value when base (and indel) frequencies are estimated by the program, estimating the q matrix, and using an "f5" model (allows all base and indel frequencies to vary) with GTR + I + Γ (–ln L = 55451.88). Both topologies are mostly congruent with the optimal tree, except for the positions of *Schizochiton incisus* and *Lepidozona mertensii* (both species in bold), and internal relationships of Acanthochitonina.

discussion of the results, we will refer to these three clades as Lepidopleuridae, *Callochiton*, and Chitonida.

Lepidopleuridae is monophyletic under all analyzed conditions, and the sister group relationship between *Callochiton septemvalvis* and Lepidopleuridae is obtained under all parameter sets except 241 for the parsimony analyses (in this case, the unstable species *Schizochiton incisus* appears as sister group to Lepidopleuridae, followed by *Callochiton*). The sister group relationship of *Callochiton* + Lepidopleuridae is also found in the maximum-likelihood analysis under the selected model.

Relationships within Chitonida are parameter dependent, and therefore different analyses under parsimony as well as the maximum-likelihood analysis suggest different relationships. Most analyses find three main clades within the "Chitonida" (Fig. 8): one (clade A) composed of *Plaxiphora*, *Nuttallochiton*, *Acanthochi-*

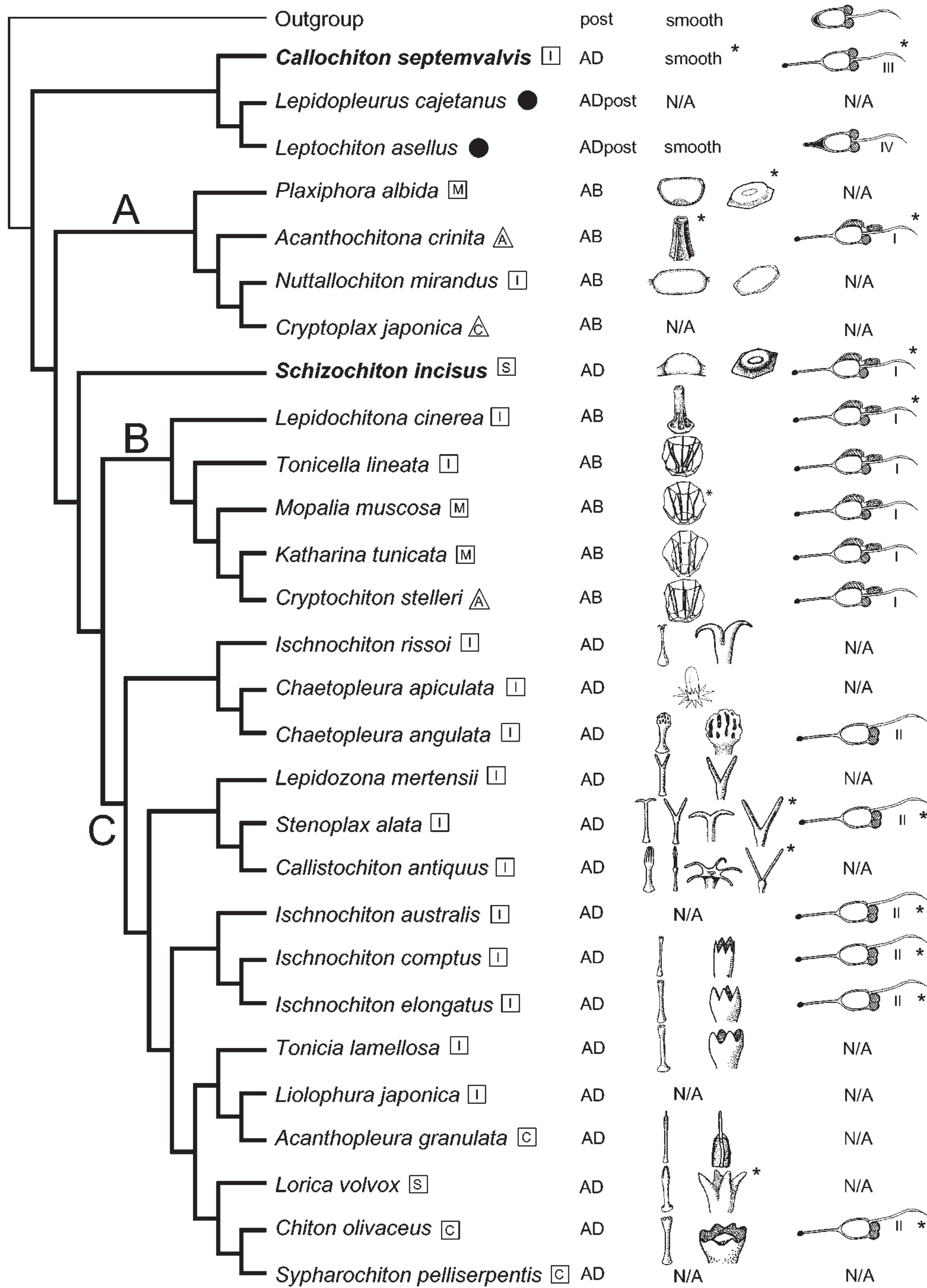

Fig. 8. Egg hull, sperm, and gill morphology mapped onto the optimal parsimony tree for polyplacophorans (Fig. 6). Egg characters redrawn from Sirenko (1993), for *Cryptochiton stelleri* based on Eernisse (1984). Gill placement as follows: AB = abanal, AD = adanal, ADpost = adanal posterior. Sperm reconstructed from SEM and light microscopy images based on Buckland-Nicks (1995). Missing data are indicated by N/A. Asterisks indicate congeneric proxies. The letters A, B and C refer to clades discussed in the text. Familiar coding based on traditional classification of chitons sensu Kaas & Van Belle (1985a) are as follows: ● = Lepidopleurina, Lepidopleuridae; □ = Ischnochitonina (I = Ischnochitonidae, C = Chitonidae, M = Mopaliidae, S = Schizochitonida); △ = Acanthochitonina (A = Acanthochitonidae, C = Cryptoplacidae).

tona, and *Cryptoplax*; a second (clade B) composed of *Lepidochitona*, *Tonicella*, *Mopalia*, *Katharina*, and *Cryptochiton*; and a third clade (clade C), corresponding to Chitonina sensu Sirenko, that includes members of most other represented genera. The positions of *Schizochiton incisus* and *Lepidozona mertensii*, and whether clades A and B form a monophyletic group (= Acanthochitonina sensu Sirenko) constitute the major disagreements between the two optimal parsimony analyses and the maximum-likelihood analysis. Furthermore, few of these nodes receive jackknife support above 50%, and only a few are stable to parameter variation.

In the optimal tree (Figs. 6, 8), Acanthochitonina is paraphyletic with clade B being the sister group to Chitonina. The paraphyly of Acanthochitonina is also obtained in the maximum-likelihood topology (Fig. 7B), whereas under equal-weighted parsimony Acanthochitonina is monophyletic (Fig. 7A). Within Acanthochitonina, monophyly of Mopalioidea and Cryptoplacoidea is not supported in any topology under any analytical conditions. Within the Chitonina, *Ischnochiton rissoi* is the sister group to *Chaetopleura* in all these three topologies, separated from the other ischnochitonids. The position of the chitonid *Schizochiton incisus* varies among the three analyses; the optimal parsimony tree positions it between the two acanthochitonine clades (A and B), while the equal-weights tree positions it as a sister group to clade C (= Chitonina), and the maximum-likelihood tree nests it within clade A. In the maximum-likelihood tree, both the position of the chitonid *Lepidozona mertensii* (nests within clade B) and the internal relationships of Acanthochitonina (*Nuttallochiton mirandus* is sister taxon to *Acanthochitona crinita*) are incongruent with those of the parsimony-based topologies, but due to computation limitations we have not been able to ascertain whether the likelihood results are model-dependent or not.

Discussion

Each partition, when analyzed independently, yields results that may seem rather incongruent with other partitions. The different loci included in this study have not been chosen to maximize congruence among partitions, as advocated by some systematists, but to include markers with "overlapping levels of resolution", as advocated e.g. by Giribet (2002a). Hence, their individual contributions should not be stressed other than for exploratory reasons; their contribution to the combined analyses of all markers is what really matters to us.

Outgroup relationships

Relationships among the outgroup taxa are, to say the least, unsatisfactory. The reason for including a broad selection of molluscan outgroups is important, because this constitutes the strictest possible test for the monophyly of chitons, as more taxa add more potential falsifiers. Obviously, the goal of the present study was not to solve molluscan relationships, which would require adequate outgroups and sampling outside molluscs. This is no excuse to explain the poor resolution of the data at such level, which only resolve monophyly of some of the known classes such as Cephalopoda, Scaphopoda, Chaetodermomorpha and Neomeniomorpha, besides Polyplacophora, but no relationship uniting any of these clades receives high support or results stable to parameter variation. Having said this, a broader study utilizing many molluscan species is underway (A. Okusu and collaborators, work in progress).

Polyplacophora

Chiton taxonomy has in the past been based mostly on morphology of valves, spicules, and girdle processes (e.g. Smith 1960, Van Belle 1983, Kaas et al. 1998). The higher systematics of chitons remains unsettled, perhaps due to the limited resolution that can be discerned from relying on those characters alone. More recently, morphology of egg hull, sperm ultrastructure, and gill placement have been explored (Eernisse 1984, Sirenko 1993, Buckland-Nicks 1995) as additional sources of morphological characters for chiton systematics. Adding these characters in combination with the more traditionally employed characters discussed above has led to fundamental changes in proposed chiton relationships. When characters such as sperm ultrastructure are employed, taxon sampling becomes an issue because data are not available for many terminals. Furthermore, many characters employed for chiton phylogeny are apomorphic for the ingroup, making outgroup polarization difficult, and the outgroup relationships are still debated (e.g. Scheltema 1996 versus Haszprunar 2000). While the morphological cladistic analysis of Buckland-Nicks (1995) illustrated in Fig. 1 constituted the basis for a classification based on egg, sperm and gill characters, molecular analysis of chiton phylogeny enables independent testing of such morphological hypotheses.

Relationships among chitons based on the combination of the five genes studied here (18S rRNA, 28S rRNA, 16S rRNA, COI, and histone H3) are mostly congruent with the results based on egg hull, sperm and gill morphology, supporting the results of modern chiton phylogenetic studies (Sirenko 1993, 1997; Buckland-Nicks 1995) (Fig. 8). For the combined data set all analyses except parameter set 241 indicate that chitons split into two main lineages, one containing Lepidopleuridae + *Callochiton*, and another clade containing the remaining Chitonida sensu Sirenko. The Chitonida includes the clade Chitonina, while the monophyly of Acanthochi-

tonina remains unclear. The positions of *Callochiton septemvalvis, Schizochiton incisus* and *Ischnochiton rissoi* deserve further discussion.

Lepidopleuridae

The monophyly of sampled species of Lepidopleuridae is a noncontroversial result (Figs. 5–8) supported by classical taxonomy (Van Belle 1983) as well as recent classification (Starobogatov & Sirenko 1975; Sirenko 1993, 1997). Only the results from the H3 partition conflict with the monophyly of the family (Fig. 4). The position of Lepidopleuridae as sister group to most other chitons (with the exception of *Callochiton*, see discussion below) is corroborated by the recent cladistic hypothesis of Buckland-Nicks (1995). Lepidopleuridae retains plesiomorphic characters (based on outgroup comparison) in sperm morphology, such as symmetrically arranged mitochondria (Buckland-Nicks 1995). Other sperm characters, such as the prominent acrosome (Buckland-Nicks 1995: Fig. 36D) are shared with members of the Neomeniomorpha, such as *Epimenia australis* (see Buckland-Nicks 1995: Fig. 23), but similar prominent acrosomes are also found in some derived pteriomorphian bivalves (Healy et al. 2000), and the plesiomorphic state within molluscs remains controversial. Lepidopleuridae has smooth eggs and adanal gills restricted to a posterior crown (Starobogatov & Sirenko 1975; Pearse 1979; Sirenko 1993, 1997) (Fig. 8), but it is not clear yet whether these are plesiomorphic or apomorphic character states. In the case of the gills, the topology from Fig. 8 favors the adanal condition to be plesiomorphic, irrespective of the optimization technique employed. The shell plates of both fossil taxa and lepidopleurids lack insertion plates (Sirenko 1997), and this has been suggested to be a plesiomorphy of the group. However, the family Hanleyidae, another putative member of the Lepidopleurida, has insertion plates in some valves, and the topology from Fig. 8 does not distinguish between plesiomorphy and apomorphy of this trait, irrespective of the position of fossil chitons lacking the insertion plates. Investigating the position of members of the Hanleyidae using molecular data may help to resolve this issue.

Callochitonidae

The position of *Callochiton septemvalvis* as sister taxon to the Lepidopleuridae (Figs. 6–8) is especially interesting, because they have a smooth, reduced egg hull similar to that of *Leptochiton* (Sirenko 1993), although data on its sperm are not available. Whether both types of eggs are homologous is uncertain based on the published drawings of *C. septemvalvis* (Sirenko 1993: Fig. 7F), but it seems that neither species has elaborate hulls. A congeneric species, *C. castaneus,* has been reported to have sperm with a long nuclear filament and a reduced acrosome (Buckland-Nicks & Hodgson 2000: Figs. 14–15) as in the other members of Chitonida sensu Sirenko, but with symmetrically arranged mitochondria (Buckland-Nicks & Hodgson 2000: Fig. 16) as in *Leptochiton* and most outgroups. *C. castaneus* also has a smooth egg like that of *C. septemvalvis* and the members of Lepidopleuridae (Buckland-Nicks & Hodgson 2000). It has been suggested that *Callochiton* may represent an 'intermediate' form between the lepidopleurids and the more 'derived' Chitonida. Another example of sperm morphology has been observed in the lepidopleurid *Deshayesiella curvata*, which has sperm with a short nuclear filament, a prominent acrosome, and symmetrically arranged mitochondria (Pashchenko & Drozdov 1998: Figs. 8B, 10, 15). *Callochiton* was recognized to be distinct from Chitonina by Buckland-Nicks (1995), and it was later suggested to be basal to Chitonida (Buckland-Nicks & Hodgson 2000). However, species of *Callochiton* have chitonid-like valves and lateral gill placement.

While the jackknife support value for the position of *C. septemvalvis* as sister taxon to Lepidopleuridae is 64% (Fig. 6), this relationship is stable to parameter variation and methods of analysis explored. With respect to the contribution of the different molecular partitions to the relationship of Lepidopleuridae and *Callochiton*, COI recognizes a clade formed by *Lepidopleurus* + *Callochiton* (*Leptochiton* not represented in the COI analysis; Fig. 3). Monophyly of *Lepidopleurus* + *Leptochiton* is obtained for all analyses based on the nuclear ribosomal genes, but none of these analyses yields monophyly of *Callochiton* + Lepidopleuridae (although several parameter sets show a convex relationship of these taxa). Monophyly of *Callochiton* + Lepidopleuridae is not supported by the histone H3 data partition under any analytical conditions.

The alternative hypothesis of monophyly of Chitonida sensu Sirenko, however, is less supported by the present data. The only partition that places *Callochiton* within a clade of Chitonida (but Chitonida appearing polyphyletic) is histone H3. With the data in hand it seems plausible that *Callochiton* does not belong with the other Chitonida, and therefore some morphological characters supporting Chitonida sensu Sirenko might be plesiomorphic. In the future, more intense sampling within *Callochiton* and Lepidopleurida (especially *Hanleya*, *Deshayesiella* and *Ferreiraella*) should contribute to better resolve relationships among these interesting chitons.

Chitonida

Our results corroborate a clade of Chitonida (excluding *Callochiton*) that is united by the presence of eggs with elaborate hull processes and sperm with the following

features: a long, filamentous nucleus, an acrosome consisting of only a small vesicle, asymmetrically arranged mitochondria, and a thickening of the flagellum at the base (Eernisse 1984, Buckland-Nicks et al. 1990, Sirenko 1993, Eernisse & Reynolds 1994, Buckland-Nicks 1995, Buckland-Nicks & Scheltema 1995, Pashchenko & Drozdov 1998) (Fig. 8). Classical characters supporting Chitonida (including *Callochiton*) include the slitted insertion plates and the lack of posterior (circumanal) gills (= lateral gills), but our molecular results conflict with the inclusion of *Callochiton* as a member of Chitonida. Monophyly of the more restricted grouping without *Callochiton* (the "elaborate hull clade") is found under most parameter sets for the combined analysis of all data, and at least for some parameter sets in the analyses of 16S rRNA, histone H3, and nuclear ribosomal genes. In some cases the position of *Callochiton* appears within Chitonida (parameter sets 421 and 441 of the ribosomal partition, and all parameter sets for H3). However, possibly due to the instability of *Schizochiton*, Chitonida receives low jackknife support (Fig. 6).

It cannot be concluded whether the clade Acanthochitonina sensu Sirenko (1993; clades A + B here) is monophyletic or paraphyletic, because support for either relationship differs among the trees obtained under the optimal and the immediately suboptimal parameter sets (Figs. 6–8). The subclades Mopalioidea and Cryptoplacoidea (Sirenko 1997) are not obtained in any analysis. Nonetheless, all topologies mostly agree in the presence of two subclades within Acanthochitonina; members of one group have simple, round to weakly hexagonal cupules of the egg hull (clade A: *Plaxiphora, Acanthochitona, Nuttallochiton, Cryptoplax*), whereas members of the other group (clade B: *Tonicella, Mopalia, Katharina, Cryptochiton*) (Sirenko 1993) have egg hulls with hexagonal cupules and projections of the hexagon edges (e.g. Sirenko 1993: Figs. 8, 10). The genus *Lepidochitona* is variable, however; egg cupules of *L. cinerea* were illustrated by Durfort et al. (1982) as being closed, but other authors have shown *L. cinerea* to have open cupules (e.g. Eernisse 1984). The specimens of *L. cinerea* sequenced for the present study were collected in an area near those illustrated by Durfort et al. (1982), therefore we chose to represent this type of egg hull in Fig. 8. Buckland-Nicks (1993) illustrated mature eggs with closed cupules for three other *Lepidochitona* species. The position of *Schizochiton incisus* is intriguing, because the species combines egg hulls with simple cupules (Sirenko 1993), characteristic of some members of Acanthochitonina, with adanal gills, characteristic of Chitonina (Figs. 6–8). *Schizochiton* is the only genus with adanal gills that has an egg hull with cupules, and it has been given its own superfamily status within Chitonina, Schizochitonoidea Sirenko, 1997. The sperm morphology of *Schizochiton incisus* is unknown. Interestingly, the position of this species is parameter dependent (see Figs. 6–7).

Members of the most speciose clade Chitonina (clade C here) are monophyletic, they have been grouped in the superfamily Chitonoidea by Sirenko (1997). The members of Chitonoidea share an adanal gill placement (also found in *Callochiton* and *Schizochiton*), various shapes of spiny egg hulls, and sperm of type II with a long, filamentous nucleus, asymmetrically arranged mitochondria, a minute acrosomal vesicle, and an offset flagellum (Fig. 8). In the clade that includes *Lepidozona, Stenoplax* and *Callistochiton* the distal ends of the egg hull are split in two or more long, finger-like projections, whereas in the clade that contains *Ischnochiton* (except for *I. rissoi*, see below), *Tonicia, Liolophura, Lorica, Chiton,* and *Sypharochiton* they are tulip-shaped with jagged edges or shorter projections (Sirenko 1993). In *Acanthopleura* the tip is further specialized into a spine (Sirenko 1993).

Ischnochiton rissoi, which has an egg hull with distal ends bifurcating with curly, long, finger-like projections, does not group with the other *Ischnochiton* species, and is instead the sister group to *Chaetopleura*, which has spiralling, complex, spiny egg hull projections (Figs. 6–8). The egg hull spines of *I. rissoi* are morphologically more similar to those of *Lepidozona* species (Sirenko 1993), and are quite distinct from the spines with tulip-shaped distal ends of other *Ischnochiton* species (*I. australis, I. comptus, I. elongatus*). The position of *Lepidozona mertensii* in the maximum-likelihood tree is incongruent with this result. Its position within Chitonina, supported by the optimal and equal-weights parsimony trees is more congruent with morphology, but jackknife support for the position of *Lepidozona* is below 50% in both analyses.

Concluding remarks

Our results indicate that there is a strong correlation of egg hull morphology with the molecular phylogenetic trees here presented. Egg hull morphology has been suggested to show little homoplasy (Eernisse 1984, Buckland-Nicks & Eernisse 1993, Sirenko 1993, Eernisse & Reynolds 1994) even when hulls are partially reduced or modified among brooding species, such as in *Lepidochitona fernaldi*, *Ischnochiton inca* (= *Ischnochiton stramineus* (Sowerby 1832), fide Kaas & Van Belle 1990), *I. mayi, Hanleyella asiatica, Schizoplax brandtii*, and *Placiphorella borealis* (Eernisse 1988, Sirenko 1993). For example, *L. fernaldi*, a brooding species of *Lepidochitona*, has egg hulls that are reduced to plates but still clearly show the strong hexagonal septa of the non-brooding species such as in *L. cinerea.* Detailed studies on sperm ultrastructure still need to be carried out for many chiton taxa.

Recent efforts in utilizing egg hull, sperm, and gill placement as indication for phylogenetic relationships revealed that classical higher taxonomical groupings are often artificial. Our topologies corroborate this finding and refute monophyly of many classical taxonomical groups sensu Kaas & Van Belle, such as Ischnochitonina and Acanthochitonina at the subordinal rank, Ischnochitonidae, Mopaliidae, Chitonidae, and Acanthochitonidae at family level, and Lepidochitoninae, Chaetopleurinae, Ischnochitoninae, Mopaliinae, and Chitoninae at subfamily level. As it was previously suggested, some members of "Ischnochitonina" have closer affinities with those members of "Acanthochitonina" that share similar egg and gill placement characters (Eernisse 1984, Eernisse & Reynolds 1994).

The phylogenetic analysis of chitons based on five molecular loci analyzed simultaneously clearly supports monophyly of Polyplacophora, and therein of Lepidopleuridae, and Chitonida, and there is evidence for the monophyly of Chitonina as well as Chitonoidea. However, the chitonid *Callochiton* is sister-taxon to Lepidopleuridae. The position of *Schizochiton* is unstable, as it falls outside of Chitonoidea under some analytical parameters. The monophyly of Acanthochitonina is unsettled, however, two distinct subclades within Acanthochitona (clades A and B) are recognized.

Phylogenetic studies of Polyplacophora based on morphology are scarce. Hence the addition of an independent source of evidence such as molecular data is important. While this analysis points towards interesting issues in chiton systematics, it mostly serves as a first step towards further analyses of morphological and molecular attributes.

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Appendix 1

Voucher data for specimens used in this study. Sequence of taxa as in Table 1. Abbreviations: AMNH = American Museum of Natural History, New York; ESC = Enrico Schwabe private collection, Munich, Germany; MCZ = Museum of Comparative Zoology, Harvard University, Cambridge, MA, USA; ZSM = Zoologische Staatssammlung München (the Bavarian State Zoological Collections), Munich, Germany.

Chaetoderma nitidulum Lovén, 1845 – Kristineberg (Sweden); January 1998; A. Okusu & A. Scheltema leg.; MCZ DNA100838
Prochaetoderma sp. – 1996; supplied by D. McHugh; MCZ DNA100839
Helicoradomenia sp. – 1998; supplied by A. Scheltema; MCZ DNA100840
Epimenia n. sp. – Amakusa (Kumamoto, Japan); August 2000; A. Okusu leg.; MCZ DNA100842
Epimenia babai Salvini-Plawen, 1997 – Amakusa (Kumamoto, Japan); August 2000; A. Okusu leg.; MCZ DNA100843
Epimenia australis (Thiele, 1897) – Madang (Papua New Guinea); A. Scheltema leg.; MCZ DNA100841
Lepidopleurus cajetanus (Poli, 1791) – Banyuls sur Mer (Languedoc-Roussillon, France); 6 June 1997; G. Giribet leg.; MCZ DNA100108
Leptochiton asellus (Gmelin, 1791) – Tjärnö (Sweden); July 2000; A. Wanninger leg.; ZSM20008014; MCZ DNA100830
Callochiton septemvalvis (Montagu, 1803) – Cabo de Gata (San José, Spain); 30 m depth; 1994; C. Palacín leg.; MCZ DNA100831
Tonicia lamellosa (Quoy & Gaimard, 1835) – 6°3′52″S;

124°23′27″E; near wreck of Nieuwkerk, north coast of Koka Atoll, Flores Sea (Indonesia); ESC 1045 (j); MCZ DNA100520

Chaetopleura apiculata (Say in Conrad, 1834) – Old Silver Beach, Falmouth (MA, USA); July 2000; A. Okusu leg.; MCZ DNA100833

Chaetopleura angulata (Spengler, 1797) – 42°17′48″N 8°49′18″W; playa de Menduíña, Ría de Aldán, (Pontevedra, Spain); 21 July 2002; G. Giribet leg.; MCZ DNA100564

Ischnochiton comptus (Gould, 1859) – Amakusa (Kumamoto, Japan); August 2000; A. Okusu leg.; MCZ DNA100834

Ischnochiton australis (Sowerby, 1840) – 33°51.18′S 151°16′00″E; Nielsen Park Shore, Port Jackson, Sydney Harbor (Sydney, NSW, Australia); 2–6 m; kelp forest; 12 April 2000; G. Giribet leg.; MCZ DNA100835

Ischnochiton elongatus (de Blainville, 1825) – 33°51.18′S 151°16′00″E; Nielsen Park Shore, Port Jackson, Sydney Harbor (Sydney, NSW, Australia); 12 April 2000; G. Giribet leg.; MCZ DNA100576

Ischnochiton rissoi (Payraudeau, 1826) – Porto Cristo (Mallorca, Spain); 45 m; November 1996; G. Giribet leg.; MCZ DNA100573

Lepidozona mertensii (von Middendorff, 1847) – Monastery Beach, Carmel (CA, USA); August 2000; G. Giribet leg.; MCZ DNA100584

Stenoplax alata (Sowerby, 1841) – 8°30′803″ S 124°03′460″E; under basalt rocks; SE Panter Island (Cape Boda, Indonesia); 5–7 m; ESC 28 (a); MCZ DNA100582

Callistochition antiquus (Reeve, 1847) – 33°51′18′S 151°16′00E; Port Jackson, Nielsen Park Shore (Sydney, NSW, Australia); 12 April 2000; G. Giribet leg.; MCZ DNA100579

Schizochiton incisus (Sowerby, 1841) – 8°14′50″S 124°6′00″E; north entrance Alor Strait (Batang Island, Indonesia); 0.02–3.8 m; ESC 208; MCZ DNA100521

Lorica volvox (Reeve, 1847) – Coles Bay, east coast (Tasmania); 12 m; ESC 1576 (c); MCZ DNA100571

Chiton olivaceus Spengler, 1797 – Tossa de Mar (Girona, Spain); 6 June 1997; G. Giribet leg.; MCZ DNA100157

Liolophura japonica (Lischke, 1873) – Amakusa (Kumamoto, Japan); August 2000; A. Okusu leg.; MCZ DNA100836

Sypharochiton pellisserpentis (Quoy & Gaimard, 1836) – Cooge Beach (Sydney, NSW, Australia); 19 March 2000; G. Giribet leg.; MCZ DNA100513

Acanthopleura granulata (Gmelin, 1791) – Cozumel Island (Quintana Roo, Mexico); January 1998; G. Giribet leg.; MCZ DNA100511

Mopalia muscosa (Gould, 1846) – Palos-Verdes (CA, USA); November 1999; G. Giribet leg.; MCZ DNA100522

Nuttallochiton mirandus (Thiele, 1906) – Station 136-1: 70°50′20″S 10°35′40″W; 271 m; M. Schrödl leg.; ZSM20008500; MCZ DNA100574

Plaxiphora albida (de Blainville, 1825) – Cooge Beach (Sydney, NSW, Australia); 19 March 2000; G. Giribet leg.; MCZ DNA100578

Katharina tunicata (Wood, 1815) – Bodega Bay (CA, USA); 2 April 2002; A. Lindgren leg.; MCZ DNA100599

Tonicella lineata (Wood, 1815) – Monastery Beach, Carmel (CA, USA); 11 August 2000; G. Giribet leg.; MCZ DNA100580

Lepidochitona cinerea (Linnaeus, 1767) – Cala Fosca, Palamós (Girona, Spain); 21 May 1993; G. Giribet leg.; MCZ DNA100832

Acanthochitona crinita (Pennant, 1777) – Blanes (Girona, Spain); 31 July 1997; G. Giribet & C. Palacín leg.; MCZ DNA100109

Cryptochiton stelleri (von Middendorff, 1847) – Bodega Bay (CA, USA); 2 April 2002; A. Lindgren leg.; MCZ DNA100592

Cryptoplax japonica Pilsbry, 1901 – Amakusa (Kumamoto, Japan); August 2000; A. Okusu, leg.; MCZ DNA100837

Cocculina messingi McLean & Harasewych, 1995 – Smithsonian Institution; M. G. Harasewych leg.; MCZ DNA100663

Entemnotrochus adansonianus (Crosse & Fischer, 1861) – Smithsonian Institution; M. G. Harasewych leg.; MCZ DNA100665

Diodora graeca (Linnaeus, 1758) – Tossa de Mar (Girona, Spain), 5 August 1997; G. Giribet & C. Palacín leg.; MCZ DNA100114

Haliotis tuberculata Linnaeus, 1758 – Tossa de Mar (Girona, Spain), 6 June 1997; G. Giribet leg.; MCZ DNA100110

Sinezona confusa Rolán & Luque, 1994 – Florida (USA), May 1998; M. G. Harasewych leg.

Viviparus georgianus (Lea, 1834) – Smithsonian Institution Acc. 420309; M. G. Harasewych leg.; MCZ DNA100112

Crepidula fornicata (Linnaeus, 1758) – Woods Hole (Massachusetts, USA), December 1997; purchased from Marine Biological Laboratory; MCZ DNA100119

Siphonaria pectinata (Linnaeus, 1758) – El Puerto de Santa María (Cádiz, Spain), 27 April 1993; G. Giribet leg.; MCZ DNA100660

Solemya velum Say, 1822 – Woods Hole (Massachusetts, USA), December 1997; purchased from Marine Biological Laboratory; MCZ DNA100116

Nucula proxima Say, 1822 – Beaufort (North Carolina, USA); DNA from D. Campbell.

Nuculana pernula (Müller, 1779) – between Blåbergsholmen and Lysekil (Gullmaren, Bohuslän, Sweden); O. Israelson leg.; MCZ DNA100121

Yoldia limatula (Say, 1831) – Woods Hole (Massachusetts, USA), December 1997; purchased from Marine Biological Laboratory; MCZ DNA100119

Mytilus edulis Linnaeus, 1758 – 28S rRNA sequence data from Woods Hole (Massachusetts, USA), December 1997; purchased from Marine Biological Laboratory [18S rRNA and COI sequence data from GenBank]; MCZ DNA100122

Limaria hians (Gmelin, 1791) – Roses (Girona, Spain), 13 May 1998; G. Giribet leg.; MCZ DNA100129

Pecten jacobaeus (Linnaeus, 1758) – Banyuls Sur Mer (Languedoc-Roussillon, France), July 2000; G. Giribet leg.; MCZ DNA100085

Neotrigonia margaritacea (Lamarck, 1804) – D'Entrecasteau channel (Tasmania, Australia), 19 April 2000; The Marine Discovery Centre (contacted through L. Turner, Tasmanian Museum); dredged [COI sequence data from GenBank]. MCZ DNA100031

Cardita calyculata (Linnaeus, 1758) – Roses (Girona, Spain), 13 May 1998; G. Giribet leg.; MCZ DNA100140

Corbicula fluminea (Müller, 1774) – South Korea, 2 February 1997 (specimens donated by D. Ó Foighil); MCZ DNA100149

Dreissena polymorpha (Pallas, 1771) – Huron River (Michigan, USA), 24 August 1998; D. Ó Foighil leg.; MCZ DNA100143

Mya arenaria Linnaeus, 1758 – Martha's Vineyard (Cape Cod, Massachusetts, USA); MCZ DNA100152

Rhabdus rectius (Carpenter, 1864) – DNA from M. G. Harasewych

Antalis pilsbryi (Rehder, 1942) – DNA from M. G. Harasewych

Dentalium vulgare (da Costa, 1778) – collection data not available

Nautilus pompilius Linnaeus, 1758 – captive specimens from Laboratoire Arago, Banyuls sur Mer (Languedoc-Roussillon, France), September 1995; DNA from M.K. Nishiguchi.

Sepia elegans d'Orbigny, 1838 – Banyuls sur Mer (Languedoc-Roussillon, France), September 1995. DNA from M. K. Nishiguchi.

Loligo pealei Lesueur, 1821 – from fish market. MCZ DNA100115

Architeuthis dux Steenstrup, 1857 – New Zealand, December 1997; AMNH

Octopus joubini Robson, 1929 – Carolina Supplies, 2002