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Evolution in the deep sea: a combined analysis of the earliest diverging living chitons (Mollusca : Polyplacophora : Lepidopleurida)

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Abstract. Lepidopleurida is the earliest diverged group of living polyplacophoran molluscs. They are found predominantly in the deep sea, including sunken wood, cold seeps, other abyssal habitats, and a few species are found in shallow water. The group is morphologically identified by anatomical features of their gills, sensory aesthetes, and gametes. Their shell features closely resemble the oldest fossils that can be identified as modern polyplacophorans. We present the first molecular phylogenetic study of this group, and also the first combined phylogenetic analysis for any chiton, including three gene regions and 69 morphological characters. The results show that Lepidopleurida is unambiguously monophyletic, and the nine genera fall into five distinct clades, which partly support the current view of polyplacophoran taxonomy. The genus *Hanleyella* Sirenko, 1973 is included in the family Protochitonidae, and Ferreiraellidae constitutes another distinct clade. The large cosmopolitan genus *Leptochiton* Gray, 1847 is not monophyletic; *Leptochiton* and Leptochitonidae *sensu stricto* are restricted to North Atlantic and Mediterranean taxa. Leptochitonidae *s. str*. is sister to Protochitonidae. The results also suggest two separate clades independently inhabiting sunken wood substrates in the south-west Pacific. Antarctic and other chemosynthetic-dwelling species may be derived from wood-living species. Substantial taxonomic revision remains to be done to resolve lepidopleuran classification, but the phylogeny presented here is a dramatic step forward in clarifying the relationships within this interesting group.

Introduction

Polyplacophora (chitons) represent a distinctive molluscan clade living in marine environments worldwide, with a fossil record extending 500 million years (Runnegar *et al*. 1979; Sigwart and Sutton 2007). The earliest derived living order (sister group to all other taxa), Lepidopleurida comprises a large assemblage of chitons that share features with fossil shells, and are morphologically supported by their special (usually posterior) adanal gill arrangement, simple gamete structures, and aesthete innervation (Sirenko 1993, 2006; Buckland-Nicks 2006; Sigwart 2008). These features separate Lepidopleurida from all other living chitons, which are in the order Chitonida (Sirenko 2006). Approximately 130 living species are known within Lepidopleurida, all within the extant suborder Lepidopleurina (Sirenko 2001, 2006); however, genera or other subgroups often lack consistent morphological synapomorphies (Fig. 1).

Molecular studies on chitons are scarce. To date, a single study has focussed on higher-level relationships within Polyplacophora using DNA sequence data (Okusu *et al*. 2003). Other studies have centred on species identification particularly within the genus *Mopalia* Gray, 1847, which excludes lepidopleuran taxa (Kelly *et al*. 2007; Kelly and Eernisse 2008), or incidentally included multiple chitons in investigating the higher-level relationships within Mollusca (e.g. Passamaneck *et al*. 2004; Giribet *et al*. 2006; Wilson *et al*. 2010). Lepidopleuran taxa in these studies are usually limited to *Lepidopleurus cajetanus* (Poli, 1791) and *Leptochiton asellus* (Gmelin, 1791), which are shallow water, European species and common compared with most species in the group.

The aim of this study was to focus on one manageable aspect of chiton phylogeny, the order Lepidopleurida, by testing the internal relationships within this clade with a far larger taxon sampling than has been included in any previous study. We included nine of the ten putative lepidopleuran genera, which are primarily deep sea species (Schwabe 2008*a*). The sequencing efforts focussed on three phylogenetically informative regions:

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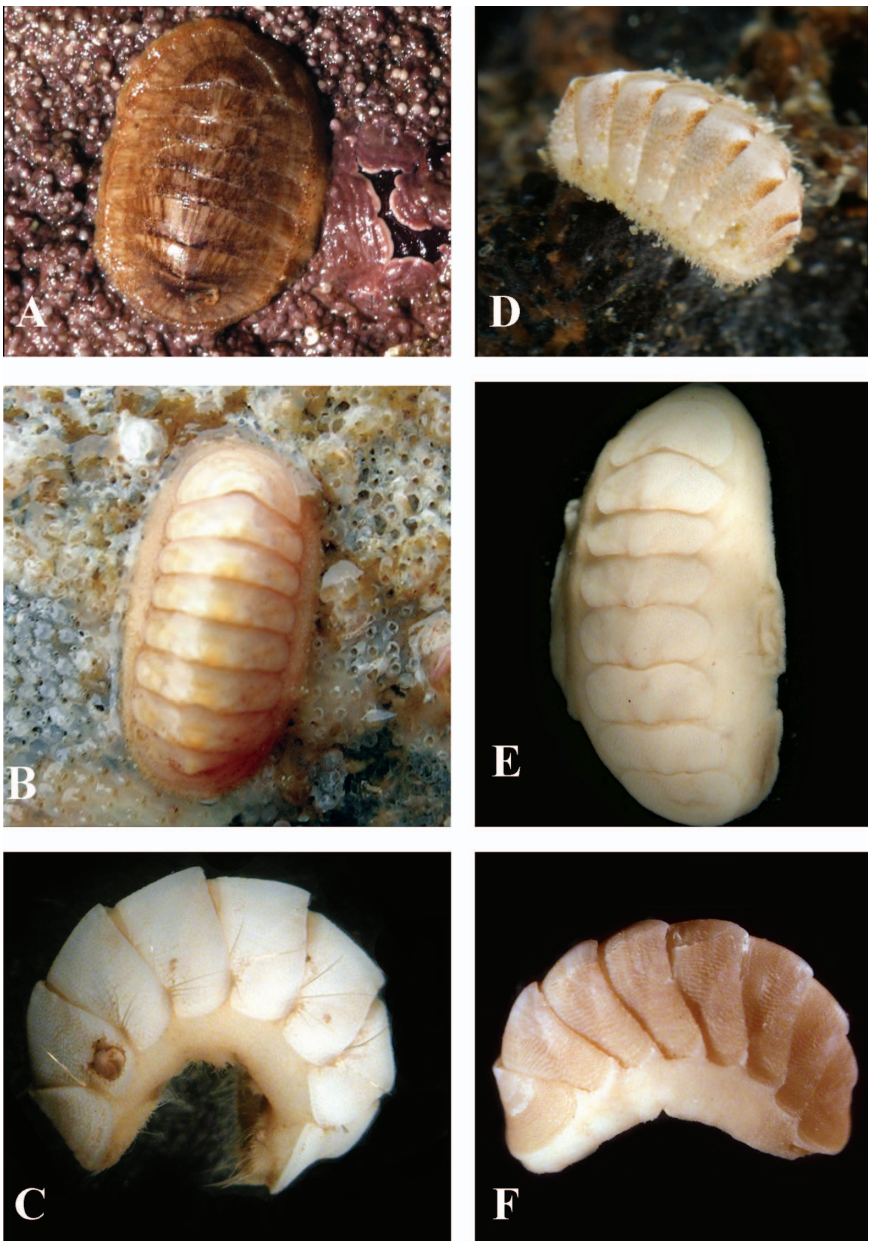

Fig. 1. Examples of chitons in the order Lepidopleurida, representing the major groups resolved in the present analysis. In all images, the anterior end is to the left, or top. (*A*) Leptochitonidae *s. str.*: *Leptochiton asellus*, Strangford Lough, Northern Ireland, intertidal. (*B*) Clade I: *Leptochiton rugatus*, Sooke, Vancouver Island, Canada, intertidal. (*C*) Clade I: *Leptochiton boucheti*, Vanuatu, 667–750 m. (*D*) Protochitonidae: *Hanleyella oldroydi*, Cortes Bank, CA, USA, 367–389 m. (*E*) Ferreiraellidae: *Ferreiraella plana*, Vanutau, 630–705 m. (*F*) Clade II: *Nierstraszella lineata*, Solomon Islands, 490–520 m. Photos by J. D. Sigwart, except *D*, photo by G. Giribet.

complete 18S rRNA (~1800 bp), a large fragment of 28S rRNA (~2200 bp, compared with the ~300 bp used by Okusu *et al.* 2003), and the mitochondrial protein-coding gene cytochrome *c* oxidase subunit I (COI; 650 bp). We also utilised a morphological data matrix for the sampled taxa and combined the morphological and molecular data in the first combined analysis for the class Polyplacophora.

Materials and methods

Taxon selection

In total, 57 specimens from 38 ingroup species were treated for this study, including museum specimens fixed in ethanol, and original field collections of live animals (Table 1). Species level identifications for all specimens were verified by their

Table 1. Taxonomic arrangement of the polyplacophoran suborder Lepidopleurina (order Lepidopleurida)
This table includes only living genera and families; genera in **bold** are included in the present study. Modified from Sirenko (2006)

Suborder	Family	Genus
Lepidopleurina Thiele, 1909	Ferreiraellidae Dell'Angelo & Palazzi, 1991	***Ferreiraella*** Sirenko, 1988
	Hanleyidae Bergenhayn, 1955	***Hanleya*** Gray, 1857
	Leptochitonidae Dall, 1889	***Lepidopleurus*** Risso, 1826
		Leptochiton Gray, 1847
		Parachiton Thiele, 1909
		Pilsbryella Nierstrasz, 1905
	Nierstraszellidae Sirenko, 1993	***Nierstraszella*** Sirenko, 1993
	Protochitonidae Ashby, 1925	***Deshayesiella*** Carpenter in Dall, 1879
		Oldroydia Dall, 1894
		[A]***Hanleyella*** Sirenko, 1973

[A]Based on the results of the present study, *Hanleyella* is tentatively included in Protochitonidae rather than Leptochitonidae.

morphology. All specimens were fixed in 70–99% EtOH and preserved in 80–99% EtOH at −80°C. Additional outgroup taxa representing Chitonida (Chitonina and Acanthochitonina) were selected to represent uncontroversial major groups, as well as the genus *Callochiton* Gray, 1847, which has previously been resolved as the immediate sister group to Lepidopleurida (Okusu *et al*. 2003), or sister to the remaining Chitonida (Buckland-Nicks 2006, 2008; Giribet *et al*. 2006; Wilson *et al*. 2010). Two specimens of *Leptochiton medinae* (Plate, 1899) were combined into a single terminal for the molecular study, as they did not provide overlapping in the amplified fragments.

DNA extraction, amplification, and sequencing

A small tissue sample was removed for each specimen from the muscle tissue of the foot or girdle. For small-bodied taxa (<6 mm long) a large portion of the animal body was used for DNA extraction. Total DNA was extracted using the DNeasy Tissue Kit (QIAGEN, Valencia, CA) using the standard protocol for extraction and purification recommended by the supplier. The purified total DNA was amplified in the target gene fragments using polymerase chain reaction (PCR; see primers in Table 2).

Two nuclear ribosomal genes (nearly complete 18S rRNA and a 2 Kb fragment of 28S rRNA) were amplified in three overlapping fragments each using the primers described in Edgecombe and Giribet (2006). In addition, the mitochondrial protein-coding gene cytochrome *c* oxidase subunit I (COI) was amplified as a single fragment using the primer pair LCO1490/HCO2198 (Folmer *et al*. 1994).

Polymerase chain reactions were performed in 50 µL volume, including: 2 µL of the purified template DNA, 1 µM of each primer (0.5 µL of 20 µm stock), 200 µM of each dNTP (Invitrogen), 1× PCR buffer containing 1.5 mM $MgCl_2$ (Perkin Elmer), 1.25 units of AmpliTaq DNA polymerase (Perkin Elmer, Norwalk, CT), and ddH_2O. The PCR were performed on a GeneAmp PCR System 9700 thermal cycler, using a thermal cycling regime based on the protocol developed by Okusu *et al*. (2003). The cycle included an initial denaturation step (5 min at 95°C) followed by 35 cycles of denaturation (95°C for 30 s), annealing (30 s at 44–46°C, experimentally determined for each sample), and extension (72°C for 1 min). After the 35 cycles were completed there was a final extension step at 72°C for 1 min. Polymerase chain reaction products were visualised by electrophoresis in a 1% agarose gel. Successfully amplified products were then purified using the QIAquick PCR purification kit (QIAGEN).

Table 2. Universal primer sequences used for DNA amplification
Each of the three fragments for the two ribosomal genes was maintained as an independent input file (see also Table 3). The relative position of primers for 18S rRNA are based on the sequence of *Limulus polyphemus* (GenBank accession L81949) and for 28S rRNA are based on the complete sequence of *L. polyphemus* (AF212167) (see map of 28S rRNA primers in Giribet and Shear 2010)

Gene fragment and primer name	Sequence position	Primer sequence (5′–3′)
18Sa: 1F	1 bp	TAC CTG GTT GAT CCT GCC AGT AG
18Sb: 3F	376 bp	GTT CGA TTC CGG AGA GGG A
18Sa: 4R	569 bp	GAA TTA CCG CGG CTG CTG G
18Sb: 7R	1421 bp	GCA TCA CAG ACC TGT TAT TGC
18Sb: 18Sbi	1319 bp	GAG TCT CGT TCG TTA TCG GA
18Sc: 18Sa2.0	1120 bp	ATG GTT GCA AAG CTG AAA C
18Sc: 9R	1781 bp	GAT CCT TCC GCA GGT TCA CCT AC
28Sa: 28S rd1a	26 bp	CCC SCG TAA YTT AAG CAT AT
28Sa: 28S rd4b	888 bp	CCT TGG TCC GTG TTT CAA GAC
28Sa: 28Sb	1220 bp	TCG GAA GGA ACC AGC TAC
28Sb: 28Sa	888 bp	GAC CCG TCT TGA AGC ACG
28Sb: 28S rd5b	1419 bp	CCA CAG CGC CAG TTC TGC TTA C
28Sc: 28S rd4.8a	1328 bp	ACC TAT TCT CAA ACT TTA AAT GG
28Sc: 28S rd7b1	2222 bp	GAC TTC CCT TAC CTA CAT
COI: LCO1490		GGT CAA CAA ATC ATA AAG ATA TTG G
COI: HCOout		CCA GGT AAA ATT AAA ATA TAA ACT TC

Purification was followed by a sequence reaction to generate single-stranded purified products for direct sequencing. Each sequence reaction, of a total volume of 10 µL, was made up of: 2 µL of the PCR product, 1 µL of one of the PCR primer pairs, 2 µL of halfTERM Dye Terminator Reagent (Genpak, Stony Brook, NY), and 2 µL of ABI BigDye™ Terminator v3.0 (Applied Biosystems, Foster City, CA), and ddH_2O. 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, single-stranded PCR products were finally cleaned with AGTC® Gel Filtration Cartridges (Edge BioSystems, Gaithersburg, MD). The sequence reaction products were then analysed using an ABI Prism 3100 Genetic Analyser (Applied Biosystems).

The chromatograms were visualised using the software Sequencher™ 4.0 (Gene Codes Corporation, Ann Arbor, MI).

Forward and reverse fragments were assembled to form double-stranded products and chromatograms were compared for consistency. For 28S and 18S rRNA, the three amplicons obtained for each gene were merged into a single sequence. Exemplars from consistent homologous regions were tested using NCBI BLAST (National Center for Biotechnology Information basic local alignment search tool) to confirm that they corresponded with known polyplacophoran sequences deposited in GenBank. Any oddities or strikingly inconsistent regions were also checked this way to ensure there was no contamination. Individual amplicon analyses were also conducted to check for possible contaminant sequences.

Final sequences were edited and aligned using the software MacGDE (Smith *et al*. 1994; Linton 2005). The datasets included additional sequences obtained from GenBank as outgroups (see Table 3). All sequences were then split into fragments using internal primers and secondary structure features (Giribet and Wheeler 2001; Giribet 2002) for subsequent analyses. From each final sequence, known external primers were excluded. Due to the lack of amplicons for some ribosomal fragments due to poor tissue preservation (mostly of the deep sea species), each of the three fragments for the two ribosomal genes was maintained as an independent input file (see also Appendix 1). The protein-coding gene COI showed no length variation among the taxa studied.

Morphology

Morphological features were coded according to the published matrix of Sigwart (2009), including 69 characters for shell, girdle, radula, and gill arrangement. All characters were non-additive. Additional outgroup taxa were coded from specimens in the Royal BC Museum (Victoria, Canada). Five ingroup taxa used by Sigwart (2009) were not included here because suitable material was unavailable: *Leptochiton alveolus* (Sars MS, Lovén, 1846), *L. binghami* (Boone, 1928), *L. inquinatus* (Reeve, 1847), *L. scabridus* (Jeffreys, 1880), and *L. thandari* Sirenko, 2001. Material coded as *L. americanus* Kaas & Van Belle, 1985 by Sigwart (2009) has subsequently been reidentified by one of the authors (ES) as *L. laurae* Schwabe & Sellanes, 2010. The present study also added four new ingroup taxa to the analysis: *Leptochiton* cf. *giganteus* (Nierstrasz, 1905), an undescribed *Leptochiton* sp. from the Gulf of Mexico, *Parachiton hodgsoni* Sirenko, 2000, and *Hanleyella oldroydi* (Bartsch MS, Dall, 1919). For details and discussion on the morphological characters see Sigwart *et al*. (2007) and Sigwart (2009*a*).

Analyses

Phylogenetic analysis was conducted in the program POY ver. 4 (Varón *et al*. 2010) for the molecular and combined analyses of morphology and molecules using parsimony under direct optimisation (Wheeler 1996). Analysis of the morphological dataset alone did not differ from the results obtained by Sigwart (2009*a*).

All genes were analysed independently and in combination under a set of 10 analytical parameters varying the indel : change ratio and the transversion : transition ratio in a sensitivity analysis fashion (Wheeler 1995). One parameter set also explored different costs for opening and extending indels (De Laet 2005). The morphological characters received a weight of 1 each when combined with the molecular data.

All phylogenetic analyses were run in a cluster of Dell Blades (8 processors per blade, 32 Gb of RAM) using 20–40 processors. A typical analysis consisted of a timed search (driven search) of two hours each with up to 100 Wagner trees. The timed search of POY implements a default search strategy that effectively combines tree building with TBR branch swapping, parsimony ratchet, and tree fusing (see Goloboff 1999). Nodal support was calculated via bootstrapping. The optimal parameter set was obtained according to a modified Mickevich–Farris character incongruence metric (ILD; Mickevich and Farris 1981).

Results

Extraction of usable DNA from Lepidopleurida was problematic. During the course of this work, DNA was extracted from more than 80 specimens representing 45 ingroup taxa; however, amplification was truly successful in only 38 ingroup species. In some cases samples did appear to amplify for some regions, but the relatively low annealing temperatures required often resulted in poor quality sequences. This poor DNA quality was most likely due to the deep sea habitat of many of the specimens and the time spent between collection and preservation of tissues, as well as the current lack of specific primers that could improve amplification quality.

In all analyses, the order Lepidopleurida is monophyletic relative to the species sampled from Chitonida, and most closely related to species in *Callochiton*. The large ingroup genus *Leptochiton* Gray, 1847 is clearly not monophyletic. Comparing the results from analyses under 10 different parameter sets, equal weights (i.e. 1 : 1 for both transversion : transition and indel : transversion ratios) minimised incongruence in the combined molecular analysis (Table 4). This combined analysis of three gene regions resulted in a single most parsimonious tree of length 6077. However, when the data were analysed including morphological characters, the optimal parameter set was 3221 (indel opening = 3; transversions = transitions = 2; indel extension = 1). This combined analysis resulted in a single most parsimonious tree of length 13 282. These two trees are shown in Fig. 2. Additional investigation of the trees resulting from single gene phylogenies had limited phylogenetic signal, but the 18S rRNA tree was most similar to that resulting from combined analyses.

These two resulting trees, from the combination of three genes (Fig. 2*A*), and three genes plus morphology (Fig. 2*B*), consistently resolve several internal clades. Ferreiraellidae, represented by two species in the genus *Ferreiraella* Sirenko, 1988, is monophyletic. The family Protochitonidae includes *Deshayesiella* Carpenter MS, Dall, 1879 and *Oldroydia* Dall, 1894 – the clade resolved here, which we label Protochitonidae also includes *Hanleyella* Sirenko, 1973. The clade that we label Leptochitonidae *sensu stricto* includes the type species of the family (*Leptochiton asellus* (Gmelin, 1791)) and other species sampled from the North Atlantic and Mediterranean. Clade I includes the genus *Parachiton* Thiele, 1909 as well as several primarily Pacific *Leptochiton* species; however, also in this clade, *L. intermedius* (Salvini-Plawen, 1968) is from the Aegean Sea, and *Leptochiton* ‘sp.’ is an undescribed species collected from

Table 3. GenBank accession numbers and collection and locality data for specimens used in this study

All specimens are deposited in museum collections: MNHN, Muséum national d'Histoire naturelle; MCZ, Museum of Comparative Zoology, Harvard University; ZSM, Zoologische Staatssammlung München; all others in National Museum of Ireland, Natural History Division, Dublin. The 'general region' refers to the coloured biogeographic regions illustrated in Fig. 2

	Specimen number/origin	18S	28S	COI	General region	Specimen locality
Lepidopleurida : Ferreiraellidae						
Ferreiraella plana	MNHN – Boa1 CP2465	HQ907740	HQ907795	HQ907844	SW Pacific	Vanuatu; 770–799 m; 2005
Ferreiraella xylophaga karenae A	MNHN – Boa1 CP2432	HQ907739	HQ907796	HQ907845	SW Pacific	Vanuatu: Big Bay; 630–705 m; 2005
Ferreiraella xylophaga karenae B	MNHN – Boa1 CP2433	HQ907738	HQ907798	HQ907846	SW Pacific	Vanuatu: Big Bay; 593–630 m; 2005
Ferreiraella xylophaga karenae C	MNHN – Solomon2 CP2212	HQ907741	HQ907797		SW Pacific	Solomon Islands: Sta Isabel; 400–475 m; 2004
Lepidopleurida : Hanleyidae						
Hanleya nagelfar	Sneli	HQ907742	HQ907799		N Atlantic/Mediterranean	Iceland: Bioice stn. 3589; 2002
Lepidopleurida : Leptochitonidae						
Lepidopleurus cajetanus	MCZ DNA100108	AF120502	HQ907802	HQ907847	N Atlantic/Mediterranean	Spain: Tossa de mar, Girona; ~10 m; 1997
Leptochiton aequispinus	Saito	HQ907743	HQ907803	HQ907848	Japan	Japan: Sagami Bay; 240–418 m; 2002
Leptochiton algesirensis	Dell'Angelo	HQ907744	HQ907804	HQ907849	N Atlantic/Mediterranean	Italy: Sardinia, S'Archittu; 2003
Leptochiton asellus A	Sneli	HQ907747	HQ907807	HQ907851	N Atlantic/Mediterranean	Norway: Aksnestangen, Trondheim; 50–200 m; 2004
Leptochiton asellus B	ZSM 20050590	HQ907748	HQ907808		N Atlantic/Mediterranean	Sweden: Gullmarsundfjord; 30 m; 2003
Leptochiton asellus C	ZSM 20008014	HQ907746	HQ907806		N Atlantic/Mediterranean	Sweden: Tjärnö; 2000
Leptochiton asellus D		AY145382	AY145414		N Atlantic/Mediterranean	Sweden: Kristineberg MRS
Leptochiton asellus E	MCZ DNA100830; ZSM 20008014	AY377631	AY377662		N Atlantic/Mediterranean	Sweden: Tjärnö; 2000
Leptochiton boucheti A	MNHN – Boa1 CP2435	HQ907750	HQ907809	HQ907853	SW Pacific	Vanuatu: Big Bay; 773–900 m; 2005
Leptochiton boucheti B	MNHN – Boa1 CP2412	HQ907751	HQ907810	HQ907854	SW Pacific	Vanuatu: Malo; 373–800 m; 2005
Leptochiton boucheti C	MNHN – Boa1 CP2435	HQ907749		HQ907852	SW Pacific	Vanuatu: Big Bay; 773–900 m; 2005
Leptochiton cancellatus	ZSM 20034176	HQ907752	HQ907811	HQ907855	N Atlantic/Mediterranean	France: Bretagne, off Roscoff; 8 m; 2003
Leptochiton deforgesi A	MNHN – Boa1 CP2435	HQ907753	HQ907812	HQ907856	SW Pacific	Vanuatu: Big Bay; 773–900 m; 2005
Leptochiton deforgesi B	MNHN – Panglao CP2362	HQ907754		HQ907857	SW Pacific	Philippines: Bohol/Sulu seas sill; 679–740 m; 2005
Leptochiton denhartogi	ZSM 20034402	HQ907755	HQ907813	HQ907858	E Atlantic	Angola: 17°9'S 11°21'E; 2004
Leptochiton foresti A	MNHN – Panglao CP2380	HQ907756	HQ907814	HQ907859	SW Pacific	Philippines: Bohol/Sulu seas sill, Dipolog Bay; 150–163 m; 2005
Leptochiton foresti B	MNHN – Panglao CP2343	HQ907757	HQ907815	HQ907860	SW Pacific	Philippines: Bohol Sea, off Pamilacan Island; 273–356 m; 2005
Leptochiton cf. *giganteus*[M]	MCZ DNA102583	HQ907779	HQ907801	HQ907873	E Pacific/N Pacific	USA: California, Cortes Bank; 367–389 m; 2007
Leptochiton hirasei	Saito	HQ907758	HQ907816	HQ907861	Japan	Japan: Shibasaki, Miura Peninsula, Japan, intertidal; 2006
Leptochiton intermedius	ZSM 20040266	HQ907759	HQ907817		N Atlantic/Mediterranean	Croatia: Istira, Rovinje, Punta Corente; 0–4 m; 2004
Leptochiton japonicus	Saito	HQ907760	HQ907818	HQ907862	Japan	Japan: Sagami Bay; 94–95 m; 2002
Leptochiton juvenis	MNHN – Boa1 CP2462	HQ907761	HQ907819	HQ907863	SW Pacific	Vanuatu; 618–641 m; 2005
Leptochiton kerguelensis	ZSM 20021483	HQ907762	HQ907820	HQ907864	Antarctica	South Georgia and South Sandwich Islands, 42.55'S 27 57.02'W; 332.3–356.0m; 2002
Leptochiton laurae	ZSM 20041460	HQ907745	HQ907805	HQ907850	Antarctica	Chile: off Concepcion, 36°21.65′S 73°44.42'W; 900–904 m; 2004

[M]Taxa that were not included in the morphological cladistic analysis of Sigwart (2009*a*).

(*continued next page*)

Table 3. (*continued*)

	Specimen number/origin	18S	28S	COI	General region	Specimen locality
Leptochiton medinae	ZSM 20021117 (=MCZ DNA100876); ZSM 20050450	HQ907763; HQ907764	HQ907821	HQ907865	Antarctica	South Georgia and Sandwich Islands, 58°44.35'S 25°10.48'W, 725–815 m (ZSM 20021117); Chile: Fuerto Bulnes, S of Punta Arenas; 2005 (ZSM 20050450)
Leptochiton cf. *pergranatus*	FMNH – GC 234–4435	HQ907773	HQ907829		W Atlantic/Gulf of Mexico	USA: Gulf of Mexico, Bush Hill vent area; 2005
Leptochiton rugatus A	Sirenko	HQ907769	HQ907826	HQ907868	E Pacific/N Pacific (Japan)	Russia: Ussuriyskiy Bay, Sea of Japan; 2–4 m; 2004
Leptochiton rugatus B	Sirenko	HQ907770		HQ907869	E Pacific/N Pacific (Japan)	Russia: Vostok Bay; 2.0–2.5 m; 2003
Leptochiton saitoi A	MNHN – Panglao CP2356	HQ907771	HQ907827	HQ907870	SW Pacific	Philippines: Bohol Sea; 1764 m; 2005
Leptochiton saitoi B	MNHN – Boa1 CP2466	HQ907772	HQ907828		SW Pacific	Vanuatu; 786–800 m; 2005
Leptochiton vanbellei	MNHN – Boa1 CP2435	HQ907775	HQ907831	HQ907871	SW Pacific	Vanuatu: Big Bay; 773–900 m; 2005
Leptochiton vaubani	MNHN – Solomon2 CP2246	HQ907768	HQ907825	HQ907867	SW Pacific	Solomon Islands: Sta Isabel; 664–682 m; 2004
Leptochiton vietnamensis A	MNHN – Panglao CP2385	HQ907776	HQ907832	HQ907872	SW Pacific	Philippines: Bohol/Sulu seas sill; 982–989 m; 2005
Leptochiton vietnamensis B	MNHN – Panglao CP2385	HQ907777	HQ907833		SW Pacific	Philippines: Bohol/Sulu seas sill; 982–989 m; 2005
Leptochiton vietnamensis C	MNHN – Panglao CP2356	HQ907778	HQ907834		SW Pacific	Philippines: Bohol Sea; 1764 m; 2005
Leptochiton n. sp. 4 A	MNHN – Boa1 CP2479	HQ907765	HQ907822	HQ907866	SW Pacific	Vanuatu; 350–358 m; 2005
Leptochiton n. sp. 4 B	MNHN – Panglao CP2380	HQ907766	HQ907823		SW Pacific	Philippines: Bohol/Sulu seas sill, Dipolog Bay; 150–163 m; 2005
Leptochiton n. sp. 5	MNHN – Boa1 CP2433	HQ907767	HQ907824		SW Pacific	Vanuatu: Big Bay; 593–630 m; 2005
Leptochiton sp.[M]	FMNH 306049	HQ907774	HQ907830		W Atlantic/Gulf of Mexico	USA: Gulf of Mexico, Bush Hill vent area; 2005
Parachiton acuminatus	ZSM 20033088	HQ907787		HQ907879	SW Pacific	Indonesia: Sulawesi, Mantehage Island; 7.5 m
Parachiton communis	Saito	HQ907788	HQ907840		SW Pacific (Japan)	Japan: Gahi-jima, Kerama Islands; 9 m; 2006
Parachiton hodgsoni[M]	ZSM 20050798	HQ907789		HQ907880	E Atlantic	South Africa: Cape Aguthas; 2005
Parachiton politus	Saito	HQ907790	HQ907841	HQ907881	SW Pacific (Japan)	Japan: Gahi-jima, Kerama Islands; 9 m; 2006
Lepidopleurida : Nierstraszellidae						
Nierstraszella andamanica	MNHN – Panglao CP2385	HQ907781	HQ907835	HQ907875	SW Pacific	Philippines: Bohol/Sulu seas sill; 982–989 m; 2005
Nierstraszella lineata A	MNHN – Panglao CP2380	HQ907782	HQ907836	HQ907876	SW Pacific	Philippines: Bohol/Sulu seas sill, Dipolog Bay; 150–163 m; 2005
Nierstraszella lineata B	MNHN – Solomon2 CP2211	HQ907783	HQ907837		SW Pacific	Solomon Islands: Sta Isabel; 313–387 m; 2004
Nierstraszella lineata C	Saito	HQ907784	HQ907838		SW Pacific (Japan)	Japan: Suruga Bay; ~500 m; 2003
Nierstraszella lineata D	ZSM 20034397	HQ907785	HQ907839	HQ907877	SW Pacific (Japan)	Japan: Suruga Bay; 1999
Lepidopleurida : Protochitonidae						
Deshayesiella curvata	Sirenko	HQ907737	HQ907794	HQ907843	E Pacific/N Pacific (Japan)	Russia: Ussuriyskiy Bay, Sea of Japan; 2–4 m; 2004
Hanleyella oldroydi[*M]	MCZ DNA102582	HQ907780	HQ907800	HQ907874	E Pacific/N Pacific	USA: California, Cortes Bank; 367–389 m; 2007
Oldroydia percrassa	ZSM 20040613	HQ907786		HQ907878	E Pacific/N Pacific	USA: California, off La Jolla; 1972

(*continued next page*)

[M]Taxa that were not included in the morphological cladistic analysis of Sigwart (2009*a*).
[*]Revision based on present analysis (previously in Leptochitonidae).

Table 3. (*continued*)

	Specimen number/origin	18S	28S	COI	General region	Specimen locality
Chitonida : Acanthochitonidae						
Acanthochitona crinita	MCZ DNA100109	AF120503	DQ279957	AF120627	N Atlantic/Mediterranean	
Acanthochitona rhodea	MCZ DNA101902	HQ907736	HQ907792		W Atlantic/Gulf of Mexico	
Chaetopleura apiculata	MCZ DNA100833 (*partim*)	AY377636	AY145398	AY377704	W Atlantic/Gulf of Mexico	
Cryptochiton stelleri	MCZ DNA100592	AY377655	AY377686	AY377720	E Pacific/N Pacific	
Cryptoplax japonica	MCZ DNA100837; DNA101109	AY377656	AY145402	HQ907842	Japan	
Chitonida : Callochitonidae						
Callochiton bouveti	MCZ DNA100873	HQ907735	HQ907791		Antarctica	
Callochiton septemvalvis	MCZ DNA100831	AY377632	DQ279952	AY377700	N Atlantic/Mediterranean	
Chitonida : Chitonidae						
Callistochiton antiquus	MCZ DNA100579	AY377645	DQ279953	AY377712	Australia	
Chiton olivaceus	MCZ DNA100157	AY377651	AY377682	AY377716	N Atlantic/Mediterranean	
Chitonida : Ischnochitonidae						
Ischnochiton comptus	MCZ DNA100834	AY145380	AY145412	AY377709	Japan	
Chitonida : Mopaliidae						
Katharina tunicata	MCZ DNA100599	AY377650	AY377681; HQ907793		E Pacific/N Pacific	
Lorica volvox	MCZ DNA100571	AY377647	DQ279954		Australia	
Mopalia muscosa	MCZ DNA100522	AY377648	DQ279956	AY377713	E Pacific/N Pacific	
Tonicella lineata	MCZ DNA100580	AY377635	AY377665	AY377702	E Pacific/N Pacific	

Table 4. Tree lengths and ILD results

The first numeral used in the parameter set (leftmost) column corresponds to the ratio between indel : transversion and the following two numbers correspond with the ratio between transversion : transition; e.g. 111 is equal weights, 121 corresponds to an indel : transversion ratio of 1 and a transversion : transition ratio of 2 : 1

	18S	28S	COI	MOL	MOR	TOT	ILD MOL	ILD TOT
111	721	2470	2713	6077	594	6875	**0.02847**	0.05484
121	1059	3646	3959	8926	594	9741	0.02935	0.04958
141	1714	5911	6331	14436	594	15252	0.03325	0.04603
211	789	2730	2719	6444	594	7256	0.03197	0.05843
221	1185	4104	3962	9593	594	10411	0.03565	0.05437
241	1965	6809	6342	15739	594	16557	0.03958	0.05116
411	903	3152	2719	7053	594	7862	0.03956	0.06283
421	1404	4903	3962	10773	594	11589	0.04678	0.06265
441	2390	8358	6346	18057	594	18896	0.05333	0.06393
3221	1472	5156	5460	12465	594	13282	0.03024	**0.04517**

cold seep habitats in the Gulf of Mexico, reported by Cordes *et al*. (2005) as *L. alveolus*. Clade II is primarily made up of species found living in sunken wood deposits and from the tropical West Pacific. The habitats of two species also included in Clade II are not well documented: *L. medinae* (Chile), and *L. kerguelensis* Haddon, 1886 (Antarctica).

The two species of *Nierstraszella* Sirenko, 1992, included in Clade II, do not form a single clade and *Nierstraszella* may include the specimen identified as *L. vietnamensis* A Sirenko, 1998. The two other genera represented by multiple species in this analysis, *Ferreiraella* and *Parachiton*, are monophyletic, but *Parachiton* includes *L. intermedius*.

There are a small number of taxa that also fall outside these groupings. *Hanleya* Gray, 1857 is clearly within Lepidopleurina but does not resolve with any of the larger clades. The same is true for the species pair *Leptochiton japonicus* (Thiele, 1909) and *L. aequispinus* (Bergenhayn, 1933). The relationships between these clades are different between the two resulting trees (Fig. 2). Sister relationships between Protochitonidae and Leptochitonidae *s. str*., and between Ferreiraellidae and Clade I, are supported by both trees and effectively every permutation of the analysis.

Discussion

This study, although taxonomically focussed on one clade within Polyplacophora, is substantially larger both in taxon sampling and in genetic sampling than any previous work on chitons. All nine accepted genera within Lepidopleurida were represented. Four additional genera or subgenera that are of interest to the definition of this group were not included here because specimens were unavailable or did not yield good quality DNA. The monotypic *Pilsbryella* was excluded from Sirenko's (2006) classification, but has several distinctive morphological characteristics that separate it from the 'typical' *Leptochiton* (Kaas and Van Belle 1985). *Hemiarthrum* Carpenter in Dall, 1876, *Weedingia* Kaas, 1988, and *Choriplax* Pilsbry, 1894 have been historically placed in Lepidopleurida, but more recent classifications have included them in the order Chitonida (e.g. Sirenko 2006 *contra* Kaas and Van Belle 1985).

The 57 ingroup specimens were selected to represent 38 nominal species, which differ slightly from those sampled by Sigwart (2009). The results demonstrate several instances of probable cryptic species: *Leptochiton vietnamensis*, *L. deforgesi* Sirenko, 2001, and *L. boucheti* Sirenko, 2001. Other species that were represented by a single specimen may also hide species complexes and this may apply to any of the species included.

We have presented two preferred trees, one from molecular data and the second including morphological characters: both resolve the same clades, but propose different relationships between them.

Distribution, habitats, and biogeography

The Japanese specimens included in this analysis demonstrate that the lepidopleuran fauna of Japan does not represent a single biogeographic province. Taxa from the southern islands of Japan (*Parachiton communis*, *P. politus*, *Nierstraszella lineata* C and D) group with other species from the tropical south-west Pacific. Those from the northern part of the Sea of Japan, on the Russian coast (*Leptochiton rugatus*, *Deshayesiella curvata*) have sister relationships with taxa from the Eastern Pacific. The fauna of central Japan consists of three different elements, northern, tropical, and temperate, in a mixing zone between the Kuroshio and Oyashio currents (Ekman 1953; Okutani 1969). The three ingroup species that we examined from central Japan do not form a clade, and the pair *L. japonicus* and *L. aequispinus* do not resolve a clear relationship with the other major clades. Substantial work remains to be done to understand the biogeography of the central Japanese fauna.

The analysis is dominated by taxa from the tropical south-west Pacific, comprising half of the ingroup terminals. These taxa occur in three areas of the tree, with the majority of taxa in Clade II, but separate from a few in Clade I, and the Ferreiraellidae. Those in Clade I are found only north of Papua New Guinea, in the Philippines (*Leptochiton foresti*) and southern Japan (*Parachiton communis*, *P. politus*). Another species, *Parachiton acuminatus* is known primarily from the Bismarck Sea but specimens have also been recovered from New Caledonia (Enrico Schwabe, unpubl. data). Eight other terminals in Clade II are also from the Philippines, but all in species that have ranges extending south to the Solomon Islands or as far as New Caledonia (Table 3).

Clade II has a biogeographic origin in the south-west Pacific, with subsequent radiation to Antarctica and Japan. *Nierstraszella*

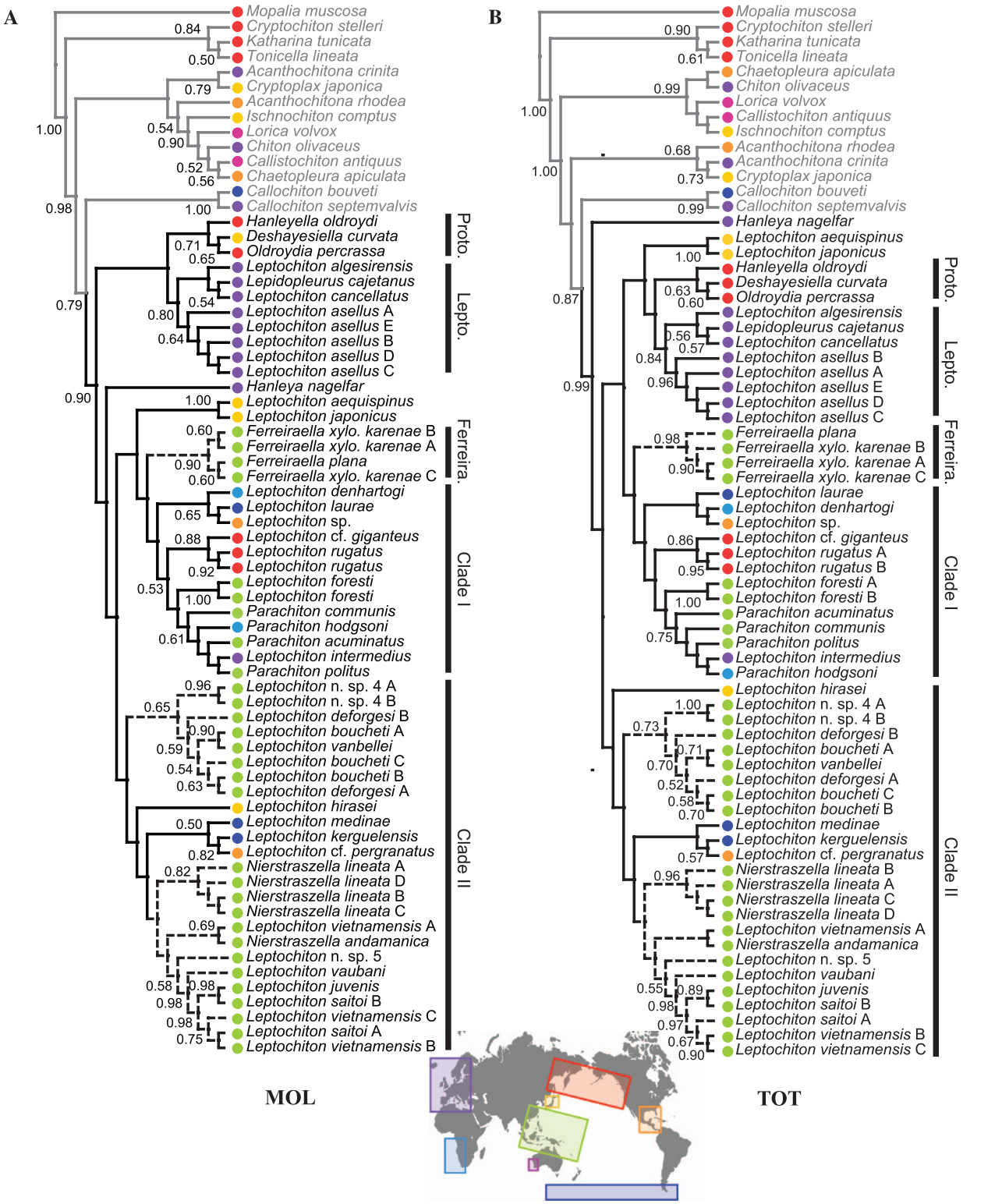

Fig. 2. Two alternative phylogenetic trees illustrating relationships within Lepidopleurida. We identified five ingroup clades: Leptochitonidae (Lepto), Protochitonidae (Proto), Ferreiraellidae (Ferreira), and two others numbered I and II. Dotted lines in the ingroup indicate species that are specialist on sunken wood substrates. Coloured dots show general geographic regions of the range of each species, as indicated in inset map. Where multiple exemplars of a species were included they are noted A, B, C (for specimen information, see Table 3). Numbers on branches indicate jackknife support values. (*A*) Combined analysis of molecular data from three loci (MOL) analysed under the optimal parameter set 111, single most parsimonious tree (MPT) length 6077 steps. (*B*) Combined analysis of all molecular data and morphological data (TOT) under the optimal parameter set 3221, single MPT length 13 282.

lineata and *Leptochiton vietnamensis* occur in Japan and in the South China Sea, so it is not surprising that this clade could also encompass species such as *L. hirasei*, which is known only from Japan.

The Antarctic species *L. kerguelensis* has a circumpolar distribution in the Southern Ocean (Schwabe 2008*b*), whereas *L. medinae* is known from Tierra del Fuego and both coasts of Patagonia (Schwabe and Sellanes 2010). Clade I contains the other Antarctic species of *Leptochiton s.l.* included in our analysis, indicating there have been at least two separate invasions of lepidopleuran chitons to the Southern Ocean, in contrast with the Antarctic as a source of radiation in other molluscs (Strugnell *et al*. 2008).

Sirenko (2004) postulated that *Ferreiraella* plays a pivotal role in the ancient origins of lepidopleuran taxa, in its morphological affinity with some of the earliest neoloricate fossils, and further that this was evidence for sunken wood as the ancestral habitat of lepidopleurans as a group. Our data suggest two separate colonisations of sunken wood habitats, with Ferreiraellidae separate from *Leptochiton s.l.* in Clade II (Fig. 2). But the wood dwelling taxa consistently occur as the earliest derived members of the local part of the tree. Sunken wood may be a critical factor in the origin and radiation of species in the south-west Pacific (in Clade II), although other members of this clade in Antarctica and possibly the Atlantic have adapted to other habitat substrates. Sunken wood has been postulated in the origins of chemosynthetic deep sea habitats (Distel *et al*. 2000). We include three species from cold seep habitats: *Leptochiton* sp. and *L. laurae* in Clade I, and *L.* cf. *pergranatus* in Clade II. These terminals consistently resolve in close proximity to sunken wood species, but without strong support.

Resolving molecules and morphology

Lepidopleuran shells typically lack insertion plates, lateral extensions of the ventral shell that anchor the shell to the girdle muscle block. But this shell feature is partially expressed in several taxa. Three genera in Lepidopleurina (*sensu* Sirenko 2006), *Ferreiraella*, *Deshayesiella*, and *Hanleya*, have shells with unslit insertion plates. Sirenko (1997, 2006) has discussed the potential for multiple evolutionary origins of shell insertion plates within Polyplacophora. Our trees (Fig. 2*A*, *B*) indicate that there have been (at least) three separate origins of insertion plates within Lepidopleurida, as these three genera occur in disparate parts of the tree.

Ferreiraella species have well developed, unslit insertion plates on both terminal valves. The genus is restricted to sunken wood habitats and is also characterised by having a ‘naked’ ventral girdle, not covered in spicules, and distinctive spatulate lateral teeth on the radula (Sirenko 1988; Saito 2006). Two of the eight described species in this genus were included in the present analysis. The family Ferreiraellidae includes only one living genus, *Ferreiraella*, and several Carboniferous fossil chitons that share the affinity for sunken wood (Sirenko 2004, 2006). The living species encompass a worldwide distribution (Caribbean, Eastern and Western Pacific) and a more detailed molecular phylogeny of this genus could test Sirenko’s (2004) hypothesis about the ancient origin of this family.

Hanleya is the only genus in the family Hanleyidae, although historical classifications have included other morphologically disparate genera that also have unslit insertion plates. This analysis has not clearly resolved the position of *Hanleya* relative to other taxa included. *Hanleya nagelfar* is interesting because it is very large for the group (up to 60 mm long, whereas the majority of lepidopleurans are less than 20 mm) and spongivorous (Todt *et al*. 2009). Its relationship to proposed congeners is worth further study (Warén and Klitgaard 1991). This genus is distinctly different from other lepidopleurans based on morphological and now also molecular data, but still resolves within Lepidopleurida.

Hanleya and *Deshayesiella* are known to differ from *Leptochiton* in several features of gamete morphology. The former two have egg hulls with a jelly coat punctured by macropores that serve as specific sites for sperm entry, whereas *Leptochiton* eggs have a completely smooth jelly coat without specific sites for sperm penetration (Buckland-Nicks 2008). The present analysis did not support a grouping that would include both *Hanleya* and *Deshayesiella*. But gamete data are not yet available for many species, and it would not be surprising to determine that *Oldroydia* and *Hanleyella* also share the same egg morphology and that this is a consistent character of Hanleyidae and Protochitonidae.

Recent work by Sirenko and Clark (2008) highlights the similarity between a resurrected species of *Deshayesiella*, and the monotypic *Oldroydia percrassa*, which have very similar shell morphology. These two genera were included as the only living genera in the family Protochitonidae in the revised taxonomy of Sirenko (2006) – we suggest that *Hanleyella* is also a member of this family. *Hanleyella oldroydi* is one of the most abundant deep water chitons in the Southern California Bight (Stebbins and Eernisse 2009); most other species in this clade are quite rarely encountered.

Nierstraszella is comprehensively defined by morphological features, particularly the characteristic fleshy proteinaceous layer that covers the dorsal shell surface (Sirenko 1992). *Nierstraszella* is also endemic to sunken wood substrates. Sigwart (2009*b*) recently revised the description of the species in *Nierstraszella*, identifying two distinct but broadly distributed species, which are both included here. Our consensus trees do not recover a monophyletic *Nierstraszella*, although some other parameter sets of the combined analysis do recover a monophyletic *Nierstraszella* including the exemplar of *Leptochiton vietnamensis* A (not figured). Although we believe this is not contamination it may represent cryptic or problematic identifications in *L. vietnamensis.*

Parachiton is identified by a dramatically enlarged tail valve and distinctive radular morphology; however, our results show a species of *Leptochiton* within the genus. Morphological cladistic analysis also failed to resolve a *Parachiton* clade with the three species examined (Sigwart 2009), and the radular morphology is not consistent in all species (Sirenko 1999).

The species pair *Leptochiton japonicus* and *L. aequispinus* are clearly closely related on the basis of morphological data. Our results further suggest that they are sister taxa and both significantly diverged from other *Leptochiton* taxa. Both species were considered to be junior synonyms of *L. belknapi* (Ferreira 1979; Kaas and Van Belle 1987), but have been reinstated

(Saito 1997). There are a number of wide ranging species of *Leptochiton* that are anecdotally accepted to contain multiple cryptic species, including particularly *L. belknapi* Dall, 1878 and allies (Ferreira 1979; Wu and Okutani 1984) and the species lumped wth *L. rugatus* (Carpenter in Pilsbry, 1892) (Ferreira 1979; Saito 2000; Stebbins and Eernisse 2009). These species complexes would particularly benefit from closer examination with molecular methods, and results could also illuminate the degree of morphological variability found within true species.

Leptochiton was anticipated to be non-monophyletic, on the basis of rather vague anatomical descriptions in the genus definition, but this analysis has also highlighted other areas in need of taxonomic revision. The species currently included in *Leptochiton* are resolved across three major clades. The type species, *L. asellus*, is included in the clade that we consider to represent Leptochitonidae *sensu stricto*. Similarly the species of *Leptochiton* in this clade are considered to be *Leptochiton s. str*., but the clade also includes the monotypic *Lepidopleurus* Risso, 1826. The taxonomic relationship between *Leptochiton* and *Lepidopleurus* has created problems since 1892 and may continue to do so.

Lepidopleurus was the first genus name proposed for lepidopleuran chitons. The genus was presented as a list including the monotypic *L. cajetanus* and two other unrelated species. Nearly twenty years later the genus name *Leptochiton* was established by Gray (1847). Both of these species were included in the family Leptochitonidae Dall, 1889 with *Leptochiton asellus* as the type species. *Lepidopleurus cajetanus* and *Leptochiton asellus* are both contained in our clade Leptochitonidae *s. str*.

Only three years later, Pilsbry (1892) listed *Leptochiton* as a junior subjective synonym of *Lepidopleurus*, and changed the family name to Lepidopleuridae. The two generic names and family names have been used more or less interchangeably for the past 100 years. Sirenko (1979) argued for the reinstatement of Leptochitonidae by priority. This convention has been followed by most workers since that time, but some contemporary authors have advocated use of Lepidopleuridae (Dell'Angelo and Palazzi 1991). The higher ranks Lepidopleurida (order) and Lepidopleurina (suborder) are used universally. The nomenclature is further confused by colloquial use of the term 'lepidopleurids' to refer to members of the order, even by workers who use Leptochitonidae as the preferred family name. To circumvent a small part of this confusion we support the use of the common name 'lepidopleuran' as an alternative.

The results of this analysis indicate that there is potentially not sufficient evidence to separate *Lepidopleurus* and *Leptochiton s. str*. as separate genera. The same topology is recovered by morphological characters alone (Sigwart 2009). *Lepidopleurus* has very distinctive shell morphology with pronounced concentric ridges on the lateral areas and terminal valves. The shell shape is in contrast with the typical flat and plain shells of most species of *Leptochiton* that might be marked with patterns of granules but generally lack strong raised sculpture.

The morphological definitions of genera and families within Lepidopleurida are described from animals that differ from the norm set by *Leptochiton asellus*. The question remains, how to interpret relationships between these very different generic groups as well as within the majority of relatively plain and character-poor species.

Morphological features clearly can resolve phylogenetic signal; however, the interpretation of morphology has not provided a suite of taxonomic characters that reliably split Lepidopleurida into subgroups. Any group that is so widespread, both in terms of geographic range and depth, and purportedly mostly belongs in a single genus, raises immediate doubts about monophyly and accuracy of classification.

The phylogenetic hypotheses generated by this study will enable future testing of the taxonomy and classification within Lepidopleurida. The major genus, *Leptochiton*, contains most of the species named, but it is not supported by morphological synapomorphies and results as paraphyletic in all molecular analyses. The phylogeny proposed here will also provide a baseline to develop further studies and interpret evolutionary patterns within the order and within Polyplacophora.

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