

Evidence for a clade composed of molluscs with serially repeated structures: Monoplacophorans are related to chitons

Gonzalo Giribet^{*†}, Akiko Okusu^{*}, Annie R. Lindgren^{*§}, Stephanie W. Huff^{*}, Michael Schrödl[¶], and Michele K. Nishiguchi[‡]

^{*}Department of Organismic and Evolutionary Biology and Museum of Comparative Zoology, Harvard University, 16 Divinity Avenue, BioLabs 1119, Cambridge, MA 02138; [†]Department of Biology, New Mexico State University, P.O. Box 30001, Las Cruces, NM 88003; and [‡]Zoologische Staatssammlung München, Münchhausenstrasse 21, 81247 München, Germany

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Monoplacophorans are among the rarest members of the phylum Mollusca. Previously only known from fossils since the Cambrian, the first living monoplacophoran was discovered during the famous second *Galathea* deep-sea expedition. The anatomy of these molluscs shocked the zoological community for presenting serially repeated gills, nephridia, and eight sets of dorsoventral pedal retractor muscles. Seriality of organs in supposedly independent molluscan lineages, i.e., in chitons and the deep-sea living fossil monoplacophorans, was assumed to be a relict of ancestral molluscan segmentation and was commonly accepted to support a direct relationship with annelids. We were able to obtain one specimen of a monoplacophoran Antarctic deep-sea species for molecular study. The first molecular data on monoplacophorans, analyzed together with the largest data set of molluscs ever assembled, clearly illustrate that monoplacophorans and chitons form a clade. This “Serialia” concept may revolutionize molluscan systematics and may have important implications for metazoan evolution as it allows for new interpretations for primitive segmentation in molluscs.

Antarctica | deep sea | Mollusca | Monoplacophora | phylogeny

Molluscs (snails, slugs, clams, mussels, squids, octopuses, chitons, etc.) exhibit the largest disparity of all animal phyla and rank second behind arthropods in species diversity. Although the majority of species still remain in the oceans, where they inhabit all types of ecosystems from the upper littoral to the abyss, they are also major components of freshwater and terrestrial habitats. Molluscan diversity can be extraordinary in tropical and temperate regions (1) but can be found at all latitudes.

The phylogenetic position of molluscs within Spiralia is supported by the presence of spiral cleavage and a trochophore larva (2, 3), although their immediate sister group remains uncertain. Although some have proposed a relationship to sipunculans (peanut worms) (4) or entoprocts (5), most researchers still consider molluscs closely related to annelids, in part because of the assumption that they retain traces of segmentation (3). The removal of arthropods and their relatives from the clade Spiralia (6) and the evolutionary importance given to segmentation in annelids have contributed to reengaging the debate about ancestral segmentation in other spiralian clades such as molluscs. This supposed segmentation in molluscs is often justified by the presence of eight sets of pedal retractor muscles and serially repeated gills in both chitons (Polyplacophora) (7) and members of the living fossil class Monoplacophora (8–10), based on the assumption that both groups are basal within their distinct lineages. Certain bivalves also exhibit multiple pedal retractor muscles (11), and caudofoveate larvae show seven transverse rows of calcareous spicules on the dorsal side (3).

Monoplacophorans are perhaps the least known members of the phylum Mollusca. They have been thought to be “primitive” forms based on their rich fossil record, which dates back to Cambrian–

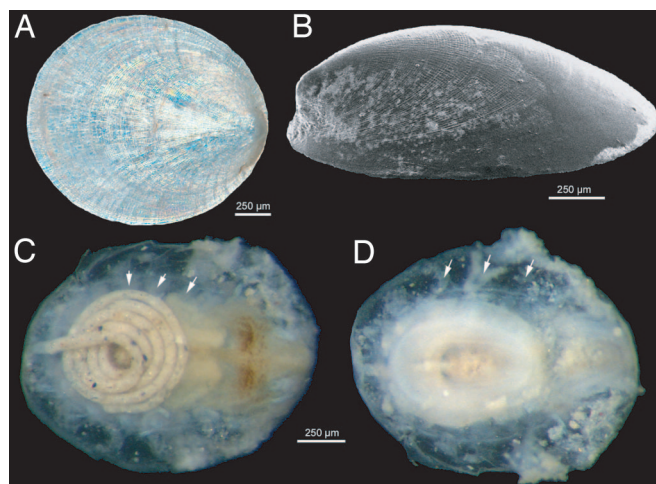


Fig. 1. Details of *L. antarctica* Warén & Hain, 1992. (A) Shell, dorsal view. Note the limpet-like shape with anterior apex and light reflection caused by prismatic and inner nacreous layers. (B) Scanning electron micrograph of the shell (dorsolateral view from left side). (C) Soft body (shell removed) (dorsal view). Note the characteristic spiral intestine (left) filled with mineral particles, brown-dotted esophageal pouches (right), and serial shell muscles (arrows). (D) Soft body, ventral view. Note the round sucker-like foot (central), serial gills (arrows), and mouth area with tentacles (right).

Devonian periods (8). After the recent discovery of the first living monoplacophoran, *Neopilina galathea*, during the second Danish *Galathea* expedition (8), it was suggested that its dorsal uncoiled cap-like shell (Fig. 1) fit the prevalent *HAM* (hypothetical ancestor mollusc) theories (12). This idea positioned monoplacophorans at the base of “Conchifera,” a clade that includes all molluscs with a true dorsal shell (the classes Monoplacophora, Gastropoda, Cephalopoda, Bivalvia, and Scaphopoda). *Neopilina*’s newly discovered anatomy [with serially repeated gills and eight sets of dorsoventral pedal retractor muscles, as those found in chitons, and serially repeated nephridia (8, 10)] suggested that serial homology was present at least in two extant molluscan lineages, Aculifera (molluscs with spicules) and Conchifera (molluscs with a true shell).

Although a generalized mollusc is portrayed as a limpet-like form with a creeping foot and a dorsal shell made of calcium carbonate

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[†]To whom correspondence should be addressed. E-mail: ggiribet@oeb.harvard.edu.

[§]Present address: Department of Evolution, Ecology, and Organismal Biology, Ohio State University, 1315 Kinnear Road, Columbus, OH 43215.

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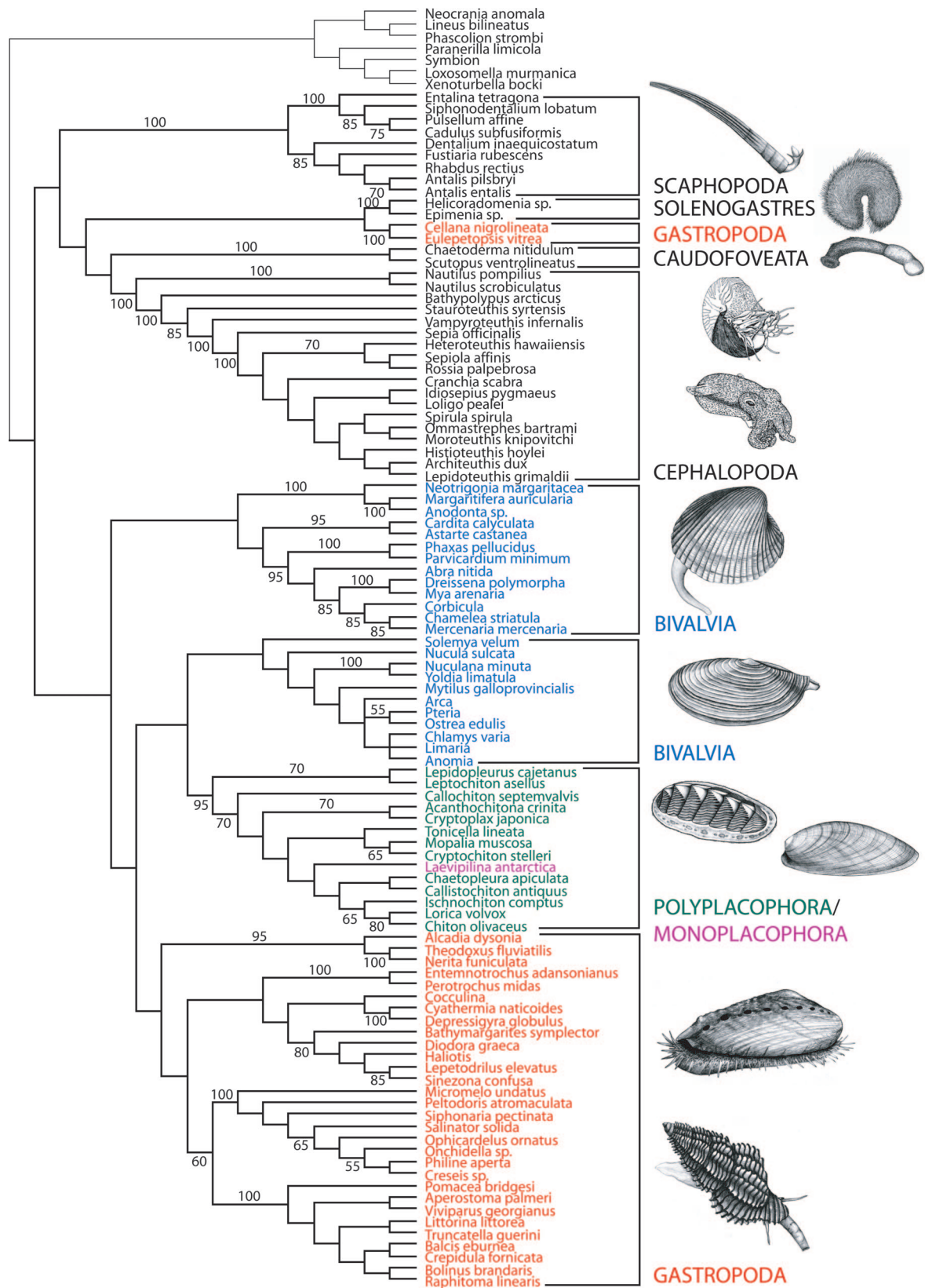


Fig. 2. Phylogenetic tree depicting the relationships of Monoplacophora to other molluscs based on the combined analysis of all molecular loci. Shown is strict consensus of two most parsimonious trees at 64,679 weighted steps (gap opening cost of 3, gap extension cost of 1, all base transformations cost 2) for the analysis of all data under direct optimization with tree fusing. Numbers on branches indicate jackknife support values. Gastropods (in red) and bivalves (in blue) appear diphyletic. Polyplacophora and Monoplacophora form a well supported clade (95% jackknife support). The monoplacophoran species (purple) appears nested within chitons (dark green), but nodal support for its exact position is low. The tree shows monophyly of molluscs, as well as that of Scaphopoda, Cephalopoda, Caudofoveata, and Solenogastres.

(as in the class Monoplacophora), other body plans such as those of the worm-like, shell-less fossorial chaetodermomorphs (class Caudofoveata) and neomeniomorphs (class Solenogastres), or the benthopelagic cephalopods (class Cephalopoda) differ radically from this prototype. Mussels, clams and their kind (class Bivalvia) are also quite divergent from this model. Furthermore, modern chitons (class Polyplacophora) have a distinct dorsal “shell” formed by eight interlocking plates. In fact, the disparity of mollusc body plans is so great that it is quite difficult to find a single trait shared by all seven classes of molluscs (13).

Our understanding of relationships among the major molluscan lineages is still in its infancy. Recent attempts to resolve their relationships by using morphological data found limitations in character homology definitions and polarization because of uncertainty regarding the molluscan sister group (4, 5, 14). Molecular attempts have not been conclusive, but they have aided to refute the “Diasoma” hypothesis (a clade uniting bivalves and scaphopods). Most recent molecular analyses suggest a relationship of scaphopods to cephalopods and gastropods (15–17), further corroborated through morphological and developmental studies (5, 18). To date, the phylogenetic position of monoplacophorans remained untested using molecular data because of difficulties in collecting live samples of these enigmatic animals.

Results and Discussion

An Antarctic Benthic Deep-Sea Biodiversity oceanographic campaign (ANDEEP III) with the RV *Polarstern* to the Weddell Sea (Antarctica), 3 km southwest of Wegener Canyon at ≈3,100-m depth, yielded one small specimen (1.7-mm shell length) of the monoplacophoran *Laevipilina antarctica* Warén & Hain, 1992 (19), one of the 26 known species of this group of molluscs (9, 20). The single specimen was obtained from an epibenthic sledge sample that had been fixed with precooled 96% EtOH for molecular studies and stored at –20°C for 48 h. The shell (ZSM Moll 20050866; Fig. 1*A* and *B*) was removed for gross anatomy and SEM examination, the soft body was photographed (Fig. 1*C* and *D*), and half of the specimen was used for molecular work.

Although monoplacophoran DNA was highly degraded, perhaps because of bulk fixation of the sediment performed in the vessel, we were able to amplify and sequence a 1.2-kb fragment of the large nuclear ribosomal subunit (28S rRNA). This gene has proven to be highly informative in recent studies on metazoan and molluscan evolution (17, 21).

Analysis of the data using a single-step phylogenetic approach with direct optimization (Fig. 2) and a two-step approach using Bayesian phylogenetics (Fig. 4, which is published as supporting information on the PNAS web site) exhibited congruent results suggesting monophyly of molluscs as well as that of the molluscan classes Caudofoveata, Solenogastres, Scaphopoda, and Cephalopoda. Resolution with high jackknife support is found mostly within the main clades of Scaphopoda (Dentaliida and Gadilida), Cephalopoda (Nautiloidea, Coleoidea, and the sister group relationship of the vampire squid to decabrachians, which include the giant squid *Architeuthis dux* and the pygmy squid *Idiosepius pygmaeus*), as well as within Bivalvia (Palaeoheterodonta and Euheterodonta) and Gastropoda (Patellogastropoda, Neritopsina, Caenogastropoda, and Heterobranchia). However, the available sequence data do not recover monophyly of Gastropoda or Bivalvia, which are both diphyetic, with patellogastropods separated from the other gastropods and heteroconchs separated from the remainder of the bivalves (protobranchs and pteriomorphians).

Nodal support for interclass relationships or for the relationships of the two clades of bivalves and gastropods is low in general, but a clade containing Monoplacophora and Polyplacophora received strong nodal support (90–100% jackknife support value depending on the analysis, as well as 1.0 posterior probability). Interestingly, this clade, which we name “Serialia,” contains the two classes whose members present a variable number of serially repeated gills and



Fig. 3. Alignment of one of the regions of 28S rRNA illustrating that *L. antarctica* does not share unique chiton synapomorphies (asterisks).

eight sets of dorsoventral pedal retractor muscles. This result clearly contrasts with previous cladistic hypotheses suggesting that Monoplacophora constitute the sister group to the remainder of the conchiferans (4, 5, 14), those molluscs with a true shell unlike that of chitons or the vermiform aplacophorans, although it finds no clear support for the exact position of Serialia. To our knowledge, this is also the first published analysis that demonstrates monophyly of the phylum Mollusca using a range of appropriate outgroups, but we caution the reader to consider that jackknife support for molluscan monophyly is low. The results further support a previous study (22) that indicates that *Xenourbella* is not a bivalve mollusc.

All analyses (including different optimality criteria and alternative models of indel and base substitutions) support a Polyplacophora plus Monoplacophora clade. However, *L. antarctica* appears nested within the chiton tree in some analyses, a result that may look suspicious at first. Evidence for including Monoplacophora within Polyplacophora is restricted to one node, which groups nonlepidopleurid chitons with the monoplacophoran species (70% jackknife support; Fig. 1), but this is not the case when considering only the 1.2-kb region of 28S rRNA amplified for *Laevipilina* (tree not shown). Furthermore, detailed examination of the DNA sequences clearly illustrates that chitons share unambiguous positions in the alignment not found in *L. antarctica* (Fig. 3). This fact eliminates the possibility of contaminant DNA in our analysis.

Evidence for a clade of serialian molluscs is important for our current understanding of molluscan relationships and may have implications for deeper metazoan evolution. This new evidence may imply that serially repeated structures (e.g., gills and pedal retractor muscles in both monoplacophorans and chitons) are not primitive for molluscs, as was previously thought (9). However, it is fair to mention that additional types of serial repetition of dorsoventral musculature have been reported in other molluscan groups (23), including the eight sets of pedal retractors of the Ordovician lucinoid bivalve *Babinka* (11), the serially repeated rows of spicules in caudofoveate larvae (3), or the two pairs of gills and nephridia in cephalopods (3). Whether these represent true seriality or not may have profound implications in reconstructing the molluscan common ancestor, but it does not contradict the evidence of our Serialia clade.

The classical hypothesis for the position of monoplacophorans as basal conchiferans relies heavily on the presence of a true dorsal shell with similar mineralogical composition to that of many basal members of each conchiferan class. However, the mode of shell deposition by the mantle edge and the microstructure and composition of the chitinous organic layer in monoplacophorans differ from those of higher conchiferans or polyplacophorans (9, 24, 25), which makes monoplacophorans apomorphic (derived) in the form of shell deposition. The rejection of conchiferan monophyly based on shell deposition would be consistent with our findings, which suggest that serial repetition of anatomical structures such as gills and muscles may have evolved once in the common ancestor of chitons and monoplacophorans. Therefore, serial repetition of these structures could constitute a derived feature that would not

Table 1. Taxon sampling and GenBank accession numbers employed in this study

Phylum/class	Species	GenBank accession nos.				
		18S rRNA	28S rRNA	H3	COI	16S rRNA
Nemertea	<i>Lineus bilineatus</i>	DQ279932	DQ279947	DQ279996	DQ280014	DQ280022
Annelida	<i>Paranerilla limicola</i>	DQ279933	DQ279948			DQ280023
Brachiopoda	<i>Neocrania anomala</i>	DQ279934	DQ279949	DQ279997		DQ280024
Entoprocta	<i>Loxosomella murmanica</i>	AY218100	DQ279950	AY218150		
Xenoturbellida	<i>Xenoturbella bocki</i>	AY291292	DQ279951			
Sipuncula	<i>Phascolion strombi</i>	DQ299984	AY210468	DQ279998		
Cycliophora	<i>Symbion americanus</i>	AY218107	AY210472	AY218153	AY218085	DQ280025
Mollusca						
Caudofoveata	<i>Chaetoderma nitidulum</i>	AY377658	AY145397	AY377763	AY377726	AY377612
	<i>Scutopus ventrolineatus</i>	X91977				
Solenogastres	<i>Helicoradomenia</i> sp.	AY21210		AY377764	AY377725	AY377613
	<i>Epimения cinerea</i>	AY377657	AY377691	AY377765	AY377723	AY377615
Polyplacophora	<i>Lepidopleurus cajetanus</i>	AF120502	AF120565	AY377735	AF120626	AY377585
	<i>Leptochiton asellus</i>	AY377631	AY145414	AY377734		AY377586
	<i>Callochiton septemvalvis</i>	AY377632	DQ279952	AY377736	AY377700	
	<i>Chaetopleura apiculata</i>	AY377636	AY145398	AY377741	AY377704	AY377590
	<i>Ischnochiton comptus</i>	AY377639	AY145412	AY377744	AY377709	AY377593
	<i>Callistochiton antiquus</i>	AY377645	DQ279953	AY377749	AY377712	AY377599
	<i>Lorica volvox</i>	AY377647	DQ279954	AY377751		AY377601
	<i>Chiton olivaceus</i>	AY377651	DQ279955	AY377755	AY377716	AY377605
	<i>Mopalia muscosa</i>	AY377648	DQ279956	AY377752	AY377713	AY377602
	<i>Tonicella lineata</i>	AY377635	AY377665	AY377739	AY377702	AY377588
	<i>Acanthochitona crinita</i>	AF120503	DQ279957	AY377759	AF120627	AY377609
	<i>Cryptochiton stelleri</i>	AY377655	AY377686	AY377760	AY377720	AY377610
	<i>Cryptoplax japonica</i>	AY377656	AY145402	AY377761		AY377611
Monoplacophora	<i>Laevipilina antarctica</i>		DQ279958			
Scaphopoda	<i>Dentalium inaequicostatum</i>	DQ279935	DQ279959	DQ279999	DQ280015	DQ280026
	<i>Rhabdus rectius</i>	AF120523	AF120580	AY377772	AF120640	AY377619
	<i>Antalis pilsbryi</i>	AF120522	AF120579		AF120639	
	<i>Antalis entalis</i>	DQ279936	AY145388	DQ280000	DQ280016	DQ280027
	<i>Fustiaria rubescens</i>	AF490597				
	<i>Entalina tetragona</i>	AF490598				
	<i>Pulsellum affine</i>	AF490600				
	<i>Siphonodentalium lobatum</i>	AF490601				
	<i>Cadulus subfusiformis</i>	AF490603				
Bivalvia	<i>Solemya velum</i>	AF120524	AY145421	AY070146	U56852	DQ280028
	<i>Nucula sulcata</i>	DQ279937	DQ279960	DQ280001	DQ280017	DQ280029
	<i>Nuculana minuta</i>	DQ279938	DQ279961	DQ280002	DQ280018	DQ280030
	<i>Yoldia limatula</i>	AF120528	AY145424	AY070149	AF120642	
	<i>Mytilus galloprovincialis</i>	L33452	AB103129	AY267748	AY497292	AY497292
	<i>Arca imbricata/A. ventricosa</i>	AY654986	AB101612	AY654989	AY654988	
	<i>Pteria hirundo/P. loveni</i>	AF120532	AB102767		AF120647	DQ280031
			AF137047/			
	<i>Ostrea edulis</i>	L49052	AF120596	AY070151	AF120651	DQ280032
	<i>Limaria hians/L. fragilis</i>	AF120534	AB102742	AY070152	AF120650	
	<i>Anomia ephippium/A. sinensis</i>	AF120535	AB102739			
	<i>Chlamys varia</i>	DQ279939	DQ279962	DQ280003		DQ280033
	<i>Neotrigonia margaritacea</i>	AF411690	DQ279963	AY070155	U56850	DQ280034
	<i>Margaritifera auricularia</i>	AY579097	AY579113	AY579137	AY579125	DQ280035
	<i>Anodonta</i> sp.	AY579090	DQ279964	AY579132	AY579122	
	<i>Cardita calyculata</i>	AF120549	AF120610	AY070156	AF120660	
	<i>Astarte castanea</i>	AF120551	AF131001	DQ280004	AF120662	
	<i>Abra nitida</i>	DQ279940	DQ279965	DQ280005		
	<i>Phaxas pellucidus</i>	DQ279941	AY145420	DQ280006	DQ280019	DQ280036
	<i>Parvicardium minimum</i>	DQ279942	DQ279966	DQ280007		DQ280037

support the hypothesis of a segmented ancestral mollusc. Again, other interpretations may exist if the pedal scars of *Bibankia* were the result of muscles homologous to the serialian dorsoventral pedal muscles.

Molluscs are undoubtedly one of the animal phyla with the largest disparity. Numerous Cambrian forms such as *Wiwaxia* and *Halkieria* or the Silurian *Acaenoplax* have been more or less ambiguously assigned to this animal phylum (26–28). *Kimberella* is another putative mollusc extending the age of the group back to the Neoproterozoic (29). Although chitons were once thought to have changed little since their first appearance in the Late Cambrian period (30), recent discoveries of articulated polyplacophorans and

multiplacophorans from the Ordovician to the Carboniferous (31, 32) suggest that a much larger disparity evolved during the Paleozoic. Perhaps such an episode of diversification is responsible for the two modern anatomies of molluscs with conspicuous serial repetition of organs, but no explanation for their divergent evolution of shell morphologies can be provided at this point. Recognition of a serialian clade comprised of chitons and multiplacophorans broadens our perspective toward new interpretations of molluscan anatomy and once more questions preconceived ideas on molluscan relationships that rely almost entirely on shell morphology.

Here we provide the first molecular test for the phylogenetic position of Monoplacophora by using sequence data from a deep-

Table 1. (continued)

Phylum/class	Species	GenBank accession nos.				
		18S rRNA	28S rRNA	H3	COI	16S rRNA
Cephalopoda	<i>Dreissena polymorpha</i>	AF120552	AF131006	AY070165	AF120663	DQ280038
	<i>Corbicula fluminea/C. japonica</i>	AF120557	AB126330	AY070161	AF120666	DQ280039
	<i>Mercenaria mercenaria</i>	AF120559	AF131019	DQ280008	AF120668	DQ280040
	<i>Chamelea striatula</i>	DQ279943	DQ279967	DQ280009		DQ280041
	<i>Mya arenaria</i>	AF120560	AB126332	AY377770	AY070140	AY377618
	<i>Nautilus pompilius</i>	AY557452	AY145417		AY557514	AY377628
	<i>Nautilus scrobiculatus</i>	AF120504	AF120567	AF033704		U11606
	<i>Stauroteuthis syrtensis</i>	AY557457	DQ279968	AY557406	AF000067	DQ280042
	<i>Vampyroteuthis infernalis</i>	AY557459	AH012197	AY557408	AF000071	DQ280043
	<i>Bathypolypus arcticus</i>	AY557465	AY557554		AF000029	DQ280044
	<i>Sepia officinalis</i>	AY557471	AY557560	AY557415	AF000062	DQ093491
	<i>Sepiolo affinis</i>	AY557474	AY557562	AY557418	AY557523	AY293667
	<i>Heteroteuthis hawaiiensis</i>	AY557472	DQ279969	AY557416	AF000044	AY293680
	<i>Rossia palpebrosa</i>	AY557473	AY557561	AY557417	AF000061	DQ280045
	<i>Spirula spirula</i>	AY557476	AY557563	AY557420	AY293709	AY293659
	<i>Idiosepius pygmaeus</i>	AY557477	AY293684	AY557421	AY293708	AY293658
	<i>Loligo pealei</i>	AT557479	AH012196	AY557423	AF120629	AF110079
	<i>Architeuthis dux</i>	AY557482	DQ279970	AY557426	AF000027	AY377629
	<i>Cranchia scabra</i>	AY557487	AY557571	AY557430	AF000035	DQ280046
	<i>Histioteuthis hoylei</i>	AY557500	AY557584	AY557442	AF000045	DQ280047
	<i>Lepidoteuthis grimaldii</i>	AY577503	AY557587	AY557445	AF000049	DQ280048
	<i>Ommastrephes bartrami</i>	AY557510	AY557594	AY557451	AF000057	DQ280049
	<i>Moroteuthis knipovitchi</i>	AY557512	AY557596	AY557453	AY557543	DQ280050
Gastropoda	<i>Cellana</i> sp.	DQ093425	DQ279971	DQ093493	DQ093515	DQ093467
	<i>Eulepetopsis vitrea</i>	DQ093427	DQ279972	DQ093495	DQ093516	DQ093468
	<i>Cocculina messingi/Cocculina</i> sp.	AF120508	DQ279973	AY377777	AY377731	AY377624
	<i>Alcaldia dysonia</i>	DQ093428	DQ279974	DQ093496		DQ093469
	<i>Theodoxus fluviatilis</i>	AF120515	DQ279975		AF120633	DQ093470
	<i>Nerita funiculata</i>	DQ093429	DQ279976	DQ093497	DQ093517	DQ093471
	<i>Cyathernia naticoides</i>	DQ093430	DQ279977	DQ093498	DQ093518	DQ093472
	<i>Depressigyra globulus</i>	DQ093431	DQ279978	DQ093499	DQ093519	DQ093473
	<i>Perotrochus midas</i>	AF120510	DQ093453	DQ093500	AY296820	DQ093474
	<i>Entemnotrochus adansonianus</i>	AF120509	DQ279979	AY377774		AY377621
	<i>Lepetodrilus elevatus</i>	DQ093432	AY145413	DQ093501	DQ093520	DQ093475
	<i>Diodora graeca</i>	AF120513	DQ279980	DQ093502	AF120632	DQ093476
	<i>Haliotis tuberculata/H. discus</i>	AF120511	AY145418	AY070145	AY377729	AY377622
	<i>Sinezona confusa</i>	AF120512	DQ279981	AY377773	AF120631	
	<i>Bathymargarites symplector</i>	DQ093433	DQ279982	DQ093503	DQ093521	DQ093477
	<i>Aperostoma palmeri</i>	DQ093435	DQ279983	DQ093505	DQ093523	DQ093479
	<i>Pomacea bridgesi</i>	DQ093436	DQ279984	DQ093506	DQ093524	DQ093480
	<i>Viviparus georginaus</i>	AF120516	AF120574	AY377779	AF120634	AY377626
	<i>Balcis eburnea</i>	AF120519	AF120576		AF120636	DQ280051
	<i>Crepidula fornicata</i>	AY377660	AY145406	AY377778	AF353154	AY377625
	<i>Littorina littorea</i>	DQ093437	DQ279985	DQ093507	DQ093525	DQ093481
	<i>Truncatella guerini</i>	AF120518	AF120575		AF120635	
	<i>Bolinus brandaris</i>	DQ279944	DQ279986	DQ280010	DQ280020	DQ280052
	<i>Raphitoma linearis</i>	DQ279945	DQ279987	DQ280011		DQ280053
	<i>Philine aperta</i>	DQ093438	DQ279988	DQ093508		DQ093482
	<i>Creseis</i> sp.	DQ279946	DQ279989	DQ280012	DQ280021	
	<i>Peltdoridius atromaculata</i>	AF120521	DQ279990	DQ280013	AF120637	DQ280054
	<i>Salinator solida</i>	DQ093440	DQ279991	DQ093510	DQ093528	DQ093484
	<i>Onchidella</i> sp.	DQ093441	DQ279992	DQ093511	DQ093529	DQ093485
	<i>Siphonaria plectinata</i>	X91973	DQ279993	AY377780	AF120638	AY377627
	<i>Ophicardelus ornatus</i>	DQ093442	DQ279994	DQ093512	DQ093530	DQ093486
	<i>Micromelo undatus</i>	DQ093443	DQ279995	DQ093513		DQ093487

sea monoplacophoran species from Antarctica. Contrary to all previously published accounts, which placed monoplacophorans as a sister group to higher, i.e., shelled, molluscs, our data strongly support a clade including Monoplacophora and Polyplacophora. This rather surprising result from a conchological perspective is congruent with soft anatomy data. It furthermore reopens the debate about the putative ancestral segmentation of molluscs (3), because serial repetition of gills and pedal retractor muscles may be derived and not primitive features within molluscs. If this were the case, little evidence would remain for the case of homology of segmentation in annelids and serial repetition in molluscs (33), as

confirmed in part by recent reevaluation of their early development (34, 35).

Materials and Methods

Species Sampling. Taxon sampling was carefully designed following original and published work on the internal phylogeny of chitons, bivalves, cephalopods, gastropods, and scaphopods (15, 16, 36–38). Outgroups were selected among other spiralian protostomes (lophotrochozoans) (39). The enigmatic *Xenoturbella* was also included because it was once postulated to be a derived mollusc, although more recent data consider it to be an ancestral deuter-

ostome (22). In total, we analyzed 101 molluscs including 2 Caudofoveata, 2 Solenogastres, 13 Polyplacophora, 1 Monoplacophora, 9 Scaphopoda, 32 Gastropoda, 24 Bivalvia, and 18 Cephalopoda (see Table 1).

Molecular Data. Molecular data were obtained from ethanol-preserved specimens following standard protocols for molluscan samples (15, 37, 38, 40). Monoplacophoran DNA samples were extracted from the half specimen preserved in 96% EtOH. DNA from preserved tissues was extracted by using the Qiagen DNeasy tissue kit. Data include complete sequences of 18S rRNA, a 3-kb fragment of 28S rRNA, the protein-coding nuclear gene histone H3, and two mitochondrial gene fragments for cytochrome *c* oxidase subunit I and 16S rRNA, totaling ≈6.5 kb per complete taxon (see Table 1). The amplified samples were purified by using the QIAquick PCR purification kit (Qiagen), labeled by using BigDye Terminator 3.0 (Applied Biosystems), and sequenced with an ABI 3730 genetic analyzer (Applied Biosystems) following the manufacturer's protocols. Chromatograms obtained from the automatic sequencer were read, and "contig sequences" were assembled by using the editing software SEQUENCHER 4.0 and further manipulated in GDE 2.2 (41).

From the five different molecular loci chosen for this study, only one yielded positive amplification for the monoplacophoran specimen. This fragment corresponds to a 1.2-kb segment of 28S rRNA obtained by amplifying two overlapping fragments using primer pairs 28Sa and 28S rd5b (5'-GACCCGTCTTGAAGCACG-3' and 5'-CCACAGCGCCAGTTCTGCTTAC-3') and 28S rd4.8a and 28S rd7b1 (5'-ACCTATTCTCAAACCTTTAAATGG-3' and 5'-GACTTCCCTTACCTACAT-3').

Data Analyses. DNA sequence data were analyzed following two approaches. First, a dynamic homology approach ("single-step phylogenetics") using parsimony as an optimality criterion for direct

optimization (42) was undertaken in the computer package POY 3.0.11 (43). Second, a static homology approach ("two-step phylogenetics") using a model-based approach was executed under Bayesian phylogenetics in MRBAYES 3.1.1 (44).

For the direct optimization analysis, tree searches were conducted by a combination of random addition sequences with multiple rounds of tree fusing (45) on a small 50-processor cluster assembled at Harvard University. Support measures were estimated by using jackknifing with a character probability of deletion of e^{-1} (46). The data were analyzed for all genes in combination as well as restricted to the 28S rRNA fragment sequenced for *L. antarctica* under different analytical parameter sets (47, 48).

Bayesian posterior probabilities were calculated by using a general time-reversible model with corrections for the proportion of invariant sites and a discrete gamma distribution, as selected in MODELTEST 3.7 (49) under the Akaike Information Criterion (50). Two runs of 10^6 generations were performed, storing 1/100th visited trees. Results from MRBAYES 3.1.1 were visualized in the program TRACER 1.3 (51), which served to determine the burnin, which differed considerably in the two runs. Aligned data were obtained from the implied alignment (52) generated in POY 3.0.11 for the analyses presented in Fig. 2.

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1. Bouchet, P., Lozouet, P., Maestrati, P. & Heros, V. (2002) *Biol. J. Linn. Soc.* **75**, 421–436.
2. Valentine, J. W. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 8001–8005.
3. Nielsen, C. (2001) *Animal Evolution: Interrelationships of the Living Phyla* (Oxford Univ. Press, Oxford), 2nd Ed.
4. Scheltema, A. H. (1993) *Biol. Bull.* **184**, 57–78.
5. Haszprunar, G. (2000) *Am. Malacol. Bull.* **15**, 115–130.
6. Aguinaldo, A. M. A., Turbeville, J. M., Lindford, L. S., Rivera, M. C., Garey, J. R., Raff, R. A. & Lake, J. A. (1997) *Nature* **387**, 489–493.
7. Eernisse, D. J. & Reynolds, P. D. (1994) in *Microscopic Anatomy of Invertebrates*, eds. Harrison, F. W. & Kohn, A. J. (Wiley-Liss, New York), Vol. 5, pp. 55–110.
8. Lemche, H. (1957) *Nature* **179**, 413–416.
9. Haszprunar, G. & Schaefer, K. (1997) in *Microscopic Anatomy of Invertebrates*, eds. Harrison, F. W. & Kohn, A. J. (Wiley-Liss, New York), Vol. 6B, pp. 415–457.
10. Lemche, H. & Wingstrand, K. G. (1959) *Galathea Rep.* **3**, 9–71.
11. McAlester, A. L. (1965) *Paleontology* **8**, 231–246.
12. Lindberg, D. R. & Ghiselin, M. T. (2003) *Proc. Calif. Acad. Sci.* **54**, 663–686.
13. Valentine, J. W. (2004) *On the Origin of Phyla* (Univ. of Chicago Press, Chicago).
14. Salvini-Plawen, L. V. & Steiner, G. (1996) in *Origin and Evolutionary Radiation of the Mollusca*, ed. Taylor, J. D. (Oxford Univ. Press, Oxford), pp. 29–51.
15. Giribet, G. & Wheeler, W. C. (2002) *Invertebr. Biol.* **121**, 271–324.
16. Steiner, G. & Dreyer, H. (2003) *Zool. Scripta* **32**, 343–356.
17. Passamanek, Y. J., Schander, C. & Halanych, K. M. (2004) *Mol. Phylogenet. Evol.* **32**, 25–38.
18. Wanninger, A. & Haszprunar, G. (2002) *J. Morphol.* **254**, 53–64.
19. Schrödl, M., Linse, K. & Schwabe, E. (2006) *Polar Biol.*, in press.
20. Schrödl, M. (2006) *Spixiana*, in press.
21. Mallatt, J. & Winchell, C. J. (2002) *Mol. Biol. Evol.* **19**, 289–301.
22. Bourlat, S. J., Nielsen, C., Lockyer, A. E., Littlewood, D. T. & Telford, M. J. (2003) *Nature* **424**, 925–928.
23. Salvini-Plawen, L. V. (1972) *Z. Wiss. Zool. (Leipzig)* **184**, 205–394.
24. Schaefer, K. & Haszprunar, G. (1997) *Zool. Anz.* **236**, 13–23.
25. Haas, W. (1981) *Biominer. Res. Rep.* **6**, 1–52.
26. Scheltema, A. H., Kerth, K. & Kuzirian, A. M. (2003) *J. Morphol.* **257**, 219–245.
27. Vinther, J. & Nielsen, C. (2005) *Zool. Scripta* **34**, 81–89.
28. Sutton, M. D., Briggs, D. E. G., Siveter, D. J. & Siveter, D. J. (2004) *Palaentology* **47**, 293–318.
29. Martin, M. W., Grazhdankin, D. V., Bowring, S. A., Evans, D. A., Fedonkin, M. A. & Kirschvink, J. L. (2000) *Science* **288**, 841–845.
30. Runnegar, B., Pojeta, J., Jr., Taylor, M. E. & Collins, D. (1979) *J. Paleontol.* **53**, 1374–1394.
31. Pojeta, J., Jr., Eernisse, D. J., Hoare, R. D. & Henderson, M. D. (2003) *J. Paleontol.* **77**, 646–654.
32. Vendrasco, M. J., Wood, T. E. & Runnegar, B. N. (2004) *Nature* **429**, 288–291.
33. Haszprunar, G. & Schaefer, K. (1996) *Acta Zool. (Stockholm)* **77**, 315–334.
34. Nielsen, C. (2004) *J. Exp. Zool. B* **302**, 35–68.
35. Wanninger, A. & Haszprunar, G. (2002) *J. Morphol.* **251**, 103–113.
36. Giribet, G. & Distel, D. L. (2003) in *Molecular Systematics and Phylogeography of Mollusks*, eds. Lydeard, C. & Lindberg, D. R. (Smithsonian Books, Washington, DC), pp. 45–90.
37. Okusu, A., Schwabe, E., Eernisse, D. J. & Giribet, G. (2003) *Org. Divers. Evol.* **3**, 281–302.
38. Lindgren, A. R., Giribet, G. & Nishiguchi, M. K. (2004) *Cladistics* **20**, 454–486.
39. Giribet, G. (2002) *Mol. Phylogenet. Evol.* **24**, 345–357.
40. Okusu, A. & Giribet, G. (2003) *J. Moll. Stud.* **69**, 385–387.
41. Linton, E. W. (2005) MACGDE: Genetic Data Environment for Mac OS X (Michigan State Univ., East Lansing).
42. Wheeler, W. C. (1996) *Cladistics* **12**, 1–9.
43. Wheeler, W. C., Gladstein, D. & De Laet, J. (2004) POY (Am. Museum of Natural History, New York), Version 3.0.
44. Ronquist, F. & Huelsenbeck, J. P. (2005) MRBAYES: Bayesian Analysis of Phylogeny (Florida State Univ., Tallahassee), Version 3.1.1.
45. Goloboff, P. A. (1999) *Cladistics* **15**, 415–428.
46. Farris, J. S., Albert, V. A., Källersjö, M., Lipscomb, D. & Kluge, A. G. (1996) *Cladistics* **12**, 99–124.
47. Wheeler, W. C. (1995) *Syst. Biol.* **44**, 321–331.
48. Giribet, G. (2003) *Syst. Biol.* **52**, 554–564.
49. Posada, D. (2005) MODELTEST (Univ. of Vigo, Vigo, Spain), Version 3.7.
50. Posada, D. & Buckley, T. (2004) *Syst. Biol.* **53**, 793–808.
51. Rambaut, A. & Drummond, A. (2003) TRACER: MCMC Trace Analysis Tool (University of Oxford, Oxford), Version 1.3.
52. Wheeler, W. C. (2003) *Cladistics* **19**, 261–268.