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

Akiko Okusu1,∗, Enrico Schwabe2, Douglas J. Eernisse3, Gonzalo Giribet1

1 Department of Organismic and Evolutionary Biology and Museum of Comparative Zoology, Harvard University, Cambridge, MA, USA
2 Zoologische Staatssammlung München, Germany
3 Department of Biological Science, California State University, Fullerton, CA, USA

Received 15 May 2003 · Accepted 9 September 2003

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 + I 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 anal gills and a spiny egg hull. Schizochiton, the only genus with anal 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-

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: Lepidopleurida, Acanthochitonina (including Acanthochitonidae and Cryptoplacidae), and Ischnochitonidae (the most speciose group, including Ischnochitonidae, Mopaliidae, Chitonidae, and Schizochitonidae). Gowllett-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 extra-cellular 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 Acanthochitonina 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-
organized subclades within Acanthochitonina, separating it into Mopaliaidea and Cryptoplacoidea. He also divided the other suborder of Chitonida, Chitonina, into Chitonina and Schizochitonina.

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 supported 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).

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).

<table>
<thead>
<tr>
<th>Taxon</th>
<th>GenBank Accession Numbers</th>
<th>18S rRNA</th>
<th>28S rRNA</th>
<th>16S rRNA</th>
<th>H3</th>
<th>COI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chaetodermomorpha</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chaetoderma nitidulum</td>
<td>AY377658</td>
<td>AY377692</td>
<td>AY377612</td>
<td>AY377673</td>
<td>AY377726</td>
<td></td>
</tr>
<tr>
<td>Scutopus ventrolineatus</td>
<td>X91977</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prochaetoderma sp.</td>
<td>AY377762</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Neomeniomorpha</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Helicoradomenia sp.</td>
<td>AY21210</td>
<td>AY377688</td>
<td>AY377613</td>
<td>AY377764</td>
<td>AY377725</td>
<td></td>
</tr>
<tr>
<td>Epimenia n. sp.</td>
<td>AY377657</td>
<td>AY377691</td>
<td>AY377615</td>
<td>AY377765</td>
<td>AY377723</td>
<td></td>
</tr>
<tr>
<td>Epimenia babai</td>
<td>AY377690</td>
<td>AY377616</td>
<td>AY377766</td>
<td>AY377724</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epimenia australis</td>
<td>AY377689</td>
<td>AY377614</td>
<td>AY377767</td>
<td>AY377722</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Polyplacophora</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lepidopleurida</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lepidopleuridae</td>
<td>Lepidopleurus cajetanus</td>
<td>AY120502</td>
<td>AY120565</td>
<td>AY377585</td>
<td>AY377735</td>
<td>AY120626</td>
</tr>
<tr>
<td>Leptochiton asellus</td>
<td>AY377631</td>
<td>AY377662</td>
<td>AY377586</td>
<td>AY377734</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Neomeniomorpha</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Helicoradomenia sp.</td>
<td>AY21210</td>
<td>AY377688</td>
<td>AY377613</td>
<td>AY377764</td>
<td>AY377725</td>
<td></td>
</tr>
<tr>
<td>Epimenia n. sp.</td>
<td>AY377657</td>
<td>AY377691</td>
<td>AY377615</td>
<td>AY377765</td>
<td>AY377723</td>
<td></td>
</tr>
<tr>
<td>Epimenia babai</td>
<td>AY377690</td>
<td>AY377616</td>
<td>AY377766</td>
<td>AY377724</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epimenia australis</td>
<td>AY377689</td>
<td>AY377614</td>
<td>AY377767</td>
<td>AY377722</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Polyplacophora</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lepidopleurida</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lepidopleuridae</td>
<td>Lepidopleurus cajetanus</td>
<td>AY120502</td>
<td>AY120565</td>
<td>AY377585</td>
<td>AY377735</td>
<td>AY120626</td>
</tr>
<tr>
<td>Leptochiton asellus</td>
<td>AY377631</td>
<td>AY377662</td>
<td>AY377586</td>
<td>AY377734</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Neomeniomorpha</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Helicoradomenia sp.</td>
<td>AY21210</td>
<td>AY377688</td>
<td>AY377613</td>
<td>AY377764</td>
<td>AY377725</td>
<td></td>
</tr>
<tr>
<td>Epimenia n. sp.</td>
<td>AY377657</td>
<td>AY377691</td>
<td>AY377615</td>
<td>AY377765</td>
<td>AY377723</td>
<td></td>
</tr>
<tr>
<td>Epimenia babai</td>
<td>AY377690</td>
<td>AY377616</td>
<td>AY377766</td>
<td>AY377724</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epimenia australis</td>
<td>AY377689</td>
<td>AY377614</td>
<td>AY377767</td>
<td>AY377722</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Polyplacophora</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lepidopleurida</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lepidopleuridae</td>
<td>Lepidopleurus cajetanus</td>
<td>AY120502</td>
<td>AY120565</td>
<td>AY377585</td>
<td>AY377735</td>
<td>AY120626</td>
</tr>
<tr>
<td>Leptochiton asellus</td>
<td>AY377631</td>
<td>AY377662</td>
<td>AY377586</td>
<td>AY377734</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Neomeniomorpha</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Helicoradomenia sp.</td>
<td>AY21210</td>
<td>AY377688</td>
<td>AY377613</td>
<td>AY377764</td>
<td>AY377725</td>
<td></td>
</tr>
<tr>
<td>Epimenia n. sp.</td>
<td>AY377657</td>
<td>AY377691</td>
<td>AY377615</td>
<td>AY377765</td>
<td>AY377723</td>
<td></td>
</tr>
<tr>
<td>Epimenia babai</td>
<td>AY377690</td>
<td>AY377616</td>
<td>AY377766</td>
<td>AY377724</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epimenia australis</td>
<td>AY377689</td>
<td>AY377614</td>
<td>AY377767</td>
<td>AY377722</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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. The 16S 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 GENE CLEAN 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 the two forward primers, and 1 µM of one of the two reverse primers. The double-stranded PCR products were verified by agarose gel electrophoresis (1% agarose), and purified using GENE CLEAN 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 the two forward primers, and 1 µM of one of the two reverse primers.
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 ‘pre-aligned’ 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 (-pvm –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
terion (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 + $\Gamma$ 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 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 cri-
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 Lepidopleurus cajetanus


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.
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 analyzed supports the following clades: (Lepidopleurus, Callochiton); (Chaetopleura, Lepidozona); and (Acanthochitona, Cryptoplax). Of these clades, (Loquima, Architeuthis), (Nuculana, Yoldia), and (Mya, Dreissenia) 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 clade. For the putative outgroup taxa, all parameter sets support the monophyly of *Epimenes*, Coleoidea, and a clade including certain bivalves + Coleoidea.

Within Polyplacophora, the strict consensus tree of all parameter sets supports monophyly of two large 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, Nuttalochitona, 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 Haliothis + Diadona with jackknife frequencies above 50%. Within Polyplacophora, the optimal parameter set supports, in addition to the clades mentioned above, (Loria (Chiton, Sypharochiton)); Acanthopleura as sister taxon to Lorica – I. comptus complex; and (Katharina, Mopalia, Tonicella, Cryptochiton) as sister taxon to (Acanthochitona, Nuttalochitona, Plaxiphora, Lepidozona, 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, Epimenes, Chaetodermomorpha, Veti gastropoda, (Li maria, Pecten), Euheterodonta, and Nuculanoidea. Within Polyplacophora, all trees agree in the monophyly of clades such as Lepidopleuridae (Lepidopleurus, Lepto choiton); 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-
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 Chaetodermoderma, Polyplacophora, Neomeniomorpha, *Epinema*, Cephalopoda, Coleoidea, Nuculanoidea, a bivalve-heterodont clade containing *Corbula, Mya*, and *Dreissenia*, 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-
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 + \( \Gamma \)) 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,
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
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, Nuttalochiton, Acanthochiton*,...
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 Eenisse (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 Cryptitonina; 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 Lepidodroma mertensi, 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. 7A). Within Acanthochitonina, monophyly of Mopalia and Cryptoplacoidea is not supported in any topology under any analytical conditions. Within the Chitonina, Ichschonchiton rissoti is the sister group to Chaetopleura in all these three topologies, separated from the other ichschonchitoniids. The position of the chitonoid 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 Lepidodroma mertensi (nests within clade B) and the internal relationships of Acanthochitonina (Nuttallochiton mirandus is sister taxon to Acanthochitonina 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 *Epimena 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 *De- shayesiella 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 clad 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 clad 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 clad 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 Mopaliaidea 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, Acanthochitonina, Nuttalochiton, 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 Lepidochiton 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 Lepidochitonina 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 superfamilial status within Chitonina, Schizochitonoida 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 L. 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 L. 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 (L. austraulis, 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 insca (= Ischnochiton stramineus (Sowerby 1832), fide Kaas & Van Belle 1990), L. mayi, Hanleyella asiatica, Schizoplax brandtii, and Placiphorella borealis (Eernisse 1988, Sirenko 1993). For example, L. fernaldi, a brooding species of Lepidochitonina, 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 Acanthochitonina (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.

Acknowledgements

We thank Michele Nishiguchi, Annie Lindgren and Cruz Palacín for assisting in collecting specimens of chitons, and Janet Voight and Damhnait McHugh for supplying Helicoradomenia and Prochaetoderma specimens. Jerry Harasewych, Diarmaid Ó Foighil, Olle Israëlsson and Liz Turner assisted with collecting some of the outgroup taxa. Also special thanks to Amélie Scheltema, Andy Knoll, and Damhnait McHugh for their comments on earlier versions of this manuscript, and to Gerhard Haszprunar and two anonymous reviewers for their helpful comments and discussions.

References


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;


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 nitidulun Lovén, 1845 – Kristineberg (Sweden); January 1998; A. Okusu & A. Scheltema leg.; MCZ DNA100838

Prochaetoderma sp. – 1996; supplied by D. McHugh; MCZ DNA100839

Helicoradomena 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. Okusu leg.; MCZ DNA100842

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. Okusu leg.; MCZ DNA100842

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. Okusu leg.; MCZ DNA100842

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. Okusu leg.; MCZ DNA100842

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 (f); MCZ DNA100520

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

*Chaetopleura angulata* (Spengler, 1797) – 42°17'48"N 8°49'18"W; playa de Menduíña, Ria de Aldán, (Pontevedra, Spain); 21 July 2002; G. Gitibet leg.; MCZ DNA100564

*Ischnochiton comptus* (Gould, 1859) – Amakusa (Kumamoto, Japan); August 2000; A. Okusу 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. Gitibet 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. Gitibet leg.; MCZ DNA100576

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

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

*Stenoplaix alata* (Sowerby, 1841) – 8°30'30"S 124°03'46"E; under basalt rocks; SE Panter Island (Cape Boda, Indonesia); 5-7 m; ESC 28 (a); MCZ DNA100582

*Calliostomum antiquum* (Reeve, 1847) – 33°51'18"S 151°16'00"E; Port Jackson, Nielsen Park Shore (Sydney, NSW, Australia); 12 April 2000; G. Gitibet 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 (a); MCZ DNA100582

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

*Liochlamys japonica* (Lischke, 1873) – Amakusa (Kumamoto, Japan); August 1997; G. Giribet & C. Palacín leg.; MCZ DNA100832

*Chiton olivaceus* Spengler, 1797 – Tossa de Mar (Girona, Spain); 24 August 1998; D. Ó Foighil leg.; MCZ DNA100143

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

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

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

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

*Pecten jacobaeus* Linnaeus, 1758 – Banyuls Sur Mer (Languedoc-Roussillon, France), July 2000; G. Gitibet 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 DNA100311

*Cardita calculata* (Linnaeus, 1758) – Roses (Girona, Spain), 13 May 1998; G. Gitibet leg.; MCZ DNA100140

*Carcinoplax gouldi* Robson, 1929 – Carolina Supplies, 2002

*Chaetopterus variopedatus* (Ehrenberg, 1831) – Hokianga Harbour (Northland, New Zealand), 16 August 1999; G. Giribet leg.; MCZ DNA100107

*Limaria hians* (Gmelin, 1791) – A. Okusу leg.; MCZ DNA100571

*Sypharochiton pellisserpentis* (Quoy & Gaimard, 1836) – Coogee Beach (Sydney, NSW, Australia); 19 March 2000; G. Gitibet leg.; MCZ DNA100836

*Pecten maximus* (Gould, 1846) – Palos-Verdes (CA, USA); November 1998; G. Gitibet leg.; MCZ DNA100522

*Nuttallotrochus mirandus* (Thiele, 1906) – Station 136-1: 70°50 ′S 151°16′ W; 271 m; M. Schrödl leg.; ZSM20008500; MCZ DNA100574

*Plaqiphora albida* (de Blainville, 1825) – Cooge Beach (Sydney, NSW, Australia); 19 March 2000; G. Gitibet 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. Gitibet leg.; MCZ DNA100580

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

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

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

*Clypeolopha japonica* Pilsbry, 1901 – Amakusa (Kumamoto, Japan); August 2000; A. Okusу leg.; MCZ DNA100837

*Coccinina messingi* M.Clean & Harasewych, 1995 – Smithsonian Institution; M. G. Harasewych leg.; MCZ DNA100663

*Entemmochitona 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

*Hallitotus tuberculatus* 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.