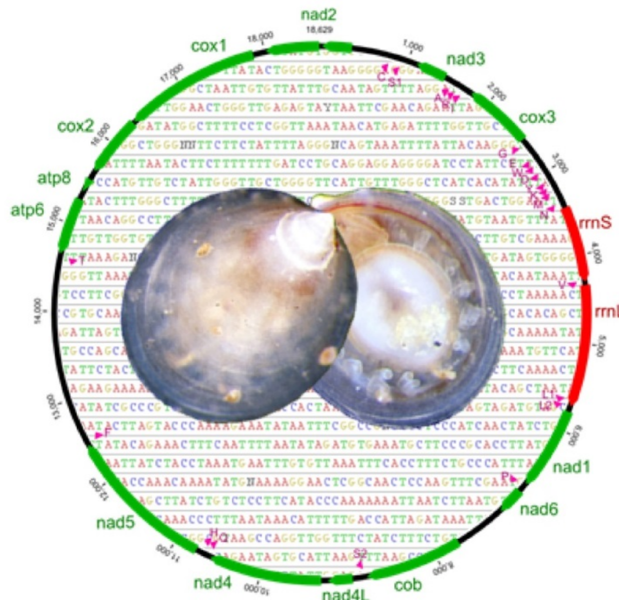
Phylogeny and evolution of Monoplacophora and Mollusca



Dissertation

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Titelbild: *Laevipilina* (Monoplacophora; Foto: Michael Schrödl) mit mitochondrialem Genom (Isabella Stöger) und Ausschnitt eines Mollusken-Alignments (Isabella Stöger); angefertigt in Zusammenarbeit mit Zeyuan Chen.

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Für meine Eltern Charlotte und Franz

Table of Contents

1. Abstract……………………………………………………………………………………………………1

2. Introduction………………………………………………………………………………………………..3

2.1. The phylum Mollusca and its classes…………………………………………………………3

2.2. Class level relationships of Mollusca…………………………………………………………8

2.3. Position of Mollusca within (Lopho-) Trochozoa…………………………………………11

2.4. Timing of the molluscan tree…………………………………………………………………12

2.5. Aims of the thesis……………………………………………………………………………………19

3. Results………………………………………………………………………………………………………21

3.1. **Isabella Stöger**, Julia D. Sigwart, Yasunori Kano, Thomas Knebelsberger, Bruce A. Marshall: **The continuing debate on deep molluscan phylogeny: evidence for Serialia (Mollusca, Monoplacophora + Polyplacophora). 2013.** BioMed Research International……………………………………………………………………………………………21

3.2. Katharina M. Jörger, **Isabella Stöger**, Yasunori Kano, Hiroshi Fukuda, Thomas Knebelsberger, Michael Schrödl: **On the origin of Acochlidia and other enigmatic euthyneuran gastropods, with implications for the systematics of Heterobranchia. 2010.** BMC Evolutionary Biology……………………………………………..61

3.3. Julia D. Sigwart, **Isabella Stöger**, Thomas Knebelsberger, Enrico Schwabe: **Chiton phylogeny (Mollusca: Polyplacophora) and the placement of the enigmatic species *Choriplax grayi* (H. Adams & Angas). 2013.** Invertebrate Systematics….85

3.4. **Isabella Stöger**, Michael Schrödl: **Mitogenomics does not resolve deep molluscan relationships (yet?). 2013.** Molecular Phylogenetics and Evolution..107

3.5. **Isabella Stöger**, Kevin M. Kocot, Albert J. Poustka, Nerida G. Wilson, Dimitry Ivanov, Kenneth M. Halanych, Michael Schrödl: **Monoplacophoran mitochondrial genomes: convergent gene arrangements and little phylogenetic signal. 2016.** BMC Evolutionary Biology…………………………………………………………………………..129

3.6. Michael Schrödl, **Isabella Stöger**: **A review on deep molluscan phylogeny: old markers, integrative approaches, persistent problems. 2014.** Journal of Natural History……149

3.7. Kevin M. Kocot, Albert J. Poustka, **Isabella Stöger**, Kenneth M. Halanych, Michael Schrödl: **New data from Monoplacophora and a carefully-curated dataset resolve molluscan relationships. 2020.** Scientific Reports……183

4. Discussion……201

4.1. Phylogenetic markers……201

4.1.1. (Limitation of) combined mitochondrial and nuclear standard markers……202

4.1.1.1. Phylogeny of Heterobranchia (Gastropoda)……202

4.1.1.2. Phylogeny of Chitons (Polyplacophora)……203

4.1.1.3. Phylogeny of Mollusca……205

4.1.2. Mitogenomics – phylogenetic analysis and gene arrangements……206

4.1.2.1. Heterobranchia (Gastropoda)……206

4.1.2.2. Chitons (Polyplacophora)……207

4.1.2.3. Mollusca……208

4.1.3. Whole genome approach – support for Aculifera/Conchifera……209

4.2. Dating the evolution of Mollusca……211

5. Conclusion and Outlook……215

6. Acknowledgements……217

7. References……218

8. Declaration of contributions to each publication……233

9. Curriculum Vitae……235

10. Statutory Declaration and Statement……239

1. Abstract

The Mollusca comprises eight classes which are highly diverse in their morphology as well as in molecular appearance. The class level relationships in molluscs were hotly debated during decades and are still under discussion as there is no overall support for one single concept. Morphological and recent phylogenomic studies support the hypothesis of Aculifera (Solenogastres, Caudofoveata and Polyplacophora) and summarize Bivalvia, Cephalopoda, Gastropoda, Monoplacophora and Scaphopoda as the Conchifera. Alternative concepts as Testaria (Bivalvia, Cephalopoda, Gastropoda, Monoplacophora, Scaphopoda and Polyplacophora) or Serialia (Monoplacophora + Polyplacophora) were suggested in the past based on morphological analyses (Testaria) or mainly molecular evidence (Serialia). In order to bring resolution to the class relationships and the early evolution within Mollusca we compiled several comprehensive taxon sets comprising different molecular datasets: combined nuclear and mitochondrial markers obtained via Sanger sequencing ("standard markers"), mitochondrial genomes (analyzing the phylogenetic information of the sequence data as well as comparing the gene arrangements) and phylogenomic data obtained via Next Generation Sequencing. We were able to generate novel data of several species of the elusive class Monoplacophora. Based on the set of standard markers, we found support for Serialia whereas the phylogenomic approach leads to Aculifera and Conchifera, providing first molecular evidence for Monoplacophora sister to Cephalopoda plus other conchiferans; a clade of Gastropoda and Scaphopoda is also supported. Both phylogenetic analyses were used for time estimations and resulted in congruent ages for the molluscan stem (Precambrian) and the diversification of Mollusca (584Mya). We were the first to present a complete mitochondrial genome of a monoplacophoran species ever. Analyzing the mitochondrial gene arrangements we were able to detect potential synapomorphies for Mollusca. Standard marker analyses on comprehensive taxon sets provided novel phylogenetic hypotheses on several molluscan subgroups, such as chitons and gastropods, in particular heterobranchs, challenging mitogenomic approaches and results in the latter. Overall, our studies addressed the phylogeny and evolution of Mollusca and subgroups with a variety of markers and methods and helped to pave the way from using multilocus markers and mitogenomics towards whole genomes.

2. Introduction

2.1. The phylum Mollusca and its classes

The phylum Mollusca is one of the largest groups of animals. It comprises at least 130,000 living species (Haszprunar et al. 2008). Species are highly diverse, not only in respect to their body structures but also regarding their feeding habits and natural habitats. Molluscs are present and often abundant in almost all ecosystems except the airspace and permanent ice, although the highest diversity is recorded in marine waters. They even colonize extreme localities as for example hot vents in the deep sea. Many taxa are of great economic and ecological importance (Ponder and Lindberg 2008). The Mollusca comprise eight recent classes: Gastropoda, Bivalvia, Cephalopoda, Polyplacophora, Scaphopoda, Solenogastres, Caudofoveata and Monoplacophora. The monophyly of each of the classes is undisputed but the relationships between classes as well as the early evolution of Mollusca are still under debate (Haszprunar et al. 2008, Salvini-Plawen 1981, Salvini-Plawen and Steiner 2014, Sigwart and Lindberg 2015, Telford and Budd 2011, Wanninger and Wollesen 2019).

Molluscs have been studied for centuries. Modern phylogenetic research was based on cladistic analyses of morphological characters (e.g. Salvini-Plawen and Steiner 1996, Haszprunar 2000). The first molecular trees were generated in the 1990s (e.g. Winnepenninckx et al. 1996) with poor support values in many of the deeper nodes. But techniques improved and many efforts were made in the field of molecular biology of the Mollusca which end up in Next Generation Sequencing (NGS) data of nuclear genomes (Kocot et al. 2011, Smith et al. 2011, Riesgo et al. 2012, Zapata et al. 2014, González et al. 2015, Combosch et al. 2017b).

Gastropoda is the most diverse molluscan group with around 100,000 species that range in size from 0.5mm up to 1m (Aktipis et al. 2008, Haszprunar and Wanninger 2012). It is the only class that invaded the land (Aktipis et al. 2008). Their shell is coiled, limpet-like, internalized or entirely missing; even bivalved shells are known in Juliidae (Aktipis et al. 2008). Six major groups are usually distinguished within the gastropods: Patellogastropoda, Vetigastropoda, Neritimorpha, Cocculinida, Caenogastropoda, and Heterobranchia. Traditionally, Patellogastropoda were the most basal offshoot of gastropods and sister to Orthogastropoda, the rest of the gastropod groups (e.g. Haszprunar 1988). This scenario was supported by mitogenomic data (Uribe et al. 2019), although it was rejected by

transcriptomic approaches: analyses conducted by the Giribet Lab (Harvard University, USA) offer an alternative relationship of patellogastropods + vetigastropods as most basal groups in sistergroup relationship to Neritimorpha and Apogastropoda (Caenogastropoda + Heterobranchia) (Zapata et al. 2014, Cunha and Giribet 2019). Particularly heterobranch groups were rearranged substantially in the last decade (e.g. Salvini-Plawen 1980, Haszprunar 1985, Salvini-Plawen and Steiner 1996, Ponder and Lindberg 1997). Traditionally, Euthyneura, Opisthobranchia, and Pulmonata were regarded as monophyletic - albeit informal - groups (Haszprunar 1985, Bouchet and Rocroi 2005). Klussmann-Kolb et al. (2008) were the first to recover non-monophyletic opisthobranchs and pulmonates with their multilocus analyses but did not yet trust their significance. Adding more taxa, Jörger and colleagues (2010) redefined these groups, respectively reclassified several superfamilies and established Euopisthobranchia, including Umbraculoidea, Cephalaspidea s.s., Runcinacea, Anaspidea and Pteropoda, but excluding Acteonoidea and Nudipleura, as well as Sacoglossa and Acochlidia, and Panpulmonata, comprising Siphonarioidea, Sacoglossa, Hygrophila, Amphiboloidea, Pyramidelloidea, Glacidorboidea, Eupulmonata and Acochlidia (see chapter 2.2). This new classification is supported by analyses of genomic and transcriptomic data (Kocot et al. 2013, Zapata et al. 2014, Cunha and Giribet 2019).

The second largest class of molluscs is the Bivalvia with up to 10,000 species. They inhabit freshwater habitats as well as marine waters from the intertidal to the abyssal. Their characteristic (and eponymous) feature is the bisected shell, the two valves connected dorsally via a hinge. The diversity of bivalve classifications was high. There was no broad agreement on valid names for groups and the classification of species (Bieler and Mikkelsen 2006, Plazzi et al. 2011). Usually, recent publications distinguish the following groups: the probably oldest group of bivalve species, the Protobranchia, including Solemyoidea, Nuculoidea, and Nuculanoidea, which is sistergroup to all other bivalves (Autolamellibranchiata): Pteriomorpha, the mussels, scallops, oysters, and arks; Palaeoheterodonta, mainly freshwater mussels and trigoniids; Archiheterodonta; and Euheterodonta, including Anomalodesmata (Bieler and Mikkelsen 2006, Giribet 2008, Sharma et al. 2012). The classification into these groups is based on morphology as well as molecular data (e.g. Waller 1998, Millard 2001, Giribet 2008, González and Giribet 2014, Combosch et al. 2017a).

Some of the autolamellibranch groups (Mytiloida, Unionoida, Veneroida) display a special molecular feature: they inherit their mitogenomes doubly uniparental (Doucet-Beaupré et al. 2010), that means, female bivalves transmit their mitogenome to male and female descendants, and male species transmit it to male offspring only. This arouses interest to investigate mitogenomics on species-, as well as, individual level (e.g. Doucet-Beaupré et al. 2010, Zouros 2013, Gusman et al. 2016, Capt et al. 2018, Plazzi and Passamonti 2018).

The almost 1,000 living species of the class Cephalopoda are present in all marine waters and can be classified in Nautiloidea, including one family, and Coleoidea with the two groups Octopodiformes and Decapodiformes (Nishiguchi and Mapes 2008). The systematic classification beyond this level is controversial in numbers of orders, suborders, superfamilies and their rank. The systematic question is getting even more complex if fossil taxa are included, as cephalopods have a high number of fossil species (ca. 30,000) over a time span of 450 million years (Nishiguchi and Mapes 2008). Analysis of a morphological character matrix divided octopods in Incirrata and Cirrata and placed Vampyromorpha as their sistergroup but the analysis could not resolve the decapod part of the tree (Young and Vecchione 1996). Especially the position of Vampyromorpha was frequently questioned by molecular data. Either this group was sister to Octopoda (Allcock et al. 2011, Lindgren et al. 2012, Groth et al. 2015, Uribe and Zardoya 2017) or to Decapodiformes (Lindgren et al. 2004, Strugnell and Nishiguchi 2007, Zhang et al. 2016). The recent study by Sanchez and colleagues analyses a comprehensive set of 124 cephalopod species and a combined data matrix of mitochondrial and nuclear markers, spanning almost 16,000 bp in length (Sanchez et al. 2018). They had some problems to resolve decapod internal relationships but receive good support for monophyly of octopod families and their relationships. The decapod part of the tree is supported by several morphological characters that were mapped to the resulting phylogeny (Sanchez et al. 2018). Still, more information on taxon level as well as on molecular marker level is needed.

Polyplacophoran species, commonly called chitons, are dorsoventrally flattened animals with usually eight overlapping dorsal shell plates (Todt et al. 2008). The plates are surrounded by the so-called perinotum, a thick marginal girdle that is covered by a cuticle with embedded sclerites (Todt et al. 2008). The cuticle is chitinous whereas sclerites are of calcium carbonate (Todt et al. 2008). These shell plates cover a ventral body that is divided in a broad foot and a head (Todt et al. 2008). There are about 920 described living species of

chitons (Schwabe 2008); all of them are living in marine water exclusively (Schwabe 2008). They were traditionally classified in four suborders: Lepidopleurina, Choriplacina (monotypic), Ischnochitonina, and Acanthochitonina (see Todt et al. 2008). This classification was revised and chitons have been divided in two orders, Lepidopleurida and Chitonida (Sirenko 1993, 1997), what is supported by the molecular study based on five standard markers (18s, 28s, 16s, COI, H3; Okusu et al. 2003). Chitonida are subdivided in Chitonina and Acanthochitonina by morphological data (Sirenko 2006).

Scaphopoda are marine "global-players" and are commonly known as "tusk-shells"; the shells of all the extant species (more than 500) are conical tubes which are curved and open on both sides (Reynolds and Steiner 2008). Their body size ranges from a few millimeters to several centimeters. Scaphopod taxa are classified in two orders: Dentaliida and Gadilida (Reynolds and Steiner 2008). Monophyly of both orders is supported by morphological (Steiner 1998, Reynolds and Okusu 1999) as well as molecular datasets (Steiner and Dreyer 2003, Steiner and Reynolds 2003, Kocot et al. 2019b), but classifications below order level vary (Reynolds and Steiner 2008).

The two shell-less, worm-shaped groups Solenogastres (Neomeniomorpha) and Caudofoveata (Chaetodermomorpha) both usually had class-level status and were not regarded as sistergroups (Salvini-Plawen 1985, Salvini-Plawen and Steiner 1996, Haszprunar 2000); alternatively, these two worm-shaped molluscan groups were seen as subclasses of the monophyletic class Aplacophora (Pelseneer 1906, Scheltema 1978, 1988, 1993). Both, Solenogastres and Caudofoveata, can be distinguished clearly from each other (Todt 2013) but the phylogenetic relationships within each of the groups are not known very well. Both clades possess characteristic calcareous sclerites instead of a shell or shell plates.

Solenogastres (Neomeniomorpha) comprises up to 300 species, with an exclusively marine habitat and a global distribution. Based on external morphological characters four orders were distinguished (Salvini-Plawen 1978): Neomeniamorpha, Sterrofustia, Pholidoskepia, and Cavibelonia. Problems of solenogaster systematics are the high diversity of morphological characters, and the limited number of taxa included in the studies (Todt et al. 2008). Molecular markers of the members of this group are hard to amplify because nuclear genes bear complex secondary structures therefore conventional primers for mitochondrial markers do not work properly (Bergmeier et al. 2017). Available molecular data need to be

treated with care because many sequences are contaminated (Meyer et al. 2010, own observations). Using two carefully treated mitochondrial markers, Bergmeier et al. (2019) recovered non-monophyletic Pholidoskepia and Cavibelonia. This result was validated partly within a phylogenomic study (Kocot et al. 2019a). Both studies dramatically improved the availability of sequence data for Solenogastres and also Caudofoveata. Moreover, solenogaster mitogenomic analyses are in progress (personal communication Franziska Bergmeier).

The Caudofoveata (Chaetodermomorpha) include ca. 130 species in three families: Chaetodermatidae, Prochaetodermatidae, and Limifossoridae which are hard to distinguish from each other (Todt 2013, Mikkelsen and Todt 2018). The up-to-date study of mitogenomic data recovered Chaetodermatidae as monophyletic, but not Limifossoridae (Mikkelsen et al. 2018). Analyses of molecular versus morphological data did not lead to consistent phylogenies within this group (Mikkelsen et al. 2018, 2019, Kocot et al. 2019a).

Monoplacophora were thought to be extinct since the Devonian period (Lindberg 2009) until a living individual of *Neopilina galatheae* was found in 1952 in the Pacific Ocean off Costa Rica (Lemche 1957). In fact, Tryblidia (Lindström 1884) is the more precise name for extant monoplacophorans, as the term Monoplacophora includes also fossil taxa, which are, however, not all doubtlessly related to the extant species; so Monoplacophora is probably a non-monophyletic grouping (Haszprunar 2008). Nevertheless, we keep to the term Monoplacophora herein as it is more common, and its inclusiveness might be adjusted. Their habitat is the deep sea (Schwabe 2008, Kano et al. 2012), what makes it difficult to collect these tiny “living fossils” (body size approximately 1 to 40mm, see Haszprunar 2008), as they were referred to since their “re”-discovery in the 1950s (e.g. Lindberg 2009, Kano et al. 2012). According to Haszprunar (2008), 29 species were arranged in two families: Neopilinidae and Micropilinidae (but see Kano et al. 2012 for alternative grouping of the genera *Veleropilina* and *Rokopella*). The monoplacophorans have cap-shaped shells with a nacreous shell structure. As these characters are usually lacking in fossils due to poor preservation, it is often difficult to distinguish them from patellogastropods (Haszprunar 2008). Several organ systems, e.g. shell muscles, nephridia, and ctenidia, are serially repeated in monoplacophorans (Haszprunar 2008).

2.2. Class level relationships of Mollusca

Although the phylum Mollusca is highly diverse, all classes have some features in common. Molluscs possess a unique radula (rasping tongue), which is reduced in bivalves and a couple of other molluscs, and a mantle cavity that is used for breathing and excretion; the molluscan body is organized in head, foot and visceral sac and a calcareous shell secreted by shell glands in the mantle. The circulatory system is usually an open system. Molecular studies recovered monophyletic Mollusca, based on e.g. a ribosomal multigene dataset (Meyer et al. 2011), housekeeping genes (Vinther et al. 2011), and two EST (expressed sequence tag) studies (Kocot et al. 2011, Smith et al. 2011, 2013). Nevertheless, class level relationships in molluscs were hotly debated during decades and are still under discussion as there is no overall support for one single concept, neither by morphological nor by molecular data (see e.g. Haszprunar and Wanninger 2012). Mollusca include a high number of problematic taxa which show reduction of certain character states and/or complete loss. That fact leads to the question if such a character is either primitive or highly derived (Lindberg et al. 2004). Furthermore, most analyses do not include morphological data of fossils (Lindberg et al. 2004). As molluscan classes date back at least to the Early Cambrian, a huge quantity of data might be lost or has at least not been considered. Otherwise the inclusion of fossil data (e.g. Sutton and Sigwart 2012) might be misleading due to the fact that mainly fossil molluscs with hard part structures can be included in such analyses.

Based on morphology, those classes that possess a single shell composed of an organic layer, the periostracum, and one thick mineralized layer made of calcium carbonate, were summarized as the Conchifera (Bivalvia, Cephalopoda, Gastropoda, Monoplacophora, Scaphopoda; e.g. Runnegar 1996, Waller 1998, Haszprunar 2000; Fig. 1) with monoplacophorans at the base (e.g. Runnegar 1996, Waller 1998, Haszprunar 2000). Other conchiferan apomorphies are one pair of statocysts and the absence of a protecting cuticle on top of the at least two shell layers (Haszprunar 2000, Haszprunar et al. 2008). Within Conchifera the position of Scaphopoda is under debate. This class might be sister to bivalves, a combination that is called Diasoma (Runnegar and Pojeta 1985, 1992, Runnegar 1996, Salvini-Plawen and Steiner 1996; Fig. 3). According to Haszprunar (2000) and Wanninger and Haszprunar (2001, 2002), Scaphopoda are sister to so-called Cyrtosoma: Gastropoda plus Cephalopoda (Fig. 3). The palaeontological view (Pojeta 1971, Pojeta and Runnegar 1976, Runnegar 1996) groups extinct Rostroconchia with the “initially laterally compressed

Diasoma (Rostroconchia + Bivalvia + Scaphopoda)" (Parkhaev 2008) versus "initially dorsoventrally elongated Cyrtosoma (Cephalopoda + Gastropoda)" (Parkhaev 2008). A scaphopod-cephalopod clade was discovered by Waller (1998) and Steiner and Dreyer (2003).

Salvini-Plawen and Steiner (1996) suggested a class relationship of Bivalvia, Cephalopoda, Gastropoda, Monoplacophora, Scaphopoda and Polyplacophora and summarized them as the Testaria, based on morphological characters (Fig. 1). All the included classes possess a shell (regardless of its exact composition). The hypothesis of Testaria was confirmed by Haszprunar (2000) based on a large morphological dataset but it was never supported in any of the molecular studies. Caudofoveata and Solenogastres, both with a rather simple morphology and possession of aragonitic sclerites instead of a true shell (e.g. Salvini-Plawen 1980, Salvini-Plawen and Steiner 1996, Haszprunar 2000), were considered as most basal molluscan groups (e.g. Salvini-Plawen 1980, Salvini-Plawen and Steiner 1996, Haszprunar 2000). Interpretation of morphological characters suggested either the Caudofoveata as the most basal offshoot of Mollusca and Solenogastres at the base of Testaria (Adenopoda concept; Salvini-Plawen 1985; Fig. 2) or the Solenogastres as earliest molluscan branch with Caudofoveata integrated at the base of Testaria (Hepagastralia concept; Salvini-Plawen and Steiner 1996, Haszprunar 2000; Fig. 2). An alternative view summarized both groups as the monophyletic taxon Aplacophora and put it as sister group to Testaria (Scheltema 1993, Waller 1998).

An alternative concept of molluscan class relationships unites worm-like molluscs (Aplacophora; Solenogastres and Caudofoveata) that possess sclerites, and places Polyplacophora as sister to Aplacophora (Aculifera concept; Scheltema 1993, Ivanov 1996, Scheltema and Taylor 1996; Fig. 1). Monophyletic Aculifera (Caudofoveata, Polyplacophora, Solenogastres) are in conflict with the Testaria hypothesis (see Fig. 1).

Several attempts were made to find molecular evidence for or against the one or the other molluscan phylogenetic hypothesis. First relevant analyses were based on relatively small taxon sets and limited datasets of nuclear and mitochondrial DNA fragments (e.g. Ghiselin 1988, Lecanidou et al. 1994, Winnepenninckx et al. 1994). These studies could not or could hardly support any of the molluscan hypotheses (e.g. Passamanneck et al. 2004). Extremely high rate heterogeneity within the Mollusca, partially high substitution rates and difficulties

in obtaining uncontaminated sequence material were some of the problems worth mentioning when dealing with the molecular biology of Mollusca (e.g. Passamanneck et al. 2004, Giribet et al. 2006, see also Wägele et al. 2009, Meyer et al. 2010). Datasets were improved and extended on species level as well as on class level, as some molluscan classes were extremely underrepresented or missing at all in former studies (e.g. Winnepenninckx et al. 1994, Winnepenninckx et al. 1996, Passamanneck et al. 2004). A set of seven housekeeping genes (=genes that code for fundamentally important cell respiratory elements; selected because of their phylogenetic accuracy and the range of evolutionary rates that they include (Sperling et al. 2009)) could confirm Aculifera: monophyletic Aplacophora with Polyplacophora at their base (Vinther et al. 2011). Cephalopoda were recovered as sister to Aculifera and therefore monophyletic Conchifera were rejected based on this dataset (Vinther et al. 2011). This scenario was found in other studies based on large-scale datasets (Dunn et al. 2008, Lieb and Todt 2008, Hejnol et al. 2009, Meyer et al. 2011).

Next generation sequencing (NGS) gave rise to large scale datasets with increasing number of species (Kocot et al. 2011, Meyer et al. 2011, Smith et al. 2011). Using a subset of genes of an EST (=expressed sequence tag) approach the phylum Mollusca as well as the molluscan classes were recovered monophyletic (Meyer et al. 2011). Gastropoda and Bivalvia were sister groups (Meyer et al. 2011), a relationship that already appeared in a large-scale analysis of up to 77 metazoans (Dunn et al. 2008) as well as based on housekeeping genes (Vinther et al. 2011) and was named Pleistomollusca by Kocot and colleagues (Kocot et al. 2011). Unfortunately, three of the eight molluscan classes were missing (Meyer et al. 2011); therefore, no reliable statement could be made concerning the hypotheses of molluscan class relationships (Meyer et al. 2011). The transcriptomic approaches of Kocot et al. (2011, 2017) and Smith et al. (2011) supported the Aculifera-/Conchifera-hypothesis, although the relationships within Conchifera were conflictive (see Kocot et al. 2017). Strong support was found for Pleistomollusca (Gastropoda + Bivalvia; Kocot et al. 2011) but also for Gastropoda + Scaphopoda (Smith et al. 2011). The Aculifera have been confirmed several times by molecular data (Kocot et al. 2011, 2017, Smith et al. 2011, Vinther et al. 2011, 2017).

Giribet et al. (2006) came up with the unprecedented hypothesis of Serialia: Monoplacophora plus Polyplacophora (Fig. 1). The topology was based on the standard set of nuclear and mitochondrial markers and could be supported by additional morphological evidence. Both classes have serially repeated gills and nephridia, as well as eight sets of

dorsoventral pedal retractor muscles (Giribet et al. 2006). Due to the contradicting position of Polyplacophora, Serialia was not compatible with the Testaria nor the Aculifera-/Conchifera-hypothesis.

2.3. Position of the Mollusca within (Lopho-)Trochozoa

Not only the inner-class relationships of molluscan taxa have been hotly disputed but also their exact placement within Lophotrochozoa (Haszprunar 1996, Giribet et al. 2000, Peterson and Eernisse 2001, Passamaneck and Halanych 2006, Dunn et al. 2008).

In the modern perspective Mollusca together with Nemertea, Entoprocta, Sipuncula, and Annelida form the clade Trochozoa; all these groups originally possess a trochophore larva in their life cycle (Haszprunar et al. 2008, Edgecombe et al. 2011, Dunn et al. 2014). Trochozoa together with lophophorate taxa were merged in the Lophotrochozoa, a clade that was originally based on 18s rDNA data (Halanych et al. 1995). The monophyly of lophotrochozoan groups was recovered several times with different molecular datasets (e.g. Halanych et al. 1995, Philippe et al. 2005, Struck et al. 2014), and supported by large genomic datasets (Dunn et al. 2008, Struck et al. 2014). Although monophyly of Trochozoa is usually well supported and widely accepted, the relationships within that clade are still under debate (Haszprunar et al. 2008). Affiliated ingroups changed since Roule originally established the clade Trochozoa; he included Annelida, Brachiopoda, Bryozoa, Echiura, Mollusca, Phoronida, Rotifera, and Sipuncula (Roule 1891). Recent studies integrated Echiura and Sipuncula into Annelida (Struck et al. 2007, 2011, Dunn et al. 2008, Edgecombe et al. 2011), and completely excluded Rotifera from Trochozoa (Dunn et al. 2008, 2014). Moreover, Nemertea were included in the trochozoan clade based on molecular markers (Turbeville et al. 1992). The sistergroup of Mollusca within Trochozoa is unclear (Fig. 1). Some authors proposed Entoprocta as sistergroup of Mollusca (e.g. Bartolomaeus 1993, Haszprunar 1996, Ax 1999). This grouping – Tetraneuralia – is based on (1) apomorph features of the larvae (Wanninger 2009): these are the complex architecture of the apical organ in both groups and a typical tetraneurous condition of the entoproct creeping larva, which was known from molluscs only (Wanninger et al. 2007), (2) characters of the entoproct creeping larva which can be found in the molluscan groundpattern, e.g. a distinct creeping foot with a ciliated gliding sole and epidermal mucous cells and a large pedal gland

(Haszprunar and Wanninger 2008, Wanninger 2009). Alternative names of the grouping have been "Lacunifera" (Ax 1999) or "Sinusoida" (Bartolomaeus 1993). Tetraneuralia was recovered in one molecular approach only so far (Marletaz et al. 2019).

Neotrochozoa, combining Mollusca and Annelida, two groups that were already connected by Lamarck in 1809, were set in close relationship because of the similarity of the trochophore larval morphology (Peterson and Eernisse 2001). This result was recovered with some molecular datasets that comprise a broad metazoan taxon selection (Halanych et al. 1995, Aguinaldo et al. 1997, García-Machado et al. 1999, Boore and Brown 2000) but was rejected in all analyses based on large lophotrochozoan transcriptomic datasets (Peterson et al. 2008, 2009, Sperling et al. 2009, Kocot et al. 2017).

A sistergroup relationship between brachiopods and molluscs is supported by the fact that both groups have the potential to build shells (Taylor et al. 2010). The shell is formed by the mantle in both cases, but the composition of the shell differs (Luo et al. 2015). Brachiopoda mainly form their shell of calcium phosphate (Luo et al. 2015); in molluscan shells calcium carbonate is the dominant material (Simkiss and Wilbur 1989, Luo et al. 2015). There is some molecular support for this relationship between Brachiopoda and Mollusca when analyzing and comparing genomic data (Struck et al. 2014, Luo et al. 2015). Evidence for this relationship can also be found from comparison of the mitochondrial gene orders: the brachiopod gene order of *Terebratulina* is very similar to the arrangement of the chiton *Katharina* (Stechmann und Schlegel 1999). Still it is questionable if both groups are related very closely, as for example investigation of Hox genes and shell forming cells do not identify common ancestry of brachiopods and molluscs (Shimizu et al. 2017).

To date, there is no consensus on the origin of Mollusca and their placement within Trochozoa is still unresolved (reviewed by Kocot 2016).

2.4. Timing of the molluscan tree

The fossil record of Mollusca reaches back to the Cambrian/Precambrian boundary (Ponder and Lindberg 2008, Parkhaev 2008). First undisputed fossil molluscs or at least molluscan remains are included in the so-called small shelly fauna (SSF) from the Cambrian (e.g. Parkhaev 2008, Parkhaev and Demidenko 2010). Components of these SSF are for example

helcionellids. They have a single conical shell with a central, subcentral, or posterior apex (Parkhaev 2008). Formerly they were interpreted as monoplacophorans (Geyer 1994), due to the fact of all being bilateral symmetrical untorted molluscs, but Peel recognized two distinct groups, Tergomya and Helcionelloida (Peel 1991a, b). Tergomya, with an anterior apex, includes the classic fossil and extant monoplacophoran species, whereas more strongly torted fossils with a posterior apex are members of Helcionelloida (Peel 1991a, b, see also Gubanov and Peel 2001). Helcionelloids appear from the Early Cambrian (with *Oelandiella,* Gubanov and Peel 1999) to the Ordovician (with *Chuiliella*, Gubanov and Peel 2003).

Kimberella, a soft-bodied fossil from the Ediacarian, was initially interpreted as a jellyfish (Wade 1972). Later, Fedonkin together with several colleagues favoured a molluscan – at least a molluscan-like affiliation (Fedonkin and Waggoner 1997, Ivantsov and Fedonkin 2001, Fedonkin et al. 2007). The fossils that were found of this organism resemble a molluscan-like bauplan, reflecting e.g. a distinct foot with a surrounding mantle and a mantle cavity as well as a dorsal structure that can be interpreted as a non-mineralized shell (Fedonkin and Waggoner 1997, Seilacher 1999, Seilacher et al. 2003). Moreover, *Kimberella* probably possessed sclerites (Ivantsov 2009). Unfortunately, it is not circumstantiated doubtlessly that the feeding traces that are visible in the immediate area around *Kimberella* localities, are attributed to the potential radulae of the individuals (Jenkins 1992, Butterfield 2006, 2008). Edgecombe et al. (2011) considered *Kimberella* to be too old to be a mollusc. Although discussion on the affiliation of *Kimberella* is ongoing, it is certainly a bilaterian organism (Fedonkin and Waggoner 1997, see also Parkhaev 2017).

Dating the molluscan tree is difficult, as long as the fossils that are used for calibrating the tree cannot be clearly assigned to a certain group and it is even more difficult when there is no stable phylogenetic backbone for the phylum. Obtaining a reliable phylogeny of the molluscan tree could help assigning fossils that are still dubious in their classification.

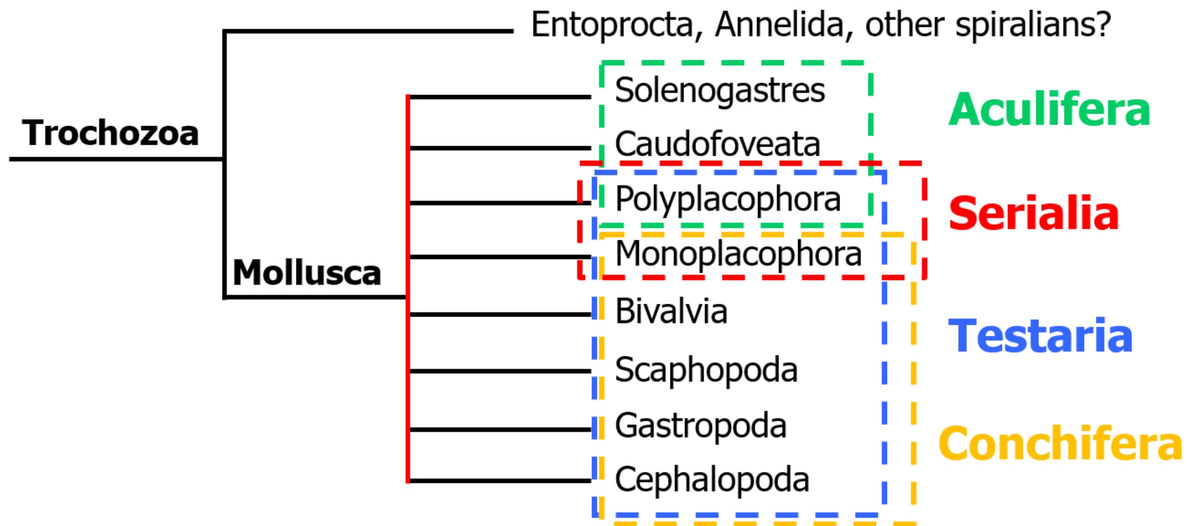

Fig. 1: Major competing hypotheses of molluscan class relationships: Aculifera, Conchifera, Testaria and Serialia, and possible outgroups of the Mollusca within Trochozoa.

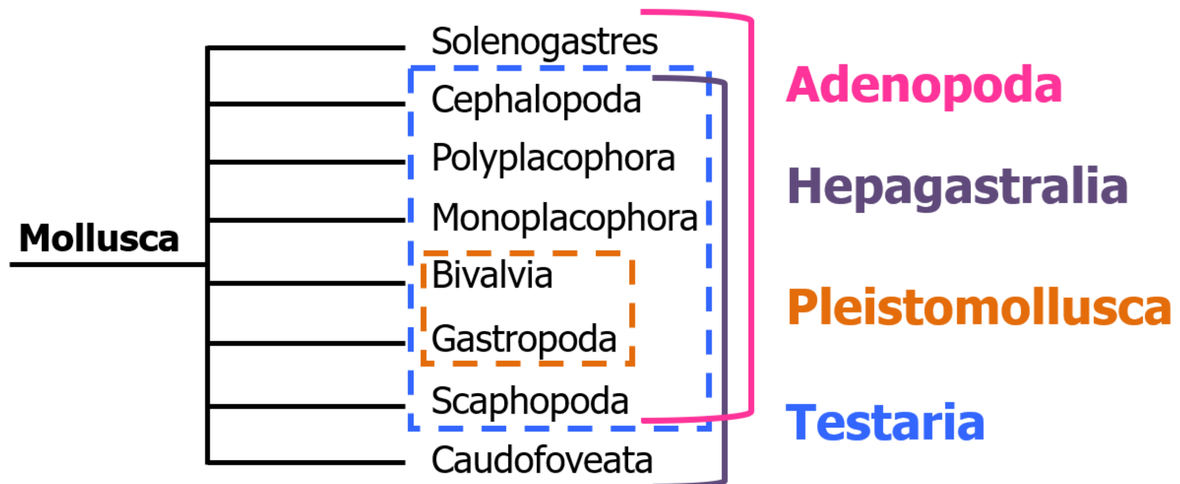

Fig. 2: Some further hypotheses on molluscan interclass relationships: Adenopoda concept: Solenogastres are sistergroup of Testaria and Caudofoveata are the most basal offshoot of Mollusca. Hepagastralia concept: Caudofoveata are sistergroup of Testaria and Solenogastres are the most basal offshoot of Mollusca. The Pleistomollusca concept unites Bivalvia and Gastropoda.

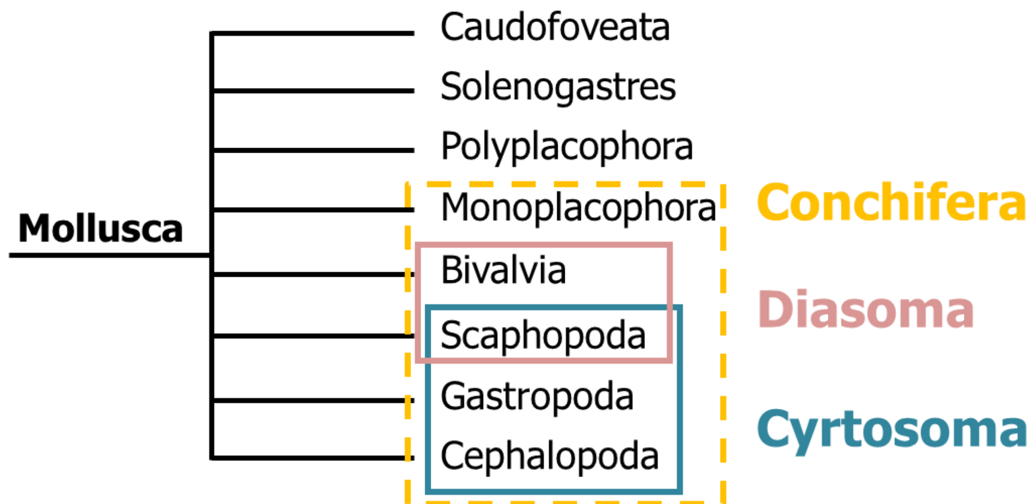

Fig. 3: Position of Scaphopoda within Conchifera: Diasoma unites Scaphopoda and Bivalvia. Cyrtosoma puts Scaphopoda as sistergroup to Gastropoda plus Cephalopoda. See chapter 2.2. for details.

2.5. Aims of the thesis

The aims of the thesis are:

1. Exploring various molecular markers with respect to their ability to resolve molluscan relationships. These are combined "standard markers" (COI, 16s, 18s, 28s, H3), mitochondrial genomes, thereof considering the phylogenetic signal of protein coding genes and the gene arrangements, and parts of nuclear genomes. All analyses conducted in the context of this work are based on broader taxon sets than available at the beginning of the thesis. Moreover, we tried to adjust the taxon selection towards a more balanced representation of major groups, covering all eight molluscan classes.

2. Resolving the phylogeny of the eight molluscan classes and subgroups thereof, with special emphasis on the elusive Monoplacophora. The datasets were analyzed with several methods and the resulting phylogenetic trees were compared to each other. Moreover, hypothesis testing was performed to assess reliability of the various molluscan class relationships in the light of the data.

3. Evaluating the various and partly contradicting concepts on the phylogeny and evolution of Mollusca, molluscan classes and subgroups thereof, in the light of molecular, anatomical and palaeontological evidence. Moreover, two chapters of my work are dealing with the origin and diversification of Mollusca and the class Gastropoda. Dating the Maximum Likelihood phylogenies with a molecular clock approach by using reliable fossil calibration points, a sensitive time estimation of a large molluscan, respectively heterobranch, taxon set was performed.

The Mollusca is a very old group that evolved in the (Pre-)Cambrian, therefore not all molecular markers were useful to resolve those relationships. Usually, there is a tradeoff regarding data versus taxon sampling and some molluscan taxa such as Monoplacophora are rare and hard to get for anatomical and genetic examination. The beginning chapters

(Chapters 1-3) deal with the analyses of molecular “standard markers” of molluscs. These are nuclear 18s, 28s and H3 genes as well as the mitochondrial 16s and COI genes. Chapter 1 analyzes the most comprehensive molluscan taxon set available in the year of publication (2013), comprising uncontaminated sequence data of multiple representatives of all eight molluscan classes for the first time. This publication supported the Serialia hypothesis (Monoplacophora + Polyplacophora) and was intended to serve as a backbone for following studies. Chapter 2 re-evaluates traditional concepts within the Heterobranchia, a major group of Gastropoda, based on a combined dataset of nuclear 18s rRNA, 28s rRNA and mitochondrial 16s rRNA and COI. Based on these four markers as well, chapter 3 resolves internal relationships of Chitonida (Polyplacophora) and clarifies the position of some enigmatic species.

As mitochondrial genomes of Metazoa can be highly conserved (see review by Gissi et al. 2008), they may be suitable markers to resolve old relationships. Chapters 4 and 5 thus analyze the phylogenetic signal of mitogenomes for Mollusca as well as molluscan gene arrangements. Chapter 4 shows the first mitochondrial genome of a monoplacophoran species ever published. Furthermore, certain gene rearrangements were proposed as potential synapomorphies for molluscs. In Chapter 5 no less than five monoplacophoran species are included in the analyses and unique arrangements of mitochondrial protein coding genes in Monoplacophora and some – but not all – chiton species were discovered. Chapter 6 gives a critical overview of the status quo of molluscan research on class-level relationships and on the origin of Mollusca in 2014. The final chapter, No. 7, presents a recent phylogenomic study with a broad taxon sampling that includes data from whole genome approaches on two monoplacophoran species for the first time. Moreover, a time estimation dates the origin of Mollusca to the Precambrian and the diversification of most molluscan classes to the Cambrian.

All chapters were published (for details see Results section).

3. Results

3.1. Isabella Stöger, Julia D. Sigwart, Yasunori Kano, Thomas Knebelsberger, Bruce A. Marshall: **The continuing debate on deep molluscan phylogeny: evidence for Serialia (Mollusca, Monoplacophora + Polyplacophora). 2013.** BioMed Research International, 2013.

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Research Article

The Continuing Debate on Deep Molluscan Phylogeny: Evidence for Serialia (Mollusca, Monoplacophora + Polyplacophora)

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Molluscs are a diverse animal phylum with a formidable fossil record. Although there is little doubt about the monophyly of the eight extant classes, relationships between these groups are controversial. We analysed a comprehensive multilocus molecular data set for molluscs, the first to include multiple species from all classes, including five monoplacophorans in both extant families. Our analyses of five markers resolve two major clades: the first includes gastropods and bivalves sister to Serialia (monoplacophorans and chitons), and the second comprises scaphopods sister to aplacophorans and cephalopods. Traditional groupings such as Testaria, Aculifera, and Conchifera are rejected by our data with significant Approximately Unbiased (AU) test values. A new molecular clock indicates that molluscs had a terminal Precambrian origin with rapid divergence of all eight extant classes in the Cambrian. The recovery of Serialia as a derived, Late Cambrian clade is potentially in line with the stratigraphic chronology of morphologically heterogeneous early mollusc fossils. Serialia is in conflict with traditional molluscan classifications and recent phylogenomic data. Yet our hypothesis, as others from molecular data, implies frequent molluscan shell and body transformations by heterochronic shifts in development and multiple convergent adaptations, leading to the variable shells and body plans in extant lineages.

1. Introduction

Molluscs are a morphologically megadiverse group of animals with expansive body plan modifications. There is no doubt about the monophyly of Mollusca as a whole or of any of the eight extant molluscan classes, based on strong morphoanatomical evidence and the consensus of molecular studies [1]. Despite a number of important recent studies, resolving ingroup molluscan topology remains contentious (Figure 1(a)) and a major challenge of invertebrate evolution [2].

Other studies have not had access to suitable material for broad taxon sampling, in particular for monoplacophorans, a class of small deep-sea molluscs that still remain rare and largely inaccessible [3, 4]. Among several recent studies on molluscan phylogeny, most use a subset of classes [5–7]; only one phylogenomic study so far has included all eight classes [8].

Multigene studies on ribosomal proteins [6] and housekeeping genes [7] and two broad phylogenomic (EST-based) data sets [5, 8] supported a monophyletic clade Aculifera. This clade comprises those molluscs with a partial or entire body covered by a cuticle with calcareous spicules or scales and is composed of shell-less vermiform molluscs (aplacophoran) and shell-plate bearing Polyplacophora (chitons). The opposing clade Conchifera (incorporating the five classes

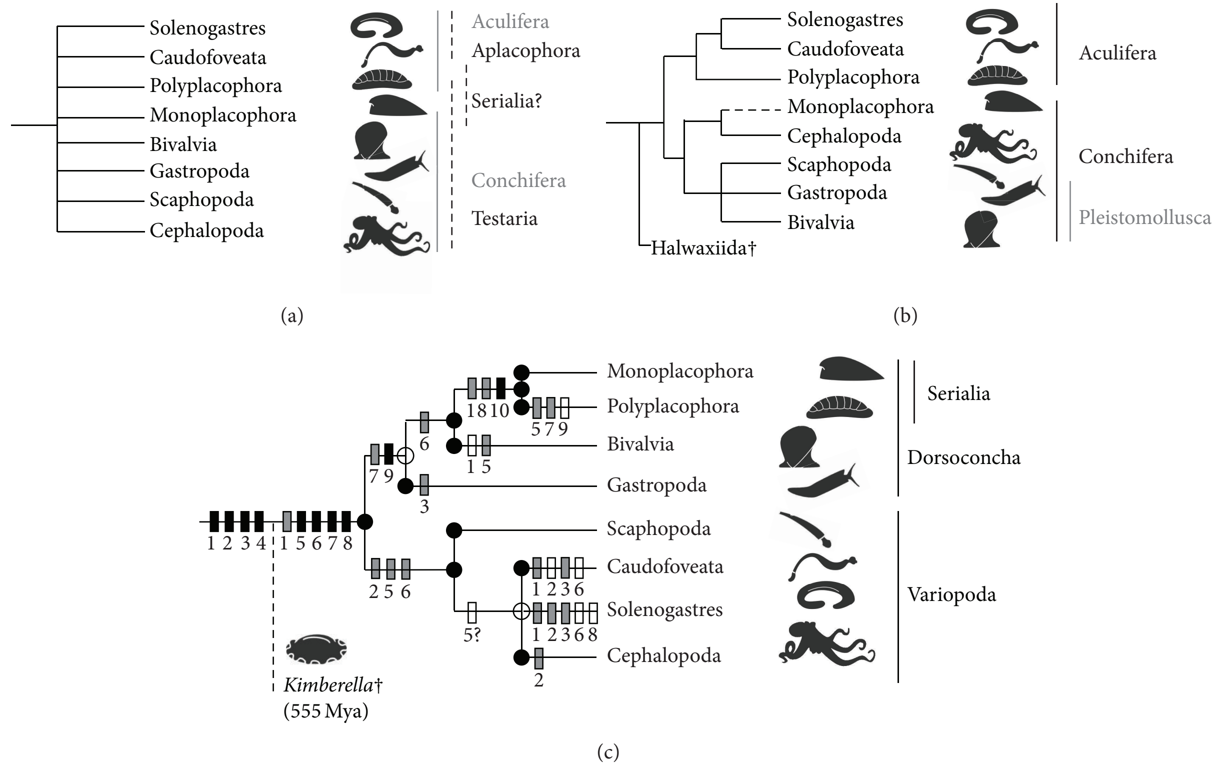

Figure 1: Schematic trees of molluscan relationships. (a) showing traditional proposed subdivisions. (b) consensus tree of two recent molluscan phylogenies inferred from large-scale genomic data by Kocot et al. [5] and Smith et al. [8]. The traditional concepts of Aculifera and Conchifera are supported but with differing positions of scaphopods. Monoplacophora is missing in the data set of Kocot et al. [5] (dotted line reflects the position of Monoplacophora in Smith et al. [8]). (c) the preferred multilocus tree with morphological features indicated numerically on branches. Unfilled dots indicate maximum Bayesian node support, filled dots additional high (>75%) bootstrap support in ML analyses. The Ediacaran fossil genus *Kimberella* corresponds to the description of molluscan stem-group features (1–4, below); crown group taxa originating in the Cambrian and later are united by additional features. Black boxes indicate first appearance of features; grey boxes indicate significant adaptive change; unfilled boxes indicate trait reversals: (1) radula: bipartite in stem molluscs and paedomorphic aplacophorans; broadened, on cartilages and specialised in crown molluscs, stereoglossate-like in Serialia; lost in Bivalvia (and several gastropods); (2), foot with broad gliding sole: transformed into digging foot in variopods (and derived bivalves), narrowed and reduced in aplacophorans, and forming the funnel in cephalopods; (3) circumpedal mantle cavity, miniaturised and anteriorly dislocated in torted gastropods while placed posteriorly in vermiform molluscs; (4) separate mantle covered with cuticula (with calcareous spicules in chitons, aplacophorans, and probably *Kimberella*); (5) dorsal shell: duplicated/fragmented in bivalves and chitons, lost in aplacophorans (and members of most other classes); (6) head with paired appendages: multiplied into feeding tentacles in variopods; trait for head reduction in bivalves plus Serialia and aplacophorans; (7) pericardium: heart fused around intestine in Dorsoconcha; (8) paired ctenidia: expanded to serially repeating gills in Serialia (and nautiloid cephalopods) and reduced in Solenogastres and some gastropod lineages; (9) complex stomach with style (reduced in carnivorous subgroups and chitons; convergently (?) present in a caudofoveate family); (10) paired eightfold dorsoventral muscles; (11) (not shown) statocysts (lost convergently in chitons and aplacophorans); (12) (not shown) suprarectal visceral commissure (subrectal convergently in chitons and aplacophorans).

with a "true" shell) remains controversial; phylogenomic studies recovered a monophyletic clade Conchifera [5, 8], but ribosomal protein multigene and housekeeping gene analyses showed paraphyletic Conchifera [6, 7].

A contradictory alternative hypothesis was proposed by earlier ribosomal RNA-dominated multilocus studies that included Monoplacophora and recovered this class as the sister to Polyplacophora [4, 9, 10]. This clade "Serialia" combines conchiferan and aculiferan members and is thus incompatible with results of recent molecular studies or the morphological Testaria (i.e., Conchifera + Polyplacophora) hypothesis (Figure 1). This result was widely criticised in the literature (e.g., [11]). Yet initial deficiencies [12] of the study by Giribet et al. [9] were addressed by Wilson et al. [10] and Serialia recovered again in a partially overlapping data set by Meyer et al. [13] and independently by Kano et al. [4].

The single phylogenomic data set with a monoplacophoran species also indicated some signal for Serialia, though weaker than that supporting a relationship of cephalopods and monoplacophorans within Conchifera [8]. Phylogenomic data sets cannot yet cover the same density of taxon sampling relative to targeted gene approaches, and while systematic errors of phylogenomic analyses have been explored recently (e.g., [14–16]), there is already a suite of

tools available for addressing well-known pitfalls of ribosomal RNA-based sequences (e.g., [17–20]). All data sets may still contribute to ongoing investigations of phylogeny if used and interpreted with care.

Where published topologies differ radically from concepts born from morphoanatomical hypotheses, these results have often been dismissed as artefacts even by the studies' own authors. In addition to the "Serialia" concept, several studies over the last decade have repeatedly recovered Caudofoveata sister to Cephalopoda (e.g., [6, 9, 10, 21–23]). But this pattern has low support values [6, 12]. The position of scaphopods is also highly variable, sometimes in a clade with gastropods and bivalves [5, 7, 8] or sister to aplacophorans and cephalopods [9, 10, 21]. With only eight major clades to rearrange, it could be a serious handicap that many studies exploring molluscan topology have had to exclude one (e.g., [5, 7, 21]) to three (e.g., [4, 6]) classes, and all but one previous study [10] used single-taxon exemplars for at least one [9] to as many as three [7, 8] of those clades. More and better quality data from the monoplacophorans are necessary to resolve molluscan relationships and particularly the two mutually exclusive hypotheses Serialia and Aculifera. We assembled a large multilocus data set for molluscs, including novel sequences of three monoplacophoran species (added to previously published data for only two species, *Veleropilina seisuimaruae* and *Laevipilina hyalina*). To determine the plausibility of this new topology, we applied several tests for phylogenetic informativity, saturation of sites, and compositional heterogeneity within the molecular data sets and have also considered our results against other molecular, morphological, and fossil evidence. Finally we calculated a new time tree via a relaxed molecular clock approach, using multiple sets of fossil calibration points.

Applying carefully calibrated molecular clocks on broad extant taxon sets and reconstructing characters on dated ancient lineages are indispensable for interpretation of enigmatic key fossils such as *Halkieria* or *Nectocaris* that may form part of the early evolutionary history of the group (e.g., [24–27]). We present an alternative view on molluscan evolution that supports the Serialia hypothesis and demonstrates that the debate on pan-molluscan relationships is still in progress.

2. Material & Methods

2.1. DNA Extraction, PCR, and Sequencing. DNA from 12 molluscan taxa, including 3 previously unsampled monoplacophoran species, was extracted using the Qiagen Blood and Tissue Kit (Qiagen, Hilden) by following the manufacturer's instructions. Amplifications of the four standard marker fragments, partial 16S, partial 18S, partial 28S, and complete H3, were carried out under PCR conditions and with primer pairs shown (see Supplementary Material available online at http://dx.doi.org/10.1155/2013/407072). Sequencing reactions were operated on an ABI 3730 48 capillary sequencer of the sequencing service of the Department of Biology of the LMU Munich by using the amplification primers. Newly generated sequences were edited in Sequencher version 4.7 (Gene Codes Inc., Ann Arbor, MI, USA).

2.2. Taxon and Gene Sampling. To compile a comprehensive and dense taxon sampling for resolving deep molluscan relationships, we expanded earlier published data sets [9, 10] by our own and archived (Genbank) data, including a broad selection of outgroups and initially including any molluscs with substantial sequence information available for five standard marker fragments (partial 16S rRNA, partial or complete 18S rRNA, partial 28S rRNA, complete H3, and partial COI). In some poorly sampled but significant ingroup clades we also included species with fragmentary sequence data. Previously unpublished, partial 16S, complete 18S and 28S, complete H3, and partial COI sequences of *Veleropilina seisuimaruae* were provided separately by one of the authors (YK). The total initial data set comprised 158 taxa (141 molluscan and 17 outgroup taxa; Suppl. Table 2).

2.3. Data Cleaning and Alignment. All the downloaded and new single sequences, including all 28S sequences, and all individual amplicons for 18S sequences in Solenogastres, were cross-checked against the nucleotide database of BLAST [29] by using the blastn algorithm. Potentially aberrant or problematic fragments were removed from the data sets (Suppl. Table 3A).

In some bivalve 28S sequences a dubious part of ca. 500 bp was detected in an otherwise homogeneous molluscan alignment. This portion differed substantially in most bivalve taxa but not in all and was highly heterogeneous also in closely related species. No pattern could be observed, so we removed the dubious region (Suppl. Table 3B).

The 18S sequences of Solenogastres were partially excluded due to contamination. Retained sequences of *Epimenia* species (*E.* sp., *E. australis,* and *E. babai*) were aligned separately with the first uncontaminated sequences of Meyer et al. [13], and resulting large gaps were cut by hand according to the template sequences of *Micromenia fodiens, Simrothiella margaritacea* and *Wirenia argentea* (Meyer sequences in [13]).

Patellogastropoda has aberrant 18S and 28S sequences with many indels causing highly incongruent alignments (own observations), leading to long branches and attraction artefacts in previous [13] and our own analyses. Patellogastropoda clustered with long branched Cephalopoda and Solenogastres under different regimes (Table 1). To verify the correct position of Patellogastropoda within or outside other Gastropoda a more focused data set was generated comprising only gastropod taxa plus some selected, short-branched outgroup taxa, that is, two bivalves, two polyplacophorans, one annelid, and one kamptozoan. This alignment is more homogeneous, and patellogastropods appear as a moderately long branch in a rather derived position within the Gastropoda (Suppl. Figure 2). So we confirm that patellogastropods show aberrant evolution leading to long branch attraction artefacts in broader data sets [13]; therefore we excluded this clade from the main analyses.

Single alignments (per fragment) were created with Mafft version 6.847b [30] with the implemented E-INS-i algorithm. Alignments of 16S, 18S, and 28S rRNA were masked with Aliscore version 5.1 [17, 31] by running 10,000,000,000 replicates.

Table 1: Preanalyses comparing different taxon sampling and masking strategies; Mafft [30] and RNAsalsa [18] are alignment methods; Aliscore [17, 31] and Gblocks [35] are masking methods.

Dataset	Alignment treatment	Alignment length (bp)	Major changes in tree topology, compared to main topology (Figure 2, Supplementary Figure 1)
Total set (158 taxa)	Mafft-cut and paste inconsistent blocks in 18S and 28S fragments-Aliscore	10318	Annelida *s.l.* sister to Mollusca; Aplacophora monophyletic (Caudofoveata sister to Solenogastres); Patellogastropoda clusters with Cephalopoda
Total set (158 taxa)	Mafft-RNAsalsa-Aliscore	7597	Mollusca non-monophyletic; Caudofoveata, Solenogastres, Cephalopoda, and Scaphopoda cluster with Annelida *s.l.*; Neritimorpha basal sister to remaining Gastropoda; Patellogastropoda sister to partial Vetigastropoda (Lepetelloida + Vetigastropoda *s.s.*)
Total set (158 taxa)	Mafft-RNAsalsa-Gblocks	4083	Nemertea + Entoprocta + Cycliophora is sister to Mollusca; Heterobranchia is sister to remaining Mollusca; Patellogastropoda clusters with Solenogastres and Cephalopoda
Large set (142 taxa, excluding Patellogastropoda)	Mafft-Gblocks	5550	Annelida *s.l.* + Entoprocta + Cycliophora is sister to Mollusca
Large set (142 taxa, excluding Patellogastropoda)	Mafft-Aliscore	8721	Main analyses (Figure 2, Supplementary Figure 1)

All ambiguous positions were automatically cut with Alicut version 2.0 [17, 31] to remove highly variable positions that could lead to aberrant phylogenetic signals. The alignments of protein coding genes H3 and COI were manually checked for stop codons using MEGA5 [32]. The single data sets were concatenated automatically using FASconCAT version 1.0 [33]. This procedure resulted in a total alignment of 142 taxa with 8721 bp in length and a proportion of 60% gaps (Suppl. Table 5). Where taxon sampling had to be modified, for example, removing taxa or dubious gene fragments, this was done in the initial single data sets and the complete procedure of alignment, masking and concatenation was carried out again.

Final analyses were computed with the large data set excluding Patellogastropoda (142-taxon set), a targeted taxon subset (81-taxon set, alignment length 8367 bp, proportion of gaps 57%) after pruning fast-evolving species or derived members of densely sampled undisputed clades, and the gastropod data set (all gastropods including Patellogastropoda plus selected slowly evolving outgroups). Moreover, we generated and analysed diverse data sets for control reasons to test interclass topologies: the 142- and 81-taxon sets without Aplacophora, the 142-taxon set without long-branched Cephalopoda and Solenogastres, the 142-taxon set with COI and H3 coded as amino acids (142-taxon set amino acid), and one data set that comprises only 18S, 28S, and H3 fragments of the 142-taxon set (Suppl. Table 5). The concatenated sequence matrices of the two main analyses (142-taxon set and 81-taxon set) were deposited at TreeBase (http://purl.org/phylo/treebase/phylows/study/TB2:S14594). New sequences generated herein were deposited at Genbank (Suppl. Table 2).

2.4. Preanalyses of the Data. Since saturated sequences have minimal or no phylogenetic signal and could even lead to anomalous results, we measured substitution saturation of the protein coding genes, namely, H3 and COI, with Xia's method implemented in DAMBE version 5.2.31 [37]. We used default parameters, and the proportion of invariable sites was specified. The method was executed for all three codon positions together, for combined first and second codon positions, and for third codon position separately. In both cases, H3 and COI, the index of substitution saturation (Iss) values of all three codon positions in combination were significantly smaller than critical index of substitution saturation (Iss.c) values. This was also true for the alignments of first and second codon positions. This assumes that those positions conserve phylogenetic signal and are useful for further analyses. In the case of third codon positions only, substantial saturation could be observed (Iss significantly higher than Iss.c). All results are shown in Supplementary Table 6. Although substitution saturation was observed in third codon positions of H3 and COI, we ran additional analyses with the complete sequence information (1st, 2nd, and 3rd codon positions) to implement potential phylogenetic signal for lower taxonomic levels.

To crosscheck the phylogenetic results of the data sets with and without excluded third codon positions of protein coding genes we conducted the same analyses with all three codon positions included, using distinct models of evolution for the three different codon positions and without third codon positions of H3 and COI.

Testing the evolutionary models for all genes and in case of COI and H3 for every single codon position and for codon positions one and two versus position three was carried out with the programs Modeltest version 3.7 [38] (for complete alignments) and MrModeltest version 2.3 [39] (for codon positions) by the help of PAUP* version 4b10 for Windows [40]. With the amino acid alignments of H3 and COI we additionally tested for the best fitting amino acid model of evolution using ProtTest version 2.4 [41]. As RAxML provides only a part of the models that can potentially be tested by ProtTest we only selected those models in our ProtTest

analysis (DAYHOFF, DCMUT, JTT, MTREV, WAG, RTREV, CPREV, VT, BLOSUM62, and MTMAM). The resulting best models for all genes (16S, 18S, 28S, H3, and COI), distinct codon positions of H3 and COI, and amino acid alignments of H3 and COI as well as the corresponding proportions of invariant sites and the gamma distribution shape parameters are shown in Supplementary Table 4.

2.5. Phylogenetic Analyses. Maximum Likelihood (ML) analyses for all data sets were executed using RAxML-HPC for Windows [28] and RAxML version 7.2.6 [28] on the Linux cluster of the Leibniz Computer Centre. Parameters for the initial rearrangement settings and the rate categories were optimised under the GTRCAT model of evolution and a partition by genes (16S, 18S, and 28S) and codon positions (COI, H3) by conducting the hardway analysis described by Stamatakis [42].

First, a set of 10 randomised Maximum Parsimony (MP) starting trees was generated. Second, based on this set of starting trees, the ML trees with a specified setting of initial rearrangements (−i 10) and with an automatically determined initial rearrangement setting had to be inferred. Third, the number of rate categories was adjusted. Initial setting –c 10 was augmented by increments of 10 up to –c 50 for all MP starting trees. The fourth step was to execute 200 inferences on the original alignments. Finally, values of 1000 bootstrap topologies were mapped on the best-scoring ML tree.

Bayesian analyses for selected data sets were conducted with MrBayes v. 3.1.2 [43]. Partitioning with corresponding models of evolution, substitution rates and nucleotide frequencies were applied according to the results of Modeltest [38], MrModeltest [39], and ProtTest [41]. One tree was sampled every 1000 generations. If the average standard deviation of split frequencies declined 0.01 after 5 million generations the analysis was stopped. If not, analysis was continued with another 5 million generations. If the average standard deviation of split frequencies still did not decrease, the log likelihood values were examined with Tracer version 1.5 [44]. If the run reached stationarity, the analysis was stopped. Burn-in was set to 2500 after 5 million generations and to 5000 after 10 million generations.

2.6. Molecular Clock Analyses. Time estimations were performed with the software package BEAST version 1.6.1 [34]. The program is based on the Bayesian Markov Chain Monte Carlo (MCMC) method and therefore can take into account prior knowledge of the data. That is used when nodes in the topology are calibrated and the rate of molecular evolution along the branches is estimated.

We used nine fossil calibration points (Suppl. Table 7) with their corresponding prior distributions and assumed a relaxed clock with a lognormal distribution [45] of the rates for each branch (Suppl. Table 7). This setting is recommended because it additionally gives an indication of how clock-like the data are [46]. Calibration points were set with a minimum bound according to Jörger et al. [47]. To reduce computing time we used the targeted (81-taxa) data set for time estimations. The topology was constrained according to the resulting tree of the phylogenetic analyses.

An Xml-file with all information on data, calibration points, priors and the settings for the MCMC options was created with BEAUti version 1.4.7 [34]. Gamma-shaped priors for all nine calibration points were used (Suppl. Table 7). We assumed that the lower bound of each calibration point is not more than 10% of its maximum age. In case that the next older fossil is within these 10% boundary we used the maximum age of that fossil as lower bound for the younger fossil [48].

Detailed partitioning of genes (16S, 18S, and 28S) and codon positions of COI and H3 and the constraint tree topology were added by hand to the Xml-file. The analysis was executed for 30 million generations, sampling one tree every 1000 generations on the Linux cluster of the Leibniz Computer Centre. The implemented program Tracer version 1.5 [44] was used to confirm that posterior probabilities had reached stationarity. Burn-in was set to 25% (7500), so 22,500 trees were effectively analysed with TreeAnnotator version 1.6.1 [34] to form the summary tree. Further, to check the reliability of our fossils, we repeated the same analysis several times and always omitted one calibration point (Table 2; Suppl. Table 7).

2.7. Testing Hypotheses. Several existing hypotheses about the molluscan interrelationships (Table 3) were tested by executing Approximately Unbiased tests (AU tests) implemented in Treefinder version of October 2008 [36]. Therefore the input constraint trees were computed with RAxML-HPC [28] by using the –g-option and the associated partition by genes and codon positions. Those input tree topologies were tested in Treefinder with maximum number of replicates under the GTR model.

3. Results and Discussion

3.1. Analyses. Analysing traditional multilocus markers for several large taxon sets with Maximum Likelihood and Bayesian methods under different alignment and masking regimes (Table 1, Suppl. Table 5), we recovered consistent phylogenetic trees (Figure 1(c)) with monophyletic Mollusca in contrast to other studies with similar markers [9, 10, 19, 21] and strong support for the monophyly of all molluscan classes, including Bivalvia (also in contrast to some earlier studies [9, 19, 21]).

Our approach included rigorously testing of all amplicons before and after alignment, which led to the exclusion of aberrant or problematic, previously published sequences from the data set (Suppl. Table 3). Criticism of previous accounts using the same set of markers has included the incomplete representation of taxa and the varying extant of missing data [12, 49]. Missing data is a common burden of multilocus studies and will be more severe for phylogenomic approaches [14, 15]. Our preanalyses showed that dubious sequences or ambiguous parts of alignments had much greater effect on the outcome than selecting taxa with the highest amount of data available. Rather than maximizing sequences per species,

we concentrated on increasing taxon sampling to minimise potential branch lengths. Our quality controlled 158-taxon set includes 17 lophotrochozoan outgroups. Analytical trials on different subsets of nonmolluscan outgroups altered outgroup topology and support values of some basal ingroup nodes but did not change the ingroup topology (Figure 1).

Alignment issues involved in ribosomal RNA data were addressed by an array of measures proven to be beneficial ([20]; see Section 2). Potential homoplasy in protein coding genes (especially the third codon positions) in our preferred multilocus analysis was addressed by additionally running the analysis with those fragments (COI and H3) encoded as amino acids. This had little effect on the topology but supported monophyletic Aplacophora. We applied a variety of alignment tools, including masking (Aliscore [17]) and refinement algorithms based on secondary structures (RNAsalsa [18]) and applied compartmentalised analyses of taxon clusters causing obvious alignment problems. Excluding patellogastropods (142-taxon set, Suppl. Figure 1; see Section 2) did not change our molluscan backbone topology (Figure 1(c)) but improved alignments. Separately analysing gastropods plus some slowly evolving outgroup taxa shows patellogastropods cluster with vetigastropods (Suppl. Figure 2). Our main aim was to elucidate molluscan relationships at the class level; thus we further pruned outgroups and fast-evolving members from more densely sampled ingroups (such as heterobranch gastropods) and used an 81-taxon set presented here in our main analysis (Figure 2).

3.2. The Basal Molluscan Dichotomy. In our new tree, the phylum Mollusca is divided into two clades (Figure 1(c), Figure 2, Suppl. Figure 1). The first clade is composed of Gastropoda sister to a clade of Bivalvia and Serialia (Monoplacophora + Polyplacophora). For convenience we will refer to this clade as "Dorsoconcha"; the name refers to the (plesiomorphic) presence of a dorsal shell for members of this clade, though modified to two lateral valves in bivalves and to (7-)8 dorsal plates in chitons, and the shell internalised or lost multiple times especially among gastropods.

Gastropods, bivalves, and monoplacophorans are commonly considered to be united by their single shell (secondarily split in bivalves) built by a shell gland at the mantle border (and by the entire mantle roof secreting organic matrix and calcareous layers letting the shell grow thicker, or repair damage). Chitons are traditionally excluded from the hypothetical clade "Conchifera" on the basis of their eight shell plates. The chiton girdle is also covered by a cuticle with embedded calcareous and organic sclerites, similar to the body cuticle of the shell-less aplacophorans, but according to our results, this is convergent and may reflect the different, single versus multicellular spicule formation in these taxa [50]. That chitons cluster with monoplacophorans rather than aplacophorans is congruent to previous molecular approaches that included monoplacophoran exemplars [4, 9, 10, 13]. The exception is the phylogenomic study by Smith et al. [8], in which a single monoplacophoran, *Laevipilina hyalina*, robustly clustered with cephalopods in the main analyses, though parts of the genes used also showed signal supporting an association with chitons.

In the second major molluscan clade, Scaphopoda are sister to a clade of vermiform Caudofoveata and Solenogastres, plus Cephalopoda. Herein we will call this clade "Variopoda," referring to the various derived foot attributes of its members: the digging foot in Scaphopoda, reduced narrow gliding sole or completely lost in (adult) aplacophorans, and transformed in cephalopods possibly building parts of tentacles and funnel. Dorsoconcha appears as a monophyletic group although bootstrap support is low (60%), and the Variopoda is strongly supported in all Maximum Likelihood analyses; Bayesian posterior probabilities are high for both nodes (Figure 2, Suppl. Figure 1).

The placement of aplacophorans within Variopoda is unconventional, but a sister relationship between Scaphopoda and Cephalopoda has been previously put forward [51, 52]. Previous multilocus approaches with broad taxon sampling (i.e., more than one exemplar of each aplacophoran class) are actually not in general disagreement with Variopoda, since contaminated aplacophoran sequences may account for occasionally aberrant topologies [9, 10, 13]. Inner scaphopod topology resolves the two currently recognised groups Dentaliida and Gadilida, as does Cephalopoda splitting into modern Nautilida and Coleoidea, and is congruent with previous classifications [53].

We calculated time trees with a Bayesian molecular clock approach (Figure 3) using a mix of younger and older calibration points (Suppl. Table 7). We also tested sets of calibrations successively excluding each single calibration point used (Table 2) to minimise circularity involved by calculating individual node times [54]. All our time trees confirm a Precambrian origin of Mollusca (Table 2, Suppl. Table 8) in agreement with previous studies [7], and 95% confidence time bars of all our time trees allow for a Cambrian origin of those classes with a reliable fossil record (Figure 3). As a further sensitivity test we also calculated a time tree from a data set excluding aplacophorans; the topologies are congruent and node ages almost identical, confirming general time estimates (not shown).

Molluscan diversification occurred at an extremely rapid pace after the initial origination of the shell (Figure 3). Short branches at the base of the ingroup can be artefacts of signal erosion in deep nodes [55], but as we discuss below, the rapid early evolution of Mollusca is also supported by the fossil record. Our molecular clock indicates a potential time frame of only around 20–40 million years from the first shelled molluscs (ca. 560–540 Ma) to the presence of differentiated variopod, dorsoconch, gastropod, bivalve, and serialian stem lineages (ca. 520 Ma). The shell was central for rapid evolutionary success of molluscs, and shell modification and divergence are correlated with adaptive radiations during this early period.

3.3. Evaluating Molecular Data Sets. All recent multigene and phylogenomic studies [5–8] have tested the effects of gene sampling, analytical methods, and inference programs; like our results, their topologies were more or less robust, also

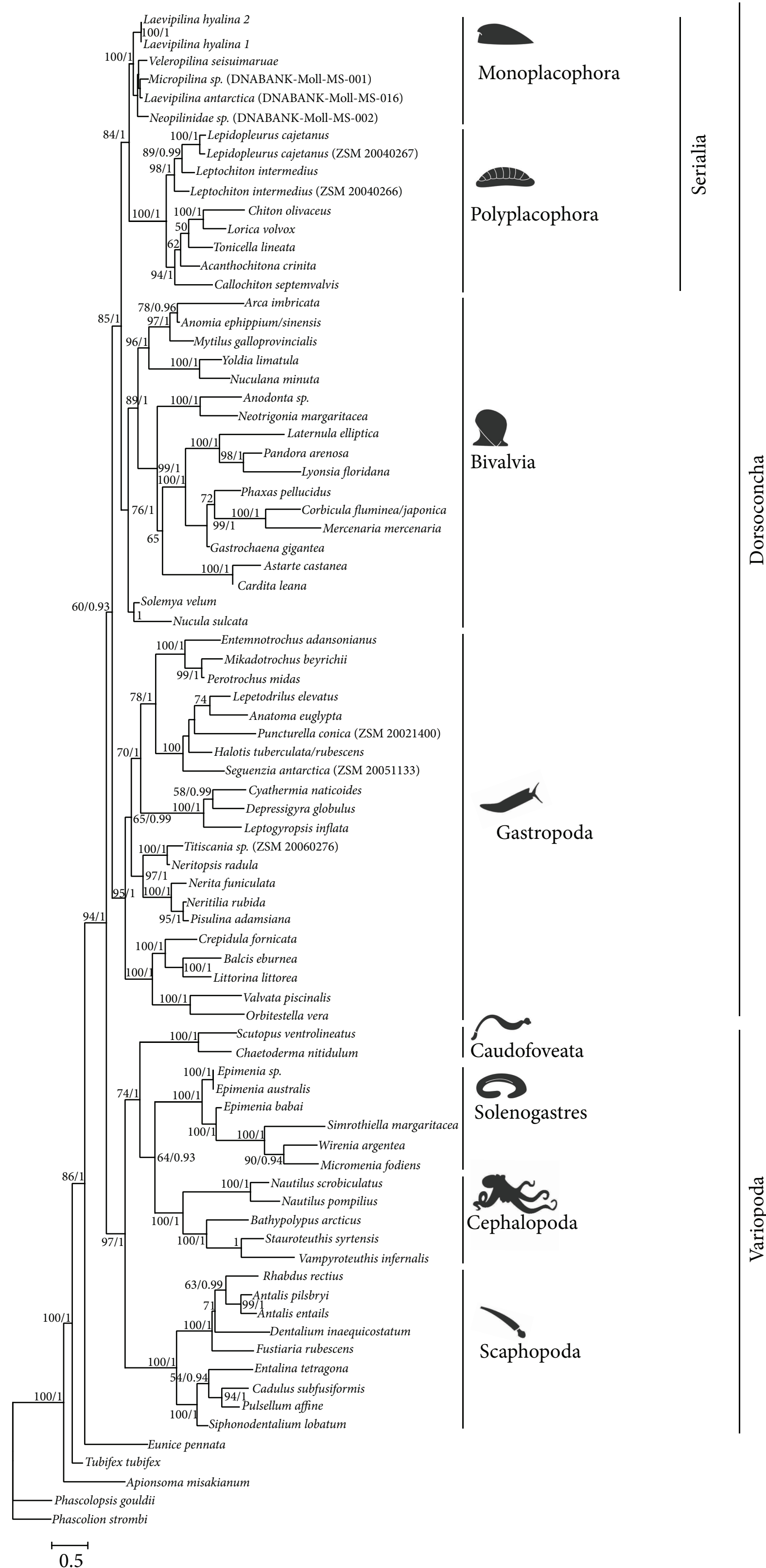

Figure 2: Preferred molluscan tree. Maximum Likelihood analysis (RAxML [28], hardway) of pruned 81-taxon set; values at nodes refer to bootstrap support (1000 pseudoreplicates, first value) and posterior probabilities obtained from the Bayesian analysis (second value).

Table 2: Sensitivity tests of individual calibration nodes used for relaxed molecular clock time estimates of major molluscan groups. Table shows influence of single calibration points on node ages of all other calibration points.

Calibrated nodes	Excluded calibration point: None	Diversification of Mollusca	Split Serialia/ Bivalvia	Origin of Cephalopoda	Split Polyplacophora/ Monoplacophora	Origin of Pteriomorpha	Origin of Caenogastropoda	Diversification of Scaphopoda	Split *Astarte/Cardita*	Diversification of Polyplacophora
Diversification of Mollusca	551.02	**683.50***	550.58	549.76	551.52	551.55	551.59	551.68	552.10	551.72
Split Serialia/Bivalvia	530.93	533.98	**523.58***	530.80	530.88	530.36	530.82	531.01	530.82	531.01
Origin of Cephalopoda	504.92	511.40	504.40	**431.05***	504.26	504.85	504.75	503.96	504.16	504.35
Split Polyplacophora/ Monoplacophora	493.06	493.44	491.79	493.68	**431.68***	493.47	493.13	493.47	493.79	493.14
Origin of Pteriomorpha	475.06	474.81	474.20	474.43	474.97	**376.65***	474.56	475.20	474.16	474.82
Origin of Caenogastropoda	421.49	422.82	421.77	421.91	422.61	421.54	**326.50***	421.95	420.85	421.61
Diversification of Scaphopoda	359.95	360.02	359.97	359.55	360.12	359.59	359.37	**382.50***	359.19	359.60
Split *Astarte/Cardita*	325.43	325.40	325.20	324.98	325.45	324.55	325.37	325.09	**44.34***	325.63
Diversification of Polyplacophora	233.44	233.49	233.37	233.18	233.75	233.42	233.63	233.57	232.71	**243.67***

Bold ages marked with an asterisk (∗) indicate time estimations without calibration of this node.

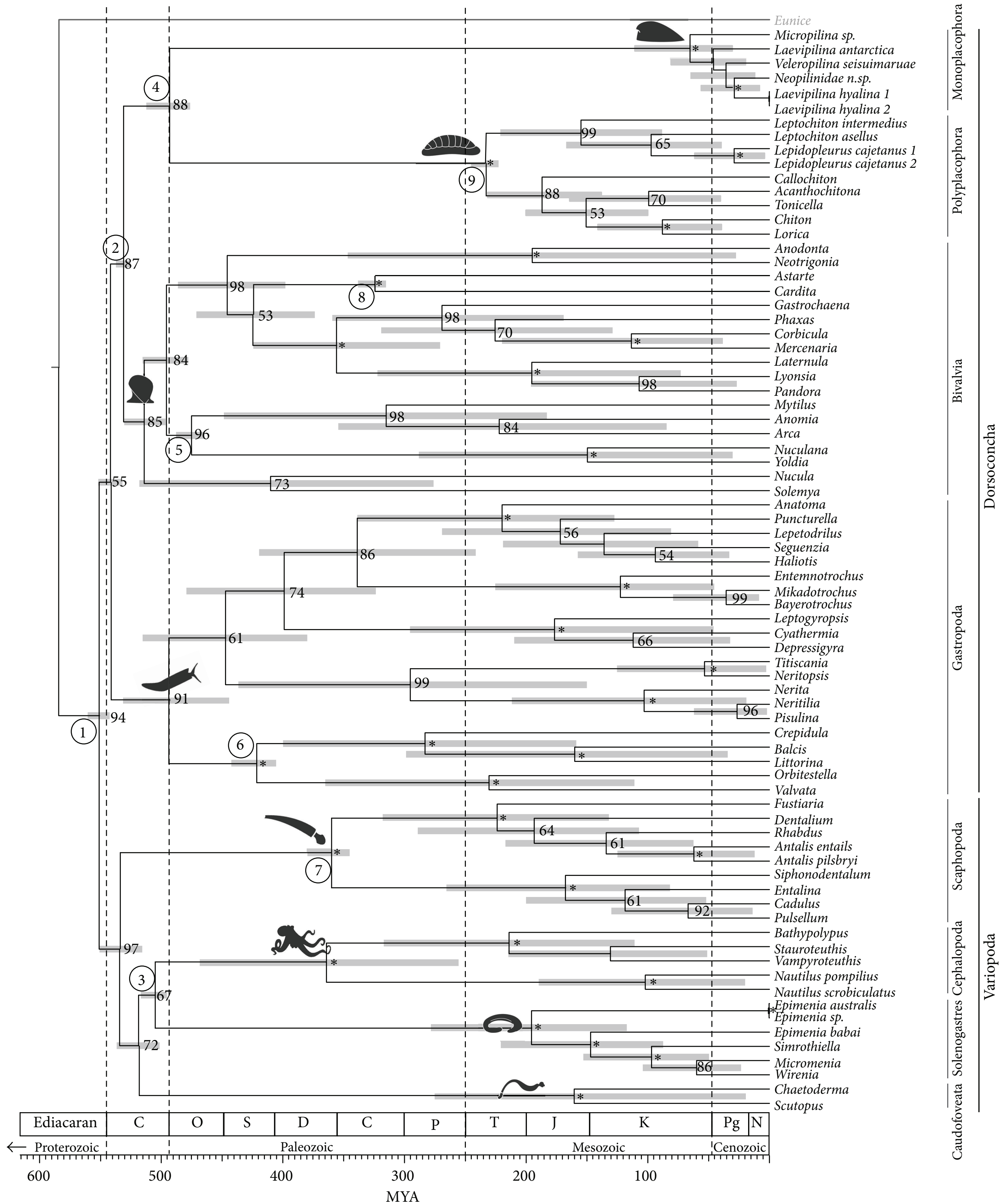

Figure 3: Chronogram of molluscan evolution. Divergence times (million years before present, Ma) estimated from BEAST version 1.6.1 [34] under an uncorrelated lognormal relaxed clock model; bars refer to the 95% highest posterior density. All nodes show maximum posterior probabilities (1.0, not indicated) from a run with 10^8 generations (25% burn-in). Numbers at nodes refer to bootstrap support values (>50%; asterisks are 100%) obtained from separate Maximum Likelihood analysis (RAxML [28], hardway, 1000 pseudoreplicates) of the same data set. Circled digits indicate calibrated nodes. Details of calibration can be found in Supplementary Table 7. Omitting Cambrian calibrations shifts molluscan diversification deeper into the Precambrian (for sensitivity analyses see Table 2).

against varying outgroup selection. Sensitivity analyses do not attribute the major split into Variopoda (or parts thereof) and Dorsoconcha or the recovery of Serialia to LBA effects. Yet our multilocus study uses fewer markers and nucleotides than "next-generation sequencing" studies [5–8], so it may be more prone to inadequate signal of certain markers or stochastic errors.

Split decomposition analyses of an earlier multilocus set [9] usually recovered the single monoplacophoran species among bivalves [12], consistent with a Dorsoconcha clade. Splitstree analyses (not shown) of our improved data set still show overall polytomy and some individual taxa are clearly misplaced in the network (e.g., the gastropod *Crepidula* clusters with cephalopods). Overall, most dorsoconch terminals are separated from variopods. Within Dorsoconcha, monoplacophorans cluster with chitons and bivalves. A lack of tree-like structure and *a priori* split support, especially in a large and heterogeneous taxon set, may not necessarily mean that there is too little signal for phylogenetic analyses; it just means that there is conflict that may or may not be resolved applying current models of sequence evolution.

Nuclear ribosomal RNA genes were shown to be informative even on deeper levels than basal molluscs, if treated adequately [20]. Other, supposedly faster-evolving mitochondrial markers (partial COI, 16S) were stringently masked herein, partitioned when necessary or excluded when saturated (Suppl. Tables 4–6). Combined analysis incorporates multiple tempos of evolution experienced by the different loci and is therefore more representative of deep evolutionary patterns. Our backbone topology is robust against varying the taxon and marker sets, masking and partitioning regimes, models of evolution, and methods of analyses (Table 1, Suppl. Tables 4 and 5).

3.4. Evaluating Alternative Morphological and Molecular Concepts. We directly evaluated the statistical fit of major competing morphology- or molecular-based concepts constraining our topologies and calculating their likeliness according to our data set. Using our preferred 81-taxon set with all markers, but also under most other schemes, the AU test rejects all the higher molluscan textbook concepts [1]: the Testaria, Aculifera, Conchifera, Cyrtosoma, and Diasoma hypotheses, with the highest possible statistical support; the same AU tests do not reject Dorsoconcha nor Variopoda (Table 3). We also tested our data against three new molecular concepts (Figure 1(b)): Pleistomollusca (Bivalvia + Gastropoda) established by Kocot et al. [5] and the clades of Monoplacophora and Cephalopoda [8] versus other conchiferans (Scaphopoda, Gastropoda, and Bivalvia) [5, 8]. Only the clade of Monoplacophora and Cephalopoda was not rejected with significant support in any of the main analyses, but all these groups received much lower AU values than our unconstrained topology.

While several recent phylogenomic studies recover Aculifera [5, 7, 8], the Serialia concept has been tested only by Smith et al. [8], by inclusion of a single monoplacophoran species. Though association with cephalopods is preferred, there is a weaker signal also for Serialia [8]. Kano et al. [4] recovered Serialia but did not include any aplacophoran taxa in their data set. The Serialia as a concept cannot be dismissed yet, and our dense taxon sampling herein, though based on far fewer sequences than recent phylogenomic approaches [5, 8], still may allow for a more differentiated and perhaps more correct view on molluscan interclass relationships.

The association of cephalopods and aplacophorans has been recovered previously but dismissed as an artefact of high substitution rates in rRNA genes [6, 13, 21]. But our results cannot easily be explained by long branch attraction (LBA) effects (*contra* [13]). Branch lengths of scaphopods and caudofoveates are moderate, and the variopod node is stable against removal of putative long branched taxa showing accelerated evolutionary rates or biased base compositions [13], such as the branches of Solenogastres or Cephalopoda or both (trees not shown).

Molluscan evolution, whatever the underlying tree, is known to be laden with convergence at all taxon levels, including morphological features previously suspected to be informative for deep phylogeny (e.g., [56–58]). Conclusions derived from single organ systems, or the shell alone, are not able to exclude alternative interpretations. Coding hypothetical bauplans rather than existing representatives has been criticised [59, 60] and may lead to erroneous assumptions especially in groups with uncertain internal topology such as gastropods or aplacophorans. Morphocladistic approaches to date (e.g., [61–63]) all recovered Testaria, but this hypothesis is not supported by any molecular approaches.

Our proposed topology and any other nontestarian hypothesis imply that ancestral molluscs were complex rather than simple. This means that many anatomical characters inherited by descendants may be plesiomorphic and thus not informative, or could have been reduced or lost repeatedly, implying a high level of homoplasy. In fact, early molluscan phylogeny may have been shaped by habitat-induced selective pressure combined with heterochronic processes (e.g., [64]). This combination may lead to concerted morphological parallelisms powerful enough to obfuscate any phylogenetic signal, which has been found to be the case in heterobranch gastropods (e.g., [47, 65, 66]). It is possible to disentangle even highly homoplastic and heterochronic groups (e.g., [67–69]) if detailed and reliable microanatomical data are available on a dense ingroup taxon sampling, which is, however, not yet available for most molluscs. Unfortunately none of the many competing morphology-based hypotheses on molluscan class interrelationships available at present appears to represent a reliable benchmark for evaluating molecular topologies.

3.5. Topologies Tested against the Fossil Record. Molluscan diversification has been widely assumed to originate from a basal "monoplacophoran" bauplan [59], although early single shelled molluscs cannot be reliable separated from gastropods or any nonmonoplacophoran univalve [70]. The earliest calcareous molluscan-like shells, including undisputed molluscs, appear in the uppermost Precambrian, in the late Nemakit-Daldynian ca. 543 Ma [70]. Polyplacophoran shell plates first appear in the Late Cambrian, almost 50 My

Table 3: Testing alternative topologies against various data sets. Results of Approximately Unbiased (AU) tests with Treefinder [36], various schemes. P-values of AU Test executed on selected taxon and data sets. Tested tree topologies were constrained in RAxML [28]. Only meaningful tests have been executed. P-value > 0.05: constrained topology is not rejected; P-value < 0.05: constrained topology is rejected significantly; P-value = 0: constrained topology is rejected with high significance.

Constrained topology	142-taxon set. all markers	81-taxon set. all markers	142-taxon set. 18S + 28S + H3	Aplacophora removed from 142-taxon set. all markers
Sinusoida	0.4244	Not tested	0.2652	0.0383
Mollusca + Kamptozoa	0.0	Not tested	0.0	0.0
Mollusca + Annelida	0.7421	0.7097	0.4090	0.3876
Testaria	0.0	0.0	0.0	Not tested
Aculifera	0.0	0.0	0.0	Not tested
Aplacophora	0.6908	0.3651	0.7730	Not tested
Conchifera	0.0	0.0	0.0	0.0333
Pleistomollusca	0.6665	0.0	0.0863	0.1927
Monoplacophora + Cephalopoda	0.1389	0.0632	0.0	0.2779
Scaphopoda + Gastropoda + Bivalvia	0.0154	0.0	0.0	0.1065
Scaphopoda + Cephalopoda	0.1913	0.0	0.2527	0.6914
Scaphopoda + Cephalopoda + Gastropoda	0.0	0.0	0.0	0.7232
Scaphopoda + Gastropoda	0.8850	0.9452	0.0573	0.8271
Diasoma (Scaphopoda + Bivalvia)	0.0	0.0	0.0	0.0
Monophyletic Protobranchia	0.0219	0.0	0.1085	0.0188
Dorsoconcha	0.6830	0.1097	0.3503	0.4048
Variopoda	0.3170	0.8903	0.6497	0.5952

later [7, 71]. This does not support the Testaria hypothesis that would suggest that chitons evolved before the invention of a true "conchiferan" shell. There are dubious disarticulated microscopic chiton-like plates [72] from the early Meishuchunian (likely Early Tommotian) of China, but these still appeared later rather than earlier than the very first undisputed conchiferan shells. The Aculifera concept with monoplacophorans sister to other members of Conchifera or our molecular basal dichotomy are both fully compatible with the origin of molluscan shells latest at the Precambrian/Cambrian boundary.

The earliest tryblidian monoplacophorans are recorded from the Late Cambrian [73]. Older, nontryblidian "monoplacophorans" do not show serialised muscle scars and thus cannot be considered part of the crown-group. Yet the earliest reliable bivalves with elaborated hinge and ligament (*Fordilla*, *Pojetaia*) appear earlier, in the Early Tommotian ([74]; ca. 535 Ma). Both Aculifera and our basal dichotomy are not contradicted by the early appearance of bivalves. Under an Aculifera topology, chiton-like stem members could appear soon after a terminal Precambrian split separating Aculifera and Conchifera. Interpreting Early to Middle Cambrian sachitids (halwaxiids) as stem aculiferans would help fill this gap [7], but these taxa show a chronological sequence of shell plate loss rather than acquisition, which may be contrary to a progressive transition to chitons. The mosaic taxon *Phthipodochiton*, which has been proposed as a stem aplacophoran, does not appear until the Ordovician [75, 76]; other fossils from the Silurian, combining aplacophoran and polyplacophoran features with some soft tissue preservation, have also been used to support the Aculifera hypothesis [77]. These could also simply represent further disparity in extinct Polyplacophora. Regardless, there is compelling evidence from molecular systematics as well as fossil evidence that aplacophorans lost their ancestral shell (or shell plates) secondarily, and many other groups show repeated shell-loss or evolution to a vermiform body plan.

The topologies recovered by Vinther et al. [7] and Kocot et al. [5] support Aculifera but also imply that cephalopods are sister to Aculifera [7] or represent the earliest-diverging conchiferans [5] (excluding monoplacophorans from the analysis). However, there is no evidence for cephalopod-like fossils appearing earlier than, for example, bivalves. Similarly, bivalves are derived within Conchifera in the topology of Smith et al. [8], which is contradicted by the early fossil record of bivalves. In contrast, our basal dichotomy could fit with the many univalve small shelly fossils occurring earlier in the fossil record than bivalves, and both monoplacophorans and polyplacophorans appear later, actually at a similar time in the Latest Cambrian, and as predicted by a split of Serialia into Monoplacophora and Polyplacophora.

3.6. The Timing of Early Molluscan Evolution. The molluscan stem is Precambrian according to all our molecular time trees. The Vendian (555 Ma) body fossil *Kimberella* was discussed as a mollusc [78], but not widely accepted as such, and rather treated as lophotrochozoan stem member or "no more specifically than as a bilaterian" [79]. According to previous constrained (e.g., [7]) and our less constrained time trees (Table 2, Suppl. Table 8), however, *Kimberella* appears late enough in the fossil record to be considered as a potential

stem mollusc. The other recent molecular clock for Mollusca puts the stem Mollusca even deeper [4], but *Kimberella* is within the 95% HPD interval for the split of the basal dichotomy also recovered herein. Having confirmed the conceptual basis of our proposed topology is not rejected by evidence in the fossil record, we further consider the timing of the radiation of specific clades proposed by our molecular clock analyses (Figure 3).

Cap-shaped Helcionellidae from the terminal Precambrian (e.g., *Latouchella*) are putative monoplacophorans according to the seminal study by Runnegar and Pojeta [80] or a separate molluscan class [81] or, based on nonserial muscle scars, gastropods [70]. Our time tree suggests that Nemakit-Daldynian and Earliest Tommotian molluscs with symmetrical cap-shaped shells with large openings are stem molluscs (or in the stem of one part of the basal dichotomy). In contrast, helicoid shells from the same period such as Aldanellidae (e.g., [82]) could well be gastropods, whether or not the animal was torted [70, 82].

Early Tommotian *Watsonella*, formerly known as *Heraultipegma* (the putatively earliest rostroconch), is a laterally compressed, bivalve-like univalve [70], possibly with dorsomedially decalcified or even bivalved shell [83]. This and other laterally compressed Watsonellidae may pre-date the first reliable Bivalvia (Early to Middle Tommotian *Fordilla*; [74] versus [70]) by some million years and thus could well be stem bivalves (or offshoots of the dorsoconch stem) according to our time tree (Figure 3).

It is important to note that neither reliable Monoplacophora (*sensu* Tryblidia) nor reliable Polyplacophora (i.e., Paleoloricata) are known before Late Cambrian, and this is confirmed in our chronograms (Figure 3). Yu [84] interpreted the Early Cambrian Merismoconchia as having eight pairs of muscles on a pseudometameric shell, linking 8-plated chitons with single shelled monoplacophorans in a transitional row of shell fusion. The similarity of merismoconchs with both serialian classes is curious, and their early occurrence in the pretrilobite Meishucun Stage suggests they could be early stem Serialia. The microscopic merismoconchs with their ventrally still connected shell segments and seven observed pairs of muscle scars may have been a transitional stage in how to make a foot efficient for sucking and a shell more flexible to adapt to uneven hard substrates. According to our time tree (Figure 3), chiton-like shell "fragmentation" into fully separated plates occurred much later, after splitting from single-shelled monoplacophoran-like ancestors.

The Cambrian (Atdabanian) *Halkieria* and related Middle Cambrian halwaxiids could also be interpreted as stem Serialia (Figure 3). A role as ancestral lophotrochozoans for halwaxiids as suggested by Edgecombe et al. [79] is not supported by our analysis.

According to our time tree (Figure 3), Yochelcionellidae, conspicuous Tommotian to Middle Cambrian shells that have a "snorkel," could be part of the gastropod radiation as suggested by Parkhaev [70], or members of the dorsoconch stem lineage, or variopod stem members. The latter possibility is especially intriguing, since Yochelcionellidae evolved a "flow-through" water system with two shell openings; a dorsal shell elongates laterally and fuses ventrally, and the body axis shifts towards anterior growth extending head and foot out of a now tube-like shell. This condition is displayed by living and fossil variopods (i.e., scaphopods, cephalopods, and nonwatsonellid Rostroconchia).

Our results show that scaphopods could have split off from the variopod stem earlier, that is, in the Early Cambrian, but the oldest potential scaphopods in the familiar modern tusk-like shape are from the Ordovician [85] or even post-Devonian [86]. There is a vast record of Middle Cambrian tube-like shells that may be unrecognised parts of the early scaphopod diversification that started much earlier and morphologically less constrained than previously expected [87].

Knightoconus, a Middle to Late Cambrian large "monoplacophoran" conical shell with internal septa but no siphuncle [88], was described as a stem cephalopod [80] but subsequently questioned (e.g., [89]) and ultimately suspected to be a brachiopod [90]. *Knightoconus* could fit stratigraphically with stem cephalopods based on our evidence (Figure 3), but its morphological interpretation remains in doubt. The earliest reliable cephalopod fossils are the small bodied, septate, and siphuncle-bearing *Plectronoceras* from the Late Cambrian. Some versions of our analysis used *Plectronoceras* as a soft bound calibration point; by not using *Plectronoceras*, the origin of cephalopods shifts considerably towards the Silurian (Table 2).

Recently, shell-less and coleoid-shaped Lower Cambrian *Nectocaris pteryx* was regarded as a cephalopod [24], but this was immediately rejected on several lines of argument [91, 92]. Other putative Early Cambrian nectocaridids such as *Vetustovermis* [93] are superficially similar to *Nectocaris* in having a pair of long cephalic tentacles and stalked eyes but show a ventral foot separated from the supposedly wing-like mantle. Interpreting *Nectocaris* as having an axial cavity with gills and a funnel would provide synapomorphies for interpreting Nectocarididae as stem cephalopods [24, 94]. Molecular clock estimates can provide further insight to such contentious interpretations; according to our time estimates (which excluded nectocaridids as potential calibration points), *Nectocaris* is too ancient to be a cephalopod (Figure 3). If *Nectocaris* could be accepted as molluscan based on its contentious morphological interpretation, our time trees would be compatible with the idea that nectocaridids are stem variopods or within the stem of an aplacophoran/cephalopod or aplacophoran clade. Nectocaridid features with superficial similarities to coleoid cephalopods [24, 94] instead could be ancestral attributes of variopods: an anteriorly elongated body with head, long and flexible head tentacles, putative preoral hood, and a more or less reduced foot.

The fossil record offers shells and body fossils which, by their occurrence and morphology, at least hypothetically fill our time tree with life. The topology and timing of our hypothesis of early molluscan evolution is not rejected by fossil evidence.

3.7. Dorsoconcha. Molecular, morphological, and palaeontological evidence support (or fail to reject) our basal molluscan dichotomy. The clade Dorsoconcha includes most

shelled molluscs and 98% of living species in four classes: Gastropoda, Bivalvia, Polyplacophora, and Monoplacophora.

We note two inferred potential morphological synapomorphies of Dorsoconcha, both relating to the digestive system and both somewhat ambiguous: the intestine is surrounded by the pericardium in basal lineages of gastropods, bivalves, and in monoplacophorans and may be positionally homologous in chitons (Figure 1(c) character 7) and a rotating enzymatic crystalline style (or protostyle; Figure 1(c) character 9). Many basal, noncarnivorous molluscs have a more or less well-developed stomach separated into sorting zones, but only dorsoconchs (and a family of caudofoveates [61]) have the complex style; this was secondarily lost in chitons, which have a derived position in our proposed topology.

Most previous studies on the phylogeny of molluscs have been driven by the Conchifera concept [1, 95] and emphasised the opinion that Serialia violates putative conchiferan synapomorphies [12]. Such features all are plesiomorphic for dorsoconchs in our topology (Figure 1(c)). We note several potential apomorphies for Serialia (Figure 1(c)): the serial (seven or) eightfold (octoserial) dorsoventral pairs of muscle bundles, with two pairs of intertwined muscle bundles in chitons and also partly present in large *Neopilina* [95, 96]; serial gills in a circumpedal mantle cavity; a highly similar cerebral nerve cord; and a longitudinal elongation of the dorsoventrally flattened body, to mention just some (Figure 1(c)). The most prominent feature of Serialia, serial paired foot retractors, is also present in bivalves, but octoserial retractors appear in Ordovician *Babinka* and not in the earliest known bivalves in the Cambrian [97] (Figure 1(c) character 10). While head and buccal apparatus are reduced almost completely in bivalves, Serialia elaborated the buccal mass evolving highly similar radulae and the radula bolster. Similar foot and radula structures in patellogastropod limpets [61] could be either plesiomorphic or convergent, because Patellogastropoda are either an isolated early-diverging gastropod group or relatively recently derived within Vetigastropoda [98, 99].

From this topological result and the available fossil evidence, we propose that the last common ancestor of monoplacophorans and chitons was cap-shelled and adapted to epibenthic life in shallow waters, rasping algae or other microorganisms from rocky substrates (Figure 1). In this scenario, chitons are not primitive molluscs but rather a derived group, potentially adapted to high-energy marine shores. Monoplacophorans initially also were shallow water dwellers [73] but could have colonised deeper waters during the Palaeozoic, where modern monoplacophorans still occur [100]. The Cenozoic or Late Cretaceous molecular dating of the diversification of living monoplacophorans and their short inner branches ([4], Figure 3) are compatible with earlier assumptions of pronounced anagenetic changes in the long stem line of these so-called "living fossils" [4, 100].

3.8. Variopoda. The clade Variopoda (Figure 1(c)) groups the scaphopods, aplacophorans, and cephalopods together in all our analyses, and it is very well supported. We infer several features of variopods, including an apparent propensity for habitat-induced transformations (noted in the taxon epithet; Figure 1(c) character 2). Some other roughly hypothesised apomorphies may refer to a clade of scaphopods and cephalopods only, that is, to variopods only under the assumption that aplacophorans represent highly paedomorphic and thus aberrant offshoots (see below): lateral extension of a primitively dorsal cap-like shell forming a tube; twisting the growth axis during ontogeny from initial dorsoventral to an anterior body extension, translocating head foot and mantle cavity with anal opening anteriorly; formation of a ring-like dorsoventral muscle insertion; multiplication of cephalic tentacles into prey-capturing feeding tentacles; and at least partly using muscle antagonist rather than merely hydrostatic systems in these tentacles (convergently in gastropod cephalic sensory tentacles); a hood is formed anterior to the mouth; and muscular retraction of the foot is used to pump water, waste, and gametes through/out the mantle cavity.

A clade of scaphopods and cephalopods repeatedly has been proposed based on morphological data, sometimes with one or the other or both together allied with gastropods [1], and was recovered by molecular data [52] and broadly within some pan-molluscan molecular phylogenies [10, 21]. In contrast, morphocladistic neontological [101] and palaeontological studies (e.g., [80, 102]) advocated the Diasoma concept suggesting scaphopods as sister to bivalves with a rostroconch ancestor. Developmental data showing different ontogeny of shells have not supported the latter opinion [103]. Diasoma has been equivocally recovered within one mitogenomic analysis ([104], but see [105] for limitations of protein coding mitochondrial genes), and in one supplementary analysis of transcriptome data [8]. Similar features such as a digging foot could be interpreted as convergent adaptations to infaunal life.

The two aplacophoran classes Caudofoveata and Solenogastres have never been associated with either scaphopods or cephalopods in morphological studies. In our analyses aplacophorans are usually paraphyletic, but some permutations, in particular when excluding (the faster-evolving, but stringently masked) COI and 16S markers, recover a clade Aplacophora sister to Cephalopoda. Aplacophora as a clade is not rejected by AU analyses of the combined 5-marker set either (Table 3). A single origin of vermiform body plans in the cephalopod stem lineage could arguably be more parsimonious than arising twice independently. Monophyly of Aplacophora is indicated by all recent studies using multiple nuclear protein coding genes and phylogenomic data sets ([5, 7, 8]; Figure 1(b)) but not neuroanatomy [106].

Aplacophorans may share an inferred tendency of modifying the ancestral foot, they have an elongated body with a foot (or head) shield with strong retractor muscle in caudofoveates, and the atrial cavity especially in Solenogastres could be interpreted as a modified preoral hood, as remnants of a hypothesised variopod body plan. Yet there is no morphological indication for a specifically aplacophoran-cephalopod clade. Interpretation of the vermiform molluscan morphology as progenetically derived rather than reflecting a basal molluscan condition (also assumed under the Aculifera concept) actually allows for hypotheses that

resolve them at any position in the molluscan tree (or makes their position impossible to recover using currently available anatomical data). Assuming that aplacophorans (once or twice independently) initially evolved into interstitial secondary worms could be correlated with precerebral ganglia present in caudofoveates [106]; these transformations have evolved many times independently in interstitial worm-like gastropod groups, which are likely progenetic [47]. Calcareous spicules also evolved many times convergently in different interstitial shell-less gastropod lineages [47] and a protective dorsal cuticle covering the body evolved within progenetic corambid sea slugs [67, 68]. "Regressive" [*sensu* [107]] traits in aplacophorans such as miniaturisation, losses of shell, tentacles, and cephalisation have been attributed to progenesis [64]. The serial dorsoventral muscle grid of aplacophorans resembles early ontogenetic stages observed in other molluscs [108] and could be paedomorphic, but it is still an adaptive innovation for nonlarval stages. The narrow bipartite radulae of aplacophorans are specialised tools for microcarnivory but also resemble some stem molluscan radula types [56]; evidence from Cambrian fossils is more congruent with an ancestral unipartite radula [109].

Our topology places aplacophorans in an unconventional position; however, there is consensus among all recent molecular studies that aplacophorans represent derived rather than plesiomorphic members of Mollusca (Figure 1). These notes on the specific feature of aplacophorans therefore are of general interest to resolving the pattern and tempo of molluscan evolution, regardless of differences between our new topology and other studies.

3.9. Molluscan Ancestors. The origin of molluscs is a long-standing question, and speculations on the "hypothetical ancestral mollusk" depend on character-polarity and even topological assumptions [1, 59]. Broad genomic analyses (e.g., [14–16, 22]) recovered molluscs as an early-derived offshoot of Lophotrochozoa (Spiralia), as had been proposed on morphological grounds [110]. Modern morphological studies suggest entoprocts as sister to molluscs [1], a view supported by mitochondrial genomics [105]. MicroRNA data [111] suggest Annelida is the sister to Mollusca, as recovered (but never robustly supported) by most of our analyses with a large outgroup taxon set (Suppl. Figure 1A). Our analyses did not resolve a consistent sister group to Mollusca. Yet permutations and pruning of our outgroup sampling did not affect ingroup topologies. We regard the molluscan sister group as an unanswered question, but not necessarily problematic to the question of internal molluscan phylogeny (if ingroup taxon sampling is sufficiently dense).

Our initial morphological character mapping (Figure 1(c)) suggests that the last common ancestor of living molluscs ("LAM") was a single-shelled conchiferan with a complex body, single (or few) paired shell retractors, single paired gills in a circumpedal mantle cavity, and an elaborated (cephalised) anterior body portion. There is little reason to assume that this hypothetical LAM resembled a chiton or monoplacophoran (e.g., [25]) or to suspect a segmented body organisation (e.g., [112]). Instead, the LAM may have resembled an untorted gastropod with a cap-like shell, perhaps similar to *Latouchella*, as assumed by morphologists before the discovery of the supposedly "living fossil" *Neopilina* and still advocated by some palaeontologists [70].

Our assessment of potential morphological apomorphies (Figure 1(c)) and the molecular clock results (Figure 3) would suggest that the Vendian (555 Ma) *Kimberella* [78] is a candidate stem-group mollusc appearing before the evolution of a dorsal shell field. The interpretation of *Kimberella* is controversial [113], but the true stem molluscs probably did have a large, bilaterally symmetrical body with subapical mouth on a snout with a likely bipartite radula [114], a broad ventral foot, many dorsoventral muscle bundles, and a dorsal mantle covered with a resistant dorsal cuticle with mineralised spicules, which are all molluscan features, but lacking a shell [115, 116]. During the latest Precambrian rise of predators and successive development of sediment bottoms [25], molluscan larvae or early juveniles may have calcified their plesiomorphic cap-shaped mantle cuticle for protective reasons. Answering Yochelson [117], the mollusc made a shell, but then the shell made the molluscs.

4. Conclusions

Only one (if any) of the dozens of proposed hypotheses on molluscan phylogeny reflects the true tree. Both the traditional palaeontological concept, with monoplacophorans giving rise to all other molluscan lineages, and the widely accepted morphocladistic Testaria hypothesis, with progressive evolution from vermiform molluscs to chitons and conchiferans [62, 118], are not supported by molecular evidence and are apparently incompatible with the chronological appearance of reliable fossils representing major molluscan lineages.

The Aculifera concept has been supported by phylogenomic results [2, 119], whose dichotomy is not inherently contradicted by the available fossil record if the last common molluscan ancestor was small and complex and had a shell (i.e., was conchiferan rather than chiton-like). Yet the branching patterns of living clades in available phylogenomic topologies appear to be incongruent with stratigraphic evidence. The debate on molluscan phylogeny can only be progressed using all available evidence, integrating morphological, fossil, and molecular data. To provide meaningful insights, molecular approaches must include all eight molluscan classes and cover the well-known diversity of living taxa.

Our results, despite using traditional markers that cover arguably less data than next-generation approaches, are based on a comprehensive taxon set with data quality checked exhaustively at all levels. Topologies recovered still may suffer from poor sampling especially of aplacophoran lineages and from heterogeneous evolution of ingroup clades such as cephalopods or patellogastropods. The data available, while extensive and of high quality, are small in comparison to the total genetic diversity of the phylum under study.

Nevertheless, our data sets, regimes, and analyses support and refine the Serialia hypothesis [9]. The topological results inferred herein cannot be refuted by recent research on shell building gene expression and mollusc palaeontology. In many well-studied molluscan taxa, shells are reduced or duplicated, bodies adapted to different environments and life styles such as benthic, interstitial, or pelagic realms, and features such as mantle cavities and radulae repeatedly were transformed, often drastically and rapidly. Heterochronic processes could already have occurred in the Palaeozoic, which would be consistent with the disparity known in living molluscs but which could also obscure deeper phylogenetic signal in morphological analyses. Ultimately, such complex diversification could have led to the fossil and extant molluscs that stand apart from other (noninsect) animals in terms of species diversity, body disparity, and variation of life traits. The true reconstruction of the early radiation of molluscs still is one of the major unresolved issues in evolutionary biology. Independent molecular evidence, such as microRNAs or phylogenomic data on a similarly comprehensive and dense taxon sampling as used herein, will be needed to further test these hypotheses.

Authors' Contributions

I. Stöger carried out the molecular genetic studies, performed the sequence alignments and the phylogenetic analyses, and drafted the paper. J. D. Sigwart participated in the design of the study and contributed to writing the paper; Y. Kano participated in original fieldwork and in the molecular genetic studies; T. Knebelsberger participated in the molecular genetic studies; B. A. Marshall carried out original fieldwork and helped draft the paper; E. Schwabe participated in original fieldwork and helped draft the paper; M. Schrödl conceived and designed the study, participated in original fieldwork, contributed to molecular genetic studies, and contributed to writing the paper.

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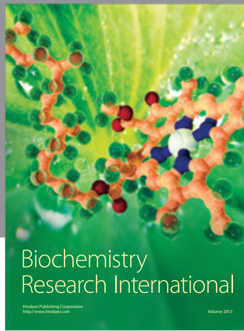

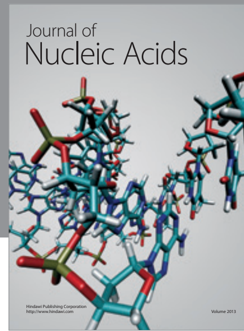

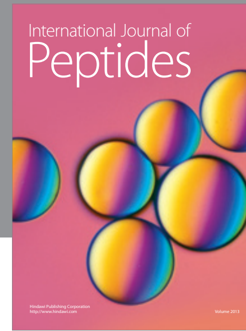

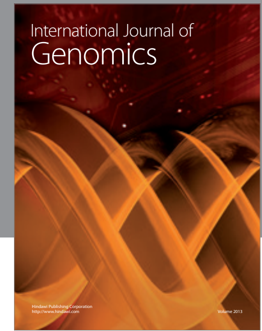

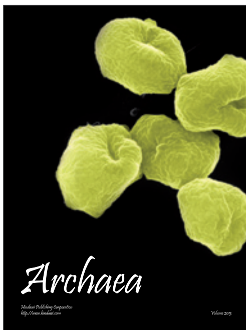

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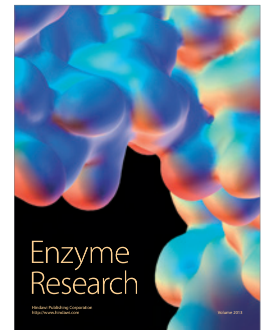
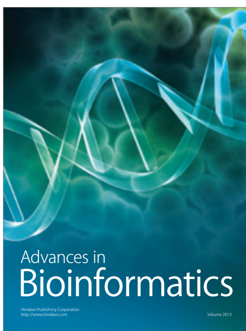
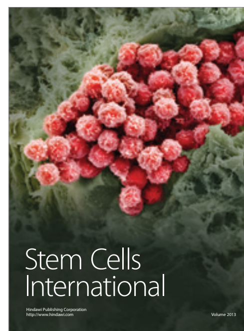
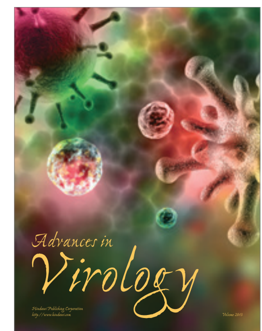
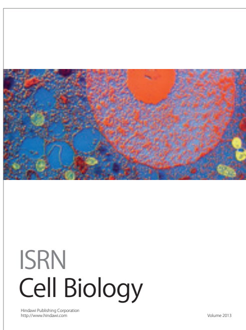
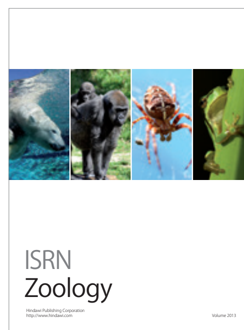


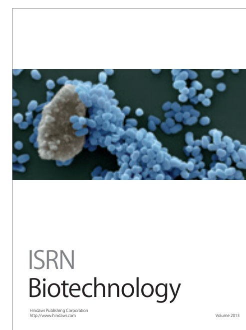




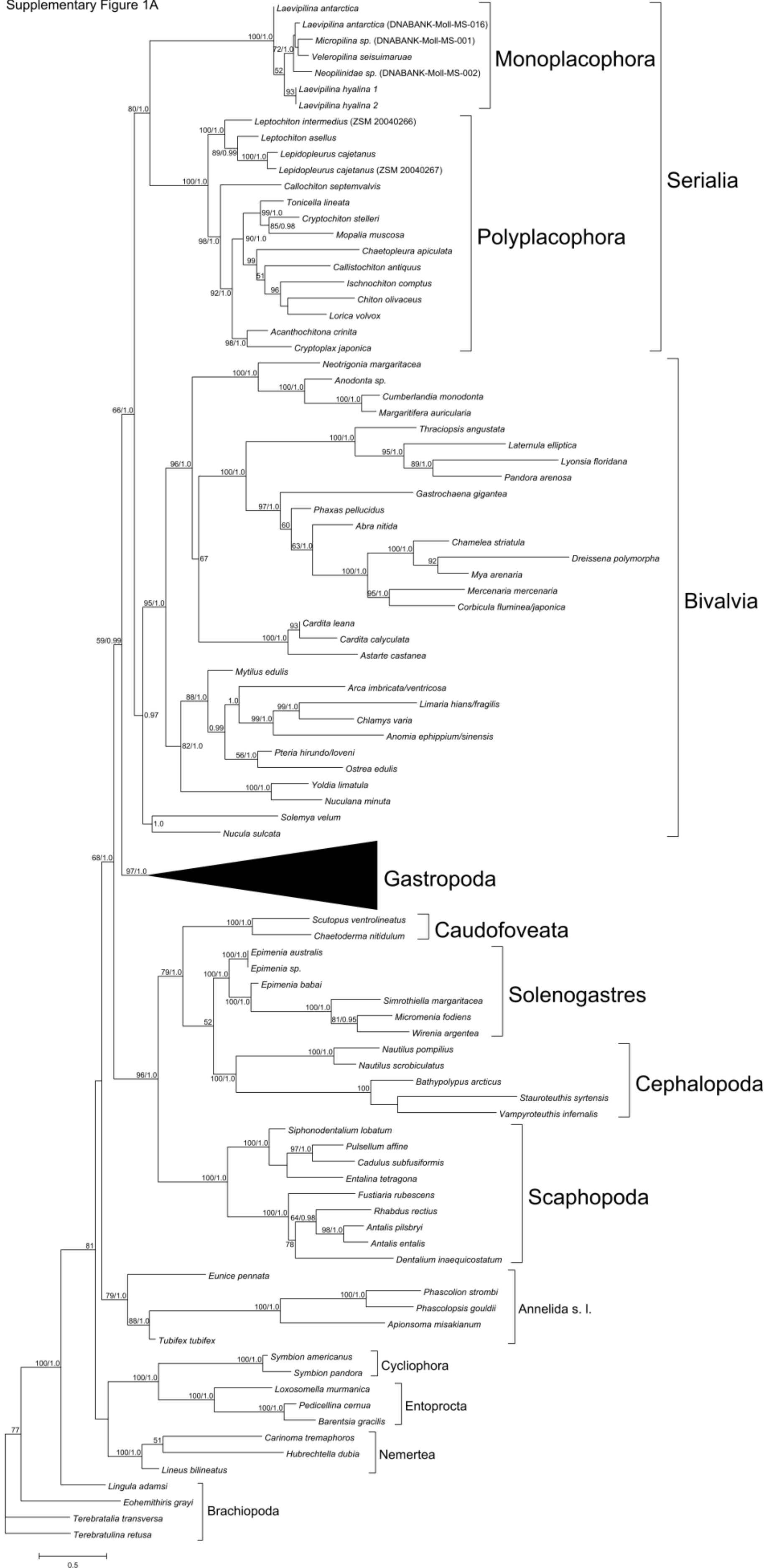




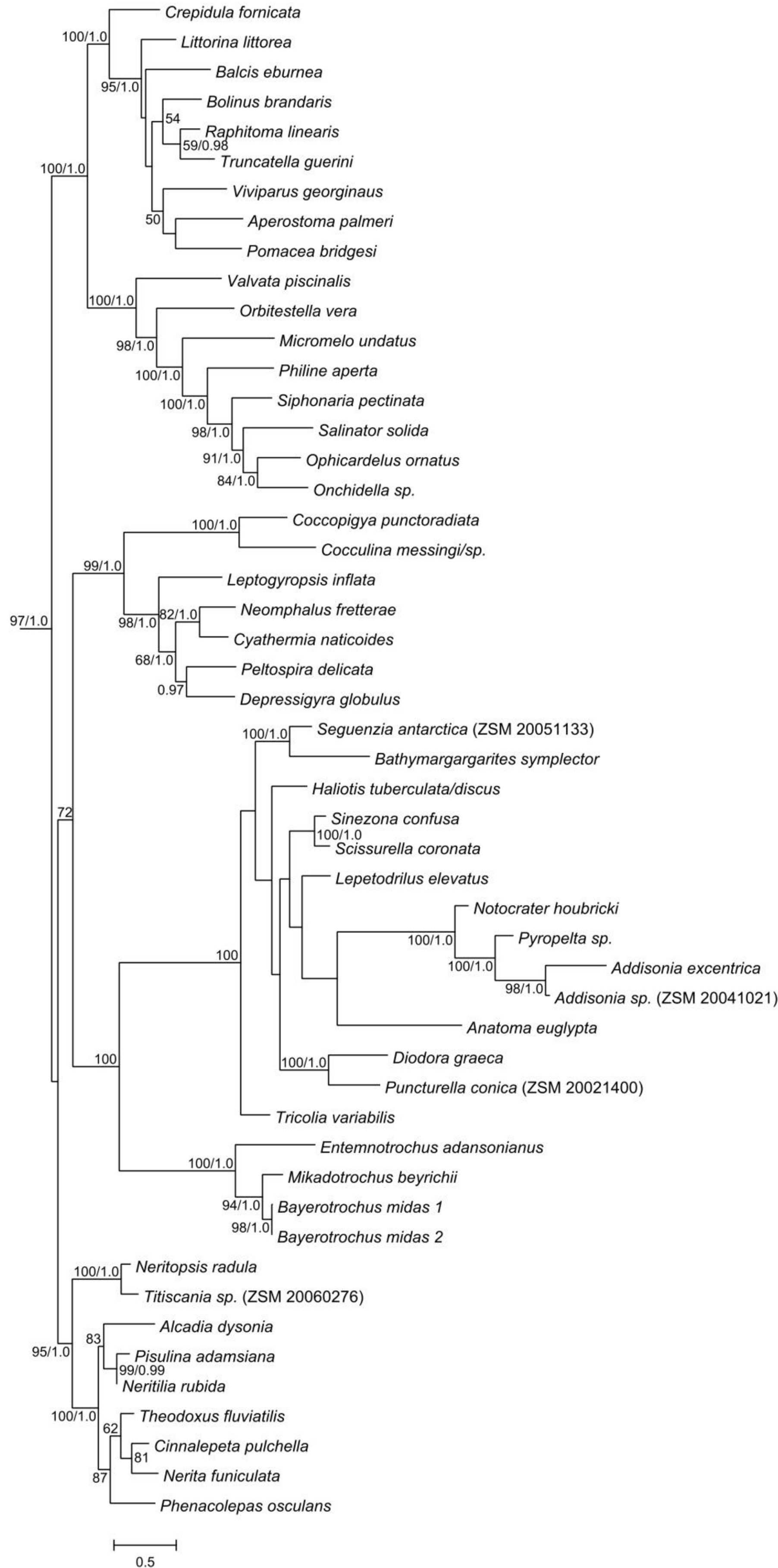




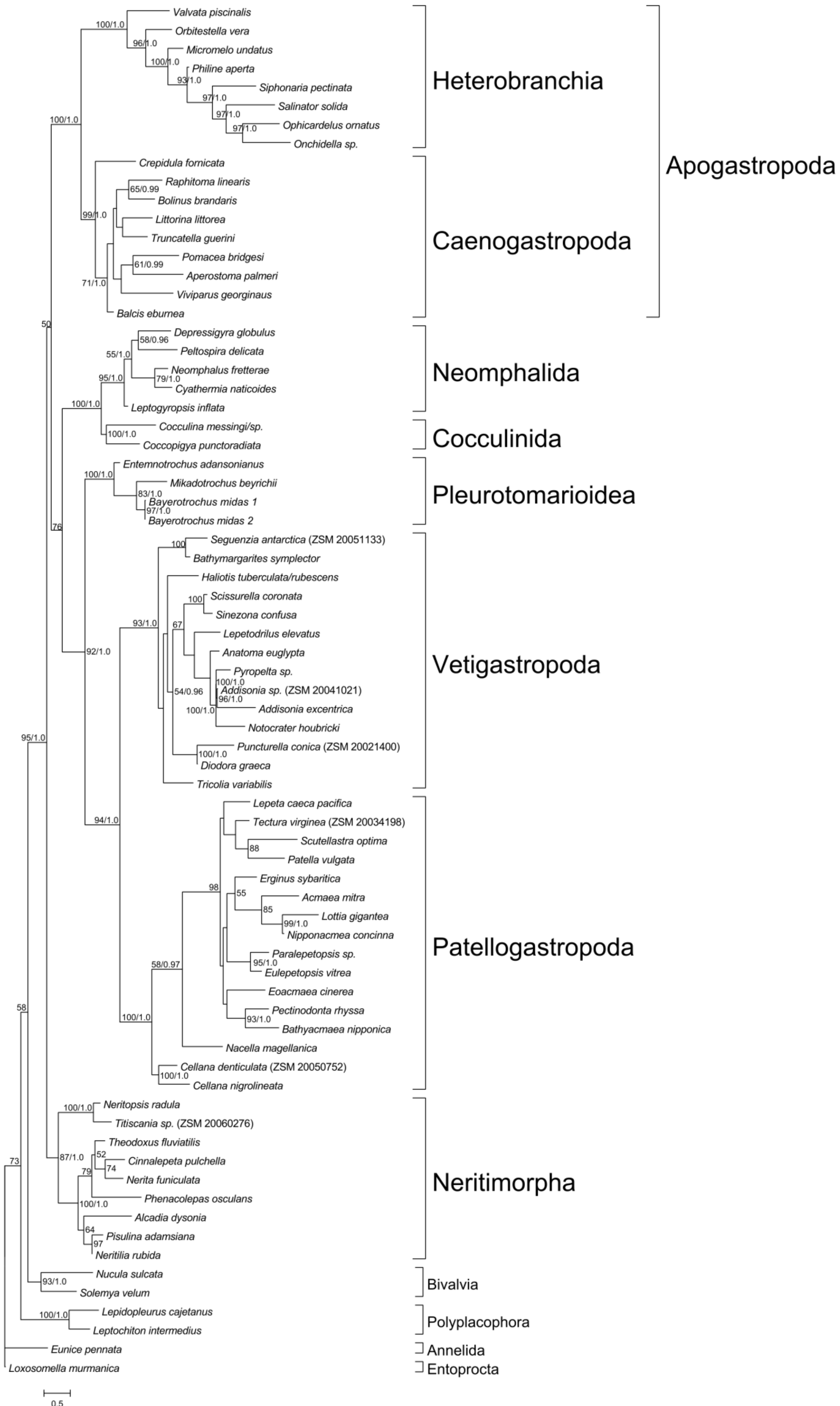
Supplementary Figure 1A



Supplementary Figure 1B



Supplementary Figure 2



Supplementary Table 1

Fragment	Primer	Primer sequence
28S rRNA small	28SF	5'-GAC CCG TCT TGA AGC ACG-3'
	28SR	5'-CCA CAG CGC CAG TTC TGC TTA C-3'
28S rRNA large	28SF2	5'-ACC TAT TCT CAA ACT TTA AAT GG-3'
	28SR2	5'-GAC TTC CCT TAC CTA CAT-3'
18S rRNA part A	18Sa2.0	5'-ATG GTT GCA AAG CTG AAA C-3'
	18S9R	5'-GAT CCT TCC GCA GGT TCA CCT AC-3'
18S rRNA part B	18S1F	5'-TAC CTG GTT GAT CCT GCC AGT AG-3'
	18S5R	5'-CTT GGC AAA TGC TTT CGC-3'
18S rRNA part C	18S3F	5'-GTT CGA TTC CGG AGA GGG A-3'
	18Sbi	5'-GAG TCT CGT TCG TTA TCG GA-3'
16S rRNA	16Sa	5'-CGC CTG TTT ATC AAA AAC AT-3'
	16Sb	5'-CTC CGG TTT GAA CTC AGA TCA-3'
H3	H3aF	5'-ATG GCT CGT ACC AAG CAG ACV GC-3'
	H3aR	5'-ATA TCC TTR GGC ATR ATR GTG AC-3'

PCR conditions

Initial step	94°C	6min
Denaturation	94°C	1min
Annealing	50°C	1min
Elongation	72°C	1min 30sec
Final elongation	72°C	6min
Cycles		45

Supplementary Table 2

	Species	18S	28S1	28S2	28S3	28S4	H3	COI	16S
Outgroups									
Annelida	*Eunice pennata*	AY040684	AY340391				DQ779731		AF321418
	Tubifex tubifex	GQ355437	GQ355465	GQ355465				EF179544	EU117545
Brachiopoda	*Eohemithiris grayi*	AF025936	AY839242	AY839242	AY839242				
	Lingula adamsi	U08329							
	Terebratalia transversa	FJ196115	AF342802	AF342802	AF342802	AF342802		FJ196085	
	Terebratulina retusa	U08324	AY839244	AY839244	AY839244		DQ779768		AF334238
Cycliophora	*Symbion americanus*	EF142068		EF142087	EF142087			EF140778	EF140771
	Symbion pandora	Y14811		AY218133				AY218084	
Entoprocta	*Barentsia gracilis*	FJ196109	AY210456	AY210456	AY210456	AY210456		FJ196079	
	Loxosomella murmanica	AY218100	DQ279950	DQ279950	DQ279950	DQ279950	AY218150		
	Pedicellina cernua	FJ196111						FJ196081	
Nemertea	*Carinoma tremaphoros*	AY039675	AJ436888	AJ436888			AJ436986	AJ436943	AJ436833
	Hubrechtella dubia	AY039674	AJ436889	AJ436889				EU489495	AJ436834
	Lineus bilineatus		DQ279947	DQ279947			DQ279996	DQ280014	DQ280022
Sipuncula	*Apionsoma misakianum*	AY210440	AY210454	AY210454	AY210454	AY210454	DQ300052	EU267000	
	Phascolion strombi	DQ299984	AY210468	AY210468	AY210468	AY210468	DQ279998		
	Phascolopsis gouldii	AF342796	AF342795	AF342795	AF342795	AF342795	AF519297	DQ300134	
Mollusca									
Bivalvia	*Abra nitida*	DQ279940	DQ279965		DQ279965	DQ279965	DQ280005		
	Anodonta sp.	AY579090	DQ279964				AY579132	AY579122	
	Anomia ephippium/sinensis	AF120535	AB102739		AB102739	AB102739			
	Arca imbricata/ventricosa	AY654986	AB101612		AB101612	AB101612	AY654989	AY654988	
	Astarte castanea	AF120551	AF131001				DQ280004	AF120662	
	Cardita calyculata	AF120549		AF120610				AF120660	
	Cardita leana	AM774481	AM779655						
	Chamelea striatula	DQ279943		DQ279967	DQ279967	DQ279967	DQ280009	AF120668	DQ280041

	Chlamys varia	DQ279939	DQ279962		DQ279962	DQ279962	DQ280003		DQ280033
	Corbicula fluminea/japonica	AF120557	AB126330		AB126330	AB126330	AY070161	AF120666	DQ280039
	Cumberlandia monodonta	AY579105					AY579144	AY579131	AY579089
	Dreissena polymorpha	AF120552	AF131006				AY070165	AF120663	DQ280038
	Gastrochaena gigantea	AM774515	AM779689						
	Laternula elliptica	AY192687	EU734752						GU227003
	Limaria hians/fragilis	AF120534	AB102742		AB102742	AB102742	AY070152	AF120650	
	Lyonsia floridana	AF120540						AF120654	
	Margaritifera auricularia	AY579097		AY579113			AY579137	AY579125	DQ280035
	Mercenaria mercenaria	AF120559	AF131019				DQ280008	DQ399403	DQ280040
	Mya arenaria	AF120560	AB126332		AB126332	AB126332	AY377770	AY070140	AY377618
	Mytilus galloprovincialis	L33452	AB103129		AB103129	AB103129	AY267748	AY497292	AY497292
	Neotrigonia margaritacea	AF411690	DQ279963		DQ279963		AY070155	U56850	DQ280034
	Nucula sulcata	DQ279937	DQ279960		DQ279960	DQ279960	DQ280001	DQ280017	DQ280029
	Nuculana minuta	DQ279938	DQ279961		DQ279961	DQ279961	DQ280002	DQ280018	DQ280030
	Ostrea edulis	L49052	AF137047/AF120596				AY070151	AF120651	DQ280032
	Pandora arenosa	AF120539		AF120601					
	Phaxas pellucidus	DQ279941	AY145420		AY145420	AY145420	DQ280006	DQ280019	DQ280036
	Pteria hirundo/loveni	AF120532	AB102767		AB102767	AB102767		AF120647	DQ280031
	Solemya velum	AF120524	AY145421		AY145421	AY145421	AY070146	U56852	DQ280028
	Thraciopsis angustata	AM774491	AM779664						
	Yoldia limatula	AF120528	AY145424		AY145424	AY145424	AY070149	AF120642	
Caudofoveata	*Chaetoderma nitidulum*	AY377658			FJ445775		AY377763	AY377726	AY377612
	Scutopus ventrolineatus	X91977							
Cephalopoda	*Bathypolypus arcticus*	AY557465		AY557554				AF000029	DQ280044
	Nautilus pompilius	AY557455		AY145417				AY557514	AY377628
	Nautilus scrobiculatus	AF120504		AF120567			AF033704		U11606
	Stauroteuthis syrtensis	AY557457					AY557406	AF000067	DQ280042
	Vampyroteuthis infernalis	AY557459	AH012197	AH012197	AH012197		AY557408	AF000071	DQ280043
Gastropoda	*Acmaea mitra*	AB282760	AB282781	AB282781	AB282781			AB238459	AB106518

Addisonia excentrica	AY603096							
Addisonia sp. (ZSM 20041021)	KF527260		KF527269	KF527269		KF527283		
Alcadia dysonia	DQ093428		DQ279974	DQ279974		DQ093496		DQ093469
Anatoma euglypta	AY923897					AY923971	AY923934	
Aperostoma palmeri	DQ093435	DQ279983	DQ279983	DQ279983		DQ093505	DQ093523	DQ093479
Balcis eburnea	AF120519		AF120576				AF120636	DQ280051
Bathyacmaea nipponica	AB282772	AB282793					AB238588	AB238451
Bathymargarites symplector	DQ093433	DQ279982	DQ279982	DQ279982	DQ279982	DQ093503	DQ093521	DQ093477
Bayerotrochus midas 1	AF120510		DQ093453			DQ093500	AY296820	DQ093474
Bayerotrochus midas 2	FJ977637	FJ977668	FJ977668	FJ977668				
Bolinus brandaris	DQ279944	DQ279986	DQ279986	DQ279986		DQ280010	DQ280020	DQ280052
Cellana nigrolineata	DQ013353		DQ279971			DQ093493	DQ093515	DQ093467
Cinnalepeta pulchella			AB087192	AB087192				
Coccopigya punctoradiata	AB282774	AB282795					AB238590	AB238453
Cocculina messingi/sp.	AF120508	DQ279973	DQ279973	DQ279973		AY377777	AY377731	AY377624
Crepidula fornicata	AY377660					AY377778	AF353154	AY377625
Cyathermia naticoides	DQ093430	DQ279977	DQ279977	DQ279977		DQ093498	DQ093518	DQ093472
Depressigyra globulus	DQ093431	DQ279978	DQ279978	DQ279978	DQ279978	AF033689	DQ093519	DQ093473
Diodora graeca	AF120513	DQ279980	DQ279980	DQ279980	DQ279980	DQ093502	AF120632	DQ093476
Entemnotrochus adansonianus	AF120509	DQ279979	DQ279979	DQ279979		AY377774		AY377621
Eoacmaea conoidalis	AB282757	AB282778					AB238505	AB238375
Erginus sybaritica	AB282761	AB282782	AB282782	AB282782			AB238461	AB238350
Eulepetopsis vitrea	DQ093427	DQ279972	DQ279972			DQ093495	DQ093516	DQ093468
Haliotis tuberculata/discus	AF120511	AY145418	AY145418	AY145418	AY145418	AY070145	AY377729	AY377622
Lepeta caeca pacifica	AB282759	AB282780	AB282780	AB282780			AB238458	AB238347
Lepetodrilus elevatus	DQ093432	AY145413	AY145413	AY145413	AY145413	DQ093501	DQ093520	DQ093475
Leptogyropsis inflata	AB365313					AB365300	AB365258	
Littorina littorea	DQ093437	DQ279985	DQ279985			DQ093507	DQ093525	DQ093481
Lottia gigantea	AB282762	AB282783	AB282783	AB282783			AB238466	AB106498
Micromelo undatus	DQ093443		DQ279995			DQ093513		DQ093487

Mikadotrochus beyrichii	AM048636	AM048695	AM048695	AM048695		AM049331	
Nacella magellanica	AB282769	AB282790	AB282790		AB433689	EU870985	AB238433
Neomphalus fretterae	AY090806						
Nerita funiculata	DQ093429		DQ279976	DQ279976		DQ093517	DQ093471
Neritilia rubida			AB087190	AB087190			
Neritopsis radula			AB087186				
Nipponacmea concinna	DQ013354					AB238486	AB106511
Notocrater houbricki	L78881				AF033700	AY296822	
Onchidella sp.	DQ093441		DQ279992		DQ093511	DQ093529	DQ093485
Ophicardelus ornatus	DQ093442				DQ093512	DQ093530	DQ093486
Orbitestella vera	FJ917207	FJ917239	FJ917239		EF561623	FJ917268	FJ917250
Paralepetopsis sp.	FJ977635	FJ977665	FJ977665		FJ977728	FJ977752	FJ977699
Patella vulgata	AB282770	AB282791	AB282791	AB282791		AB238580	AB238445
Pectinodonta rhyssa	AB282773	AB282794	AB282794	AB282794		AB238589	AB238452
Peltospira delicata	AY923893				AY923967	AY923931	
Phenacolepas osculans	AY923890					AY923928	
Philine aperta	DQ093438	DQ279988	DQ279988	DQ279988	DQ093508		DQ093482
Pisulina adamsiana			AB087191	AB087191			
Pomacea bridgesi			DQ279984	DQ279984	DQ093506	DQ093524	DQ093480
Puncturella conica (ZSM 20021400)	KF527257		KF527266	KF527266	KF527278		KF527251
Pyropelta sp.	FJ977636	FJ977666	FJ977666	FJ977666	FJ977729	FJ977753	FJ977700
Raphitoma linearis	DQ279945		DQ279987		DQ280011		DQ280053
Salinator solida	DQ093440	DQ279991	DQ279991	DQ279991	DQ093510	DQ093528	DQ093484
Scissurella coronata	AM048637	AM048696	AM048696				
Scutellastra optima	AB282771	AB282792				AB238585	AB106482
Seguenzia antarctica (ZSM 20051133)	KF527261		KF527270	KF527270	KF527279		KF527253
Sinezona confusa	AF120512	DQ279981	DQ279981	DQ279981	AY377773		
Siphonaria pectinata	X91973	DQ279993	DQ279993		AY377780	AF120638	AY377627
Theodoxus fluviatilis	AF120515		DQ279975	DQ279975		AF120633	DQ093470
Titiscania sp. (ZSM 20060276)	KF527262		KF527271	KF527271	KF527280		KF527254

	Tricolia variabilis	AB365304					AB365267	AB365219	
	Truncatella guerini	AF120518		AF120575				AF120635	
	Valvata piscinalis	FJ917222	FJ917224	FJ917224	FJ917224	FJ917224		FJ917267	FJ917248
	Viviparus georginaus	AF120516		AF120574			AY377779	AF120634	AY377626
Monoplacophora	*Laevipilina antarctica*			DQ279958					
	Laevipilina antarctica (DNABANK-Moll-MS-016)	KF527265		KF527274	KF527274		KF527276		KF527256
	Laevipilina hyalina 1	FJ445774	FJ445777	FJ445777	FJ445777	FJ445777	FJ445778	FJ445781	FJ445782
	Laevipilina hyalina 2	FJ449542	FJ449541	FJ449541	FJ449541	FJ449541		FJ449540	FJ449543
	Micropilina sp. (DNABANK-Moll-MS-001)	KF527263		KF527272	KF527272		KF527277		KF527255
	Neopilinidae sp. (DNABANK-Moll-MS-002)	KF527264		KF527273	KF527273		KF527275		
	Veleropilina seisuimaruae	AB669192	AB669193	AB669193	AB669193	AB669193	AB669194	AB669195	AB669196
Polyplacophora	*Acanthochitona crinita*	AF120503	DQ279957	DQ279957	DQ279957		AY377759	AF120627	AY377609
	Callistochiton antiquus	AY377645	DQ279953	DQ279953	DQ279953		AY377749	AY377712	AY377599
	Callochiton septemvalvis	AY377632		DQ279952	DQ279952		AY377736	AY377700	
	Chaetopleura apiculata	AY377636	AY145398	AY145398	AY145398	AY145398	AY377741	AY377704	AY377590
	Chiton olivaceus	AY377651		DQ279955	DQ279955		AY377755	AY377716	AY377605
	Cryptochiton stelleri			AY377686			AY377760	AY377720	AY377610
	Cryptoplax japonica	AY377656	AY145402	AY145402	AY145402	AY145402	AY377761	FJ445780	AY377611
	Ischnochiton comptus	AY377639	AY145412	AY145412	AY145412	AY145412	AY377744	AY377709	AY377593
	Lepidopleurus cajetanus	AF120502	FJ445776	FJ445776	FJ445776		AY377735	AF120626	AY377585
	Lepidopleurus cajetanus (ZSM 20040267)	KF527259		KF527268	KF527268		KF527282		KF527252
	Leptochiton intermedius (ZSM 20040266)	KF527258		KF527267	KF527267		KF527281		
	Leptochiton asellus	AY377631	AY145414	AY145414	AY145414	AY145414	AY377734	FJ461256	AY377586
	Lorica volvox	AY377647	DQ279954	DQ279954	DQ279954		AY377751		AY377601
	Mopalia muscosa	AY377648	DQ279956	DQ279956	DQ279956		AY377752		AY377602
	Tonicella lineata	AY377635		AY377665			AY377739	AY377702	AY377588
Scaphopoda	*Antalis entalis*	DQ279936	AY145388	AY145388	AY145388	AY145388	DQ280000	DQ280016	DQ280027
	Antalis pilsbryi	AF120522		AF120579				AF120639	
	Cadulus subfusiformis	AF490603							
	Dentalium inaequicostatum	DQ279935		DQ279959	DQ279959		DQ279999	DQ280015	DQ280026

	Entalina tetragona	AF490598				
	Fustiaria rubescens	AF490597				
	Pulsellum affine	AF490600				
	Rhabdus rectius	AF120523	AF120580	AY377772	AF120640	AY377619
	Siphonodentalium lobatum	AF490601				
Solenogastres	*Epimenia sp.*	AY377657	AY377691	AY377765	AY377723	AY377615
	Epimenia australis		AY377689	AY377767	AY377722	AY377614
	Epimenia babai	AY212107	AY377690	AY377766	AY377724	AY377616
	Micromenia fodiens	FJ649601				
	Simrothiella margaritacea	FJ649600				
	Wirenia argentea	FJ649599				

Supplementary Table 3A

	Species	18S	28S1	28S2	28S3	28S4	H3	COI	16S
Annelida	*Eunice pennata*							AY838870	
	Lineus bilineatus	DQ279932			DQ279947	DQ279947			
Bivalvia	*Cardita calyculata*						AY070156		
	Cumberlandia monodonta		AF305382						
Brachiopoda	*Eohemithiris grayi*							AB053200	
	Lingula adamsi							AB128054	
	Terebratulina retusa							NC000941	
Cephalopoda	*Nautilus pompilius*		AY145417		AY145417	AY145417			
	Stauroteuthis syrtensis			DQ279968	DQ279968				
Cycliophora	*Symbion pandora*						AY218153		
Gastropoda	*Coccopigya punctoradiata*						AB365301		
	Crepidula fornicata		AY145406	AY145406	AY145406	AY145406			
	Nerita funiculata						DQ093497		
	Neritilia rubida							AB102712	
	Neritopsis radula				AB087186				
	Ophicardelus ornatus		DQ279994	DQ279994	DQ279994				
	Pomacea bridgesi	DQ093436							
	Scissurella coronata							AM049332	AM116867
	Sinezona confusa							AF120631	
Nemertea	*Hubrechtella dubia*						AJ436987		
Polyplacophora	*Mopalia muscosa*							AY377713	

Supplementary Table 3B

	Species	28S2
Bivalvia	*Gastrochaena gigantea*	AM779689
	Thraciopsis angustata	AM779664
	Solemya velum	AY145421
	Nucula sulcata	DQ279960
	Nuculana minuta	DQ279961
	Yoldia limatula	AY145424
	Mytilus galloprovincialis	AB103129
	Arca imbricata/ventricosa	AB101612
	Pteria hirundo/loveni	AB102767
	Ostrea edulis	AF137047 + AF120596
	Limaria hians/fragilis	AB102742
	Anomia ephippium/sinensis	AB102739
	Chlamys varia	DQ279962
	Neotrigonia margaritacea	DQ279963
	Anodonta sp.	DQ279964
	Abra nitida	DQ279965
	Phaxas pellucidus	AY145420
	Dreissena polymorpha	AF131006
	Corbicula fluminea / japonica	AB126330
	Mercenaria mercenaria	AF131019
	Mya arenaria	AB126332
	Astarte castanea	AF131001
	Cardita leana	AM779655

Supplementary Table 4

Data set	Number of taxa	Total length of alignment (aligned with Mafft, masked with Aliscore)	Model of sequence evolution (AIC)	Proportion of invariable sites	Gamma distribution shape parameter
16S (142 taxa)	87	519	TVM+I+G	0.1554	0.6522
18S (142 taxa)	133	3617	GTR+I+G	0.1739	0.5009
28S (142 taxa)	118	3583	GTR+I+G	0.2126	0.5255
H3 (142 taxa) all codon positions	94	322	TVM+I+G	0.5221	1.0204
H3 (142 taxa) 1st codon position	94	322	SYM+I	0.8382	equal
H3 (142 taxa) 2nd codon position	94	322	GTR+G	-	1.7209
H3 (142 taxa) 3rd codon position	94	322	GTR+I+G	0.5797	2.5094
COI (142 taxa) all codon positions	92	678	GTR+G	-	0.2944
COI (142 taxa) 1st codon position	92	678	GTR+I+G	0.1649	0.6211
COI (142 taxa) 2nd codon position	92	678	GTR+G	-	0.7885
COI (142 taxa) 3rd codon position	92	678	GTR+I+G	0.2253	0.8226
16S (81 taxa)	51	459	GTR+I+G	0.1784	0.7234
18S (81 taxa)	78	3449	GTR+I+G	0.1271	0.4028
28S (81 taxa)	68	3465	GTR+I+G	0.2235	0.4999
H3 (81 taxa) all codon positions	55	327	GTR+I+G	0.5395	1.3596
H3 (81 taxa) 1st codon position	55	327	SYM+I	0.8289	equal
H3 (81 taxa) 2nd codon position	55	327	SYM+I+G	0.0332	2.0347
H3 (81 taxa) 3rd codon position	55	327	GTR+I+G	0.5940	7.1635
COI (81 taxa) all codon positions	51	666	GTR+G	-	0.2723
COI (81 taxa) 1st codon position	51	666	GTR+I+G	0.2019	0.6262
COI (81 taxa) 2nd codon position	51	666	GTR+G	-	0.4874
COI (81 taxa) 3rd codon position	51	666	GTR+I+G	0.2320	0.7413
16S (Gastropods)	53	531	TVM+I+G	0.1968	0.9294
18S (Gastropods)	67	1959	TrN+I+G	0.1772	0.4059
28S (Gastropods)	62	2808	GTR+I+G	0.2945	0.4736

H3 (Gastropods) all codon positions	47	326	GTR+I+G	0.5564	1.3578
H3 (Gastropods) 1st codon position	47	326	SYM+I+G	0.7580	0.1501
H3 (Gastropods) 2nd codon position	47	326	GTR+I+G	0.0330	0.7695
H3 (Gastropods) 3rd codon position	47	326	GTR+I	0.6461	equal
COI (Gastropods) all codon positions	51	672	GTR+I+G	0.2489	0.4266
COI (Gastropods) 1st codon position	51	672	GTR+I+G	0.2541	0.6279
COI (Gastropods) 2nd codon position	51	672	GTR+G	-	0.6405
COI (Gastropods) 3rd codon position	51	672	GTR+I+G	0.2950	0.7110

Supplementary Table 5

Data set	Variations on data sets	Partition	Alignment length	proportion of gaps
142 taxa	-	9: 16S, 18S, 28S, COI 1st, 2nd, 3rd codon position, H3 1st, 2nd, 3rd codon position	8721	60%
81 taxa	-	9: 16S, 18S, 28S, COI-1st, -2nd, -3rd codon position, H3-1st, -2nd, -3rd codon position	8367	57%
134 taxa, without Aplacophora	exclusion of Aplacophora; exclusion of 3rd codon positions of COI and H3	5: 16S, 18S, 28S, COI, H3	8122	58%
74 taxa, without Aplacophora	exclusion of Aplacophora; exclusion of 3rd codon positions of COI and H3	5: 16S, 18S, 28S, COI, H3	7812	55%
142 taxa	COI and H3 coded as amino acids	5: 16S, 18S, 28S, COI (aa), H3 (aa)	8052	62% (16S-18S-28S); 25% (COI-H3)
142 taxa	only fragments 18S, 28S and H3 are used	5: 18S, 28S, H3-1st, -2nd, -3rd codon position	7527	61%
Gastropods (67 gastropods including patellogastropods, 6 outgroups)	only gastropod taxa are used (including Patellogastropoda)	9: 16S, 18S, 28S, COI-1st, -2nd, -3rd codon position, H3-1st, -2nd, -3rd codon position	6296	53%

Supplementary Table 6

Data set	**saturation**	**Iss**		**Iss c Sym (P)**
H3 (142 taxa) all codon positions	no	0,330	<	0,692 (0,0000)
H3 (142 taxa) 1st and 2nd codon positions	no	0,215	<	0,749 (0,0000)
H3 (142 taxa) 3rd codon position	no	0,192	<	0,949 (0,0000)
COI (142 taxa) all codon positions	no	0,410	<	0,697 (0,0000)
COI (142 taxa) 1st and 2nd codon positions	no	0,239	<	0,683 (0,0000)
COI (142 taxa) 3rd codon position	yes	0,918	>	0,720 (0,0000)
H3 (81 taxa) all codon positions	no	0,392	<	0,689 (0,0000)
H3 (81 taxa) 1st and 2nd codon positions	no	0,045	<	0,722 (0,0000)
H3 (81 taxa) 3rd codon position	no	0,814	<	1,039 (0,0001)
COI (81 taxa) all codon positions	no	0,418	<	0,703 (0,0000)
COI (81 taxa) 1st and 2nd codon positions	no	0,251	<	0,685 (0,0000)
COI (81 taxa) 3rd codon position	yes	0,925	>	0,704 (0,0000)
H3 (Gastropods) all codon positions	no	0,414	<	0,687 (0,0000)
H3 (Gastropods) 1st and 2nd codon positions	no	0,046	<	0,715 (0,0000)
H3 (Gastropods) 3rd codon position	no	0,842	<	1,009 (0,0001)
COI (Gastropods) all codon positions	no	0,466	<	0,706 (0,0000)
COI (Gastropods) 1st and 2nd codon positions	no	0,164	<	0,687 (0,0000)
COI (Gastropods) 3rd codon position	yes	0,929	>	0,700 (0,0000)

Supplementary Table 7

	Calibration node on preferred tree	Fossil calibration	Date range (Ma)	Reference	Prior settings in BEAST v. 1.6.1 (distribution; gamma shape, gamma scale, zero offset)
1	Mollusca	first shell record	~ 545	Parkhaev 2008	Gamma; 2.5, 2.0, 542.0
2	Serialia/Bivalvia	*Fordilla*	~ 530	Parkhaev 2008	Gamma; 3.3, 2.2, 525.0
3	Cephalopoda/Solenogastres	*Plectronoceras*	~ 505	Nishiguchi and Mapes 2008	Gamma; 2.4, 7.0, 495.0
4	Polyplacophora/Monoplacophora	*Orthriochiton*	~ 490	Vendrasco and Runnegar 2004	Gamma; 5.0, 5.0, 470.0
5	Origin of Pteriomorpha	Cyrtodontidae	~ 475	Pojeta 1978	Gamma; 1.9, 9.5, 465.0
6	Origin of Caenogastropoda	Sublitoidea	~ 418	Nützel et al. 2000	Gamma; 2.3, 9.0, 405.0
7	Scaphopoda	*Dentalium*	~ 353	Yochelson 1999	Gamma; 2.2, 6.7, 345.0
8	*Astarte/Cardita*	*Astartella concentrica*	~ 322	Hoare et al. 1989	Gamma; 2.6, 5.0, 315.0
9	Polyplacophora	*Leptochiton davolii*	~ 231	Laghi 2005	Gamma; 5.0, 2.7, 220.0

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Supplementary Table 8

	time estimation (BEAST v. 1.6.1)
Calibrated nodes	
Diversification of Mollusca	551,02 (542,88-559,94)
Split Serialia/Bivalvia	530,93 (525,89-536,72)
Split Cephalopoda/Solenogastres	504,92 (495,72-516,34)
Split Polyplacophora/Monoplacophora	493,06 (476,69-511,76)
Origin of Pteriomorpha	475,07 (465,17-487,24)
Origin of Caenogastropoda	421,49 (405,93-441,94)
Diversification of Scaphopoda	359,95 (345,61-379,28)
Split *Astarte/Cardita*	325,43 (315,69-337,77)
Diversification of Polyplacophora	233,44 (223,22-244,78)
Major molluscan groups	
Diversification of Dorsoconcha	541,69 (532,24-551,28)
Diversification of Bivalvia	495,69 (478,15-514,89)
Diversification of Gastropoda	493,14 (444,21-531,35)
Diversification of Monoplacophora	65,29 (30,36-110,8)
Diversification of Variopoda	534,2 (515,99-550,29)
Split Caudofoveata/Cephalopoda+Solenogastres	518,59 (501,75-536,16)
Diversification of Cephalopoda	364,19 (255,97-467,95)
Diversification of Solenogastres	195,61 (117,78-277,8)
Diversification of Caudofoveata	160,52 (19,5-274,97)

3.2. Katharina M. Jörger, **Isabella Stöger**, Yasunori Kano, Hiroshi Fukuda, Thomas Knebelsberger, Michael Schrödl: **On the origin of Acochlidia and other enigmatic euthyneuran gastropods, with implications for the systematics of Heterobranchia. 2010.** BMC Evolutionary Biology, 10.

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On the origin of Acochlidia and other enigmatic euthyneuran gastropods, with implications for the systematics of Heterobranchia

Katharina M Jörger[1*], Isabella Stöger[1], Yasunori Kano[2], Hiroshi Fukuda[3], Thomas Knebelsberger[1], Michael Schrödl[1]

Abstract

Background: A robust phylogenetic hypothesis of euthyneuran gastropods, as a basis to reconstructing their evolutionary history, is still hindered by several groups of aberrant, more or less worm-like slugs with unclear phylogenetic relationships. As a traditional "order" in the Opisthobranchia, the Acochlidia have a long history of controversial placements, among others influenced by convergent adaptation to the mainly meiofaunal habitats. The present study includes six out of seven acochlidian families in a comprehensive euthyneuran taxon sampling with special focus on minute, aberrant slugs. Since there is no fossil record of tiny, shell-less gastropods, a molecular clock was used to estimate divergence times within Euthyneura.

Results: Our multi-locus molecular study confirms Acochlidia in a pulmonate relationship, as sister to Eupulmonata. Previous hypotheses of opisthobranch relations, or of a common origin with other meiofaunal Euthyneura, are clearly rejected. The enigmatic amphibious and insectivorous Aitengidae *incerta sedis* clusters within Acochlidia, as sister to meiofaunal and brackish Pseudunelidae and limnic Acochlidiidae. Euthyneura, Opisthobranchia and Pulmonata as traditionally defined are non-monophyletic. A relaxed molecular clock approach indicates a late Palaeozoic diversification of Euthyneura and a Mesozoic origin of the major euthyneuran diversity, including Acochlidia.

Conclusions: The present study shows that the inclusion of small, enigmatic groups is necessary to solve deep-level phylogenetic relationships, and underlines that "pulmonate" and "opisthobranch" phylogeny, respectively, cannot be solved independently from each other. Our phylogenetic hypothesis requires reinvestigation of the traditional classification of Euthyneura: morphological synapomorphies of the traditionally defined Pulmonata and Opisthobranchia are evaluated in light of the presented phylogeny, and a redefinition of major groups is proposed. It is demonstrated that the invasion of the meiofaunal habitat has occurred several times independently in various euthyneuran taxa, leading to convergent adaptations previously misinterpreted as synapomorphies. The inclusion of Acochlidia extends the structural and biological diversity in pulmonates, presenting a remarkable flexibility concerning habitat choice.

Background

Since the introduction of the Heterobranchia concept by Haszprunar [1,2], considerable advances have been achieved, solving the phylogeny of certain heterobranch groups (i.e. "families" or "orders") on morphological (e.g. Mikkelsen [3] on Cephalaspidea; Jensen [4] on Sacoglossa; Wägele and Willan [5] on Nudibranchia, Klussmann-Kolb [6] on Aplysiidae) and molecular levels (e.g. Wollscheid-Lengeling et al. [7] on Nudibranchia; Wade et al. [8] on Stylommatophora; Klussmann-Kolb and Dinapoli [9] on Pteropoda). Members of the Euthyneura - the major heterobranch clade - have conquered marine, limnic and terrestrial habitats from the deep sea to the high mountains. As a result they form one of the most successful and diverse groups within Gastropoda, and even within Mollusca as regards species numbers and ecological diversity. Quite some effort has been dedicated to revealing relationships in the taxon, and to

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supporting or rejecting the respective monophyly of traditional higher groupings such as Pulmonata and Opisthobranchia. Nevertheless, the phylogeny of the Euthyneura has remained partially unresolved and heavily discussed [see e.g. [10-17]]. While morphological analyses face the problem of convergent developments that might mask the true phylogenetic signal, and depend on the coding procedure for morphological characters [18], single-marker molecular analyses are challenged in choosing a suitable marker, and multi-locus molecular studies stand and fall with the available taxon sampling.

One major problem in molecular studies is that highly aberrant or derived taxa of uncertain taxonomic relationships "jump around" in phylogenetic analyses and weaken the phylogenetic signal for higher taxa. Members of such groups are often hard to obtain (especially for molecular purposes); thus, the groups are frequently either excluded from phylogenetic analyses or only included with a low number of representatives, resulting in poor overall taxon sampling. One attempt to support future phylogenetic approaches on a higher taxonomic level (i.e. Heterobranchia or Gastropoda) is to provide data on small enigmatic groups and their phylogenetic relationships step by step.

The Acochlidia, a traditional "order" of the Opisthobranchia since their establishment by Odhner [[19]; as Acochlidiacea], form one of the unsolved mysteries within Euthyneura [18]. Being a small group with only 28 valid species worldwide, these slugs are morphologically and biologically highly aberrant and diverse, comprising a series of unusual characters (e.g. secondary gonochorism, lack of copulatory organs, asymmetric radulae) [see e.g. [20-23]]. Most acochlidians live interstitially in marine sands, while some have conquered limnic systems (uniquely within opisthobranch gastropods). Their monophyly is widely accepted [20,22,24,25] especially since a proposed sister group relationship of the acochlidian family Ganitidae with Sacoglossa (based on the dagger-shaped radula teeth, see [26]) could be rejected based on a comprehensive parsimony analysis of morphological characters [22]. During the last years a series of studies have redescribed key acochlidian taxa in great detail, including 3D reconstructions [27-32], and added considerably to the morphological and biological knowledge of this previously little understood group. A first comprehensive cladistic analysis of their phylogeny is now established [22], but the identity of their sister group remains uncertain. Most recent morphological analyses suggested a common origin with either the equally enigmatic Rhodopemorpha [10], the diaphanid cephalaspidean *Toledonia* [25], or with runcinid or philinoid cephalaspideans [22,33]. However, morphology-based analyses by Schrödl and Neusser [22], demonstrated that Acochlidia usually group with other mesopsammic taxa, if any were included (i.e. with the sacoglossan *Platyhedyle*, the rhodopemorph *Rhodope* or the cephalaspideans *Philinoglossa* or *Philine exigua*). Thus, it is likely that convergent adaptations to the interstitial habitat mask the truly phylogenetic signals. Molecular markers independent from direct ecological pressures suggested an unresolved basal opisthobranch origin for Acochlidia ([34] based on nuclear 18S rRNA and 28S rRNA). A first combined multi-gene dataset led to the surprising result of Acochlidia clustering in a pulmonate relationship, united in a clade with Pyramidelloidea, Amphiboloidea and Eupulmonata [17]. However, only three derived acochlids [see [22]] were included, with partially missing data. Therefore this unexpected result requires re-examination based on complete multi-locus data and a more focused taxon sampling, including all previously suggested potential sister groups of Acochlidia. Most recently, another curiosity with potential affinities to Acochlidia has been described: the amphibious and insectivorous sea slug *Aiteng ater* from mangrove mud in Thailand [35]. Due to its unusual combination of morphological characters (prepharyngeal nerve ring, presence of ascus, uniseriate radula) it was placed in a new family, Aitengidae, with unclear phylogenetic relationships and affinities to Sacoglossa, Acochlidia and Cephalaspidea. A similar but still undescribed species was found in Japan, which was available for the present study. Morphologically it clearly belongs to the Aitengidae, but shows differences to *A. ater* at genus or species level (own unpublished data). Its affinity to *A. ater* is confirmed by comparison of the mitochondrial 16S rRNA-sequences (K. Händeler, pers. comm.).

The present study aims to clarify the origins and phylogenetic relationships of Acochlidia and potentially related enigmatic taxa such as Aitengidae, based on a combined molecular dataset from nuclear and mitochondrial markers. For the first time, representatives of six out of seven acochlidian families [22] are analysed in the context of a broad taxon sampling that includes other meiofaunal slugs (*Philinoglossa praelongata*, *Philine exigua*, *Smeagol phillipensis*) and most euthyneuran sub-groups. Furthermore, the potentially related *Gascoignella nukuli* (as a representative of Platyhedylidae) and an undescribed species of Aitengidae are included in the present study. Since there is no fossil record of Acochlidia or any other mesopsammic Euthyneura, we apply a molecular clock approach to estimate divergence times for these groups. On the basis of our phylogenetic hypothesis we discuss evolutionary trends and potential consequences for euthyneuran classification in general.

Results

Neighbournet analysis

The neighbournet graph created by SplitsTree 4 (see Additional File 1) visualises a generally high conflict in

the data (shown by a netlike structure with edges of similar length), and high substitution rates displayed by long terminal branches in many taxa. There is no clade-supporting pattern for the monophyly of Opisthobranchia or of Pulmonata on the basis of our dataset. Of the major traditional heterobranch taxa only Acteonoidea and Nudipleura show a clear split support (visualised by long parallel edges); some split support is present for Pyramidelloidea, Cephalaspidea s.s., Anaspidea, Umbraculoidea, pteropod Gymnosomata and Thecosomata, Amphiboloidea and Siphonarioidea. No pattern supporting any of the other opisthobranch or pulmonate groups can be found, mainly due to affinities of individual species to neighbouring groups. No split pattern indicates a relationship between the different meiofaunal heterobranchs such as Acochlidia, *Smeagol phillipensis* and Philinoidea (*Philinoglossa praelongata* and *Philine exigua*) (see Additional File 1).

The monophyly of the Acochlidia receives no split support. A very weak signal supports a grouping of Acochlidia together with some pulmonate taxa, but there is no indication for affinities to other opisthobranch taxa. The acochlidian subgroups Hedylopsacea and Microhedylacea receive no split support, due to some common support for *Hedylopsis* (Hedylopsacea) and *Asperspina* (Microhedylacea). The enigmatic Aitengidae sp. receives split support grouped with acochlidian Pseudunelidae and Acochlidiidae, and shows no affinity to Sacoglossa or Cephalaspidea.

Phylogenetic analysis

Examination of differences in incongruence length between the four genetic markers - 18S rRNA, 28S rRNA, 16S rRNA and cytochrome *c* oxidase subunit I (COI) - using the ILD-test implemented in PAUP* [36] revealed that the phylogenetic signal is improved in the combined data set (p-value of 0.01). Thus a concatenated dataset was used for phylogenetic analyses. The likelihood values of the different partitions of the dataset were compared via the Akaike Information Criterion (AIC) and the separation into 5 partitions (one each for 18S, 28S and 16S; COI separated in the two partitions 1st and 2nd position and 3rd position) improved the likelihood significantly (see Additional File 2). The dataset aligned with MAFFT, masked with Gblocks and analysed in 5 partitions led to the best likelihood value, thus it is presented herein as the most probable phylogenetic hypothesis based on our data (see Figure 1). For comparison of the different analytical approaches and the resulting differences in tree topology and related support values, see Table 1.

The Euthyneura form a monophyletic group without significant bootstrap support (BS) in ML-analyses, or posterior probability (PP) in Bayesian analyses. They do not include the Acteonoidea (sister to "lower heterobranch" Rissoelloidea) in most of our analyses, but include the Pyramidelloidea and Glacidorboidea as sister group to Amphiboloidea. Within the Euthyneura the Opisthobranchia clearly result as non-monophyletic. At the basis of the Euthyneura the Nudipleura split off, with high internal support. The clade of the remaining euthyneuran taxa receives good support (85 BS/1.0 PP). First, an opisthobranch clade (no significant BS/1.0) is composed of Umbraculoidea, Runcinacea, Cephalaspidea s.s., Anaspidea and Pteropoda, with Umbraculoidea as the most basal branch. The runcinid *Runcina africana* forms the sister group to the Anaspidea and the well backed (82/1.0) Pteropoda (Gymnosomata and Thecosomata), and the above combined are sister to the remaining Cephalaspidea s.s., with high support for monophyly of Cephalaspidea s.s. (100/1.0). Internally the Cephalaspidea s.s. are poorly resolved, and their internal topology differs between the RAxML and Bayesian analyses (see Table 1). The mesopsammic *Philine exigua* and *Philinoglossa praelongata* do not form a clade: *P. praelongata* clusters with *Scaphander lignarius*, whereas no clear sister group relationship could be identified for *P. exigua*.

The Pulmonata as traditionally defined result as non-monophyletic due to the inclusion of the opisthobranch groups Sacoglossa and Acochlidia and of the "lower" heterobranch Pyramidelloidea and Glacidorboidea. The pulmonate clade is significantly supported (75/1.0), but internally characterised by an unstable topology, with no or low support concerning the sister group relationships between the major groups. Siphonarioidea and Sacoglossa form a clade (lacking significant support) sister to the remaining taxa (see Figure 1). In the analyses of the ALISCORE dataset Siphonarioidea form the most basal group, followed by a split-off of the Sacoglossa (see Table 1). The monophyletic Sacoglossa (98/1.0) combine clades with shelled and shell-less representatives, with *Gascoignella nukuli* (Platyhedylidae) as the most basal offshoot of the latter. Siphonarioidea + Sacoglossa are recovered as sister group to a clade composed of (Glacidorboidea + (Amphiboloidea + Pyramidelloidea)) + (Hygrophila + (Eupulmonata + Acochlidia)). Apart from Acochlidia, the monophyly of all higher taxa is well supported: Amphiboloidea (100/1.0), Pyramidelloidea (99/1.0), Hygrophila (86/1.0) and Eupulmonata (93/1.0). However, relations between these taxa are poorly resolved, not supported, and vary within the different analyses (see Table 1). In all our analyses Amphiboloidea cluster with Glacidorboidea and Pyramidelloidea. Thus Thalassophila (= Siphonarioidea and Amphiboloidea) and Basommatophora (= Thalassophila and Hygrophila) are left as polyphyletic. The Eupulmonata (Stylommatophora, Systellommatophora, Ellobioidea, Trimusculoidea and Otinoidea) are recovered sister to

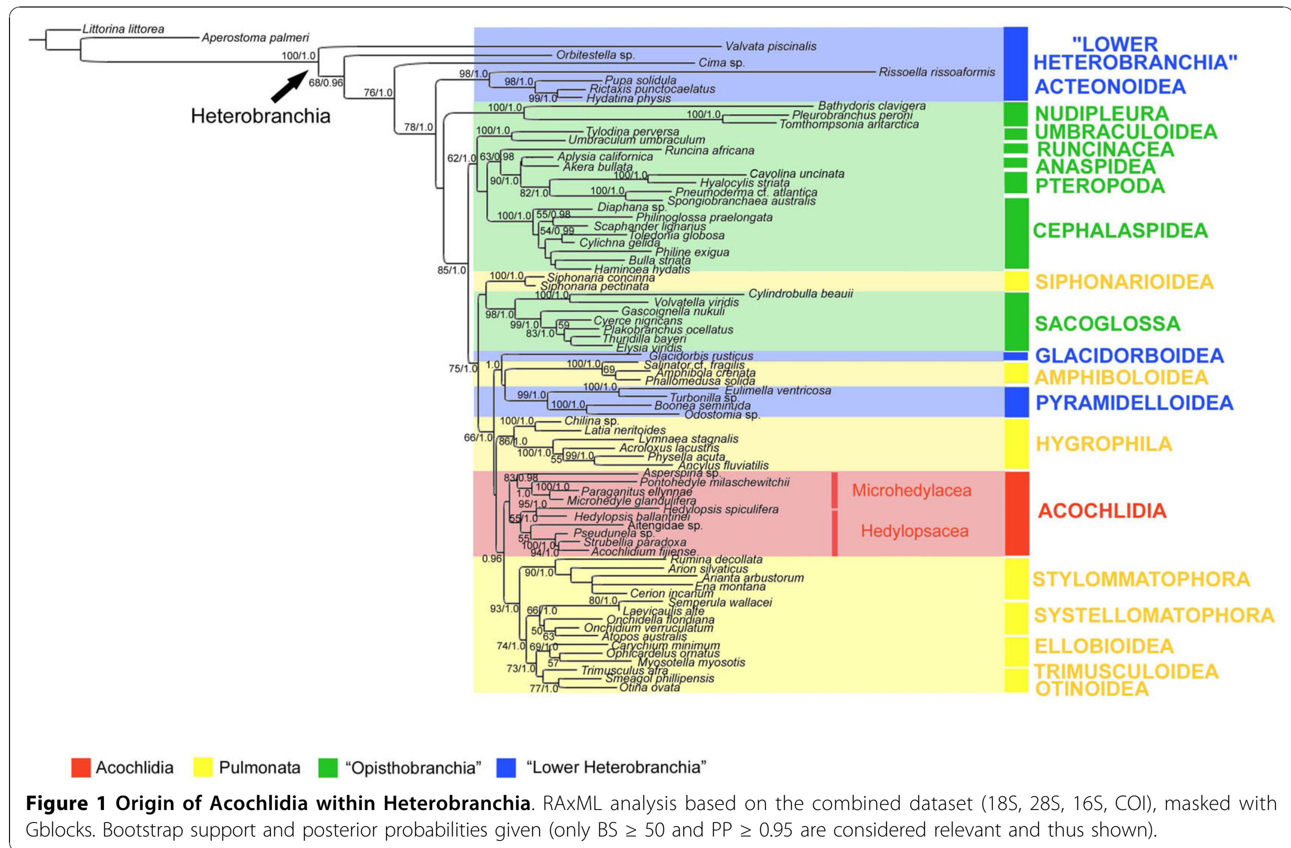

Figure 1 Origin of Acochlidia within Heterobranchia. RAxML analysis based on the combined dataset (18S, 28S, 16S, COI), masked with Gblocks. Bootstrap support and posterior probabilities given (only BS ≥ 50 and PP ≥ 0.95 are considered relevant and thus shown).

Table 1 Summary of the different analyses conducted

Sequence alignment/ masking/ phylogenetic analysis	Model of sequence evolution	Length of alignment	Monophyly of Acochlidia and sister group relationship	Changes within the tree topology compared to Figure 1
MAFFT + Gblocks + RAxML	GTRCAT GTR + GAMMA	3641 bp	see Figure 1	see Figure 1
MAFFT + ALISCORE + RAxML	GTRCAT GTR + GAMMA	3926 bp	Acochlidia monophyletic (no BS support) Aitengidae basal within Hedylopsacea; Acochlidia sister to (Hygrophila + (Glacidorboidea + (Amphiboloidea + Pyramidelloidea))) (no BS support)	Anaspidea non-monophyletic; different internal topology of Cephalaspidea s.s. (Philine exigua basal to remaining taxa); Siphonarioidea and Sacoglossa form no clade, but Siphonarioidea + (Sacoglossa + remaining pulmonate taxa)
MAFFT + Gblocks + MrBayes	GTR + G + I	3641 bp	Acochlidia monophyletic (no significant PP); sister group to Eupulmonata (0.96 PP)	basal tritomy within Euthyneura: (Acteonoidea + Rissoelloidea)/Nudipleura/remaining Euthyneura; different internal topology of Cephalaspidea s.s. (Philine exigua basal to remaining taxa), ((Glacidorboidea + Amphiboloidea) + Pyramidelloidea)
MAFFT + ALISCORE + MrBayes	GTR + G + I	3926 bp	Acochlidia monophyletic (no significant PP) Aitengidae basal within Hedylopsacea; Acochlidia sister to (Hygrophila + (Glacidorboidea + Amphiboloidea + Pyramidelloidea)) (no significant PP)	Anaspidea non-monophyletic; different internal topology of Cephalaspidea s.s. (Philine exigua basal to remaining taxa); Siphonarioidea and Sacoglossa form no clade, but Siphonarioidea + (Sacoglossa + remaining pulmonate taxa); Nudipleura form a basal clade with (Acteonoidea + Rissoelloidea)

The table lists the different methods of masking the alignment, phylogenetic approaches and models of sequence evolution used for the different analyses, as well as the resulting differences in tree topology (bootstrap support = BS; posterior probability = PP).

Acochlidia. Within Eupulmonata Stylommatophora (90/1.0) form the basal group; Systellommatophora (no significant BS/1.0) is sister to a clade Ellobioidea + (Trimusculoidea + Otinoidea), the latter comprising *Smeagol phillippensis* and *Otina ovata*.

Acochlidia are recovered as monophyletic but with no significant support. The internal phylogeny of the Acochlidia is composed of the two monophyletic traditional suborders Hedylopsacea (with Hedylopsidae, Pseudunelidae and Acochlidiidae) and Microhedylacea (with Asperspinidae and Microhedylidae including Ganitidae), and is congruent with the morphology-based phylogeny of Acochlidia proposed by Schrödl and Neusser [22]. Additionally the enigmatic Aitengidae sp. clusters within the Hedylopsacea as sister group to Pseudunelidae and Acochlidiidae (see Figure 1) or basal within Hedylopsacea.

In analyses of Gblock datasets Acochlidia are sister to Eupulmonata (see Figure 1), in ALISCORE based analyses they cluster sister to Hygrophila + (Glacidorboidea + Amphiboloidea + Pyramidelloidea) (see Table 1). To assess the level of confidence of the "best" tree (i.e. pulmonate relationship of Acochlidia), we calculated the p-values of an alternative topology (Acochlidia cluster within Opisthobranchia) in combination with the "best" tree topology. Based on the resulting p-values of the AU test the alternative hypothesis is highly significantly rejected (AU value = 0).

Molecular clock

The phylogenetic hypothesis obtained with the software BEAST (see Figure 2) based on the concatenated four-marker Gblocks dataset largely confirms the topology obtained from RAxML and MrBayes (see Figure 1). Based on the three fossil calibration points the Euthyneura originated already in the Palaeozoic, probably in the Carboniferous or Permian. The diversification of Euthyneura with the rise of many extant taxa started approximately in the late Palaeozoic (Permian) and major divergence events occurred in the Mesozoic. On the basis of our analysis the pulmonate clade (also including Sacoglossa, Acochlidia, Pyramidelloidea and Glacidorboidea) first appeared in the late Palaeozoic to early Mesozoic, approximately at the Permian/Triassic transition. The split between Eupulmonata and Acochlidia took place in the Mesozoic, between the Triassic and Jurassic periods. The diversification of Acochlidia is estimated to have happened in the Jurassic with the split between Hedylopsacea and Microhedylacea. Aitengidae split off from Pseudunelidae and Acochlidiidae in the Cretaceous. The transition to limnic habitats within Acochlidiidae appears as a comparatively recent event dating to the Palaeogene.

According to our data, major opisthobranch groups originated also in the Mesozoic (e.g. Cephalaspidea s.s. estimated to the Jurassic, Sacoglossa approximately Triassic/early Jurassic period, Pteropoda to the Cretaceous).

For comparison and to evaluate the impact of removing ambiguous parts of the alignment on molecular clock analyses, we repeated the analysis with the raw (i.e. uncut) alignment of our data (again using the concatenated four-marker dataset in five partitions). Even though the topology varied slightly from the one in the previous analysis, the estimated divergence times stayed surprisingly constant, supporting the rough estimate given above.

Discussion

Implications for the phylogeny of Heterobranchia

Our results on the origin of Acochlidia - in congruence with previous molecular studies on Euthyneura based on the same molecular markers [14,17] - necessitate the reconsideration of current classification concepts. Redefinitions below aim to observe continuity in traditional nomenclature and cause the unavoidable minimum of changes in terminology.

Euthyneura

The monophyly of Euthyneura (traditionally uniting Opisthobranchia and Pulmonata) has been widely accepted and well supported [13,18,37], even though their eponymous apomorphy - the euthyneury - has been revealed as convergent development [1,2]. Euthyneuran monophyly was recently questioned due to inclusion of "lower Heterobranchia" Pyramidelloidea unresolved within Pulmonata [13,15,16] or sister to Amphiboloidea [14,17]. Some other morphological studies place Pyramidelloidea as sister to Euthyneura [10,33]. Dinapoli and Klussmann-Kolb [14] argued to include them within Euthyneura, which has also been supported by morphological analysis [13]. Latest molecular data on Pyramidelloidea support an euthyneuran origin and indicate a relationship with Glacidorboidea and Amphiboloidea [38]. Our data again recovers Pyramidelloidea as sister to Amphiboloidea within pulmonates (see Figure 1), but with no significant support. In addition to nucleotide sequences [[14,15,17], present study], data from mitochondrial gene arrangements [16], a "morpho-molecular" synapomorphy (20 bp deletion in 16S rRNA helix of Pyramidelloidea and Euthyneura, see [11]) as well as morphology (presence of a euthyneurous nervous system with giant nerve cells) all support the inclusion of Pyramidelloidea within Euthyneura. When first describing Glacidorboidea, Ponder [39] placed them within Pulmonata and discussed a relationship to Amphiboloidea. However, Haszprunar [2] moved them to "lower Heterobranchia". The first molecular data on Glacidorboidea confirmed a pulmonate relationship [14]. This is again supported by our data.

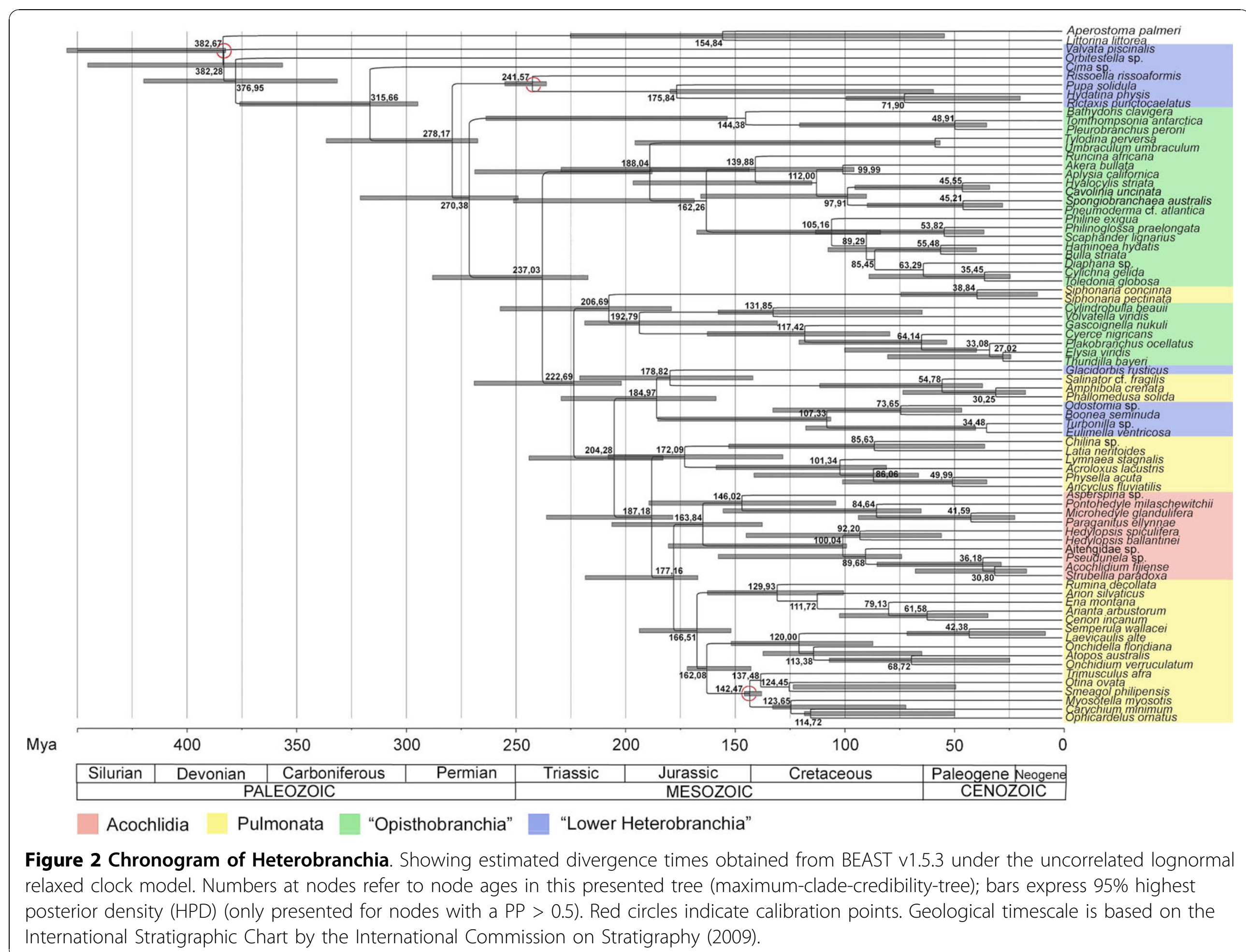

Figure 2 Chronogram of Heterobranchia. Showing estimated divergence times obtained from BEAST v1.5.3 under the uncorrelated lognormal relaxed clock model. Numbers at nodes refer to node ages in this presented tree (maximum-clade-credibility-tree); bars express 95% highest posterior density (HPD) (only presented for nodes with a PP > 0.5). Red circles indicate calibration points. Geological timescale is based on the International Stratigraphic Chart by the International Commission on Stratigraphy (2009).

"Opisthobranchia"

While the monophyly of several opisthobranch subgroups (e.g. Pteropoda, Cephalaspidea s.s., Nudipleura) receives good support, the monophyly of the Opisthobranchia in a traditional sense is rejected in all recent studies, regardless of whether the latter are molecular or morphological [e.g. [14,17,40]]. This is confirmed by our multi-locus molecular approach (see Figure 1) and supported by the results of the AU test. Thus, "Opisthobranchia" as traditionally defined should be considered as non-monophyletic.

As in previous studies we can clearly distinguish at least two clades (i.e. basal Nudipleura and Umbraculoidea + Runcinacea + Anaspidea + Pteropoda + Cephalaspidea s.s.) within "Opisthobranchia" that lead towards the pulmonate level of organisation.

Only one of our analyses indicates the Acteonoidea sister to Nudipleura (see Table 1). This clade that had resulted repeatedly in molecular studies with still limited "lower heterobranch" taxon sampling, either in a derived position [34,41] or as a basal offshoot within Euthyneura [15,17]. A recent molecular phylogeny on Acteonoidea suggest a common origin with lower heterobranch Rissoelloidea and a sister group relationship to Nudipleura [42]. While the basal position of Acteonoidea was commonly accepted [33,40], some authors doubted the basal position of Nudipleura, which was originally considered as a highly derived taxon, and suspect rate heterogeneity and deviant base composition as causing this unnatural grouping [17,34]. Based on potential synapomorphies in the reproductive system (presence of a ciliary stripe within the ampulla, androdiaulic or triaulic pallial gonoduct), Ghiselin [43] already suggested a relationship between Acteonoidea and Nudipleura. However, Acteonoidea form a well-supported "lower heterobranch" clade with Rissoelloidea, (see Figure 1; Table 1), confirming results by Aktipis et al. [44] and Dinapoli and Klussmann-Kolb [14]. The latter authors also recovered Nudipleura as the first offshoot of Euthyneura, which is confirmed by our study. Salvini-Plawen and Steiner [10] grouped Umbraculoidea with Nudipleura, but none of the recent molecular or morphological studies support such a relationship [17,33,34].

A common clade including Umbraculoidea, Anaspidea, Cephalaspidea s.s. and Pteropoda was already well supported in previous molecular analyses [9,14,17], and monophyly of a clade Anaspidea + Pteropoda received strong support in one previous study [12]. The present results confirm Cephalaspidea s.s., including Diaphanidae, but excluding Runcinidae as suggested in a previous analysis [45]. In our study *Runcina africana* groups with Anaspidea and Pteropoda, as in the Bayesian analysis of the concatenated 18S rRNA, 28S rRNA and COI dataset of the more comprehensive cephalaspidean phylogeny by Malaquias et al. [45]. The latter authors thus proposed to reinstate Runcinacea as a taxonomic category equivalent to Cephalaspidea s.s.. However, different analyses of the same authors led to different placements of Runcinacea, e.g. as sister to the remaining Cephalaspidea s.s.; hence the group's origin was left unresolved. Surprisingly our study indicates independent origins for the mesopsammic *Philine exigua* (Philinidae) and *Philinoglossa praelongata*(Philinoglossidae). The internal topology of Cephalaspidea s.s. is weakly supported in our study, but a more complete cephalaspidean sampling also rendered Philinoidea paraphyletic (based on 18S and 28S) [45].

Based on our results and in congruence with the topology in previous studies [14,17], we suggest to unite Umbraculoidea, Anaspidea, Runcinacea, Pteropoda and Cephalaspidea s.s. in the new clade Euopisthobranchia (see Figure 3), presenting a monophyletic remainder of the "Opisthobranchia" as traditionally defined. Previous studies [9,18] discussed the gizzard (i.e. a muscular oesophageal crop lined with cuticula) with gizzard plates as homologous apomorphic structures supporting a clade composed of Cephalaspidea s.s., Pteropoda and Anaspidea. A gizzard with gizzard plates probably originated in herbivorous taxa in which it worked like a grinding mill, thus might be secondarily reduced in carnivorous groups within Cephalaspidea s.s. and Gymnosomata [9]. Klussmann-Kolb and Dinapoli [9] considered the gizzard in Umbraculoidea as non-homologous with the one in the previous groups, on account of the absence of gizzard plates or spines. This contradicted Salvini-Plawen and Steiner [10], who had proposed the gizzard to be a synapomorphy of the larger clade of Paratectibranchia (Pteropoda, Cephalaspidea and Anaspidea) and Eleutherobranchia, secondarily lost in Nudipleura but still present in Umbraculoidea. As coded in Wägele and Klussmann-Kolb [33], our phylogenetic hypothesis supports homology of the gizzard in Umbraculoidea with the gizzard with gizzard plates and spines in the other euopisthobranchian taxa. Thus, the structure is proposed as a synapomorphy of Euopisthobranchia.

"Pulmonata"

The monophyly of Pulmonata as traditionally defined has been well supported in morphological analyses (see e.g. [10,13]) and molecular studies [8,46]. However, doubts have arisen recently due to molecular studies which recovered additional taxa (e.g. Pyramidelloidea, Sacoglossa or Acochlidia) within "Pulmonata" [14,17], or to novel studies based on mitochondrial gene arrangements [16] which rendered "Pulmonata" polyphyletic. Based on our phylogenetic hypothesis (Figure 1) "Pulmonata" as traditionally defined is non-monophyletic due to the inclusion of Pyramidelloidea, Glacidorboidea, Sacoglossa and Acochlidia. On the premise of monophyletic Euthyneura, with basal Nudipleura and monophyletic Euopisthobranchia (see discussion above), the remaining euthyneuran taxa necessarily form a clade, in our study supported with maximum posterior probability (1.0) and significant bootstrap support (75%) (see Figure 1). Even though the topology within this pulmonate clade is unstable and not well resolved (see Table1), for practical reasons and due to the assumptions of monophyletic Euthyneura and Euopisthobranchia we suggest the new taxon Panpulmonata to unite Siphonarioidea, Sacoglossa, Glacidorboidea, Pyramidelloidea, Amphiboloidea, Hygrophila, Acochlidia and Eupulmonata (see Figure 3). The scientific meaning of the name "Pulmonata" and the corresponding major feature of those animals being "air-breathers" surely are not applicable to the novel panpulmonate groups Acochlidia, Sacoglossa and Pyramidelloidea, but also not for traditional pulmonate taxa such as Siphonarioidea or Hygrophila, most members of which lack permanently air-filled lungs. The term Panpulmonata is chosen for continuity in terminology. While certain pulmonate groups are well supported morphologically and molecularly (i.e. Eupulmonata and Hygrophila), unambiguous synapomorphies for Panpulmonata are hard to find (see discussion below).

Siphonarioidea and Sacoglossa form a clade sister to the remaining Panpulmonata (see Figure 3). While Haller [47] classified Siphonarioidea as opisthobranchs (e.g. on account of the presence of a gill), nowadays they are usually considered as "primitive" pulmonates, either grouped at the basis of the remaining Pulmonata [37,46] or united with Amphiboloidea as basommatophoran Thalassophila [48]. Molecular studies rendered Basommatophora and Thalassophila paraphyletic and indicated a close relationship of Siphonarioidea to Sacoglossa, either both within Opisthobranchia [16], at their basis [15], or basal to the remaining Pulmonata [[14,17], present study] as sister groups or separate clades. However, all studies show weak support at these nodes, and the positions of siphonariids and sacoglossans as well as

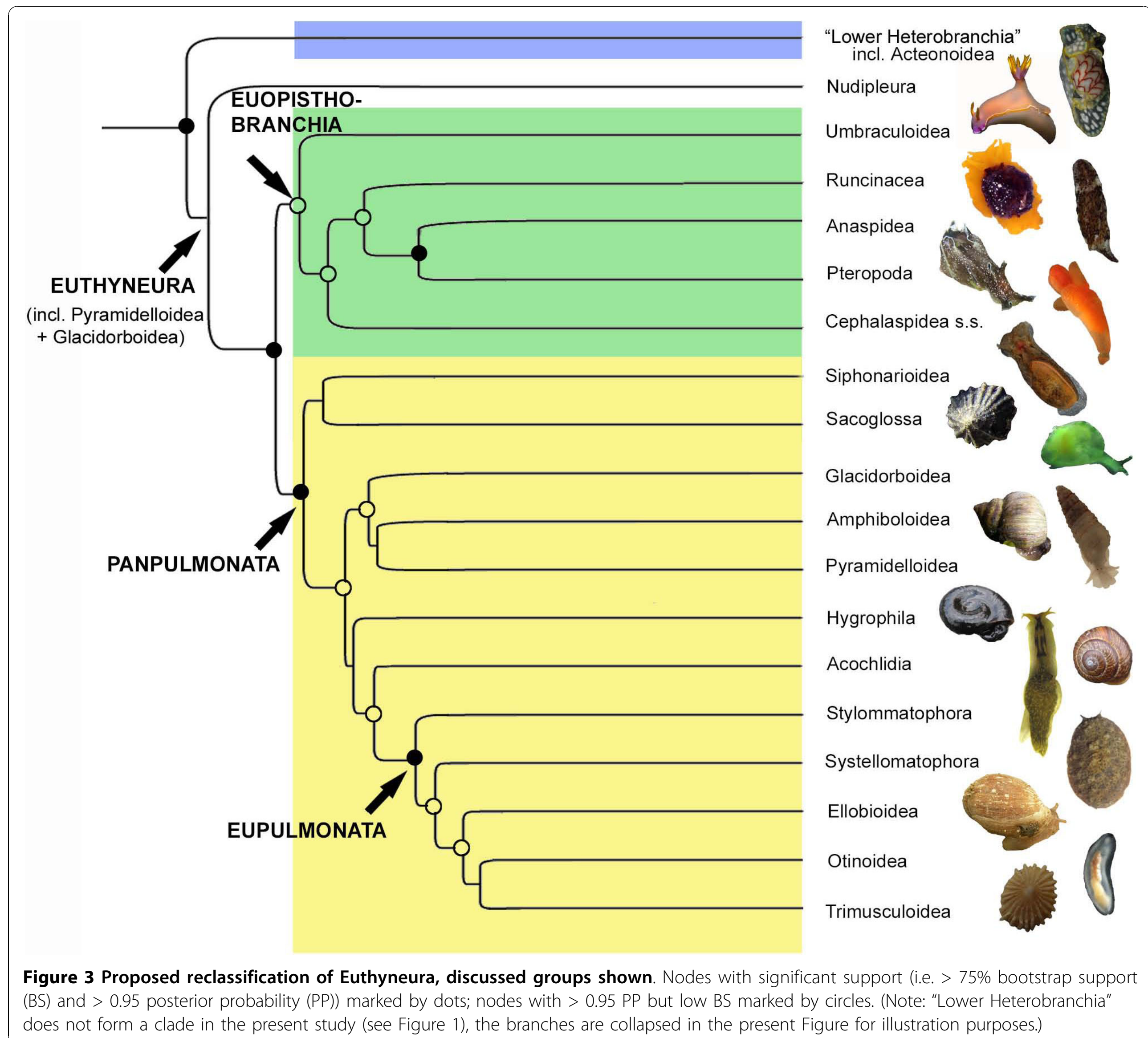

Figure 3 Proposed reclassification of Euthyneura, discussed groups shown. Nodes with significant support (i.e. > 75% bootstrap support (BS) and > 0.95 posterior probability (PP)) marked by dots; nodes with > 0.95 PP but low BS marked by circles. (Note: "Lower Heterobranchia" does not form a clade in the present study (see Figure 1), the branches are collapsed in the present Figure for illustration purposes.)

their relationship still need confirmation by other character sets and improved taxon sampling.

In the present study the monophyly of Sacoglossa is well supported and also the split into shelled Oxynoacea and Plakobranchacea is well backed (see Figure 1). Both suborders are also well supported morphologically [4]. Platyhedylidae stand basally within the latter, as sister to Limapontioidea plus the remaining Plakobranchoidea. Jensen [4] placed Platyhedylidae at the basis of Plakobranchoidea but already pointed out their unclear relationships.

Hygrophila, Amphiboloidea and Eupulmonata are all well supported monophyletic groups in the present study, but their sister group relationships are not well resolved and receive little to no support.

Origin of Acochlidia

All groups previously discussed as having an affinity or closer relationship to Acochlidia were included in the present study to reveal their phylogenetic relationships. Only the enigmatic Rhodopemorpha are lacking, but a recent molecular phylogeny based on nuclear and mitochondrial markers shows no affinities between Acochlidia and Rhodopemorpha [49], and the morphological characters common to both groups can be explained as convergent developments (see discussion below and [22]). A phylogenetic relationship of Acochlidia with the diaphanid *Toledonia*, which was suggested based on similar radula characteristics [25], is rejected by the present molecular data and also resulting from morphological analyses [22]. Morphological studies indicated a

common origin for small Runcinacea and Cephalaspidea (i.e. mesopsammic *Philinoglossa* and *Philine exigua*) with Acochlidia [22,33]. However, Schrödl and Neusser [22] showed the liability of the topology to inclusion of other interstitial taxa such as *Rhodope* and *Platyhedyle*, which always resulted as direct sister groups to Acochlidia in various analyses. The authors thus concluded that the convergent adaptations to the interstitial habitat (e.g. worm-shaped body, development of spicules, loss of pigmentation) mask the true phylogenetic signal. This interpretation is supported by our SplitsTree analysis (see Additional File 1) and the present molecular results (see Figure 1), which clearly signal independent evolutionary origins for all the different mesopsammic Heterobranchia included here.

Previous molecular analyses placed the Acochlidia basally in an unresolved opisthobranch level [34] or surprisingly clustered them in an unresolved pulmonate relationship [17]. While any opisthobranch affinities are rejected based on split support (see Additional File 1), based on the AU test and based on phylogenetic analysis, the pulmonate relationship of Acochlidia is confirmed in this study (see Figure 1), which presents a much better acochlidian taxon sampling and highly likely topology within Acochlidia (see discussion below). Even though support for their direct sister group relationships are low and the topology varies between the different analyses, all analyses performed in the present study placed Acochlidia within pulmonates (see Table 1). This grouping based on molecular markers requires a re-evaluation of morphological characters and earlier, potentially biased homology assumptions, and a search for potential synapomorphies uniting Acochlidia with pulmonates. Three anatomical characters are generally accepted as true synapomorphies of the "Pulmonata" as traditionally defined: the pallial cavity opening by means of a pneumostome, presence of a procerebrum (with cerebral gland and double cerebro-connectives) and the existence of medio-dorsal (cerebral) bodies [13,50].

1) Pallial cavity opening by means of a pneumostome

Although denied by some earlier authors, the pulmonary cavity of "Pulmonata" is today generally considered as homologous to the pallial cavity of non-pulmonate gastropods [51]. Whereas the loss of a gill and the presence of a "lung" certainly is a matter of multiple convergence paralleled in several prosobranch clades, the acquisition of a pneumostome (i.e. a small respiratory opening) is considered as synapomorphic for "Pulmonata" [13,18,48]. Dayrat and Tillier [[13], see also references therein] pointed out that the pneumostome of Siphonarioidea is not contractile, and their phylogenetic hypothesis [13] favoured homology with the pneumostome of the remaining Pulmonata. On the other hand, at least some siphonariids are reported to open and close their pneumostome [e.g. [52]]. A morphocline from a wide open pallial cavity to a narrow, nearly closed one (i.e. presence of pneumostome) is present in both "Opisthobranchia" and "Pulmonata"; thus the presence of a pneumostome in general cannot be considered as a pulmonate synapomorphy [53]. Barker [53] also questioned the synapomorphic contractile pneumostome, which might have evolved independently in different pulmonate taxa, e.g. in Eupulmonata and some Siphonarioidea. The presence of a small opening seems to be variable, indeed, and might depend on the habitat. For example, the truly subtidal marine *Williamia* (Siphonarioidea) have a wide opening [54], while intertial *Siphonaria* have a small one (i.e. a contractile or non-contractile pneumostome). The opening is wide also in subtidal shell-bearing Sacoglossa [3], whereas the pallial cavity is usually reduced in shell-less Sacoglossa. Pyramidelloidea also have a wide opening. In general within "Pulmonata" the "lung" undergoes a series of reductions; e.g., the tiny *Smeagol climoi* only has a small pallial cavity without respiratory function [51], as do larger Onchidiidae. A small, reduced pallial cavity can still be found in the quite basal acochlidian *Hedylopsis ballantinei* [55] (as *Hedylopsis* sp.), while all remaining Acochlidia studied so far entirely lack such a cavity [22,30]. All hedylopsacean nervous systems described in detail contain an osphradial ganglion [25,29,31,32], which can be interpreted as a remainder of an osphradium that was reduced in the course of the reduction of the pallial cavity. A group of derived, benthic and limnic acochlidians have developed a sensory, osphradium-like organ [56] like the one reported for the basal ellobiid *Ovatella* [57].

2) Presence of a procerebrum

The procerebrum of "Pulmonata" is defined as an accessory lobe linked to the cerebral ganglion via two connectives, associated to the optic, tentacular and peritentacular nerves [58]. Its homology with the opisthobranch rhinophoral ganglion has long been discussed [2,47,59]. The configuration of the cerebral nerves and associated ganglia is complex in Acochlidia. The labiotentacular nerve arises ventrally from the cerebral ganglion; the rhinophoral ganglion usually gives rise to the rhinophoral nerve (with Hancock's nerve branching off), and the optic ganglion to the optic nerve ([31,32,56] and own unpublished data). However, in *Pseudunela cornuta* the optic nerve splits off from the rhinophoral nerve, and no nerves arise from the optic ganglion [29]. A similar arrangement occurs in *Hedylopsis spiculifera* and *H. ballantinei*, except that the optic ganglion is lacking [25,60]. In the microhedylaceans *Pontohedyle* and *Microhedyle* the rhinophoral nerve emerges directly from the cerebral ganglion, and eyes nestle directly on it ([27], own unpublished data); thus the additional ganglion might refer to either the

rhinophoral or the optic ganglion. Tillier et al. [46] discussed a potential homology between the optic ganglion in "Opisthobranchia" and the pulmonate procerebrum. In Acochlidia double cerebral connectives could be identified for the rhinophoral ganglion in *Tantulum elegans* [60], the optic (but not the rhinophoral) in *Strubellia paradoxa* [56], and for the unclear optic/rhinophoral ganglion in *Pontohedyle milaschewitchii* and *Microhedyle glandulifera* ([27] as rhinophoral ganglion, own unpublished data). The variable development of cerebral features in Acochlidia makes homologisation difficult at this time. Rhinophoral and optic ganglia are closely related to and might develop from the cerebral ganglion, and they share common features with the pulmonate procerebrum. Based on our phylogenetic hypothesis, the plesiomorphic state for Panpulmonata might be separate rhinophoral and optic ganglia that have been fused various times independently. However, the presence of so-called "globineurons" - neurons with densely packed, small, round nuclei - in Eupulmonata [58,61] appears to be a synapomorphy for this clade.

Additionally, the presence of a cerebral gland - a small, tube-like structure involved in the formation of the procerebrum - is considered as characteristic for the pulmonate nervous system [58,61]. This ectodermal structure may form a tube-like process from the procerebrum towards the lateral head region, or it may be reduced to a small epithelial cavity attached or enclosed within the procerebrum [58,61]. No structure similar to the cerebral gland has been described for Acochlidia, but due to the small size of the cerebral gland and the previously unknown pulmonate affinities of Acochlidia it might have been overlooked in morphological studies; hence, ultrastructural reinvestigations of acochlidian nervous systems are needed in the future. The cerebral gland is lacking also in other pulmonate taxa, e.g. Amphiboloidea [58], which either raises doubts about their pulmonate affinities [46] or suggests that the structure might have been lost secondarily. Moreover, Tardy [62,63] described a similar invagination involved in the formation of the rhinophoral ganglion in different nudibranchs. In light of the present phylogenetic hypothesis, with Nudipleura as the most basal euthyneuran offshoot, this might indicate that the formation of the rhinophoral ganglion (and the homologous procerebrum) involving an ectodermal invagination is plesiomorphic within Euthyneura, and that there are remnants (or paedomorphotic reinstatements) of this structure in adults of (some) pulmonate taxa.

3) Presence of medio-dorsal (= cerebral) bodies

(Medio-)dorsal bodies (also termed cerebral bodies) are endocrine organs situated dorsally of the cerebral ganglia in "Pulmonata" [13], but considerable variation exists within the main pulmonate groups as regards the structure and innervation of the dorsal bodies [58,61,64]. Similar structures closely attached to the cerebral ganglia have been found in several Acochlidia: First described as "dorsal bodies" [25], they were later renamed "lateral bodies" by Neusser et al. [60], due to their more lateral position to the central nervous system and the unclear homology to pulmonate dorsal bodies. Since dorsal bodies in Pulmonata play a role in female reproduction [64], they might be fully developed in female adults only, thus might have been overlooked in some studies of gonochoristic acochlidian species or of hermaphrodites with "sex change". Further ultrastructural data on acochlidian "lateral bodies" and their potentially neurosecretory function are needed to evaluate homology with pulmonate structures. Moreover, pulmonate dorsal bodies might be homologous to the juxtaganglionar organs of some opisthobranchs [60], and thus might represent a plesiomorphic character of Panpulmonata and a potential synapomorphy of Euthyneura.

In addition, the presence of an unpaired dorsal jaw, which probably originated through the fusion of the paired lateral jaws [65], has been discussed as a potential synapomorphy of "Pulmonata" [18,48]. The presence of a pair of dorso-lateral jaws is a plesiomorphic character state for Euthyneura [13,65], but that condition has been reduced various times independently in "Opisthobranchia" and "Pulmonata" [18]. A dorsal, unpaired jaw might have evolved at the basis of Panpulmonata, and then have been secondarily reduced various times independently (e.g. in Onchidiidae, *Amphibola*) [18]. In Acochlidia, jaw-like structures are reported only for the derived microhedylacean family Ganitidae (as paired jaws), and as unclear "cuticular elements" for *Microhedyle glandulifera* (see [22] for citations). According to the derived position of Ganitidae in morphological [22] and molecular analyses (present study), these structures may represent either secondary developments (potentially related to the specialised dagger-shaped radula) or paedomorphic structures; however, studies of Acochlidia larvae are still overdue.

The only potential synapomorphy of "Opisthobranchia" is the presence of a rhinophoral nerve with a thickened basis (i.e. rhinophoral ganglion) and of associated sensory structures such as Hancock's organ [66]. Based on our phylogenetic hypothesis the presence of a rhinophoral nerve has to be considered as a plesiomorphic character within Euthyneura, and thus for Panpulmonata. The rhinophoral ganglion, and potentially the optic ganglion, is considered as homologous with the pulmonate procerebrum. Rhinophoral nerve and Hancock's organ have been reduced various times independently, probably correlated with the reduction of the rhinophores and/or habitat changes.

In summary, we are currently unable to find clear morphological synapomorphies which support a placement of Acochlidia within pulmonate taxa, as sister to Eupulmonata. In the light of our phylogenetic hypothesis, conventional pulmonate synapomorphies appear to be plesiomorphies or convergences within pulmonate taxa. On the other hand, no morphological characters currently contradict that molecular phylogenetic hypothesis, nor do they favour any alternative relationships, since morphological characters common to the mesopsammic heterobranchs are shown to be convergent developments, and the potential synapomorphy of Acochlidia with "Opisthobranchia" has to be considered as plesiomorphic.

The aberrant morphology of Acochlidia in relation to its proposed sister groups remains problematic. In his ontological studies on the nudibranch *Aeolidiella alderi*, Tardy [62] reported an abnormal development in some larvae that leads to a visceral hump separated from the head-foot complex in juvenile stages, thereby closely resembling external morphology in Acochlidia (see fig. 20 in [62]). According to Tardy [62] these abnormal developmental forms are also known from pulmonate Stylommatophora. Progenesis is discussed as a principle in the evolution of meiofaunal taxa [67], and acochlidian morphology might have evolved by retention of the juvenile characters of an aberrant developmental form of an early pulmonate.

Monophyly and phylogeny of Acochlidia

The monophyly of Acochlidia is well supported morphologically [20,22,24] and also backed by previous molecular studies [17,34]. Our study, which includes all valid acochlidian families except for the monotypic Tantulidae, also recovers Acochlidia as monophyletic but with low posterior probability and bootstrap support. The low bootstrap values for Acochlidia and some internal acochlidian taxa (e.g. Hedylopsacea) might be caused by their relatively early (Mesozoic) divergence times (see Figure 2): recent acochlidian taxa probably constitute but a remnant of much larger diversity in evolutionary history.

The acochlidian internal topology confirms the morphological analysis of Schrödl and Neusser [22], showing the same family relationships, but with better resolution within Microhedylacea: the genus *Pontohedyle* splits off at the basis of the Microhedylidae s.l. (including Ganitidae) with the closely related genera *Microhedyle* and *Paraganitus*. The hedylopsacean family Acochlidiidae includes the genera *Strubellia* and *Acochlidium* as proposed by Arnaud et al. [68] and Schrödl and Neusser [22]. Puzzling is the position of the enigmatic Aitengidae within Acochlidia, either as sister to Pseudunelidae and limnic Acochlidiidae (see Figure 1) or basal within Hedylopsacea (see Table 1). Aitengidae shows some of the general, but not unique, features of Acochlidia, such as the lack of a shell, reduction of mantle cavity, the praepharyngeal (circumpharyngeal) nerve ring, and the radula with a descending and ascending limb. This taxon also shares some features with limnic Acochlidiidae: the radula with a strong rhachidian tooth specialised in egg feeding, as also reported for *Strubellia* sp. [56]; the large, internal lateral eyes closely associated with the cerebral ganglia; and the presence of a foot groove and a branched digestive gland like reported for the genera *Acochlidium* and *Palliohedyle* [69,70]. On the other hand, Aitengidae lacks several acochlidian characteristics: the division of the body into head-foot complex and visceral hump; presence of 1-2 head appendages (with characteristic innervation of the rhinophores); and the ability to retract the head-foot complex into the visceral hump. However, in the absence of a separated visceral hump *A. ater* is able to retract its head under the notum. The presence of spicules is confirmed for Aitengidae sp., and the "parasites" described for *A. ater* might represent spicules instead (T. Neusser, pers. comm.). Re-examination of the doubtful "ascus" in *A. ater* is necessary; examination of Aitengidae sp. showed no true (i.e. sacoglossan-like) ascus containing old teeth, just a radula slightly bent at the end (own unpublished data). The presence of an ascus is currently accepted as a unique synapomorphy of Sacoglossa [4], and any sacoglossan relationship is clearly rejected by SplitsTree analysis (see Additional file 1) and phylogenetic analyses in the present study.

At the present stage of knowledge, molecular data suggests an inclusion of Aitengidae within Acochlidia, as sister to Pseudunelidae and Acochlidiidae. Detailed description by semithin serial sectioning and 3D reconstruction of the Aitengidae sp. used in the present study, together with focused redescription of *A. ater*, are needed as a basis to evaluating phylogenetic relationships of Acochlidia and Aitengidae in the future. This should be supported by a comprehensive molecular phylogeny of Acochlidia, including the two known species of Aitengidae.

Evolutionary traits in Euthyneura

Invasion of the interstitial habitat

Our study supports earlier assumptions that invasion of the interstitial habitat has occurred various times independently within the Euthyneura [22,68,71], probably by benthic, sand-dwelling or temporarily (i.e. juvenile) mesopsammic ancestors of the nudibranch genera *Embletonia* and *Pseudovermis*, the cephalaspidean *Philinoglossa* and *Philine exigua*, the sacoglossan *Platyhedyle*, some members of the Rhodopemorpha *incertae sedis* (*Helminthope* and some *Rhodope*), and the Acochlidia

[22,68]. The pulmonate genus *Smeagol* is found in gravel or pebble beaches on the undersides of stones; due to the relatively large body size in some species (e.g. up to 14 mm in *S. manneringi*[72]), it cannot be generally assigned to the meiofauna.

Major convergent adaptations to this spatially limited and unstable habitat are the worm-shaped body, loss of shell, and reduction of head appendages and pigmentation [21]. The development of subepidermal, calcareous spicules in Acochlidia, Rhodopemorpha and potentially *Platyhedyle* can also be considered as an adaptation to the interstitial habitat, probably serving to stabilise certain body parts during movements through the interstices [27], even though the occurrence of spicules is not limited to the mesopsammon. As far as is known, Acochlidia represent the most successful group of Heterobranchia in the mesopsammon concerning species diversity and abundance [27]. Key features for their success probably are an initial heterochronic miniaturisation and two different evolutionary trends towards a rapid, imprecise sperm transfer [23]. Additionally, adaptation to (temporarily) brackish waters with the development of a complex excretory system in Hedylopsacea [22,29] allows colonisation of shallow sands with freshwater impact (by groundwater or rain), overcoming limitations to deeper, truly marine sands.

Colonisation of freshwater and terrestrial habitats

It is undisputed and again confirmed by the present study that the "Pulmonata" have a marine origin [see e.g. [17,18]]. The hygrophilian radiation in the freshwater system is the most successful within "Pulmonata" [17], in terms of diversity and abundance, but not a unique event in pulmonate evolutionary history. Dinapoli and Klussmann-Kolb [14] already showed that the invasion of freshwater within pulmonate taxa took place at least twice, in Hygrophila and in the enigmatic *Glacidorbis*. According to our study, the colonisation of freshwater in Panpulmonata has occurred at least one more time in Acochlidia. Schrödl and Neusser [22] showed that within Acochlidia the freshwater colonisation already occurred twice independently, with a radiation of the Indo-Pacific Acochlidiidae and the single Caribbean *Tantulum elegans* (Tantulidae, not included in the present study). Thus, the development of a complex kidney within Hedylopsacea [29] as an adaptation to (temporarily) brackish water can be considered as a precursor to the invasion of limnic systems in Acochlidia. Acochlidian invasion of freshwater originated probably from a mesopsammic ancestor with temporary freshwater tolerance [32], or via a semi-terrestrial habitat as reported for Aitengidae [35]. Our study thus highlights the high diversity and flexibility of pulmonate habitats ranging from marine to temporarily brackish, permanently brackish, limnic and terrestrial environments. The still enigmatic *Aiteng ater* (Aitengidae) lives "amphibiously" and tolerates marine to brackish waters, but there are no observations of these animals truly leaving the water [35]. The species' mangrove habitat is comparable to that of representatives of, e.g., the pulmonate Onchidiidae, and is classified as marginal zones from which the transition to terrestrial habitat probably originated [17]. Similar to the limnic habitat, terrestrial environments have been colonised various times independently [53]. The present study indicates a least four independent pathways to the terrestrial habitat: in Amphiboloidea, Stylommatophora, Systellommatophora and Ellobioidea.

Molecular clock and estimation of divergence times in Acochlidia

The use of molecular clocks to estimate divergence times is controversially debated, due to conflicting results from different studies and disparities with paleontological or archaeological data [73-76]. Criticism focuses on the major problems such as faulty calibration, impact of rate heterogeneity among lineages, and "time dependency of molecular rates" [73,75-77]. Some of the problems could be solved by the relaxed clock approach [78], and despite all pitfalls and criticism, molecular clock approaches have helped considerably to reveal the evolutionary history of life, especially when it comes to divergence times of groups with poor or no fossil record [75,76,79]. Thus, we consider it a valuable methodology to roughly estimate divergence times for tiny, sluggish gastropods for which there is no fossil record. Molecular clock dating stands and falls with the accuracy with which genetic distances can be estimated [80]; thus we consider the removal of ambiguous (i.e. potentially non-homologous) sites from the alignment as problematic. It seems common use to run the molecular clock analyses with reduced datasets (e.g. [14,81-83]), but the crucial question, how this will affect the molecular dating, has remained unaddressed. The exclusion of highly saturated positions - e.g., in some cases the 3rd codon position of the COI sequence (see e.g. [84]) - can be justified by the biasing effect of saturation on the molecular clock. It can be argued that ambiguous parts of the alignment are often highly variable and might suffer from saturation, but on the other hand the exclusion of a series of non-saturated sites might result in underestimated divergence times. However, our Beast analysis of the raw, uncut dataset provided estimations of divergence times very similar to those from the Gblocks dataset (not shown). Nevertheless, we recommend to critically compare data from masked and raw alignments for molecular clock analyses, and to stay mindful of the potentially underestimating effect on divergence times.

The only molecular clock data on Heterobranchia [14] available prior to the present study suffers from

unreliable calibration, which is considered as the most sensible and critical part of divergence time estimations [76]. There is no objective way to assign fossils to a certain point of a stem line in a recent phylogeny, thus the age of the fossil has to be taken as the minimum age of the split between the extant taxon it is assigned to and its sister group [80]. In [14] the fossil ages were assigned to the diversification of Heterobranchia, Acteonoidea and Omalogyridae, respectively, rather than to the splits from the corresponding sister groups, which led, e.g., to the surprising Pre- to early Cambrian split between Vetigastropoda and Apogastropoda. Our molecular clock was calibrated to the split between Caenogastropoda and Heterobranchia; thus molecular dating of this node is biased (i.e. depends directly on calibration features). However, fossil data shows two clearly different lineages by the mid-Devonian, thus indicating a pre- or early Devonian split of Apogastropoda [85,86]. According to our study euthyneuran gastropods already emerged in the Palaeozoic Permian, diverting from the "Lower Heterobranchia", but all major radiations of Euthyneura occurred in the early Mesozoic. According to paleontological data the oldest opisthobranchs appeared in the Triassic (about 220 Mya), the earliest pulmonates in the Jurassic (about 190 Mya) [85,86].

Based on their phylogenetic hypothesis from morphological data and the fossil record of cephalaspidean outgroups, Schrödl and Neusser [22] suspected a Jurassic time frame for the origin of Acochlidia. Their inferred sister group relationships are different from the present study, but the early divergence time is supported by our molecular clock approach, which places the origin of Acochlidia in the late Triassic to early Jurassic and their major diversification in the Jurassic. In the present study the Eupulmonata as sister group to Acochlidia show similar origin and diversification times, and so do the Hygrophila. Tillier et al. [46] inferred divergence times from branch lengths in a molecular distance tree (based on partial 28S sequences), indicating a similar Jurassic time frame for Eupulmonata and slightly younger for Hygrophila. This corresponds with fossil data, which reports a first occurrence in the late Jurassic (approx. 150 Mya) [46]. Based on fossils, diversification times of eupulmonate groups such as Stylommatophora can be dated to the late Cretaceous, when most extant families appear [87].

According to our data most acochlidian families appeared in the Jurassic or Cretaceous, only Ganitidae, Pseudunelidae and Acochlidiidae have a Palaeogene origin. These old splits on the family and even genus levels (see *Hedylopsis*, Figure 2, diverging in the Cretaceous) might indicate either that the extant diversity of Acochlidia is only a small remnant of high diversity in former times, or that known acochlidian diversity is just the tip of the iceberg still waiting to be discovered.

Based on fossil data the major diversification of "opisthobranch" taxa in a traditional sense took place comparatively recently, at the beginning of the Cenozoic (around 60 Mya), with the first records of Sacoglossa, Anaspidea and Thecosomata [86]. However, due to more or less reduced shells the fossilization probability is low. Our study suggests that most extant "opisthobranch" taxa, e.g. Sacoglossa, Cephalaspidea s.s., Pteropoda, Umbraculoidea and Anaspidea, have a Mesozoic origin. Ambiguous is the basal euthyneuran position of the Nudipleura and the resulting estimates of an old age (late Palaeozoic) and diversification (middle Mesozoic). This contradicts previous molecular clock analyses on Nudipleura, which indicated a Triassic origin and Jurassic diversification [82]. These discrepancies clearly result from major differences in tree topology (basal vs. derived position). Moreover, while our study includes only three nudipleuran representatives (poor ingroup taxon sampling), Göbbeler's and Klussmann-Kolb's [82] analysis lacks comprehensive heterobranch outgroup sampling. Future studies are needed to resolve the origin of Nudipleura within the Heterobranchia.

Conclusions

Our multi-locus molecular study including six out of seven acochlidian families and the recently established Aitengidae confirms a pulmonate relationship of Acochlidia, which was traditionally placed within Opisthobranchia. The enigmatic Aitengidae cluster within Acochlidia. Previously assumed morphological synapomorphies of Pulmonata (pallial cavity with pneumostome, procerebrum with cerebral gland, and presence of medio-dorsal bodies) appear as either homoplastic or plesiomorphic in light of the present phylogenetic hypothesis, as does the potential opisthobranch synapomorphy (presence of rhinophoral nerve). At present, morphological characters neither justify a placement of Acochlidia within Pulmonata, nor do they favour any opisthobranch relationships that would contradict the molecular hypothesis. The aberrant acochlidian morphology might have resulted from ancestral progenesis and paedomorphic retention of the morphology of an abnormally developed juvenile.

The present study once more underlines the respective non-monophyly of Euthyneura, Opisthobranchia and Pulmonata as defined traditionally. We demonstrate the necessity for inclusion of small, enigmatic groups to solve deep-level phylogenetic relationships, and highlight that the "pulmonate" and "opisthobranch" phylogenies cannot be solved independently from each other. Clarification of remaining enigmas such as Rhodopemorpha, and of well supported taxa with unclear relationships such as Pyramidelloidea or Sacoglossa, is needed for future advances. The reclassification suggested herein

defines 1) Euthyneura as including Pyramidelloidea and Glacidorboidea; 2) Euopisthobranchia as including Umbraculoidea, Cephalaspidea s.s., Runcinacea, Anaspidea and Pteropoda, but excluding Acteonoidea and Nudipleura, as well as Sacoglossa and Acochlidia; and 3) Panpulmonata as composed of Siphonarioidea, Sacoglossa, Hygrophila, Amphiboloidea, Pyramidelloidea, Glacidorboidea, Eupulmonata and Acochlidia. The present results based on standard molecular markers require confirmation from other character sets (e.g. rare genomic changes, mitochondrial gene arrangements, additional molecular markers) and careful (re-)examination of morphological characters and homology assumptions in the light of the new phylogenetic hypothesis. Our molecular clock analysis estimates a Mesozoic origin for all major panpulmonate taxa. The poorly supported topology within Panpulmonata might be promoted by the old age of this group, which potentially stands for a series of radiation and extinction events in history, resulting in poor taxon representation in present times.

The present study shows that the mesopsammon was colonised various times independently within Euthyneura, resulting in a series of convergent adaptations to the interstitial habitat. The inclusion of Acochlidia within pulmonate taxa extends the structural and biological diversity of the pulmonate clade, which exhibits remarkable flexibility in habitat choice, with various transitions from marine to limnic and terrestrial habitats.

Methods

Taxon sampling

A total of 78 gastropod taxa were investigated in the present study. As new material, nine acochlidian taxa and five additional enigmatic and hard-to-obtain euthyneuran taxa with potential acochlidian relationships were included (see Table 2). Specimens were collected by hand or extracted from sand samples following the method described by Schrödl [88], usually anaesthetised with $MgCl_2$, and fixed in 96% ethanol. Reference specimens and DNA vouchers of sequences generated in this study are deposited at the Bavarian State Collection for Zoology (ZSM); sampling localities, reference material and DNA Bank accession numbers (http://www.dna-bank-network.org) of our own data are listed in Table 2. Other sequences were retrieved from GenBank (for accession numbers see Table 3). Outgroups were chosen to include all major euthyneuran and several further heterobranch taxa. Special focus was given to mesopsammic representatives and groups previously discussed as potentially related to Acochlidia. Of these potential relatives only Rhodopemorpha are missing in our study, but a Rhodopemorpha-Acochlidia relationship can be clearly rejected based on molecular markers [49].

DNA extraction, PCR and sequencing

Genomic DNA was extracted from tissue samples of the foot or from entire specimens using the DNeasy Blood and Tissue Kit (Qiagen, Hilden Germany). Four markers were amplified: nuclear 18S rRNA (approx. 1800 bp), 28S rRNA (approx. 1020 bp), mitochondrial 16S rRNA (approx. 300-400 bp), and cytochrome *c* oxidase subunit I (COI - approx. 650 bp). For PCR protocols and primers used, see additional file 3. Successfully amplified PCR products were purified using ExoSapIT (USB, Affymetrix, Inc.). Cycle sequencing and the sequencing reaction were performed by the sequencing service of the Department of Biology Genomic Service Unit (GSU) of the Ludwig-Maximilians-University Munich, using Big Dye 3.1 kit and an ABI 3730 capillary sequencer. All fragments were sequenced in both directions using the PCR primers. All sequences have been deposited at GenBank (see Table 3 for accession numbers). The Gblock alignment and the resulting tree were deposited in TreeBASE (http://www.treebase.org, accession number 10801).

Sequence editing and alignment

All sequences generated in this study were checked for contaminations with BLAST searches [89] implemented in the GenBank database on the NCBI webpage (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Reconciliation of forward and reverse reads was carried out in BioEdit 7.0.5. [90]. MAFFT v6 [91] was used to generate sequence alignments for each gene region, using the default settings (automatically chosen models for 18S, 28S, COI: FFT-NS-i; for 16S: L-INS-i). The alignment of the protein coding COI gene was corrected manually according to the amino acids. The individual MAFFT alignments were parsed 1) using Gblocks [92,93] with the default settings for less stringent selection, 2) with ALISCORE v1.0 [94] using the default parameters, or c) left unmasked.

Phylogenetic analysis

For an *a priori* analysis of variation in the phylogenetic signal a split-decomposition analysis was performed using SplitsTree v4.6 [95].

The best-fit model of nucleotide substitution for each gene was selected using Modeltest 3.7 [96] via the Akaike Information Criterion (AIC). The incongruence length difference (ILD) test [97] was carried out in Paup 4.0b10 [36]. This test was conducted with heuristic searches and 100 replicates to evaluate incongruence between single markers.

Table 2 Information on the material generated for the present study

Taxon	Family	Locality	Museums Nr.	DNA Bank voucher Nr.
Acochlidia				
Hedylopsis spiculifera	Hedylopsidae	Istria Croatia/Corse France, Mediterranean Sea	ZSM Mol 20080951/ZSM Mol 20080955	AB35081816 AB35081817
Hedylopsis ballantinei	Hedylopsidae	Sinai, Egypt, Red Sea	ZSM Mol 20090244	AB34858170
Pseudunela sp.	Pseudunelidae	Mounparap Island, Vanuatu, Pacific	ZSM Mol 20080393	AB35081809
Strubellia paradoxa	Acochlidiidae	Ambon, Indonesia, Indo-Pacific	Berlin Moll 193944	AB34858174
Acochlidium fijiense	Acochlidiidae	Vitilevu, Fiji, Pacific	ZSM Mol 20080063	AB34404244
Asperspina sp.	Asperspinidae	Kamtschatka, Russia, North Pacific	ZSM Mol 20090171	AB35081833
Microhedyle glandulifera	Microhedylidae	Istria, Croatia, Mediterranean Sea	ZSM Mol 20081019	AB35081799
Pontohedyle milaschewitchii	Microhedylidae	Istria. Croatia, Mediterranean Sea	ZSM Mol 20080054/ZSM Mol 20080925	AB34404241
Paraganitus ellynnae	Ganitidae	Guadalcanal, Solomons, Pacific	ZSM Mol 20080170	AB34404203
Sacoglossa				
Gascoignella nukuli	Platyhedylidae	Pak Phanang Bay, Thailand, Gulf of Thailand	ZSM Mol 20090182	AB344011928
Volvatella viridis	Volvatellidae	Bonotsu, Kagoshima, Japan, Pacific	-	-
Aitengidae sp.	Aitengidae	Hisamatsu, Miyako Island, Okinawa, Japan, Pacific	-	-
Cephalaspidea				
Philine exigua	Philinidae	Guadalcanal, Solomons, Pacific	ZSM Mol 20080752	AB34401927
Philinoglossa praelongata	Philinoglossidae	Istria, Croatia, Mediterranean Sea	ZSM Mol 20080917	AB34500041

The table lists the species names, collecting localities, reference numbers of museum vouchers (ZSM = Bavarian State Collection for Zoology; Berlin = Museum of Natural History, Berlin) and DNA vouchers deposited in the DNA Bank of the ZSM.

Maximum likelihood analyses were performed using RAxML 7.0.3 [98] adapting the program parameters to the alignment as described in the manual ("hard & slow way" - with 10 parsimony starting trees and 6 different rate categories). Additionally 200 multiple inferences were executed on the original alignment and 1000 bootstrap replicates were generated. Analyses were run under the GTR Gamma model as recommended in the manual [98] and the caenogastropod taxa *Littorina littorea* and *Aperostoma palmeri* were defined as outgroups. The alignment was analysed in different partition sets: one partition, two partitions (18S + 28S + 16S combined; COI separate), three partitions (18S + 28S + 16S combined; COI with codons partitioned to 1st + 2nd separate from 3rd), four partitions (separated by gene regions), and five partitions (18S, 28S, 16S, COI 1st + 2nd, COI 3rd). To test whether partitioning significantly improves the likelihood values of the dataset, we compared the likelihood values of all partitions via the Akaike Information Criterion.

Bayesian phylograms were generated from the Gblocks and ALISCORE alignments with MrBayes 3.1.2 [99]. The general time-reversible model was used for both datasets, with invariant site frequency and gamma-shape parameter estimated from the data (GTR + I + G). The 'shape', 'proportion of invariant sites', 'state frequency' and 'substitution rate' parameters were estimated for each gene separately. Each codon position in the amino-acid coding COI was also allowed to have different parameters; hence the alignments had six partitions of parameters. Two parallel runs were made for 5×10^6 generations (with a sample frequency of 1000), using a default value of four Markov chains. Quality and ESS values (effective sampling size) of each run were checked in Tracer 1.5.3. The first 2000 trees for each run were discarded to ensure that the four chains reached stationarity. The consensus tree and posterior probabilities were computed from the remaining 6000 trees (3000 trees × 2 runs).

To evaluate support for our tree topology an alternative topology (grouping Acochlidia with Opisthobranchia) was tested in comparison to the "best" tree topology by using the Approximately Unbiased Test [100]. The hypothetic topology was computed with RAxML [98] using the -g option for the constraint ML tree. The p-values of the sitewise log likelihoods combined with the "best" topology were estimated using Treefinder [101].

Molecular clock

Approximate divergence times were calculated using the relaxed molecular clock approach [78] implemented in the software BEAST 1.5.3 [102]. For molecular clock analysis the concatenated Gblock-dataset was analysed in five partitions as for the phylogenetic analyses.

Calibration points were chosen for groups with stable and well supported nodes in the phylogenetic

Table 3 GenBank accession numbers of the sequences used in the present study

Taxon	Family	Species	18S	28S	16S	COI
Caenogastropoda	Cyclophoridae	*Aperostoma palmeri*	DQ093435	DQ279983	DQ093479	DQ093523
	Littorinidae	*Littorina littorea*	X91970	AJ488672	DQ093481	AY345020
"Lower" Heterobranchia	Orbitestellidae	*Orbitestella* sp.	EF489352	EF489377	EF489333	EF489397
	Valvatidae	*Valvata piscinalis*	FJ917223/FJ917222	FJ917224	FJ917248	FJ917267
	Cimidae	*Cima* sp.	FJ917206.1	FJ917228.1	FJ917260.1	FJ917279.1
	Rissoellidae	*Rissoella rissoaformis*	FJ917214.1	FJ917226.1	FJ917252.1	FJ917271.1
	Pyramidellidae	*Turbonilla* sp.	EF489351	EF489376	EF489332	EF489396
	Pyramidellidae	*Boonea seminuda*	AY145367	AY145395	AF355163	-
	Pyramidellidae	*Eulimella ventricosa*	FJ917213.1	FJ917235.1	FJ917255.1	FJ917274.1
	Pyramidellidae	*Odostomia* sp.	AY427526.1	AY427491.1	FJ917256.1	FJ917275.1
	Glacidorbidae	*Glacidorbis rusticus*	FJ917211.1	FJ917227.1	FJ917264.1	FJ917284.1
Acteonoidea	Acteonidae	*Pupa solidula*	AY427516	AY427481	EF489319	DQ238006
	Aplustridae	*Hydatina physis*	AY427515	AY427480	EF489320	GQ845174.1
	Acteonidae	*Rictaxis punctocaelatus*	EF489346	EF489370	EF489318	EF489393
Nudipleura	Bathydorididae	*Bathydoris clavigera*	AY165754	AY427444	AF249222	AF249808
	Pleurobranchidae	*Tomthompsonia antarctica*	AY427492	AY427452	EF489330	DQ237992
	Pleurobranchidae	*Pleurobranchus peroni*	AY427494	AY427455	EF489331	DQ237993
Umbraculoidea	Umbraculidae	*Umbraculum umbraculum*	AY165753	AY427457	EF489322	DQ256200
	Tylodinidae	*Tylodina perversa*	AY427496	AY427458	-	AF249809
Anaspidea	Akeridae	*Akera bullata*	AY427502	AY427466	AF156127	AF156143
	Aplysiidae	*Aplysia californica*	AY039804	AY026366	AF192295	AF077759
Pteropoda	Pneumodermatidae	*Pneumoderma cf. atlantica*	DQ237970	DQ237989	-	DQ238003
	Pneumodermatidae	*Spongiobranchaea australis*	DQ237969	DQ237988	-	DQ238002
	Cavoliniidae	*Hyalocylis striata*	DQ237966	DQ237985	-	-
	Cavoliniidae	*Cavolinia uncinata*	DQ237964	DQ237983	-	DQ237997
Runcinacea	Runcinidae	*Runcina africana*	DQ923473	DQ927240	-	DQ974680
Cephalaspidea s.s.	Bullidae	*Bulla striata*	DQ923472.1	DQ986694.1	DQ986632.1	DQ986567.1
	Phillinoglossidae	*Philinoglossa praelongata*	AY427510	AY427475	HQ168411*	-
	Scaphandridae	*Scaphander lignarius*	EF489348	EF489372	EF489324	-
	Haminoeidae	*Haminoea hydatis*	AY427504	AY427468	EF489323	DQ238004
	Philinidae	*Philine exigua*	HQ168425*	HQ168438*	HQ168412*	HQ168450*
	Diaphanidae	*Diaphana* sp.	-	EF489373	EF489325	EF489394
	Diaphanidae	*Toledonia globosa*	EF489350	EF489375	EF489327	EF489395
	Cylichnidae	*Cylichna gelida*	EF489349	EF489374	EF489326	-
Sacoglossa	Volvatellidae	*Volvatella viridis*	HQ168426*	HQ168439*	HQ168413*	HQ168451*
	Cylindrobullidae	*Cylindrobulla beauii*	EF489347	EF489371	EF489321	-
	Platyhedylidae	*Gascoignella nukuli*	HQ168427*	HQ168440*	HQ168414*	HQ168452*
	Caliphyllidae	*Cyerce nigricans*	AY427500	AY427463	EU140843	DQ237995
	Plakobranchidae	*Plakobranchus ocellatus*	AY427497	AY427459	DQ480204	DQ237996
	Elysiidae	*Thuridilla bayeri*	AF249220	AY427461	DQ480206	DQ471271
	Elysiidae	*Elysia viridis*	AY427499	AY427462	AY223398	DQ237994
Sacoglossa (?)	Aitengidae	*Aitengidae* sp.	HQ168428*	HQ168441*	HQ168415*	HQ168453*
Acochlidia	Hedylopsidae	*Hedylopsis ballantinei*	HQ168429*	HQ168442*	HQ168416*	HQ168454*
	Hedylopsidae	*Hedylopsis spiculifera*	HQ168430*	HQ168443*	HQ168417*	HQ168455*
	Pseudunelidae	*Pseudunela* sp.	HQ168431*	HQ168444*	HQ168418*	HQ168456*
	Acochlidiidae	*Strubellia paradoxa*	HQ168432*	HQ168445*	HQ168419*	HQ168457*
	Acochlidiidae	*Acochlidium fijiense*	HQ168433*	HQ168446*	HQ168420*	HQ168458*
	Asperspinidae	*Asperspina* sp.	HQ168434*	HQ168447*	HQ168421*	-
	Microhedylidae	*Pontohedyle milaschewitchii*	HQ168435*	AY427484	HQ168422*	HQ168459*
	Ganitidae	*Paraganitus ellynnae*	HQ168436*	HQ168448*	HQ168423*	HQ168460*
	Microhedylidae	*Microhedyle glandulifera*	HQ168437*	HQ168449*	HQ168424*	HQ168461*

Table 3 GenBank accession numbers of the sequences used in the present study *(Continued)*

Siphonarioidea	Siphonaridae	*Siphonaria pectinata*	U86321	DQ279993	AY377627	AF120638
	Siphonaridae	*Siphonaria concinna*	EF489334	EF489353	EF489300	EF489378
Amphiboloidea	Amphibolidae	*Amphibola crenata*	EF489337	EF489356	EF489304	-
	Amphibolidae	*Phallomedusa solida*	DQ093440	DQ279991	DQ093484	DQ093528
	Amphibolidae	*Salinator cf. fragilis*	-	EF489355	EF489303	EF489381
Hygrophila	Latiidae	*Latia neritoides*	EF489339	EF489359	EF489307	EF489384
	Chilinidae	*Chilina* sp.	EF489338	EF489357	EF489305	EF489382
	Acroloxidae	*Acroloxus lacustris*	AY282592	EF489364	EF489311	AY282581
	Lymnaeidae	*Lymnaea stagnalis*	EF489345	EF489367	EF489314	EF489390
	Physidae	*Physella acuta*	AY282600	EF489368	AY651241	AY282589
	Planorbidae	*Ancylus fluviatilis*	AY282593	EF489365	EF489312	AY282582
Stylommatophora	Arionidae	*Arion silvaticus*	AY145365	AY145392	AY947380	AY987918
	Helicidae	*Arianta arbustorum*	AY546383	AY014136	AY546343	AY546263
	Enidae	*Ena montana*	AY546396	-	AY546356	AY546276
	Cerionidae	*Cerion incanum*	-	AY014060.1	-	-
	Subulinidae	*Rumina decollata*	-	13794085:464-1292	AY345050	AY345050
Systellommatophora	Onchidiidae	*Onchidium verruculatum* (§)	AY427522	AY427487	EF489316	EF489391
	Onchidiidae	*Onchidella floridiana*	AY427521	AY427486	EF489317	EF489392
	Veronicellidae	*Laevicaulis alte*	X94270.1	AY014151.1		
	Veronicellidae	*Semperula wallacei*	-	DQ897671.1	DQ897675.1	DQ897673.1
	Rathouisiidae	*Atopos australis*	-	AY014152.1	-	-
Trimusculoidea	Trimusculidae	*Trimusculus afra*	EF489343	-	EF489309	EF489388
Otinoidea	Otinidae	*Otina ovata*	EF489344	EF489363	EF489310	EF489389
	Smeagolidae	*Smeagol phillipensis*	FJ917210	FJ917229	FJ917263	FJ917283
Ellobioidea	Carychiidae	*Carychium minimum*	EF489341	EF489361	EF489308	EF489386
	Ellobiidae	*Ophicardelus ornatus*	DQ093442	DQ279994	DQ093486	DQ093486
	Ellobiidae	*Myosotella myosotis*	EF489340	EF489360	AY345053	EF489385

Sequences generated within this study are marked with *; (§) in GenBank as "*O. verrucosum*", which is not a valid name, thus treated as *O. verruculatum*. (" - " indicates missing sequences).

hypothesis and decently documented fossil record with clear identification to recent taxa. Minimum constraints for three nodes were chosen based on the fossil record: 1) split between Caenogastropoda and Heterobranchia based on the oldest known fossil of the Heterobranchia (*Palaeocarboninia janke*) recorded from the Middle Devonian (390 Ma) [85]; 2) the split between Acteonoidea and its sister group based on acteonoid fossils with a minimum age of 240 Ma ([103], A Nützel pers. comm.) and 3) the split of Ellobioidea and their sister group based on ellobiid fossils with a minimum age of 140 Ma ([86], A Nützel pers. comm.). We calibrated using a hard minimum bound (i.e. the divergence data cannot be younger than the oldest known fossil); the probability that the divergence event occurred above the minimum date declines according to a gamma distribution, such that 95% of the posterior density falls within the range [x - x + 10%] [see [104]]. Calibration nodes were not fixed as monophyletic.

The analyses were run with the relaxed uncorrelated lognormal clock model under the Yule process using the GTR+G+I substitution model (chosen from Modeltest 3.7 [96] via the Akaike Information Criterion) for all markers. The MCMC was run ten times independently, generating 10^6 generations each, and sampled every 1000 steps. The single runs were combined with LogCombiner 1.5.3, with the first 10^5 samples each discharged as burn-ins. The runs were checked for quality and sufficient ESS (effective sample size) in Tracer 1.5.3. All trees were combined to produce a consensus tree using TreeAnnotator 1.5.3, with the first 1000 trees of each dataset discharged as burn-in.

To evaluate the potential effect on molecular dating of removing ambiguous sites from the alignment, the BEAST runs were repeated with the raw alignments (i.e. mainly uncut; only longer ends of some sequences removed due to the use of different primers) alignments, generating 10×10^6 generations and following the method described above.

Additional material

Additional file 1: Neighbournet graph on the origin of Acochlidia. Generated with Splits Tree v4.6 from the concatenated, four marker dataset masked with Gblocks, visualising highly conflicting signal at the basis of the Acochlidia. Representatives of meiofaunal taxa highlighted in boldface, showing the absence of a common phylogenetic signal.

Additional file 2: Likelihood values of different partitions

Additional file 3: PCR protocols and primers used [105-107].

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Authors' contributions

KMJ, MS, YK and HF sampled the material. KMJ, IS, TK and YK generated the molecular data. KMJ and YK conducted the phylogenetic and network analysis. KMJ performed the molecular clock approach. KMJ wrote the initial version of the manuscript; all authors contributed to the discussion of the results and the preparation of the final manuscript. MS planned and supervised the study. All authors have read and approved the final manuscript.

Competing interests

The authors declare that they do not have competing interests.

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Gene region	Primer	Sequence 5´ - 3´	Reference	PCR program
18S	18A1	CCT ACT TCT GGT TGA TCC TGC CAG T	[105]	98°C 30sec (98°C 5sec, 48-65°C 5sec, 72°C 20-25sec) x 28-40, 72°C 60sec (Phire polymerase, New England Biolabs)
	700R	CGC GGC TGC TGG CAC CAG AC	[34]	
	470F	CAG CAG GCA CGC AAA TTA CCC	[34]	
	1500R	CAT CTA GGG CAT CAC AGA CC	[34]	
	1155F	CTG AAA CTT AAA GGA ATT GAC GG	[34]	
	1800	TAA TGA TCC TTC CGC AGG TT	[105]	
28S	28SC1	ACC CGC TGA ATT TAA GCA T	[12]	98°C 30sec (98°C 5sec, 48-65°C 5sec, 72°C 20-25sec) x 28-40, 72°C 60sec (Phire polymerase, New England Biolabs + Q-solution, Qiagen)
	28SD2R	CCT TGG TCC GTG TTT CAA GAC GGG	[34]	
	28SC2F	GAA AAG AAC TTT GAA GAG AGA GT	[34]	
	28SD3	GACGAT CGA TTT GCA CGT CA	[34]	
16S	16S-H	CGC CTG TTT ATC AAA AAC AT	[106]	98°C 30sec (98°C 5sec, 48-55°C 5sec, 72°C 25sec) x 35-40, 72°C 60sec (Phire polymerase, New England Biolabs)
	16S-R	CCG GTC TGA ACT CAG ATC ACG T	[106]	
	16Sf-50	GGC CGC AGT ACC TTG ACT GT	present study	
	16Sr-380	TCC ACC ATC GAG GTC ACA AG	present study	
COI	LCO1490	GGT CAA CAA ATC ATA AAG ATA TTG G	[107]	94°C 3min (94°C 60sec, 48-52°C 60sec, 72°C 90sec) x 35-40, 72°C 3min (Taq polymerase, Sigma)
	HCO2198	TAA ACT TCA GGG TGA CCA AAA AAT CA	[107]	

3.3. Julia D. Sigwart, **Isabella Stöger**, Thomas Knebelsberger, Enrico Schwabe: **Chiton phylogeny (Mollusca: Polyplacophora) and the placement of the enigmatic species *Choriplax grayi* (H. Adams & Angas). 2013.** Invertebrate Systematics, 27, 603-621.

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Chiton phylogeny (Mollusca : Polyplacophora) and the placement of the enigmatic species *Choriplax grayi* (H. Adams & Angas)

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Abstract. Shallow marine chitons (Mollusca : Polyplacophora : Chitonida) are widespread and well described from established morphoanatomical characters, yet key aspects of polyplacophoran phylogeny have remained unresolved. Several species, including *Hemiarthrum setulosum* Carpenter in Dall, 1876, and especially the rare and enigmatic *Choriplax grayi* (Adams & Angas, 1864), defy systematic placement. *Choriplax* is known from only a handful of specimens and its morphology is a mosaic of key taxonomic features from two different clades. Here, new molecular evidence provides robust support for its correct association with a third different clade: *Choriplax* is placed in the superfamily Mopalioidea. *Hemiarthrum* is included in Cryptoplacoidea, as predicted from morphological evidence. Our multigene analysis of standard nuclear and mitochondrial markers demonstrates that the topology of the order Chitonida is divided into four clades, which have also been recovered in previous studies: Mopalioidea is sister to Cryptoplacoidea, forming a clade Acanthochitonina. The family Callochitonidae is sister to Acanthochitonina. Chitonoidea is resolved as the earliest diverging group within Chitonida. Consideration of this unexpected result for *Choriplax* and our well-supported phylogeny has revealed differing patterns of shell reduction separating the two superfamilies within Acanthochitonina. As in many molluscs, shell reduction as well as the *de novo* development of key shell features has occurred using different mechanisms, in multiple lineages of chitons.

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Introduction

Chitons (Polyplacophora) are a relatively small clade of living molluscs, with ~960 Recent and 390 fossil species described (Schwabe 2005; E. Schwabe, unpubl. data). The position of this class is singularly important in the ongoing debate on the phylogenetic topology within Mollusca (Giribet *et al*. 2006; Meyer *et al*. 2010; Kocot *et al*. 2011; Smith *et al*. 2011; Stöger *et al*. in press). But there have been only a handful of molecular phylogenetic studies on chitons themselves (Okusu *et al*. 2003; Kelly and Eernisse 2008; Sigwart *et al*. 2011; Sirenko *et al*. 2013). Molecular tools have been used to examine chiton population genetics (Kelly and Eernisse 2007; Kelly and Palumbi 2010; Kelly *et al*. 2010; Doonan *et al*. 2012) and cryptic or enigmatic species (Bonfitto *et al*. 2011; Sirenko *et al*. 2013), yet the literature remains very sparse. While chiton systematics has actually been substantially improved in the last 10 years on the morphological front (e.g. Saito 2004; Buckland-Nicks 2006; Vendrasco *et al*. 2008), there have been no further molecular investigations into intraclass relationships.

The systematic arrangement within Polyplacophora is well resolved, in that there are two major clades separated by morphological, anatomical and genetic features. Members of the order Lepidopleurida are usually small (up to 2 cm long), and mostly found in deep sea habitats; in contrast, Chitonida contains around 80% of living species, in a broader diversity of size, body shapes and lifestyles and generally with more complex shell and girdle structures (Sirenko 2006). Variation, here, is relative to a rather constrained chiton norm.

The most recent systematic revision for the class (Sirenko 2006) divides the majority order (Chitonida) into two nominal suborders, Chitonina (containing two superfamilies, Chitonoidea and Schizochitonoidea) and Acanthochitonina (with two superfamilies: Mopalioidea and Cryptoplacoidea) (Table 1). Recent morphological and molecular analyses have recognised several distinct and well-defined clades, which agree in part with traditional systematics (Okusu *et al*. 2003; Buckland-Nicks 2008). The relative phylogenetic position of these groups and the membership of several critically important taxa remain unresolved (Fig. 1).

Because of the superficial similarity among most species, chitons are usually considered a 'difficult' group. More importantly, the highly constrained nature of the chiton body plan in modern taxa produces uncertainty over which characters provide useful phylogenetic signal, or are static, or convergent

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Table 1. Polyplacophoran species sampled in this study

GenBank numbers are given for the four gene fragments in each species. For specimens where new sequences were generated we have noted the specimen voucher number, collection date and general origin (further detailed specimen data are available from the holding institutions). Other taxa, which require further taxonomic revision beyond this study, are retained in their positions according to Sirenko (2006) but noted with a dagger (†); the sampled members of Tonicellidae indicate this family is not monophyletic, and *Plaxiphora* and *Nuttallochiton* are resolved in Cryptoplacoidea not Mopalioidea. We make two systematic changes based on the results of the present analysis (noted with a double dagger ‡): *Cryptochiton* is placed in the family Mopaliidae; *Choriplax* and Choriplacidae are placed in the superfamily Mopalioidea; we erect a new superfamily, Callochitonoidea, in recognition of the clear distinction between members of Callochitonidae and other Chitonida. Previously published sequences are from Okusu *et al.* (2003; accession numbers AY-), Kelly and Eernisse (2008; accession number EU-) and Sigwart *et al.* (2011; accession numbers HQ-)

	COI	16S	18S	28S	
Order Lepidopleurida Thiele, 1910					
Leptochitonidae Dall, 1889					
Leptochiton asellus (Gmelin, 1791)	HQ907851	AY377586	HQ907747	HQ907807	
Lepidopleurus cajetanus (Poli, 1791)	HQ907847	AY377585	AF120502	HQ907802	
Order Chitonida Thiele, 1910					
Suborder Chitonina Thiele, 1910					
Superfamily Chitonoidea Rafinesque, 1815					
Chitonidae Rafinesque, 1815					
Chiton (*Chiton*) *pelliserpentis* Quoy & Gaimard, 1835	AY377718	AY377607	AY377653	AY377684	
Chiton (*Rhyssoplax*) *olivaceus* Spengler, 1797	AY377716	AY377605	AY377651	AY377682	
Ischnochitonidae Dall, 1889					(not sampled)
Callistoplacidae Pilsbiy, 1893					(not sampled)
Chaetopleuridae Plate, I899					
Chaetopleura angulata (Spengler, 1797)	AY377703	AY377591	AY377637	AY377668	
Chaetopleura apiculata (Say in Conrad, 1834)	AY377704	AY377590	AY377636	AY377667	
Loricidae Iredale & Hull, 1923					
Lorica volvox (Reeve, 1847)	–	AY377601	AY377647	AY377678	
Callochitonidae Plate, 1901					
Callochiton euplaeae (O.G. Costa, 1829)	KC887247	KC887228	KC887254	KC887271	ZSM-Mol-20080841: Croatia, 2008 (96% EtOH)
Callochiton gaussae Thiele, 1908	–	KC887229	KC887255	KC887272	ZSM-Mol-20021258: Antarctica, 2002 (96% EtOH)
Callochiton puniceus (Couthouy MS, Gould, 1846)	KC887246	KC887230	KC887256	KC887273	ZSM-Mol-20050295: Chile, 2005 (96% EtOH)
Callochiton schilfi Schwabe & Ruthensteiner, 2001	–	KC887231	KC887257	KC887274	ZSM-Mol-20033136: Indonesia, 2003 (96% EtOH)
Callochiton septemvalvis (Montagu, 1803)	KC887245	–	KC887258	KC887275	ZSM-Mol-20031152: France, 2003 (96% EtOH)
Callochiton subsulcatus Kaas & Van Belle, 1985*b*	–	KC887232	KC887259	KC887276	ZSM-Mol-20033131: Indonesia, 2003 (96% EtOH)
Callochiton sulcatus Nierstrasz, 1905	–	–	KC887260	KC887277	ZSM-Mol-20033123: Indonesia, 2003 (96% EtOH)
Superfamily Schizochitonoidea Dall, 1889					
Schizochitonidae Dall, 1889					(not sampled)
Suborder Acanthochitonina Bergenhayn, 1930					
Superfamily Mopalioidea Dall, 1889					
†Tonicellidae Simroth, 1894					
Cyanoplax dentiens (Gould, 1846)	KC887250, KC887251	KC887240, KC887241	KC887266, KC887267	KC887284, –	Bamfield, Canada, 2011 (96% EtOH)
Lepidochitona cinerea (Linnaeus, 1767)	AY377701	–	AY377633	AY377664	
Tonicella lineata (Wood, 1815)	–	EU406998	–	EU407117	
Schizoplacidae Bergenhayn, 1955					(not sampled)
Mopaliidae Dall, 1889					
Katharina tunicata (Wood, 1815)	AY377715	AY377604	AY377650	AY377681	
Mopalia hindsii (Sowerby MS, Reeve, 1847)	EF159594	EU406911	–	EU407033	

(*continued next page*)

Table 1. (*continued*)

	COI	16S	18S	28S	
Superfamily Mopalioidea (continued)					
Mopalia muscosa (Gould, 1846)	EF159577	EU406891	–	EU407018	
	EF159580	EU406894	–	EU407021	
†*Nuttallochiton mirandus* (Smith MS, Thiele, 1906)	AY377705	AY377592	AY377638	AY377669	
†*Plaxiphora albida* (Blainville, 1825)	AY377714	–	AY377649	AY377680	
‡*Cryptochiton stelleri* (von Middendorff, 1847)	EF159619	EU406933	– AY377655	EU407053	
	AY377720	AY377610		AY377686	
‡Choriplacidae Ashby, 1928					
Choriplax grayi (H. Adams & Angas, 1864)	KC887244	KC887234	KC887262	KC887279	SAMA D 16542: Tasmania, 1972 (original fixative unknown)
Superfamily Cryptoplacoidea H. & A. Adams, 1858					
Acanthochitonidae Pilsbry, 1893					
Acanthochitona crinita (Pennant, 1777)	AF120627	AY377609	AF120503	DQ279957	
Choneplax indica Odhner, 1919	–	KC887233	KC887261	KC887278	ZSM-Mol-20052202: Seychelles, 2005 (96% EtOH)
Craspedochiton laqueatus (Sowerby, 1842)	KC887252	KC887235	KC887263	KC887280	ZSM-Mol-20033119: Indonesia, 2003 (96% EtOH)
Craspedochiton tesselatus Nierstrasz, 1905	KC887249	KC887236	KC887264	KC887281	ZSM-Mol-20033137: Indonesia, 2003 (96% EtOH)
Cryptoconchus porosus (Blainville MS, Burrow, 1815)	–	KC887237	–	KC887282	ZSM-Mol-20100239: New Zealand, 1988 (78% EtOH)
Leptoplax coarctata (Sowerby, 1841)	KC887248	KC887243	KC887269	KC887286	ZSM-Mol-20033124: Indonesia, 2003 (96% EtOH)
Leptoplax curvisetosa (Leloup, 1960)	KC887253	KC887238	KC887270	–	ZSM-Mol-20050839: Egypt, 2003 (96% EtOH)
Cryptoplacidae H. & A. Adams, 1858					
Cryptoplax oculatus (Quoy & Gaimard, 1835)	–	KC887239	KC887265	KC887283	ZSM-Mol-20040632: Indonesia, 1999 (96% EtOH)
Hemiarthridae Sirenko, 1997					
Hemiarthrum setulosum Carpenter in Dall, 1876	–	KC887242	KC887268	KC887285	ZSM-Mol-20100171: Argentinia, 1992 (78% EtOH)

(Sigwart 2009). The two most important anatomical distinctions that separate the two orders are the gill arrangements and the insertion laminae on the shell plates. Polyplacophoran gills form paired series of individual ctenidia in the pallial cavities on either side of the foot, and gills are continuously added as the animal grows (Hunter and Brown 1965). Where the growth is bi-directional (adanal condition) specimens have multiple gills posterior of the nephridiopore; if the gills grow only on the anterior end of the row (abanal condition) there is always a single gill posterior to the nephridiopore (Sirenko 1997). Previous literature used these terms in subtly different ways. Adanal gills (*sensu* Sirenko 1997, i.e. bi-directional) result in the posterior arrangement of gills typical of Lepidopleurida. Chiton shell valves are composed of a ventral articulamentum and a dorsal tegmentum, which is the exposed part of the shell in the living animals. The articulamentum can extend laterally forming insertion plates that anchor the valve to the muscular girdle; these insertion plates are perforated by slits where the aesthete nerve channels connect to the surrounding tissue (Eernisse and Reynolds 1994). The absence of slitted insertion plates is considered plesiomorphic and typical of Lepidopleurida, yet several genera that are unambiguously part of Lepidopleurida have unslit insertion plates that have apparently developed convergently (Sirenko 1997; Sigwart *et al*. 2011).

Among chitons, *Choriplax grayi* (H. Adams & Angas, 1864) is one of the rarest species, known from only eleven individuals (Table 2; Gowlett-Holmes 1987). The species was named from a single specimen from New South Wales, Australia; a second unique specimen collected in South Australia in 1918 was nominated as a separate species *C. pattisoni* Ashby, 1921. A further seven specimens allowed its comprehensive redescription (Gowlett-Holmes 1987). Herein, we examined two further specimens. It is an apparent mosaic taxon with features of both orders and a quite unusual morphology (Fig. 2). The primary distinctive features were a reduced shell tegmentum (found in Acanthochitonina), adanal gills (found in Chitonida in general), unslit insertion plates (found in Lepidopleurida) and the ventral girdle lacking spicular armature (only otherwise known from one

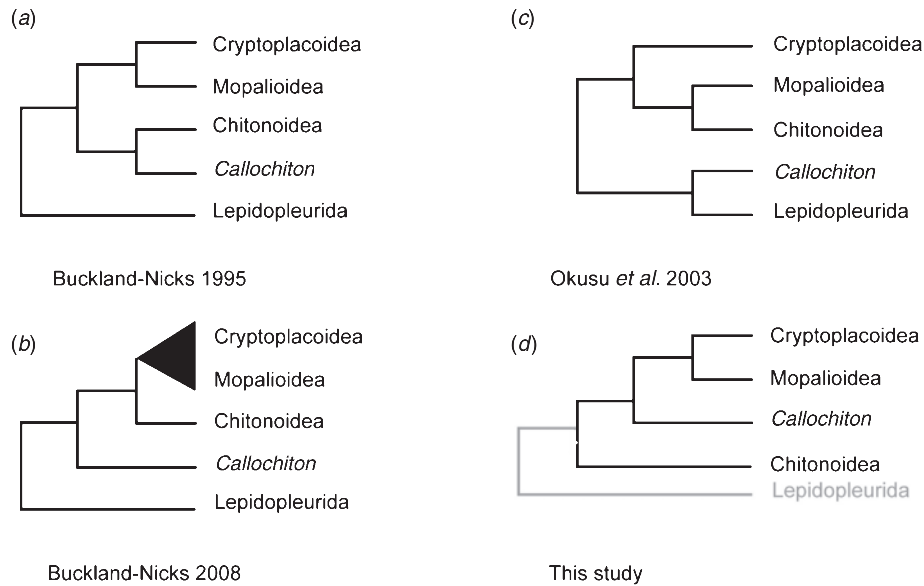

Fig. 1. Topological summary of relationships between the major clades of Polyplacophora based on numerical phylogenetic analyses of morphology (*A*, Buckland-Nicks 1995; *B*, Buckland-Nicks 2008) and molecular data (*C*, Okusu *et al*. 2003; *D*, this study). These are redrawn and summarised based on the position of genera in currently established superfamilies (Sirenko 2006; Table 1). The present study does not explicitly test the position of Lepidopleurida; other studies used non-polyplacophoran outgroups.

Table 2. Compilation of all known specimens of *Choriplax grayi* (H. Adams & Angas, 1864)

OZCAM (2008) lists additional records, but without indication whether the records were checked by a taxonomic expert, so they are not included here. Entries in **bold** are those specimens examined during the present study; the asterisk (*) indicates material for molecular data

Specimens	Specimen number	Locality	Habitat	Depth	Source
1	**NHMUK 1877.11.7.2 holotype: *Microplax grayi***	**Australia, NSW, Sydney Harbour [33°52′S 151°15′E]**	**Under stones at low water**	**Unknown**	**Gowlett-Holmes (1987), herein**
1	SAMA D15019 holotype: *Choriplax grayi pattisoni*	South Australia, Near Cape Banks Lighthouse [37°54′S 140°22′E]	Washed ashore after heavy storm, amongst large kelp	Unknown	Gowlett-Holmes (1987)
1 *	**SAMA D16542**	**Tasmania, Fluted Cape, Bruny Island [43°22′S 147°22′E]**	**Living on red alga *Sonderopelta coriacea***	**10 m**	**Gowlett-Holmes (1987), herein**
1	SAMA D17443	South Australia, Racecourse Bay, Port MacDonnell [38°04′S 140°45′E]	Washed ashore with kelp	Unknown	Gowlett-Holmes (1987)
2	SAMA D16543	South Australia, Cape Northumberland [38°04′S 140°40′E]	Living on red alga *Sonderopelta coriacea*	Unknown	Gowlett-Holmes (1987)
2	NMV F51767	Western Australia, Carnac Island, Perth [32°07′S 115°40′E]	On an unknown red algae	Unknown	Gowlett-Holmes (1987)
1	**AM C151131**	**West Australia, off Freemantle, West side of Carnac Island (32°7′S 115°40′E)**	**On brown algae, cryptic fauna on sponge**	**6 m**	**Gowlett-Holmes (1987), herein**
1	**WAM S16380**	**West Australia, Jurien Bay, SW of Essex Rocks (30°21′09″S 114°59′18″E)**	**Weed washing**	**7–11 m**	**Herein**
1	**WAM S16289**	**West Australia, Dry Lump, West of Green Head (30°07′19″S 114°56′47″E)**	**Unknown**	**5–6.2 m**	**Herein**

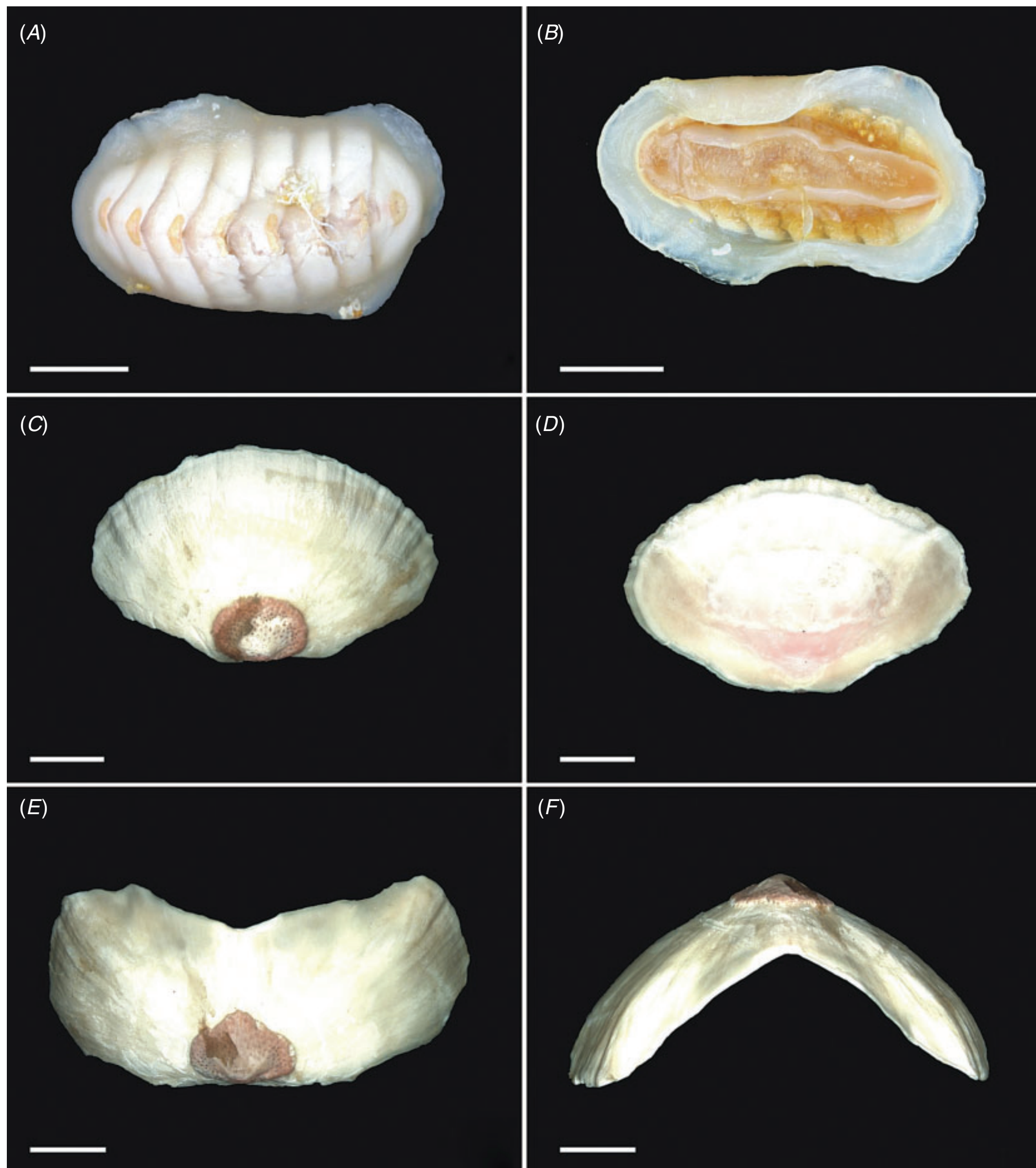

Fig. 2. *Choriplax grayi* (H. Adams & Angas, 1864). (*A*) Dorsal view of a complete specimen (AMS C151131), anterior at left side; (*B*) ventral view of a complete specimen (AMS C151131), anterior at left side; (*C*) dorsal view of head valve (SAMA D16542), anterior at top; (*D*) ventral view of head valve (SAMA D16542), anterior at top; (*E*) dorsal view of valve ii (SAMA D16542), anterior at top; (*F*) frontal view of valve ii (SAMA D16542). Scale bars: *A*, *B* = 5 mm; *C*–*F* = 1 mm. Photos *A*, *B* by Marianne Müller (ZSM).

genus in Lepidopleurida endemic to sunken wood, *Ferreiraella*). This has been the subject of lively discussions about the species' systematic position and it has been moved between Lepidopleurida and Acanthochitonina, alternating through its taxonomic history. There is hardly another chiton species that has been redescribed and reinterpreted so often, from so little material; we review this history, below. Recent findings of subadult specimens have allowed us to re-evaluate *Choriplax grayi*, and we present new information on these and additional characters as well as molecular markers.

Another enigmatic taxon, *Hemiarthrum setulosum* Carpenter in Dall, 1876 also has abanal gills, and shells with well-developed but unslit insertion plates (Sirenko 1993, 2006). This genus is also classified in Acanthochitonina, but its biology and anatomy are much better understood so it is less controversial than *Choriplax.* The phylogenetic position of both of these species is therefore particularly important illuminating patterns of character evolution in Polyplacophora as a whole.

Repeated shell loss and regrowth throughout evolution is a fundamental process in molluscan evolution. Chitons make a

particularly useful testing ground for hypotheses about shell evolution; they are morphologically constrained yet show obvious divergences, such as the internalised valves in *Cryptochiton*, and the taxa discussed here have been postulated to demonstrate multiple origins of shell insertion plates (Sirenko 2006). Further evidence on the flexibility or constraint of shell growth in chitons may provide a framework for interpreting broader patterns through the Mollusca.

This new analysis addresses the monophyly and topology of the major taxonomic clades of Chitonida: Chitonoidea, Mopalioidea, Cryptoplacoidea, the genus *Callochiton*, and the position within Polyplacophora for the two key genera *Hemiarthrum* and *Choriplax*.

Taxonomy of Choriplax

Adams and Angas (1864) created the new genus *Microplax* for the first specimen *Microplax grayi*, unaware that the genus name was preoccupied by a group of insects (Fieber 1861). While Adams and Angas (1864) placed their new genus into the Chitonidae Rafinesque, 1815, Pilsbry (1892) treated the genus under the family Lepidopleuridae Pilsbry, 1892 (= Leptochitonidae Dall, 1889) due to the absence of slits in the ventral shell insertion plates. A little later, Pilsbry (1894*a*) recognised that the characters of the genus warranted placement into a distinct family and renamed the genus (due to its homonymy) *Choriplax*. It was Ashby (1921) who doubted Pilsbry's classification of the genus under Lepidopleuridae, and he argued for a transfer of *Choriplax* into the family Acanthochitonidae Pilsbry, 1893 under the new subfamily Microplaxinae. This was accepted by Iredale and Hull (1925) but they used the name Cryptoconchidae Iredale, 1914 instead of Acanthochitonidae. To underline the unusual morphology of the genus again, Ashby (1928) adjusted the subfamily name Microplaxinae by renaming it Choriplacinae under the family Acanthochitonidae. Cotton and Weeding (1939), obviously not aware of the previously erected subfamily, introduced the 'new' family Choriplacidae, but did not change the general placement of *Choriplax* among the acanthochitonids (which they named Isoplacophora).

Bergenhayn (1955) ranked the genus *Choriplax* (under a new family Choriplaxidae [*sic*]) in close relationship to *Hanleya* Gray, 1857 and *Hemiarthrum* Carpenter in Dall, 1876 under the order Lepidopleurida Thiele, 1909, but warned that the placement was tentative, due to the scarce information available from the type species. Later authors adopted the interpretation that *Choriplax* was allied to plesiomorphic forms (Smith 1960; Van Belle 1975, 1983; Kaas and Van Belle 1980). Some included *Choriplax* as the only living member of the family Afossochitonidae Ashby, 1925 (Ferreira 1981; Kaas and Van Belle 1985*a*), with several fossil genera that had unslit insertion plates, in Lepidopleurida.

Starobogatov and Sirenko (1975) erected Choriplacina as an entirely separate suborder under the Neoloricata Bergenhayn, 1955, at the same rank as Lepidopleurina. This separation was retained by subsequent authors (Van Belle 1983). In her redescription of *C. grayi*, Gowlett-Holmes (1987) also evaluated the characters of the suborder and redefined it again with new information on *Choriplax grayi* from seven specimens (the first new material discovered since the holotypes of the two nominal species). Her interpretation was generally accepted (e.g. Kaas and Van Belle 1990, 1998; Gowlett-Holmes 1998, 2001; Van Belle 1999), but Sirenko (1997) later placed the suborder Choriplacina again under the order Lepidopleurida.

Most recently, Sirenko (2006) grouped the genus and family in Acanthochitonina – effectively returning to a similar interpretation as that proposed by Ashby (1921) and later authors – when he split Chitonida into superfamilies (noted above). The primary reason presented for including the species in Chitonida was the abanal condition of the gills, as this species had been described as having only one gill posterior to the nephridiopore (Gowlett-Holmes 1987; Sirenko 1993), which is the general condition in Chitonida. Sirenko (2006) further presented an argument that the lack of slits in the insertion plates as the end point of a 'lineage' of genera in Chitonida with relatively few slits per valve and reduced tegmentum.

There are two, contradictory, proposed hypotheses for the placement of *Choriplax.* They are: that it may be a member of Acanthochitonina, with secondary loss of slit insertion plates; or, it may be a member of Lepidopleurida, with an independent gain of (unslit) insertion plates. The resolution of this should give us new insights to the evolution of shell form within Polyplacophora.

Materials and methods

Taxon selection for phylogenetic analysis

Taxa were selected for analysis from the extensive polyplacophoran collection in the Bavarian State Collection of Zoology (ZSM, Munich, Germany) and augmented with previously published sequences. DNA material for *Choriplax grayi* was successfully amplified from a single specimen from the South Australian Museum (D 16542). Specimens were chosen at genus level to represent the three major clades of Chitonida; thirty-one ingroup taxa were included in the final analyses presented here, including *Hemiarthrum* and *Choriplax*. Taxa were included only where two or more fragments were available that could be added to alignment with our new sequences. The total species cover 10 of the 14 currently recognised living families in the order Chitonida (Table 1). Two representatives of Lepidopleurida were selected as outgroup taxa; we decided *a priori* to conduct an initial analysis of Chitonida as the ingroup, and to later expand taxon selection to total-group Polyplacophora if either of the target species (*Hemiarthrum* and *Choriplax*) were not clearly resolved in a derived position within Chitonida.

DNA extraction, amplification and sequencing

DNA was extracted from 18 specimens that were preserved in 96% ethanol (Table 1); the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) was used for *Choriplax grayi* and all *Callochiton* spp. For *Leptoplax coarctata*, *Leptoplax curvisetosa*, *Craspedochiton tesselatus*, *Choneplax indica*, *Craspedochiton laqueatus*, *Cryptoconchus porosus*, *Cryptoplax oculatus* and *Hemiarthrum setulosum* we applied the Nucleo Spin Tissue Kit (Macherey-Nagel, Dueren, Germany). For extraction procedures we followed the manufacturers' instructions.

Standard markers COI (partial), 16S (partial), 18S (partial) and 28S (partial) were amplified with three different polymerase

Table 3. Primers used for sequence amplification and relevant references using these primers for polyplacophoran sequences

Fragment and primer name	Primer sequence (5′ → 3′)	Original source	Application in Polyplacophora
COI: LCO1490	GGT CAA CAA ATC ATA AAG ATA TTG	Folmer *et al*. 1994	Okusu *et al*. 2003
COI: HCO2198	TAA ACT TCA GGG TGA CCA AAA AAT CA	Folmer *et al*. 1994	Okusu *et al*. 2003
COI: HCOout	CCA GGT AAA ATT AAA ATA TAA ACT TC	Carpenter and Wheeler 1999	Sigwart *et al*. 2011
16s: 16sa	CGC CTG TTT ATC AAA AAC AT	Xiong and Kocher 1991	Okusu *et al*. 2003
16s: 16sb	CTC CGG TTT GAA CTC AGA TCA	Xiong and Kocher 1991	Okusu *et al*. 2003
18s: 18sa2.0	ATG GTT GCA AAG CTG AAA C	Whiting *et al*. 1997	Okusu *et al*. 2003
18s: 18sa9R	GAT CCT TCC GCA GGT TCA CCT AC	Giribet *et al*. 1996	Okusu *et al*. 2003
28s part 1: 28sF	GAC CCG TCT TGA AGC ACG		Giribet *et al*. 2006
28s part 1: 28sR	CCA CAG CGC CAG TTC TGC TTA C		Giribet *et al*. 2006
28s part 2: 28sF2	ACC TAT TCT CAA ACT TTA AAT GG		Giribet *et al*. 2006
28s part 2: 28sR2	GAC TTC CCT TAC CTA CAT		Giribet *et al*. 2006

chain reaction (PCR) systems (Table 3). For all fragments of *Callochiton* spp. and of *Choriplax grayi* Sigma Taq Polymerase (Fermentas, Burlington, Canada) was applied. Per sample we added 2.5 µL 10 × buffer (supplied by manufacturer), 2.0 µL $MgCl_2$ (supplied by manufacturer), 0.125 µL polymerase, 2.5 µL dNTPs (conc. 2 mM each, Fermentas) and 0.5 µL of each primer (conc. 10 pM, Metabion, Martinsried, Germany); 1.0 µL of genomic DNA was added and the mix was filled with molecular water up to 25 µL. For PCR conditions we applied 94°C for 360 s for the initial step, then 94°C for 60 s, 50°C for 60 s, 72°C for 90 s for 40 cycles, with a final elongation of 72°C for 360 s.

For all fragments of the superfamily Cryptoplacoidea the Multiplex PCR Kit (Qiagen) was used. Per sample we used: 1.0 µL Q-solution (supplied by manufacturer), 5.0 µL Multiplex solution, 0.8 µL of each primer (conc. 10 pM, Metabion), 1.4 µL molecular water, and added 1.0 µL of genomic DNA. For PCR conditions we applied 95°C for 900 s for the initial step, then 95°C for 30 s, 50°C for 90 s, 72°C for 90 s for 40 cycles, with a final elongation of 72°C for 360 s.

All fragments of *Cyanoplax dentiens* (two individuals) were amplified using Illustra PuRe Taq Ready-To-Go PCR beads (GE Healthcare, Little Chalfont, UK). A mix of 0.5 µL of each primer (conc. 10 pM, Metabion) plus 23 µL of molecular water was added to 1.0 µL of genomic DNA. For PCR conditions we applied 95°C for 300 s for the initial step, then 95°C for 45 s, 50°C for 50 s, 72°C for 200 s for 36 cycles, with a final elongation of 72°C for 600 s.

In *Leptoplax coarctata* and *L. curvisetosa* we used the primer HCOout instead of the universal primer HCO2198. Sequencing reactions used the same primers as for amplification. The complete sequencing process was carried out on an ABI 3730 48 capillary sequencer by the Sequencing Service Unit of the Ludwig-Maximilians-Universität Munich.

Sequence selection and phylogenetic analyses

Taxa were included for analysis only where there were at least two gene regions with large overlap with the main dataset. All sequence amplicons and previously published sequences were subjected to BLAST search to exclude contamination.

Single stranded sequence contigs were assembled with CAP3 (Huang and Madan 1999) and subsequently checked with BLAST for contamination. Alignments for analyses under MrBayes were determined via ClustalW2 (Larkin *et al*. 2007). (Analyses in POY used unaligned sequences using the dynamic parsimony criterion.) Previously published sequences were trimmed to the overlap length of *de novo* sequences.

Bayesian inference was implemented in MrBayes ver. 3.2.1 (six chains, 5 million generations, sampling one tree every 1000 generations, temperature 0.02, discarding *a priori* 25% burn-in). Data were partitioned by gene and a separate most-appropriate model of evolution was determined for each gene region (partition) using the Akaike Information Criterion (AIC) determined under jModelltest v0.1.1 (Posada 2008). Three partitions (COI, 18S, 28S) used GTR+I+Γ; the other (16S) partition used HKY+I+Γ. Convergence was only computationally achievable under low temperature; however, the analyses resulted in low posterior probabilities (pp) of convergence at the end part of the analysis (pp = 0.05).

As a second independent line of evidence, separate parsimony analyses on the aligned sequences were conducted under POY ver. 4.1.2 (Varón *et al*. 2010). A modified Incongruence Length Difference (ILD) test (Mickevich and Farris 1981) was used to assess sensitivity of the results to different parameter values as well as incongruence among the separate results from independent gene regions. Data were analysed under nine different parameter sets: three different indel : transversion ratios (1 : 1, 2 : 1 and 4 : 1) and three different transversion : transition ratios (1 : 1, 2 : 1, 4 : 1). The minimum ILD $((L_{COI+16S+18S+28S} - L_{COI+L16S+L18S+L28S})/L_{COI+16S+18S+28S}$, where L_i refers to the length of the most parsimonious trees for a given partition i) parameter set (equal weights) was used for final analysis.

The combined analysis of four data partitions used 250 random addition sequence replicates followed by subtree-pruning and regrafting (SPR), tree-bisection-reconnection (TBR) branch-swapping and tree-fusing (Goloboff 1999). Jackknife support values were calculated from 1000 replicates of randomly removing half of the characters, building 10 trees by random addition sequence followed swapping (TBR/SPR), with up to five minimum-cost trees retained (i.e. up to 50 stored trees per replicate).

Morphological examination of Choriplax grayi

Ethanol preserved material from some of the museums listed below were used for this study. From one specimen (SAMA

D16542, the specimen which was also used in DNA amplification) the terminal valves and valve ii were dissected, and the radula as well as a part of the gonads were carefully removed.

Light microscopic photographs were taken with a Jenoptic (Jena, Germany) ProgRes C12P^{plusP} digital camera mounted on an Olympus SZX12 stereo microscope (Tokyo, Japan). Image acquisition was controlled by ProgRes Capture Pro 1.0.0-control software, and afterwards z-stacks were processed with Auto-Montage (Synoptics) software. For the examination of the gonad part, it was dehydrated using the AXA method of Kees van Achterberg (Leiden, The Netherlands). His method is based on the alcohol-ethyl acetate method used for the preparation of Syrphidae (Vockeroth 1966). In van Achterberg's modified version the ethyl acetate was replaced by amyl acetate. The soft part was placed in a bowl with a 40 : 60 mixture of xylene and 96% ethanol for 24 h. Then the liquid was poured off and replaced with 100% amyl acetate, where it was permitted to evaporate for at least 24 h.

For scanning electron microscopy (SEM), specimens were sputter coated for 135 s (Polaron Equipment Ltd, Watford, UK) and were examined with a LEO 1430VP SEM (Electron Microscopy Ltd, Cambridge, UK).

One specimen was sent to Dr Peter Bartsch (Museum fuer Naturkunde der Humboldt-Universitaet zu Berlin, Germany) for an X-ray study of the animal.

Geographic coordinates for localities not available from original datasets were established from the Gazetteer Client (2004) and added in square brackets.

Abbreviations

AMS – Australian Museum, Sydney, Australia.
NHMUK – Natural History Museum, London, United Kingdom.
NMV – Museum of Victoria, Melbourne, Australia.
SAMA – South Australian Museum Adelaide, Australia.
WAM – Western Australian Museum, Perth, Australia.

Results

Phylogenetic analyses

The consensus tree obtained with MrBayes supports four major clades (Fig. 3), and these clades are also recovered with parsimony analysis under POY. Parsimony analysis produced four most parsimonious trees (MPTs) of cost 6064, with broadly the same topology (specific deviations are explained below). The points of difference are the arrangement of the four clades, and some topological differences among individual taxa, especially within the Cryptoplacoidea. These are indicated in Fig. 3 where jackknife support values are unavailable.

The results of our analyses support the monophyly of three major superfamilies within Chitonida, and that Mopalioidea + Cryptoplacoidea form a clade (suborder) Acanthochitonina based on the genera sampled (Fig. 3). Parsimony analysis also resolved Acanthochitonina but with poor support (jackknife support 0.34) and excluding *Plaxiphora*. (The only taxon of possibly uncertain placement in terms of clade membership is *Plaxiphora*, as under parsimony it resolves within Chitonoidea).

In contrast to all previous phylogenetic hypotheses, we find *Callochiton* as sister to Acanthochitonina (Fig. 3). However, support for this aspect of topology under parsimony was also low (jackknife support 0.34). Chitonoidea including *Callochiton* is paraphyletic; we refer to Chitonoidea *sensu stricto* to mean the suborder excluding *Callochiton* (Fig. 3). Chitonoidea *s.s.* is sister to the remaining Chitonida.

Nuttallochiton, which was previously included in Mopaliidae (Mopalioidea), is resolved in Cryptoplacoidea. *Hemiarthrum* is confirmed as a member of Cryptoplacoidea, as proposed by Sirenko (2006), in a derived position within that superfamily. *Choriplax* is unambiguously resolved within Mopalioidea (Fig. 3).

Systematic taxonomy

Order **CHITONIDA** Thiele, 1909

Suborder **ACANTHOCHITONINA** Bergenhayn, 1930

Superfamily **MOPALIOIDEA** Dall, 1889

Family **CHORIPLACIDAE** Ashby, 1928

Genus ***Choriplax*** Pilsbry, 1894*a*

Microplax H. Adams & Angas, 1864 (*non* Fieber, 1861): 194.
Choriplax Pilsbry, 1894*a* (*nom. nov.* pro *Microplax* H. Adams & Angas, 1864): 139.
Choryplax [sic] Sirenko, 1993 [*lapsus calami* for *Choriplax* Pilsbry, 1894*a*]: 115.
Type species: *Microplax grayi* H. Adams & Angas, 1864, by original designation.

Material examined

Holotype. NHMUK 1877.11.7.2: New South Wales, Port Jackson, Sydney Harbour, under stones at low water.

Additional material. One specimen AMS C151131: West Australia, off Freemantle, west side of Carnac Island (32°7′S 115°40′E), on brown algae, cryptic fauna on sponge, in 6 m, leg. Neville Coleman, 18.xii.1971, det. T. Cochran 1985 (as 'cf.'); 1 specimen SAMA D16542: Tasmania, Fluted Cape, Bruny Island [43°22′S 147°22′E], living on red alga *Sonderopelta coriacea* [on older label identified as '*Ethelia australis*'], in 10 m, leg. S. A. Shepherd, 2.ii.1972, det. K. L. Gowlett [now Gowlett-Holmes], 21.vi.1983; 1 specimen WAM S16380: West Australia, Jurien Bay, SW of Essex Rocks (JWAM08/Q3) (30°21′09″S 114°59′18″E), weed washing, 7–11 m, Sampey *et al.* 1.v.2005; 1 specimen WAM S16289: West Australia, Dry Lump, West of Green Head (JWAM11/Q1) (30°07′19″S 114°56′47″E), 5–6.2 m, Sampey *et al.* 2.v.2005. (Total of five specimens examined; Table 2.)

Original diagnosis

For *Microplax*: *Testa elongata. Valvæ partibus externis parvis, cordiformes, disjunctæ; laminæ insertionis magnæ. Limbus nudus.* In this genus the exposed parts of the valves are very small, and are completely disunited, the distance between them in *Microplax grayi* being nearly equal to the length of the valves. (H. Adams and Angas 1864: p. 194.)

For *Choriplax*: This is an extremely peculiar and isolated genus, and forms, I am disposed to believe, a distinct family of the

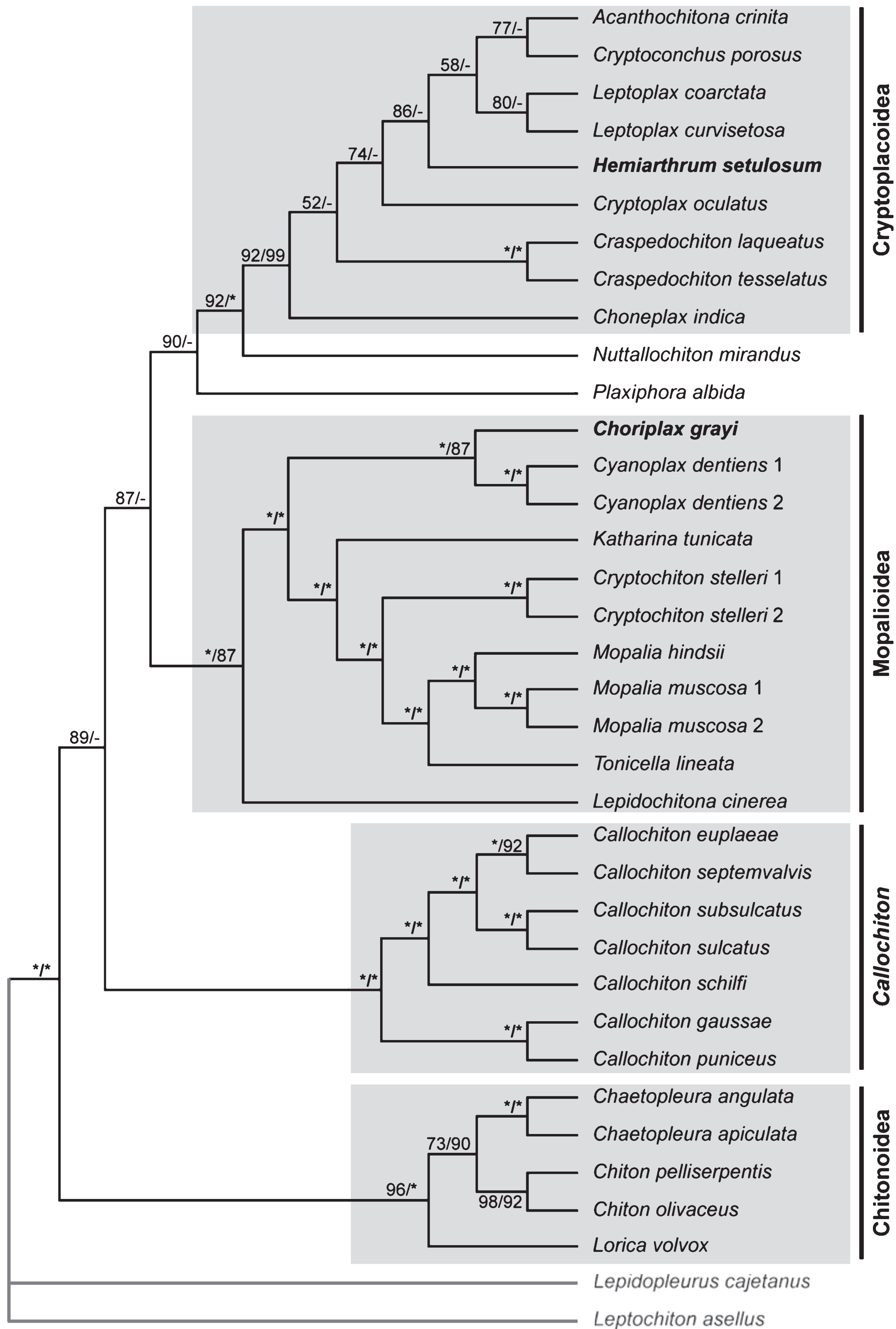

Fig. 3. Phylogeny of Chitonida; preferred tree based on MrBayes results. Support values for nodes (a/b) show results from two independent analyses from (a) posterior probabilities from MrBayes and (b) jackknife support values for parsimony analysis in POY. The position of *Plaxiphora* is equivocal between the two analyses.

Eoplacophora or slitless Chitons – that is, if the slits really prove to be completely absent, for the unique type has not been disarticulated. In some features it recalls the *Acanthochitidæ* [sic]. (Pilsbry 1894*a*: p. 139.)

Distribution

Southern, eastern and western part of Australia, south of 30°, also in Tasmania. Recent.

Choriplax grayi (H. Adams & Angas, 1864)

Microplax grayi H. Adams & Angas, 1864: 194.

Chresonymy provided by Gowlett-Holmes (1987), with additional records as follows:

Microplax grayi: Tryon, 1883: 340; Haddon, 1886: 9; Fischer, 1887: 877; Pilsbry, 1894*b*: 69; Van Belle, 1975: 143; Gowlett-Holmes, 1987: 105; 2001: 44; Kaas & Van Belle, 1998: 85.

Choriplax grayi: Pilsbry, 1894*b*: 70; Nierstrasz, 1905: 13; Thiele, 1909: 106; Iredale, 1910*a*: 90; 1910*b*: 158; Iredale & McMichael, 1962: 29; Hyman, 1967: 125, fig. 55D; Van Belle, 1975: 143, pl. 2, fig. 11; Gowlett-Holmes, 1987: 105, figs 1–2; 1998: 180, fig. 3.33; 2001: 44; Ludbrook & Gowlett-Holmes, 1989: 509; Kaas & Van Belle, 1990: 23, fig. 8; 1998: 85; Slieker, 2000: 102, pl. 39, fig. 6; Sirenko, 2006: 35; Lay, 2006: 11, 39, 41, 42, 58, figs 22, 24; Todt *et al*., 2008: 83; Sigwart, 2009: 96.

Choriplax grayi pattisoni: Gowlett-Holmes, 1987: 106; 2001: 44; Kaas & Van Belle, 1990: 23; 1998: 139.

Type material: NHMUK 1877.11.7.2, holotype of *Microplax grayi*: New South Wales, Port Jackson, Sydney Harbour, under stones at low water; SAMA D15019, holotype of *Choriplax grayi pattisoni* (not seen): South Australia, Cape Banks Lighthouse, among storm-washed material on a beach among large kelp.

Type locality: Australia, New South Wales, Port Jackson, Sydney Harbour [33°52′S 151°15′E].

Morphological and anatomical remarks

Detailed descriptions were reported by Ashby (1921), Kaas and Van Belle (1985*a*), and Gowlett-Holmes (1987), but here we present a summary to give context for new observations.

Preceding authors mentioned a granulose tegmentum surface, which is true, but none of these mentioned the occurrence of black pigmented aesthetes (Figs 4*A*, 5*A*, *E*). This character is clearly visible in smaller specimens, but also in the specimen examined by Gowlett-Holmes (1987: fig. 2A, SAMA D16542), which we had the opportunity to re-examine. Where the perinotum extends over the tegmentum margin some ‘granules’ occur at the perinotum (Fig. 6*D*), which correspond to the subsurface aesthetes. Removing the second and the terminal valves allowed a side view of the tegmentum, which is extensive and shows very spongy eaves well perforated with aesthete canals (Fig. 4*F*).

A visual inspection of the articulamentum gives the impression that the unslit condition of the valves is merely an artefact of the holoperipheral growing of this valve layer, where the slits are fused in the lateral growth of the valves. It appears that earlier growth stages could have been slitted (Fig. 2*C*). We attempted to examine this via X-ray in one specimen (AMS C151131). Some structures of the valves may be interpreted as slit rays, but damage to the valves and the wide coverage of the valves by the perinotum make the available pictures unsuitable to definitively prove this hypothesis. If more material becomes available in future this could be clarified by semi-thin sectioning of the valves or disarticulation of early juvenile specimens.

Previous redescriptions of this species report dorsal perinotum elements in a size range of 20–30 µm (Kaas and Van Belle 1985*a*; Gowlett-Holmes 1987); however, the smooth curved needles may attain a length of at least twice the size (Fig. 6). In addition some structures (Fig. 6*C*) deeply embedded in the perinotum could be sensory structures. These ‘granules’ have a diameter of ~5 µm and show small lateral perforations, similar to the lens structures of ocelli (compare Schwabe 2004: fig. 9B). These previous authors failed to find ventral girdle elements, or at least none mentioned the occurrence of them. Ventrally the girdle is sparingly beset all over with straight, sharply pointed conical spicules measuring 15×3.5 µm. They are longitudinally ribbed and deeply embedded in the cuticle (Fig. 7*A–C*).

One specimen (AMS C151131) 18.7 mm in length has an ~3.6 mm long radula membrane with at least 42 transversal teeth rows (Fig. 7*D*, *E*). Of these, 29 rows show mineralised teeth. The cartilage length is 2.1 mm. The present examination confirms the central tooth is asymmetric and the widening of the first lateral tooth. The third uncinal tooth is elongate (omitted by previous authors). Gowlett-Holmes (1987) described the central radula tooth as ‘small weak’, instead it appears that her photo was taken slightly lateral or that an underdeveloped tooth row was used for examination. In contrast, the tooth is tulip-shaped and quite broad (Fig. 7).

Removing tissue for the present genetic analysis revealed the occurrence of immature eggs in the gonads (Fig. 7F). These have a diameter of ~115 µm, are smooth and do not show any chorion processes.

In one specimen (SAMA D16542) ~22 mm in length (Gowlett-Holmes 1987: fig. 2A, B) there are 26 ctenidia at the right and 28 ctenidia at the left side. They are arranged holobranchially and adanal (*sensu* Sirenko 1993, having multiple gill pairs posterior of the nephridiopore), with the gonopores situated between ctenidia 7–8 from posterior and nephridiopore at gill 6–7. The search for a pigment patch in the mantle cavity under the mouth lappet (see Todt *et al*. 2008) was negative.

Discussion

Polyplacophoran phylogeny

The present study addresses the definition and arrangement of the major clades (superfamilies) within Chitonida, and we report several important results to polyplacophoran systematics. The phylogeny recovered in our analyses broadly supports the systematic revision of Sirenko (2006) and other phylogenetic hypotheses that have been published in recent years. With the exception of *Callochiton*, the largest order of living chitons, Chitonida, is divided into two clades: Chitonina and Acanthochitonina. The latter is subdivided into two clades, which conform to the proposed superfamilies Mopalioidea and Cryptoplacoidea (Sirenko 2006). This systematic arrangement also gives morphological support for the first molecular phylogeny for Polyplacophora (Okusu *et al*. 2003). The three major clades in that study align with our tree: their clade ‘A’

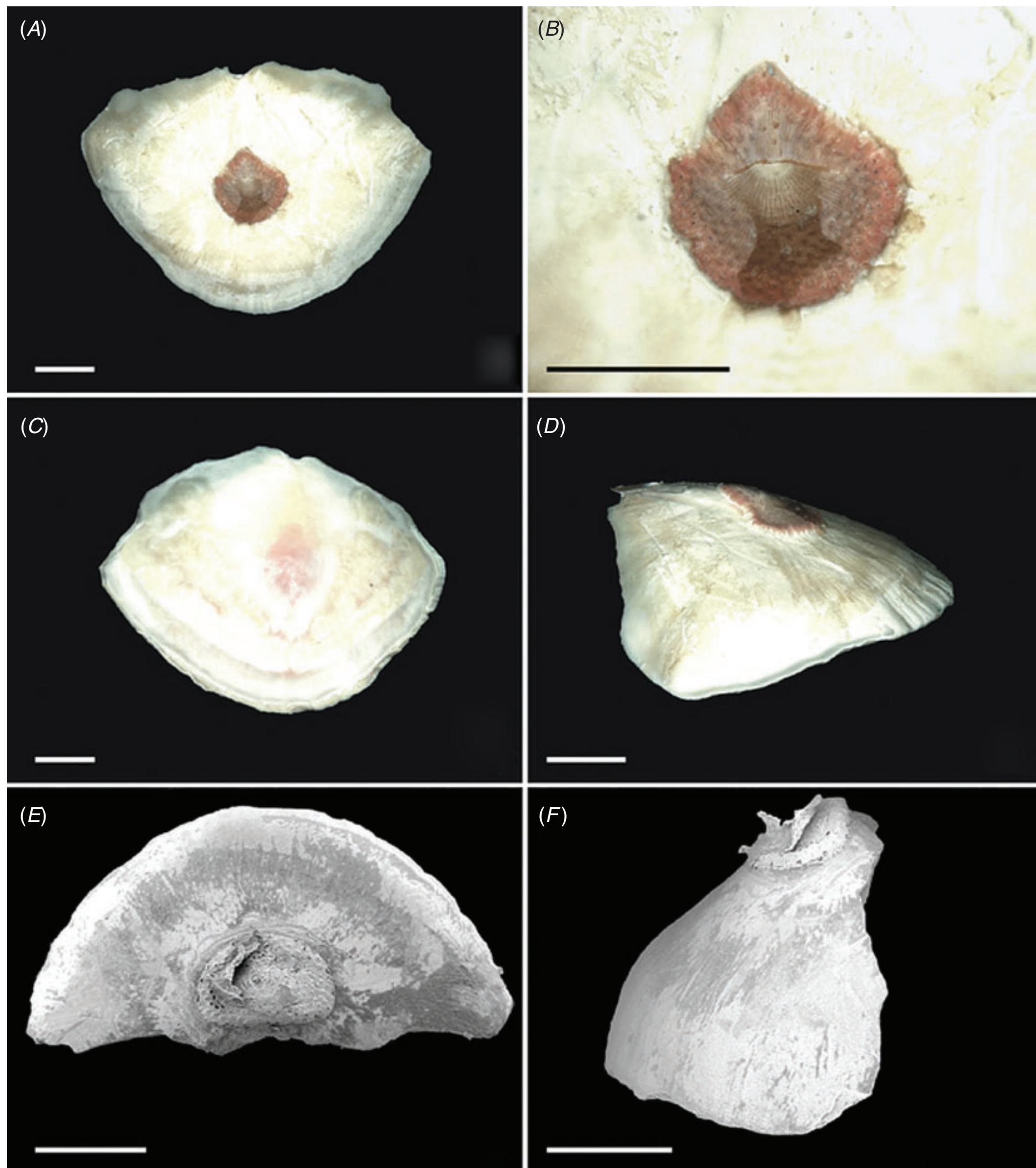

Fig. 4. *Choriplax grayi* (H. Adams & Angas, 1864). (*A*) Dorsal view of tail valve (SAMA D16542), anterior at top; (*B*) tegmentum detail of *A*; (*C*) ventral view of tail valve (SAMA D16542), anterior at top; (*D*) left lateral view of tail valve (SAMA D16542), anterior at left side; (*E*) dorsal view of head valve (SAMA D16542), anterior at top; (*F*) left lateral view of head valve (SAMA D16542), anterior at left side. Scale bars: *A*–*F* = 1 mm.

represents Cryptoplacoidea, clade ‘B’ is Mopalioidea, and clade ‘C’ is Chitonoidea *s.s*.

Two genera are resolved in Cryptoplacoidea that may be classified in Mopalioidea: *Nuttallochiton* and *Plaxiphora*. Placement in Cryptoplacoidea agrees with the findings of Okusu *et al*. (2003). However, this result is equivocal in the case of *Plaxiphora* and it is the only terminal that resolves in a different clade; under parsimony *Plaxiphora* is in Mopaliodea (in agreement with Sirenko 2006). Many of the previously published sequences incorporated into this analysis were generated by Okusu *et al*. (2003) including *Plaxiphora* and *Nuttallochiton*. Another study, also using the same previously published COI sequence, recovered *Nuttalochiton* in Mopalioidea (Sirenko *et al*. 2013). In our systematic presentation of the species considered we have therefore conservatively retained these two genera in Mopalioidea (Table 1; Fig. 3). Other revisions to the most recent systematic classification of the class (Sirenko 2006) are inevitably necessary to achieve an accurate phylogenetic systematic approach. Further data are required to resolve the positions of those specific taxa with confidence, and denser taxon sampling will further determine finer (family-) level systematics.

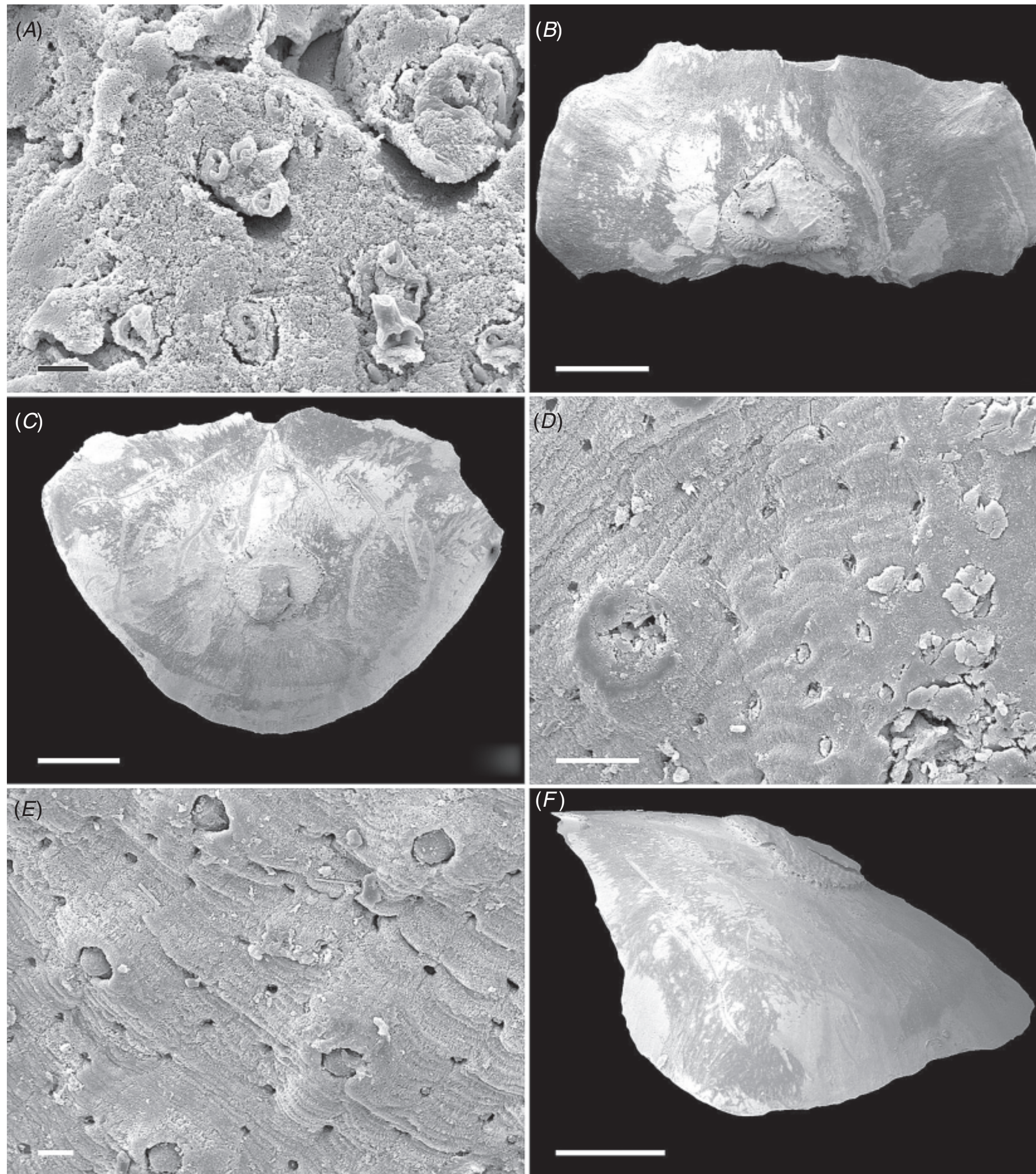

Fig. 5. *Choriplax grayi* (H. Adams & Angas, 1864). (*A*) Detail of head valve (SAMA D16542), anterior at top; (*B*) dorsal view of valve ii (SAMA D16542), anterior at top; (*C*) dorsal view of tail valve (SAMA D16542), anterior at top; (*D*) antemucronal area (SAMA D16542), anterior at top; (*E*) postmucronal area (SAMA D16542), anterior at top; (*F*) left lateral view of tail valve (SAMA D16542), anterior at left side. Scale bars: *A*, *E* = 10 µm; *B*, *C*, *F* = 1 mm; *D* = 20 µm.

Another genus that is transferred from one superfamily to the other within Acanthochitonina is the popular, well studied, giant Pacific chiton *Cryptochiton.*

The idea that *Cryptochiton* is not in Acanthochitonidae is supported by multiple lines of evidence, and it has been included in Mopaliidae in recently published systematic and phylogenetic treatments (Eernisse *et al*. 2007; Kelly *et al*. 2007; Kelly and Eernisse 2008; Sirenko *et al*. 2013) and is generally accepted (e.g. Lord 2011; Schwabe 2012). The key argument to retain *Cryptochiton* in Acanthochitonidae was that early stage juveniles, where the larval shell is still emergent through the dorsal girdle tissue, the tufts of bristles in the girdle armature may be arranged in evenly distributed pairs on either side of the valves and with four tufts around the head valve, as in *Acanthochitona* (Sirenko 2006: 41). Examination of additional material demonstrates this is not a consistent pattern between individuals and the distribution of girdle bristles appears to be random, as in the adults (Lord 2011: fig. 6; J.D. Sigwart, pers. obs.).

The most enigmatic clade of chitons may be *Callochiton*. We find, in contrast to other studies, that *Callochiton* is in a derived position (Fig. 1). Morphological systematics included Callochitonidae within Chitonoidea (Sirenko 2006). Yet previous numerical phylogenetic approaches, from molecular or

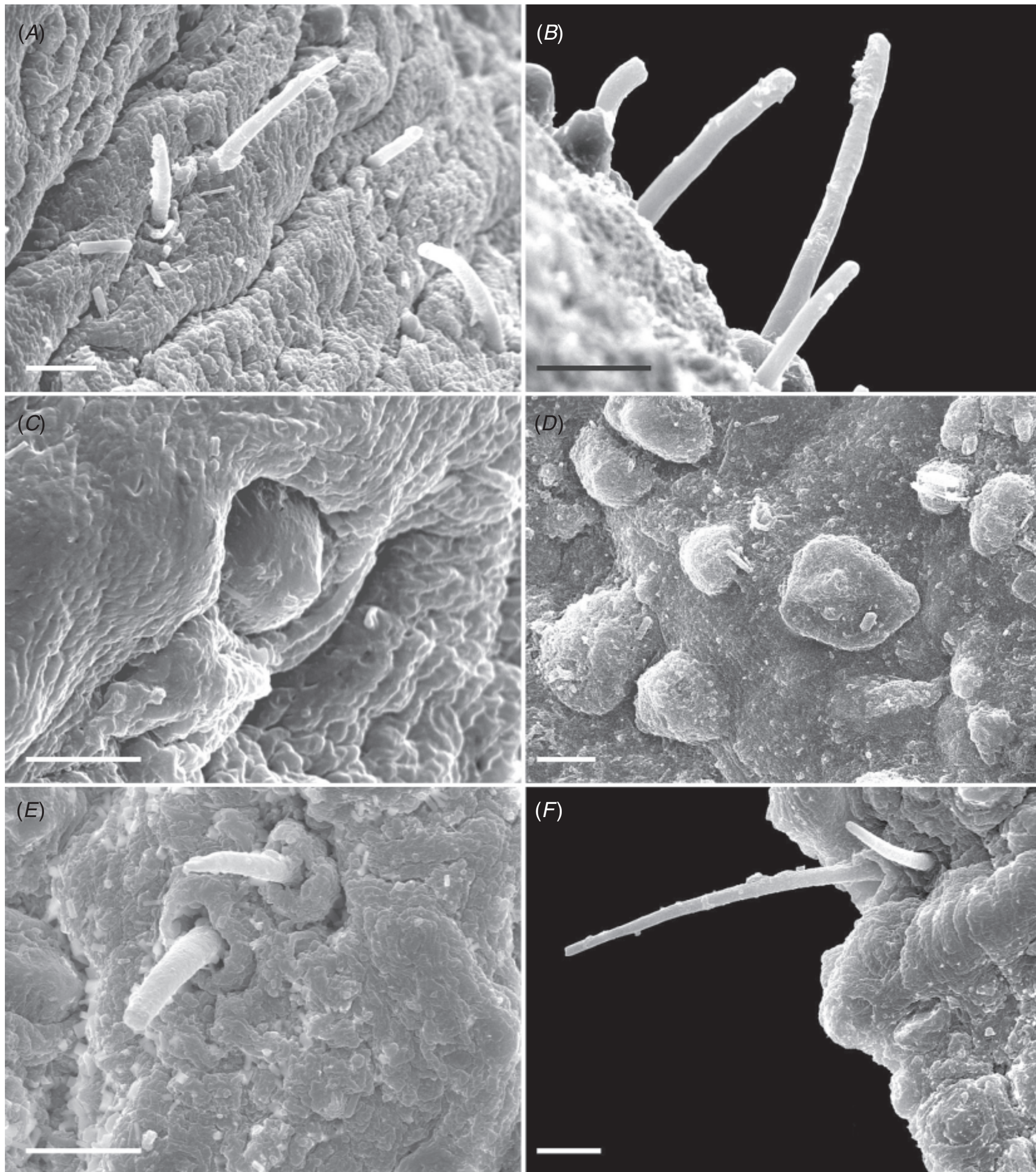

Fig. 6. Dorsal girdle elements of *Choriplax grayi* (H. Adams & Angas, 1864) (SAMA D16542). (*A–C*) Taken from left side of specimen at level of valve ii, close to margin. (*D–F*) Part of perinotum that covered in the complete specimen the left hind margin of valve ii; perinotum very thin. It is assumed that *C* shows a sensoric organ and the knobbles in *D* correspond to the aesthetes of valves. Scale bars: *A*, *B*, *D–F* = 10 µm; *C* = 5 µm.

morphological data, have found *Callochiton* to be sister to Chitonoidea *s.s.* (Buckland-Nicks 1995), sister to all other Chitonida (Buckland-Nicks 2008), or actually outside Chitonida (Okusu *et al*. 2003). On the basis of available evidence, it is clear that *Callochiton* is phylogenetically markedly different to other members of Chitonida. There are four (or five) genera in Callochitonidae Plate, 1901: *Callochiton* Gray, 1847, *Eudoxochiton* Shuttleworth, 1853, *Leloupia* Kaas & Van Belle, 1990, *Vermichiton* Kaas, 1979 and questionably *Quaestiplax* Iredale & Hull, 1929 (Schwabe 2013). To resolve this aspect of chiton phylogeny and systematics would require additional sampling from especially these taxa.

Callochiton presents several unusual features, including pigmented shell eyes with a transparent lens (Baxter *et al*. 1990). Members of *Callochiton* have oocytes that lack the projecting chorion processes found on most Chitonida, a smooth egg hull is the plesiomorphic condition for chitons and is common to the earlier-derived order Lepidopleurida (Sirenko 1993; Buckland-Nicks 1995). However, both of these features – pigmented shell aesthetes and smooth eggs – are also shared with *Choriplax* and may be independently derived multiple times in polyplacophoran evolution (see below).

Shell 'eyes' or extrapigmented aesthetes are known from several groups of chitons. The most complex of these are

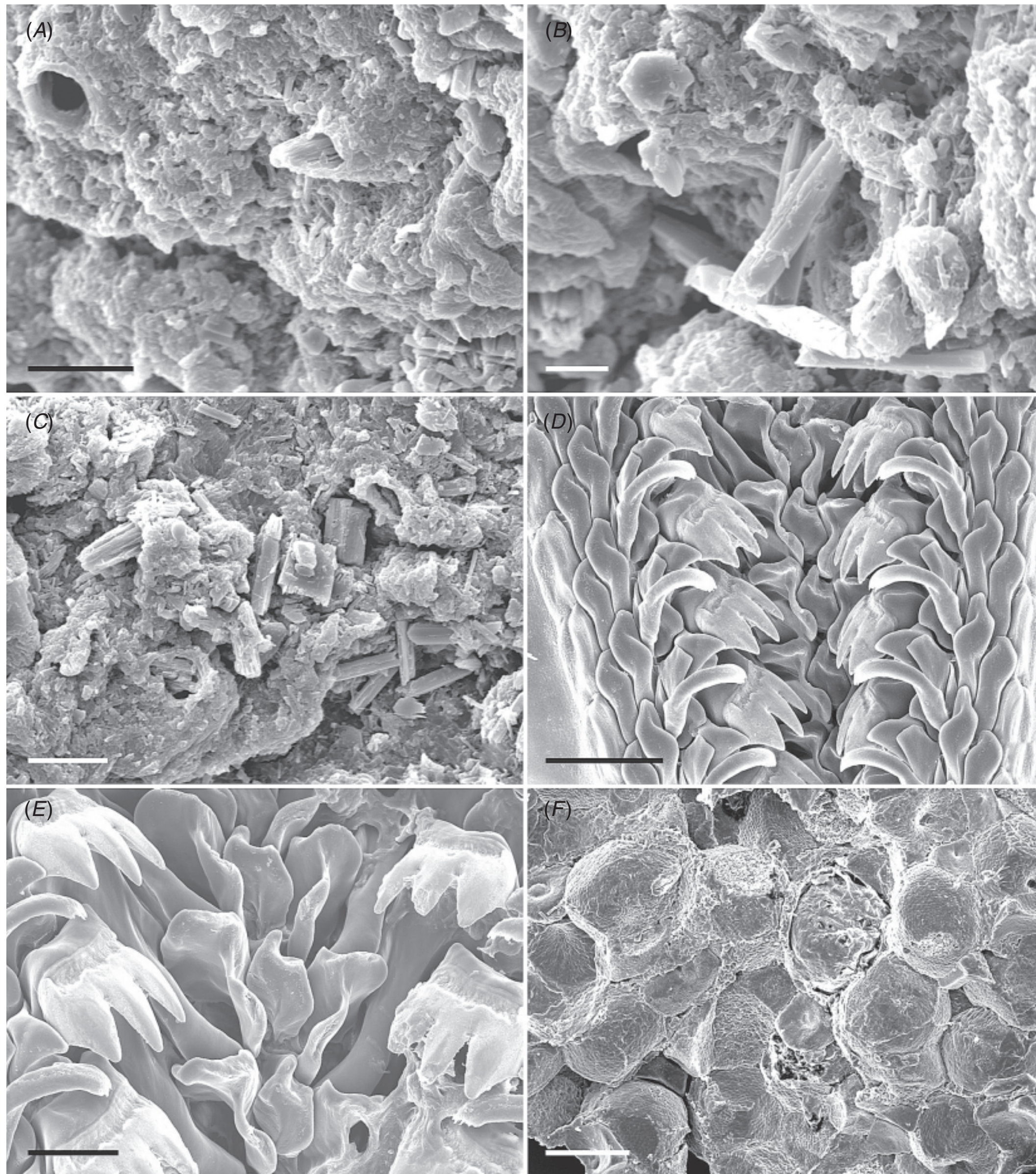

Fig. 7. *Choriplax grayi* (H. Adams & Angas, 1864). (*A*–*C*) Ventral girdle elements of the specimen (SAMA D16542), taken from left side of specimen at level of valve ii, close to margin; (*D*) radula (AMS C151131), anterior portion; (*E*) detail of *D*, showing the central, first, and second lateral tooth; (F) immature eggs (SAMA D16542), *in situ*. Scale bars: *A*, *C* = 10 µm; *B* = 5 µm; *D*, *F* 1= 00 µm; *E* = 50 µm.

found in Chitonoidea *s.s.*, in members of *Stenochiton* H. Adams & Angas, 1864, *Ischnochiton* Gray, 1847, *Chiton* Linnaeus, 1758, and especially *Acanthopleura* Guilding, 1829 (Schwabe 2010; Speiser *et al*. 2011). The presence of shell eyes in species of *Callochiton* represents an independent origin of pigmented ocelli within chitons. And pigmented aesthetes are also present in *Choriplax*; yet, so far, no species in Cryptoplacoidea is known to show such modified aesthetes.

We conclude from the balance of evidence that *Callochiton* is probably not a member of Chitonoidea. This has been repeatedly suggested from morphological (Fig. 1*A, B*) and molecular data (Figs 1*C*, 3). Whether Chitonoidea *s.s.* represents the earliest-diverging group of Chitonida (Fig. 3), or whether Chitonoidea *s.s.* and *Callochiton* may represent sister groups (parsimony analysis not shown), is equivocal.

Whither *Choriplax?*

Choriplax represents an apparent mosaic taxon that has confounded classification. The results of our analysis show *Choriplax* in a derived position aligned to Mopalioidea (Fig. 3), which represents a different new placement contrasting to

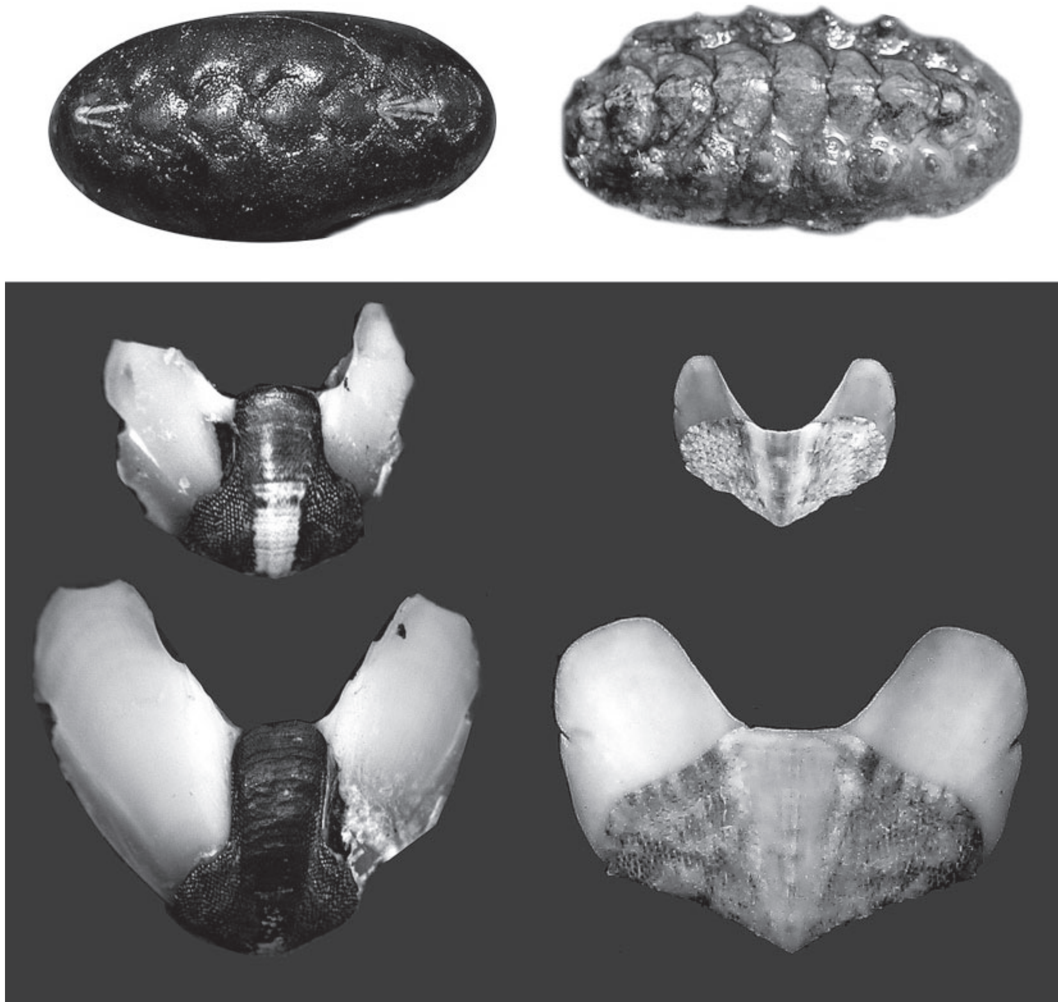

Fig. 8. Valves of *Katharina tunicata* (left, Mopalioidea) and *Acanthochitona crinita* (right, Cryptoplacoidea) in dorsal view. Each species is shown with a live specimen (top) and representative intermediate valve of a juvenile (middle) and adult (bottom). The dark inner area is the tegmentum, the exposed valve surface *in vivo*; the lighter, outer area is the insertion plate formed by the articulamentum. The nature of ontogenetic growth of valves in Mopalioidea (left) maintains a roughly equal size tegmentum while articulamentum expands; in Cryptoplacoidea (right) valve growth is isometric.

any phylogenetic hypothesis previously proposed from morphological evidence.

Kaas and Van Belle (1985*a*) followed the convention at the time, placing *Choriplax* within the basal clade Lepidopleurida, because it lacks shell insertion plates. The most recent, broadly accepted systematic revision of Polyplacophora placed it in Acanthochitonina on the basis of gill arrangements and discounting reduced shell insertion plates as probably convergent (Sirenko 2006). There are few apparent morphological features to tie *Choriplax* to other species in Mopalioidea; however, the synapomorphies of Mopalioidea are not presently well defined and do not exclude this classification.

The radula in *Choriplax* is asymmetric, a character which is also known from *Cryptochiton* von Middendorff, 1847 and members of *Notoplax* A. Adams, 1861, as well as callochitonids (cf. Saito 2004). Thus there are apparently isolated instances of radular asymmetry in Mopalioidea, Cryptoplacoidea and *Callochiton*; this asymmetry is likely an adaptation associated with feeding strategy in particular lineages or taxa (Hickman 1980).

We found that the gill arrangement in *Choriplax* is not abanal but adanal (*contra* Gowlett-Holmes 1987), because there is more than one gill behind the nephridiopore, indicative of bi-directional growth of the gill row (as defined in Sirenko 1993, 2006). This is the typical characteristic in Lepidopleurida; all other members of the order Chitonida have abanal gill rows (unidirectional, anterior growth of the gill row). Within Lepidopleurida, the position of gonopores and nephridiopores is highly variable between species (Sigwart 2008). Yet of all the species studied to date, *Choriplax* is the only member of Chitonida with multiple ctenidia posterior to the nephridiopore.

The shell valves show a quite spongy eave, which without doubt enables a lot of nerve connections with the aesthetes. In most species within Chitonida, the aesthete canals diverge from a

diagonal perforation in line with the insertion slits, connecting to the body muscle through pores along this diagonal line in the ventral shell (Vendrasco *et al*. 2008). However, there are no apparent perforations in the articulamentum of *Choriplax*. Interestingly, Ashby (1921: 139) was seemingly unsure about the slitless condition in *Choriplax*, referring to a '. . . partial or entire absence of slits' and continued: 'While in the undissected shell under examination I cannot detect any slits in any of the insertion plates, I cannot say that they do not exist in a modified form'. He even goes so far that, one page later, he speculated 'I would suggest the probability that in the juvenile stage some evidence of slits may exist and disappear in the mature or senile form' (Ashby 1921: 140). This appears to have been overlooked in subsequent literature, but seems to be the correct interpretation of the ontogeny.

Another key species in this analysis, *Hemiarthrum*, is also in a derived position, but not related to *Choriplax*. The placement of *Hemiarthrum* agrees with the established morphological classification (Sirenko 2006). This supports the idea that insertion plates have been lost at several independent points in polyplacophoran evolution (Sirenko 1997; Sirenko 2006). Based on the taxa sampled here, we identify at least one shell-reduction event within the stem of Cryptoplacoidea and two separate independent shell-reductions within Mopalioidea, as independently derived apomorphies of *Cryptochiton* and *Choriplax* (and potentially independently again in other taxa not included in this analysis). This is further evidence of the plasticity of shell form in Mollusca, which is well known. However, it is somewhat unsatisfying as evidence for a radical systematic reclassification of *Choriplax.* We therefore considered what patterns of shell reduction – including, by extension, the loss of insertion plate slits – can be observed in the two clades, Mopalioidea and Cryptoplacoidea.

We speculate that the evolutionary process of shell reduction can be visualised as occurring in two distinct ways, by reduction of the tegmentum (exposed dorsal aspect), or by extension of the articulamentum (the ventral, internal shell aspect). These are not identical: in the former, the total valve size and shape perhaps stays the same, but the tegmental area of the valve is reduced in proportion to the whole valve profile. In the latter scenario, the plesiomorphic valve extends outwards at one or all margins, so that the derived state achieves the same final ratio of insertion lamina to tegmentum, but by a different mechanism. The difference between these two processes should be quite clear in ontogeny. That is, in the former case (reducing tegmentum) the ratio of tegmentum to insertion lamina should remain constant through post-larval ontogeny. In the alternative scenario (increasing articulamentum) the ration of tegmentum to insertion lamina should progressively decrease through growth – that is, the tegmental area grows more slowly than the spreading of the articulamentum, so there is relatively more and more insertion plate as the valve grows.

In comparing the totality of available specimens for *Choriplax grayi*, the tegmental area of the valves remains nearly constant through ontogeny, while the articulamentum increases in all directions (Gowlett-Holmes 1987: 107). This followed a remark by Kaas and Van Belle (1985*a*) that a feature separating the second proposed epithet, *C. grayi pattisoni*, was its proportionately smaller tegmentum, which was revealed to be an artefact of ontogeny.

Within Mopalioidea, *Cryptochiton* has a tiny area of tegmentum that is retained through the first few months of life (Lord 2011), which is subsumed by the girdle, and the tegmentum is present only as a holoperipheral apex of the dissected shell. This represents a more extreme reduction of proportional tegmentum than *Choriplax*. Another taxon with a distinctively reduced tegmentum, *Katharina*, follows the same pattern but to a lesser extreme (Fig. 8), where the width of the tegmentum stays approximately the same during growth while the articulamentum expands around it. By contrast, *Acanthochitona* spp. also have a girdle that encroaches on the tegmentum, in comparison with the 'typical' valve shape in Chitonoidea *s.s.*, *Callochiton* and most Lepidopleurida. However, comparison of valves from younger and older individuals of *Acanthochitona crinita* reveals that the proportion of tegmentum to insertion laminae is constant; both aspects grow at the same rate meaning the valve growth appears isometric (Fig. 8).

We propose this is a morphological synapomorphy that unites many members of Mopalioidea – within Mopalioidea, shell reduction was achieved via expansion of the articulamentum. By contrast, members of Acanthochitonoidea represent a separate evolutionary experiment in shell reduction, using the opposite mechanism of tegmental reduction. This is not to suggest that there is a monophyletic group of reduced-shell-bearing species within each of the two superfamilies (e.g. *Katharina* and *Cryptochiton* both have 'reduced' tegmentum, but *Tonicella* has 'normal' rectangular shell valves; therefore within Mopalioidea shell reduction is clearly paraphyletic, as illustrated in Fig. 3). But from available phylogenetic data, the mechanism to achieve reduced tegmentum appears to be different in the two superfamilies. This requires further investigation both in terms of morphometrics and increased taxon sampling for the phylogeny of suborder Acanthochitonina.

The position of specific enigmatic taxa, particularly *Choriplax*, is clearly critically important to understanding deeper patterns in polyplacophoran evolution. Single morphological features (shells, radula) are not informative in isolation. It is well known that shell reduction has occurred multiple times within molluscs (e.g. cephalopods, opisthobranch and pulmonate gastropods, teredinid bivalves) and several times independently within some of those groups (e.g. Wägele and Klussmann-Kolb 2005). That chitons gained or lost insertion plates several times is in line with this general pattern of molluscan evolution. This new phylogeny supports a growing consensus on chiton systematics, and an important shell-based synapomorphy for two major clades.

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Mitogenomics does not resolve deep molluscan relationships (yet?)

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ABSTRACT

The origin of molluscs among lophotrochozoan metazoans is unresolved and interclass relationships are contradictory between morphology-based, multi-locus, and recent phylogenomic analyses. Within the "Deep Metazoan Phylogeny" framework, all available molluscan mitochondrial genomes were compiled, covering 6 of 8 classes. Genomes were reannotated, and 13 protein coding genes (PCGs) were analyzed in various taxon settings, under multiple masking and coding regimes. Maximum Likelihood based methods were used for phylogenetic reconstructions. In all cases, molluscs result mixed up with lophotrochozoan outgroups, and most molluscan classes with more than single representatives available are non-monophyletic. We discuss systematic errors such as long branch attraction to cause aberrant, basal positions of fast evolving ingroups such as scaphopods, patellogastropods and, in particular, the gastropod subgroup Heterobranchia. Mitochondrial sequences analyzed either as amino acids or nucleotides may perform well in some (Cephalopoda) but not in other palaeozoic molluscan groups; they are not suitable to reconstruct deep (Cambrian) molluscan evolution.

Supposedly "rare" mitochondrial genome level features have long been promoted as phylogenetically informative. In our newly annotated data set, features such as genome size, transcription on one or both strands, and certain coupled pairs of PCGs show a homoplastic, but obviously non-random distribution. Apparently congruent (but not unambiguous) signal for non-trivial subclades, e.g. for a clade composed of pteriomorph and heterodont bivalves, needs confirmation from a more comprehensive bivalve sampling. We found that larger clusters not only of PCGs but also of rRNAs and even tRNAs can bear local phylogenetic signal; adding *trnG–trnE* to the end of the ancestral cluster *trnM–trnC–trnY–trnW–trnQ* might be synapomorphic for Mollusca. Mitochondrial gene arrangement and other genome level features explored and reviewed herein thus failed as golden bullets, but are promising as additional characters or evidence supporting deep molluscan clades revealed by other data sets. A representative and dense sampling of molluscan subgroups may contribute to resolve contentious interclass relationships in the future, and is vital for exploring the evolution of especially diverse mitochondrial genomes in molluscs.

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1. Introduction

Mollusca are a megadiverse phylum with first reliable molluscan shell records known from Precambrian/Cambrian border. Since then molluscs conquered almost all habitats and dramatically increased their body plan disparity, and diversity of sizes and food habits. Despite the wealth of shelled fossils, basal molluscan palaeontology is hotly disputed (see review by Parkhaev (2008)), and surprising findings with the potential to change earlier paradigms are made (e.g. Smith and Caron, 2010). Earlier morphological and molecular approaches on the origin and deep phylogeny of molluscs lead to contradictory results.

Morphological analyses usually proposed one of two major hypotheses. The first is the Testaria concept, with small worm-like molluscs without a shell (Solenogastres and Caudofoveata) as sister or paraphyletic ancestral grade of Polyplacophora (chitons, with shell plates) sister to shell-bearing Conchifera (Monoplacophora, Scaphopoda, Bivalvia, Gastropoda, Cephalopoda) (Salvini-Plawen and Steiner, 1996; Haszprunar, 2000). The second is the Aculifera hypothesis (Ivanov, 1996; Scheltema, 1993, 1996; Scheltema and Schander, 2006). In the latter, Conchifera are sister to aculiferan classes (aplacophorans and Polyplacophora), i.e. those without a true shell built by a shell gland but having a mantle covered by a cuticle with sclerites. The competing hypotheses have massive implications on the evolution of molluscan body plans. The Testaria hypothesis implies progressive evolution from worm-like to shell-bearing molluscs, while under the Aculifera concept complex ancestors had a true shell or at least polyplacophoran-like shell plates, and aplacophoran worm-like body plans are secondarily simplified.

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Older molecular studies using nuclear ribosomal genes or multi-locus markers did not usually recover monophyletic Mollusca or molluscan classes, and interclass relationships were unconventional and heterogeneous (Passamaneck et al., 2004; Giribet et al., 2006; Wilson et al., 2010); those studies including Monoplacophora recovered a taxon Serialia, i.e. Polyplacophora plus Monoplacophora, contradicting morphology-based hypotheses. For the first time using true rather than contaminated 18S rRNA sequences of Solenogastres Meyer et al. (2010) recovered monophyletic Mollusca and Serialia; the limits of 18S as single marker were reflected by recovering non-monophyletic bivalves, and increased substitution rates with biased base composition were detected in Patellogastropoda, Cephalopoda and Solenogastres (Meyer et al., 2010). Analyzing sets of 79 respectively 18 ribosomal protein coding genes Meyer et al. (2011) recovered monophyly of Mollusca and of all the five molluscan classes included (Monoplacophora, Solenogastres, Scaphopoda were missing). However, interclass relationships were unconventional, with a clade of cephalopods and the single caudofoveate sister to Polyplacophora plus a clade of Gastropoda and Bivalvia. Further recent molecular analyses based on selected housekeeping genes (Vinther et al., 2011), large-scale EST data on all classes except Monoplacophora (308 gene regions, Kocot et al., 2011), and transcriptomes of Mollusca including Monoplacophora (1185 gene regions, Smith et al., 2011). All recovered monophyletic Mollusca, Aculifera and Aplacophora, and all rejected the Testaria concept. Apart from such congruence, however, each of the recent studies recovered different conchiferan interclass relationships, e.g. with gastropods and bivalves forming a species-rich taxon Pleistomollusca (Kocot et al., 2011; Vinther et al., 2011; Meyer et al., 2011), Cephalopoda sister to Aculifera (Vinther et al., 2011), or Monoplacophora plus Cephalopoda clustering at the base of Conchifera (Smith et al., 2011). There is broad consensus on the lophotrochozoan relationships of Mollusca. Usually annelids (including Sipuncula) and, more recently, entoprocts (Kamptozoa) were suggested as potential sisters to Mollusca on morphological grounds (Haszprunar and Wanninger, 2008). However, none of the recent or any other, earlier molecular studies resolved the sistergroup relationship of Mollusca convincingly. Broad transcriptome-based analyses (Dunn et al., 2008; Philippe et al., 2009; Pick et al., 2010) recovered molluscs as basal offshoot of (non-platyzoan) lophotrochozoans. Among metazoans, the origin and early evolution of molluscs still is one of the greatest mysteries.

The gene content of metazoan mitochondrial genomes is highly conserved (see review by Gissi et al. (2008)). Usually there are roughly 15 kb referring to 13 protein coding genes (PCG), 2 ribosomal RNA (rRNA) and 22 transfer RNA (tRNA) genes plus a variably long non-coding "control-region" arranged on the two strands of the circular mt genome (Boore and Brown, 1994). Mitochondrial genomic sequence data are widely used for resolving phylogenetic relationships of several animal taxa, e. g. in placental mammals (Janke et al., 1994) or modern birds (Pacheco et al., 2011), but also were successfully applied to deep phylogeny. Annelida rather than sister of arthropods were shown to be related with Mollusca (Boore and Brown, 2000; Boore and Staton, 2002). Mitochondrial genome analyses also contributed to resolving Sipuncula, previously considered as potential sistergroup of molluscs, as part of Annelida. While supporting the monophyly of Lophotrochozoa sister to Ecdysozoa, unfortunately, mitochondrial sequence analyses did not provide much signal for resolving inner lophotrochozoan and deep molluscan relationships (Boore et al., 2004). At that time, only 10 complete molluscan mitogenomes were available, covering 5 of 8 classes. Mitogenomic sequence analyses by Dreyer and Steiner (2004) and Waeschenbach et al. (2006) showed non-monophyletic molluscs in variable outgroup relationships, letting the former authors doubt on the usefulness of mitochondrial genes for resolving deep relationships, while considerable potential was stated for lower level bivalvian taxa (Dreyer and Steiner, 2006). Yokobori et al. (2008) provided the first complete mitogenomes of entoprocts. Their sequence analyses of protein coding genes failed to recover monophyletic Mollusca in a broader lophotrochozoan sampling but recovered Mollusca in a sampling pruned by putative long branches such as Chaetognatha and Nemertea; however, platyzoan phyla were not considered and only 9 molluscan species covering 5 classes were selected. The single dentaliid scaphopod and unionid bivalve species formed a clade consistent with a Diasoma hypothesis, sister to a clade composed of the chiton *Katharina* plus gastropods (single vetigastropod and caenogastropod), and Cephalopoda (4 species). Until the last review on molluscan mitogenomics the number of sampled mollusc species increased to 40, but with strong bias to cephalopods, euthyneuran gastropods and bivalves (Simison and Boore, 2008). In early 2011, already over 100 complete molluscan mitogenomes were available (including some species with multiple sequences), and all classes but Monoplacophora and Solenogastres were covered by at least a single representative. Analyzing 13 protein coding genes for this large though still fragmentary and biased taxon set we hoped for some insights on deep molluscan nodes.

In some molluscan subgroups, analyses of mitogenomic sequence data lead to good resolution, for example in bivalves and cephalopods (Doucet-Beaupré et al., 2010; Akasaki et al., 2006; Allcock et al., 2011) and the results are at least partly congruent with other phylogenetic reconstructions (reviewed in Ponder and Lindberg (2008)). In other cases, however, analyses of selected or almost complete sets of protein coding mitochondrial genes generated results that are far from convincing. For example, addressing gastropod phylogeny Grande et al. (2008) recovered a strongly supported topology with Patellogastropoda rather than Caenogastropoda as sister to Heterobranchia, with species-rich stylommatophoran land pulmonates more basal than freshwater pulmonates and sea slugs. Based on expanded sea slug sampling, Medina et al. (2011) resurrected monophyletic Opisthobranchia and Pulmonata, combined both as Euthyneura (including "lower heterobranch" Acteonoidea) and estimated an Early Cambrian origin of their last common ancestor (but see Schrödl et al., 2011a). Long branch attraction (LBA) artifacts were assumed to misroot and constrain such mitochondria-based euthyneuran trees (Schrödl et al., 2011a,b). Adding 10 further pulmonate mitogenomes the topology by White et al. (2011) comes closer to a recent reclassification of Euthyneura (Jörger et al., 2010; Schrödl et al., 2011b). However, it still implies an evolution from pulmonate body plans to sea slugs, which is contradicted by multi-locus marker analyses using a successively improved and representative heterobranch taxon sampling (e.g. Dinapoli and Klussmann-Kolb, 2010; Jörger et al., 2010; Schrödl et al., 2011b), and also by recent studies using a broad variety of nuclear genes and transcriptomes (e.g. Kocot et al., 2011). There are still technical problems to obtain complete mitogenomic sequences from a dense enough taxon sampling including potentially basal heterobranch taxa with tiny representatives. More problematic, mitochondrial substitution rates are generally higher than in nuclear genes. Evolutionary rates may greatly differ even among closely related lineages, and base composition may be biased. In most animals including molluscs, mitochondrial genes usually are AT rich (Kurabayashi and Ueshima, 2000), while some may be not (e.g. the pulmonate *Cepaea*, Terrett et al., 1996), and still other lophotrochozoan taxa exceptionally may be GT rich, such as certain brachiopods (Helfenbein et al., 2001) or the bivalve *Mytilus* (Boore et al., 2004). According to genes on different strands, often, but not always, there is a skew on base composition also affecting amino acid composition and, thus, phylogenetic reconstruction (Hassanin et al., 2005). Obviously, these are systematic rather than stochastic drawbacks that could perma-

nently negatively affect phylogenetic analyses of mitochondrial sequences, in particular on nucleotide level.

In addition to sequences, mitochondria offer a number of "genome-level" features that could be useful for phylogenetic purposes. For example, genes can be coded on one or the other strand having different directions for transcription, genes can overlap, and stop codons can vary. In molluscs, we can find unique features such as doubly uniparental inheritance (DUI) in unioid bivalves und certain members of palaeoheterodont and heterodont bivalve taxa (Doucet-Beaupré et al., 2010) that may be involved in sex determination (Breton et al., 2011). Mitogenomes vary in size, especially among bivalves and cephalopods (Gissi et al., 2008; Akasaki et al., 2006). Gene content and gene arrangement can vary within genera (e.g. Wu et al., 2010), and even intraspecifically in certain bivalves with female and male type mitochondria, and amino acids may diverge with up to 50% among sexes (Doucet-Beaupré et al., 2010). Genes can be duplicated and/or missing (e.g. Vallès and Boore, 2006). For example, *atp8* is absent in *Mytilus*, *Crassostrea*, *Venerupis* and partially in *Inversidens* (only in the male type mitogenome) (Serb and Lydeard, 2003; Mizi et al., 2005). Duplicated genes may remain functional and almost identical in oegopsid cephalopods (Yokobori et al., 2004; Akasaki et al., 2006) or degrade into non-functional pseudogenes, e.g. in oysters of the genus *Crassostrea* (Wu et al., 2010). Also, "non-coding" sequence(s) can greatly vary in numbers, lengths and positions, including noticeable long and highly conserved motifs with tandem repeats that are likely involved in controlling replication and transcription (e.g. Akasaki et al., 2006). The use of such genome level data for characterizing certain clades crucially depends on correct annotation that is, however, often erroneous in commonly used databases such as DOGMA or at least controversial in many cases (Jühling et al., 2012; Bernt et al., 2013c).

Special attention for resolving deep nodes was given to the relative arrangement of mitochondrial genes (e.g. Boore and Brown, 1995; Kurabayashi and Ueshima, 2000). While mt gene arrangement is stable over long evolutionary times in certain lineages, e.g. vertebrates with identity of all 37 genes in sharks and humans (Boore, 1999), rates of rearrangements can be quite high in lophotrochozoan lineages (Gissi et al., 2008). In particular, tRNAs can be frequently rearranged (Bernt et al., 2013c). Protein coding genes are similarly arranged and thus presumably plesiomorphic in arthropod *Drosophila* and the polyplacophoran *Katharina*, while radically differing from the bivalve *Mytilus edulis* (Boore and Brown, 1994). Compared to other phyla, molluscs show accelerated rates of mitochondrial rearrangements (Gissi et al., 2008). Within cephalopods both highly derived and very plesiomorphic arrangements occur. *Octopus*, *Vampyrotheutis* and also the vetigastropod *Haliotis* have an almost identical arrangement to *Katharina*. In contrast, among Apogastropoda, caenogastropods show moderate rearrangements while heterobranchs appear radically aberrant; both higher groups show little internal variation. The gene arrangement of the patellogastropod *Lottia* is dissimilar to any other animal, and also known members of some molluscan classes such as scaphopods and bivalves are aberrant and highly variable (Simison and Boore, 2008). There have been promising attempts to resolve and characterize inner class relationships via gene order data, e.g. in bivalves (Serb and Lydeard, 2003; Doucet-Beaupré et al., 2010) and in cephalopods (Akasaki et al., 2006; Allcock et al., 2011). In a broader taxon approach, Yokobori et al. (2008) used breakpoint and Maximum Parsimony analyses of shared boundaries of mitochondrial genes of selected metazoans and recovered monophyletic Lophotrochozoa, with the molluscs *Katharina* and *Octopus* in a clade with the entoproct *Loxocorone* or clustering together with representatives of Entoprocta, Phoronida and Nemertea. However, neither the origin of molluscs nor inner molluscan phylogeny could be resolved based on gene arrangements yet. Different gene arrangements are assumed to be shaped by translocations, inversions, inverse translocations, and random duplication of partial mtDNA and subsequent random loss of gene copies (Bernt et al., 2013c), but tandem duplication of whole genomes with random or nonrandom loss processes also might occur (Lavrov et al., 2002). Whatever the mechanisms, metazoan data sets coding gene rearrangement events were once thought to be nearly free of homoplasy (e.g. Boore, 1999; Boore and Staton, 2002). In 2008, metazoan datasets available still were stated to include only very few cases of convergent rearrangements of coding genes, i.e. arrangements are variable enough to be informative but events are rare enough to provide phylogenetic signal also in fast evolving groups such as molluscs (Simison and Boore, 2008).

By reason of the numerous problems that might occur in molluscan mitogenomics, there exists no contemporary phylogenetic analysis of mitochondrial DNA comprising a broad and large sampling of molluscs. Although there are already 104 complete mitogenomes of molluscs in molecular databases (e.g. GenBank/RefSeq) available (summer 2011) and six out of eight molluscan classes are represented, the taxon sampling is still far from convincing for most classes. Gastropod and bivalve mitochondrial genomes are examined far best, followed by cephalopods. The other classes are covered poorly (Polyplacophora, Scaphopoda, Caudofoveata) or lack at all (Solenogastres, Monoplacophora). Herein we address the task to resolve the phylogeny of Mollusca in the light of a complete metazoan taxon set and with a molluscan subsampling as comprehensive as available in July 2011.

2. Material and methods

Two taxon sets were analyzed herein: one comprising 668 metazoan and 16 non-metazoan mitochondrial genomes, which were used as outgroup references (for details see Bernt et al., 2013b). 657 complete mitogenomic sequences were retrieved from the RefSeq database (Pruitt et al., 2007) release 41, and eleven so far unpublished genomes were added by authors herein (for details see Bernt et al., 2013b). Only 52 molluscan taxa are included in the metazoan analysis, and the topology failed to recover monophyletic Mollusca and other undisputed subgroups (Fig. 1). To reduce potential long branch artifacts we designed a second taxon set with expanded ingroup sampling, i.e. all 96 molluscan mitogenomes available in July 2011. Outgroups were constrained to 16 lophotrochozoans, representing Bryozoa, Platyhelminthes, Brachiopoda, Entoprocta, Annelida, Sipuncula, Echiura, and Nemertea. Sequences were obtained from RefSeq.

For both taxon sets the following procedure was performed: mitogenomic sequences were annotated with a new algorithm (Bernt et al., 2013a). Single amino acid alignments for each protein coding gene (PCG) were produced using MAFFT v. 6.716 (Katoh et al., 2002); tRNA and rRNA genes were not taken into account for phylogenetic analyses. Then the concatenated alignments were masked with Noisy v. 1.5.9 (Dress et al., 2008) and Maximum Likelihood (ML) analyses were executed using RAxML v. 7.2.8 (Stamatakis et al., 2008) under a mixed model for proteins, CAT+MTZOA+F. For more details about the alignment procedures and phylogenetic reconstruction see Bernt et al., 2013b.

We performed three additional ML analyses of the second taxon set in our lab (resulting trees not shown). One reanalysis of the amino acid data set without a preceding masking procedure and two analyses based on the nucleotide alignment. For the two nucleotide analyses single data sets of each of the protein coding genes were aligned with MAFFT v. 6.847b (Katoh, 2009) applying the implemented E-INS-i algorithm. One ML analysis was performed with a masked nucleotide alignment. Therefore masking procedures of the single alignments were conducted via the

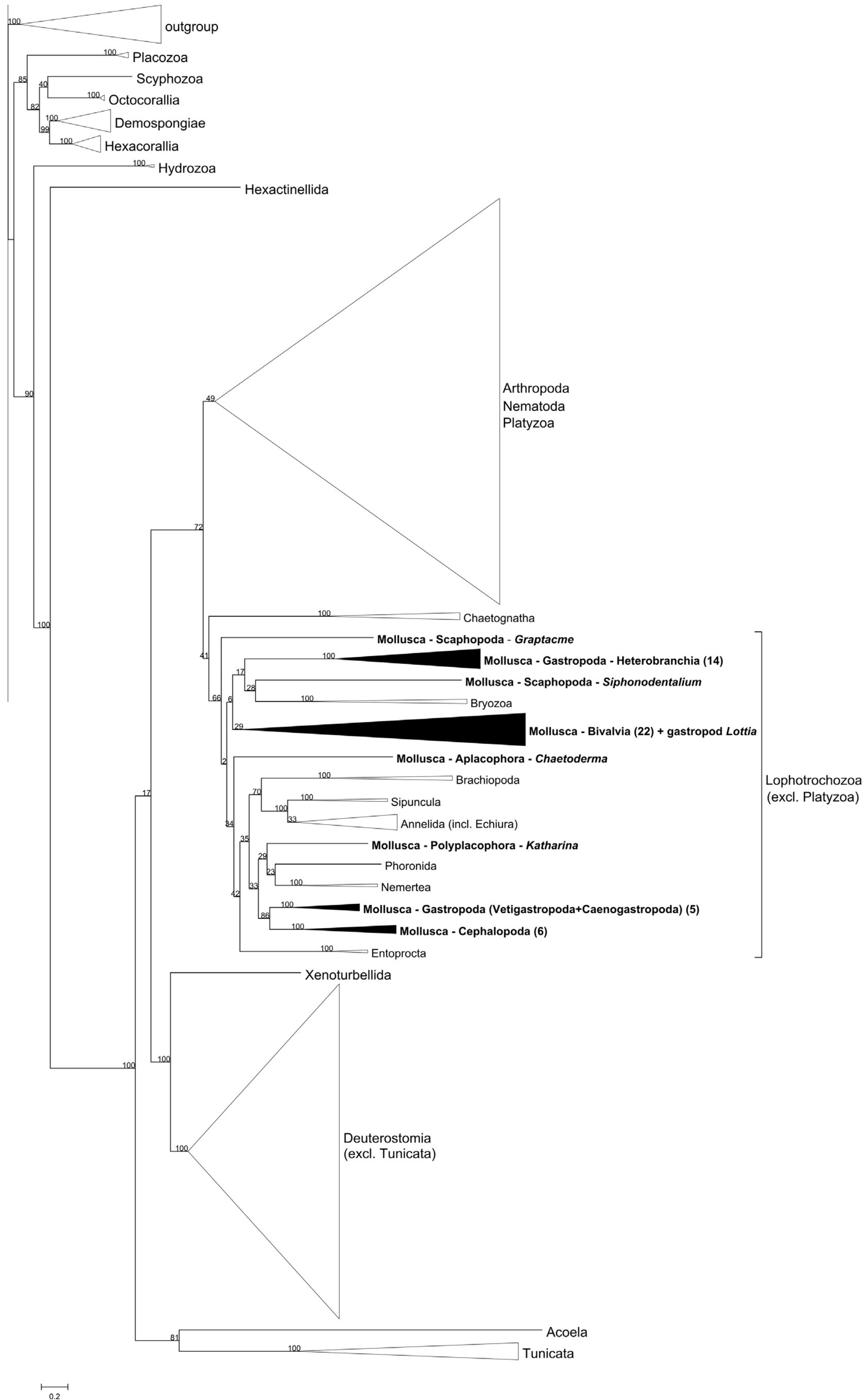

Fig. 1. Phylogenetic tree of metazoans, molluscs are highlighted in bold. Analysis is based on the amino acid sequences of all mitochondrial protein coding genes. ML analysis was performed with RAxML v. 7.2.8 under the mixed model for proteins CAT+MTZOA+F and 300 bootstraps. Support values are indicated above branches. Black triangles indicate collapsed molluscan groups. Numbers of included taxa per molluscan class are displayed in parentheses.

Gblocks server (Castresana, 2000) by using all options for a less stringent selection of poorly aligned positions and divergent regions before tree reconstruction. Single nucleotide alignments (masked or unmasked) were subsequently concatenated using FASconCAT (Kück and Meusemann, 2010). The unmasked concatenated nucleotide alignment was then translated in MEGA5 (Tamura et al., 2011) using the codon table for mitochondrial invertebrate DNA for the analysis of the amino acid data set without masking. Phylogenetic ML reconstructions of the unmasked amino acid and the masked, respectively the unmasked nucleotide, concatenated alignments were computed with RAxML v. 7.2.8 on the Linux cluster of the Leibniz Computing Department of the Ludwig-Maximilian-University Munich running 500 bootstraps for each analysis. For amino acid analysis we used the PROTCATGTR model, the two nucleotide data sets were executed by applying the GTRCAT model. After analyses the resulting consensus trees were rooted with Bryozoa (sensu Ectoprocta) assuming it is a distant outgroup in reference to Mollusca.

Gene arrangements were compared across lophotrochozoans and more exhaustively within our comprehensive set of molluscs. The overall gene arrangements comprised 93 molluscan and 16 outgroup taxa. Arrangements were annotated by Mathias Bernt with MITOS, an improved pipeline constructed by Bernt et al. (2013a). Because of the overall heterogeneity of gene arrangements within and between molluscan clades we did not try calculating phylogenies from coding shared single gene boundaries. Similarly, the generally disputed deep molluscan phylogeny paired with fragmentary sampling does not permit us to simply plot gene arrangements on a reliable tree and infer rearrangements node by node. Instead, we screened the literature and the entire dataset for similar gene clusters, identified blocks with high a priori probability of homology, i.e. more complex (longer) portions of identical arrangements, and analyzed their distribution among and within undisputed taxa. We also screened the dataset for putative cases of convergence, i.e. patterns of similar gene clusters occurring in non-related taxa.

3. Results

3.1. Phylogeny

In the metazoan analysis Mollusca are not recovered monophyletic, but are mixed up with other lophotrochozoan taxa (Fig. 1). *Siphonodentalium* (Scaphopoda) is sister to Bryozoa and Polyplacophora appear basal to Phoronida and Nemertea. Regarding molluscan classes, Caudofoveata and Polyplacophora are only represented by one taxon each and therefore monophyly cannot be tested. The two scaphopod taxa are diphyletic. *Graptacme* appears at the base of Lophotrochozoa whereas *Siphonodentalium* together with Bryozoa is sister to partial Gastropoda, namely Heterobranchia, in a derived position. The only molluscan class that can be recovered monophyletic is Cephalopoda with basal *Nautilus* as the only representative of Nautiloidea. Within Coleoidea we find two clusters, one comprising Vampyromorpha and Octopoda, the second consisting of two teuthid taxa *Todarodes* and *Loligo*. Cephalopoda is sister group to the second cluster of Gastropoda, comprising Vetigastropoda and Caenogastropoda. *Lottia* as the single representative of Patellogastropoda is nested within pteriomorph bivalves.

The tree resulting from the masked amino acid data of our 112 taxa set is similar to the topology of the metazoan tree (Fig. 2). Mollusca are still non-monophyletic. Almost all outgroup taxa except Bryozoa (trees are rooted with Bryozoa) are recovered within the ingroup taxa. Sipuncula, Annelida, Echiura, Brachiopoda and Entoprocta (Kamptozoa) cluster together with *Chaetoderma* (Caudofoveata) (Suppl. Figs. 1–3). Gastropoda and Bivalvia, both represented by a high number of taxa, still cannot be recovered monophyletic (Suppl. Figs. 1–2). Gastropods appear in two larger clusters, Heterobranchia (with pulmonates and opisthobranchs) at the root of the tree and Caenogastropoda (represented by neogastropods) together with Vetigastropoda (*Haliotis*) as sister to Nemertea in a more derived position. The patellogastropod *Lottia* and Platyhelminthes cluster within Bivalvia (Suppl. Fig. 2). Palaeoheterodonta (represented by unionids) split off basally from the bivalve stem line, and then successively Veneroida as the only heterodont group, and Pteriomorphia with Mytiloida, Ostreoida and Pectinoida. All included bivalve families are recovered monophyletic (Suppl. Fig. 1). This is also the case for gastropod families. The inner heterobranch topology recovers paraphyletic Panpulmonata basal to all other heterobranch clades. Acteonoidea (*Pupa*, *Micromelo*, *Hydatina*) is sister to Nudipleura, and both are sister to Euopisthobranchia (Suppl. Fig. 2). Cephalopods are again monophyletic with Nautiloidea sister to coleoids, comprising Decabrachia versus Octobrachia (Suppl. Fig. 3).

Analysis of the unmasked amino acid data set recovers a similar topology (tree not shown). At the root of the tree the heterobranch clade is replaced by the scaphopod *Siphonodentalium*, whereas Heterobranchia switch slightly to the base of all other molluscan classes and included outgroups. *Graptacme*, the second scaphopod taxon, is sister to a clade comprising outgroup taxa, *Chaetoderma* (Caudofoveata), Cephalopoda, partial Gastropoda (Vetigastropoda and Caenogastropoda) and *Katharina* (Polyplacophora). Platyhelminthes are sister to the Ostreoida clade of Bivalvia with the unmasked data set rather than sister to Mytiloida as with the masked data set.

The analysis of the masked nucleotide alignment recovers Annelida, Sipuncula, Echiura and Brachiopoda at the root of the tree (tree not shown). Entoprocta, Nemertea, and Platyhelminthes still cluster within the Mollusca as it was the case in the amino acid based trees. Entoprocta and Nemertea form a cluster with *Katharina*, and Platyhelminthes are again nested within Pteriomorphia. The gastropod groups Heterobranchia, Vetigastropoda, Caenogastropoda and Patellogastropoda are recovered monophyletic, but again are distributed over the tree. Vetigastropoda are the sistergroup to Caenogastropoda. Bivalves form a cluster, but again disturbed by *Lottia* (Patellogastropoda) and Platyhelminthes. The latter are sister to Ostreoida plus Pectinoida. Disregarding *Lottia* and Platyhelminthes, the branching order of considered bivalves is Palaeoheterodonta at the base of bivalves, followed by Veneroida (Heterodonta) and Pteriomorphia in derived position with basal Mytiloida and Pectinoida sister to Ostreoida. *Chaetoderma* (Caudofoveata) is sister to Cephalopoda. Cephalopod topology is identical to the amino acid analysis. *Katharina* (Polyplacophora) can be found within a cluster with Entoprocta and Nemertea. Scaphopoda again are not recovered monophyletic. *Graptacme* is basal to *Siphonodentalium* plus Heterobranchia.

The topology from unmasked nucleotides shows only minimal differences (tree not shown). The position of *Lottia* is still within Pteriomorphia but is now recovered as sister to pectinoid bivalves. Platyhelminthes change from sister group relationship with Pectinoida plus Ostreoida to sister of Ostreoida only.

3.2. Reannotation

Available mitogenomes of the RefSeq database were reannotated with a newly designed method in Leipzig (Bernt et al., 2013a). The molluscan rearrangements of mitogenomes are not investigated exhaustively herein; we mainly focused on certain features e.g. absence or presence of genes or formation of special gene-/RNA-clusters.

Polyplacophora and Aplacophora are represented by only one mitogenome each: *Katharina tunicata* and *Chaetoderma nitidulum*.

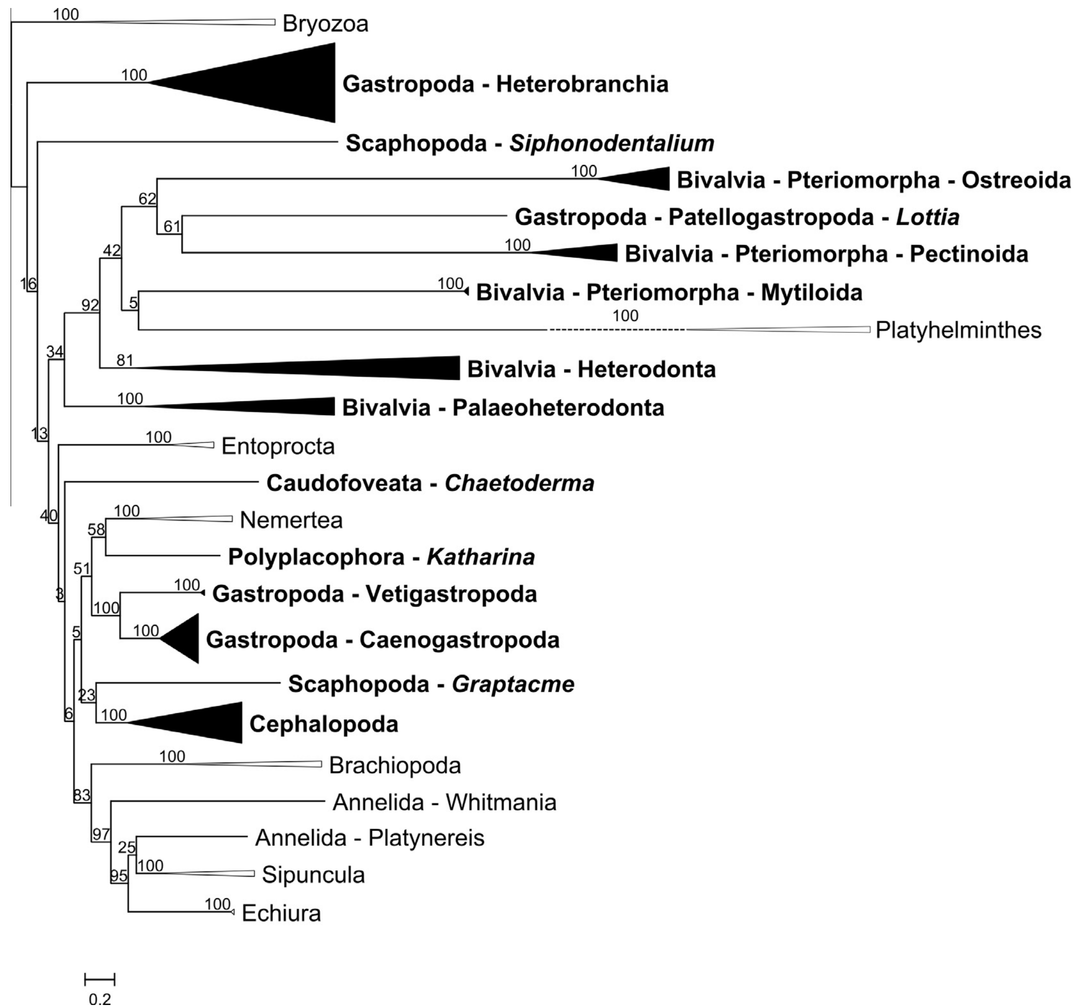

Fig. 2. Preferred phylogenetic tree of the expanded molluscan taxon set. Amino acid sequences of all protein coding genes were aligned with MAFFT v. 6.716 and masked with Noisy v. 1.5.9. ML analysis was performed with RAxML v. 7.2.8 under the mixed model for proteins CAT+MTZOA+F and 300 bootstraps. Support values are indicated above branches. Groups are collapsed; black triangles show molluscan classes with more than one representative. The detailed topology is shown in Supplementary Fig. 1–3. The branch length of Platyhelminthes is compressed (dashed line).

Cephalopoda are represented by a single nautiloid and 14 coleoid mitogenomes. All gene arrangements in those classes are congruent with the published annotations in GenBank. Duplicated *cox2* is also recovered with the new annotation method in *Chaetoderma* as well as duplication events of *cox1*, *cox2*, *cox3*, *nad2*, *atp6*, *atp8*, and *trnD* in several cephalopod taxa (Ommastrephidae, Enoploteuthidae and Architeuthidae).

Scaphopoda are represented by two genomes, one gadilid (*Siphonodentalium*) and one dentaliid (*Graptacme*). Regarding *Siphonodentalium* the new annotation is congruent with the published. In *Graptacme* there is a slight difference in the orientation of the small subunit of rRNA. According to the published annotation in NCBI it is located on the plus strand whereas it is recovered on the minus strand in our reannotation.

The 30 bivalve taxa with mitogenomes available comprise 5 unionid Palaeoheterodonta, 8 Heterodonta and 17 Pteriomorphia. Protobranchia is not represented by any mitogenome. Within Heterodonta we found *atp8* present in all taxa in our reannotations, while in the NCBI annotations the taxa *Venerupis* (submission to NCBI: Okazaki et al., 2011, unpublished), *Sinonovacula* (submission to NCBI: Zheng et al., 2008, unpublished), *Meretrix* (*M. meretrix* (submission to NCBI: He et al., 2010, unpublished) and *M. petechialis* (Ren et al., 2009)) and *Acanthocardia* (Dreyer and Steiner, 2006) are supposed to lack *atp8*. According to Wang et al. (2010) *atp8* is present in *Meretrix lusoria*, a veneroid taxon that is not included in our bivalve taxon set. To clarify the functionality of *atp8* in those questionable taxa we checked the amino acid sequences. None of them inhabits stop codons within the sequence. The lengths of the protein range between 39 sites (*Venerupis*), 42 sites (*Sinonovacula*) and 44 sites (*Meretrix*) whereas the length of the *Acanthocardia atp8* protein is noticeably short (26 sites). Average number of sites in the *atp8* gene is ca. 49 sites within Mollusca. Within Pteriomorphia *atp8* was detected in 5 pectinoid (*Mimachlamys nobilis*, *Chlamys farreri*, *Placopecten magellanicus*, *Mizuhopecten yessoensis*, *Argopecten irradians* NC_012977) and two ostreoid taxa (*Crassostrea hongkongensis*, *Crassostrea iredalei*) in a total of 17 taxa. In *Mizuhopecten yessoensis* we actually detected a duplication of *atp8*, but it is not clarified yet, if both gene copies are functional. Interestingly, in *Argopecten irradians*, which is represented by two individuals, only one possesses detectable *atp8*. This pattern of absence and presence of *atp8* is in contrast to former results (Ren et al., 2009; Doucet-Beaupré et al., 2010). Furthermore our arrangement of *Crassostrea iredalei* shows two copies of *nad2* of which one is not implemented in the NCBI annotation but is mentioned in Wu et al. (2010). In *Crassostrea gigas* our annotation method did not detect any of the supposed two parts (602 bp and 713 bp long) of *rrnL* and only one part of such an assumed split-

ted rRNA gene in *Crassostrea virginica* (Yu et al., 2008). Instead we found two copies of *rrnS* in *Crassostrea hongkongensis and C. virginica*.

Gastropoda are covered by 44 mitochondrial genomes, i.e. Caenogastropoda, Heterobranchia, Vetigastropoda, and Patellogastropoda; three further major groups are not represented, i. e. Neritimorpha, Neomphalina, and Pleurotomariidae. The published mitogenomes reflect an unbalanced coverage of the groups. Heterobranchia, and Caenogastropoda are represented by 24 respectively 17 genomes, patellogastropods are covered by only one taxon (*Lottia*) and vetigastropods by two individuals of *Haliotis*. The reannotations of the regarded gene arrangements did not show any aberrant features to the arrangements in the NCBI database except the orientation of genes *atp8* and *rrnS* of *Platevindex* (NC_013934); both genes are annotated on the plus strand in NCBI but are located on the minus strand in our reannotation.

3.3. Conserved gene clusters

The polyplacophoran taxon *Katharina* reflects an arrangement of PCGs and rRNAs that is found in certain (but usually not all) members of three other molluscan classes (Cephalopoda, Gastropoda, and Caudofoveata) as well (Fig. 3A). This is the only pattern that can be found in more than one molluscan class. Within Cephalopoda the arrangement is present in *Octopus* and *Vampyroteuthis*. Shared differences of the two cephalopod taxa in contrast to *Katharina* are the position of *trnD* and the strand orientation of *trnP*. In the gastropod *Haliotis* the position of *trnD* and the orientation of *trnP* are different to *Katharina*, too. Whereas the position of *trnP* is congruent in *Octopus* and *Haliotis*, *trnD* is translocated in *Octopus* to a position between *cox2* and *atp8* but still adjacent to *cox2* as in *Katharina*, and in *Haliotis* it is shifted to the tRNA complex *trnK–trnA–trnR–trnI*. Furthermore *trnN* and *trnY*/*trnC* are allocated in *Haliotis*. It is *trnY–trnC* in *Haliotis* whereas in *Katharina* we detect the order *trnC–trnY*. *Chaetoderma* (Caudofoveata) also shows identical arrangement of PCGs and rRNAs as *Katharina*, but *rrnS* and *rrnL* appear in inverse order (*rrnL–rrnS* in *Katharina*, *rrnS–rrnL* in *Chaetoderma*); both taxa share the positions of *trnH*, *trnK*, *trnL1*, *trnL2*, *trnP*, *trnS2*, and *trnT*. Other tRNAs (*trnA*, *trnC*, *trnD*, *trnE*, *trnF*, *trnG*, *trnI*, *trnM*, *trnN*, *trnQ*, *trnR*, *trnS1*, *trnW*, *trnY*) change their positions within the genome, but not necessarily in relative position to each other or to coding genes. *TrnV* is related to *rrnL* in both genomes, *trnS1* is connected to *nad3*, *trnM*, *trnC*, *trnQ*, *trnY* stick to *rrnS* and *trnA*, *trnR* and *trnI* build a complex in both mitogenomic orders. Special attention should be directed to two tRNA clusters which occur frequently within groups. This is *trnK–trnA–trnR–trnN–trnI* which is detected in *Katharina* as well as in *Octopus*. In *Haliotis* and *Chaetoderma* we found reduced parts of it (*trnK–trnA–trnR–trnI* in *Haliotis*, *trnA–trnR–trnI* in *Chaetoderma*). Interestingly, the complex appears in non-molluscan lophotrochozoans as well. In entoprocts the complete sequence is apparent in inverse direction (*trnI–trnN–trnR–trnA–trnK*). Besides the inversed tRNA complex the linked segment of PCGs *cox3–nad3–nad2* (order in *Katharina*) is found in inverse orientation in entoprocts, too (*nad2–nad3–cox3*). In the nemertean *Cephalotrix* the tRNA complex is present, within the second nemertean *Lineus* we found the complex with embedded *trnF* and *trnQ* (*trnK–trnA–trnF–trnQ–trnR–trnN–trnI*). The other complex comprises tRNAs *trnM–trnC–trnY–trnW–trnQ–trnG–trnE* in *Katharina*, *Octopus* and *Haliotis* but with an inversion of *trnC* and *trnY* in the latter one, so it is *trnM–trnY–trnC–trnW–trnQ–trnG–trnE*. The complex or at least residues of it are present in almost all cephalopods apart from Loliginidae where only *trnC–trnY–trnE* is extant. In *Nautilus* we detected *trnM–trnC–trnY–trnW–trnQ*. *Vampyroteuthis* is congruent with *Octopus*. Partitioning of that complex is observed in all other cephalopod taxa. In *Sepia trnC–trnY–trnQ–trnG* is split up from *trnM–trnW–trnE*, in *Watasenia* and *Architeuthis trnY–trnW–trnG–trnE* and *trnM–trnC–trnQ* are partitioned and in all ommastrephid taxa we found the parts *trnM–trnY–trnW–trnG–trnE* and *trnC–trnQ*. *Chaetoderma* presents only parts of the complex with a different order (*trnM–trnC–trnQ–trnY*). In entoprocts we detected *trnC–trnY–trnW–trnQ*.

Mitochondrial gene orders of Cephalopoda differ in arrangements between groups but common features that occur in the gene order of *Katharina* as well are detectable. *Nautilus*, as the single representative of Nautiloidea, shows several translocations in comparison to the gene arrangement of *Katharina*. These are translocation of *trnG*, *trnT*, and of the protein coding gene *atp6* and translocation of the complex *trnL2–trnL1–rrnL–trnV–rrnS–trnM–trnC–trnY–trnW–trnQ*. *TrnP* is subject to a reverse transposition between the plus strand in *Katharina* and the minus strand in *Nautilus*. Taxa within Sepiida have congruent gene arrangements to *Katharina* as well as Ommastrephidae. Within Loliginidae a transposition of two sections happened between the order of *Loligo* and *Sepioteuthis* (*trnA–trnD–atp8–atp6–trnH–trnL1–cox3–nad3–trnS2–cob–nad6–trnP–nad1–trnQ* and *trnI–rrnL–trnV–rrnS–trnW*). *Vampyroteuthis* shows the same arrangement as *Octopus* (Fig. 3A). Across all cephalopod gene arrangements some gene clusters occur frequently. This is *cox1–cox2–trnD–atp8–atp6* which is not present in this combination in Loliginidae. In comparison to *Katharina* a shift of *trnD* occurred from *trnD–cox2* to *cox2–trnD* in all arrangements where the cluster is present. *Atp8* is transposed in *Nautilus* and *trnD* in both *Sepia* taxa. *Nad5–trnH–nad4–nad4L–trnT–trnS2–cob–nad6–trnP* is unchanged in all taxa but with translocated *trnT* in *Nautilus* and splitted in two sections in Loliginidae (*nad5–nad4–nad4L–trnT* and trn*S2–cob–nad6–trnP*). *Nad1–trnL2–trnL1–rrnL–trnV–rrnS–trnM–trnC–trnY–trnW–trnQ–trnG–trnE* occurs unchanged in *Vampyroteuthis* and the two *Octopus* species. It appears without *nad1* and with partial *trnM*, *trnC*, *trnY*, *trnW*, *trnQ*, *trnG*, *trnE* complex in *Nautilus* (*trnM–trnC–trnY–trnW–trnQ*) and with only *trnC–trnY–trnQ–trnG* in *Sepia*. In *Watasenia* the complex is divided into *nad1–trnL2–trnL1–rrnL–trnY–trnW–trnG–trnE* and *trnV–rrnS–trnM–trnC–trnQ*. The sequence is also splitted in *Architeuthis* and all *Ommastrephidae*: *nad1–trnL2–trnL1–rrnL–trnM–trnY–trnW–trnG–trnE* and *trnV–rrnS–trnC–trn Q*. The complex appears in the most aberrant version in Loliginidae where only the partial sequence of *rrnL–trnV–rrnS* is still present; the remaining parts of the complex are distributed throughout the complete genome arrangement. The two mitogenomes that represent the Scaphopoda (*Siphonodentalium*, *Graptacme*) differ substantially in their gene orders, although some of the PCGs build certain complexes (Fig. 3C). This is *cox1–nad2*, furthermore *cob–cox2–cox3*. In *Siphonodentalium* the genes of the latter complex are located on one strand while *cob* is on the opposite strand than *cox2–cox3* in *Graptacme*. In comparison to *Katharina* there can be found one common feature in *Siphonodentalium*, the combination of *rrnL* and *trnL1* and five similar complexes in *Graptacme*, that is *nad5–nad4–nad4L*, the alliance of *nad6* with *trnP*, both located on the same strand in *Katharina*, but on opposite strands in *Graptacme*, as well as *trnG* and *trnQ*, *trnL1–trnL2*, which are in inverse direction and on the opposite strand than in *Katharina,* and *rrnL* in combination with *trnV*.

The three major bivalve groups, Pteriomorphia, Palaeoheterodonta and Heterodonta contain taxa with highly variable gene orders. We could not detect any obvious common pattern in the arrangement of PCGs and rRNAs in Bivalvia; Pteriomorph gene arrangements are even variable on genus level, see for example NC_012138 *Chlamys farreri* and NC_006161 *Mytilus edulis* (Fig. 3D). Within the conserved gene order in Palaeoheterodonta and that of *Katharina* several genes and tRNAs appear on the same strands: *nad2*, *nad4*, *nad4L*, *nad5*, *trnH*, *trnT*, *trnP*, *trnK*, *trnA*, *trnR*, *trnN*, *trnI*, and *trnS1*. Some small gene complexes respectively gene-tRNA complexes of *Katharina* can be

recovered in palaeoheterodonts: *nad4–nad4L*, *trnL1–rrnL–rrnS* in reverse orientation (*rrnS–rrnL–trnL1*) and with *trnM–trnW–trnR* between the two rRNAs instead of *trnV* in *Katharina*, furthermore the complex *nad3–nad2*, in which both genes are located on opposing strands and frame the tRNA sequence *trnH–trnA–trnS2–trnS1–trnE* whereas in *Katharina* both genes enclose the tRNA complex *trnK–trnA–trnR–trnN–trnI*, and *nad1–trnL2*, this complex again in reverse orientation in palaeoheterodonts (*trnL2–nad1*). Although variability in gene arrangement is high, there are some representative tRNA aggregations within several groups. For Unionoida (Palaeoheterodonta) complexes *trnA–trnS2–trnS1–trnE*, *trnM–trnW–trnR*, *trnK–trnT–trnY*, *trnL1–trnN–trnP*, and *trnQ–trnC–trnI–trnV–trnL2* are typical orders which are present in all mitogenomes except *Hyriopsis*. All mytiloid taxa (Pteriomorphia) represent three characteristic tRNA clusters: *trnG–trnN–trnE–trnC–trnI–trnQ–trnD*, *trnK–trnM–trnL1–trnL2*, and *trnR–trnW–trnA–trnS1–trnH–trnP*. Further specific combinations of tRNAs can be found in Ostreoida (Pteriomorphia). These are *trnI–trnT–trnE*, *trnM–trnS1–trnL2–trnM–trnS2*, and *trnL1–trnF–trnA*.

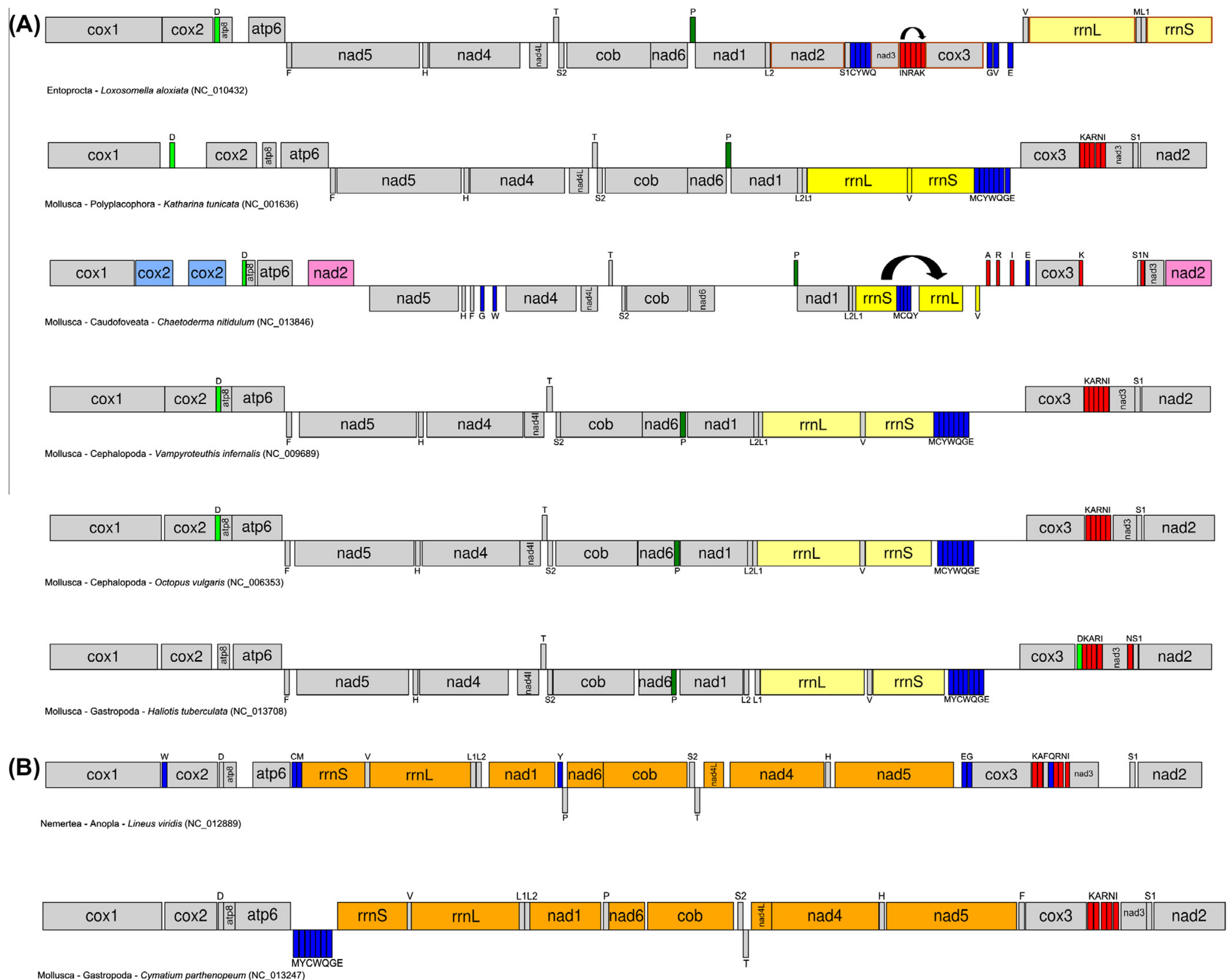

Fig. 3. Mitochondrial gene orders of different molluscan taxa covering 6 of 8 classes (no mitochondrial genomes are available of Monoplacophora and Solenogastres) and two lophotrochozoan outgroups. Arrangements are annotated with MITOS and linearized and rotated to *cox1* for display reasons. Gene lengths of coding and non-coding regions correspond to relative lengths of the genomes. Transfer RNAs are displayed according to the one-letter code. NCBI accession numbers are given in parentheses. (A) Highly similar, putatively conserved gene arrangements of four different molluscan classes and one entoproct. Positions of *trnD* (light green) and *trnP* (dark green) are differing among taxa. Two complexes of tRNAs, this is *trnK–trnA–trnR–trnN–trnI* (red) and *trnC–trnY–trnW–trnQ–trnG–trnE* (blue) or at least remnants of them appear in all taxa. *TrnK–trnA–trnR–trnN–trnI* is in reverse order in *Loxosomella aloxiata* (indicated by a black dart). Sequence of *rrnS* and *rrnL* as it is visible in *Katharina tunicata* appears in inverse direction in *Chaetoderma nitidulum* (yellow; inversion is indicated by a black dart). All other taxa show the genes in the same direction as *Katharina tunicata* (light yellow). Inverse transposition of the PCG complex *nad2–nad3–cox3* versus *rrnL–rrnS* in *Loxosomella aloxiata* is highlighted by brown boxes. Duplicated gene *cox2* in *Chaetoderma nitidulum* is shown in light blue, duplicated gene *nad2* in *Chaetoderma nitidulum* is marked in rose. (B) Caenogastropod *Cymatium parthenopeum* and nemertean *Lineus viridis* showing identical, but probably convergent rearrangement of mitochondrial protein coding genes. The order of PCGs is in inverse order (orange) compared to other molluscs and putative lophotrochozoan outgroups. Transfer RNAs *trnK–trnA–trnR–trnN–trnI* form a complex in both taxa, too (red). Transfer RNAs of the second complex (*trnC–trnY–trnW–trnQ–trnG–trnE*) (blue) are distributed over the mt-genome in *Lineus viridis*. (C) Aberrant gene arrangements of two scaphopod taxa. *Siphonodentalium lobatum* and *Graptacme eborea* share only two short sequences of protein coding genes (purple). (D) Aberrant genome arrangements of two bivalve taxa; *Mytilus edulis* (Mytiloida) and *Chlamys farreri* (Pectinoida) reflect the high variation on genus level in Pteriomorphia. Typical tRNA complexes shared by all mytiloid taxa are highlighted in pink in *Mytilus* arrangement. (E) Aberrant genome arrangements of three gastropod taxa; *Micromelo undatus* (lower Heterobranchia) and *Albinaria coerula* (Panpulmonata) are largely congruent in gene order but differ to other gastropod arrangements; *Lottia digitalis* (Patellogastropoda) is highly aberrant to all mitochondrial gene orders known to date. Gene lengths of Heterobranchia are ca. 14.6 kb, length of *Lottia* is ca. 26.8 kb.

Fig. 3. (*continued*)

Comparing the gene orders of all gastropod groups one noticeable feature has to be mentioned: The vetigastropod and caenogastropod gene order is identical in two complexes, one comprising *cox1–cox2–atp8–atp6* and the other *cox3–nad3–nad2*, a feature that is also evident in *Katharina* (Fig. 3A, 3B). Caenogastropoda show low internal variability of gene orders. Within caenogastropods a conserved order is present, which does only vary in the positions of few tRNAs. While tRNAs may vary substantially, most heterobranch taxa have a congruent order and strand orientation of PCGs and rRNAs (Fig. 3E): *cox1–rrnL–nad6–nad5–nad1–nad4L–cob–cox2–atp8–atp6–rrnS–nad3–nad4–cox3–nad2*. The gene order of *Lottia* (Patellogastropoda) differs substantially from all other gastropod orders (Fig. 3E).

Among molluscs, some gene clusters appear frequently in certain major taxa, but do not always reflect the gene orders of the complete group. For example, vetigastropods (*Haliotis*) and caenogastropods (*Cymatium* as representative) have two complexes of PCGs in common: *cox1–cox2–atp8–atp6* and *cox3–nad3–nad2*. All genes in between are arranged in inverse direction and on the opposite strand within the two groups (Fig. 3A, 3B). But this pattern is not visible in all gastropod taxa. Differences can thus be explained by a single event of reverse transposition of the genes to the opposite strand and two transpositions of *trnD* and *trnN*; considering that *Haliotis* has the same arrangement as *Octopus* and *Katharina*, this arrangement is very likely plesiomorphic for gastropods and the rearrangement event occurred in ancestral caenogastropods. Including the lower heterobranch *Micromelo* into comparison, dissimilarity is even more substantial; complex tandem duplication random loss events could be responsible but cannot be reconstructed herein. Only one complex of *cox2–atp8–atp6* is still present in all heterobranch taxa. We are not aware of any events relating *Lottia* to other known animal gene arrangements. *Atp8* and *atp6* are combined only in Vetigastropoda and Caenogastropoda. In most Heterobranchia, *trnN* splits those two regulatory genes; in lower Heterobranchia and Euopisthobranchia we found *trnC* in between.

3.4. Strand and size variation in mitochondrial genomes

Molluscs and palaeoheterodont bivalves in our annotation use both strands for transcription. All Pteriomorphia except for one individual of *Argopecten irradians* transcribe their mtDNA from the same strand; Individual *A. irradians* NC_009687 has *trnE* encoded on the minus strand, whereas in the second individual of *A. irradians* NC_012977 all genes and tRNAs are located exclusively on the plus strand. Within Caenogastropoda, all PCGs and most of the tRNAs share the same strand, excluding *trnT* and the complex *trnM–trnY–trnC–trnW–trnQ–trnG–trnE*.

The size of molluscan mitochondrial genomes analyzed herein ranges between 13.6 kb (*Biomphalaria*, Gastropoda) and 31.5 kb (*Placopecten*, Bivalvia). Scaphopod genome sizes are situated at the lower bound with ca. 14.4 kb in *Graptacme* respectively 13.9 kb in *Siphonodentalium* as well as the polyplacophoran *Katharina* (ca. 15.5 kb). *Chaetoderma* (Caudofoveata) ranges at the upper bound with ca. 20 kb. Within Gastropoda the largest genome refers to the patellogastropod *Lottia* (ca. 26.8 kb), and the smallest to the heterobranch *Biomphalaria* (13.6 kb). Average sizes of mitogenomes in Gastropods are 14.6 kb in Heterobranchia, 15 kb in Cae-

nogastropoda and 16.7 kb in Vetigastropoda. Cephalopoda range in genome lengths from 14.8 kb (*Vampyroteuthis*) up to ca. 18 kb (some teuthid taxa, i.e. *Dosidicus*, *Sthenoteuthis*, *Architeuthis*). The smallest bivalve mt-genome is recorded in *Cristaria* (15.7 kb). Palaeoheterodont mitogenomes possess an average length of 16 kb, whereas Heterodonta and Pteriomorpha reflect a mean length of 18.5 kb respectively 18.6 kb. *Placopecten* ranges between 30 and 40 kb, depending on the number of copies of several tandem repeats; this number changes within the individuals (see Smith and Snyder (2007) for further details).

4. Discussion

4.1. Deep molluscan phylogeny

There were several attempts of analyzing lophotrochozoan and deep molluscan phylogeny using (almost) complete sets of protein coding and ribosomal mitochondrial genes, e.g. by Boore et al. (2004), Dreyer and Steiner (2004, 2006) and Yokobori et al. (2008), but results were not conclusive. Among others, fragmentary and unrepresentative taxon sets, generally fast evolving mitochondrial genes, base composition bias also affecting amino acid composition, and heterogeneous rates of evolution causing artificial attraction of unrelated taxa were assumed to be responsible for dubious results. Problems were expected to become worse with increasing ages of divergences to be explored in a common framework. Nevertheless, in the Deep Metazoan Phylogeny framework we analyzed molluscs via mitochondrial markers. We hoped for the beneficial effects of using (1) the currently best possible taxon sampling, i.e. all metazoan taxa with mitogenomes available, including 52 molluscs of 6 (of 8) classes, (2) advanced and homogeneous gene annotations (Jühling et al., 2012; Bernt et al., 2013a, c), (3) conservative amino acid sequences of protein coding genes (rather than more homoplastic nucleotides), (4) alignment masking, (5) ML models relaxing evolutionary rates. However, such expectations were disappointed. Neither the origin of molluscs, nor any of the hotly debated deeper molluscan relationships were resolved in the 684 metazoan taxa analysis (Fig. 1). Molluscs are mixed up with other lophotrochozoan taxa, and none of the molluscan classes with more than single representatives was recovered monophyletic in any of the various analyses. The sole exception is Cephalopoda, showing *Nautilus* sister to coleoid taxa as was recovered also in virtually all recent morphology-based analyses, multi-locus marker studies also using nuclear rRNA genes (Giribet et al., 2006; Nishiguchi and Mapes, 2008), and broad phylogenomic/transcriptome data (Kocot et al., 2011; Smith et al., 2011).

Extending our taxon sampling to 96 molluscs and a selection of putatively related lophotrochozoans (16) lead to slightly different topologies but did not significantly improve the plausibility of the results (Fig. 2). Using masked versus unmasked amino acid alignments showed little influence on resulting topologies. Surprisingly, analyzing nucleotides rather than supposedly more conservative amino acids of our 112 taxa set recovered a slightly more plausible topology, with most of the outgroup taxa recovered outside of still paraphyletic Mollusca, and gastropods split into three distant clades. Remarkably, this is true even for the analyses of the unmasked, highly heterogeneous nucleotide alignment, still including poorly aligned and divergent parts.

Some of the lophotrochozoan outgroup taxa clustering within molluscs can be explained by assuming long branch attraction (LBA) artifacts, e.g. long-branched platyhelminths cluster as sister of the pteriomorph bivalve *Mytilus* in our preferred tree (Fig. 2), while clustering with long-branched nematodes and acari in the large metazoan analysis (Fig. 1). The single patellogastropod *Lottia digitalis* appears to be attracted by relatively long pteriomorph bivalve branches in all analyses. The very basal positions of long-branched heterobranch gastropods and of one or both scaphopods in the amino acid trees also may be attributed to LBA (Fig. 2 and Suppl. Figs. 1–3). However, other obviously erroneous relationships, such as of nemertean taxa sister to the chiton *Katharina* in our masked amino acid analysis, refer to relatively short branches (Fig. 2 and Suppl. Figs. 1–3). Disregarding outgroups and obviously misplaced molluscan clades discussed above, interclass relationships of molluscs recovered in our masked amino acid tree, i.e. (Bivalvia (Caudofoveata ((Scaphopoda with Dentaliida only, Cephalopoda) (Polyplacophora, Gastropoda))), still are in contrast to previous analyses based on mitochondrial genes with, however, much smaller taxon sets (Boore et al., 2004; Dreyer and Steiner, 2006; Yokobori et al., 2008). Our topology also differs from any other of the many previously proposed phylogenies based on other data sets such as nuclear genes, and refers to very short internal branches not showing any significant bootstrap support. Our nucleotide-based molluscan interclass topologies still differ, are clearly artificial, and neither show significant internal branch lengths nor node support. We conclude that analyses of mitochondrial gene sequences available on a comprehensive taxon set at present fail to resolve the origin of Mollusca among lophotrochozoans and fail to resolve deep molluscan phylogeny. Adding further mitogenomes of unsampled or poorly represented classes is desirable, but because of the obvious lack of phylogenetic signal in basal branches, this will probably not resolve the origin of molluscs or deep molluscan nodes reliably.

Yokobori et al. (2008) have shown that a selection regime towards slowly evolving taxa can be beneficial for recovering monophyletic Mollusca. Also, the basal position of Mollusca among (non-platyzoan) lophotrochozoans recovered by analyses of mitochondrial protein coding genes (Yokobori et al., 2008) parallels results of recent nuclear multigene or EST-based studies (e.g. Dunn et al., 2008; Pick et al., 2010; Meyer et al., 2011). Remarkably, none of the broader molecular studies support morphology-based hypotheses of a direct sister group relationship of molluscs with either annelids (including Sipuncula) or entoprocts. Instead, both taxa could be part of a more complex sister clade of molluscs. Addressing deep molluscan evolution, future mitochondrial taxon sets thus should be pruned to just a few short-branched outgroups. Of course, representatives of all major ingroup taxa should be included and, in better sampled undisputed groups such as gastropods, it seems reasonable to select slowly evolving vetigastropods and caenogastropods rather than aberrant euthyneurans or patellogastropods. However, on molluscan class level, both available scaphopod mitogenomes are aberrant in the light of forming long branches in all our analyses, and bivalves all appear to have comparatively long branches. Therefore, a strict ingroup selection regime as done by Yokobori et al. (2008) is likely to produce a tree that appears more plausible than ours, but may hide intrinsic problems in the data and still misses its genuine goal, i.e. resolving molluscan interclass relationships. Using alternative, i.e. nuclear markers with better signal to noise ratio, or complex characters with little probability of convergence, i.e. rare genome level changes, may be better options.

4.2. Phylogeny of molluscan subgroups

Our preferred analysis (Fig. 2) included mitochondrial genome sequences of single caudofoveate and chiton species, of two highly diverging scaphopods, and of multiple representatives of three molluscan classes, i.e. cephalopods (14), bivalves (31) and gastropods (47). As mentioned above, inner cephalopod monophyly was recovered by all our analyses, regardless whether they were based on amino acids or nucleotides, and independent from using

masked or unmasked alignments (Fig. 2 and Suppl. Figs. 1–3). Cephalopod monophyly and inner cephalopod subdivision into the single nautiloid and coleoids receive maximum support in all analyses and reflect current knowledge (e.g. Nishiguchi and Mapes, 2008; Allcock et al., 2011). Coleoids (Neocolioida) divide into Decabrachia (Decapodiformes) and Octobrachia (Octopodiformes), receiving maximum support in all analyses but moderate support (80%) in the masked amino acid analysis. Octobrachia with Vampyromorpha represented by *Vampyroteuthis infernalis* and Octopoda (two *Octopus* species) thus form a clade herein that was recovered by multi-locus analyses (e.g. Strugnell et al., 2005) and mitochondrial gene data (Akasaki et al., 2006; Yokobori et al., 2007; Allcock et al., 2011) earlier, while other molecular, morphology-based or combined studies suggested paraphyletic Octobrachia, with *Vampyroteuthis* sister to Decabrachia (see Nishiguchi and Mapes (2008) for review). Mitogenomic markers thus may be informative for resolving cephalopod relationships. This is quite remarkable considering the old age of the group; oldest reliable cephalopod fossils date back into the Late Cambrian, and the split of Nautiloida from Coleoida was dated to the mid-Palaeozoic in molecular clock approaches (Kröger et al., 2011). The currently available cephalopod mitogenome sampling still is much too fragmentary to be conclusive on the disputed phylogeny of coleoids.

Bivalve protein coding mitochondrial genes appear to evolve at a higher pace than in most other molluscs, as inferred from long branches in our nucleotide and amino acid analyses. Interestingly, all well-established bivalve family level groups and major taxa such as Palaeoheterodonta (represented by unionids), Pteriomorphia and Heterodonta were recovered, if ignoring obviously misplaced long branched platyhelminths and *Lottia* (Fig. 2 and Suppl. Figs. 1–3). Consistent with previous analyses of mitochondrial genes, unionids are sister to a clade of pteriomorphs and heterodonts (e.g. Dreyer and Steiner, 2006), contrasting to the Heteroconchia hypothesis uniting palaeoheterodonts with heterodonts (e.g. Giribet, 2008). Indeed, even an "edited monophyly" of autolamellibranch bivalves is remarkable for mitochondrial nucleotide and amino acid analyses herein, since bivalve mitochondria are among those animals showing most aberrant evolution (Gissi et al., 2008). Also, Bivalvia are very old, with first reliable fossils known already from the Early Cambrian (Giribet, 2008), and first autolamellibranchs were present latest in Early Ordovician. Adding mitogenomes of representatives of yet missing, likely basal protobranch and other undersampled taxa appears promising for resolving inner bivalve relationships.

Gastropods may be as old as or even older than bivalves, perhaps dating back into the terminal Precambrian (e.g. Parkhaev, 2008). They are the by far most diverse molluscan class, count with the greatest number of species considered in our molluscan analyses, and also display greatest sequence heterogeneity. In all our analyses, gastropods split into three distant clades, (1) Patellogastropoda (represented by *Lottia*), (2) Heterobranchia (including pulmonates and opisthobranchs), and (3) Vetigastropoda (*Haliotis*) and Caenogastropoda (several species). Patellogastropoda are usually thought to be sister to all other gastropods (Ponder and Lindberg, 1997), as represented herein by *Haliotis* as single member of Vetigastropoda, and Apogastropoda, composed of caenogastropods and heterobranchs. *Lottia*, however, has a highly aberrant mitogenome, and behaved enigmatic in any sequence analyses (Fig. 2 and Suppl. Figs. 1–3). Previous analyses of mitochondrial genes recovered a well-supported clade of *Lottia* and heterobranchs that was seriously discussed for its potential phylogenetic implications (Grande et al., 2008). Our analyses with the most complete set of mitogenomes available suggest that *Lottia* represents a long branch with a strong tendency for LBA (Fig. 2 and Suppl. Figs. 1–3). Patellogastropods show notoriously long branches also in multi-locus datasets including nuclear genes and a more representative patellogastropod sampling (Giribet et al., 2006; Meyer et al., 2010). Recently, analysis of broad EST data succeeded in recovering *Lottia* as a gastropod, i.e. in a basal position, with, however, limited gastropod sampling (3 species) and showing rather weak node support (Kocot et al., 2011). Other recent works, with slightly broader gastropod sampling, recovered *Lottia* within gastropod subclades, closely related or sister to *Haliotis* instead, using selected ribosomal protein genes (Meyer et al., 2011), housekeeping genes (Vinther et al., 2011) and broad phylogenomic EST data (Smith et al., 2011). The latter results are consistent with multi-locus data analyzed in a more focused, i.e. large gastropod taxon sampling, in which patellogastropods also cluster in a basal position to vetigastropods (e.g. Aktipis and Giribet, 2010, 2012). It thus appears that long branch artifacts are an issue, and mitochondrial sequences are not prime candidates for resolving patellogastropod relationships.

Similarly, heterobranch gastropods form a well-supported but long branched clade that is, however, pulled away from their supposed sister group Caenogastropoda and all other gastropods towards the base of the lophotrochozoan tree in all our molluscan analyses (Fig. 2 and Suppl. Figs. 1–3), or clustering with a scaphopod and bryozoans in the large metazoan tree (Fig. 1). None of these entirely artificial relationships obtains significant support values though. Earlier studies on heterobranch phylogeny using selected mitochondrial genes or mitochondrial genomes always recovered unconventional topologies, e.g. recovering stylommatophoran pulmonates as basal euthyneuran offshoot (e.g. Grande et al., 2002, 2008; Knudsen et al., 2006) as herein, or rejecting the otherwise well-established monophyly of Nudibranchia (Grande et al., 2004a,b). Inner heterobranch topology herein shows paraphyletic panpulmonates (i.e. stylommatophoran pulmonates as most basal offshoot of a sequence of further traditional pulmonate, pyramidellid, sacoglossan and siphonarian clades), followed by a dichotomy of actenoidean (=lower heterobranch) and nudipleuran taxa with monophyletic Nudibranchia on one branch, and of Euopisthobranchia (including Cephalaspidea and Anaspidea sampled herein) on the other (Suppl. Fig. 2). Our topology resembles a recently published tree (White et al., 2011) from mitogenomes of 27 gastropods, including 10 pulmonate mitogenomes that were not yet considered herein. These analyses confirm several important aspects found using multi-locus markers on much more representative heterobranch samplings (e.g. Dinapoli and Klussmann-Kolb, 2010; Jörger et al., 2010), e.g. neither Pulmonata nor Opisthobranchia as traditionally defined are monophyletic, and formerly lower heterobranch Pyramidellidae and opisthobranch sacoglossans cluster among traditional pulmonate clades. However, these mitogenomic results neither recover traditional Euthyneura, nor newly established clades such as Tectipleura and Panpulmonata (Jörger et al., 2010; Schrödl et al., 2011a). Most intriguing is the fact that mitogenomic heterobranch trees (e.g. by Grande et al. (2008) and Medina et al. (2011)), and even more so trees by White et al. (2011) and recovered herein (Suppl. Fig. 2) are virtually up side down or reversed when compared to multi-locus topologies, i.e. with Acteonoidea outside Euthyneura, Nudipleura sister to Tectipleura, the latter splitting into Euopisthobranchia and Panpulmonata (reviewed by Schrödl et al. (2011b)). The latter topology has been confirmed recently by analyses using broad sets of various nuclear genes and ESTs (Kocot et al., 2011; Smith et al., 2011; Vinther et al., 2011), while the topologies from entirely mitochondrial genes are clearly rejected. This new, multiple and independent evidence confirms an earlier assumption that mitogenomic euthyneuran trees are misrooted (Schrödl et al., 2011b), with longest internal branches (such as derived stylommatophorans, or in their absence, other derived pulmonates) pulled to the tree base. As shown herein, euthyneuran stems of all amino acid or nucleotide analyses are very long compared to internal branches, and caenogastropod or other gastropod out-

groups with mitogenomes available obviously are too divergent to root heterobranchs adequately. Generating mitogenomic data on basal caenogastropods and on still unsampled "lower heterobranchs" (i.e., lower than Acteonoidea) we predict this situation will change, long branches will be split and the euthyneuran topology is likely to reverse. Then, there will be no more need to redefine and reinterpret taxa according to predefined concepts, such as adjusting the pulmonate *Siphonaria* to be an opisthobranch (Medina et al., 2011; White et al., 2011). Considering topological and morphological evidence, *Siphonaria* is a basal panpulmonate, and similarities with euopisthobranchs rather than synapomorphies may be plesiomorphic or convergent (e.g. Jörger et al., 2010; Schrödl et al., 2011a,b). In the light of all the new evidence from mitochondrial, mixed and nuclear datasets, there is no more case for monophyletic Opisthobranchia and Pulmonata. Sticking to (or reshaping) such concepts, which appear entirely artificial, hides the crucial point, i.e. that evolution of roughly 50,000 species of euthyneuran slugs and snails likely was absolutely different to centenary paradigms.

4.3. Genome level characters

Genome level characters such as gene order and composition were promoted as having potential to resolve molluscan relationships (Simison and Boore, 2008). A precondition is that genes to be compared are correctly annotated. Our dataset of molluscan mitochondrial genes generally confirms previous annotations although some discrepancies emerged. Within heterodont bivalves several taxa display duplicated *cox2* (*Meretrix*, *Loripes*, *Venerupis*). This is in clear contrast to the published sequences in *Meretrix* species and *Loripes* (Ren et al., 2009; Dreyer and Steiner, 2006) whereas a duplication of that gene was reported in Doucet-Beaupré et al. (2010). Copies of that gene are between 321 bp and 366 bp length (*Meretrix*). *Venerupis* even has three copies of that gene with an extension of 294–342 bp. These lengths are half of the normal size of that gene (about 680 bp). We could not determine if the copies are still functional or if they reflect a duplication event followed by reduction or a division of the normal sized *cox2* gene occurred. Although duplication of *cox2* was detected in other groups before, e.g. several cephalopods (Yokobori et al., 2004) and *Chaetoderma* (Caudofoveata) (submission to NCBI: Dreyer and Steiner, 2010, unpublished), neither duplicated and reduced nor divided *cox2* is reported in other molluscan classes so far. Several characters in bivalve taxa seem to be connected to their unique pattern of inheriting mitochondrial DNA (doubly uniparental inheritance (DUI), see e.g. Curole and Kocher, 2005; Doucet-Beaupré et al., 2010); duplication of *cox2* is probably one of those correlated features since it is apparent only in the female individual of *Venerupis* (Doucet-Beaupré et al., 2010). Unfortunately we could not determine the sex of our individuals and therefore cannot support this assumption. Nevertheless, the characteristic pattern of duplicated and reduced or, alternatively, partitioned *cox2* seems to be synapomorphic to Heterodonta.

A second dissimilarity between our rearrangements and annotations in NCBI concerns bivalve rearrangements of all reviewed *Crassostrea* species herein. Our reannotated mitochondrial genomes of *Crassostrea* individuals share the same typical distribution of rRNAs. *RrnL* is reduced to 719 bp (*C. sikamea*)-834 bp (*C. angulata*) instead of a general size of ca. 1300 bp, whereas *rrnS* is duplicated in each individual with both copies span about 945 bp in length which is similar to the common size of that gene. All individuals possessing duplicated *rrnS* have mitogenome sizes between 18.2 kb (*C. angulata*) and 22.4 kb (*C. iredalei*). An exception is *C. virginica*, the only individual with just a single *rrnS* gene present accompanied by slightly smaller overall size of the genome (17.2 kb) compared to the other individuals. The second exception is *C. gigas* which lacks *rrnL* completely according to our reannotation, although complete genome size covers a similar size (18.2 kb) as those individuals with duplicated *rrnS*. Since splitted *rrnL* was recovered earlier in *C. gigas* and *C. virginica* and only one copy of *rrnS* in *C. hongkongensis* (Milbury and Gaffney, 2005; Yu et al., 2008) it has to be reinvestigated if our reannotation failed in those individuals. Shortened *rrnL* is also detected in *Saccostrea* but neither in palaeoheterodont nor in heterodont taxa. Mapped on the topology of Doucet-Beaupré et al. (2010), shortened *rrnL* is probably synapomorphic for ostreoid taxa, and duplicated *rrnS* is a synapomorphy for all *Crassostrea* species except *C. virginica*, which is the most basal taxon within *Crassostrea* (see Wu et al., 2010).

As it is apparent in *Crassostrea* species the size of mitochondrial genomes is correlated with duplication events. Metazoan mitochondrial genome sizes range from 14 to 20 kb (Dreyer and Steiner, 2006), and molluscs cover the entire range. There is no obvious phylogenetic signal regarding interclass relationships. Sizes of poorly sampled aculiferan classes vary between the chiton *Katharina* (15.7 kb) and the caudofoveate *Chaetoderma* (20 kb). As an exception among conchiferans, both scaphopod genomes available are small; sizes as small as 13.9 kb require special adaptations as severely reducing non-coding regions in *Graptacme* (Boore et al., 2004) or shortening gene lengths as it is observed in *Siphonodentalium* (Dreyer and Steiner, 2004). Other, better sampled conchiferan classes show enormous internal variation, roughly as great as between them. Cephalopods range from ca. 15 to 20 kb. Enlarged mitogenomes in teuthid taxa are correlated with the duplication events of genes (see Yokobori et al., 2004) what might be of phylogenetic interest (Yokobori et al., 2004; Akasaki et al., 2006). Multiplication of non-coding regions is also visible in taxa with smaller genomes, e.g. *Sepia* possesses two non-coding regions each ca. 570 bp long and a mitogenome length of just about 16 kb (Yokobori et al., 2004; Akasaki et al., 2006). The most striking size is tracked in the bivalve *Placopecten*, ranging from 32 to 40 kb between individuals. Size in this taxon depends on the number of copies of a 1.4 kb repeat, which is duplicated up to eight times depending on the individual, and on the amount of non-coding DNA, which can account up to 25 kb of the complete mitochondrial genome (Smith and Snyder, 2007). Similarly large non-coding DNA regions are reported from the brachiopod *Lingula* (Endo et al., 2005) and multiple non-coding regions were detected in other bivalve taxa (Ren et al., 2010 and references therein, Wu et al., 2010). Typically, in metazoan mitogenomes the origins of replication and transcription are situated within the longest non-coding region. As Yokobori et al. (2004) suggested, multiplied non-coding regions could imply more replication origins and mitochondrial genomes can therefore be replicated more often or faster than those with only one single initiation region. These regions should hold high AT content and conserved sequence patterns as well as distinct secondary structures and adjacent tandem repeats, but still such features are not investigated thoroughly within invertebrates (Smith and Snyder, 2007; Ren et al., 2010); in several cases it is unclear if the multiplied non-coding regions include the origin of replication and transcription or whether these genome parts have other functions. Understanding the mechanisms and processes of duplication events in mitochondrial genomic regions clearly would strengthen their potential use for molluscan phylogeny.

Among gastropods, *Lottia* (Patellogastropoda) shows extremely long non-coding regions, 1500 and 7000 bp long (Simison et al., 2006), which elongate the genome to more than 26 kb. This is in contrast to all other gastropods, which have rather small and compact genomes with an average size of 15.5 kb (without Patellogastropoda) due to small sized and overlapping genes and short intergenetic spacers (Grande et al., 2008). *Lottia* not only has a different mitogenome size but nuclear rRNA genes of patellogastro-

pods also show massive insertions and behave aberrantly in phylogenetic analyses (e.g. Meyer et al., 2010). Comparing average sizes of mitogenomes among gastropod subgroups shows Vetigastropoda (16.7 kb) larger than Apogastropoda, with 15 kb in Caenogastropoda and only 14.6 kb in Heterobranchia; thus, there seems to be a trend for reducing mitogenome size towards Heterobranchia. Size changes of mitochondrial genomes thus might characterize certain lineages, but distribution of e.g. large mitogenome sizes over several molluscan classes and also more distant groups of Metazoa, i.e. Brachiopoda, Coleoptera or Nematoda (Boore, 1999 and references therein) suggests considerable homoplasy, and different processes might be involved. In contrast to mere sizes, comparisons of special duplication (or reduction) events might be of considerable phylogenetic significance.

Most metazoan taxa exhibit a more or less uniform distribution of genes between the two strands of mitochondrial DNA. But in all groups the switch from transcription of double to single strand is present (Gissi et al., 2008). As other molluscs, unionid bivalves transcribe their complete set of mitochondrial genes on two strands, while other bivalves use a single strand. Thus Doucet-Beaupré et al. (2010) inferred palaeoheterodonts as retaining the original two strand condition, while the ancestor of pteriomorphs and heterodonts switched genes on a single strand. However, plotting the feature on Giribet's (2008) new bivalve tree either requires two independent switches to a single strand, or one switch to single strand in the common ancestor of (at least non-protobranch) bivalves and distribution on both strands in ancestral Palaeoheterodonta. This scenario is further complicated considering that several molluscan outgroups like brachiopods and annelids including sipunculids all transcribe the mitochondrial genome from just a single strand. Depending on their exact origin, molluscs thus evolved from a single or double strand condition, with both scenarios requiring multiple changes. Mechanisms such as head to tail genome duplication were proposed leading to homogeneous gene blocks arranged on one or the other strand (Lavrov et al., 2002) that also could force for single strands ("ratchet", Vallès and Boore, 2006); in contrast, genome duplication, including control regions, head to head may lead from a single stranded ancestor to descendents with genes on two strands (Lavrov et al., 2002) suggesting considerable likeliness for convergence. Ren et al. (2010) conclude that all marine representatives of bivalves use only one single strand for transcription whereas all freshwater taxa transcribe from both strands. This is not entirely congruent with our results based on an enlarged taxon set, since *Argopecten irradians* (NC_009687) encodes one tRNA on the minus strand.

Atp8 is a short protein coding gene that is nevertheless variable in length and conserved in just a short fragment at the 5′ region (Smith and Snyder, 2007; Gissi et al., 2008). This makes it challenging to annotate the gene correctly. Indeed, in that point our reannotated genomes differ substantially from the published arrangements. We detected *atp8* in five heterodont and seven pteriomorph taxa. In *Venerupis* (Heterodonta) the gene was already detected earlier, although the question of functionality emerged, because the gene is clearly reduced in length in comparison to other metazoan *atp8* sequences (Dreyer and Steiner, 2006) and the correct annotation is difficult. The issue of functionality remains to be examined exhaustively in those taxa, which lack *atp8* according to NCBI annotations, but display the gene in our reannotations. *Atp8* clearly belongs to the complete set of animal mitochondrial genes (Gissi et al., 2008), but is the fastest evolving mitochondrial gene and was lost or transferred to the nuclear genome independently in various metazoan clades e.g. in platyhelminths, nematodes, poriferans, and chaetognaths (Gissi et al., 2008). *Atp8* was lost or reduced in pteriomorph and heterodont bivalves (Doucet-Beaupré et al., 2010; Dreyer and Steiner, 2006), which were inferred as several independent incidents and the absence/presence of *atp8* in bivalves was stated as "labile" (Doucet-Beaupré et al., 2010) or even "dispensable" (Gissi et al., 2008). Ren et al. (2010) concluded that it is characteristic for marine bivalves to lack *atp8*. However, this issue has to be reinvestigated, since we detected *atp8* or at least a remnant of this gene in mitochondrial genomes of several marine bivalves. These findings also affect evolutionary scenarios reconstructed by Doucet-Beaupré et al. (2010); based on their topology the presence of (putative) *atp8* would be part of the ancestral state of Bivalvia (or at least of all non-protobranch bivalves), and *atp8* is symplesiomorphic for Palaeoheterodonta and Heterodonta. *Atp8* likely was lost or dislocated to the nuclear genome independently in basal pteriomorph lineages and retained or relocated to the mitochondrial genome by pectinoid taxa and *Crassostrea hongkongensis*. Alternatively, mitochondrial *atp8* already could have lacked in the pteriomorph ancestor but then must have been regained later; the process of regaining a lost gene is unclear.

Further genome level characters refer to coupling and decoupling events of gene pairs. Some pairs of PCGs are known to be co-located in mitochondrial DNA, e.g. *atp8*/*atp6* and *nad4*/*nad4L*. Both genes, *atp8* and *nad4L*, produce transcripts which are possibly too short for efficient interaction with the small subunits and therefore cannot be translated correct (Taanman, 1999). Nevertheless, coupling of *atp8* and *atp6* was lost in annelids including sipunculans (Boore and Staton, 2002). The latter authors assumed this decoupling is an apomorphy of Eutrochozoa sensu Ghiselin, i.e. including Mollusca. This is not necessarily so. Mollusca are a (potentially basal) member of Trochozoa (Edgecombe et al., 2011), and conditions are heterogenous among trochozoan and molluscan clades. Among brachiopods the two genes appear in both states, coupled and decoupled (Helfenbein et al., 2001). Most cephalopod and gastropod groups such as Octobrachia, Decabrachia, Caenogastropoda and Vetigastropoda have *atp8* associated with *atp6*. But decoupled taxa appear frequently in those classes, that is *Nautilus* within Cephalopoda and *Lottia* in gastropods. Among bivalves, Palaeoheterodonta have *atp8* and *atp6* coupled in contrast to all other bivalve groups. On a new bivalve tree according to Giribet (2008), and provided that the unexplored protobranchs show the coupled condition, decoupling could have occurred twice, in the pteriomorph and the heterodont ancestor. Based on the topology of Doucet-Beaupré et al. (2010) and assuming that coupled *atp8* and *atp6* is plesiomorphic for bivalves it would need just a single decoupling event, i.e. in the common ancestor of Heterodonta and Pteriomorphia.

The second pair of PCGs coupled in most animals is *nad4* and *nad4L*. Seperated by genes in non-unionid bivalves, one incident of decoupling occurred in the common ancestor of Pteriomorphia and Heterodonta (Doucet-Beaupré et al., 2010). Again, if underlying the bivalve topology by Giribet (2008) with Pteriomorphia (uncoupled) sister to Palaeoheterodonta (coupled) plus Heterodonta (uncoupled), this would require either two independent losses, or one loss in the common ancestor of (at least non-protobranch) bivalves and reinstatement in ancestral Palaeoheterodonta. *Nad4* and *nad4L* are not separated in all gastropod groups except for Heterobranchia; decoupling thus is apomorphic. Even the patellogastropod *Lottia* showing an otherwise highly aberrant gene order displays both genes adjacent. All other molluscan classes retain the cluster of *nad4* and *nad4L* with the sole exception in Scaphopoda, with coupled genes in *Graptacme* but decoupled genes in *Siphonodentalium*. Since the majority of lophotrochozoan ouptgroup taxa displays coupled *nad4*/*nad4L* this is probably the ancestral state for at least Lophotrochozoa, and decoupling events occurred several times during evolution. However, the case of the non-basal cephalaspidean heterobranch taxon *Sagaminopteron* (see Brenzinger et al., 2012), which displays *nad4* adjacent to *nad4L*, only divided by *trnY*, suggests that secondary coupling may occur among molluscs.

We did not investigate all potential molluscan genome level characters exhaustively herein, and signal is promising but not unambiguous. The four characters of bivalve genome level features that were investigated herein are in conflict with the Heteroconchia hypothesis by Giribet (2008) and Sharma et al. (2012), suggesting that Pteriomorphia is sister to a clade of Palaeoheterodonta and Heterodonta. Instead genome level features support the bivalve topology with Palaeoheterodonta sister to Pteriomorphia and Heterodonta as inferred by Doucet-Beaupré et al. (2010). Better taxonomic sampling, i.e. inclusion of protobranch bivalves, and better hypotheses on the origin of bivalves will be necessary to confirm the potential of genome level features for resolving phylogeny. Variation observable in the distribution of genome level features indicates that molluscs are excellent models to investigate the evolution of mitochondrial genomes.

4.4. Phylogenetic significance of conserved gene clusters

Gene arrangements on smaller scales, i.e. referring to sharing one or a few common gene boundaries, were given phylogenetic significance in older works, e.g. by Serb and Lydeard (2003), and character state changes were reconstructed and discussed as potential apomorphies. However, single gene boundaries, especially if including heterogeneous molluscan subtaxa and "mobile" tRNAs, have little probability of homology. In our comprehensive molluscan dataset we find obvious cases of convergence. In addition to homoplastic coupling of *atp8*/*atp6* and *nad4*/*nad4L* discussed above, this is for example the gene boundary of *trnA* and *trnS2*, or gene/tRNA boundary *nad2*–*trnM*, which appear to be synapomorphies for Palaeoheterodonta (Serb and Lydeard, 2003), but the tRNA boundary is also detectable in annelids and echiurids. Combination of PCGs *cob*–*cox2*–*cox3*, disregarding tRNAs in between or adjacent, was considered as synapomorphy for scaphopods. However, this complex is also present in the bivalve *Placopecten magellanicus* (NC_007234), and differing tRNAs included might weaken homology of PCG clusters. There may be many more examples in our molluscan dataset showing that there is a considerable level of homoplasy. Future studies have to show whether or not such noise can disturb cladistic analyses of metazoan and especially molluscan gene arrangements.

We thus explored whether or not larger gene clusters (sharing two or more boundaries) are less homoplastic. In fact, in our data set there is only one striking case of a more complex gene cluster occurring in certainly distant taxa. It refers to the nemertean *Lineus* and caenogastropods, e.g. *Cymatium*, which have dissimilar tRNA arrangements but share identical orders of protein coding and rRNA genes (Fig. 3B; Chen et al., 2012), i.e. sharing an inversion of the PCG block from *nad5* to *rrnS* compared to other molluscs and putative lophotrochozoan outgroups. Rather than suspecting this arrangement was inherited from the last common ancestor of *Lineus* and *Cymatium*, but reinvented independently in all other lineages since, it is much more likely to assume such inversed arrangement refer to simple inversion events in convergence.

To our surprise, there are no other obvious convergent cases of PCG clusters sharing two or more gene boundaries observed in our data set, and not a single case of convergence of identical larger gene clusters (considering tRNAs and strand positions). This indicates that convergence of larger gene portions is very rare even among the generally heterogeneous molluscs. In agreement with earlier studies we conclude that large scale similarities in gene arrangements/gene clusters are highly likely due to homology.

In fact, wherever we found larger PCG clusters across or within molluscan subgroups these are similar to the arrangement found in some other lophotrochozoans and the chiton *Katharina*, i.e. these arrangements seem plesiomorphic and thus do not bear signal for reconstructing deep molluscan phylogeny. Major molluscan groups deviating from plesiomorphic PCG arrangements, such as scaphopods and bivalves and subgroups thereof, are too heterogeneous to detect unambiguous synapomorphies. In contrast, the gastropod taxon Heterobranchia is characterized by a unique, apomorphic PCG arrangement (Fig. 3E), supporting its well-established monophyly (Haszprunar, 1985; Jörger et al., 2010). Mixed PCG and tRNA clusters were found being diagnostic and thus apomorphic for Caenogastropoda (Fig. 3B), i.e. the complete gene order from *rrnS* to *trnF* is an inversion of *Katharina* arrangement transformed to the opposite strand; furthermore the tRNA complex *trnM*–*trnY*–*trnC*–*trnW*–*trnQ*–*trnG*–*trnE* passed a translocation. We also found that certain tRNA clusters are characteristic and putatively apomorphic for some recognized molluscan subclades, e.g. bivalve Palaeoheterodonta, mytiloids and oysters. Quite unexpectedly, tRNAs thus bear considerable potential for reconstructing molluscan phylogeny, from shallow to deeper levels.

4.5. Deep Molluscan gene arrangements

It has long been noted that the chiton *Katharina* shares much of its PCG arrangement with distantly related animals such as arthropods or vertebrates (Boore and Brown, 1994; Boore, 1999), and even more with some other lophotrochozoan taxa such as *Phoronis* (Helfenbein and Boore, 2004) or the nemertean *Lineus* (that has an inversion of a large gene cluster relative to *Katharina*). Though other lophotrochozoans such as annelids and brachiopods and especially platyzoan taxa show more dissimilar arrangements, the gene arrangement of *Katharina* was implicitly or explicitly used as a proxy for the ancestral molluscan pattern. Indeed, the chiton *Katharina* and the coleoid cephalopods *Octopus* and *Vampyrotheutis* share almost identical gene arrangement (e.g. Yokobori et al., 2004, 2007; Akasaki et al., 2006), i.e. 35 of 37 mitochondrial genes are in the same position (Fig. 3A). However, one additional tRNA is on the opposite strand and one tRNA is transposed, and non-coding regions have dissimilar lengths and positions (Yokobori et al., 2004). These differences rather than by simple translocations were explained by a complex tandem duplication random loss involving 12 genes of an *Octopus*-like (just *trnP* on opposite strand) hypothetical molluscan ancestor (Yokobori et al., 2004). If so, the sequence of *Octopus*, though almost identical to *Katharina*, would in considerable part (non-coding region and following *cox2*–*trnD*) not be homologous to that of *Katharina*.

In 2012, the origin of molluscs among lophotrochozoans is still obscure, and inner molluscan relationships are not yet resolved; chitons rather than a basal molluscan offshoot are recovered as a more or less derived clade among Serialia (e.g. Giribet et al., 2006; Wilson et al., 2010) or Aculifera (e.g. Kocot et al., 2011; Smith et al., 2011). *Katharina* still is the only chiton with mitogenomic data available, but is a derived rather than a basal polyplacophoran clade (Okusu et al., 2003; Sigwart et al., 2011); its gene arrangement thus might neither represent the diversity of polyplacophoran mitogenomes nor reflect a basal chiton condition. Our survey of available molluscan mitogenomes using modern annotation algorithms shows that the *Katharina* arrangement almost identically occurs not only in certain cephalopods but also in the gastropod *Haliotis* and, with some modification, in the caudofoveate *Chaetoderma*. This does not completely exclude that partial rearrangements and subsequent reversals into the original order occurred independently within each of these classes, since functional or evolutionary constraints may exist (Rawlings et al., 2003), but further reduces the chance of homoplasy. Whether *Katharina* or *Octopus*/*Haliotis* (both with transposed *trnD* and reversal transposition of *trnP* and *Haliotis* with additional transposed *trnN*) reflects closer the most ancestral state in molluscs cannot be decided here, because of heterogeneous ingroup and outgroup conditions.

Molluscan PCG arrangements share several gene clusters with other non-platyzoan phyla. This is consistent with recent large-scaled molecular results confirming that molluscs are non-platyzoan lophotrochozoans (Dunn et al., 2008; Sperling et al., 2009; Pick et al., 2010). The proposed apomorphic inversion of *cob–nad6* in *Phoronis* (Phoronida) and *Katharina* (or ancestral molluscs) relative to *Limulus* by Helfenbein and Boore (2004) is shared by entoprocts (see Yokobori et al., 2008), but not by other lophotrochozoans such as *Lineus*. As revealed herein, molluscs and entoprocts as well as nemerteans share the tRNA complex *trnK–trnA–trnR–trnN–trnI* (inverse in entoprocts) while *trnA–trnR–trnN* is already present in *Limulus* and *trnK–trnA–trnR–trnN* in *Phoronis*. Based on the topology of Dunn et al. (2008) at least the ancestor of non-platyzoan Lophotrochozoa had the complete complex *trnK–trnA–trnR–trnN–trnI*. One inversion in the ancestor of entoprocts and complete loss of the complex in ancestral annelids (including Sipuncula) occurred. The loss (translocation) of single *trnI* happened in the ancestor of Phoronida, Brachiopoda and Nemertea followed by a loss of the residues of the complex in the brachiopod stem line. A second cluster of tRNAs (*trnM–trnC–trnY–trnW–trnQ*, or subsets thereof) is shared between *Limulus*, molluscs, and entoprocts. Within *Limulus trnQ–trnM* and *trnW–trnC–trnY* are splitted by *nad2*, in entoprocts the complex is shorter (*trnC–trnY–trnW–trnQ*) and with adjacent *nad2*. Plotted on the topology of Dunn et al. (2008) and assuming that *trnM–trnC–trnY–trnW–trnQ* is the lophotrochozoan ancestral state of that cluster, it would need loss of *trnM* in the entoproct stem line, loss of the cluster in the ancestor(s) of Annelida, Sipuncula, Phoronida, Brachiopoda, and Nemertea and gain of *trnG* and *trnE* in the common ancestor of Mollusca; the latter synapomorphic for molluscs. Secondary rearrangements of tRNAs can be frequent, and multiple modification or loss of such clusters also occurred within molluscan subtaxa. Molluscs may be a basal rather than derived non-platyzoan lophotrochozoan clade (Dunn et al., 2008; Pick et al., 2010; Philippe et al., 2011); the complex *trnK–trnA–trnR–trnN–trnI* and *trnC–trnY–trnW–trnQ* clusters could be symplesiomorphic for polyzoans and trochozoans and were secondarily modified in nemerteans and some other higher clades.

5. Conclusion

Concluding, we could not detect any unambiguously apomorphic rearrangements supporting the sistergroup relationship of Mollusca among Lophotrochozoa, nor supporting one of the competing higher molluscan classifications. On the other hand, adding tRNAs G–E to the end of the cluster *trnM–trnC–trnY–trnW–trnQ* may refer to a herein discovered genome level synapomorphy for Mollusca. Mitogenome evolution, including rearrangements of supposedly mobile tRNAs, remained highly stable in some but not other lophotrochozoan clades. It greatly differed among molluscan lineages, remaining virtually unchanged for more than 500 million years, e.g. in the vetigastropod *Haliotis*, while rearranging completely in the patellogastropod *Lottia*; periods of rapid rearrangement and virtual stasis occur in certain lineages such as cephalopods. Most interestingly, as displayed by cephalopods, not always the most basal clades retain the most plesiomorphic gene arrangement. In fact, those taxa with the *Katharina*-like mt gene order tend to have the shortest branches in our ML trees pointing to a low or normal substitution rate, whereas taxa with a very aberrant gene order such as bivalves, or the gastropods *Lottia* and Heterobranchia exhibit rather long branches. This might indicate a correlation between multiple rearrangements and increased substitution rate and could explain the problems occurring in phylogenetic analyses of lophotrochozoans and especially molluscs based solely on mitochondrial sequences. However, we do not know adequately about mitochondria evolution and the present molluscan taxon sampling still is way too fragmentary to be conclusive. Adding gene arrangement and other genome level data on unsampled monoplacophorans and solenogastres, as well as protobranch bivalves, lepidopleurid chitons and some supposedly basal gastropods such as neritimorphs, neomphalids and further patellogastropods and vetigastropods, plus adding further species of scaphopods and caudofoveates might well give a clue on basal molluscan phylogeny.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2012.11.017.

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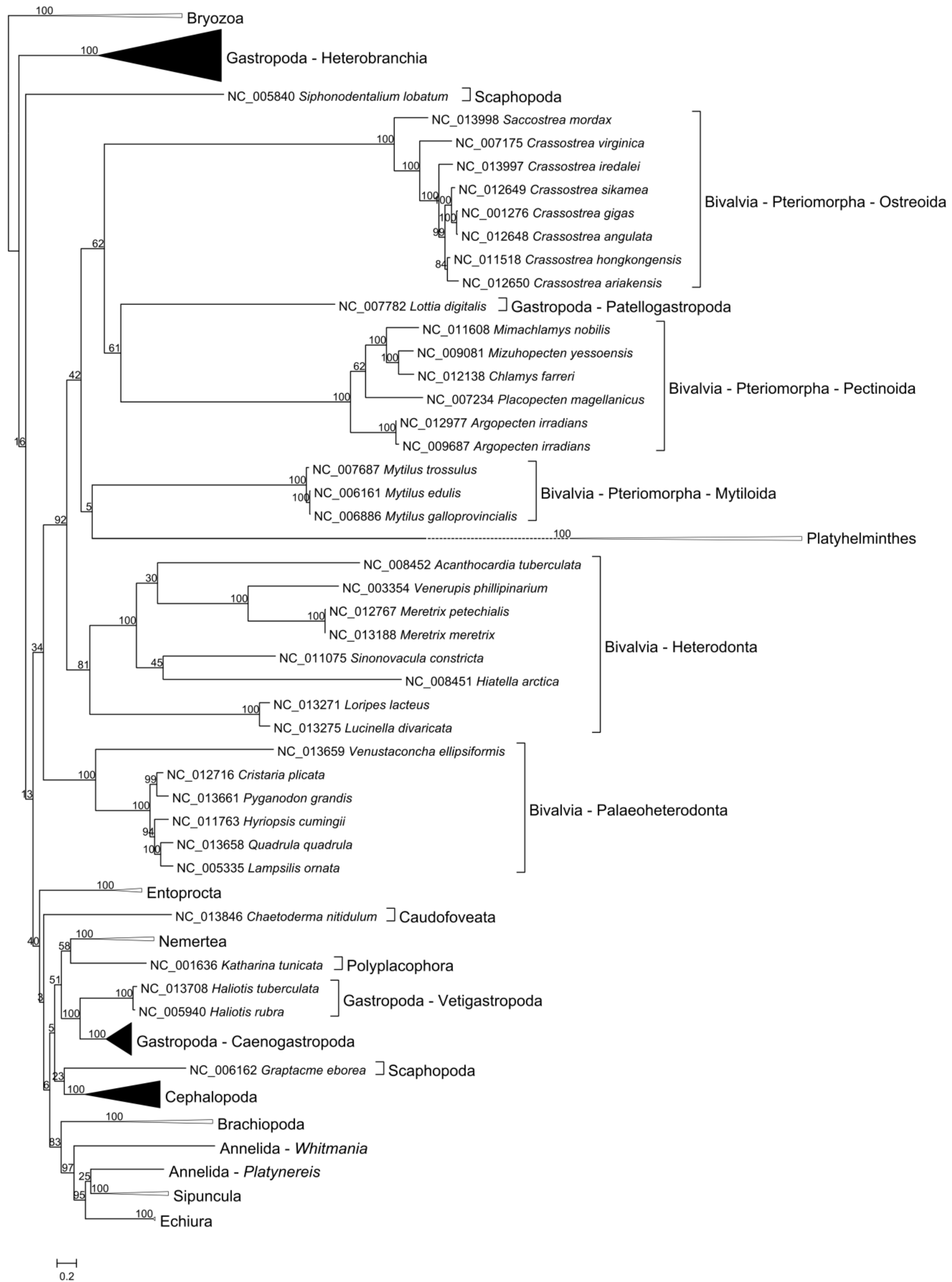
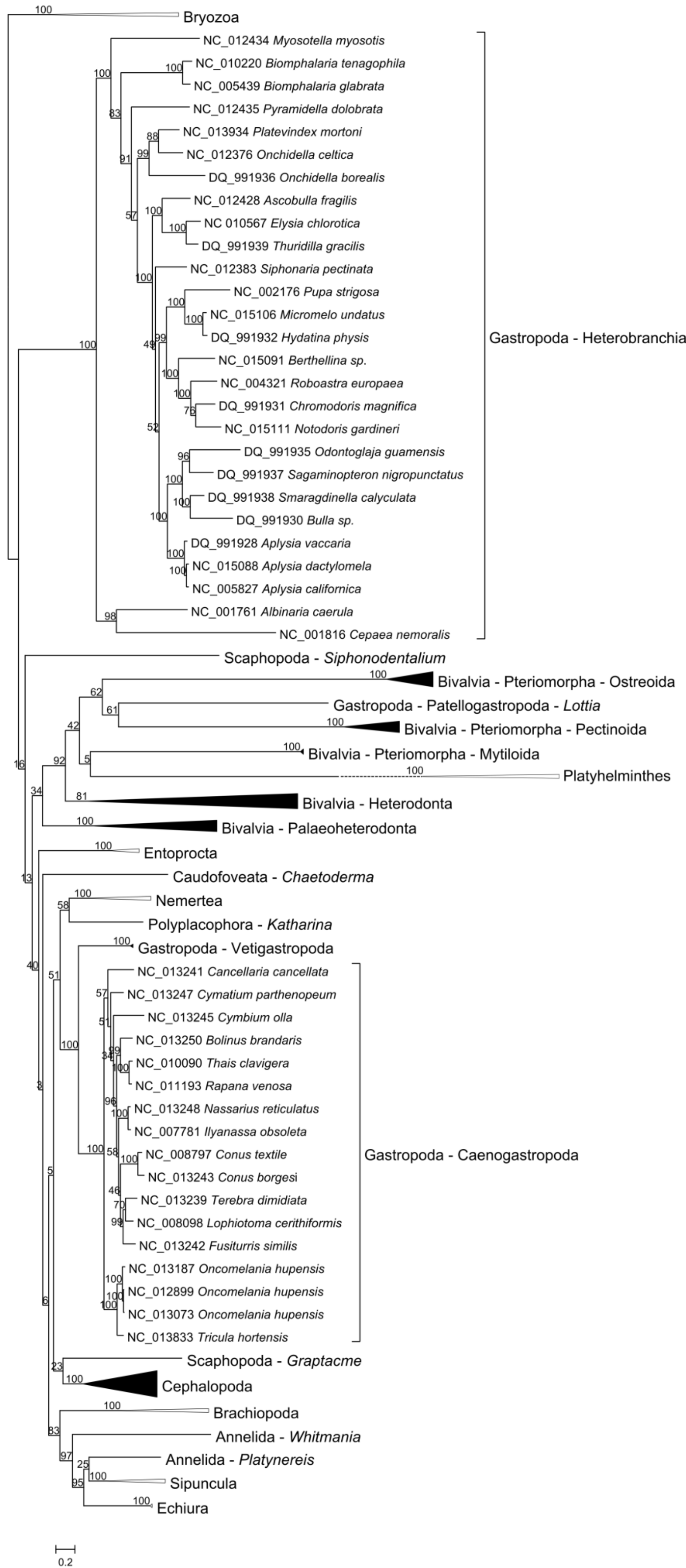
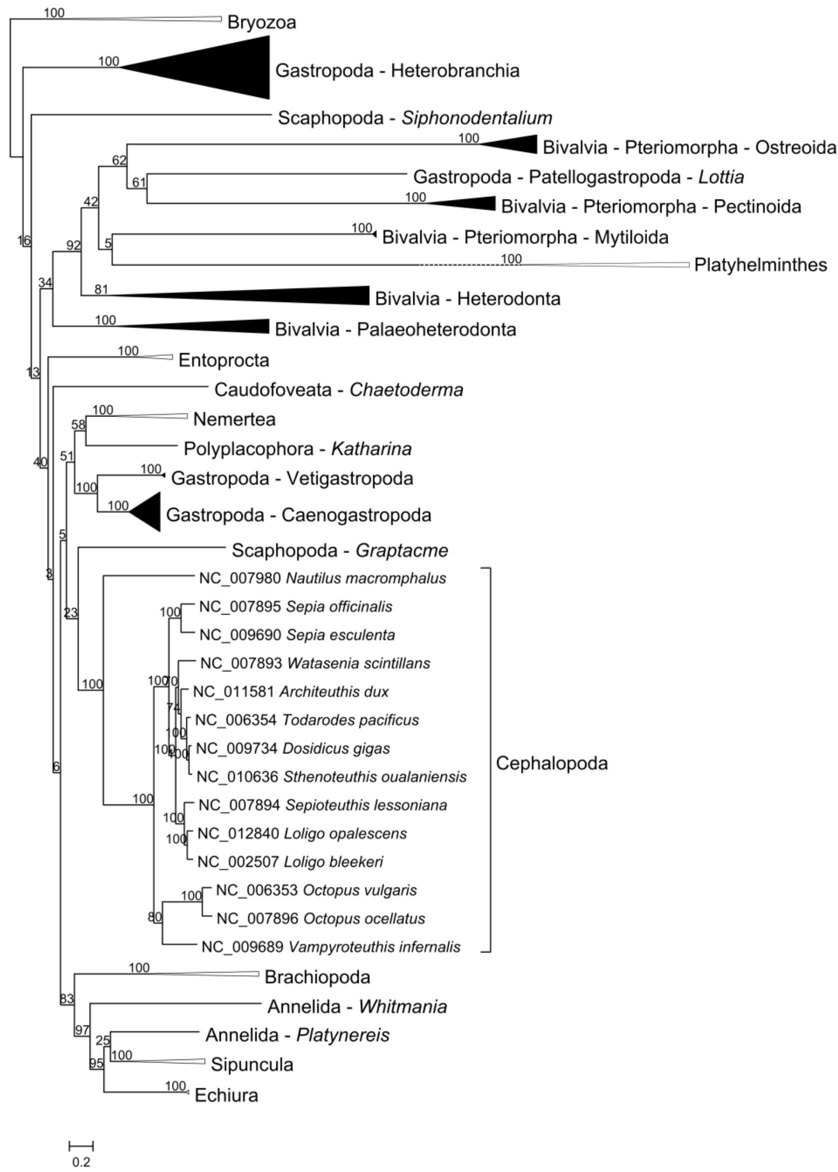


Stöger and Schrödl 2013 Supplementary Figure 1



Stöger and Schrödl 2013 Supplementary Figure 2



Stöger and Schrödl 2013 Supplementary Figure 3

3.5. Isabella Stöger, Kevin M. Kocot, Albert J. Poustka, Nerida G. Wilson, Dimitry Ivanov, Kenneth M. Halanych, Michael Schrödl: **Monoplacophoran mitochondrial genomes: convergent gene arrangements and little phylogenetic signal. 2016.** BMC Evolutionary Biology, 16.

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Monoplacophoran mitochondrial genomes: convergent gene arrangements and little phylogenetic signal

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Abstract

Background: Although recent studies have greatly advanced understanding of deep molluscan phylogeny, placement of some taxa remains uncertain as different datasets support competing class-relationships. Traditionally, morphologists have placed Monoplacophora, a group of morphologically simple, limpet-like molluscs as sister group to all other conchiferans (shelled molluscs other than Polyplacophora), a grouping that is supported by the latest large-scale phylogenomic study that includes *Laevipilina*. However, molecular datasets dominated by nuclear ribosomal genes support Monoplacophora + Polyplacophora (Serialia). Here, we evaluate the potential of mitochondrial genome data for resolving placement of Monoplacophora.

Results: Two complete (*Laevipilina antarctica* and *Vema ewingi*) and one partial (*Laevipilina hyalina*) mitochondrial genomes were sequenced, assembled, and compared. All three genomes show a highly similar architecture including an unusually high number of non-coding regions. Comparison of monoplacophoran gene order shows a gene arrangement pattern not previously reported; there is an inversion of one large gene cluster. Our reanalyses of recently published polyplacophoran mitogenomes show, however, that this feature is also present in some chiton species. Maximum Likelihood and Bayesian Inference analyses of 13 mitochondrial protein-coding genes failed to robustly place Monoplacophora and hypothesis testing could not reject any of the evaluated placements of Monoplacophora.

Conclusions: Under both serialian or aculiferan-conchiferan scenarios, the observed gene cluster inversion appears to be a convergent evolution of gene arrangements in molluscs. Our phylogenetic results are inconclusive and sensitive to taxon sampling. Aculifera (Polyplacophora + Aplacophora) and Conchifera were never recovered. However, some analyses recovered Serialia (Monoplacophora + Polyplacophora), Diasoma (Bivalvia + Scaphopoda) or Pleistomollusca (Bivalvia + Gastropoda). Although we could not shed light on deep evolutionary traits of Mollusca we found unique patterns of gene arrangements that are common to monoplacophoran and chitonine polyplacophoran species but not to acanthochitonine Polyplacophora.

Keywords: Mollusca, Mitogenome, Monoplacophora, Serialia, Aculifera, Conchifera, Gene arrangement, Phylogeny, Evolution

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Background

Mollusca, comprising eight extant classes, has high diversity and an origin that dates back more than 540 million years [1, 2]. One of the most enigmatic classes, Monoplacophora, was thought to be extinct since the Palaeozoic until a living exemplar of *Neopilina galatheae* was found during the Galathea expedition in 1952 [3]. The significance of "living fossil" monoplacophorans for deep molluscan systematics was soon recognized [3], and Monoplacophora (with about 30 recent members called Tryblidia [4]) were central in several palaeontological, morphological and cladistic analyses (e.g., [5–8]) that tried to resolve the phylogeny of Mollusca. These analyses resulted in a number of different phylogenetic placements being hypothesized for Monoplacophora. Under the Conchifera/Aculifera hypothesis, Monoplacophora were traditionally viewed as the sister group to all other conchiferans with and as the sister group of Aplacophora (Caudofoveata + Solenogastres; [9]).

Early molecular analyses based on nuclear ribosomal DNA did not include monoplacophorans [10, 11]. Later analysis of a data set dominated by nuclear ribosomal genes and including all eight extant molluscan classes placed Monoplacophora within Polyplacophora, Serialia [12]. The single 28S sequence from *Laevipilina antarctica* used in that study was a chimera between monoplacophoran and chiton 28S [13], subsequent studies based on the same markers but free of contamination recovered Monoplacophora as sister to Polyplacophora but retained the term Serialia [2, 13, 14]. However, relationships among molluscan classes in these studies were unconventional, recovering Serialia as sister group to bivalves and gastropods, and clustering scaphopods together with aplacophorans and cephalopods. The Serialia hypothesis, which is based on ribosomal DNA dominated data, is provocative, since it challenges traditional taxonomic text-book hypotheses.

Both Aculifera and Conchifera are strongly supported by phylogenomic studies [15–17] and became a new paradigm in molluscan systematics [18–22]; but see [23–25]. Schrödl and Stöger [26] recently emphasized that there is some conflict between the consensus topology (Fig. 1 in [26]), and any of the several phylogenomic [15–17, 27–30] and other nuclear sequence sets [31, 32]. All these molecular datasets cover substantial sequence data, but represent a limited taxon sampling. Smith et al. [16] present the first phylogenomic study including representatives of all eight molluscan classes, and thus it directly addressed placement of Monoplacophora [16, 17]. Although the authors detected many sites in their dataset with weak signal for Serialia and some sites with strong signal for Serialia, the sister group relationship of the one sampled monoplacophoran species and Cephalopoda is clearly favored [16, 17]. A more recent phylogenomic analysis [33] placed the sole monoplacophoran representative employed as the most basal lineage of conchiferans, albeit with low nodal support, but in line with some traditional morphological hypotheses.

An alternative to studying multiple genes is exploring the information content of mitochondrial (mt) genomes [34]. In Metazoa, mitochondrial genomes usually consist of a highly conserved set of 13 protein-coding genes (PCGs), two ribosomal RNAs (rRNAs), and 22 transfer RNAs (tRNAs) [35, 36]. Furthermore, metazoan mtDNA includes at least one (sometimes more in molluscs) noncoding region of which the largest typically contains the control region, the site of initiation for transcription and/or replication [10]. All known mt genomes in molluscs are circular, with orthologs readily identifiable, making them easy to compare. Analyses of mitochondrial protein-coding genes have been successfully used to resolve phylogenetic relationships as for example the affiliation of Sipuncula and Annelida [37–39]. Although the analysis of mitochondrial sequence data provides good resolution in some molluscan subgroups, e.g. Bivalvia [40] or Cephalopoda [41, 42], the resolution for deep molluscan class-relationships is generally poor [35, 43]. Even the analysis of all protein-coding genes of 96 available mt genomes covering six molluscan classes (lacking Monoplacophora and Solenogastres) lacked sufficient phylogenetic signal to robustly resolve relationships among the major lineages of Mollusca [44]. The known problem of increased rates of sequence evolution [45] in some subclades such as bivalves and scaphopods [46] in addition to the Precambrian split of Mollusca from the closest outgroups [2, 30] not surprisingly leads to long-branch attraction ([44]. Taxa showing massive gene rearrangements also show faster nucleotide evolution [26, 34, 44], creating analytical challenges. Stöger and Schrödl [44] recommended analyses of a more representative molluscan taxon set, with fast-evolving taxa at both sequence and gene rearrangement level excluded from analyses. Osca et al. [47] followed this strategy, excluding bivalves and including a second caudofoveate taxon, *Scutopus ventrolineatus*, resulting in an aculiferan/conchiferan topology, although with low support in Maximum Likelihood (ML) analyses. Support for Aculifera is strong for their Bayesian topology, but the clade is nested within outgroup taxa. Plazzi et al. [48] published the first mitogenome of Protobranchia, which are putatively basal lineage of bivalves. This genome appears more conservative relative to the inferred ancestral molluscan and lophotrochozoan arrangements compared to other bivalves, which show greater rearrangement [34, 44]. More recently, mitogenomes of five further chiton species were published in 2014 [49, 50]. According to the authors [49], gene orders are highly congruent with the earlier published mt genome of *Katharina* [35], showing a

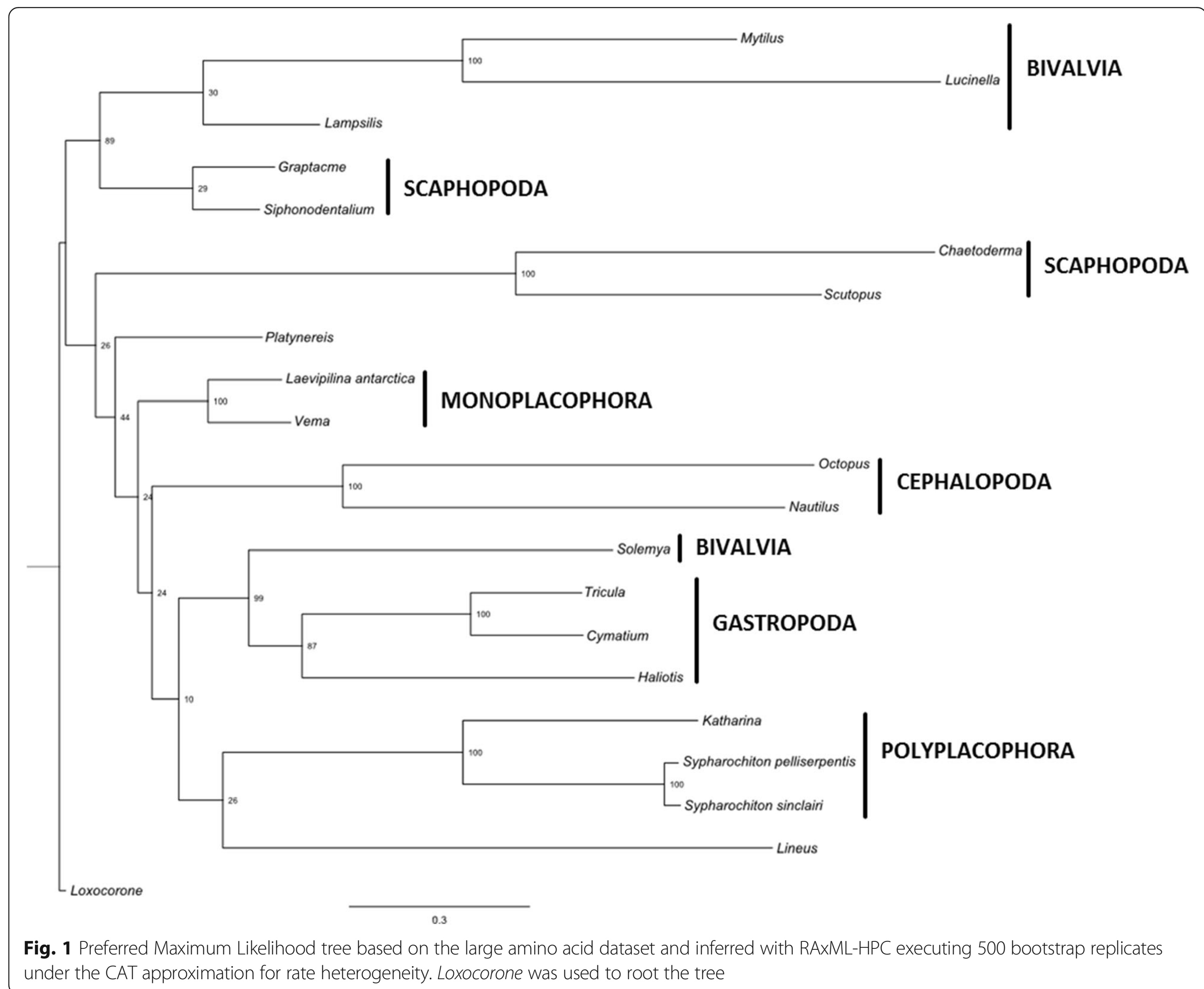

Fig. 1 Preferred Maximum Likelihood tree based on the large amino acid dataset and inferred with RAxML-HPC executing 500 bootstrap replicates under the CAT approximation for rate heterogeneity. *Loxocorone* was used to root the tree

plesiomorphic arrangement for lophotrochozoans, but this interpretation is not correct.

Here we contribute to the class-level taxon sampling of molluscan mitochondrial genomes by sequencing two Recent monoplacophorans (*Laevipilina antarctica* and *Vema ewingi)* and an almost complete mitogenome of *Laevipilina hyalina*. By generating the first mitogenomes for Monoplacophora our aims were 1) to explore the origin of the enigmatic Monoplacophora, 2) to evaluate whether or not a more balanced taxon excluding rapidly-evolving taxa improved resolution of deep molluscan phylogeny and 3) to compare monoplacophoran gene arrangements with a lophotrochozoan ground pattern [34].

Results

General structure/architecture of the monoplacophoran mitogenomes

Mitogenomes of *Vema ewingi* and *L. antarctica* are 17,910 bp and 18,642 bp in length, respectively. Both genomes include the complete set of 37 bilaterian mitochondrial genes: 13 protein-coding genes (PCGs), two ribosomal RNAs (rRNAs), and 22 transfer RNAs (tRNAs). Distribution of PCGs between the two strands is almost equal: ATP synthase subunits (*atp6*, *atp8*) and cytochrome c oxidase subunits (*cox1*, *cox2*, *cox3*), as well as *nad2* and *nad3* are located on the plus strand, whereas NADH dehydrogenase subunits (*nad1, nad4, nad4L, nad5, nad6*) and cytochrome *b* (*cob*) are on the minus strand. Ribosomal genes, *rrnS* and *rrnL*, as well as most of the tRNAs (15 in *L. antarctica*, 16 in *Vema ewingi*) are located on the plus strand. Only seven tRNAs in *L. antarctica* and six in *Vema ewingi* can be found on the opposite (minus) strand. Long-PCR fragments of *L. hyalina* were assembled into 1 contig totaling 15,102 bp and comprising 12 PCGs (*atp8* is missing), both rRNAs and 16 of 22 tRNAs (*trnT, trnC, trnW, trnG, trnH, trnE* are missing). We detected two copies of *trnK* in *L. hyalina*. One copy with a lower e-value (5.223e-05) is

located within the tRNA complex *DYKNM* and the second *trnK* with an e-value of 0.6443 is adjacent to *cox2*. In comparison, that *trnK* with a lower e-value is more probable. Both copies of *trnK* show typical cloverleaf secondary structures, similar to that of the two other monoplacophoran *trnK* structures, and the typical anticodon for lysine (UUU), so both copies are potentially functional. All PCGs that could be detected by MITOS are evenly distributed between both strands whereas *rrnS* and *rrnL* are exclusively located on the positive strand. Twelve tRNAs can be found on the plus strand, five are on the minus strand.

For *L. antarctica*, the GC content of the complete mitochondrial genome is 35.5%. GC content of individual PCGs ranges between 33.9% in *atp8* and 39.8% in *cox2* and values for ribosomal RNAs are slightly below the average of the complete genome with 34.4% for *rrnS* and 31.7% for *rrnL*. Transfer RNAs show considerable variation in their GC content with values ranging from 16.1% (*trnH*) to 46.8% (*trnY*). The GC content of the complete mitochondrial genome of *Vema ewingi* is 36.7% with a GC content of PCGs between 33.9% (*nad3*) and 40.4% (*nad6*). Both ribosomal RNAs have a value of 33.8% and tRNAs range between 17.5% (*trnH*) and 55.6% (*trnY*). GC content of the mitogenome of *L. hyalina* is 38.8%. GC content of PCGs is minimum 36.0% in *nad3* and maximum 45.8% in *cox2*. For ribosomal RNAs the GC content is 37.5% for *rrnS* and 34.3% for *rrnL*, within tRNAs range from 22.7% in *trnS2* to 50.0% in *trnY*.

Based on the MITOS results, we identified 28 non-coding regions (NCR) within the mitogenome of *L. antarctica*. Six are less than 10 bp long, 16 are between 10 and 100 bp in length and only six are larger than 100 bp. The largest NCR between *trnF* and *trnT* is 2012 bp long and contains a pattern with the regular expression TATA[TC]ATATATA[GT]A[CT][AT][TA][AT][TCG][GC], we refer to that pattern hereinafter as motif 1. Motif 1 includes an $(AT)_6$ repetition (see Table 1). Moreover, some repetitive motifs occur in that NCR (not shown). Motif 1 is additionally detected within the NCR between *trnG* and *trnE* (181 bp) with $(AT)_7$. A second motif with the regular expression CCTCGAAATCGTTGCATC (motif 2, Table 1), is visible in the NCR between *nad2* and *trnC* (478 bp). Moreover the NCR between *trnF* and *trnT* includes remains of *atp6*. In the NCR between *nad6* and *cob* MITOS detects residual sequence parts of *nad6*.

In *Vema ewingi* we found 27 non-coding regions; five regions are less than 10 bp long, 18 are 10-100 bp long and four are larger than 100 bp. The largest NCR between *trnF* and *trnT* (2287 bp) as well as the NCR located between *trnG* and *trnE* (151 bp) contain motif 1, which is already described for *L. antarctica*. Between *trnF* and *trnT* the motif contains $(AT)_6$ with a discontinuity of one (CA), and between *trnG* and *trnE* we count $(AT)_9$ (Table 1). Motif 2 was detected in the NCR between *nad2* and *trnS1* (108 bp) (Table 1). Moreover, repetitive motifs are visible in this largest NCR between *trnF* and *trnT* of *Vema ewingi* (not shown).

Within the partial mitogenome of *L. hyalina* we found 21 NCRs, one of which is less than 10 bp long. Sixteen regions are 10 to 100 bp and four are more than 100 bp in length. Within the NCR between *cox1* and *trnK* (299 bp) motif 1 with $(AT)_{10}$ is visible.

The largest NCRs of *L. antarctica* and *Vema ewingi* are located between *trnF* and *trnT* in both mtDNAs and in both NCRs the congruent motif 1 which includes AT-repetitions occurs at almost the same relative positions (Table 1). This motif 1 is recovered in a second NCR in each mitogenome again at congruent relative positions. Motif 2 can be found in NCR between *nad2* and *trnC* in *L. antarctica* and in NCR between *nad2* and *trnS1* of *Vema ewingi.* This motif 2 is located at almost identical relative positions (Table 1). Neither comparisons of these two NCRs to the BLAST nucleotide database results in any similarities to gene regions of other taxa nor are the 2D-foldings informative, which were computed in Geneious with default parameters.

Comparing the relative gene borders of the non-coding regions of the three monoplacophoran species, we discovered 13 NCRs that are embedded between the same genes in all three monoplacophoran genomes (Fig. 3). This number might be even higher since we do not know all NCR borders of *L. hyalina*. Identical positions of NCRs relative to gene order between *L. antarctica* and *Vema ewingi* are 11 whereas only one NCR has the same position between both *Laevipilina* species (Fig. 3). All three monoplacophoran species appear to share two NCRs with the cephalopod *Nautilus* [51]. This is NCR

Table 1 Table shows motifs 1 and 2, their location in the mitogenome and the specific motif sequence

Motif no.	Occurrence	NCR border	Starting position within NCR	Motif sequence
1	*L. antarctica*	trnG/trnE	55	TATATATATATAGATATATG
1	*Vema ewingi*	trnG/trnE	78	TATATATATATATACATATG
1	*L. antarctica*	trnF/trnT	893	TATATATATATAGACTATCG
1	*Vema ewingi*	trnF/trnT	898	TATACATATATATACTTAGC
2	*L. antarctica*	nad2/trnC	23	CCTCGAAATCGTTGCATC
2	*Vema ewingi*	nad2/trnS1	22	CCTCGAAATCGTTGCATC

between *cox1* and *nad2*, and NCR between *nad1* and *trnP* (Fig. 3).

We detected six overlapping regions that occur in all three monoplacophoran mt genomes. These overlaps are located between gene pairs *trnY/trnK*, *trnM/rrnS*, *rrnS/trnV*, *rrnL/trnL1*, and *trnP/nad6* (Fig. 3). Two pairs, *trnV/rrnL* and *rrnL/trnL1*, are overlapping with more than 25nts according to the MITOS annotation output.

Gene order within Monoplacophora

Gene arrangements of *L. antarctica* and *Vema ewingi* are shown in Fig. 3. They appear in two clusters (cluster means a group of genes in the following), this is *trnT-atp6-atp8-cox2-cox1-nad2-trnC-trnS1-nad3-trnA-trnR-trnI-cox3-trnG* on one strand and *trnE-trnW-trnD-trnY-trnK-trnN-trnM-rrnS-trnV-rrnL-trnL1-trnL2-nad1-trnP-nad6-cob-trnS2-nad4L-nad4-trnQ-trnH-nad5-trnF* on the opposite strand for *L. antarctica* (Fig. 3). The difference in *Vema ewingi* is the position of *trnC,* which is not located between *nad2* and *trnS1* as in *L. antarctica*, but is found within the tRNA complex *GEWDCYKNM*. The two gene clusters, *nad4/nad4L* and *atp6/atp8* are known to appear adjacent to each other in many animals [40, 52], which is detected here, too.

Within the partial mt genome of *L. hyalina* we observed a very similar gene order and orientation as in *L. antarctica* and *Vema ewingi*, although there are some differences (aside from missing genes). *TrnC* as well as tRNAs *GEW* are missing in the cluster *GEWD[C]YKNM* in the gene order of *L. hyalina* (Fig. 3). Though *trnK* is present within that complex, a second *trnK* with a much more reliable e-value appears adjacent to *cox2*. *TrnH*, adjacent to *trnQ* in *Vema* and *L. antarctica,* is missing in *L. hyalina*, as well as *trnF* and *atp8*.

The gene order in monoplacophoran PCGs and rRNA genes investigated herein is highly similar, therefore we summarize these arrangements and refer to it as the monoplacophoran plesiomorphic state.

Gene order within Polyplacophora

In addition to the mitogenome of the black chiton *Katharina* [35] five more chiton mitogenomes are available now [49, 50]. The three acanthochitonine mt gene arrangements (*Cryptochiton*, *Cyanoplax*, *Nuttalina*) are in line with the *Katharina* arrangement except the two tRNA complexes *KARNI* and *MCYWQGE*, which are present in *Katharina* and *Cryptochiton*. Both complexes appear in inversed orders in *Nuttalina* and *Cyanoplax*. Although mitogenomes of the chitonine taxa *Sypharochiton pelliserpentis* and *S. sinclairi* have already been published their gene order is not thoroughly examined [49]. The authors claim that the gene arrangements of their chitonine species resemble that of other chitons, but did not show the actual arrangement, so we have reexamined these mitogenomes (Fig. 3). Both *Sypharochiton* mitogenomes are congruent to each other in their gene arrangement but *contra* [49] the gene order is not "almost identical to that found in *Katharina tunicata*" ([49], Fig. 3 herein). The genes of *Sypharochiton* are arranged in the two clusters of genes that are already described for Monoplacophora (Fig. 3). Moreover, these two clusters have identical orientation as in the monoplacophoran arrangement (Table 4). Differences to the monoplacophoran gene order are restricted to the two tRNA complexes: one is *INRAK* in *Sypharochiton*, the second is *EGQWYCM*, which are exactly inverse to the *Katharina* order (Fig. 3, Table 4), but congruent to the order of *Nuttalina* and *Cyanoplax*.

Phylogenetic analyses

Our initial taxon set based on the amino acid alignment of all protein-coding genes includes 18 molluscs and three lophotrochozoan outgroup taxa (Table 5, Fig. 1, Additional file 1: Figure S1). The entoproct *Loxocorone* was used to root the tree as it represents the most distant related of the non-mollusc taxa employed [33]. Maximum Likelihood (ML) analysis of this taxon set recovers Mollusca as non-monophyletic with *Platynereis* (Annelida) and *Lineus* (Nemertinea) nested within Mollusca. Monoplacophora, Polyplacophora, Caudofoveata, and Cephalopoda were recovered monophyletic with maximal bootstrap support (bs) whereas support for gastropod monophyly was moderate (bs = 87%) and support for scaphopod monophyly was weak (bs = 29%). Relationships among higher level taxa were generally poorly supported. Also, Scaphopoda together with three non-protobranch bivalves form a moderately well-supported clade (bs = 89%; Fig. 1, Table 2).

Phylobayes analysis of this dataset recovered a topology that is unresolved at its base. All classes of Mollusca except Scaphopoda were recovered monophyletic with strong support (posterior probabilities, pp = 0.99-1.00). Pleistomollusca was also strongly supported (pp = 0.99) and Monoplacophora was recovered sister to Caudofoveata (albeit with weak support by Bayesian standards, pp = 0.84; Additional file 1: Figure S1, Table 2).

Exclusion of the outgroup taxa *Lineus* and the more distant outgroup *Loxocorone* and the reduction of bivalve taxa to the protobranch taxon *Solemya*, which is the most basal bivalve group, lead to a ML topology with a strongly supported Pleistomollusca (bs = 99) and a moderately supported sister group relationship of Caudofoveata and Scaphopoda (bs = 73; Fig. 2). Phylobayes analysis of this trimmed down dataset (Additional file 1: Figure S1, Table 2) yielded similar results with Scaphopoda (pp = 0.72) being the most weakly supported class and Pleistomollusca recovered (pp = 0.96).

In our test on saturation of the alignments TreSpEx calculated *cox1* as the least saturated and *nad6* as the

Table 2 Table gives an overview on all Maximum Likelihood (pre-) analyses and the resulting molluscan hypotheses; taxon sets aa-1 – aa-11 are based on amino acis data, taxon sets nuc-1 – nuc-11 are based on nucleotide datasets; main analyses based on aa-1 and aa-2 were additionally analyzed with Phylobayes which is indicated in the first column; Numbers are bootstrap support values of the corresponding hypothesis that appeared in that analysis, numbers followed by "pp" are posterior probabilities of the Phylobayes analysis; "-"means that the hypothesis did not appear in that analysis

Taxon set	Inclusion/exclusion of taxa	Monophyletic Mollusca	Aculifera	Conchifera	"Diasoma" (non-protobranch Bivalvia + Scaphopoda)	Monoplacophora + Cephalopoda	"Pleistomollusca" (Solemya + Gastropoda)	Serialia
aa-1	initial and largest taxon set, comprising 18 molluscan taxa and 3 lophotrochozoan outgroup taxa	-	-	-	81	-	100	-
aa-1 Phylobayes	initial and largest taxon set, comprising 18 molluscan taxa and 3 lophotrochozoan outgroup taxa, analyzed with Phylobayes	-	-	-	-	-	Bivalvia + Gastropoda: 0.99 pp	-
aa-2	initial taxon set except non-protobranch Bivalvia and outgroups reduced to *Platynereis* (Annelida) only	0	-	-	-	-	100	-
aa-2 Phylobayes	initial taxon set except non-protobranch Bivalvia and outgroups reduced to *Platynereis* (Annelida) only, analyzed with Phylobayes	-	-	-	-	-	0.96 pp	-
aa-3	initial taxon set except non-protobranch Bivalvia, Scaphopoda and outgroups reduced to *Platynereis* (Annelida) only	0	-	-	-	-	100	-
aa-4	aa-1 without any outgroups	-	-	-	8	-	-	-
aa-5	aa-2 without any outgroups	-	-	-	-	-	-	-
aa-6	aa-3 without any outgroups	-	-	-	-	-	-	-
aa-7	aa-1 plus *Scutopus*	-	-	-	-	11	99	-
aa-8	aa-2 plus *Scutopus*	0	-	-	-	-	-	-
aa-9	aa-1 plus *Scutopus* and two *Sypharochiton* species	-	-	-	-	-	99	-
aa-10	aa-2 plus *Scutopus* and two *Sypharochiton* species	0	-	-	-	-	99	-
aa-11	aa-10 *Solemya* excluded	0	-	-	-	-	-	-
nuc-1	initial and largest taxon set, comprising 18 molluscan taxa and 3 lophotrochozoan outgroup taxa	-	-	-	-	-	-	-
nuc-2	initial taxon set except non-protobranch Bivalvia and outgroups reduced to *Platynereis* (Annelida) only	0	-	-	-	-	-	100
nuc-3	initial taxon set except non-protobranch Bivalvia, Scaphopoda and outgroups reduced to *Platynereis* (Annelida) only	0	-	-	-	-	-	56
nuc-4	nuc-1 without any outgroups	-	-	-	92	-	-	100
nuc-5	nuc-2 without any outgroups	-	-	-	-	-	-	-
nuc-6	nuc-3 without any outgroups	-	-	-	-	-	-	100
nuc-7	nuc-1 plus *Scutopus*	-	-	-	-	-	-	100

Table 2 Table gives an overview on all Maximum Likelihood (pre-) analyses and the resulting molluscan hypotheses; taxon sets aa-1 – aa-11 are based on amino acis data, taxon sets nuc-1 – nuc-11 are based on nucleotide datasets; main analyses based on aa-1 and aa-2 were additionally analyzed with Phylobayes which is indicated in the first column; Numbers are bootstrap support values of the corresponding hypothesis that appeared in that analysis, numbers followed by "pp" are posterior probabilities of the Phylobayes analysis; "-"means that the hypothesis did not appear in that analysis *(Continued)*

nuc-8	nuc-2 plus *Scutopus*	0	-	-	-	-	-	100
nuc-9	nuc-1 plus *Scutopus* and two *Sypharochiton* species	-	-	-	-	-	-	-
nuc-10	nuc-2 plus *Scutopus* and two *Sypharochiton* species	0	-	-	-	-	-	-
nuc-11	nuc-10 *Solemya* excluded	0	-	-	-	-	-	-

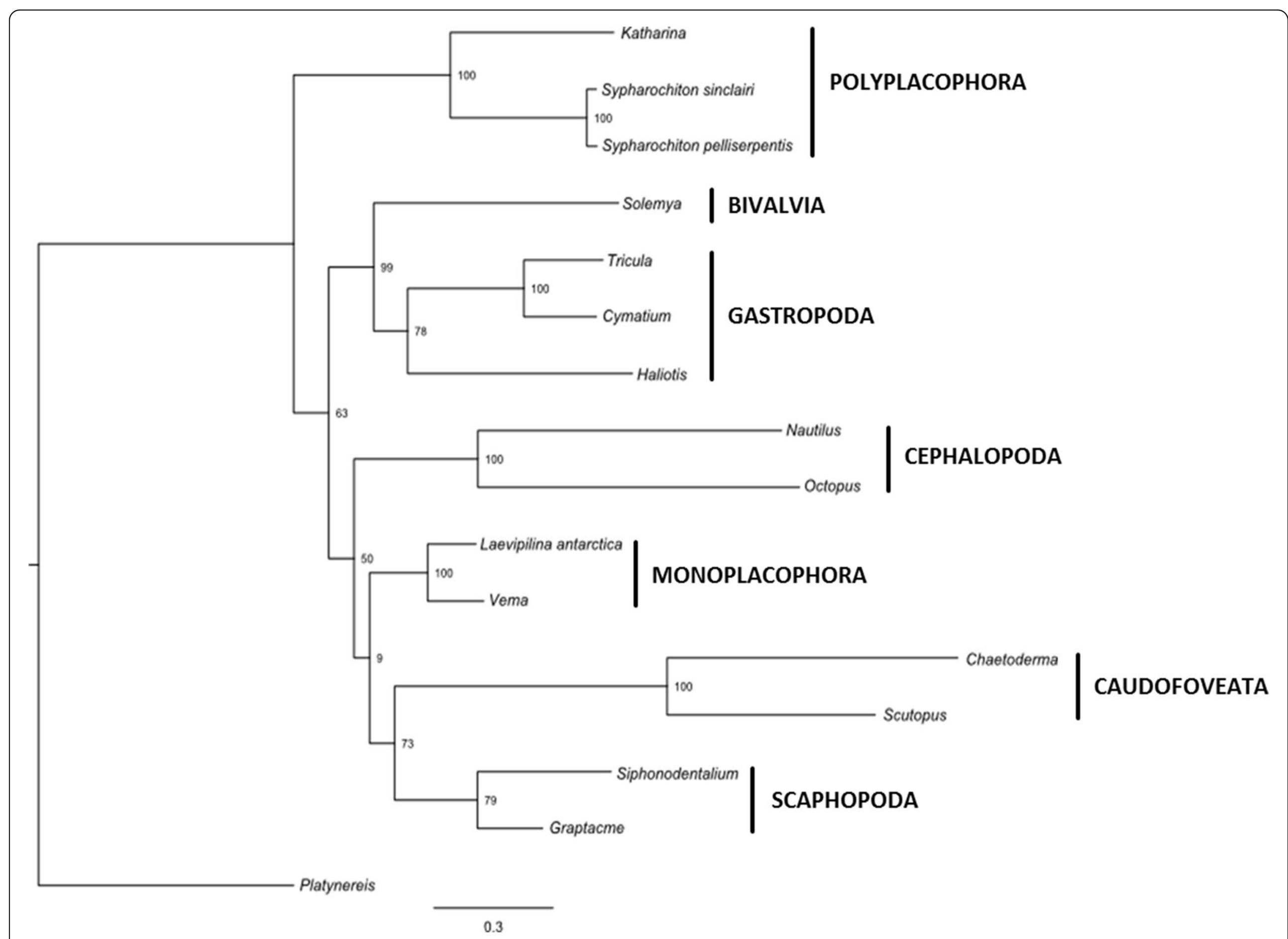

Fig. 2 Maximum Likelihood tree based on the amino acid dataset without the two outgroup taxa *Lineus* and *Loxocorone* and the reduction of bivalve taxa to the protobranch taxon *Solemya*. Tree was inferred with RAxML-HPC executing 500 bootstrap replicates under the CAT approximation for rate heterogeneity. *Platynereis* was used to root the tree

most saturated. There is a gradual decline in the slope value from the best to the worst so cutting out particular genes does probably not improve the tree topology. BaCoCa measures rate heterogeneity and again detects *cox1* as the “best” gene but there is a gradual decline. *Platynereis* and *Nautilus* are the most compositionally heterogeneous taxa in the datasets but not extremely so. Overall, we were not able to identify certain genes or taxa that are particularly problematic.

Hypothesis testing using the Shimodaira-Hasegawa (SH) test and the Approximately Unbiased (AU) test failed to reject Aculifera, Conchifera, Monoplacophora as the sister taxon to the rest of Conchifera, Monoplacophora sister to Cephalopoda, Serialia, or Testaria as being significantly less likely than the most likely tree recovered in either of the two ML analyses (Table 3). Hypothesis testing was performed on both main datasets (aa-1 and aa-2 in Table 2).

Discussion

Gene order

The gene arrangement of Monoplacophora revealed herein is either highly conserved or the taxa here recently diverged from each other. *L. antarctica* and *Vema ewingi* differ only in the position of *trnC* which is adjacent to *trnS1* in *L. antarctica* but is embedded in the tRNA complex *GEWDCYKNM* in *Vema ewingi* (Fig. 3).

Presence of two conserved gene blocks was confirmed in these monoplacophoran species (Table 4). One conserved block, *rrnS-rrnL-nad1-nad6-cob,* was defined previously for Lophotrochozoa [34], and the second block that is putatively conserved in Lophotrochozoa, *nad4L-nad4-trnH-nad5*, could be detected adjacent to *rrnS-rrnL-nad1-nad6-cob* (shown as combined cluster 2 in Table 4), although in a somehow aberrant appearance concerning tRNAs, since in *L. antarctica* and *Vema ewingi* there is *trnQ* nested between *nad4* and *trnH* and in *L. hyalina trnH* is missing (Fig. 3). The part of yet

Table 3 Results of SH and AU hypothesis testing

Analysis	Constraint	Log-likelihood	AU-test p-value	SH-test p-value
aa-1	Unconstrained	−99817.64	0.852	0.935
aa-1	Aculifera monophyletic	−99837.74	0.113	0.392
aa-1	Conchifera monophyletic	−99854.34	0.113	0.113
aa-1	Monoplacophora sister to rest of Conchifera	−99859.16	0.069	0.089
aa-1	Monoplacophora sister to Cephalopoda	−99825.77	0.467	0.704
aa-1	Serialia monophyletic	−99839.30	0.062	0.354
aa-1	Testaria monophyletic	−99860.90	0.053	0.083
aa-2	Unconstrained	−99817.64	0.854	0.940
aa-2	Aculifera monophyletic	−99837.74	0.130	0.390
aa-2	Conchifera monophyletic	−99854.34	0.118	0.120
aa-2	Monoplacophora sister to rest of Conchifera	−99859.16	0.066	0.092
aa-2	Monoplacophora sister to Cephalopoda	−99825.77	0.449	0.703
aa-2	Serialia monophyletic	−99839.30	0.063	0.353
aa-2	Testaria monophyletic	−99860.90	0.053	0.083

another lophotrochozoan conserved gene block (*cox3-nad3-nad2-cox1-cox2-atp8-atp6*) usually appears in the forward direction. In our monoplacophoran species the part *nad2-cox1-cox2-atp8-atp6* is inverted (Table 4). In *L. hyalina atp8* is missing but *trnK* is included. Presence of these conserved lophotrochozoan gene blocks and a relatively high percentage of divergence between the PCGs of the monoplacophoran species (22.4%) leads to the assumption that gene order in Monoplacophora is conserved.

A potential synapomorphy for Mollusca [44], aggregation of *trnG-trnE* with the tRNA complex *MCYWQ*, is present in Monoplacophora, although the complex is reversed in its order (Fig. 3). A second tRNA complex that appears frequently in Lophotrochozoa is *KARNI* [44]. Within our monoplacophoran taxa we instead find *ARI* which is also present in the caudofoveate *Chaetoderma*. A clade of caudofoveates and monoplacophorans is recovered by some of our sequence analyses, but not by any other analyses including nuclear data (for review see [26]); we thus assume that congruency in the tRNA order *ARI* is convergent.

Focusing on the gene order of protein-coding genes (PCGs) and ribosomal RNAs, the ancestral state for both PCG clusters is forward in the lophotrochozoan ground pattern (cluster 1 and 2, see Table 4). Within Mollusca, the order of PCGs that is observed in *Katharina* and other Acanthochitonina [35, 50] is hypothesized to represent the ancestral arrangement for at least molluscs, since this arrangement is recurring with no or almost no modifications in other molluscan classes [44]. In reference to the lophotrochozoan pattern, we show that the orientation of cluster 1 of the Acanthochitonina gene order is ancestral, whereas cluster 2 is derived (Table 4). This order is opposite in Monoplacophora: Their gene orders reflect a derived orientation for cluster 1, but the plesiomorphic state for cluster 2, which appears to be a unique condition among lophotrochozoans. We confirm a plesiomorphic gene arrangement in Acanthochitonina but a monoplacophoran-like derived gene order in Chitonina (Table 4). Rearrangements of PCG clusters are considered to be rare events, and thus are given high phylogenetic significance [51, 52]. Accordingly, the uniquely derived arrangement of cluster 2 could be interpreted as a synapomorphy, supporting Serialia; because of the undisputed monophyly of Polyplacophora, the heterogeneous arrangement within chitons implies homoplasy. Unfortunately, no information is available on mitogenomes of the Lepidopleurida, the morphologically most plesiomorphic chiton clade [53, 54]. Under the Aculifera-Conchifera concept we find this derived condition of gene order in some but not all members of both major clades, also implying convergence within Mollusca. Such convergent rearrangements of large PCG complexes have rarely been detected in invertebrates [52] but not in vertebrates [55]. One such example is known from Caenogastropoda, which shares a congruent gene order of PCGs with the nemertean *Lineus* [44]. We could not find any similar examples within molluscs in the literature and we anticipate that denser sampling may reveal more cases.

Gene architecture

Mitogenome lengths of *L. antarctica* and *Vema ewingi* are consistent with other molluscan mitochondrial genomes, which range between 13.6 kb in *Biomphalaria* (Gastropoda) to 31.5 kb in *Placopecten* (Bivalvia) [44]. Nevertheless, both range at the upper bound of animal mtDNA length, which is typically less than 20 kb [56]. Both

Fig. 3 Gene arrangements of selected molluscan taxa; arrangements are annotated with MITOS and linearized and rotated to *cox1* for display reasons. Gene lengths of coding and non-coding regions correspond to relative lengths of the genomes. The directions of the genes are given by arrows. Green arrows indicate protein coding genes (PCGs); red arrows indicate ribosomal genes (rRNAs); pink arrows indicate transfer RNAs (tRNAs), which are named corresponding to the one-letter code. *Sypharochiton sinclairi* and *S. pelliserpenti*s showed identical gene order

mitogenomes contain the complete gene complement of a typical bilaterian mitogenome [52]. *L. hyalina* lacks *atp8* and six tRNAs. *Atp8* is conserved in just a short fragment at the 5′ region [36, 57], which makes it rather difficult to identify. That might explain the absence of *atp8* in *L. hyalina* since that gene is not located at the boundaries of the contig sequence that was used as input for MITOS, where we would expect missing data in an incomplete mitochondrial genome.

We detected two copies of *trnK* in *L. hyalina,* both highly similar to the *trnK* of *L. antarctica* and *Vema ewingi* in their structure. Duplication of tRNAs is not uncommon and has been reported before (e.g., [37]). A partial inversion of at least *cox1-cox2-trnK* of a conserved lophotrochozoan gene complex could explain the duplicated *trnK* detected in *L. hyalina*, since MITOS additionally detected relics of *cox1* in a row with *trnK* and *cox2* in that individual arrangement. This could also indicate a tandem duplication random loss event.

The three monoplacophoran mitogenomes analyzed herein exhibit almost the same number of non-coding regions; 21 in the incomplete mtDNA of *L. hyalina* and 27 and 28 in *Vema ewingi* and *L. antarctica,* respectively. Several non-coding regions are larger than 100 bp, distributed throughout the genomes and differing substantially in lengths within the same genome. This occurs frequently in molluscan mitogenomes. For example, in the class Gastropoda, some families possess many small NCRs [58, 59], as well as in Cephalopoda, which show intergenic regions that may be longer than 900 bp

Table 4 Directions of PCGs and rRNAs in the two clusters; tRNAs are not considered. Based on the lophotrochozoan ground pattern [34] we find two evolutionary lines. One is evident in *Katharina,* as well as in *Octopus,* with an inversion of PCGs in cluster 2. From this derived arrangement we can infer the *Nautilus* gene order with a "simple" translocation of rRNAs. The second line is an inversion of cluster 1 of the lophotrochozoan ground pattern, which leads to the monoplacophoran (and the *Sypharochiton*) pattern of gene arrangement. We could not detect this arrangement of PCGs in another lophotrochozoan group so far (see e.g., [80])

	Cluster 1: cox3-nad3-nad2-cox1-cox2-atp8-atp6	Cluster 2: rrnS-rrnL-nad1-nad6-cob-nad4L-nad4-nad5	Remarks
Lophotrochozoan ground pattern (Bernt et al. [34])	→	→	
Monoplacophoran plesiomorphic state	←	→	Cluster 1 missing *atp8* in *L. hyalina* as it was not sampled
L. antarctica	←	→	
Vema ewingi	←	→	
L. hyalina	←	→	Cluster 1 misses *atp8*
Sypharochiton spp.	←	→	
K. tunicata	→	←	

[51]. *Katharina* (Polyplacophora) also has several NCRs [35], and the bivalve taxon *Placopecten* contains NCRs up to 10,000 bp [57]. Almost half of the NCRs in Monoplacophora are located between the same genes in all three mtDNAs. *L. antarctica* shares more relative gene boundaries of NCRs with *Vema ewingi* than with *L. hyalina;* this is unexpected since it suggests a closer relationship of *L. antarctica* to *Vema ewingi* than to *L. hyalina*, but this might also be due to information missing in *L. hyalina*. The congruent relative location of two NCRs found in *Nautilus* and Monoplacophora with two identical or even highly similar sequence motifs might be synapomorphic and thus indicate common ancestry for monoplacophorans and cephalopods as it is proposed by Smith and colleagues [16, 17]; however, the motifs are very short and could also be either plesiomorphic or convergent.

Each of the two complete mitogenomes of *L. antarctica* and *Vema ewingi* has its largest NCR between *trnF* and *trnT* (see Fig. 3). These NCRs are 2012 bp and 2287 bp long respectively and both contain the AT-rich motif 1 that is almost identical in both mitogenomes concerning nucleotide composition, length, and position within the NCR (Table 1). A very similar motif is visible in *L. hyalina* in the NCR between *trnK* and *cox1* (Table 1). The long and unassigned regions could be the potential origins of transcription of our monoplacophoran mtDNAs since AT-rich motifs are usually evidence for the control region of mitogenomes [59, 60]. Several other repetitive motifs are visible in the largest NCRs of *L. antarctica* and *Vema ewingi,* which provide even more evidence that this region is the control region. Motif 1 is repeated between *trnG* and *trnE* in *L. antarctica* and *Vema ewingi*, again with almost congruent starting points and very similar positions within the NCRs (Table 1, Fig. 3). We hypothesize that the initiation region was partially duplicated to have two starting points for the replication process which would lead to an increased transcription rate as was suggested for cephalopods before [61]. Although we found evidence for the potential control region in *L. hyalina*, too, we were not able to detect its duplication in this incomplete mt genome.

MITOS annotated fragmentary *cox1* in *L. hyalina* and parts of *atp6* in *L. antarctica* in the potential initiation regions. These protein-coding gene fragments are located near their functional copies. A possible scenario could be that part of the mitogenome, consisting minimally of the respective PCGs, was duplicated, and this is still visible in both *Laevipilina* individuals through residual PCG fragments. These duplicated copies might be in the process of being lost. Whether in *Vema ewingi* the loss is already finished, or the duplication event never took place is not clear. Nevertheless, we identified a region of accelerated rearrangement rate and this is third indication for locating the origin of replication in these NCRs in *Laevipilina*. Such a control region is usually described as the longest non-coding region within the mitogenome that is rich in AT, often including repetitive motifs, and seems to be a hotspot for rearrangements [59, 62]. The existence of duplicated control regions or parts thereof could be seen as a similarity for Monoplacophora and Cephalopoda (see [63]), since this feature is not known from other molluscs so far but is observed in other metazoan mitogenomes [64–66].

The second repetitive sequence motif (motif 2), is found in *L. antarctica* in the unassigned region between *nad2* and *trnC* as well as in *Vema ewingi* in the non-coding part between *nad2* and *trnS1* (Table 1). This motif starts in both NCRs at almost the same position. Unassigned regions are known to be extremely variable because they do not underlie any selective pressure. Independent evolution of two identical 18 bp long

nucleotide motifs in the same position is unlikely, so this motif is probably an apomorphy inherited from the common ancestor of these two taxa.

Phylogeny

Several phylogenetic approaches resulted in ambiguous topologies, which were sensitive to taxon sampling. Neither nucleotide nor amino acid taxon sets supported Aculifera (Polyplacohora + Aplacophora) or Conchifera (comprising all other shell-bearing classes), in contrast to Osca and colleagues [47] (see Table 2). A trend in amino acid analyses is the repeated recovery of a highly supported Pleistomollusca, whereas nucleotide based analyses supported Serialia (Table 2). In the data set with 3 non-molluscan outgroups, neither the amino acid nor nucleotide datasets supported the monophyly of Mollusca, which is, however, well-established [2, 15, 16, 27, 28, 32]. Molluscan non-monophyly is a common result of phylogenetic analyses based on mt protein coding genes [34, 44, 46] which was unaffected by the addition of more taxa here (Table 2).

Analyses recovered a monophyletic Monoplacophora and tended to support monophyly of other molluscan classes, except for bivalves. Non-protobranch bivalves have longer branches and rearranged gene orders compared to the protobranch *Solemya*. Such high levels of gene rearrangements were suggested to be linked with high rates of nucleotide substitution [26, 34, 44].

In amino acid datasets, the lamellibranch bivalves cluster as the sister group to scaphopods, but *Solemya* clusters with gastropods (Fig. 1, Table 2). The latter relationship was also recovered by Plazzi et al. 2013 [48] but was interpreted as an artifact due to limited phylogenetic signal in the bivalve lineage of Opponobranchia (including Nuculida and Solemyida). *Solemya* is the only bivalve in our dataset that has its genes arranged on both strands, a fact that leads to different substitution skew between plus and minus strands of the mt genome. Such differences in nucleotide composition might influence phylogenetic analyses and could be an explanation for our diphyletic clustering of bivalve taxa [67, 68].

Pruning non-protobranch bivalves recovers *Solemya* as the sister group to gastropods, i.e. a taxon Pleistomollusca ([15], Fig. 2; Additional files 1 and 2: Figures S1 and S2) in most amino acid analyses. Excluding the remaining protobranch bivalve, *Solemya*, from our analyses did not result in an aculiferan topology (Table 2). That is in contrast to Osca et al. [47] who excluded Bivalvia and recovered Aculifera (although Solenogastres was not sampled) either with poor support (ML) or with strong support but not as part of a monophyletic Mollusca (BI). In the taxon set in Osca et al. [47], Conchifera were lacking Bivalvia, which were pruned, and Monoplacophora.

As Osca et al. [47] recovered Aculifera and Conchifera we expected that adding further, taxa such as protobranchs and monoplacophorans might be beneficial to resolve further aspects of deep molluscan evolution. Within this study we employed different taxon sets to explore the robustness of the data. However, the diversity of topologies recovered herein is striking and suggests there is limited phylogenetic signal in this data. By modifying datasets we recovered several formerly proposed and currently disregarded hypotheses of higher taxa, but never the preferred Aculifera or Conchifera [47].

Conclusion

This mitogenomic study includes three members of two monoplacophoran genera. Our phylogenetic results of analyzing the protein coding supermatrix of 13 genes of 18 selected molluscan taxa across 7 of 8 classes stay ambiguous. Common and highly accepted molluscan hypotheses as the Aculifera or Conchifera concepts never appear in any of our phylogenetic permutations.

Our finding of unique protein gene arrangements in Monoplacophora and chitonine but not acanthochitonine Polyplacophora is remarkable because it may support the Serialia hypothesis, which is in conflict with the Aculifera/Conchifera hypothesis, but more likely it represents a plesiomorphic genome structure for molluscs. Any topology would imply convergent evolution of identical PCG clusters within Mollusca. On one hand, this clearly weakens the significance of supposedly rare gene rearrangement events and single genome level characters. On the other hand, this demonstrates the existence of further genome level characters that may become useful if mitogenomes are explored densely over molluscan (and other) taxa. Unfortunately, phylogenetic analyses of the mtDNA provided little information for resolving mollusc phylogeny. Furthermore, we need to expand our yet limited knowledge on mitochondrial evolution and data from the molluscan class Solenogastres (=Neomeniomorpha) is still lacking. High throughput sequencing as used here is a powerful and accurate way to add further mitogenomes of taxa that are small or with limited material available.

Methods

Preparation of *Vema ewingi*

Vema ewingi was collected on R/V "Dimitry Mendellev" at 8°S 81°W in 5800 m depth. DNA was extracted and purified using the Qiagen DNeasy kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. DNA concentration was measured using a Qubit with the double-stranded DNA broad range kit. DNA quality was evaluated using a 1% SB agarose gel. Gel electrophoresis revealed that the DNA was degraded with an average

fragment size of around 500 bp. However, some large fragments of DNA up to around 10,000 bp were present.

An Illumina Nextera (Illumina, San Diego, CA, USA) library was prepared following the manufacturer's protocol. However, the resulting library had a low size distribution because the template DNA was degraded. Additional attempts were made to prepare Nextera libraries using more template DNA than recommended by the Illumina protocol. This produced better quality libraries based on size distribution with the optimal library using four times the recommend amount or 200 ng total.

Sequencing was conducted using a 2 × 250 bp paired-end (PE) v2 kit on the Illumina MiSeq at Auburn University. The *Vema* libraries were sequenced in parallel with libraries for other projects with around eight dual-indexed libraries sequenced at a time. Several attempts at sequencing various *Vema* Nextera libraries were made using different amounts of template DNA, combining all of the *Vema* genomic data collected to that point, and assembling the paired-end reads using Ray 2.2.0 with a k-mer of 31 on the Auburn University SkyNet server never yielded a complete mitochondrial genome.

Therefore, we abandoned the Nextera approach and prepared libraries using the NEB Next Ultra kit (New England Biolabs, Ipswich, MA, USA) for Illumina sequencing. As the DNA was already degraded to an average size of around 500 bp, no shearing was necessary. End-repair, adapter ligation, and barcode incorporation via PCR were conducted following the manufacturer's protocol. As above, sequencing was conducted using a 2 × 250 bp paired-end (PE) v2 kit on the Illumina MiSeq at Auburn University. Again, around eight indexed libraries were sequenced at a time and after two runs, a complete mitochondrial genome could be assembled for *Vema*.

In order to identify the complete mitochondrial genome, the assembly was searched against a nucleotide BLAST database constructed from the complete mitochondrial genome of *Katharina tunicata* (Polyplacophora) using BLASTN and TBLASTX using an e-value cutoff of 0.01.

Preparation of *Laevipilina antarctica*

Total genomic DNA was extracted from a piece of tissue of one specimen of *Laevipilina antarctica* (ZSM-Mol-20090330, DNABANK-Mol-MS-016), which was collected during the expedition with R/V Polarstern in Antarctica, using the NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany) following the instructions in [69].

Ten nanogram of DNA was used for multiple strand replacement based DNA amplification using the illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare Life Sciences, Freiburg, Germany) using the manufacturers instruction followed by standard ethanol precipitation. Subsequently the DNA was purified using the Qiagen MinElute system (Qiagen, Hilden, Germany), DNA concentration was determined using the Qubit® 2.0 Fluorometer. 1 μg of DNA was used to create a standard fragment DNA sequencing library with the TruSeq DNA Sample Preparation Kit v2 (Illumina, San Diego, CA, USA); the experimental average insert size was 250 bp. Two lanes of 101 bp paired-end-reads were sequenced on the Ilumina HiSeq2000 system. About 90 Gigabase-pairs (Gbp) were obtained. These were filtered for quality, PCR duplicates, and adaptor sequences and corrected using SOAPfilter_v2.0 (https://github.com/tanghaibao/jcvi-bin/blob/master/SOAP/SOAPfilter_v2.0) using default settings. We subsetted 5–200 million paired reads in K-mer iterations of 23–99 and using various parameters for mitogenome assembly using SOAPdenovo2 [70]. The best assembly of the complete mitogenome was discovered using 50 million paired reads and settings other than default –R –u.

Preparation of *Laevipilina hyalina*

Total genomic DNA was extracted from a single specimen collected off California [13] using the Qiagen DNeasy kit (Qiagen, Hilden, Germany), following manufacturer's protocols. Standard PCR protocols were used to generate sequences from Cytochrome c oxidase I (COI), 16S rDNA (16S) (see [13]) plus Cytochrome oxidase B (cob) using universals 424f + 876R [71] and Cytochrome c oxidase III (COIII) [72]. All amplifications were done using illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare Life Sciences, Freiburg, Germany) following the manufacturer's protocols. PCR products were cleaned using USB ExoSAP-IT, and sequenced by Retrogen Inc. (San Diego, CA, USA). Sequencher v4 was used to inspect and trim sequences. Sequences from these mitochondrial genes were used to design *Laevipilina*-specific primers for long PCR amplification. The Primer3 algorithm was used to design these primers [73].

Various primer combinations were tested, and a final set of MCOIf + MCytbr (5′-ATTGGCTGGGGCAGTTACTA-3′ + 5′-TGTGGAGAGGGGTAACAAGG-3′) and MCOX3f + MCOIR (5′-GATGTTTCGGTTGGGATACG-3′ + 5′-AAAGGAACCCGCTCAAGAGT-3′) resulted in two overlapping fragments (approximately 7 kb and 3 kb respectively). All long PCR products were amplified using Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Waltham, MA, USA) following the manufacturer's specifications. The PCR products were visualized on 1% agarose gels run at 80 V for 90 min. PCR products were cleaned using GelElute Extraction kit (5 Prime, South San Francisco, CA, USA) and outsourced to Engencore (Selah Genomics, Greenville, SC, USA) for

sequencing and assembly with the Roche 454 platform and Newbler v2.3.

Annotation of mitogenomic consensus sequences

Mitogenomic sequences were filtered from the whole genome assemblies via BLAST searches and by alignment to known sequences of mitochondrial genes. The MITOS web server [74] was used to annotate mitogenomic data of *L. antarctica* and *Vema ewingi* as well as the partial consensus sequence of *L. hyalina.* Mitogenomic consensus sequences of the bivalve *Solemya velum* (NC_017612 [48]), the caudofoveate *Scutopus ventrolineatus* (KC_757645 [47]) as well as *Sypharochiton pelliserpentis* (KJ_534307 [49]) and *S. sinclairi* (KJ_534306 [49]) were downloaded from GenBank and newly annotated via the MITOS web server as well. Recommended default parameters [74] and the invertebrate mitochondrial genetic code (translation Table 5) were used for all annotations of protein coding genes, transfer and ribosomal RNAs. Annotated single sequences were imported in Geneious version 6.1.7 to work on GC content, extract and examine non-coding regions as well as overlaps, to visualize secondary structures of tRNAs of special interest (default parameters in Geneious were used), and to compile the different datasets for phylogenetic analyses (please see section "Phylogenetic analyses" for details). As Tomita et al. [75] proposed for non-coding regions in *Loligo*, we conducted BLAST searches of all non-coding regions larger than 100 bp of our three monoplacophoran genomes to find possible similarities to other mt genomes but we did not find any noticeable hits. Moreover, we checked the largest NCRs (>2 kb) of *L. antarctica* and *Vema ewingi* for group II transposons. This phenomenon was found in the annelid *Nephtys* [76] but also in insects [77] and might give an explanation for the unusually long NCRs in our species. We conducted DNA foldings of the non-coding sequences via the Mfold web server under default options, but could not find any similarities to the described secondary structure of *Nephtys* which is described as a central core with six radiating helical domains [76]. Both NCRs were compared to the Dfam database [78], but no hits were detected.

MITOS detected genes *atp6, cob, cox3, nad3*, and *nad4* in the *L. hyalina* consensus sequence divided in two parts, *nad2* in three parts. The parts of *atp6*, *cob*, and *cox3* are overlapping (*atp6*, *cob*) or are at least adjacent (*cox3*) and therefore were combined manually; *nad2*, *nad3*, and *nad4* do actually have non-annotated nucleotides in reverse order between the annotated gene

Table 5 Table shows all taxa that were used in this study with their corresponding GenBank accession numbers

	Class	Taxon	GenBank acc. no.
Outgroup taxa	Annelida	*Platynereis dumerilii*	NC 000931
	Entprocta	*Loxocorone allax*	NC 010431
	Nemertea	*Lineus viridis*	NC 012889
Mollusca	Bivalvia	*Lampsilis ornata*	NC 005335
		Lucinella divaricata	NC 013275
		Mytilus edulis	NC 006161
		Solemya velum	NC 017612
	Caudofoveata	*Chaetoderma nitidulum*	NC 013846
		Scutopus ventrolineatus	KC 757645
	Cephalopoda	*Nautilus macromphalus*	NC 007980
		Octopus vulgaris	NC 006353
	Gastropoda	*Cymatium parthenopeum*	NC 013247
		Haliotis rubra	NC 005940
		Tricula hortensis	NC 013833
	Monoplacophora	*Laevipilina antarctica*	KY 244020
		Laevipilina hyalina	KY 284344
		Vema ewingi	KY 244019
	Polyplacophora	*Katharina tunicata*	NC 001636
		Sypharochiton pelliserpentis	NC 024174
		Sypharochiton sinclairi	NC 024173
	Scaphopoda	*Graptacme eborea*	NC 006162
		Siphonodentalium lobatum	NC 005840

parts. These non-annotated parts turned out to be reverse complement parts and were corrected by hand in Geneious version 6.1.7.

Annotated gene arrangements of all three monoplacophoran species were compared to each other and to other molluscan taxa (*Katharina tunicata* (NC_001636 [35]), *Sypharochiton* spp. (KJ_534306, KJ_534307 [49]), *Nautilus macromphalus* (NC_007980 [51]), *Octopus vulgaris* (NC_006353 [61]) by eye. Furthermore we searched for sequence motifs that occur in more than one monoplacophoran species with MEME Suite version 4.9.1 via the MEME web server [79].

Phylogenetic analyses

Newly generated data for *Vema ewingi* and *Laevipilina antarctica* and reannotated mt data of *Solemya velum* [48], *Scutopus ventrolineatus* [47], *Sypharochiton pelliserpentis* and *S. sinclairi* [49] were added to a taxon-subset of the 13 mitogenomic protein coding genes (PCGs) from Stöger and Schrödl [44], comprising 18 molluscan and three lophotrochozoan outgroup taxa (Table 5). Due to visibly exceptionally long branches and unusual attraction of outgroup taxa to ingroups in previous studies [34, 44, 46, 80] and in own pre-analyses, we excluded all outgroup taxa except *Platynereis* (Annelida) that showed a short branch in pre-analyses with more outgroup taxa, and excluded all bivalve taxa but the basal protobranch *Solemya* [81]. To reduce potential long-branch attraction artifacts that are already known from previous studies (e.g., [47]), we removed the two scaphopod taxa *Graptacme* and *Siphonodentalium*. Moreover, we also ran analyses based on nucleotide (nuc) and amino acid (aa) datasets of all taxon sets without any outgroups. All single nucleotide PCG sets were translated into amino acids using the invertebrate mitochondrial genetic code. Single nucleotide and amino acid datasets of PCGs were aligned using MAFFT version 7.017 [82] implemented in Geneious under the E-INS-i algorithm with a gap open penalty of 3. In pre-analyses we masked all single gene-alignments (nuc and aa) with Aliscore version of 5th February 2008 [83, 84] by running 10.000.000.000 replicates. Hypervariable positions were trimmed with Alicut version 2.0 [83, 84]. Moreover, we ran pre-analyses where we eliminated poorly aligned and hypervariable regions of all aa single alignments via Gblocks [85] since this program is more restrictive than Aliscore. In Gblocks we applied default options except for the *atp8* alignment because this dataset would have been subsequently eliminated completely and we wanted to include the complete set of protein-coding genes; for *atp8* alignments we chose all options using a less stringent selection. The Gblocks masked single alignments were tested for best fitting evolutionary models with ProtTest version 2.4 [86] by choosing from those models that are available in RAxML (DAYHOFF, DCMUT, JTT, MTREV, WAG, RTREV, CPREV, VT, BLOSUM62, and MTMAM). We further tried to improve the aa single alignments by refining the MAFFT-alignment via Muscle version 3.8 [87]. The resulting nucleotide and amino acid individual PCG-gene alignments under the different treatments were concatenated in Geneious with the following order: *atp6, atp8, cob, cox1, cox2, cox3, nad1, nad2, nad3, nad4, nad4L, nad5, nad6*. All *atp8* alignments produced herein are missing the sequence for *Mytilus*, since this taxon lacks the *atp8* gene [43]. All Maximum Likelihood (ML) analyses were performed with the program RAxML-HPC [88], executing 500 bootstrap replicates under the CAT approximation for rate heterogeneity and the GTR model. Masking with Aliscore or Gblocks or no masking procedure as well as partitioning the concatenated dataset or not did not make any difference in the resulting tree topology and will not be discussed below. Further analyses of selected concatenated alignments were carried out with the program SplitsTree version 4 [89] to test for potential conflicts of the data.

For the two preferred datasets (aa-1, aa-2) we carried out additional analyses with Phylobayes MPI on the CIPRES Science Gateway (https://cushion3.sdsc.edu/portal2/) using the CAT-GTR model and running 4 chains for each of the datasets. Analysis of datset aa-1 was executed for 79.839, respectively aa-2 for 105.593 generations until stationarity was reached. Burn-in was set to 2000 for each of the chains. Maxdiff for aa-1 was 0,07, for aa-2 it was 0,1.

Competing phylogenetic hypotheses run on the two main datasets aa-1 and aa-2 were evaluated using the Shimodaira Hasegawa test [90] and the Approximately Unbiased test [91] in RAxML 8.2.4 [92] and Consel [90]. The PROTG AMMAGTR model was used for these analyses.

Since the phylum Mollusca diverged in the Cambrian or earlier, non-phylogenetic signal in the molecular datasets could lead to anomalous topologies due to compositional biases, substitution saturation or increased substitution rates [93, 94]. Therefore we tested our preferred single gene alignments (amino acid only) for saturation and rate heterogeneity with the programs TreSpEx [95] and BaCoCa [96].

Additional files

Additional file 1: Figure S1. Bayesian Inference tree based on the large amino acid dataset. The tree was inferred with Phylobayes running four chains and 79.839 generations until stationarity was reached. *Loxocorone* was used to root the tree. (PDF 2 kb)

Additional file 2: Figure S2. Bayesian Inference tree based on the amino acid dataset without the two outgroup taxa *Lineus* and *Loxocorone* and the reduction of bivalve taxa to the protobranch taxon *Solemya*. The tree was inferred with Phylobayes running four chains and 105.593 generations until stationarity was reached. *Platynereis* was used to root the tree. (PDF 2 kb)

Abbreviations
aa: Amino acid; *atp6-8*: ATP synthase subunits 6–8; bp: Base pair; *cob*: Cytochrome oxidase b; *cox1-3*: Cytochrome c oxidase subunits 1–3; Gbp: Gigabase pair; ML: Maximum likelihood; mt: mitochondrial; *nad1-6*: *4L*, NADH dehydrogenase subunits 1–6, 4L; NCR: Non-coding region; nuc: Nucleotide; PCG: Protein-coding gene; PCR: Polymerase chain reaction; PE: Paired-end; rRNA: Ribosomal RNA; *rrnL*: Large ribosomal RNA; *rrnS*: Small ribosomal RNA; tRNA: Transfer RNA; *trnX*: Transfer RNA for amino acid X (denoted by the one-letter IUPAC code)

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Availability of data
The main datasets and tree topologies of this article will be available at TreeBase (http://purl.org/phylo/treebase/phylows/study/TB2:S20256). New mitogenomic sequence data of the three monoplacophoran species generated herein will be deposited at Genbank (see Table 5 for accession numbers).

Authors' contributions
IS performed analyses and drafted the manuscript; AJP, KMK and NGW carried out the molecular lab work and genome assemblies; DI, KMH, NGW and MS did the field work and provided monoplacophorans; KMH, NGW and MS conceived the study and contributed to writing the paper. All authors contributed to read and approved the final manuscript version.

Competing interests
The authors declare that they have no competing interests.

Ethics approval and consent to participate
The authors confirm that all experiments conducted in this study comply with institutional, national, or international guidelines.

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A review on deep molluscan phylogeny: old markers, integrative approaches, persistent problems

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Molluscs are diverse and important enough to receive considerable scientific attention. We herein question recent opinions that the molluscan origin, deep inner relationships, and early evolution have been largely resolved. The traditional Testaria concept implied progressive evolution from worm-like aplacophoran to polyplacophoran and then conchiferan body plans; sole evidence for this came from morphocladistic analyses, which we conclude were confounded by homoplasy. The recently preferred Aculifera-Conchifera concept is supported by some but not all analyses using massive sequence data on rather small and uneven taxon sets. Recent results from mitogenomics indicate that gene-rearrangement events could cause sequence biases overriding potential phylogenetic signal. We discuss recent progress regarding multilocus marker analyses, particularly refining the neglected Serialia hypothesis with Monoplacophora sister to Polyplacophora. This third hypothesis for molluscan relationships is supported by an integrative interpretation and is roughly compatible with available fossil evidence if the first molluscs were small and had a true shell rather than a chiton-like body organization. Despite some claims of consensus, there are substantial discrepancies among recent molecular studies regarding class-level topologies. These may be symptomatic of a plethora of factors and evolutionary processes – obvious or more hypothetical – that might hinder successful reconstruction of early molluscan diversification.

Keywords: Lophotrochozoa; morphology; palaeontology; molecular systematics; genome; evolution

Introduction

'Bringing order to the molluscan chaos' (Telford and Budd 2011) has been identified as one of the greatest challenges in invertebrate evolution. The problem of resolving deep molluscan phylogeny largely refers to resolving molluscan class relationships, which always have been disputed. In 2011, multi-gene studies on ribosomal proteins (Meyer et al. 2011) and housekeeping genes (Vinther et al. 2012) and two broad phylogenomic data sets (Kocot et al. 2011; Smith et al. 2011) all recovered monophyletic Mollusca, and the latter three studies supported monophyletic Aculifera. This group comprises molluscs having the partial or entire body covered by a cuticle which integrates calcareous spicules or scales, and is composed of shell-less 'worm-molluscs' (aplacophoran Solenogastres and Caudofoveata) and shell-plate bearing

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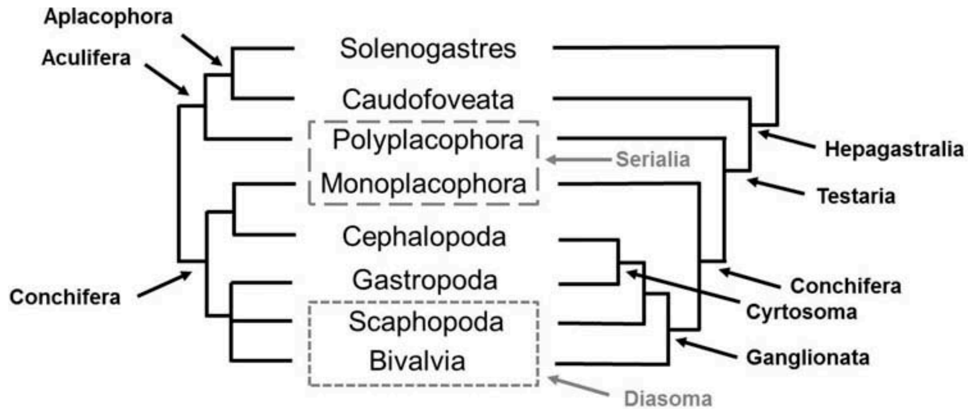


Figure 1. Major competing hypotheses on molluscan phylogeny and evolution. Left: Consensus tree from phylogenomics according to Telford and Budd (2011) and Kocot (2013), showing a basal split into Aculifera and Conchifera, and Monoplacophora sister to Cephalopoda; suggesting that worm-like body constructions of aplacophorans are secondary simplifications of a more complex ancestor (Scheltema 1993). Right: Morphocladistic tree from Haszprunar (2000), showing basal paraphyletic aplacophorans, monophyletic Testaria and Conchifera with basal Monoplacophora, implying progressive evolution from worm-molluscs to shell plate-bearing chitons and towards molluscs with true shell (Salvini-Plawen 2006). The taxon Serialia (Giribet et al. 2006) is incompatible with traditional Testaria, Aculifera and Conchifera concepts.

Polyplacophora (chitons). The two phylogenomic studies recovered monophyletic Conchifera (molluscs having a true shell built by a shell gland) as sister to Aculifera (Figure 1). The 'firm establishment' of the basal split of molluscs into aculiferan and conchiferan taxa was considered by Telford and Budd (2011) as the most important recent achievement, and supports an earlier proposal based on morphological grounds (e.g. Scheltema et al. 2003). The Aculifera is usually recovered with worm-like molluscs forming a clade Aplacophora sister to Polyplacophora (Kocot 2013). This suggests that ancestral molluscs had shells or shell plates covering a quite complex body construction, and that shell-less and simpler organized aplacophoran bodies evolved secondarily.

The alternative, morphocladistic Testaria hypothesis (e.g. Salvini-Plawen 1980; Salvini-Plawen and Steiner 1996; Haszprunar 2000) assumes a simple worm-like molluscan ancestor (Haszprunar and Wanninger 2012), basal and usually paraphyletic worm-molluscs, and Polyplacophora sister to Conchifera (Figure 1). Under a Testaria hypothesis, molluscs evolved continuously and progressively towards increasing body complexity, and a true shell evolved just once (Salvini-Plawen 2006). However, Conchifera was recovered paraphyletic by both Meyer et al. (2011) and Vinther et al. (2012), and also in earlier studies on haemocyanin sequences (Lieb and Todt 2008) and phylogenomic expressed sequence tag (EST) markers (Dunn et al. 2008). In all these studies Cephalopoda is a basal clade, in the latter three studies sister to (or among) Aculifera, implying that ancestral molluscs had a true shell rather than a body covered with calcareous spines or shell plates (Vinther et al. 2012). In contrast, cephalopods clustered with the single species of putative

'living fossil' Monoplacophora in a study based on broad EST data (Smith et al. 2011). Within Conchifera, the relationship of Scaphopoda remained ambiguous, clustering sister to Gastropoda (Smith et al. 2011) or sister to a clade of Gastropoda and Bivalvia (Pleistomollusca) (Kocot et al. 2011; Vinther et al. 2012). While using broad sequence data, all these recent approaches are based on limited taxon sampling, with Smith and colleagues (2011) the only workers including at least one member of each of the eight molluscan classes. In their review, Telford and Budd (2011) relied on the power of recent phylogenomic approaches, although taxon sets are still small, topologies are not fully compatible, and, as noted by Kocot (2013), the proposed sister group relationship of Monoplacophora and Cephalopoda is somewhat unexpected.

The Aculifera hypothesis became the favoured paradigm (e.g. Kocot 2013), although the Testaria hypothesis still has its proponents (e.g. Haszprunar and Ruthensteiner 2013). A third major hypothesis on deep molluscan relationships, with a 'Serialia' clade of monoplacophorans sister to chitons (Giribet et al. 2006), as recovered by ribosomal gene dominated multilocus studies, has been given less attention in recent reviews. The Serialia clade combines conchiferan and aculiferan members (Figure 1) and is thus incompatible with results of recent phylogenomic studies, and also is in conflict with the morphology-based traditional views of Testaria subdivided into Polyplacophora and Conchifera. Recovering serialian classes as a derived clade among (non-monophyletic) molluscs also contradicted earlier hypotheses of molluscan evolution (e.g. Nielsen et al. 2007). While there were problems with the study by Giribet et al. (2006) (e.g. Haszprunar 2008; Wägele et al. 2009), these were addressed by Wilson et al. (2010), still recovering Serialia. Meyer et al. (2010) were the first to provide uncontaminated 18S sequences of aplacophoran Solenogastres and their single gene analysis recovered monophyletic Mollusca and, again, Serialia. Using multilocus markers and a small molluscan taxon set with full data from two monoplacophoran species, Kano et al. (2012) also recovered well-supported Serialia. Interestingly, the completely independent, single recent phylogenomic data set with a monoplacophoran species included also showed a signal for Serialia (Smith et al. 2011), though weaker than that for a cephalopod-monoplacophoran clade.

Earlier mitochondrial genomic approaches based on sequence analyses of protein-coding genes recovered unresolved lophotrochozoan and molluscan trees (e.g. Boore et al. 2004), non-monophyletic molluscan classes (e.g. Dreyer and Steiner 2004) and unconventional interclass relationships (e.g. Dreyer and Steiner 2006). Yokobori et al. (2008) recovered monophyletic Mollusca as a basal lophotrochozoan clade and monophyletic molluscan classes, with Scaphopoda and Bivalvia forming a clade Diasoma as sister to a clade with Cephalopoda sister to Polyplacophora plus Gastropoda. However, apart from cephalopods, very few representatives of major molluscan and non-molluscan groups were selected, and sampling was generally inadequate and uneven. Using mitochondrial gene arrangements for reconstructing phylogeny was proposed as a powerful approach (e.g. Boore and Brown 1998; Boore 2006; Simison and Boore 2008), but none has recovered the placement nor the inner topology of the molluscs.

In the framework of the Deep Metazoan Phylogeny priority programme of the German research foundation (DFG), we concentrated on two DNA-based approaches for recovering deep molluscan phylogeny, using mitochondrial genomes and multilocus

data (mitochondrial COI and 16S fragments and the nuclear 18S and 28S ribosomal RNA and histone 3 genes). We anticipated that more and better quality molecular data, in particular from the elusive monoplacophorans, could help to resolve molluscan relationships. Results from increased taxon sampling should also be evaluated within molecular, morphological and palaeontological frameworks.

Herein we summarize and discuss recent results from our research group on (1) molluscan mitogenomics, and (2) phylogenetic analyses using multilocus markers. We then present an extensive comparison of major hypotheses on early molluscan phylogeny, including reanalyses of some published morphological and phylogenomic data, and class-level interrelationships. In the synthesis presented here, we evaluate issues that could have confounded past and current morphocladistic and phylogenomic approaches.

Molluscan mitogenomics

The placement of molluscs within the lophotrochozoans as well as the interclass relationships within this phylum is still a contentious issue. Because mitogenomes are considered to be suitable for resolving deep nodes of Mollusca (e.g. Simison and Boore 2008), we used all 96 molluscan mitogenomes of six molluscan classes and 16 lophotrochozoan outgroup taxa publicly available (GenBank in July 2011; the classes Solenogastres and Monoplacophora were not represented). Mitogenomes were reannotated with the MITOS pipeline designed by the work group of Peter Stadler (Leipzig) (Bernt, Bleidorn, et al. 2013; Bernt, Braband, et al. 2013; Bernt, Donath et al. 2013). Resulting gene arrangements were compared by eye and screened for conserved gene clusters and other mitogenomic features such as gene duplications, lengths of genomes as well as non-coding regions, and whether genes are transcribed from both strands or merely from a single strand. Maximum Likelihood (ML) analyses were computed with the paralellized RAxML v. 7.2.8 (Stamatakis 2006) using a metazoan and an expanded molluscan taxon set, comprising 13 mitochondrial protein coding genes. In case of the molluscan taxon set we compared resulting trees of each masked and unmasked amino-acid alignments and nucleotide alignments (for details see Stöger and Schrödl 2013).

Similar to other mt-marker-based deep molluscan analyses (e.g. Grande et al. 2008; Plazzi et al. 2013), the trees are very unconventional (Stöger and Schrödl 2013; see Figure 2). Regardless of the taxon set, alignment masking, amino acid or nucleotide sequences, or partitions or models applied, monophyletic Mollusca was not recovered. The only molluscan class represented with multiple members and recovered monophyletic was Cephalopoda. Ingroup relationships of cephalopods and bivalves (e.g. Palaeoheterodonta, Heterodonta and Pteriomorpha) are congruent with topologies from other mt-based analyses (Akasaki et al. 2006; Doucet-Beaupré et al. 2010). In all our published analyses, gastropod families were recovered monophyletic, but the class Gastropoda is non-monophyletic and members are distributed all over the tree (Stöger and Schrödl 2013; Figure 2). Long-branched Patellogastropoda and Heterobranchia are separated from Caenogastropoda and Vetigastropoda, which are sisters with maximum support. The latter relationship has already been recovered based on mitogenomes and other molecular markers in earlier studies (Aktipis et al. 2008; Grande et al. 2008), but is in conflict with morphological evidence (Ponder and Lindberg 1997) and other molecular analyses

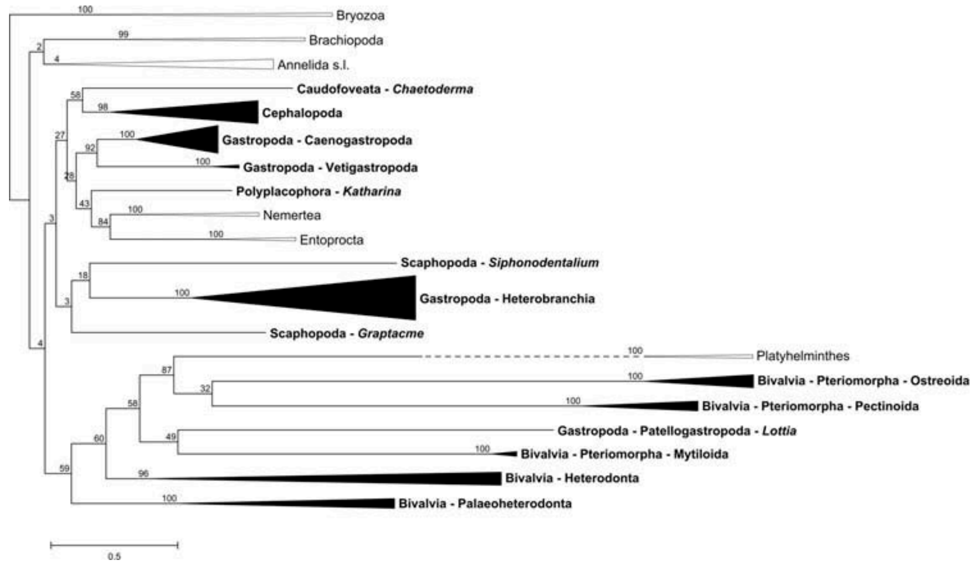


Figure 2. Collapsed molluscan phylogenetic tree based on mitochondrial genomic nucleotide sequence data (analysis published by Stöger and Schrödl 2013; 13 protein mt genes analysed as nucleotides; 16 lophotrochozoan outgroup and 96 molluscan species; alignment masked using GBlocks with less stringent options; ML analysis was performed with RAxML v. 7.2.8 under the GTRCAT model of evolution and 500 bootstraps). Support values are indicated above branches; molluscan groups are in bold and indicated with black triangles.

where Caenogastropoda forms the clade Apogastropoda with Heterobranchia (Colgan et al. 2007; Aktipis and Giribet 2010, 2012; Castro and Colgan 2010; Stöger et al. 2013; Zapata et al. 2014). Improving the representation of major gastropod clades (but excluding fast-evolving patellogastropods), a recent mitogenomic study recovers an unconventional clade of paraphyletic caenogastropods, including Vetigastropoda and a single neritimorph, as sister to 'Euthyneura', i.e. heterobranchs (Williams et al. 2014).

Analyses on Euthyneura relying on mitochondrial markers usually (but see Gaitán-Espitia et al. 2013) recovered inner-heterobranch topologies either more or less compatible with traditional Opisthobranchia and Pulmonata (e.g. Medina et al. 2011), or Opisthobranchia nested within paraphyletic pulmonates (White et al. 2011; Stöger and Schrödl 2013; Williams et al. 2014). These relationships are rejected by analyses including nuclear genes (e.g. Klussmann-Kolb et al. 2008; Jörger et al. 2010; Kocot et al. 2011; Kocot, Halanych, et al. 2013; Zapata et al. 2014). Conflicting mt-based topologies (e.g. Grande et al. 2008; Medina et al. 2011) were suspected to suffer from uneven taxon sampling and aberrant mt gene evolution leading to ambiguous alignments and long-branch artefacts (Schrödl, Jörger, Klussmann-Kolb, et al. 2011, Schrödl et al. 2011).

Indeed, the mt-nucleotide based ML trees seem to be biased by long-branch attraction (Stöger and Schrödl 2013). Unexpected groupings, such as heterobranchs nesting within scaphopods (Figure 2), usually indicate long branches, though not all long branches refer to clearly artificial groups; e.g. the inner bivalve or cephalopod

topologies recovered can be disputed but not readily rejected. A similarly, perhaps even more artificial and implausible molluscan topology resulted from amino acid-based analyses, which show long-branched molluscan subgroups associated with non-molluscs or as alien inner branches of well-established molluscan taxa in the broad metazoan and focussed molluscan taxon sets (Stöger and Schrödl 2013). It is remarkable that adverse effects of heterogeneous mt nucleotide evolution in molluscs cannot be corrected or partially compensated for, by using supposedly more conservative amino acids and available models of protein evolution (Bernt, Bleidorn, et al. 2013). We found long molluscan branches are associated with unstable gene arrangement (Stöger and Schrödl 2013), causing strand biases such as different GC skews (Bernt, Bleidorn, et al. 2013). We thus postulate that major topological inconsistencies with nuclear gene-based trees can be explained as formerly unrecognized artefacts from mitogenomic processes, such as gene duplications, rearrangements, mutations and loss events on different strands, generating systematic errors. At an extreme, a major rearrangement event, such as towards heterobranchs and patellogastropods, can cause biases that override any potential phylogenetic signal in an affected deep branch. While mitogenomic sequence analyses seem promising between or within groups with conservative gene arrangement, future research will show whether or not deleterious effects of mt rearrangements can be addressed by a denser taxon sampling and using more suitable models of mt evolution.

Multilocus markers: Serialia resurrected

Stöger et al. (2013) compiled a multilocus (mitochondrial COI and 16S, nuclear 18S, 28S and H3 genes) molecular data set on lophotrochozoan outgroups (up to 17) and a comprehensive sampling of molluscan ingroup taxa (up to 141). Based on rigorously quality-optimized sequence sets of representative molluscan taxon samplings, they tested the monophyly of Mollusca and its major subclades (classes), and evaluated the many competing hypotheses of class interrelationships (e.g. Haszprunar et al. 2008; Lindberg 2008, 2009; Telford and Budd 2011; Haszprunar and Wanninger 2012). In particular, the question of whether or not Serialia (Giribet et al. 2006) is a natural group was considered crucial for understanding molluscan evolution. Stöger et al. (2013) recovered phylogenetic trees (see Figure 3) with monophyletic Mollusca (but see Boore et al. 2004; Mallatt et al. 2010, Mallat et al. 2012; Wilson et al. 2010) and monophyletic Bivalvia (but see Passamaneck et al. 2004; Giribet et al. 2006; Meyer et al. 2010). In all those data sets and under all alignment masking, partitioning, modelling and analyses regimes, the five included monoplacophoran species robustly clustered as sister clade of chitons, confirming earlier results (Giribet et al. 2006; Wilson et al. 2010; Kano et al. 2012). Major traditional taxa such as Aculifera, Testaria and Conchifera concepts (Haszprunar et al. 2008) are incompatible with the presence of Serialia (Figure 1). In all analyses by Stöger et al. (2013), the Serialia form a clade with bivalves and gastropods, opposed by a well-supported clade with scaphopods sister to aplacophorans and cephalopods (Figure 3).

While usually interpreted as plesiomorphies for serialian taxa within Testaria and Conchifera (Salvini-Plawen 2006; Wägele et al. 2009), the topology recovered (Figure 3) implies several putative apomorphies for Serialia (see Stöger et al. 2013). Striking features such as the (1) highly similar radulae, (2) special radula bolster with radula

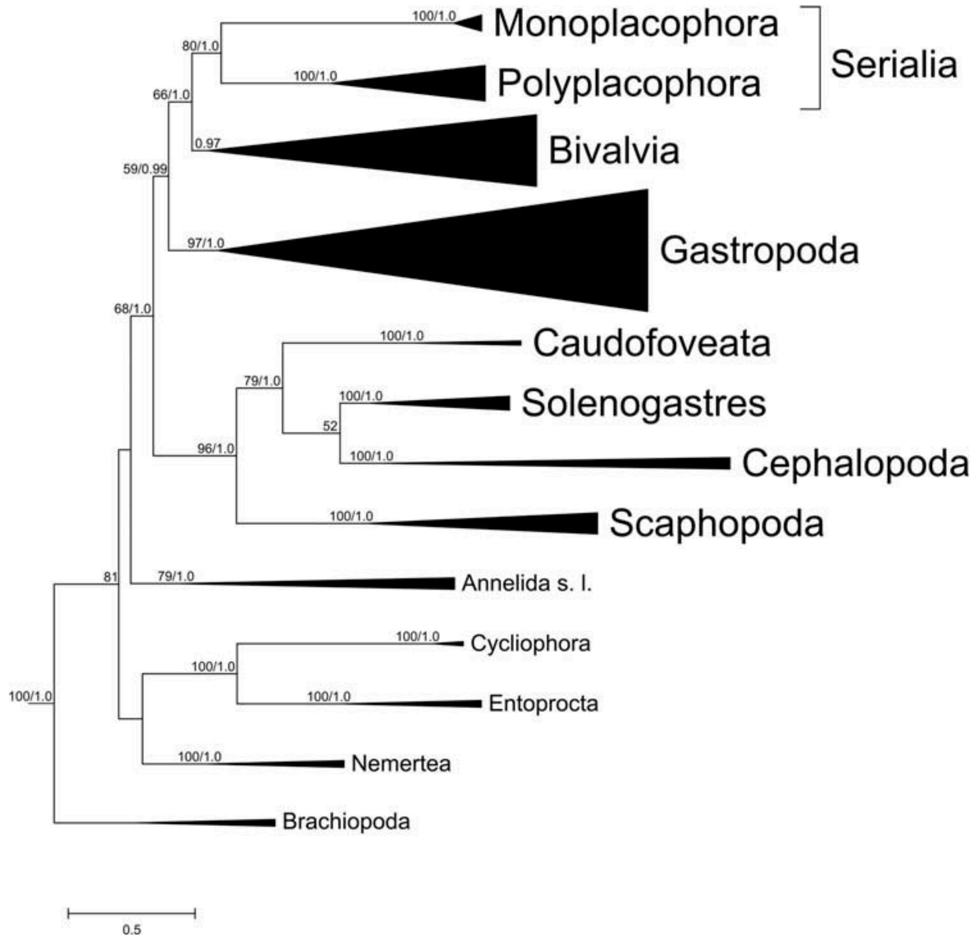


Figure 3. Molluscan class tree based on rRNA-dominated multilocus markers (five-gene nucleotide analysis published by Stöger et al. 2013; 17 lophotrochozoan outgroup and 125 molluscan species, MAFFT alignment masked using ALISCORE; ML analysis was performed with RAxML v. 7.2.8 under the GTRCAT model of evolution and 1000 bootstraps, respectively with MrBayes v. 3.1.2). Support values are indicated above branches (first value is bootstrap support, second value is posterior probability). Molluscan classes are in bold.

vesicle between lateral and median cartilages, and (3) a horizontal approximator muscle were emphasized as homologies earlier (e.g. Wingstrand 1985; Salvini-Plawen 2006). Similar stereoglossate radulae in topologically distant (Stöger et al. 2013; Zapata et al. 2014) but ecologically analagous patellogastropods were considered as results of different evolutionary pathways (Guralnick and Smith 1999). Chitons and monoplacophorans show further potential synapomorphies such as (4) serial (2×) 8-fold dorso-ventral pairs of muscle bundles, (5) anteroventrally elongated and dorsoventrally flattened body with (6) broad sucking foot, (7) serial gills in a circumpedal mantle cavity, reduction of the head with (8) mouth lappets rather than tentacles, and (9) a similar neural cord-like organization of the central nervous system

(Shigeno et al. 2007; Sigwart et al. 2014), among others. Such potential serialian apomorphies under an Aculifera-Conchifera scenario would be convergently derived in monoplacophorans and chitons or inherited from the ancestral mollusc.

That previous study (Stöger et al. 2013) also quantitatively tested competing concepts biasing various topologies and calculating their likeliness according to the data set. Under all schemes (Table 1) the Approximately Unbiased (AU) test (Shimodaira 2002) rejects (p <0.05) all higher molluscan concepts, such as Aculifera, Testaria, Conchifera, Cyrtosoma, Diasoma hypotheses. Those data, as well as previous analyses using similar markers and taxon subsets, suggest that Serialia is a clade, which is incompatible with the recently reinstated Aculifera-Conchifera paradigm.

Dorsoconcha

Serialia are placed in a derived rather than basal position (Figure 3) by all previous multilocus analyses (see Stöger et al. 2013), which is important for the interpretation of early fossils and reconstruction of molluscan evolution. Positions within each class provide a qualitative measure of confidence in the overall topology. The inner-chiton topology recovered Lepidopleurida as sister to Chitonida, consistent with more focused studies (Sigwart et al. 2011, 2013). The sister to Serialia is Bivalvia (Figure 3). While earlier studies (Passamaneck et al. 2004; Giribet et al. 2006) using similar marker sets resulted in diphyletic bivalves, this was likely an artefact caused by aberrant D-loop regions of the 28S gene. In trees recovered by Stöger et al. (2013) protobranchs are basal but paraphyletic, which resembles Giribet's (2008) bivalve classification with unresolved protobranchs rather than more recent, broader data sets recovering monophyletic Protobranchia (Sharma et al. 2012). Note the analysis resulting in Figure 3 excludes Patellogastropoda because their aberrant rRNA sequences cause long-branch effects (Stöger et al. 2013). The 'dorsoconch' clade of Gastropoda, Bivalvia and Serialia is stable in all main analyses, strongly supported in Bayesian analyses, but just moderately supported by ML bootstrap values, and we failed to detect any conspicuous, ubiquitous apomorphies (Stöger et al. 2013).

Variopoda

The second, 'variopod' basal molluscan clade (Figure 3) comprises Scaphopoda, aplacophoran 'worm-molluscs', and cephalopods. This clade is unconventional at least, but well supported in all analyses by Stöger et al. (2013). It was recovered in a single gene (18S) analysis by Meyer et al. (2010), but assumed to be caused by long-branch attraction (LBA) effects. Stöger et al. (2013) showed that branch lengths of scaphopods and caudofoveates are moderate, and the node is stable against removal of putative long-branched taxa showing accelerated evolutionary rates or biased base compositions, such as Solenogastres and/or Cephalopoda.

The internal scaphopod topology showing a split into Gadilida and Dentaliida is consistent with former morphological and molecular analyses (Steiner 1998, 1999; Steiner and Dreyer 2003), although the phylogenetic relationships within Gadilida still need further examination. Based on 18S sequences only, *Entalina* is basal within Gadilida (Steiner and Dreyer 2003), whereas in analyses including five molecular markers *Siphonodentalium* is in a basal position (Stöger et al. 2013). Within

Table 1. Major hypotheses on the origin and deep phylogeny of Mollusca, and their fit with different data sets. (+) = supported, (-) = contradicted, (~) = more or less compatible, ( ) not applicable or no information available. Node support here means pp >0.95 and/or bootstrap >75%. AU test results: $p > 0.05$ (~; hypothesis not rejected), $p < 0.05$ (-; hypothesis rejected). *Reanalysed means *Drosophila* and columns with entirely missing data removed from the alignment.

Analysis Hypothesis	Morphoanatomy, cladistic analysis, molluscan class ground-patterns (Haszprunar 2000)/ modified reanalysis (herein)	Multilocus markers, topology/ node support (herein)	AU tests based on multilocus markers (Stöger et al. 2013)	7 housekeeping genes, 31 molluscan taxa (Vinther et al. 2012)	79 ribosomal genes, 16 molluscan taxa (Meyer et al. 2011)	EST data set, 308 markers, 42 molluscan taxa (Kocot et al. 2011)	EST data set, 1185 markers, 35 molluscan taxa (Smith et al. 2011)	Smith et al. (2011) 301 gene subset, reanalysed* with/ without Monoplaco-phora (herein)	AU tests based on Smith et al. (2011) 301 gene subset reanalysed* (herein)	Chronology of reliable fossils (see Stöger et al. 2013)
Mollusca + Kamptozoa + Cycliophora	−/	−/−	~			+	–	−/−		
Sinusoida (Mollusca + Kamptozoa)	−/−	−/−	–	–	–	–	–	−/−		
Neotrochozoa (Mollusca + Annelida)	−/−	+/+	~	–	–	+	–	−/−	~	
Mollusca	+/+	+/+	~	+	+	+	+	+/+		+
Testaria (Polyplacophora + Conchifera)	+/−	−/−	–	–	–	–	–	−/−	~	–
Aculifera (Polyplacophora + aplacophorans)	−/−	−/−	–	+	–	+	+	+/+		~ (acceptable if aculiferan ancestor was shelled)
Aplacophora	−/−	−/−	~	+		+	+	+/+		
Conchifera	+/−	−/−	–	–	–	+	+	+/+		~ (acceptable if gastropods and bivalves are basal)
Pleistomollusca (Gastropoda + Bivalvia)	−/−	−/−	~	+	+	+	–	−/−	~	~
Monoplacophora + Cephalopoda	−/−	−/−	~				+	+/		~
Scaphopoda + Gastropoda + Bivalvia (Ganglionata)	−/−	−/−	–			+	+	+/+		~ (acceptable if gastropods or bivalves are basal)
Scaphopoda + Cephalopoda	−/−	~/−	~			–	–	–		~
Scaphopoda + Cephalopoda + Gastropoda	+/+	−/−	–			–	–	−/−		~ (acceptable if gastropods basal)
Cyrtosoma (Cephalopoda + Monoplacophora + Gastropoda)	−/−	−/−	~				–	−/−	~	~ (acceptable if gastropods are basal)

(*Continued*)

Table 1. (Continued).

Analysis Hypothesis	Morphoanatomy, cladistic analysis, molluscan class ground-patterns (Haszprunar 2000)/ modified reanalysis (herein)	Multilocus markers, topology/ node support (herein)	AU tests based on multilocus markers (Stöger et al. 2013)	7 housekeeping genes, 31 molluscan taxa (Vinther et al. 2012)	79 ribosomal genes, 16 molluscan taxa (Meyer et al. 2011)	EST data set, 308 markers, 42 molluscan taxa (Kocot et al. 2011)	EST data set, 1185 markers, 35 molluscan taxa (Smith et al. 2011)	Smith et al. (2011) 301 gene subset, reanalysed* with/ without Monoplaco-phora (herein)	AU tests based on Smith et al. (2011) 301 gene subset reanalysed* (herein)	Chronology of reliable fossils (see Stöger et al. 2013)
Cephalopoda + Gastropoda (Cyrtosoma or Visceroconcha, sensu Haszprunar 2000)	+/+	−/−		–	–	–	–	−/−	~	~
Scaphopoda + Gastropoda	−/−	−/−	~			–	+	+/+		~
Diasoma (Scaphopoda + Bivalvia)	−/−	−/−	–			–	–	−/−	~	~
Monophyletic Protobranchia (all superfamilies)		−/−	–				+	+		
Serialia (Monoplacophora + Polyplacophora)	−/+	+/+	~				–	−/	~	+
Bivalvia + Serialia	−/−	+/+					–	−/−		+
Gastropoda + Bivalvia + Serialia (Dorsoconcha)	−/−	+/(+)	~				–	−/−	~	+
Scaphopoda + aplacophorans + Cephalopoda (Variopoda)	−/−	+/+	~			–	–	−/−	~	~
Aplacophorans + Cephalopoda	−/−	+/+		–	+	–	–	−/−		

Cephalopoda a strongly supported basal dichotomy into *Nautilus* and coleoids was recovered, which is noncontroversial (Bonnaud et al. 2004; Lindgren et al. 2004; Kröger et al. 2011). The taxon sampling within coleoids was optimized for selecting some basal and slowly evolving members rather than to reconstruct inner relationships (Allcock et al. 2011).

Stöger et al. (2013) tentatively called this topological concept the 'Variopoda', since the foot of its members is not a broad gliding sole but a digging foot in Scaphopoda (convergent with many bivalves), reduced to a narrow ciliated gliding sole in vermiform Solenogastres, further reduced to a suture which is not functional or lost completely in Caudofoveata, and modified into forming the funnel in cephalopods.

The two aplacophoran classes form a clade in some, but not all those analyses, but always are sisters to Cephalopoda. An association of aplacophorans and cephalopods is highly suspicious from a morphologist's view, but was also recovered repeatedly in several independent molecular data sets (Lieb and Todt 2008; Meyer et al. 2011; Vinther et al. 2012; Struck et al. 2014), and thus should not be instantly dismissed. Parsimony-based and likelihood-based character reconstructions with our preferred multilocus topology (Figure 3) using Mesquite (Maddison and Maddison 2011) indicate the possibility that the (external) shell had already been lost in the joint ancestor of cephalopods and paraphyletic aplacophorans, suggesting the nautiloid and any other (external) cephalopod shells could be secondary. The worm-like shape and many regressive features especially in the digestive, excretory and genital systems of both Solenogastres and Caudofoveata are either symplesiomorphic or convergent, possibly adaptations to life in sediments.

Comparison of data sets

We are well aware that the multilocus results (Figure 3) supporting Serialia, Dorsoconcha and Variopoda are unconventional, and may be wrong, after all. Yet we conclude that a densely sampled and carefully quality-checked multilocus data set bears enough phylogenetic signal to recover monophyletic Mollusca, all molluscan classes, and most of the previously argued subtaxa. In the present study, we compiled and compared evidence on deep molluscan phylogeny from various data sets and analyses, in a roughly formalized, integrative approach (Table 1). Out of dozens of hypotheses on the origin of molluscs and class interrelationships available in the literature, we used traditional taxa and also a selection of named and unnamed concepts and nodes from recent studies. We assessed all these hypotheses for fit with morphological, palaeontological and molecular evidence according to literature. We also performed a reanalysis and sensitivity tests of major morphocladistic and phylogenomic studies, implementing Splitstree (Huson and Bryant 2006) neighbornet analyses to visualize conflict in such molecular data sets, and AU tests with Treefinder (Jobb et al. 2004) to evaluate whether or not alternative, constrained topologies are rejected significantly by other, already published data sets.

Mollusca: origin unresolved

The origin of molluscs from a Sinusoida (or synonymous Lacunifera or Tetraneuralia) clade as proposed by morphologists (e.g. Bartolomaeus 1993; Ax

1999) was recently revitalized and strengthened by ontogenetic evidence (Nielsen et al. 2007; Wanninger 2009). However, such a clade of molluscs and entoprocts (usually associated with Cycliophora) has not been recovered in any molecular phylogenetic analysis to date. The highly similar mt gene arrangement of some sinusoid taxa (Yokobori et al. 2008) likely reflects the plesiomorphic condition of non-platyzoan lophotrochozoans (Stöger and Schrödl 2013). Alternatively, morphologists assumed a molluscan sistergroup relationship with Annelida, which currently includes Sipuncula, Echiura and pogonophorans. This concept called Neotrochozoa is supported by some molecular studies, e.g. using housekeeping genes (Peterson et al. 2008, 2009; Sperling et al. 2009) or EST data (Kocot et al. 2011) with, however, still limited taxon sampling. Overall, Neotrochozoa was recovered sporadically rather than consistently and reliably, e.g. recovered in rRNA gene analyses (Mallatt et al. 2010; Mallat et al. 2012), Bayesian (but not ML) analyses of EST data of ribosomal genes (Struck and Fisse 2008) as well as in a multigene study by Lartillot and Philippe (2008) under the WAG but not the CAT model of evolution.

Recent, comprehensive phylogenomic analyses by Struck et al. (2014) do not resolve the origin of molluscs unambiguously, recovering Mollusca sister to a clade of annelids and nemerteans, or sister to brachiopods, or as a more basal offshoot among lophotrochozoans. Earlier phylogenomic approaches on broad metazoan sets also recovered Mollusca as a basal offshoot of a lophotrochozoan (spiralian) clade excluding platyzoan taxa (e.g. Dunn et al. 2008; Smith et al. 2011). A more basal rather than derived origin of Mollusca among Lophotrochozoa is consistent with their Precambrian dating in molecular clock approaches.

This timing prompts a consideration that Late Ediacaran *Kimberella* Wade, 1972 is a stem-group mollusc (Stöger et al. 2013) rather than a basal bilaterian (e.g. Edgecombe et al. 2011). Former reports of Early Ediacaran bilaterian trace fossils, such as assumed worm burrows (e.g. Seilacher et al. 1998) might refer to protozoans (Matz et al. 2008). More recent data, however, indicate that first putative fossil bilaterians (Chen et al. 2004) and burrows of macroscopic infaunal worms (Pecoits et al. 2012) date back to strata from the Early Ediacaran, more than 580 Mya, and this is compatible with an Ediacaran origin of molluscs. Common features in adult entoprocts and molluscs, such as the sinusoidal body cavity (e.g. Ax 1999), which may be a common situation for non-(eu)coelomate lophotrochozoans (see Jenner 2004a), thus may be plesiomorphic for *Kimberella* and other molluscs. A ventrally flattened body with ciliated foot with crossing dorsoventral muscles, which is present in adult molluscs and some entoproct creeping larvae, plus a tetraneural nervous system were considered apomorphies for Sinusoida (as Tetraneuralia, Wanninger 2009).

Alternatively, in the absence of any molecular support for Sinusoida, all these features may be plesiomorphies retained by entoprocts and molluscs. According to our preferred multilocus tree (Figure 3), at least Nemertea and Annelida lost such features and became worm-like, the latter segmented and eucoelomatic, with secondary modifications in Sipuncula, including a (progenetic?) creeping larva in some species. Cells with the ability to produce chitinous cuticle (but becoming collagenous and flexible in annelids) and biomineralize calcareous particles, spicules or plates, however, under a topology with basal Mollusca clearly are plesiomorphic for lophotrochozoans (Vinther 2009), or at least non-platyzoan lophotrochozoans (Zhang et al. 2013). Though possibly more basal and thus older than previously expected, the exact origin of Mollusca remains unknown.

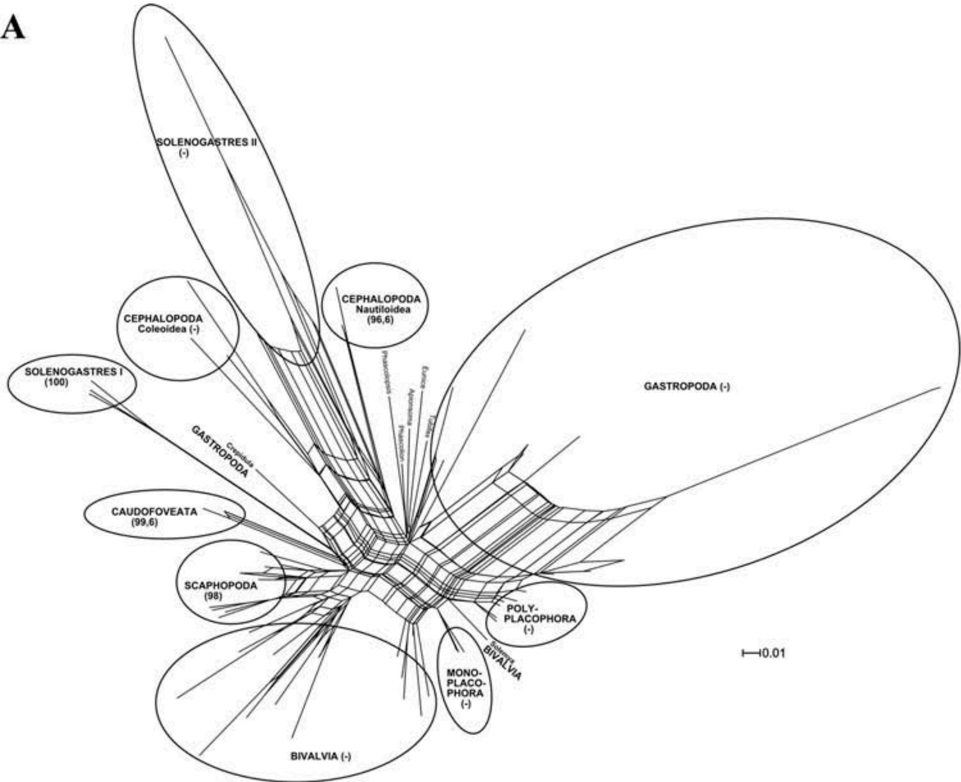
'Wormy' Testaria?

Aplacophorans were not widely recovered as monophyletic in previous morphological studies, but monophyly is usually implied under the Aculifera concept (e.g. Scheltema 1993). Under the Testaria hypothesis, Caudofoveata was considered as the earliest offshoot of Mollusca (Adenopoda concept) in earlier studies (e.g. Salvini-Plawen 1990); later Solenogastres was regarded as sister to other molluscs (Salvini-Plawen and Steiner 1996), so-called Hepagastralia (Haszprunar 2000). Previously published multilocus analyses are ambiguous, recovering paraphyletic aplacophorans in most cases (Figure 3; Stöger et al. 2013), while recent phylogenomic and housekeeping gene-based analyses, on fewer taxa, clearly support Aplacophora (Kocot et al. 2011; Smith et al. 2011; Vinther et al. 2012).

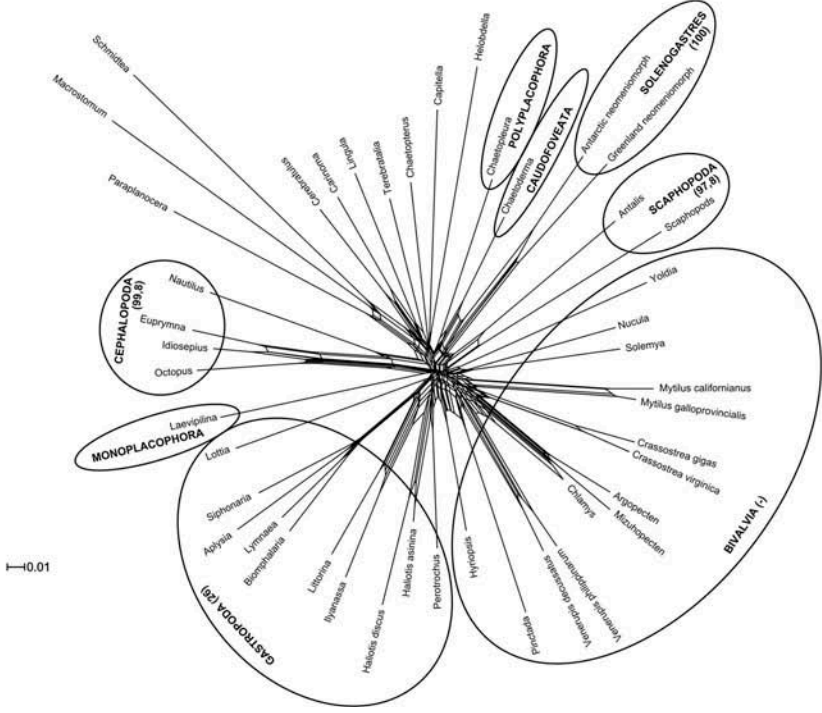
Regardless whether monophyletic or paraphyletic, aplacophorans as the putative most basal offshoot(s) of the molluscan tree were central to the traditional Testaria (Eumollusca) hypothesis. This hypothesis is advocated in several textbooks (e.g. Westheide and Rieger 1996; Ax 1999) and in comparative morphological analyses (e.g. Salvini-Plawen 1980, 1990, 2006; Haas 1981). Testaria has been strongly supported by morphocladistic analyses (e.g. Salvini-Plawen and Steiner 1996; Haszprunar 2000), and until recently considered valid by leading experts (e.g. Haszprunar et al. 2008; Wägele et al. 2009; Haszprunar and Wanninger 2012). However, modifying the selection of taxa and characters influences the results (e.g. Jenner 2004a). Using shells, sclerites and other molluscan features that can be fossilized in a taxon sampling with shelled and shell-less outgroups led to shelled molluscs being basal and shell-less molluscs being derived clades (Sigwart and Sutton 2007; Sutton et al. 2012). Still, under parsimony principles, using mainly soft part anatomical features and shell-less worm-like outgroup taxa, molluscan 'worms' are recovered basal (Haszprunar 2000). Relaxing *a priori* groundpattern, homology assumptions and character selection of the latter analysis, i.e. using a non-patellogastropod ancestral snail (as implied e.g. from Stöger et al. 2013, suppl. fig. 2; Zapata et al. 2014) and adding potential synapomorphies for Serialia listed above, leads to different ingroup relationships, i.e. the recovery of monophyletic rather than paraphyletic Serialia (Table 1). However, aplacophorans remain basal in all such morphoanatomy-based permutations, even when including recently discovered similarities in myogenesis of solenogasters and polyplacophorans (Scherholz et al. 2013). Under parsimony principles shelled (or shell plate-bearing) molluscs have a strong tendency to cluster together rather than allowing secondary reduction of shells and associated features. The latter, however, is a process that is well known to have occurred multiple times in several conchiferan clades (e.g. Wägele and Klussmann-Kolb 2005; Jörger et al. 2010; Brenzinger, Haszprunar, et al. 2013).

Secondary character loss (or substantial reduction and modification) was suggested as a major factor confounding morphology-based metazoan phylogenetic reconstruction by Jenner (2004b). Testaria thus could be another example of non-parsimonious evolution misleading parsimony-based morphocladistics. In fact, the Testaria hypothesis is rejected by all the many molecular analyses available to date (Table 1). Furthermore, the Testaria concept appears neither compatible with fossil stratigraphy nor with molecular molluscan chronograms available on different data sets (Table 1; Vinther et al. 2012; Stöger et al. 2013). The Testaria hypothesis implying a progressive evolution from simple, worm-like molluscs to more complex

A



B



conchiferans (Salvini-Plawen 2006) and always higher body complexity in conchiferan subtaxa thus appeared most parsimonious and plausible in morphoanatomical frameworks, but seems no longer supported in the light of current, integrative data, from multiple perspectives.

Serialia versus Aculifera/Conchifera

Ingroup molluscan hypotheses may be focused on two, Serialia versus Aculifera/Conchifera. A main criticism of the original work by Giribet et al. (2006) was the supposedly chimerical nature of the 28S fragment available for *Laevipilina antarctica* (Schrödl et al. 2006). Wilson et al. (2010) confirmed that one amplicon of the combined sequence includes true monoplacophoran, while the other is a chiton. However, the 'chiton' fragment is not identical to any confirmed chiton sequence. While the 'monoplacophoran' fragment of abyssal *L. antarctica* is similar to a newly generated 28S sequence of bathyal *L. antarctica* (Stöger et al. 2013), it is not identical (2% p-distance). There is thus a certain chance that the abyssal specimen is cryptic and closely related to rather than conspecific with bathyal *L. antarctica*.

Some further problems typical for initial multilocus data sets have also been documented (Wägele and Mayer 2007; Wägele et al. 2009; Meyer et al. 2010) and corrected in other studies (Meyer et al. 2010; Wilson et al. 2010; Kano et al. 2012; Stöger et al. 2013). We realize that even the data sets with the largest taxon sampling (Stöger et al. 2013) still have undersampled groups such as aplacophorans and, though stringently masked, considerable missing data (Table 1). In a key problem emphasized by Wägele et al. (2009), those data still exhibit considerable conflict in neighbornet analyses of individual or concatenated markers, as is visualized by a central netlike structure with edges of similar length (Figure 4A). However, lack of tree-like structures referring to deep molluscan phylogeny has been typical for all other data sets regardless of which markers and how many loci were used (Figure 4B), and Serialia is never contradicted by other well-supported splits. None of the Cambrian or earlier molluscan class or lophotrochozoan relationships received convincing split support, thus we suspect that a considerable degree of conflict may be intrinsic to data sets involving deep Cambrian divergences.

In summary, 18S and multilocus phylogenetic analyses with single or multiple monoplacophoran species all recovered Serialia, usually with robust support (Giribet et al. 2006; Meyer et al. 2010; Wilson et al. 2010; Kano et al. 2012; Stöger et al. 2013). This affinity cannot be explained by the influence of a single aberrant gene or gene class, since BLAST searches herein showed individual nuclear 18S and 28S rRNA

Figure 4. Neighbornet graphs computed in SplitsTree 4 (version 4.11.3) visualizing conflict (net-like or star-like structure) versus potential signal (tree-like structure) in primary data. (A) Splitstree analysis of the concatenated nucleotide alignment of the 81-taxon set of Stöger et al. (2013); molluscan classes are outlined by circles; most of the taxon names were removed for display reasons. Numbers in parentheses display bootstrap support of 500 replicates for classes with more than one representative; (-) means no support. (B) Analysis of the 301-gene set (amino acids) of Smith et al. (2011); the distant outgroup *Drosophila* and columns with entirely missing data were removed from the data set. Molluscan classes are outlined by circles. Numbers in parentheses display bootstrap support of 500 replicates for classes with more than one representative; Bivalvia is not supported.

sequences, and also the mitochondrial COI sequences of the monoplacophoran *L. hyalina* are similar to those of chitons, while H3 and 16S behave unspecifically. None of these five genes show any specific affinity to certain conchiferan groups as would be expected under a conchiferan/aculiferan concept. Competing hypotheses such as Testaria, Aculifera and Conchifera were rejected by AU tests of several different variants of the main data set (Stöger et al. 2013; Table 1). Serialia thus had a problematic start in science, but has been successively refined and now is clearly supported by multilocus analyses. Other marker sets are needed to test Serialia.

Recent studies using housekeeping genes (Vinther et al. 2012), multiple ribosomal protein coding genes (Meyer et al. 2011) or phylogenomic data (Struck et al. 2014) are based on small molluscan taxon sets lacking any monoplacophorans. Therefore, although some resolve Aculifera, they cannot test the Serialia hypothesis and, interestingly, all these studies failed to recover monophyletic Conchifera. These and some broader EST studies (Dunn et al. 2008; Hejnol et al. 2009) recovered Cephalopoda sister to or among aculiferan taxa. Increasing the taxon sampling of such phylogenomic studies places Cephalopoda basal in a monophyletic Conchifera sister to Aculifera (Pick et al. 2010), a molluscan topology that is congruent with the recent EST based study of Kocot et al. (2011), which also excluded Monoplacophora. However, the conchiferan versus aculiferan relationship of cephalopods in Kocot et al. (2011) is sensitive to outgroup sampling.

Smith et al. (2011, 2013) included a monoplacophoran species in their EST analyses (sets with 1185 and 301 gene fragments used) and recovered it sister to cephalopods. Such a clade was never recovered in any multilocus marker permutation (Stöger et al. 2013); it was not rejected by the AU tests of the larger taxon samplings of that study either (Table 1), but significantly rejected in the 'best' taxon sampling, i.e. the 81 taxa subset in which long inner branches of molluscan subclades have been partly removed. While close fossil links of monoplacophorans and cephalopods have been proposed in the study of early molluscan palaeontology (Runnegar and Pojeta 1974), these are neither structurally undisputed nor fit with the timing of molluscan class evolution indicated by stratigraphy or molecular time trees (Stöger et al. 2013).

To test whether or not the single monoplacophoran attracts cephalopods into a basal position we excluded the monoplacophoran from the 301-gene analysis of Smith et al. (2011). The RAxML reanalysis herein recovered the same topology (not shown), with cephalopods sister to other conchiferans. However, cephalopods as basal conchiferans appear implausible considering that first putative fossil bivalves (*Fordilla, Pojetaia*) appeared some 30 million years earlier than the first undisputed cephalopods (Stöger et al. 2013). Cephalopoda as a basal conchiferan, aculiferan or molluscan offshoot is, up to now, a feature of small taxon sets using vast sequence data, and may be artificial.

Conflict in inner conchiferan concepts

A morphology-based tree of paraphyletic Serialia, with chitons sister to Monoplacophora plus Ganglionata, i.e. all other conchiferan classes, was recovered (Haszprunar 2000) and accepted as a phylogenetic hypothesis (e.g. Salvini-Plawen 2006; Wägele et al. 2009). Such topology would explain similar features of serialian taxa as plesiomorphies (see Wägele et al. 2009), but has not been recovered by molecular analyses yet. Cyrtosoma in its original palaeontological meaning

(Monoplacophora plus Cephalopoda and Gastropoda) and in a neontological subset version (Cephalopoda and Gastropoda only; synonyms are Visceroconcha and Rhacopoda) has not been recovered in comprehensive molecular analyses either. Rather than being apomorphic, similarities like differentiated head, cephalization, and cerebral eyes may either have evolved convergently, or, may remain from common molluscan ancestors having complex, elaborate rather than simple bodies (Stöger et al. 2013).

The Diasoma concept, proposed by palaeontologists and including bivalves, scaphopods and extinct rostroconchs, in its neontological form is supported in some morphocladistic analyses (Simone 2009) but not in others (e.g. Waller 1998; Haszprunar 2000). Apart from mitochondrial sequence studies (Dreyer and Steiner 2006; but see Stöger and Schrödl 2013) there is little molecular indication for such a clade (i.e. limited to single permutations of Kocot et al. 2011 and Smith et al. 2013; Table 1). Similarities of bivalves and scaphopods are indeed compelling (Simone 2009), but apparently are either plesiomorphic or, according to topologies with scaphopods more closely related to cephalopods and gastropods than to bivalves, rather convergent adaptations to infaunal life (Wanninger and Haszprunar 2001; Steiner and Dreyer 2003).

Pleistomollusca was a taxon name proposed for Gastropoda together with Bivalvia, thus including >95% of recent molluscan species diversity (Kocot et al. 2011). Pleistomollusca was robustly supported by tree statistics and also recovered by independent, taxon-limited multigene analyses (Meyer et al. 2011; Vinther et al. 2012). A sister-group relationship is not rejected significantly by AU tests on the multilocus data (Stöger et al. 2013) or on the 301-gene set by Smith et al. (2011) herein (Table 1). However, no molecular study with a more representative taxon sampling has recovered Pleistomollusca yet. In contrast, Smith et al. (2011) and our present reanalysis of their 301-gene set under the PROTGAMMAWAG model of evolution, but with Monoplacophora and the distant outgroup taxon *Drosophila* removed, all recovered Gastropoda as sister to Scaphopoda, with equally high node support as Pleistomollusca in Kocot et al. (2011). The most recent phylogenomic study on a comprehensive lophotrochozoan sampling by Struck et al. (2014) includes nine molluscs, representing five classes. While all their analyses recover monophyletic Mollusca and Pleistomollusca, none recovers Aculifera or Conchifera. In fact, the relative positions of Polyplacophora, Cephalopoda and Solenogastres vary across ML and Bayesian analyses of different gene sets, adding further diversity to the already large set of available hypotheses on molluscan class relationships.

While a unified molluscan tree was presented by Telford and Budd (2011) and also by Kocot (2013) (see Figure 1), clearly there are substantial contradictions between independent phylogenomic and other molecular approaches. Remarkably, any conchiferan topology with bivalves and gastropods in derived positions would contradict the chronology of fossils appearing in stratigraphy (Table 1), simply because putative gastropod and bivalve fossils are much older than other, less secure members of other recent conchiferan lineages (e.g. Parkhaev 2008; Stöger et al. 2013). The consensus topology of deep molluscan phylogeny suggested by Telford and Budd (2011) is thus actually incompatible with the fossil record; it illustrates classes in derived positions (bivalves) with older reliable fossils than any of the more basal molluscan groups or potential stem offshoots (*Wiwaxia*, *Halkieria*) shown. In contrast, a serialian topology with basal dichotomy into dorsoconchs and variopods and

time trees on further diversification fits well even with fine-scale fossil data available (Stöger et al. 2013). Obviously, in palaeontology 'not found' does not necessarily mean 'absent', especially when it refers to (Pre)Cambrian soft bodies or tiny shells, and the latter may still be difficult to interpret. However, it may be more problematic to ignore the fossil evidence already available than to consider it, comparing and integrating fossils with other lines of evidence, each suffering from specific drawbacks.

Synthesis and outlook

Morphology alone or combined with molecular data available at present cannot resolve deep molluscan phylogeny reliably. The Testaria hypothesis is no longer supported. The Serialia hypothesis (Giribet et al. 2006) now is based on quite comprehensive taxon sampling and quality improved sequences and alignments. But it still relies on limited and fragmentary gene sampling with little *a priori* signal in the data; despite all efforts, analyses still may suffer from unrecognized artifacts such as LBA effects (see Wägele and Mayer 2007; Kück et al. 2012; Stöger et al. 2013). Covering all molluscan classes with vast phylogenomic data, Smith et al. (2011) completed the paradigm shift towards the 'Aculifera-Conchifera' hypothesis. As expected, Splitstree neighbornet graphs (Figure 4B) from amino acids of the 301-gene set by Smith et al. (2011) appear more tree-like than from multilocus nucleotide data on a larger molluscan sampling (Figure 4A). However, both networks are essentially similar in showing little signal, with no support for any of the debated deep molluscan splits. Even more surprising, AU analyses of the 301-gene set by Smith et al. (2011) neither rejected Serialia nor most other alternative class interrelationships (Table 1). The presence of a certain signal for Serialia detected by Smith et al. 2011, suppl. fig. 8), and the lack of signal for Aculifera/Conchifera in Stöger et al. (2013), may be additional evidence of a problem.

Regardless of the topological differences, all molecular trees imply that aplacophorans are secondarily worm-like and morphoanatomically simplified. This is in agreement with recent ontogenetic results (Scherholz et al. 2013). We suspect that meiofaunal ancestors or at least ontogenetic stages evolved a 'meiofaunal syndrome' as also occurred multiple times within heterobranch gastropods (Brenzinger, Haszprunar, et al. 2013).

Integrating evidence from molecular phylogenetic analyses, reconstructed or implied morphological innovations, and times of diversification by molecular clock dating with palaeontological and palaeoecological data (e.g. Caron et al. 2006, 2007; Ivantsov 2009, 2010; Smith and Caron 2010) presented some novel perspectives on early molluscan evolution (Stöger et al. 2013). Such a refined scenario (Figure 3) would fit with the stratigraphic chronology of the fossil record, if accepting that the last common ancestor of living molluscs was neither aplacophoran nor chiton-like, but a small, cap-shelled, untorted gastropod-like animal (Table 1). Early Cambrian merismoconchs (Yu 1984a, 1984b) and halwaxiids (Conway Morris and Caron 2007) could be offshoots of serialian or aculiferan stems, referring to different stages of shell fragmentation towards a chiton-like body organization in the Cambrian, and aplacophorans adapted to infaunal life progenetically later (Stöger et al. 2013). Reconstruction of early molluscan evolution thus may converge. In contrast, because none of the various molecular topologies available is completely congruent (Table 1),

we should consider the possibility that the molluscan class-level phylogeny is still unresolved.

1001 reasons against paradigms

Here we have examined recent progresses in resolving deep molluscan phylogeny by molecular data and advanced bioinformatics. Apart from mitochondrial sequence analyses, all the recent multilocus and phylogenomic data sets and analyses recover monophyletic Mollusca, monophyletic molluscan classes, and some of the supposedly reliable inner-class relationships. This is surprising considering the geological time-scale and frequent arguments against the power of certain molecular marker sets, such as rRNA-dominated sets. In contrast, molluscan phylogeny based on class-level relationships remains notoriously uncertain even in our integrative approach. This can be explained by (1) an incomplete fossil record and problematic interpretation of early fossils, (2) bias and preconceptions in traditional molluscan systematics, (3) genetic divergences evolving in a very short time, (4) the erosion of sparse original signal, combined with extinction of basal lineages and anagenetic change, (5) long-branch artefacts in old and/or heterogeneously evolving lineages, (6) stochastic errors in non-phylogenomic sequence sets, (7) uneven and sparse taxon sampling (see e.g. Roure et al. 2013), and (8) difficulties of data quality and orthology assessments in huge phylogenomic data sets.

But there also may be several other, not commonly recognized or still speculative, general weaknesses in sequence and other data sets that we want to elaborate further.

Phylogenomic molluscan sets have moderate average gene occupancy (40–50%) (Kocot 2013), but coverage of taxa may be uneven. In re-examining published data in the course of this study, we also found heterogeneous alignments, with many genes well aligned, while others appear highly variable, similar to unmasked 18S and 28S rRNA alignments. Bioinformatic methods of optimizing gene selection in large EST data sets are promising but not yet fully adequate (von Reumont et al. 2012). Despite the huge amount of data, careful gene selection (for coverage, base or amino acid compositional biases, and alignment quality), stringent alignment masking and application of more suitable substitution models (e.g. Philippe et al. 2011) will likely be helpful to reduce noise and errors in phylogenomic data sets (e.g. Nosenko et al. 2013). The high degree of missing data in amino acid sequences (e.g. 34% in the degapped 301-gene set by Smith et al. 2011, gaps account for 59%; own data) was partly explained by a concatenation artefact and corrected (Smith et al. 2013).

Contradictions between robustly supported published phylogenomic molluscan topologies first point to the need for reconsidering the relevance of maximum node supports in phylogenomic-scale studies (e.g. Simmons 2012). One way of testing and curing such problems is to include more slowly evolving taxa, densely representing all major lineages. Outliers, or groups with suspected aberrant evolution should be identified and analysed separately. Exclusion of data must be documented; we advocate successive filtering and selection of taxa, models and analyses and comparing results critically.

There is, however, also contradiction within data from a given taxon set. Nosenko et al. (2013) pointed out that different phylogenomic-scale functional gene sets tell different highly supported stories in deep metazoan phylogeny. Ribosomal

protein coding genes were the slowest evolving, resulting in a more conventional tree than non-ribosomal protein genes. In molluscs, effects of gene function on deep topologies remain to be tested in a taxon set representing all major clades. Analyses of 79 ribosomal protein coding genes of a molluscan subset with five out of eight classes by Meyer et al. (2011) recovered a highly unconventional though strongly supported topology. Smith et al. (2011) had conflicting signal among their EST data, overall stronger for a cephalopod–monoplacophoran relationship, weaker for Serialia; however, many genes show a strong signal for Serialia. Whether or not such differences are due to functional gene classes, slow versus fast-evolving genes, gene location on different chromosomes, or other common attributes remains to be studied. It is clear though that there is conflicting signal in the genome, that selection of gene sets, regardless of their phylogenomic-scale sizes, potentially influences the resulting topology, and that tree statistics alone are not good measures for the quality of competing molluscan trees.

The fact that different genes can tell different stories in deep phylogeny can be explained by different rates of evolution or compositional biases (see Nosenko et al. 2013). It remains to be explored whether or not similarly slow-evolving genes with similar base or amino acid composition and from the same functional groups bear signal for different trees, and whether or not there are evolutionary factors other than random mutations causing this. In mitogenomics, genome rearrangement may cause strong base composition biases and non-phylogenetic signal leading to dubious trees (Bernt, Bleidorn, et al. 2013; Stöger and Schrödl 2013). There may be no comparable strand biases in the nuclear genome; however, with only preliminary insights into molluscan and lophotrochozoan whole genomes, we suspect that there will be several surprises to be discovered regarding genome evolution (see Simakov et al. 2012). We assume that genome-scale duplication and loss events (e.g. Hallinan and Lindberg 2011), potential competition between chromosomes, regions or gene families, mechanisms of DNA modifications, and the bulk of 'junk DNA' with almost unknown properties may influence gene histories beyond what can be reflected by currently available analytical models.

We are intrigued by the fact that molluscan class relationships are resistant to phylogenetic reconstruction, while Mollusca, molluscan classes or subclades are not. Rapid class diversification (i.e. within roughly 50 million years, Stöger et al. 2013), with little time for signal development and much time for later extinction, may not be the full story. We suspect that gene trees in early Cambrian molluscs could have been different from species trees, e.g. because of incomplete lineage sorting, as seen in recent species (e.g. Maddison and Knowles 2006). Gene tree discordance is well known from recent lineages, but may also be preserved in ancient lineages, especially those that evolved from rapid radiations (Degnan and Rosenberg 2009). If such Cambrian molluscan gene lineages were not fully sorted (e.g. because of large population sizes) before diversification, then conventional phylogenetic analyses would not be able to reconstruct molluscan class relationships unambiguously, regardless of the amount of genes, because different genes actually have different histories. Very ancient incomplete lineage sorting preserved in the genome could explain signal for different species histories in different genes as described by Smith et al. (2011). After speciation, horizontal gene transfer and hybridization events could have further contributed to blur signatures of early molluscan evolution. Like other

potential genomic artefacts discussed above, these problems may affect gene sets, including multilocus or phylogenomic markers.

We also assume that aberrant phenotype evolution, rather than being independent from functionally unrelated molecular markers such as rRNA or housekeeping genes, may be correlated with aberrant molecular evolution to a certain extent. For example, habitat transitions or rare long-distance dispersal events may go along with phenotypic adaptations and with population bottlenecks causing rapid genetic drift, which may in some cases adversely affect both morphocladistic and molecular phylogenetic frameworks. Polyploidy may be beneficial especially in harsh environments and allow for habitat or niche shifts (Otto 2007). There may be thus higher incidence of duplications of genes, gene families, chromosomes, or the genome in taxa which coped with ecological stress during their history; higher genetic flexibility may accelerate and bias their molecular evolution. Intuitive support for a wider distribution of such collateral effects may come from observations that especially small, ecologically extreme or otherwise aberrant molluscan taxa, whatever their systematic rank or position, often seem to also cause problems in molecular analyses. Such putative correlated biases thus may refer to old and isolated lineages, but also to more recent and relatively diverse ones. Of course, potential examples and exceptions may be found for any scenario; here we just emphasize that unexpected connections between different types of data may exist.

Processes causing or indicating a higher risk of sequence bias include regressive evolution via heterochrony. Progenetic miniaturizations and morphological simplifications led to rampant morphological parallelism in opisthobranch sea slugs, which are a showcase group for multiple progenetic lineages adaptive to mesopsammic environments (e.g. Jörger et al. 2010, 2012, 2014). Among acochlidians, the most progenetic clade Microhedylacea has members with the most aberrant multilocus marker sequences. In rhodopemorphs, a long-branch taxon in our previous analyses, we provided evidence for extreme vermification in a mesopsammic habitat (Brenzinger, Haszprunar, et al. 2013). Progenesis has favoured rapid development in an unstable habitat and left pseudoarchaic features that obscure morphological phylogenetic signal (Martynov and Schrödl 2011; Martynov et al. 2011). We assume that progenesis is common in many molluscan lineages (Lindberg 1988).

Ontogenetic patterns have contributed to the clarification of aspects of molluscan phylogeny, e.g. recently by Scherholz et al. (2013). Loosely based on Haeckel's (1866) biogenetic law, observations of developmental sequences are usually expected to help distinguish between old and newly acquired features (e.g. Salvini-Plawen 2006). But this is not necessarily the case (i.e. heterochrony may obscure such sequences and even may have acted in different directions within a lineage). For example, heterobranch sea slugs, lineages with progressively paedomorphic (*Corambe*) and peramorphic (*Loy*) patterns occur (Martynov and Schrödl 2011; Martynov et al. 2011). Haeckel's law can be reversed by progressive progenesis simplifying ontogeny of derived members of a lineage to a pseudoarchaical condition (Martynov and Schrödl 2011; Martynov et al. 2011). If not considered, this phenomenon may have the power of inverting interpretations of ontogenetic data from ontogenetically and phylogenetically sparsely sampled and poorly known molluscs (see also Lindberg 1988; Lindberg and Ponder 1996).

How to proceed?

Our revisiting of the serialian topologies may appear as the revival of the confusion surrounding molluscan phylogeny. However, this confusion is focused on uncertain class relationships. Second, we suspect that early molluscan diversification was faster than previously thought, with an ancestrally complex body allowing for multiple, independent reductions, and molecular evolution hardly explored; early molluscan evolution should not be discussed reliably or tested without the serialian hypothesis in consideration. Third, we are convinced that future approaches must be integrative, with phylogenetic hypotheses tested against all other available lines of evidence.

Experience from current multilocus and phylogenomic approaches suggests that an accumulation of sequence data of both targeted gene approaches and broad EST sets is useful, aiming for a much denser and more representative molluscan taxon set; particularly aplacophorans, chitons, monoplacophorans and scaphopods but also some major gastropod lineages are undersampled at a phylogenomic scale to date. Especially promising for resolving deep molluscan and perhaps metazoan phylogeny are newly developed techniques of hybrid enrichment (e.g. Lemmon et al. 2012). Hundreds or thousands of conservative genomic loci can be generated for hundreds of samples, and new computational methods may help to select those most informative for deep nodes (López-Giráldez and Townsend 2011). Optimizing the quality (e.g. Kocot, Citarella, et al. 2013; Zapata et al. 2014) and signal of data and alignments (see e.g. Misof et al. 2014), and identification of 'outlier' species and markers (e.g. Leigh et al. 2011; de Vienne et al. 2012) and exclusion of artificial signal (e.g. Struck 2014) from densely sampled data sets will also be important for recovering meaningful trees from genomic data. Compartmentalized analyses of fast-evolving taxa and inclusion of more typical representatives into general taxon sets may be as promising as optimizing and analysing multiple data subsets under complex models. Novel approaches of testing phylogenetic hypotheses are also available (Church et al. 2014).

Clearly, representative whole-genomic data are needed. This is essential for both phylogenetic analysis and exploration of genome evolution. Analogous to mitogenomes there may be processes causing biases and influencing sequences beyond the capacities of available models for phylogenetic analyses. Once genomes are available for a broad range of taxa, rare genomic changes may provide at least local support for basal nodes (for a selection of possibilities see Simakov et al. 2012; Kocot 2013). Combined molecular timetrees and ancestral character state reconstructions may help to assign some more ambiguous fossils to certain clades.

Morphocladistic analyses are suitable for reconstructing the phylogeny within problematic subgroups if these are densely sampled and studied in detail (e.g. Schrödl and Neusser 2010; Martynov and Schrödl 2011), rather than expected to resolve the entire basal molluscan phylogeny. This is a half billion years' history of extinctions and multiple phenotypic transformations, of rareness and elusive members such as monoplacophorans, and of a lack of comparative microanatomical detail. Using software-based 3D reconstruction techniques from serial histological sections, morphologists are just beginning to explore representatives of molluscan classes in full microanatomical detail (e.g. Ruthensteiner et al. 2010; Brenzinger, Haszprunar, et al. 2013; Brenzinger, Padula, et al. 2013; Sigwart et al. 2014), and studying the ontogeny of major organ systems such as renopericardial complex across molluscan classes is especially rewarding (e.g. Baeumler et al. 2011, 2012). As demonstrated by

Scherholz et al. (2013), ontogenetic data can be highly useful for detecting character homologies, and will likely provide additional characters and help to understand their evolution. There is a whole arsenal of modern micromorphological techniques to be applied comparatively, and to be combined with gene expression studies (e.g. Jackson et al. 2010). Morphology in a broader sense will provide at least some support for basal nodes and is indispensable for integrating fossils.

Palaeontology will contribute by establishing global stratigraphies and rethinking fossil interpretations in the light of novel topologies, molecular time trees and reconstructed evolutionary scenarios. Also, there seem to be endless possibilities of finding more specimens, better data (e.g. Smith 2012) and further taxa (e.g. Sutton and Sigwart 2012; Sutton et al. 2012), which could be ancestors or completely new and enigmatic. In particular, fossils are the only real, though usually modified, phenotypic testimonies of the past and thus play a key role in evaluating the competing scenarios based on neontological data.

While some types of data sets may perform better in certain groups than others, none has an exclusive power to uncover evolutionary history, and thus integrative approaches are needed (Peterson et al. 2007). A hypothesis of deep molluscan phylogeny and evolution must stand the test of an integrative approach and consideration of all available evidence.

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New data from Monoplacophora and a carefully-curated dataset resolve molluscan relationships

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Relationships among the major lineages of Mollusca have long been debated. Morphological studies have considered the rarely collected Monoplacophora (Tryblidia) to have several plesiomorphic molluscan traits. The phylogenetic position of this group is contentious as morphologists have generally placed this clade as the sister taxon of the rest of Conchifera whereas earlier molecular studies supported a clade of Monoplacophora + Polyplacophora (Serialia) and phylogenomic studies have generally recovered a clade of Monoplacophora + Cephalopoda. Phylogenomic studies have also strongly supported a clade including Gastropoda, Bivalvia, and Scaphopoda, but relationships among these taxa have been inconsistent. In order to resolve conchiferan relationships and improve understanding of early molluscan evolution, we carefully curated a high-quality data matrix and conducted phylogenomic analyses with broad taxon sampling including newly sequenced genomic data from the monoplacophoran *Laevipilina antarctica*. Whereas a partitioned maximum likelihood (ML) analysis using site-homogeneous models recovered Monoplacophora sister to Cephalopoda with moderate support, both ML and Bayesian inference (BI) analyses using mixture models recovered Monoplacophora sister to all other conchiferans with strong support. A supertree approach also recovered Monoplacophora as the sister taxon of a clade composed of the rest of Conchifera. Gastropoda was recovered as the sister taxon of Scaphopoda in most analyses, which was strongly supported when mixture models were used. A molecular clock based on our BI topology dates diversification of Mollusca to ~546 MYA (+/− 6 MYA) and Conchifera to ~540 MYA (+/− 9 MYA), generally consistent with previous work employing nuclear housekeeping genes. These results provide important resolution of conchiferan mollusc phylogeny and offer new insights into ancestral character states of major mollusc clades.

Mollusca is the second most diverse animal phylum whose members exhibit an incredible array of body shapes and sizes. Many molluscs have important ecological roles in marine, freshwater, and terrestrial environments and others are culturally and/or economically important as a source of food, jewellery, or dye[1]. Despite their diversity and importance, understanding of early molluscan evolution remains incomplete and several conflicting phylogenetic hypotheses[1–9] have been proposed regarding relationships among the eight major clades (i.e., classes): Bivalvia (clams, scallops, oysters, etc.), Caudofoveata (Chaetodermomorpha), Cephalopoda (octopuses, squids, and *Nautilus*), Gastropoda (snails and slugs), Monoplacophora (Tryblidia; deep-sea, limpet-like molluscs), Polyplacophora (chitons), Scaphopoda (tusk shells), and Solenogastres (Neomeniomorpha).

Within Conchifera (Bivalvia, Cephalopoda, Gastropoda, Monoplacophora, and Scaphopoda), the clade of molluscs with uni- or bivalved shells, the deep-sea limpet-like Monoplacophora has long been thought to be important to understanding early molluscan evolution[5,10–14] with most morphology-based hypotheses placing Monoplacophora sister to a clade of all other conchiferans. However, no published molecular studies have

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supported this topology to date (but see Philippe and Roure 2012[15]). Studies of molluscan phylogeny employing datasets dominated by nuclear ribosomal and mitochondrial genes have generally had poor resolution among major lineages[10–14]. However, one finding of particular interest from these studies was the recovery of a close relationship of Monoplacophora and Polyplacophora (Serialia)[16,17]. More recent studies employing PCR-amplified fragments of nuclear protein-coding "housekeeping" genes[18] or nuclear protein-coding genes obtained from transcriptome and genome data[19,20] have instead provided strong support for a clade called Aculifera, which groups Polyplacophora with Aplacophora (Caudofoveata + Solenogastres) to form a group of molluscs with calcareous sclerites.

Smith *et al.*[19], the only published phylogenomic study to date focused on deep molluscan relationships to sample Monoplacophora (specifically *Laevipilina hyalina*), recovered it as the sister taxon of Cephalopoda. This result is inconsistent with the prevailing traditional morphological view placing Monoplacophora sister to all other conchiferans[3,21–23], but is consistent with some (but not all) palaeontological hypotheses on early molluscan diversification[24–27]. Two subsequent studies included data from *L. hyalina* but focused on relationships within Gastropoda[28] or Bivalvia[29], and thus had limited taxon sampling outside of those clades. Kocot *et al.*[30] focused on among-phylum relationships within Lophotrochozoa but had relatively broad sampling of Mollusca. Most of those analyses recovered Monoplacophora as the sister taxon of Conchifera or Cephalopoda, but support for its placement was generally weak. Phylogenomic studies have also supported a clade including Gastropoda, Bivalvia, and Scaphopoda, although there has been inconsistency in recovered relationships among these taxa[19,20,28,30]. Because conchiferan molluscs are well-represented in the early animal fossil record[31,32], understanding their phylogeny has important implications for understanding early animal evolution and the identity of enigmatic fossil taxa hypothesized to be stem-group molluscs.

Results and Discussion

We sequenced a draft genome for the monoplacophoran *Laevipilina antarctica*. Unfortunately, because of the small size of this species, there was only adequate material for paired-end Illumina sequencing library preparation with insufficient material for mate pair, long-read, or transcriptome library preparation using techniques available at the time that this work was conducted. This resulted in a rather fragmented genome assembly (427,488 contigs >500 bp; N50 = 2,167 bp; 1.26 Gbp total assembly size). Assessment of this assembly with BUSCO[33] showed that it is rather incomplete with only 14.6% of the 978 metazoa_odb9 genes recovered as complete and another 17.9% recovered as fragmented. Nevertheless, aside from transcriptome data from *Laevipilina hyalina*, these represent the only available genome data from any monoplacophoran and are thus a valuable resource for testing the phylogenetic position of this group.

We curated a dataset of 257 genes totalling 54,596 amino acids in length with data from 49 taxa of which 32 represented ingroup species (Supplementary Table 1). Care was taken to exclude possible contamination and mistranslated sequence regions (see Methods) while minimizing the amount of missing data in the final matrix (27.86% missing data). Additionally, only genes with a sequence from *L. antarctica* were sampled. Phylogenetic analyses were conducted using maximum likelihood (ML) in RAxML 8[34] with the best-fitting model for each gene, and in IQ-TREE using the posterior mean site frequency (LG + C60 + G + F) mixture model[35–37]. A Bayesian inference (BI) analysis was conducted in PhyloBayes MPI[38] with the CAT-GTR mixture model[39].

ML analysis of the partitioned dataset in RAxML (Fig. 1A) recovered Monoplacophora sister to Cephalopoda with moderate bootstrap support (bs = 88), consistent with the results of Smith *et al.*[19] and some interpretations of the fossil record[11]. However, the ML analysis in IQ-TREE using the PMSF model (Fig. 1B) and the Bayesian inference analysis in PhyloBayes using the CAT-GTR model (Fig. 1C) recovered Monoplacophora sister to the rest of Conchifera with a bootstrap support value of 94 and posterior probability of 0.99 respectively, consistent with most morphology-based hypotheses of conchiferan relationships[11].

To examine support for Monoplacophora sister to Conchifera from individual partitions, we used a multi-species coalescent approach in ASTRAL 5.6.1[40]. This analysis also recovered Monoplacophora sister to the rest of Conchifera (local posterior probability, lpp = 0.89; Fig. 1D).

Placement of Monoplacophora sister to all other conchiferans had a lower likelihood score than Monoplacophora + Cephalopoda in the RAxML analysis and could not be rejected by the Shimodaira-Hasegawa (SH) test ($p = 0.190$). This alternative topology was, however, rejected by the Approximately Unbiased (AU) test ($p = 0.001$). Both tests rejected the Serialia hypothesis (AU test $p = 0.001$; SH test $p = 0$).

A clade of all conchiferans except Monoplacophora, as recovered in most of our analyses, was originally proposed by morphologists and called Ganglionata (reviewed by Schrödl and Stöger 2014[5]). Despite the name, ganglia are neither restricted to Ganglionata nor do all species within Ganglionata show distinct pairs of ganglia[41–43]. Kocot *et al.*[20] curated a morphological character matrix for Mollusca building on that of Haszprunar[21] and conducted ancestral state reconstruction for key molluscan characters (see Methods) under a number of different phylogenetic scenarios including Monoplacophora sister to the rest of Conchifera. Our analyses placing Monoplacophora sister to the rest of Conchifera indicate that the only unambiguously apomorphic trait of Ganglionata is the reduction of adult dorsoventral muscle pairs from a hypothesized ancestral set of eight (or possibly seven[44]). Monoplacophorans also differ from other conchiferans with respect to the arrangement and structure of mantle folds, anatomy of the shell gland, and structure of the shell[23], but whether these are retained conchiferan plesiomorphies or monoplacophoran apomorphies is ambiguous.

Relationships among Gastropoda, Bivalvia, and Scaphopoda, a clade of molluscs with relatively thick, multi-layered shells[27], have been the subject of debate[3,5,7,8,31] due to incongruence among recent studies[18–20,45,46]. Whereas our RAxML and ASTRAL analyses found poor support for relationships among these taxa, our IQ-TREE and PhyloBayes analyses using mixture models strongly supported Scaphopoda + Gastropoda with this clade sister to Bivalvia, consistent with Smith *et al.*[19]. Gastropoda is an extremely diverse, morphologically disparate, and ecologically variable group of species that inhabit almost all environments on land and in the sea.

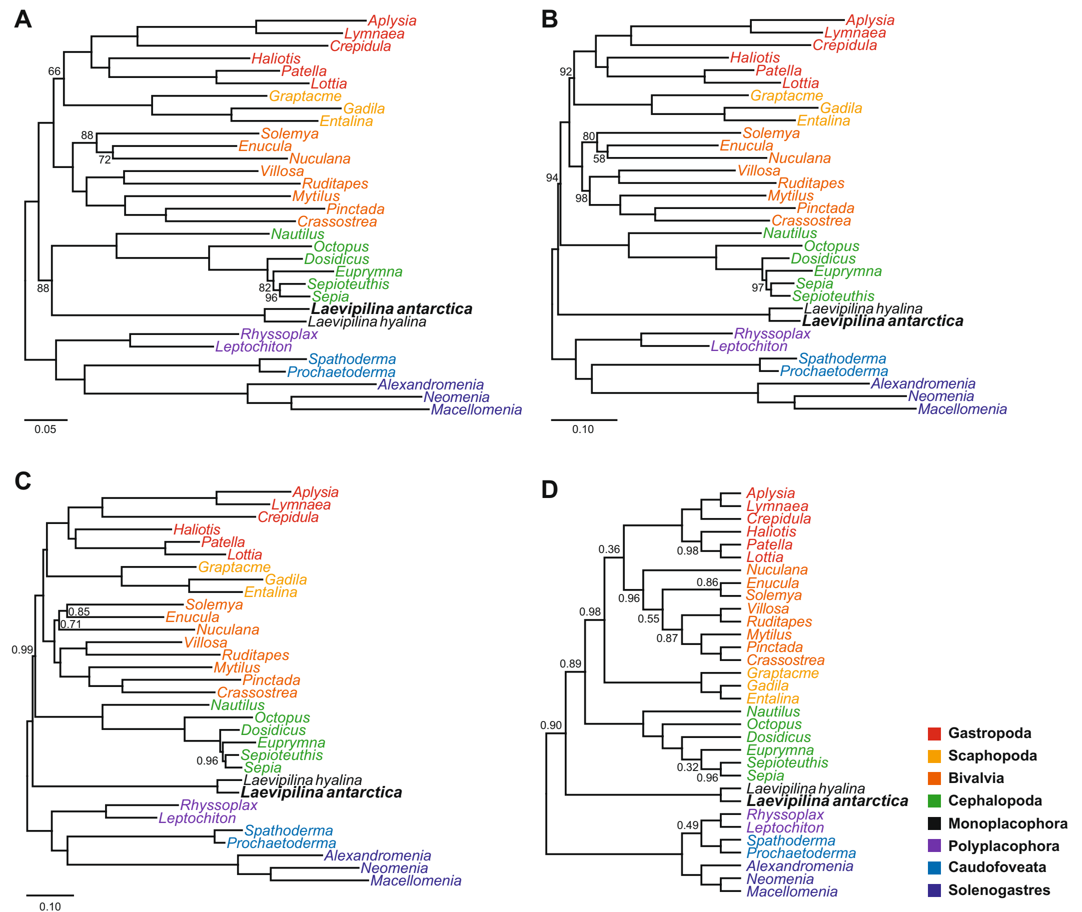

Figure 1. Results of phylogenetic analyses (outgroup taxa not shown). (**A**) RAxML maximum likelihood (ML) topology. Bootstrap support values below 100 shown. (**B**) IQ-TREE ML topology. Bootstrap support values below 100 shown. (**C**) PhyloBayes Bayesian inference (BI) topology. Posterior probabilities below 1.0 shown. (**D**) ASTRAL tree. Local posterior probabilities below 1.0 shown.

Scaphopoda, on the other hand, is a much less diverse group of relatively morphologically uniform animals that dig in marine sediments and prey upon foraminiferans and other infauna. This pair of unequal sister taxa contradicts the Cyrtosoma concept uniting Gastropoda and Cephalopoda (plus Monoplacophora by the original definition[10]; reviewed by Kocot[22]). Interestingly, a close relationship of Scaphopoda and Gastropoda was proposed based on the pronounced dorsoventral axis[47] and recent work has confirmed the morphological ventral position of the scaphopod foot[48]. Examination of published molluscan morphological data matrices[20,21,49] reveals obvious symplesiomorphies shared between these taxa (e.g., external univalved shell), but we find no clear morphological synapomorphies for the gastropod-scaphopod clade.

Consistent with other phylogenomic studies[18–20,50], all of our analyses strongly support a molluscan dichotomy with two major clades: Conchifera and Aculifera[51]. Within Aculifera, we recovered chitons (Polyplacophora) sister to the vermiform, shell-less aplacophorans. Within Aplacophora, we recovered Solenogastres and Caudofoveata reciprocally monophyletic. Aculifera contradicts the classical morphology-based Testaria hypothesis[5], which places chitons sister to Conchifera and the shell-less worm-like aplacophorans as an early-branching, paraphyletic grade. The Testaria hypothesis implies a progressive evolution from a simple unshelled worm-like ancestor towards chitons with shell plates and later with the uni- or bivalved conchiferans as the crown-group of Mollusca. Our results unequivocally reject this hypothesis (AU test p-value = 4.00E-56; SH test p-value = 0).

In light of support for placement of Monoplacophora sister to the rest of Conchifera and our earlier ancestral character state reconstruction analyses based on this phylogenetic hypothesis[20], we infer that the last common ancestor of extant molluscs was likely a dorsoventrally flattened animal that had a mantle, a dorsal cuticle, a broad foot, eight (or seven[44]) pairs of dorsoventral muscles, a circumpedal or posterior mantle cavity with serially arranged gills, and a radula as part of a longitudinally arranged, regionalized digestive system. Whether or not the last common ancestor of extant molluscs had a single shell, multiple shell plates, or no shell is ambiguous[20]. Possession of a single shell is clearly plesiomorphic for Conchifera but this was probably also the case in *Calvapilosa*, *Maikhanella*, and *Orthrozanclus*, fossil taxa inferred to be stem aculiferans[52], suggesting that the last common molluscan ancestor may have been single-shelled. Additional studies comparing development, mineralogy, and other structural aspects of chiton shells, conchiferan shells, and aculiferan sclerites would be of great

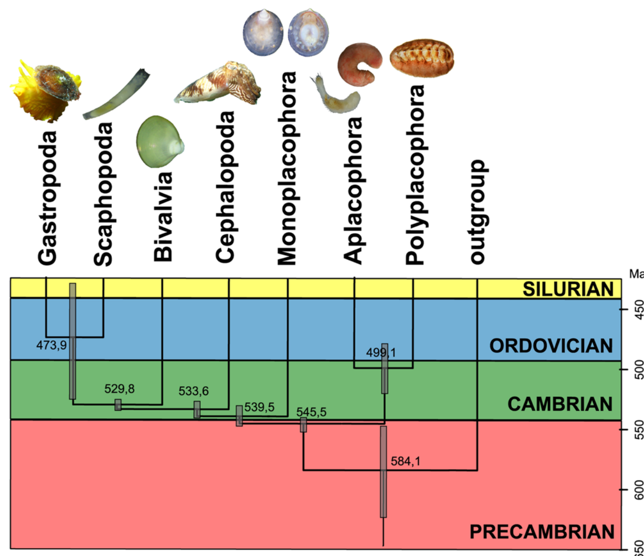

Figure 2. Summary of relaxed molecular clock analysis results. Numbers along y-axis are millions of years before present (Ma). Numbers at nodes represent the average age of the split; Error bars at nodes represent the height 95% HPD (highest posterior density). A detailed version of this tree is presented in Supplementary Fig. 1 and the raw data and uncollapsed tree are available via FigShare (see Data Availability section).

interest to further address this and other important questions about the origin(s) and homology of molluscan biomineralized structures[53].

Our molecular clock analysis (Fig. 2; Supplementary Fig. 1; Supplementary Table 3) indicates that the molluscan stem split from trochozoan relatives about 584 MYA (95% highest posterior density [HPD] = 547–623 MYA), Conchifera diversified 540 MYA (531–548 MYA), and Aculifera diversified 499 MYA (479–520 MYA), generally consistent with previous relaxed molecular clocks calculated from multilocus[18,45,52,54] and phylogenomic data[28,55], showing the molluscan stem to be Precambrian in origin. The Ediacaran fossil genus *Kimberella* has been hypothesized to represent a stem-group mollusc by some[31,56–58] but the molluscan affinity of *Kimberella* has been criticized by others who instead view it as an early-branching bilaterian[32], in part because of its old age (~555 MYA). Although broad, our and other recent estimates for the divergence of molluscs are at least compatible with hypotheses regarding *Kimberella* as an early offshoot of the molluscan stemline[31,45,59]. However, if *Kimberella* was indeed a mollusc, it differed from most extant molluscs in its lack of a shell (although sclerites may have been present) and, more significantly, a bizarre rake-like mode of feeding unlike that of any modern mollusc[32].

Late Precambrian and Cambrian small shelly fossil (SSF) assemblages consist of abundant, diverse, and tiny (0.5–5 mm) animals[60] in strong contrast to the large-bodied Vendian *Kimberella*. Our time tree is consistent with the prevailing notion that SSFs such as helcionellids and other gastropod- and monoplacophoran-like fossils were conchiferan molluscs[32], but relatively broad posterior densities preclude confident placement of these fossil taxa along any one branch. According to our time tree, molluscan SSFs would have been stem conchiferans, or less likely, belonged to the stem of Monoplacophora or the lineage that gave rise to the remaining conchiferans. As noted above, at least some fossil aculiferans had a single shell; at least some SSFs could conceivably have been aculiferans. Surprisingly, the split of gastropods and scaphopods is rather late according to our molecular clock analysis (474 MYA; 95% HDP = 479–520 MYA); this could mean that many Cambrian shells currently regarded to be gastropods were actually members of the gastropod-scaphopod stem lineage.

In conclusion, we analysed a high-quality and representative molluscan phylogenomic dataset and recovered a robust and intriguing hypothesis on molluscan class-level relationships. Analyses employing site-heterogeneous models and a coalescent approach provide support for a dichotomy dividing the molluscs into Aculifera and Conchifera, the latter with Monoplacophora sister to the rest of uni- or bivalved molluscs and gastropods sister to scaphopods, not bivalves. Our results contradict hypotheses such as Testaria, Serialia, and Monoplacophora + Cephalopoda, and have important consequences for reconstructing early molluscan evolution.

Methods

Molecular laboratory work. One specimen of *Laevipilina antarctica* (ZSM-Mol-20090330, DNABANK-Mol-MS-016) was collected with the *R/V Polarstern* in Antarctica between 70°24.00′S, 8°19.72′W and 70°23.86′S, 8°18.68′W at 597–602 m depth on 12 January 2008. DNA was extracted from the specimen using the NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany). DNA (10 ng) was used for whole genome amplification using the Illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare Life Sciences, Freiburg, Germany) followed by standard ethanol precipitation and re-purification using the Qiagen MinElute system (Qiagen, Hilden, Germany). Concentration was determined using a Qubit 2.0 Fluorometer, and 1 µg was used to create a sequencing library with the TruSeq DNA Sample Preparation Kit v2 (Illumina, San Diego, CA, USA) with an average insert size of

approximately 250 bp. Two lanes of 101 bp paired-end-reads were sequenced on the Illumina HiSeq 2000 system yielding about 90 Gbp. Raw reads were filtered for quality, PCR duplicates, and adapter sequences and corrected using SOAPfilter_v2.0 (https://github.com/tanghaibao/jcvi-bin/blob/master/SOAP/SOAPfilter_v2.0) using default settings.

Genome assembly and annotation. Reads retained by SOAPfilter_v2.0 were assembled *de novo* using SOAPdenovo2_v2.04[61]. Sparse_pregraph was used to construct the K-mer graph using the following settings: -K 31 -g 15 -z 2000000000 -d 1 -e 1 -r 0 -p 28. Contigs were computed using kmer iterations up to K = 63 (-M 3 -m 63 -p 30). The remapping step of SOAPdenovo was carried out using standard settings and the scaffolding step was used with parameters: -F -G 200 -p 28. Finally, additional gaps were filled using SOAP Gapcloser v1.12. Genescan[62] was used to generate gene predictions resulting in 83 Mb of protein-coding sequences, which were subsequently used for phylogenomic analyses.

Taxon sampling and data preparation for phylogenomic analysis. Taxon sampling (Supplementary Table 1) was selected to broadly span the diversity of Mollusca including at least two representatives of each major lineage and at least two representatives of each phylum considered a candidate for the sister taxon of Mollusca[63]. Publicly available protein sequences from complete genomes and assembled transcriptomes were downloaded when available. Dataset assembly and processing built on our established and routinely used bioinformatic pipeline[30,64–67] with a number of modifications to help reduce possible exogenous contamination and low quality data (e.g., incorrectly translated gene predictions from Genescan; see below). Unassembled publicly available transcriptome data were digitally normalized and assembled using Trinity[68]. Transcriptome assemblies were translated with TransDecoder (https://sourceforge.net/p/transdecoder/), keeping only amino acid (AA) sequences longer than 100 AAs.

Orthology inference. For orthology inference, we employed HaMStR 13[69], which infers orthology based on predefined sets of orthologous groups (OGs). We employed the Trochozoa custom core-ortholog set of Kocot *et al.*[30]. Translated transcripts for all taxa were then searched against the 2,259 Trochozoa pHMMs. Sequences matching an OG's pHMM were then compared to the proteome of *Lottia gigantea* using BLASTP[70] with the -strict option. If the *Lottia* amino acid sequence contributing to the pHMM was the best BLASTP hit in each of these back-BLASTs, the sequence was then assigned to that OG.

Dataset processing. Sequences shorter than 100 amino acids were deleted and OGs sampled for fewer than 35 taxa were discarded. Redundant identical sequences were removed with UniqHaplo (http://raven.iab.alaska.edu/~ntakebay/). In cases where one of the first or last 20 characters of an amino acid sequence was an X, all characters between the X and that end of the sequence were deleted and treated as missing data. Each OG was then aligned with MAFFT[71] (mafft–auto–localpair–maxiterate 1000). Alignments were then trimmed with Aliscore[72] and Alicut[73] to remove ambiguously aligned regions. Next, a consensus sequence was inferred for each alignment using the EMBOSS program infoalign[74]. For each sequence in each single-gene amino acid alignment, the percentage of positions of that sequence that differed from the consensus of the alignment were calculated using the infoalign's "change" calculation. Any sequence with a "change" value greater than 75 was deleted. Subsequently, a custom script (AlignmentCompare; https://github.com/kmkocot/basal_metazoan_phylogenomics_scripts_01-2015) was used to delete any likely mistranslated sequence regions of 20 or fewer amino acids in length surrounded by ten or more gaps on either side. Next, alignment columns with fewer than four non-gap characters were deleted. At this point, alignments shorter than 50 amino acids in length were discarded. Lastly, sequences that did not overlap with all other sequences in the alignment by at least 20 amino acids were deleted, starting with the shortest sequences not meeting this criterion.

In some cases, a taxon was represented in an OG by two or more sequences (splice variants, lineage-specific gene duplications [=inparalogs], overlooked paralogs, or exogenous contamination). In order to select the best sequence for each taxon and exclude any paralogs or exogenous contamination, we built trees in FastTree 2[75] and used PhyloTreePruner[76] to select the best sequence for each taxon. OGs sampled for fewer than 35 taxa and OGs lacking a sequence from *Laevipilina antarctica* were discarded. The remaining alignments were manually screened to identify and remove putative contamination or mistranslated sequences. Sequences that were obviously very different from the majority of the sequences in the alignment were blasted against NCBI NR using BLASTP and sequences that did not return an animal as the top hit were discarded. Finally, remaining OGs were then concatenated using FASconCAT[77].

Phylogenetic analyses. Maximum likelihood analyses were conducted in RAxML 8.2.4[34] and IQ-TREE 1.5.5[35]. For the RAxML analysis, matrices were partitioned by gene with the PROTGAMMAAUTO model (the best-fitting model for each gene) used for all partitions. The tree with the best likelihood score after 10 random addition sequence replicates was retained and topological robustness (i.e., nodal support) was assessed with 100 replicates of fast bootstrapping (the -f a command line option was used). For the IQ-TREE analysis, we used the posterior mean site frequency (PMSF) model[37], which is an approximation to full empirical profile mixture models for ML analysis. Specifically, the LG + C60 + G + F model was specified. Because this approach requires a guide tree to infer the site frequency model, we used the previously generated RAxML tree. Nodal support was assessed with 1000 replicates of ultrafast bootstrapping (-bb 1000). Bayesian Inference analysis was conducted with PhyloBayes 4.1b[78] using the site-heterogeneous CAT-GTR model. Two chains were run for 14,143 and 13,400 generations, respectively with the first 2,000 trees from each chain discarded as burn-in. A bpcomp maxdiff value of 0.28 indicated that the chains had converged.

To examine support for key hypotheses from individual partitions, we made trees for each gene in RAxML using the best-fitting model, used these as guide trees for IQ-TREE analyses with the LG + C20 + G + F model,

and inferred a supertree using a multi-species coalescent model in ASTRAL 5.6.1[40]. Weakly-supported nodes (bs < 50) were collapsed as advocated by Zhang *et al*.[40]. Hypothesis testing using the Approximately Unbiased test[79] and the Shimodaira Hasegawa test[80] was conducted using RAxML 8.2.4[34] and CONSEL[81] based on the RAxML analysis.

Divergence time estimates (Supplementary Table 3) were obtained in BEAST2 v.2.4.6[82] on the CIPRES Science Gateway (https://www.phylo.org/) with a log-normal relaxed clock and the WAG model of substitution. The topology of the tree was manually constrained *a priori* by defining the major splits of the BI tree analysed herein. Fossil calibrations[83–89] are presented in Supplementary Table 4. The analysis was executed for 180 million generations sampling a tree every 1,000 generations. After discarding the first 3,600 trees as burn-in, 14,401 trees were analysed with TreeAnnotator 2.4.5 to build the summary tree.

Ancestral character state reconstruction. Ancestral character state reconstruction was performed previously by Kocot *et al*.[20] using an updated and modified version of the morphological matrix of Haszprunar[21]. Because this analysis was already performed in light of numerous alternative hypotheses of molluscan class-level phylogeny including Monoplacophora sister to the remainder of Conchifera, it was not re-done here. The data matrix analysed is available via FigShare at https://figshare.com/s/934e61a053aacd8d37c1.

Data availability

Illumina paired-end genomic data for *L. antarctica* were submitted to NCBI SRA under accession number SRR6506080. The assembled *L. antarctica* genome, assembly statistics, Genescan output, molecular and morphological data matrices analysed, and other data files associated with results presented herein were submitted to FigShare: https://figshare.com/s/934e61a053aacd8d37c1. Sources of publicly available datasets used herein are presented in Supplementary Table 1.

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Author contributions
M.S. and A.J.P. conceived the project. M.S. collected the specimen of *L. antarctica*. A.J.P. prepared sequencing libraries, assembled and annotated the genome, and conducted other bioinformatic analyses. K.M.K. conducted dataset preparation for phylogenomic analysis, orthology inference, dataset processing, and most phylogenetic analyses. I.S. conducted molecular clock analyses. K.M.K., M.S., K.M.H., I.S. and A.J.P. wrote the manuscript and approved the final version.

Competing interests
The authors declare no competing interests.

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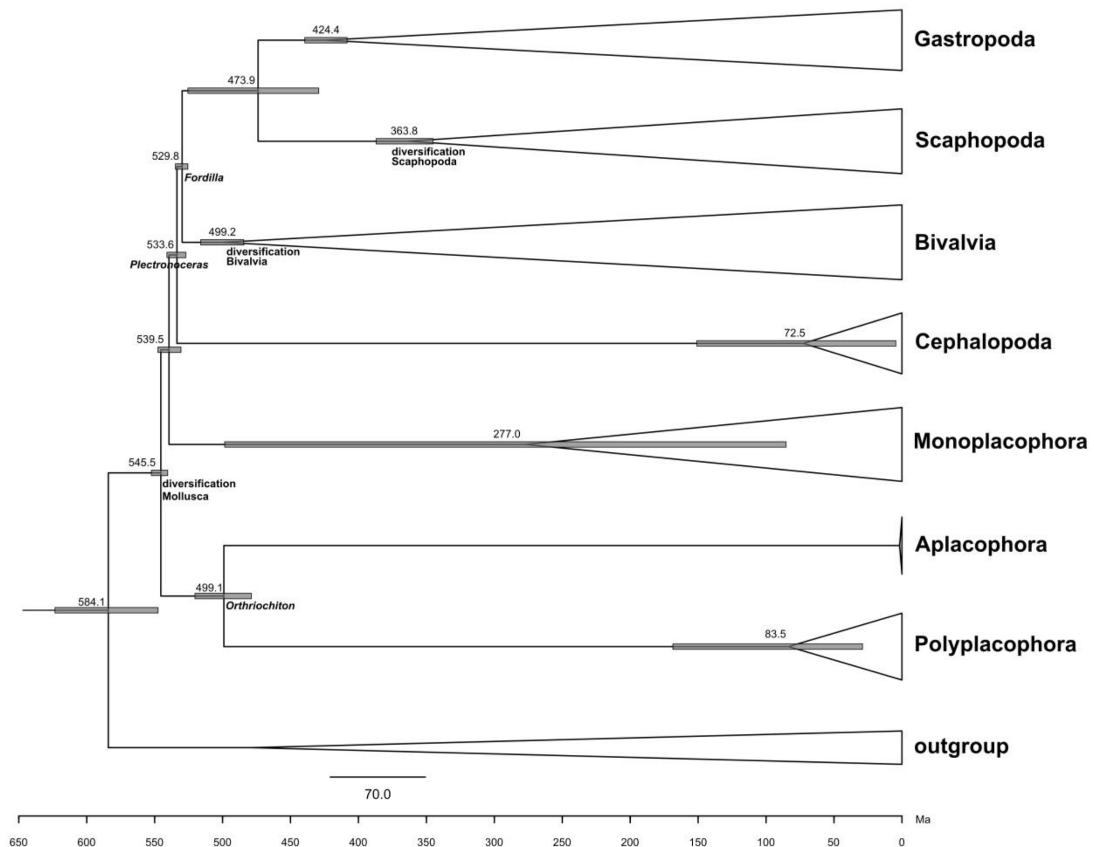
Supplementary Information

Supplemental Information includes one figure and four tables and can be found with this article online.



Supplementary Figure 1. Detailed presentation of results of relaxed molecular clock analysis (Figure 2). Numbers along x-axis are millions of years before present (Ma). Numbers at nodes represent the average age of the split; Error bars at nodes represent the 95% HPD (highest posterior density). The raw data and uncollapsed tree are available via FigShare (see Data Availability section).

Tables

Supplementary Table 1. Taxon sampling.

Taxon	Species	Abbrev.	Type	Reads	HaMStR genes	Source	Accession Number(s) / Version / URL / Citation
Solenogastres	*Alexandromenia crassa*	ACRA	Illumina	45,059,456	2,084	NCBI SRA	SRR2052564
	Macellomenia schanderi	MSCH	Illumina	49,904,154	2,130	NCBI SRA	SRR2057023
	Neomenia carinata	NCAR	Illumina	36,612,396	1,547	NCBI SRA	SRR2057026
Caudofoveata	*Prochaetoderma californicum*	PCAL	Illumina	90,561,352	2,116	NCBI SRA	SRR6926326
	Spathoderma clenchi	SCLE	Illumina	52,062,402	1,172	NCBI SRA	SRR8258011
Polyplacophora	*Leptochiton rugatus*	LRUG	Illumina	49,670,054	2,096	NCBI SRA	SRR1611558
	Rhyssoplax olivaceus	ROLI	Illumina	23,189,291	2,149	NCBI SRA	SRR618506
Gastropoda	*Aplysia californica*	ACAL	Sanger	216,556	931	NCBI UniGene	January 27, 2010 Version
	Crepidula fornicata	CFOR	Illumina	39,362,017	1,883	NCBI SRA	SRR1324873-SRR1324880
	Haliotis rufescens	HRUF	Illumina	355,678,562	2,177	Dryad	http://datadryad.org/resource/ doi:10.5061/dryad.85p80
	Lottia gigantea	LGIG	Genome	-	2,259	JGI	JGI filtered models v. 1.0
	Lymnaea stagnalis	LSTA	Illumina	81,851,004	936	NCBI SRA	DRR002012
	Patella vulgata	PVUL	Illumina	47,237,104	1,931	From authors	https://doi.org/10.1007/s10126-

							012-9481-0
Bivalvia	*Crassostrea gigas*	CGIG	Genome	-	2,199	V9 protein models	http://gigadb.org/Pacific_oyster/
	Enucula tenius	ETEN	Illumina	77,448,350	1,760	Dryad – exemplars/isotigs only	dryad.34644
	Mytilus edulis	MEDU	Illumina	72,220,824	2,238	NCBI SRA	SRX565221-SRX565224
	Nuculana pernula	NPER	Illumina	35,983,152	591	NCBI SRA	SRR2057025
	Pinctada fucata	PFUC	Genome	-	2,073	From authors	http://marinegenomics.oist.jp/ genomes/download?project_id=20
	Ruditapes philippinarum	RPHI	Illumina	41,031,443	1,911	NCBI Nucleotide Database	JO101212-JO124029
	Solemya velum	SVEL	Illumina	66,597,054	2,068	Dryad – exemplars/isotigs only	dryad.34644
	Villosa lienosa	VLIE	Illumina	162,000,000	2,118	NCBI BioProject	PRJNA75063, ID #75063
Scaphopoda	*Entalina tetragona*	ETET	Illumina	39,609,424	1,575	NCBI SRA	SRR2057018
	Gadila tolmiei	GTOL	Illumina	75,942,132	1,815	Dryad – exemplars/isotigs only	dryad.34644
	Graptacme eborea	GEBO	Illumina	61,523,742	2,156	NCBI SRA	SRR2057020
Monoplacophora	*Laevipilina antarctica*	LANT	Genome	-	2,233	NCBI SRA	SRR6506080
	Laevipilina hyalina	LHYA	454, Sanger	75,485	430	Dryad – transcripts	dryad.34644
Cephalopoda	*Dosidicus gigas*	DGIG	Illumina	37,094,323	1,663	NCBI SRA	SRR1386212
	Euprymna scolopes	ESCO	Sanger	35,420	1,179	NCBI Trace Archive	DW251302-DW286722
	Nautilus pompilius	NPOM	454	549,720	406	NCBI SRA	SRR108979

			454	112,375	464	Dryad – exemplars/isotigs only	dryad.34644
	Octopus vulgaris	OVUL	Illumina	16,501,336	1,742	Dryad – exemplars/isotigs only	dryad.34644
	Sepia esculenta	SESC	Illumina	80,947,907	1,705	NCBI SRA	SRR1386223
	Sepioteuthis lessoniana	SLES	Illumina	67,170,966	1,482	NCBI SRA	SRR1386192
Annelida	*Boccardia proboscidea*	BPRO	Illumina	63,634,426	1,722	NCBI SRA	SRR2057014
	Capitella teleta	CTEL	Genome	-	2,215	JGI	JGI v1.0
	Clymenella torquata	CTOR	Illumina	85,285,816	2,129	NCBI SRA	SRR2057016
	Glycera dibranchiata	GDIB	Illumina	82,775,880	1,299	NCBI SRA	SRR2057019
	Helobdella robusta	HROB	Genome	-	2,049	JGI	JGI filtered models v. 3
	Pectinaria gouldii	PGOU	Illumina	145,853,782	1,506	NCBI SRA	SRR2057036
	Phascolosoma agassizii	PAGA	Illumina	63,918,870	1,876	Dryad – transcripts	dryad.30k4v
Brachiopoda	*Hemithiris psittacea*	HPSI	Illumina	60,731,022	2,224	NCBI SRA	SRR1611556
	Glottidia pyramidata	GPYR	Illumina	67,613,510	2,101	NCBI SRA	SRR1611555
	Laqueus californicus	LCAL	Illumina	67,414,776	2,113	NCBI SRA	SRR1611557
	Novocrania anomala	NANO	Illumina	52,243,928	1,531	NCBI SRA	SRR1611564
Phoronida	*Phoronis psammophila*	PPSA	Illumina	58,372,182	2,223	NCBI SRA	SRR1611565
	Phoronis vancouverensis	PVAN	Illumina	69,531,036	2,211	NCBI SRA	SRR1611566
Entoprocta	*Barentsia gracilis*	BGRA	Illumina	67,947,336	1,283	NCBI SRA	SRR1611554

	Loxosoma pectinaricola	LPEC	Illumina	75,025,552	1,675	NCBI SRA	SRR1611559
Nemertea	*Malacobdella grossa*	MGRO	Illumina	30,538,858	1,878	NCBI SRA	SRR1611560
	Paranemertes peregrina	PPER	Illumina	59,441,992	1,986	NCBI SRA	SRR1611562

Supplementary Table 2. Hypothesis test results.

Constraint	**Log-likelihood**	**AU test (p-value)**	**SH test (p-value)**
Unconstrained	-1,481,197.46		
Monoplacophora sister to all other conchiferans	-1,481,202.46	0.001	0.190
Gastropoda + Bivalvia	-1,481,209.21	3.00E-78	3.00E-04
Diasoma	-1,481,227.52	0.198	0.814
Patellogastropoda sister to clade of all other gastropods	-1,481,298.44	0.210	0.744
Cyrtosoma (as Gastropoda + Cephalopoda)	-1,481,535.61	0.448	0.827
Serialia	-1,481,747.97	0.001	0.000
Testaria	-1,482,088.01	4.00E-56	0.000

Supplementary Table 3. BEAST2 molecular clock results. HPD = highest posterior density.

Node	Divergence time (Ma)	95% HPD (Ma)
Mollusca	545.449	540.386 - 552.405
Aculifera	499.060	478.787 - 520.313
Aplacophora	1.677	1.527 - 1.912
Solenogastres	0.777	0.703 - 0.887
Caudofoveata (Prochaetodermatidae)	0.127	0.070 - 0.183
Polyplacophora	83.455	29.001 - 168.611
Conchifera	539.449	530.604 - 547.625
Monoplacophora (*Laevipilina*)	276.956	85.269 - 498.414
Ganglionata	533.595	527.025 - 540.918
Cephalopoda	72.505	4.478 - 150.959
Bivalvia + Gastropoda + Scaphopoda	529.765	525.529 - 534.738
Bivalvia	499.182	484.407 - 516.073
Gastropoda + Scaphopoda	473.888	429.253 - 525.494
Scaphopoda	363.764	345.457 - 386.955
Gastropoda	424.426	408.354 - 439.456
Mollusc stem/Trochozoa	584.137	547.438 - 623.354

Supplementary Table 4. Constraints used in molecular clock analysis.

Calibration node	Fossil calibration	Date range (Ma)	Reference	Prior settings in BEAST v.2.4.6 (distribution; gamma shape, gamma scale, zero offset)
Diversification of Mollusca	first shell record	~ 545	[63]	Gamma; 2.5, 2.0, 540.0
Split of Bivalvia/Scaphopoda/Gastropoda	*Fordilla*	~ 530	[63]	Gamma; 3.3, 2.2, 525.0
Split of Cephalopoda/Bivalvia/Scaphopoda/ Gastropoda	*Plectronoceras*	~ 505	[86]	Gamma; 2.4, 7.0, 495.0
Split of Polyplacophora/ Aplacophora	*Orthriochiton*	~ 490	[87]	Gamma; 5.0, 5.0, 470.0
Diversification of Bivalvia		~ 490	[48]	Gamma; 5.0, 5.0, 470.0
Origin of Caenogastropoda	Sublitoidea	~ 418	[88]	Gamma; 2.3, 9.0, 405.0
Diversification of Scaphopoda	*Dentalium*	~ 353	[89]	Gamma; 2.2, 6.7, 345.0

4. Discussion

4.1. Phylogenetic markers

The publications presented in this thesis give a comprehensive overview of the molluscan phylogeny in the light of different datasets. We were able to contribute novel sequences for numerous taxa and tried hard to fill taxonomic gaps. In particular, we sequenced 'standard' markers, combining partial mitogenomic genes (COI, also known as the barcoding fragment, and 16s) and three fragments of the nuclear genome (18s, 28s, H3) of several monoplacophoran species (Stöger et al. 2013). The combination of these genes was chosen to resolve deep nodes, e.g. the splits of molluscan classes (see Giribet et al. 2006, Wilson et al. 2010), as well as more recent relationships on family-level (see Okusu et al. 2003, Klussmann-Kolb et al. 2008). We compiled a comprehensive set of molluscan species (Stöger et al. 2013) as well as of Heterobranchia (Gastropoda) (Jörger et al. 2010) and chitons (Polyplacophora) (Sigwart et al. 2013). Moreover, we sequenced two complete and one partial mitogenomes of Monoplacophora and integrated them in a comprehensive molluscan taxon set (Stöger and Schrödl 2013, Stöger et al. 2016). Mitogenomes were analyzed with the Maximum Likelihood method for phylogenetic relationships and by comparing gene arrangements (Stöger and Schrödl 2013, Stöger et al. 2016). We present genomic data of one more monoplacophoran species (*Laevipilina antarctica*; transcriptomic data of *L. hyalina* is already available in public databases (Smith et al. 2011)), which we combined with a data matrix of a broad molluscan taxon sampling (Kocot et al. 2020). This large phylogenomic dataset was investigated via Maximum Likelihood and Bayesian Inference methods (Kocot et al. 2020). Each of the studies reflects a comprehensive taxon set as they all contain individuals of the existing eight molluscan classes as well as a balanced sampling of trochozoan outgroup taxa (Stöger et al. 2013, Stöger and Schrödl 2016, Kocot et al. 2020). All datasets were carefully processed and checked to avoid potential contaminations. Moreover, two studies exemplify the inner relationships of gastropod and chiton phylogeny by using molecular and morphological evidence (Jörger et al. 2010, Sigwart et al. 2013).

Standard markers worked properly for inner relationships within the level of 'classes' (Jörger et al. 2010, Sigwart et al. 2013) and we still found good resolution and high support values in very deep nodes, at the root of Mollusca and the diversification of this phylum in the

Cambrian/Ordovician (Stöger et al. 2013), compared to most recent phylogenomic approaches (Kocot et al. 2020). However, neither the origin of Mollusca nor class-level relationships within Mollusca could be resolved convincingly using standard markers; the quality and quantity of nucleotide data and analyses thus was too poor and could not be compensated by sampling a large, dense and balanced taxon set.

Analyses of mitogenomic sequence data were equally insufficient when dealing with such old events as the early evolution of Mollusca, but the comparison of gene arrangements led to valuable results, such as finding potentially apomorphic rearrangements for the Mollusca and other taxa (Stöger and Schrödl 2013, Stöger et al. 2016). Phylogenomics are probably the best way to go when analyzing the evolutionary traits of Mollusca (Kocot et al. 2020).

4.1.1. (Limitation of) combined mitochondrial and nuclear standard markers

4.1.1.1. Phylogeny of Heterobranchia (Gastropoda)

Standard markers (COI, 16s, 18s, 28s) worked very well to resolve relationships on family and genus level when based on a dense and balanced taxonomic sampling. Regarding heterobranchs, we strived for a “whole-euthyneuran” taxon set, instead of restricting it to pulmonate, respectively opisthobranch, taxa only. Adding several small, enigmatic groups such as Acochlidia, we were able to challenge some widely accepted concepts within Gastropoda such as Pulmonata and Opisthobranchia, and to establish a novel view on heterobranch relationships (Jörger et al. 2010) that is by now widely accepted (Bouchet et al. 2017). Euthyneuran monophyly was presumed for a long time (e.g. Dayrat and Tillier 2002, 2003) but questioned via datasets that included or were based on molecular markers and a broader taxon set (Grande et al. 2004a, 2004b, Klussmann-Kolb et al. 2008, Dinapoli and Klussmann-Kolb 2010). By adding several euthyneuran subgroups and analyzing the molecular data, the controversial placement of Pyramidelloidea and Glacidorboidea within Euthyneura was confirmed (Jörger et al. 2010) and was supported by other phylogenetic studies since, all presenting similar tree topologies of the euthyneuran clade (Dayrat et al. 2011, Dinapoli et al. 2011, Göbbeler and Klussmann-Kolb 2011, Teasdale 2017). The monophyly of traditional Opisthobranchia was questioned in several studies dealing with molecular as well as morphological characters (Klussmann-Kolb et al. 2008, Wägele et al. 2008, Dinapoli and Klussmann-Kolb 2010). The monophyly of this group was rejected with

our multilocus-dataset (Jörger et al. 2010). Newly classified Euopisthobranchia (Jörger et al. 2010), now comprising Umbraculoidea, Runcinacea, Cephalaspidea s.s., Anaspidea and Pteropoda, is also supported by a genomic dataset of ultra-conserved elements (Moles and Giribet 2021) and morphologically supported by the apomorphic presence of a cuticularized gizzard (i.e., muscular oesophagial crop lined with cuticula) (Jörger et al. 2010, Jörger 2013, Bouchet et al. 2017). Due to the inclusion of Pyramidelloidea, Glacidorboidea, Sacoglossa and Acochlidia, the traditional group of Pulmonata is no longer monophyletic (Jörger et al. 2010, Teasdale 2017). The new classification of this group is now Panpulmonata (Jörger et al. 2010, Bouchet et al. 2017, Teasdale 2017). Panpulmonata include the traditional groups of Pulmonata plus Sacoglossa, Siphonarioidea, Pyramidelloidea and Acochlidia (Jörger et al. 2010b) and is generally supported by phylogenomics (Teasdale et al. 2016, Moles and Giribet 2021). The double rooted rhinophoral ganglion, or the homologous double rooted procerebrum are morphological apomorphies for this clade (Jörger 2013). Both groups (Euopisthobranchia and Panpulmonata) were subsequently summarized as Tectipleura (Schrödl et al. 2011a). This topology was confirmed with multi-locus data of a heterobranch taxon set by Kano et al. (2016) and was further supported by analyses using other datasets than the standard marker matrix received via Sanger sequencing, each study using an independent and broad set of various nuclear genes, ESTs, transcriptomes and ultra-conserved regions of the genome (Kocot et al. 2011, Smith et al. 2011, Vinther et al. 2011, Zapata et al. 2014, Romero et al. 2016, see also Bouchet et al. 2017 for classifications, Moles and Giribet 2021).

4.1.1.2. Phylogeny of Chitons (Polyplacophora)

Furthermore, we reconstructed the phylogeny of chitons (Polyplacophora) with a subset of the already mentioned standard markers: COI (partial), 16s (partial), 18s (partial) and 28s (partial). The phylogeny recovered in our analyses broadly supports the systematic revision of Sirenko (2006). The largest order of living chitons, Chitonida, is divided into two clades: Chitonina and Acanthochitonina. The latter is subdivided into two clades, which is in confirmation to the proposed superfamilies Mopalioidea and Cryptoplacoidea (Sirenko 2006, Sigwart et al. 2013).

Some chiton species with a so far unclear classification are now assigned (Sigwart et al. 2013). Morphological features were used to assign *Hemiarthrum* to Cryptoplacoidea (Sirenko 2006). This result is confirmed by our molecular analysis (Sigwart et al. 2013). *Choriplax* is placed in a derived position in the superfamily Mopalioidea (Chitonida, Polyplacophora) based on our molecular data, although the taxon provides taxonomic key features of two other clades (Sigwart et al. 2013). Due to the lack of shell insertion plates the taxon was previously associated with Lepidopleurida (Kaas and Van Belle 1985a). Sirenko (2006) integrated *Choriplax* in Acanthochitonina (Chitonida), because of the congruent gill arrangement (abanal, i.e. bi-directional; Sirenko 1997, see also Sigwart et al. 2013 for details); subsequently the reduced shell insertion plates are probably convergent. We found that the gill arrangement in *Choriplax* is not abanal but adanal (contra Gowlett-Holmes 1987). That is the typical characteristic in Lepidopleurida; yet, of all the species studied to date, *Choriplax* is the only member of Chitonida with that condition of gills (Sigwart et al. 2013). Unfortunately, the synapomorphies of Mopalioidea are presently not well defined and do not exclude the classification of *Choriplax* within Mopalioidea (Sigwart et al. 2013).

Both species, *Hemiarthrum* and *Choriplax*, have reduced insertion plates in their adult conditions and are slitless (Sirenko 2006, Sigwart et al. 2013). This supports the idea that insertion plates have been lost at several independent points in polyplacophoran evolution (Sirenko 1997, Sirenko 2006). We could identify at least one shell reduction event within the stem of Cryptoplacoidea and two separate independent shell reductions within Mopalioidea, as independently derived apomorphies of *Cryptochiton* and *Choriplax* (Sigwart et al. 2013). This is further evidence of the plasticity of shell form in Mollusca, which is well known (Aktipis et al. 2008, Zapata et al. 2014). The evolutionary process of shell reduction can be visualized as occurring in two distinct ways, by reduction of the tegmentum (exposed dorsal aspect), or by extension of the articulamentum (the ventral, internal shell aspect) (Sigwart et al. 2013). Within Mopalioidea, shell reduction was achieved via expansion of the articulamentum. By contrast, members of Acanthochitonoidea represent a separate evolutionary experiment in shell reduction, using the opposite mechanism of tegmental reduction (Sigwart et al. 2013).

4.1.1.3. Phylogeny of Mollusca

Regarding deep splits, as for example the root of Mollusca and the diversification of this phylum in the Cambrian/Ordovician, the set of standard markers still resolved the phylogeny with good support which was remarkable on one hand but – when compared to results of now available phylogenomic studies – obviously not reflecting phylogenetic signal.

All analyses of the five gene matrix (various methods of alignment masking, partitioning, modelling and analyses regimes were tested) that covers all molluscan classes and includes five monoplacophoran species, result in the Serialia concept that summarizes Monoplacophora and Polyplacophora (Giribet et al. 2006, Wilson et al. 2010, Stöger et al. 2013, Fig. 1 herein). Serialia is incompatible with all traditional textbook concepts of molluscan class relationships as it unites aculiferan and conchiferan taxa (Stöger et al. 2013, see also Fig. 1 in Schrödl and Stöger 2014). Moreover, major traditional taxa such as Aculifera, Testaria and Conchifera concepts (Haszprunar et al. 2008) are clearly rejected by the Approximately Unbiased Test (AU Test, Shimodaira 2002) with our multi-locus dataset. All molluscan studies that have been based on standard markers or a subset thereof resulted in the Serialia hypothesis (Giribet et al. 2006, Wilson et al. 2010, Meyer et al. 2010, Kano et al. 2012, Stöger et al. 2013), whereas phylogenomic analyses based on thousands of markers as well as morphological investigations support the split of molluscan classes in Aculifera and Conchifera (Kocot et al. 2011, Smith et al. 2011, Kocot et al. 2020).

The monophyly of Mollusca and of the molluscan classes is confirmed via standard markers (Stöger et al. 2013). The monophyly of these groups was not questioned at all and is well supported by morphological features (Haszprunar et al. 2008) but has not been detected in all molluscan studies. Some other analyses of standard markers differed from our results (Giribet et al. 2006, Wilson et al. 2010, Meyer et al. 2010) due to potentially contaminated sequences caused by the life style of the animals themselves, technical problems during the amplification/sequencing process (Giribet et al. 2006, Wilson et al. 2010) or unbalanced taxon sampling (Meyer et al. 2010). The careful handling of sequence data and the declared intention to adjust the sampling lead to monophyletic molluscan classes (Meyer et al. 2011, Vinther et al. 2011, Kano et al. 2012, Stöger et al. 2013). Based on our dataset molluscs are split in two clades: gastropods, bivalves and Serialia versus scaphopods, aplacophorans and cephalopods (Stöger et al. 2013). Although sensitivity analyses do not attribute this result to

long-branch effects, this dichotomy was problematic, as it never appeared in any other analyses of a molluscan dataset and might be a result of perturbing signal of certain markers (Stöger et al. 2013).

4.1.2. Mitogenomics – phylogenetic analysis and gene arrangements

4.1.2.1. Heterobranchia (Gastropoda)

Within a framework of molluscan mitogenomes we analyzed the protein-coding genes of several heterobranch taxa; the classification of Euthyneura and Pulmonata, respectively Panpulmonata was not found (Stöger and Schrödl 2013). The inner heterobranch topology recovered paraphyletic Panpulmonata basal to all other heterobranch clades (Stöger and Schrödl 2013), a topology similar to White et al. (2011). Medina et al. (2011) found some support for the traditional concepts of Pulmonata and Opisthobranchia but their analyses were criticized since the taxon sampling seemed to be biased (Schrödl et al. 2011a). Euthyneura was recovered by Sevigny and colleagues (2015) by mitogenomic analyses; their results were highly similar to the topology of White et al. (2011). It seems that the heterobranch topology here is highly constrained by the taxon sampling. White et al. (2011) as well as Sevigny et al. (2015) included pulmonate mitogenomes which were not considered in our analysis (Stöger and Schrödl 2013) as these sequences had not yet been approved via RefSeq when we compiled our dataset (see Bernt et al. 2013a). Recent analyses of the so far most comprehensive set of 87 heterobranch mitogenomes are still struggling with long-branch attraction and possibly extremely high rate heterogeneity (Varney et al. 2020). With choosing a suitable model that better reflects site-specific rate heterogeneity the resulting topology is more congruent with up-to-date studies on heterobranch phylogeny (Varney et al. 2020). We recovered monophyletic Euopisthobranchia in sistergroup relationship to Acteonoidea plus Nudipleura (Stöger and Schrödl 2013). This is in line with the topology based on multi-locus data (Jörger et al. 2010) and the recent mitogenomic result of Varney et al. (2020) who recovered a monophyletic euopisthobranch clade in their Bayesian Inference analysis. Other mitogenomic studies on heterobranch relationships did not find monophyletic Euopisthobranchia (Medina et al. 2011, White et al. 2011, Sevigny et al. 2015). The analysis of protein-coding genes in Heterobranchia seems to be influenced by taxon sampling and model complexity. Most intriguing is the fact that mitogenomic heterobranch

trees (e.g. by Grande et al. 2008, Medina et al. 2011, White et al. 2011, Stöger and Schrödl 2013) are virtually reversed when compared to multi-locus topologies, i.e. with Acteonoidea outside Euthyneura, Nudipleura sister to Tectipleura, the latter splitting into Euopisthobranchia and Panpulmonata (reviewed by Schrödl et al. 2011b). It seems that mitogenomic euthyneuran trees are misrooted (Schrödl et al. 2011b), with longest internal branches (such as derived stylommatophorans, or in their absence, other derived pulmonates) pulled to the tree base (Stöger and Schrödl 2013).

Some "lower heterobranchs" (Omalogyridae, Rissoellidae) were sampled by Varney et al. (2020) but caused some additional long branches and were thus excluded fom their final analyses; however, their euthyneuran topology was no longer misrooted.

4.1.2.2. Chitons (Polyplacophora)

The phylogenetic analyses of protein-coding genes of chitons within a molluscan framework did support the split into the two chitonid clades Chitonina and Acanthochitonina, although we analyzed only three chiton species (Stöger et al. 2016). The result is congruent with other studies including more taxa (Guerra et al. 2018, Irisarri et al. 2020) and using different data (Sirenko 2006, Sigwart et al. 2013). Moreover, a recent mitogenomic study confirmed the deep split of chitons in Lepidopleurida and Chitonida (Irisarri et al. 2020) what is congruent with our (Sigwart et al. 2013) and other studies (Okusu et al. 2003, Buckland-Nicks 2008, Wilson et al. 2010, Irisarri et al. 2014).

Investigation of gene orders in acanthochitonine Polyplacophora leads to the assumption, that this gene arrangement – at least the arrangement of protein coding genes and rRNAs – can be seen as ancestral for the phylum Mollusca (Stöger and Schrödl 2013, Stöger et al. 2016) as the pattern can be found in other molluscan classes as well (e.g. Akasaki et al. 2006, Yokobori et al. 2007). Within Polyplacophora the gene orders of the chitonine *Chaetopleura* (Guerra et al. 2018) and the four lepidopleurid mitogenomes (Irisarri et al. 2020) are congruent with the acanthochitonine gene arrangement, except the direction of *cox2-trnD*. These two genes are inversed in *Katharina* only (Guerra et al. 2018). Probably the plesiomorphic polyplacophoran gene arrangement is no longer reflected by the *Katharina* order (Stöger et al. 2013, Stöger et al. 2016) but now by the *Chaetopleura* order (Guerra et al. 2018). Although the gene arrangements in Polyplacophora seem to be rather conserved,

we detected an inversion of gene clusters in the chitonine *Sypharochiton* (Stöger et al. 2016, but see Guerra et al. 2018). This might be due to erroneous reading direction (Guerra et al. 2018) but own reannotation of the mitogenome via MITOS (Bernt et al. 2013b) as well as the official GenBank annotation confirm our result (own observation, Stöger et al. 2016). As the *Sypharochiton* order is congruent with the monoplacophoran order (Stöger et al. 2016) and the monophyly of chitons is undisputed, this gene arrangement of *Sypharochiton* is likely homoplastic (Stöger et al. 2016).

Studying and analyzing mitogenomes of chitons is very promising, although more taxa, especially in the order Lepidopleurida, are essential to support recent analyses.

4.1.2.3. Mollusca

Using the complete set of 13 protein coding genes (PCGs) of a broad set of molluscan mitogenomes leads to unconventional phylogenetic trees with hardly any significance. The results are not convincing, as molluscs are clustering with lophotrochozoan outgroups in all reconstructed trees and most of the molluscan classes are recovered non-monophyletic, regardless of variations on taxon sets, masking or coding regimes (Stöger and Schrödl 2013, see also Bernt et al. 2013a). The results stay ambiguous even if the molluscan taxon set is carefully preselected and 7 of 8 classes are included (Stöger et al. 2016). None of the common existing concepts of inner molluscan relationships as the Aculifera or Conchifera appears in any of the analyses, although we recovered “Diasoma” (Bivalvia + Scaphopoda) in some amino acid based analyses and one single nucleotide based analysis, and Serialia in several nucleotide based analyses (Stöger and Schrödl 2013, Stöger et al. 2016). Trees based on mitochondrial nucleotide as well as amino acid sequences seem to be heavily biased by long-branch attraction (Stöger and Schrödl 2013). Beside the fact that the taxon sampling was not perfectly balanced between classes (due to the limited availability of complete molluscan mitochondrial genomes in public databases), molluscan mitogenomes are known to frequently shuffle their gene order, promoting differences in strand bias, which in turn affects the amino acid usage (Boore 1999, Bernt et al. 2013c, d). It seems to be essential that core genes are included in phylogenetic analyses to achieve reliable results for deep nodes.

However, comparing the gene arrangements of molluscs as well as several lophotrochozoan outgroups leads to striking results. We were able to find unique arrangements of protein

coding genes in Monoplacophora and some – but not all – chiton species (Stöger et al. 2016, but see Guerra et al. 2018). Under the Aculifera-/Conchifera-hypothesis this can be explained with a convergent inversion of *cox3-nad3-nad2-cox1-cox2-atp8-atp6* (Stöger et al. 2016 cluster 1 therein). Convergence is an unusual event within lophotrochozoan PCG arrangements and is known only from the gene order comparison of *Lineus* (Nemertea) and Caenogastropoda so far; these gene orders depict congruent arrangements of the protein coding genes (Stöger and Schrödl 2013). Under the Serialia concept the observed arrangements would lead to the assumption of paraphyletic Polyplacophora. The monoplacophoran pattern of gene order is congruent with *Sypharochiton* (Chitonina) pattern, but vice versa to the *Katharina* (Acanthochitonina) pattern (Stöger et al. 2016, but see Guerra et al. 2018).

We were able to determine a potential synapomorphy for Mollusca in their mitogenomic arrangements: tRNAs *G* and *E* are located in adjacent position to the tRNA complex *MCYWQ* (Stöger and Schrödl 2013). Furthermore, Guerra and colleagues located *trnP* on the plus strand in Aculifera whereas it is found on the minus strand in all conchiferan taxa, which could be a synapomorphy for these two groupings of molluscan classes (Guerra et al. 2018).

4.1.3. Whole genome approach – support for Aculifera/Conchifera

Latest analyses of a phylogenomic dataset that include all eight molluscan classes and a concatenated datamatrix that counts 54,596 amino acid positions in length and covers 257 genes recovers the common concepts of Aculifera and Conchifera, with Monoplacophora at the conchiferan base in most of the analyses, and Polyplacophora as sister to aplacophoran taxa (Kocot et al. 2020). The split into Aculifera and Conchifera is supported by morphological studies (Runnegar and Pojeta 1985, Scheltema 1993, 1996, Ivanov 1996, Salvini-Plawen and Steiner 1996, Scheltema and Schander 2006) as well as earlier genomic approaches (Kocot et al. 2011, Smith et al. 2011). The placement of Monoplacophora as sister to the rest of the conchiferan classes is novel for molecular approaches and in line with morphology-based classifications of the Conchifera (Pojeta and Runnegar 1976, Haszprunar 2008). Monoplacophorans differ in their arrangement and structure of mantle folds, anatomy of the shell gland and structure of the shell from other conchiferan classes (Haszprunar 2008, Kocot et al. 2011). Whether these are plesiomorphic characters of the

conchiferan classes or if they are monoplacophoran apomorphies stays dubious. In our approach both analyses, Maximum Likelihood and Bayesian Inference, provide strong support for the placement of monoplacophorans at the base of Conchifera under mixture models (Kocot et al. 2020). The Maximum Likelihood analysis of Kocot et al. (2020) under a site-homogenous model results in a topology with Monoplacophora sister to Cephalopoda with moderate support. That relationship is in line with the first genomic analysis that included monoplacophoran data (Smith et al. 2011) as well as it is in line with some interpretations of the fossil record (Pojeta and Runnegar 1976). Nevertheless, the multi-species coalescent approach as well as the ancestral state reconstruction of a morphological character matrix of Mollusca (built on the matrix of Haszprunar 2000, Kocot et al. 2011) both support the position of Monoplacophora at the base of Conchifera (Kocot et al. 2020).

The phylogenetic relationships within Mollusca, a bifurcated tree which splits in Aculifera and Conchifera (Kocot et al. 2020), rejects the Testaria hypothesis of molluscan class-relationships. The Testaria hypothesis would logically lead to the assumption of a progressive evolution from an initially simple, worm-like ancestor without a shell to polyplacophorans with shell plates towards the uni- or bivalved conchiferan molluscan classes (Salvini-Plawen 2006). The Aculifera-/Conchifera-hypothesis with monoplacophorans as sister to the rest of the conchiferan classes implies an evolution from a dorsoventrally flattened molluscan ancestor with a mantle, dorsal cuticle, broad foot, 8 or 7 (Scherholz et al. 2013) dorsoventral muscle pairs, a circumpedal or posterior mantle cavity with serially repeated gills and a radula (Scheltema 1993, Kocot et al. 2020).

Scaphopoda and Gastropoda form a clade that is sister to Bivalvia (Kocot et al. 2020), a result that is again consistent with Smith et al. (2011). The placement of Scaphopoda as sister to Gastropoda rejects the Cyrtosoma hypothesis (Gastropoda + Cephalopoda, Fig. 3) as well as the Diasoma hypothesis (Scaphopoda + Bivalvia, Fig. 3) within conchiferan relationships which were stated earlier based on morphological data (Runnegar and Pojeta 1985, 1992, Runnegar 1996, Salvini-Plawen and Steiner 1996, Haszprunar 2000, Haszprunar et al. 2008). Some morphological indication can be found for the relationship of Scaphopoda and Gastropoda (Sigwart et al. 2017).

4.2. Dating the evolution of Mollusca

Dating the molluscan tree is problematic, as long as the fossils that are used for calibrating the tree cannot be clearly assigned to a certain group and it is even more difficult when there is no stable phylogenetic backbone for the phylum. Obtaining a reliable phylogeny of the molluscan tree could help assigning fossils that are still dubious in their classification.

The phylogenetic analysis based on the NGS dataset of 257 genes confirms the Aculifera-/Conchifera concepts of molluscan relationships (Kocot et al. 2020). According to our time estimation the molluscan stem is dated to the Precambrian era and molluscan diversification started ca. 584Mya in the past (Kocot et al. 2020). The split of Mollusca into the groups Aculifera and Conchifera is estimated near the Precambrian/Cambrian boundary to 546Mya (Kocot et al. 2020). Diversification of Conchifera took place 540Mya and diversification of Aculifera is set to 499Mya (Kocot et al. 2020). These results are consistent with previous molecular clock analyses (Erwin et al. 2011, Stöger et al. 2013, Zapata et al. 2014, Vinther et al. 2017). A more basal position of Mollusca within the Lophotrochozoa (see for example Dunn et al. 2008) is in line with molecular clock approaches that date the molluscan stem to the terminal Precambrian era and depict a rapid diversification of the extant molluscan classes in the Cambrian (e.g. Stöger et al. 2013, Vinther 2015, Kocot et al. 2020, but see Parkhaev 2017).

Our most recent analysis (Kocot et al. 2020) shows an Ordovician origin of the gastropod stemline (474Mya) in contrast to earlier assumptions on a Cambrian origin (Stöger et al. 2013, Zapata et al. 2014). The difference might be a result of the varying data or taxon settings, or of technical nature, how parameters of the time estimation analyses were adjusted. Fossil gastropods cannot be easily linked to extant taxa (Frýda et al. 2008) and maybe a new time frame for gastropod ages can assist to integrate dubious fossil findings in the evolutionary line of Gastropoda. The diversification of Gastropoda into the main lineages is dated to the Silurian/Devonian boundary (424Mya, not explicitly shown in the published figure of Kocot et al. 2020) as it was shown in an earlier gastropod-specific analysis (Zapata et al. 2014). Artificially reducing the number of markers used for analysis seems to push the time estimation towards the Cambrian (Stöger et al. 2013, own unpublished data).

Edgecombe et al. (2011) refuted affiliation of *Kimberella* to molluscs for their later appearance. As the molluscan stem is dated to the terminal Precambrian era in our analyses, however, the heavily discussed fossil taxon *Kimberella* can well be considered a stem mollusc; its age of 555Mya suits the dating of the molluscan origin (Stöger et al. 2013, Kocot et al. 2020). The nature of *Kimberella* differs from any modern mollusc, as *Kimberella* lacks a mineralized shell (but probably possessed sclerites (Ivantsov 2009) and a structure that can be interpreted as a non-mineralized shell (Fedonkin and Waggoner 1997, Seilacher 1999, Seilacher et al. 2003)) and probably had a deviant mode of feeding in comparison to modern molluscs (Parkhaev 2017). Still, *Kimberella* resembles a molluscan-like bauplan, with a distinct foot, a surrounding mantle and a mantle cavity (Fedonkin and Waggoner 1997, Seilacher 1999, Seilacher et al. 2003). If *Kimberella* is of molluscan nature, the last common ancestor of molluscs is probably single-shelled and more similar to a monoplacophoran rather than to a polyplacophoran bodyplan (Vinther et al. 2017, Wanninger and Wollesen 2019). That leads to the assumption that the conchiferan state of possessing a single shell is the ancestral state and that the aculiferan condition(s) with eight shell plates in Polyplacophora respectively reduced shells but spicule-bearing Aplacophora evolved secondarily (Schrödl and Stöger 2014, Wanninger and Wollesen 2019). Tiny fossils of the small shelly fauna (SSF), as for example helcionellids (see e.g. Parkhaev 2008) that appear from the Early Cambrian to the Ordovician (e.g. Gubanov and Peel 1999, Gubanov and Peel 2003), are suitable to be conchiferan molluscs (Parkhaev 2017). According to our timetree (Kocot et al. 2020), molluscan SSFs would have been stem conchiferans, or less likely, belonged to the stem of Monoplacophora or the lineage that gave rise to the remaining conchiferans (Kocot et al. 2020).

The last common ancestor of molluscs is still unknown and discussion is ongoing as long as there is no definite agreement on the evolution of molluscs and their putative lophotrochozoan sistergroup. Sinusoida (Mollusca + Entoprocta; syn. Lacunifera, Tetraneuralia; Bartolomaeus 1993, Ax 1999, Nielsen et al. 2007, Wanninger 2009, Wanninger and Wollesen 2019) depict similar gene arrangements in their mitochondrial genomes (Yokobori et al. 2008), but that is likely to be the plesiomorphic state of Lophotrochozoa as there is no other molecular evidence for the grouping (Stöger and Schrödl 2013, Schrödl and Stöger 2014). The concept of Neotrochozoa (Mollusca + Annelida s.l.; Dunn et al. 2008, Peterson et al. 2008, 2009, Sperling et al. 2009) is not reliably

supported by recent analyses (Schrödl and Stöger 2014). The comprehensive phylogenomic study by Struck et al. (2014) resolves the origin of Mollusca with ambiguous results: Mollusca sister to a clade of annelids and nemerteans, as sister to brachiopods, or as a more basal offshoot among lophotrochozoans (Struck et al. 2014). Based on our topology that shows Monoplacophora as sistergroup to the rest of Conchifera (Kocot et al. 2020), the last common ancestor of molluscs was dorsoventrally flattened with a mantle, a dorsal cuticle, a broad foot, eight (or seven) pairs of dorsoventral muscles, a circumpedal or posterior mantle cavity with serially arranged gills, and a radula as part of a longitudinally arranged, regionalized digestive system (Kocot et al. 2020). In addition to evo-devo and palaeontological approaches, the origin of Mollusca will hopefully be resolved using comprehensive whole genome analyses on a dense and balanced set of lophotrochozoans.

5. Conclusion and Outlook

The Mollusca is a very old group with a Precambrian origin. Here we compared the performance of molecular markers for resolving relationships between and within the molluscan classes. In the context of this work we were able to create novel sequence data of several monoplacophoran species, e.g. the first complete Laevipilina-mitogenomes that are published so far (Stöger and Schrödl 2013, Stöger et al. 2016). We analyzed a set of standard markers (combined mitochondrial and nuclear datasets, obtained via Sanger sequencing), that worked properly for inner class relationships (Jörger et al. 2010, Sigwart et al. 2013) and we still found good resolution and high support values in very deep nodes, as the root of Mollusca and the diversification of this phylum in the Cambrian/Ordovician (Stöger et al. 2013), as compared to most recent phylogenomic approaches (Kocot et al. 2020). However, neither the origin of Mollusca nor class-level relationships within Mollusca could be resolved convincingly; the quality and quantity of nucleotide data thus was too poor and could not be compensated by sampling a large, dense and balanced taxon set. Analyses of mitogenomic sequence data were equally insufficient when dealing with such old events as the evolution of Mollusca, but the comparison of gene arrangements led to valuable results, such as finding potentially apomorphic rearrangements for the Mollusca and other taxa (Stöger and Schrödl 2013, Stöger et al. 2016). Phylogenomics are probably the best way to go when analyzing the evolutionary traits of Mollusca (Kocot et al. 2020). Combining our newly generated genomic data of *Laevipilina antarctica* with a data matrix of a broad molluscan taxon sampling we could support the Aculifera-/Conchifera-hypothesis with Monoplacophora as sister to Conchifera. This position of Monoplacophora was never detected before in any molecular analysis but it is in line with morphological classifications of Conchifera (e.g. Haszprunar 2008).

Obtaining reliable ages for the molluscan tree is often difficult, as long as the fossils that are used for calibrating the tree cannot be clearly assigned to a certain group and it is even more difficult when there is no stable phylogenetic backbone for the phylum. Based on our time estimations of the phylogenomic analysis (Kocot et al. 2020) the molluscan stem is Precambrian and the molluscan diversification started 584Mya in the past (Kocot et al. 2020). The split of Mollusca into Aculifera and Conchifera is settled at the Precambrian/Cambrian boundary (546Mya; Kocot et al. 2020). These results are consistent

with previous time estimations (e.g. Stöger et al. 2013). The heavily discussed fossil *Kimberella* can well be considered as a stem mollusc, as its age of 555Mya suits to our dating of the molluscan origin. The affiliation of *Kimberella* to the Mollusca would imply that the last common ancestor of molluscs was probably single-shelled and was more similar to a monoplacophoran than a polyplacophoran bauplan (Vinther et al. 2017, Wanninger and Wollesen 2019). Subsequently, the conchiferan state of possessing a single shell would be probably the ancestral state rather than the aculiferan state(s) of possessing eight shell plates (Polyplacophora) respectively the shell less but spicule bearing condition of Aplacophora.

Still, collecting more data on rare molluscan classes, as for example Aplacophora, and basal taxa to connect evolutionary traits within subgroups such as the Gastropoda, is essential. The analyses of fast-evolving taxa and inclusion of typical representatives into general taxon sets is important for further research on molluscan relationships. The use of so-called ultra-conserved elements (UCEs; see Moles and Giribet 2021) and especially of whole genomes seems to be promising when dealing with such old splits as can be found in the phylum Mollusca. Equally important is the development and application of highly sensitive analyzing tools to carefully edit and optimize large scale datasets.

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8. Declaration of contributions to each publication

3.1. Isabella Stöger, Julia D. Sigwart, Yasunori Kano, Thomas Knebelsberger, Bruce A. Marshall: The continuing debate on deep molluscan phylogeny: evidence for Serialia (Mollusca, Monoplacophora + Polyplacophora). 2013. BioMed Research International, 2013.

I did the lab work, carried out the molecular genetic studies, performed the sequence alignments and the phylogenetic analyses, and drafted the paper.

3.2. Katharina M. Jörger, Isabella Stöger, Yasunori Kano, Hiroshi Fukuda, Thomas Knebelsberger, Michael Schrödl: On the origin of Acochlidia and other enigmatic euthyneuran gastropods, with implications for the systematics of Heterobranchia. 2010. BMC Evolutionary Biology, 10.

I generated the molecular sequence data and helped with the data analyses, and contributed to the discussion of the results and the preparation of the final manuscript.

3.3. Julia D. Sigwart, Isabella Stöger, Thomas Knebelsberger, Enrico Schwabe: Chiton phylogeny (Mollusca: Polyplacophora) and the placement of the enigmatic species Choriplax grayi (H. Adams & Angas). 2013. Invertebrate Systematics, 27, 603-621.

I did the lab work and generated the sequence data, performed alignments and phylogenetic analyses, and contributed to write the manuscript.

3.4. Isabella Stöger, Michael Schrödl: Mitogenomics does not resolve deep molluscan relationships (yet?). 2013. Molecular Phylogenetics and Evolution, 69, 376-392.

I contributed to analyse the data and drafted the manuscript.

3.5. Isabella Stöger, Kevin M. Kocot, Albert J. Poustka, Nerida G. Wilson, Dimitry Ivanov, Kenneth M. Halanych, Michael Schrödl: Monoplacophoran mitochondrial genomes: convergent gene arrangements and little phylogenetic signal. 2016. BMC Evolutionary Biology, 16.

I performed the analyses and drafted the manuscript.

3.6. Michael Schrödl, Isabella Stöger: A review on deep molluscan phylogeny: old markers, integrative approaches, persistent problems. 2014. Journal of Natural History, 48, 2773-2804.

I performed the molecular analyses and contributed to write the manuscript.

3.7. Kevin M. Kocot, Albert J. Poustka, Isabella Stöger, Kenneth M. Halanych, Michael Schrödl: New data from Monoplacophora and a carefully-curated dataset resolve molluscan relationships. 2020. Scientific Reports, 10.

I conducted the molecular clock analyses and contributed to write the manuscript.

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Isabella Stöger ____

9. Curriculum Vitae

PERSONAL DATA

Name Isabella Stöger

Date of birth 15 October, 1979

Place of birth Munich

STUDIES

Since 12/2009
Bavarian State Collection of Zoology, Munich
Dissertation within the DFG Priority Programme "Deep Metazoan Phylogeny", Department of Mollusca: *Phylogeny and evolution of Monoplacophora and basal Mollusca*

02/2006 - 11/2006
Bavarian State Collection of Zoology, Munich
Diploma thesis, Department of Ichthyology: *Phylogenetic relationships and age estimates of Sulawesi´s Telmatherinidae (Pisces – Teleostei – Telmatherinidae) endemic to the Malili Lake system*

2000 – 2007
Johannes-Gutenberg-University, Mainz
Studies in Biology
Major: Zoology
Minors: Botanics, Immunology
Degree: Diploma

SCIENTIFIC CONFERENCE CONTRIBUTIONS

Posters

09/2013
Stöger, I, Poustka, AJ, Wilson, N, Schrödl, M: Extended mitogenomic taxon sampling in the phylum Mollusca. Tagung der Deutschen Zoologischen Gesellschaft, Munich.

10/2011
Stöger, I, Schrödl, M: Alternative view on deep metazoan phylogeny. Deep Metazoan Phylogeny Congress, Munich.

09/2008
Knebelsberger, T, Gemeinholzer, B, Haszprunar, G, Klenk, H-P, **Stöger, I**, Wägele, J-W: Request for DNA donations. Tagung der Deutschen Zoologischen Gesellschaft, Jena.

03/2008
Stöger, I, Herder, F, Schliewen, U: Phylogenetic relationships and age estimates of Sulawesi´s Telmatherinidae (Pisces – Teleostei – Telmatherinidae) endemic to the Malili Lake system. 6. Tagung der Gesellschaft für Ichthyologie, Munich.

Oral presentations

01/2011
Stöger, I, Schrödl, M: Confirming Serialia – and its consequences for molluscan phylogeny. Workshop of the DFG Priority Programme SPP 1147 "Deep Metazoan Phylogeny", Hannover.

07/2010
Stöger, I, Marshall, BA, Schwabe, E, Knebelsberger, T, Schrödl, M: Serialia or not Serialia? Adding three monoplacophoran species to a multigene approach on basal molluscan phylogeny. World Congress of Malacology, Phuket.

SCIENTIFIC PUBLICATIONS

2020
Kocot, KM, Poustka, AJ, **Stöger, I**, Halanych, KM, Schrödl, M: New data from Monoplacophora
and a carefully-curated dataset resolve molluscan relationships. Scientific Reports.

2018
Meyer, M, Mellein, S, Moriniere, M, **Stöger, I**, Abbt, V, Gruppe, A, Gebhardt, M: Barcoding of two enigmatic Orthopteran taxa (Ensifera: Gryllidae: Trigonidiinae; Caelifera: Tridactyloidea) from the Peruvian lowland rainforest. Mitteilungen der Deutschen Gesellschaft für Allgemeine und Angewandte Entomologie.

2016
Stöger, I, Kocot, KM, Poustka, AJ, Wilson, NG, Ivanov, D, Halanych, KM, Schrödl, M: Monoplacophoran mitochondrial genomes: convergent gene arrangements and little phylogenetic signal. BMC Evolutionary Biology.

Padula, V, Bahia, J, **Stöger, I**, Camacho-García, Y, Malaquias, MAE, Cervera, JL, Schrödl, M: A test of color-based taxonomy in nudibranchs: molecular phylogeny and species delimitation of the Felimida clenchi (Mollusca: Chromodorididae) species complex. Molecular Phylogenetics and Evolution.

2014
Schrödl, M, **Stöger, I**: A review on deep molluscan phylogeny: old markers, integrative approaches, persistent problems. Journal of Natural History.

Stelbrink, B, **Stöger, I**, Hadiaty, RK, Schliewen, UK, Herder, F: Age estimates for an adaptive lake fish radiation, its mitochondrial introgression, and an unexpected sister group: Sailfin silversides of the Malili Lakes system in Sulawesi. BMC Evolutionary Biology.

2013

Stöger, I, Sigwart, JD, Kano, Y, Knebelsberger, T, Marshall, BA, Schwabe, E, Schrödl, M: The continuing debate on deep molluscan phylogeny: Evidence for Serialia (Mollusca, Monoplacophora + Polyplacophora). BioMed Research International.

Stöger, I, Schrödl, M: Mitogenomics does not resolve deep molluscan relationships (yet?). Molecular Phylogenetics and Evolution.

Bernt, M et al.: A comprehensive analysis of bilaterian mitochondrial genomes and phylogeny. Molecular Phylogenetics and Evolution.

Sigwart, JD, **Stöger, I**, Knebelsberger, T, Schwabe, E: Chiton phylogeny (Mollusca: Polyplacophora) and the placement of the enigmatic species *Choriplax grayi* (H. Adams & Angas, 1864). Invertebrate Systematics.

2012

Knebelsberger, T, **Stöger, I**: DNA Extraction, Preservation and Amplification. In: Kress, WJ, Erickson, DL (editors): Methods in Molecular Biology, DNA BARCODES: METHODS AND PROTOCOLS. Berlin: Humana Press, Springer. Science&Publishing Media.

2010

Jörger, KM, **Stöger, I**, Kano, Y, Fukuda, H, Knebelsberger, T, Schrödl, M: On the origin of Acochlidia and other enigmatic euthyneuran gastropods, with implications for the systematic of Heterobranchia. BMC Evolutionary Biology.

10. Statutory Declaration and Statement

Eidesstattliche Erklärung

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt ist.

München, den 31.05.2021

Isabella Stöger

Erklärung

Die Dissertation wurde weder ganz, noch in wesentlichen Teilen, bei einer anderen Prüfungskommission vorgelegt.

Ich habe noch zu keinem früheren Zeitpunkt versucht, eine Dissertation einzureichen oder mich einer Doktorprüfung zu unterziehen.

München, den 31.05.2021

Isabella Stöger