Molecular phylogeny of Acanthochitonina (Mollusca: Polyplacophora: Chitonida): three new mitochondrial genomes, rearranged gene orders and systematics

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Few molecular studies have addressed the phylogenetic relationships within Acanthochitonina. Here, we use high-throughput sequencing to determine three mitochondrial genomes of Acanthochitonina. We further explore phylogenetic relationships with expanded taxon sampling based on mitochondrial and nuclear sequence data. Phylogenetic analyses recover two major lineages roughly corresponding to Cryptoplacoidea and Mopalioidae, but the necessity of reassigning multiple genera challenges their conventional definition and that of the families Mopaliidae, Schizoplacidae and Lepidochitonidae. Two mitogenomes share a gene rearrangement that might represent a synapomorphy for Lepidochitonidae. Our phylogenetic results support the reinterpretation of certain morphological characters as homologous, previously assumed to be convergent. They further reveal that major Acanthochitonina lineages were restricted to particular ocean basins, where they diverged into endemisms with disparate morphology. Our results are corroborated by morphological and biogeographical evidence and contribute toward resolving the phylogenetic relationships of Acanthochitonina, and highlight the need for further phylogenetic and systematic studies.

Keywords: chiton; gene rearrangement; mitogenomics; Mollusca; mtDNA

Abbreviations

18S, small nuclear ribosomal RNA gene; 28S, large nuclear ribosomal RNA gene; atp6, atp8, ATP synthase subunits 6 and 8; AU, approximately unbiased; BI, Bayesian inference; bp, base pair; cob, cytochrome b; cox1-3, cytochrome c oxidase subunits 1–3; Kb, kilobase pair; Mb, megabase pair; ML, maximum likelihood; MCMC, Markov chain Monte Carlo; mt, mitochondrial, nad1-6, 4L NADH dehydrogenase subunits 1–6 and 4L; rRNA, ribosomal RNA; rrnL, large mitochondrial ribosomal RNA gene; rrnS, small mitochondrial ribosomal RNA gene; TDR, tandem duplication–random loss; tRNA, transfer RNA gene; trnX, tRNA genes for the amino acid X as abbreviated by IUPAC codes.

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Introduction

Chitons are an ancient lineage of marine molluscs with a fossil record tracing back to the Upper Cambrian (Vendrasco and Runnegar 2004; Vendrasco et al. 2004; Sigwart and Sutton 2007; Puchalski et al. 2008). Recent molecular phylogenies (Kocot et al. 2011; Smith et al. 2011; Vinther et al. 2011), palaeontological evidence (Sigwart and Sutton 2007; Sutton et al. 2012; Sutton and Sigwart 2012), and larval musculature conditions (Scherholz et al. 2013) favour a close relationship between Polyplacophora and Aplacophora (Chaetodermomorpha + Neomeniomorpha) to form the clade Aculifera, which is sister to all other molluscs (i.e., Conchifera). Nevertheless, some morphological studies supported Polyplacophora as sister group of Conchifera forming the clade Testaria (e.g., Salvini-Plawen and Steiner 1996) and certain molecular studies have also suggested the alternative hypothesis that Polyplacophora and Monoplacophora are sister taxa, forming the clade Serialia (Giribet et al. 2006; Stöger et al. 2013). The Serialia hypothesis is apparently unsupported by morphological evidence and possibly a result of complex signals in the data that hinder the true basal relationships among molluscs (Wägele et al. 2009).

Polyplacophora is a relatively small group compared with other mollusc classes (with ca. 940 described living species; Schwabe 2005), fairly well studied at the species level (e.g., Kaas and Van Belle 1985a, 1985b, 1987, 1990, 1994; Kaas et al. 2006). In recent years, important revisions have been accomplished for restricted geographic areas or taxonomic groups (e.g., Kelly and Eernisse 2008; Sigwart et al. 2011). In contrast, the higher-level classification of chitons has undergone drastic changes in the past (e.g., Pilsbry 1892–1894; Thiele 1909–1910; Bergenhayn 1955; Smith 1960; Van Belle 1983; Sirenko 1993, 1997, 2006), and it is still the subject of important revisions and modifications. Some higher-level classifications (e.g., Smith 1960; Van Belle 1983) have been based mostly on shell plate (valve) characters, sometimes supplemented by girdle features. Van Belle (1983) extensively reviewed previous classifications and proposed a comprehensive system, especially emphasizing valves, for both living and post-Cambrian fossil chitons then known. Kaas and Van Belle (1980, 1998) compiled nominal chiton species according to the system of Van Belle (1983) and completed much of a planned worldwide monograph series (cited above) while they were still alive. Shell characters are conspicuous and are generally the only surviving parts of fossil chitons. However, a system that does not take other potentially informative evidence into account is bound to imperfectly reflect phylogeny. Eernisse (1984) and Sirenko (1993, 1997, 2006) demonstrated the congruent pattern of gill placement and sculpturing of extracellular processes surrounding the egg (egg hull), and argued for their phylogenetic utility. Hodgson et al. (1988) and Buckland-Nicks (1995) added valuable sperm characters.

Sirenko (1993, 1997, 2006) has proposed the most recent taxonomic system for the whole class, which is based on a combination of valve, radula, girdle, egg hull, sperm, and other morphological characters. Several other studies based on these new characters notably improved our understanding of the evolution and systematics of specific chiton taxa (e.g., Bullock 1988; Saito 2003; Buckland-Nicks 2006; Vendrasco et al. 2008; Sigwart 2009). The application of molecular systematics to chitons brought an independent line of evidence that confirmed certain traditional groupings but also questioned many others. Within Acanthochitonina, previous molecular studies (Okusu et al. 2003; Kelly and Eernisse 2008; Sigwart et al. 2013) have pointed
out close relationships of both Cryptochiton von Middendorff and Tonicella Carpenter with members of Mopaliidae, thus suggesting these two genera had been misplaced in previous taxonomic classifications (Van Belle 1983; Sirenko 2006). In addition, Sigwart et al. (2013) supported Choriplax Pilsbry placed in Mopaliioidea and Hemiarthrum Carpenter in Dall within Cryptoplacoidea, and Sirenko et al. (2013) noted a close affinity of Schizoplax Dall with Cyanoplax Pilsbry. With the exception of the above, few molecular studies have addressed Acanthochitonina relationships, particularly at higher taxonomic levels.

Phylogenetic studies generally found a deep split within Polyplacophora corresponding to the orders Lepidopleurida and Chitonida; the latter diagnosed on the basis of the presence of valve insertion plates at least primitive with slits that correspond to the lateral innervation of dorsal shell pores or ‘aesthetes’ (Kaas and Van Belle 1985a; Eernisse and Reynolds 1994). Within Chitonida, both morphological and molecular evidence support the existence of two major lineages, corresponding to the suborders Chitonina and Acanthochitonina sensu Sirenko 2006. In Sirenko’s (2006) classification system, Chitonina is characterized by the adanal placement of gills (i.e., plesiomorphic as in Lepidopleurida) and numerous thin and long egg hulls, whereas Acanthochitonina possess abanal gills and fewer wide cup- or cone-like egg hulls. In the abanal condition, a single ctenidium is present just posterior to each paired nephridiopore, and these are the earliest-appearing gills in juvenile chitons, with all subsequent (and smaller) gills added to the anterior. In contrast, chitons with an adanal condition add gill pairs in both anterior and posterior directions during ontogeny (reviewed by Eernisse 1984; see also Sirenko 1993; Schwabe 2010). Despite the overall agreement between several morphology- and molecular-based studies in the recognition of these major lineages, the phylogenetic relationships within them are still poorly understood and the phylogenetic position of particular genera remain unsettled (Okusu et al. 2003; Sigwart et al. 2013). Because several classifications have been proposed for Polyplacophora and revisions are ongoing, we have tried to minimize confusion by adopting Sirenko’s (2006) system (see also Sirenko 1993, 1997) for our initial presentation unless otherwise stated.

Within Polyplacophora, several instances of morphological convergence and parallel evolution have been previously suggested (Sirenko 2006) but not yet tested with phylogenetic methods. In addition, chitons have evolved divergent morphologies despite their relatively conserved body plan. Good examples of this are the reduction or internalization of valves found in Cryptoplax Blainville and Cryptochiton Gray, respectively (Kaas and Van Belle 1985a). The presence of insertion plates (i.e., lateral or terminal extensions of the articulamentum shell layer into the surrounding girdle) has been considered of high significance in chiton classifications, even for schemes introduced in the 1800s, as are insertion plate slits (reviewed in Van Belle 1983). However, the lack of slits in some chitons that have insertion plates was long thought to be an intermediate condition between having no insertion plates and having slitted insertion plates, but slits have since been reinterpreted as primitively absent in some chitons (e.g., Hanleya) and secondarily lost in others (e.g., Hemiarthrum) (Sirenko 1997, 2006). Having a robust phylogenetic framework is fundamental for the testing of such alternative character hypotheses and can also reveal biogeographic correspondence between distribution patterns and shared ancestry.

During the last two decades, complete mitochondrial (mt) genomes have been widely used for studying phylogenetic relationships in animals (Schierwater et al.
Mt genomes of bilaterian animals possess certain properties that make them ideal candidates for such a purpose: they contain a consistent set of 13 protein-coding, two rRNA and 22 tRNA genes, which makes orthology definition straightforward; their average size is ca. 16 Kb (known to range between 13.7 and 32.1 Kb in molluscs; D’Onorio de Meo et al. 2012), and these properties provide a relatively high number of characters for robust phylogeny reconstruction with relatively easy amplification and sequencing; they are haploid and usually maternally inherited, meaning that in principle genes do not undergo recombination (Boore et al. 2005; but see Thyagarajan et al. 1996; Tsaoousis et al. 2005). Furthermore, rare genomic changes such as gene order rearrangements can retain additional phylogenetic information (Boore and Brown 1998; Rokas and Holland 2000). Nevertheless, the use of mt genomes for resolving deep divergences can impose important analytical challenges that need to be taken into account. In general, animal mt genes have higher substitution rates than nuclear genes (e.g., Brown et al. 1979; Irisarri et al. 2012). Depending on the level of divergence, the higher mt rates can produce a saturation of substitutions, and thus loss of phylogenetically informative character states. A related problem is the presence of among-lineage rate heterogeneity or non-random extinction patterns, either of which can produce the well-known long-branch attraction artefacts (Felsenstein 1978; Bergsten 2005). In molluscs, mt genome sequences have been successfully used for reconstructing robust phylogenetic hypotheses in many groups, including gastropods (e.g., Grande et al. 2008; Cunha et al. 2009), bivalves (e.g., Doucet-Beaupré et al. 2010; Yuan et al. 2012), and cephalopods (e.g., Yokobori et al. 2004; Alcock et al. 2011). A chiton, Katharina tunicata Wood, was one of the first metazoan species to have its complete mt genome sequenced (Boore and Brown 1994), but no chiton mt genomes had been published since then. During the review process of this paper, the complete mt genomes of two species of the genus Sypharochiton Thiele (suborder Chitonina) were published (Veale et al. Forthcoming 2014).

Here, we used Illumina HiSeq2000 for the massive parallel sequencing of three new complete mt genomes of chitons belonging to Acanthochitonina. The genomic features and phylogenetic performance of the new mt genomes are studied. We present evidence for a mt genome rearrangement shared by two species. Combining new mt sequences and previously available mt and nuclear gene sequence data with a broader taxon sampling, we address the phylogenetic relationships within Acanthochitonina and focus on particular questions with relevance for chiton systematics and evolution.

Materials and methods

Taxon and character sampling

The reconstruction of the Acanthochitonina phylogeny was based on a multigene matrix of partial sequences from three mt (rrnL, cox1 and cob) and two nuclear genes (18S and 28S). Taxon sampling was designed to represent main chiton lineages (Lepidopleurida and Chitonida) with emphasis on Acanthochitonina, our target group. We included 27 species in 19 genera from all families and subfamilies of Acanthochitonina, with the exception of the rarely collected Choriplacidae (represented only by Choriplax) (see Sigwart et al. 2013) and Juvenichitoninae (placed in Tonicellidae by Sirenko 1975). The multigene matrix was assembled from newly sequenced mt data and previously available mt and nuclear gene sequences. See
Table 1 for detailed information of specimens and GenBank (Benson et al. 2011) accession numbers. Further information on collectors and collection dates and localities can be found in Supplemental File 1. All specimens were collected with appropriate collection permits. The following species were selected for sequencing the three complete mt genomes, representing distinct lineages within Acanthochitonina: Cyanoplax cf. caverna Eernisse, Nuttallina californica (Nuttall MS, Reeve), and Cryptochiton stelleri von Middendorff (GenBank acc. Nos. KJ569361–J569363, respectively). Our Cyanoplax cf. caverna specimen was sampled further south than the recognized distribution for this species (Santa Catalina Island, Los Angeles Co., California) and there is evidence that it is not C. caverna but is instead a closely related species (DJE, unpublished observation). For convenience, we hereafter refer to it as C. caverna. The gastropods Haliotis rubra Leach (Vetigastropoda), Bolinus brandaris (Linnaeus) and Ilyanassa obsoleta (Say) (Caenogastropoda) were used as additional non-chiton outgroups in both complete mt genome and multilocus data sets. These species were chosen because (particularly for mt genomes) they show shorter branches than other available gastropods, aplacophorans, bivalves, cephalopods or scaphopods (Grande et al. 2008; Cunha et al. 2009; Doucet-Beaupré et al. 2010; Stöger and Schrödl 2013), and thus potentially introduce less bias into the phylogenetic analyses.

**DNA sequencing**

Total genomic DNA was isolated using the DNeasy® Blood & Tissue Kit (QIAGEN, Carlsbad, California). Partial sequences of the mt genes rrmL, cox1, and cob were amplified with appropriate universal primer pairs (see Supplemental File 1 for primer sequences and specific PCR annealing temperatures). PCR reactions contained 2.5 μl of 10× Taq Buffer advanced, 1.5 μl of MgCl2 (25 mM), 0.5 μl of dNTP mixture (10 mM each), 0.5 μl of each primer (10 μM each), 0.5 μl of template DNA (10–40 ng/μl), 0.2 μl 5PRIME® Taq DNA polymerase (5 units/μl; 5PRIME GmbH, Hamburg, Germany), and DEPC water up to 25 μl. An initial denaturation step (94°C for 5 min) was followed by 45 cycles of denaturing at 94°C for 60 s, annealing at 48–51°C for 60 s, and extending at 72°C for 90 s, and a final extension at 72°C for 5 min. PCR products were purified by ethanol precipitation (Sambrook et al. 1989), and sequenced in automated DNA sequencers (ABI PRISM® 3700) using the BigDye® Terminator v3.1 cycle-sequencing kit (Applied Biosystems, Foster City, CA, USA) and PCR primers, following the manufacturer’s instructions.

The following procedure was used for the amplification of mt genomes: after previously determined partial mt sequences, species-specific primers were designed to serve as anchors in the amplification of the remaining mt genome by long-range PCR of 2–3 overlapping fragments (see Supplemental File 1 for primer sequences and PCR annealing temperatures). Long-PCR reactions were carried out in a total volume of 25 μl, containing 2.5 μl of 10× LA Buffer II (with MgCl2), 4 μl of dNTP mixture (2.5 mM each), 0.5 μl of each primer (10 μM), 0.5 μl of template DNA (10–40 ng/μl), 0.25 μl TaKaRa LA® Taq DNA polymerase (5 units/μl; TaKaRa BioInc., Otsu, Japan). An initial denaturation step (98°C for 30s) was followed by 45 cycles of denaturation at 98°C for 10 s, annealing at 53–60°C for 30 s, and extension at 68°C for 60 s per Kb, and a final extension step at 68°C for 15 min. Long-PCR products were purified by ethanol precipitation and all fragments corresponding to each mt
Table 1. Taxa and genes used in this study. GenBank accession numbers are shown for the five partial gene sequences used in the multilocus data sets, and newly sequenced mitochondrial genes are highlighted in grey. Information on vouchers and collection data are provided in Supplemental File 1.

| Classification | Genus, species | Gene       | rrnL    | cox1    | cob    | 18S       | 28S       |
|----------------|----------------|------------|---------|---------|--------|-----------|-----------|
| CHITONIDA: ACANTHOCHITONINA | Acanthochitona avicula (Carpenter) |             | KJ574073 | KJ574093 | KJ574111 | AF120503  | AF120566  |
|                | Acanthochitona crinita (Pennant) |             | AY377609 | AF120627 |         |           |           |
|                | Amicula vestita (Broderip and Sowerby) |             | KJ574072 | KJ574092 | KJ574110 |           |           |
|                | Boreochiton beringensis (Jakovleva) |             | KJ574065 | KC776930 |         |           |           |
|                | Choneplax lata (Guilding) |             | KJ574074 | KF184983 |         |           |           |
|                | Cryptochiton stelleri von Middendorff |             | KJ569363 | KJ569363 | KJ569363 | AY377655  | AY377686  |
|                | Cryptonchus floridus (Dall) |             |         |         |         |           |           |
|                | Crypotoaxa iredae Ashby |             | KJ574076 | KJ574096 | KJ574113 |           |           |
|                | Cryptoplax japonica Pillsby |             | AY377611 | HQ907842 |         | AY377656  | AY145402  |
|                | Cyanoplax cf. caverna Earnisse |             | KJ569361 | KJ569361 | KJ569361 |           |           |
|                | Dendrochiton flectens (Carpenter) |             | EU406917 | EF159598 |         | EU406795  | EU407038  |
|                | Dendrochiton gothicus (Carpenter) |             | KJ574064 | KJ574084 | KJ574104 |           |           |
|                | Hemiarthrum setulosum Carpenter in Dall |             | KJ574075 | KJ574095 | KJ574112 | KC887268  | KC887285  |
|                | Katharina tunicata Wood |             | NC_001636 | NC_001636 | NC_001636 | AY377650  | HQ907793  |
|                | ‘Lepidochitona’ beanii (Carpenter) |             | KJ574063 | KJ574083 | KJ574103 |           |           |
|                | Lepidochitona caprea (Scacchi) |             | KJ574062 | KJ574082 | KJ574102 |           |           |
|                | Lepidochitona cinerea (Linnaeus) |             |         |         |         | AY377701  |           |
|                | Mopalia muscosa (Gould) |             | EU406894 | EF159580 |         | AY377648* | DQ279956  |
|                | Nuttallina californica (Nuttall MS, Reeve) |             | KJ569362 | KJ569362 | KJ569362 | AY377638  | AY377669  |
|                | Nuttallochiton mirandus (Smith MS, Thiele) |             | KJ574070 | KJ574089 | KJ574108 |           |           |
|                | Placiphora albida (Blainville) |             | KJ574068 | KJ574087 | KJ574106 | AY377649  | AY377680  |
|                | Placiphora biramosa (Quoy & Gaimard) |             | KJ574069 | KJ574088 | KJ574107 |           |           |
|                | Placiphorella sp. A |             | KJ574071 | KJ574090 | KJ574109 |           |           |
|                | Placiphorella velata (Carpenter MS, Dall) |             | EU406910 | EF159591 |         | EU406785  | EU407030  |

(Continued)
Table 1. (Continued).

| Genus/Marine Mollusk | GenBank Accession Numbers | GenBank Accession Numbers | GenBank Accession Numbers | GenBank Accession Numbers |
|---------------------|---------------------------|---------------------------|---------------------------|---------------------------|
| *Schizoplax brandii* (von Middendorff) | KJ574067 | KJ574086 | KJ574105 | | AY377635 | AY377665 |
| *Tonicella lineata* Wood | EU406998 | EF159682 | | | | |
| *Tonicella lokii* Clark | KJ574066 | KJ574085 | | | | |
| **CHITONIDA: CHITONINA** | | | | | | |
| *Callochiton bouveti* Thiele | KJ574080 | KJ574100 | KJ574117 | | HQ907735 | HQ907791 |
| Chaetopleura apiculata* (Say in Conrad) | AY377590 | AY377704 | | | AY377636 | AY145398 |
| *Ischnochiton boninensis* Bergenhayn | KJ574079 | KJ574099 | KJ574116 | | AY377639 | AY377673 |
| *Loricavolvox* (Reeve) | AY377601 | | | | AY377647 | DQ279954 |
| *Tonicia forbesii* Carpenter | KJ574081 | KJ574101 | KJ574118 | | | |
| **LEPIDOPLEURIDA** | | | | | | |
| *Hanleyella oldroydi* (Dall) | KJ574077 | KJ574097 | KJ574114 | | HQ907780 | HQ907800 |
| *Nierstraszella lineata* (Nierstrasz) | KJ574078 | KJ574098 | KJ574115 | | HQ907784 | HQ907838 |
| **GASTROPODA** | | | | | | |
| *Bolinus brandaris* | NC_013250 | NC_013250 | NC_013250 | | DQ279944 | DQ279986 |
| *Haliotis rubra* | NC_005940 | NC_005940 | NC_005940 | | FJ605494 | GQ160635 |
| *Ilyanassa obsoleta* | NC_007781 | NC_007781 | NC_007781 | | AY145379 | AY145411 |

* See note on Supplemental File 2.
genome were pooled together in equimolar concentrations for massive parallel sequencing. Individual, indexed and pair-ended DNA libraries were constructed for each mt genome using the TrueSeq® DNA Sample Kit (Illumina, San Diego, CA, USA) and following the manufacturer’s instructions. The three mt genomes, each carrying a different index, were pooled together with several indexed mt genomes from other projects into a single Illumina lane (HiSeq2000) and sequenced at Macrogen (Seoul, Korea).

**Assembly and annotation of mitochondrial genomes**

Raw reads from each of the three mt genomes were pulled apart by their unique indices used in library construction. Prior to assembly, the quality of the raw Illumina reads was checked with FastQC v.0.10.1 (Andrews 2011); all adapter sequences were removed with SeqPrep (St John 2013) and reads were trimmed and filtered out according to their quality scores using PRINSEQ v.0.20.3 (Schmieder and Edwards 2011). CLC Genomics Workbench® v.5.5.1 (http://www.clcbio.com) was used for the de novo assembly of mt genomes. As a quality measure of the de novo assemblies, reads were mapped back to the assembled mt genomes (treated as circular molecules), in all cases over 95.65% of the reads mapping concordantly to them. The probabilistic variant calling method implemented in CLC Genomics Workbench was used to check and correct possible disagreements among reads that mapped to the mt genome sequences.

The new mt sequences were annotated by comparison with other molluscan mt genomes using the software DOGMA (Wyman et al. 2004) and MITOS (Bernt, Donath, et al. 2013). These pipelines identify genes by BLAST (Altschul et al. 1990) searches after open reading frames of protein-coding genes are translated using the appropriate invertebrate mt genetic code, and tRNA genes are further identified based on their putative cloverleaf secondary structure. MITOS further uses covariance models and accounts for secondary structure for the identification of tRNA and rRNA genes (Bernt, Donath, et al. 2013). The start and end positions of all genes were manually checked and modified if necessary for creating a consistent annotation and to avoid unnecessary overlap between genes.

**Multiple sequence alignment and phylogenetic reconstruction**

Multiple sequence alignments were created for each individual gene. Protein-coding genes (atp6, atp8, cob, cox1-3, nad1-6, nad4L) were aligned at the amino acid level using MAFFT v.7.122 (Katoh and Standley 2013) with default settings, and consistent nucleotide alignments were generated with TranslatorX (Abascal et al. 2010). Ribosomal RNA genes (rrnS, rrnL, 18S, 28S) were aligned with MAFFT using an iterative algorithm optimized for sequences with multiple conserved domains and long gaps (E-INS-i). Alignment regions with unreliable positional homology were discarded using GBlocks (Castresana 2000), an algorithm whose thresholds depend on the number of taxa in the alignment. The following parameters were used for the whole mt genome data sets (and multilocus data sets, in parentheses): minimum number of sequences for a conserved position 4 (19), minimum number of sequences for a flanking position 8 (8), minimum length of a block 5 (5), allowed gap positions with half (with
Individual nucleotide and amino acid (for protein-coding) gene alignments of complete mt genes from four chitons and three gastropods were concatenated into (1) the mt nucleotide and (2) mt amino acid data sets, respectively. The gene alignments of partial mt (cob, cox1, rrnL) and nuclear (18S, 28S) genes from 34 chitons and three gastropods were concatenated into (3) the multilocus nucleotide and (4) multilocus amino acid data sets.

For all four matrices, best-fit models of sequence evolution and best-fit partitioning schemes were statistically selected according to the Bayesian information criterion (BIC; Schwarz 1978) using PartitionFinder and PartitionFinderProtein (Lanfear et al. 2012) (see Supplemental File 1 for results). Probabilistic phylogenetic reconstruction was performed on all four data sets using maximum likelihood (ML; Felsenstein 1981) and Bayesian inference (BI; Huelsenbeck and Ronquist 2001). Additional ML analyses were performed for the separate mt and nuclear genes from the multilocus data set, in order to assess the power of resolution of and congruence between these two different marker types. ML reconstruction used the rapid hill-climbing algorithm implemented in RAxML v.7.3.1 (Stamatakis 2006), starting from 100 randomized maximum parsimony trees. Two independent BI analyses were run using MrBayes v.3.1.2 (Ronquist and Huelsenbeck 2003), each analysis consisting of four MCMC chains and 10 million generations, sampling trees every 1000 generations. Convergence of Markov chains was checked a posteriori using Tracer (Rambaut and Drummond 2009). Trees sampled during the first million generations were discarded as burn-in to prevent sampling before stationarity of MCMC chains and post-burn-in trees were summarized into a majority-rule consensus tree. Support for internal branches was evaluated by non-parametric bootstrapping (Felsenstein 1985) (ML) and posterior probabilities (BI).

Ten alternative tree topologies (see Table 2, results and discussion) were evaluated with the non-parametric AU test (Shimodaira 2002), as implemented in Consel v.0.1i (Shimodaira and Hasegawa 2001) using one million multi-scale bootstrap replicates.

Table 2. Results from the approximately unbiased (AU) tests for 10 alternative topologies, estimated from the multilocus nucleotide data set. Similar results were obtained from the analysis of the multilocus amino acid data set (see Supplemental File 1). For each topology, the log-likelihood (lnL) and p-value of AU tests are given. Significant results (p < 0.05) are highlighted in bold italics.

| Phylogenetic hypotheses                              | lnL       | AU p-value |
|------------------------------------------------------|-----------|------------|
| Unconstrained BI tree (Figure 2)                     | −26493.589558 | 0.687 |
| Plaxiphora + Cryptoplacoidea                          | −26495.325669 | 0.139 |
| Monophyletic Acanthochitonidae                       | −26504.063533 | 0.629 |
| Monophyletic Lepidochitonidae                        | −26589.726109 | 0.177 |
| Acanthochitonidae sensu Sirenko 2006                 | −26775.794530 | 4 × 10⁻⁶ |
| Tonicellidae sensu Sirenko 2006                       | −26668.571974 | 0.006 |
| Mopaliidae sensu Sirenko 2006                        | −26822.354013 | 0.021 |
| Sirenko’s (2006) system                              | −27260.157722 | 4 × 10⁻³⁶ |
| Okusu et al. (2003)                                  | −26529.452137 | 9 × 10⁻⁵⁵ |
| Kelly and Eernisse (2008)                            | −26529.383963 | 8 × 10⁻³⁸ |
| Sigwart et al. (2013)                                | −26513.348467 | 9 × 10⁻⁵³ |
ML trees that satisfied the constraints of the alternative tree topologies were inferred with RAxML, and site-wise log-likelihoods were computed applying best-fit models and partitions. AU tests were conducted in both the multilocus nucleotide and amino acid data sets. All matrices and phylogenetic trees are deposited in Treebase (Sanderson et al. 1994) under Study ID 16189 and URL http://purl.org/phylo/treebase/phylows/study/TB2:S16189?format=html.

Results

New chiton mt genomes

The complete sequences of the mt genomes of Cryptochiton stelleri, Cyanoplax caverna, and Nuttallina californica were determined. Illumina sequencing produced 21.27, 21.02 and 8.81 Mb of data and 93.82%, 94.83% and 97.5% of the raw reads passed the adapter and quality trimming thresholds for the above mt genomes, respectively. The lengths of these mt genomes (15082, 15141 and 15604 bp, respectively) were similar to that reported for Katharina tunicata (15532 bp). All assemblies had a sequencing depth over 5000×. They all encoded the typical 37 genes found in other metazoan mt genomes (Boore 1999). Most protein-coding genes started with the codon ATG, with the following exceptions: nad4 started with TAT in C. stelleri and with AGT in N. californica, and nad5 started with TTA in N. californica. Likewise, most stop codons were complete (TAA or TAG) although incomplete stop codons (TA or T) that are likely post-transcriptionally completed (Ojala et al. 1981) were also present in some cases. All tRNA genes could be folded into cloverleaf secondary structures.

The gene arrangements of the newly determined mt genomes were identical to that of K. tunicata regarding the relative position of protein-coding genes. However, some noteworthy differences were found in the relative position of tRNA genes. A first difference was the position of the trnD gene, which appears between cox1 and cox2 in K. tunicata, but it is located between cox2 and atp8 in all other newly determined mt genomes (Figure 1). In addition, the long (424 bp) AT-rich region reported between trnD and cox2 in K. tunicata (Boore and Brown 1994) is only 4–12 bp in the newly sequenced mt genomes. Besides the position of trnD, the mt genome order of C. stelleri conforms to that of K. tunicata (Figure 1). A second major difference is shared in the mt gene orders of C. caverna and N. californica, which are alike in having seven tRNA genes that are encoded on the plus strand: trnE, trnG, trnQ, trnW, trnY, trnC and trnM (or 5’-EGQWYCM-3’ for short). This gene cluster is likely derived relative to the same cluster encoded on the minus strand in K. tunicata, C. stelleri, a bivalve, a cephalopod and a gastropod (Figure 1). For K. tunicata, C. stelleri and the cephalopod, the gene order is 5’-MCYWQGE-3’ when listed 5’ to 3’ on the plus or non-coding strand, and the bivalve and gastropod show each one additional tRNA translocation (Figure 1).

In the three newly determined mt genomes as well as in K. tunicata, most genes are close to each other or separated by very short (<12 bp) intergenic regions (Boore and Brown 1994). Remarkable exceptions to this rule were the relatively long and AT-rich intergenic regions found between genes encoded by opposite strands. The region between atp6 and trnF averaged 49.7 ± 29.3 bp and AT content of 89.9% in K. tunicata, C. stelleri, and C. caverna but only 7 bp (85.7% AT) in N. californica; in K.
Figure 1. Phylogenetic relationships and gene arrangements of available chiton mitochondrial genomes. Majority-rule consensus tree from the Bayesian analysis of the mitochondrial genome nucleotide data set (outgroup taxa is omitted for simplicity). Identical topologies were recovered from all other analyses of mitochondrial genome data sets (see main text). Numbers at nodes are posterior probabilities and maximum likelihood bootstrap proportions, respectively. Scale bar is in substitutions per site. Mitochondrial gene orders of *Haliotis rubra* (Gastropoda), *Octopus vulgaris* (Cephalopoda) and *Solemya velum* (Bivalvia) are shown for comparison. Genes encoded by the minus strand are underlined; rearranged genes are highlighted in red (translocations) and green (changes of coding strands).
tunicata and C. stelleri, the region between trnE and cox3 was 137.5 ± 5.0 bp and 89.5% AT-rich on average, and in C. caverna and N. californica the region between rrnS and trnE was 217.5 ± 33.2 bp and 79.5% AT-rich.

Molecular phylogeny of Acanthochitonina

The concatenated mt gene alignment (cob, cox1, rrnL) had a total number of 1545 positions, 901 of which were variable (845 total and 461 variable positions when protein-coding genes were analysed as amino acids), whereas the concatenated nuclear gene alignment (18S, 28S) had 1479 positions, 309 of which variable. The ML analyses of concatenated mt genes produced very similar results compared with the multilocus (mt + nuclear) data sets (Supplemental File 2). In contrast, the ML analysis of concatenated nuclear genes resulted in a poorly resolved phylogeny (Supplemental File 2), suggesting that these short fragments of nuclear rRNA genes alone lack power to resolve the phylogenetic relationships among chitons, probably due to a lower number of phylogenetically informative positions compared with mt genes. This lack of resolution might have been further exacerbated by the lower number of taxa (20 out of 37) in nuclear genes compared with the multilocus data set. Nonetheless, nuclear markers provided valuable information when analysed in combination with mt markers (combined data sets), which is reflected in higher bootstrap supports (Supplemental File 2). For the multilocus data set, both BI and ML arrived at similar tree topologies for nucleotide- and amino acid-level analyses (Figure 2 and Supplemental File 2). In all cases, groupings consistent with the orders Lepidopleurida and Chitonida were each recovered with high support, albeit including only selected non-chiton outgroups and sparse taxon representation outside Acanthochitonina. Within Chitonida, Callochiton Gray was sister group to two main lineages, corresponding to the suborders Chitonina and Acanthochitonina. The monophyly of Acanthochitonina received high support from both BI and ML analyses. With our sampling of outgroups, Plaxiphora Gray is recovered basal in Acanthochitonina, with high support from BI but only with moderate support from ML. Topology tests significantly rejected the conventional position of Plaxiphora within Mopaliidae, but could not reject a sister group relationship with Cryptoplacoidea (Table 2). The remaining Acanthochitonina were divided into two main lineages that received strong statistical support, corresponding to the superfamilies Cryptoplacoidea and Mopalioidae sensu Sirenko 2006, if Plaxiphora is excluded from Mopalioidae and certain genera are reassigned from one to the other (see below). Nuttallochiton Plate is recovered basal within Cryptoplacoidea, receiving high support from both ML and BI (Figure 2 and Supplemental File 2). Bayesian analyses of the nucleotide multilocus data set recovered Hemiarthrum (Hemiarthridae) as the sister group of Cryptoplax (Cryptoplacidae) with high (>0.95) posterior probabilities, and both together as the sister group of Acanthochitonidae (Figure 2). ML analyses and BI of the amino acid multilocus data set favoured Hemiarthrum as sister to Acanthochitonidae, but receiving low support (Supplemental File 2). Within Acanthochitonidae, Acanthochitona is recovered as paraphyletic, here represented by Acanthochitona crinita and Acanthochitona avicula, with respect to Choneplax + Cryptoconchus (Figure 2). The monophyly of Acanthochitona could not be rejected by topology tests (Table 2).
Within Mopalioida, Bayesian analyses found *Lepidochitona cinerea* and *Lepidochitona caprearum* together to be sister to *Cyanoplax*, *Schizoplax*, *Nuttallina* and ‘*Lepidochitona*’ *beanii*. The relationships (*Cyanoplax*, (*Schizoplax*, (*Nuttallina* + ‘*L.*’ *beanii’*)) received high support in all multilocus analyses (Figure 2 and Supplemental File 2). Our ML analysis of the multilocus amino acid data set did not recover the basal position of *L. cinerea* + *L. caprearum* within Lepidochitonidae, which were instead recovered basal in Mopaliidae with weak support (Supplemental File 2).

Certain members of Lepidochitonidae exhibit relatively long internal branches compared with other Mopalioida or Cryptoplacoidea. Our phylogenetic analyses recover a non-monophyletic *Lepidochitona* Gray as currently defined (Sirenko 2006), even though topology tests were unable to reject its monophyly (Table 2).

A second lineage within Mopalioida is highly supported and includes most genera conventionally placed in Mopaliidae (including the type genus *Mopalia* Gray) except for *Plaxiphora* and *Nuttallochiton* (see above). In addition, it includes various genera such as *Tonicella*, *Boreochiton* GO Sars (see Sirenko 2000), and *Dendrochiton* Berry (formerly a subgenus of *Lepidochitona*, Kaas and Van Belle 1985b; but see Berry 1917, 1918, who originally considered it a subgenus of *Mopalia*), and *Cryptochiton*, which was formerly assigned to Acanthochitonidae (Cryptochitoninae) within Cryptoplacoidea. A position

Figure 2. Molecular phylogeny of Acanthochitonina. Majority-rule consensus tree from the Bayesian analysis of the multilocus nucleotide data set, which includes three mitochondrial and two nuclear markers. Additional phylogenetic results are available in the Supplemental File 2. Note the proposed taxonomic arrangements shown by vertical lines. Numbers at nodes are support values from posterior probabilities and maximum likelihood bootstrap proportions, respectively. Scale bar is in substitutions per site.
of Cryptochiton within Acanthochitonidae was highly significantly rejected by AU tests (Table 2). Within this second mopalioid lineage, Placiphorella Dall branched off first, followed by Boreochiton + Tonicella. Katharina was sister to Amicula + Cryptochiton and Dendrochiton + Mopalia (Figure 2). These relationships received strong statistical support and were congruently recovered by the different methods (ML and BI) and data sets (nucleotides and amino acids), even though the BI on the multilocus amino acid data set did not resolve the position of Katharina (Supplemental File 2).

The nucleotide mt genome data set had 12,610 positions, 7891 of which were variable (5606 total and 3332 variable positions with protein-coding genes at the amino acid level). Both BI and ML arrived at identical topologies at both the nucleotide- or amino acid-levels (Figure 1), with slight differences in branch lengths. The phylogeny was fully congruent with the phylogenetic analyses based on the multilocus data set: Cryptochiton + Katharina together were sister taxon to Nuttallina + Cyanoplax, with all nodes receiving maximal support from both BI and ML.

Discussion

Chiton mitogenomics: next-generation sequencing, phylogenetic performance and new gene rearrangements

Complete mt genomes have increasingly been used for studying phylogenetic relationships in various groups of metazoans, and the number of such available sequences is growing rapidly (over 3600 in GenBank, including 148 molluscan mt genomes; http://www.ncbi.nlm.nih.gov/ as of November 2013). At present, next-generation sequencing techniques offer cost-effective methods for massive sequencing of mt genomes (e.g., McComish et al. 2010; Botero-Castro et al. 2013; Hahn et al. 2013). Here, we took advantage of the high number of paired and relatively long (2 × 100 bp) reads produced by Illumina HiSeq2000 to sequence several chiton mt genomes from long-PCR amplicons. The use of indexed libraries enables pooling tens of mt genomes into a single Illumina lane, producing enough reads for a high-coverage de novo assembly of mt genomes.

For about 20 years, a single mt genome has been available as representative of the molluscan class Polyplacophora (Boore and Brown 1994). Thus, our newly generated data considerably increase available mt genome information for the suborder Acanthochitonina, adding three chiton species (see also Veale et al. Forthcoming 2014). Our phylogenetic analyses demonstrated that complete mt genome sequences are useful for reconstructing robust phylogenetic hypotheses in chitons (Figure 2). In addition, gene order changes can be important phylogenetic markers (Rokas and Holland 2000), as demonstrated here for Cyanoplax and Nuttallina Carpenter MS, Dall (Figure 1). Complete mt sequences are also essential to understand the molecular mechanisms behind gene rearrangements, replication and transcription in this organelar genome (Bernt, Braband, et al. 2013). The mt gene order of Katharina tunicata has long been considered to represent that of chitons (Boore and Brown 1994; Rawlings et al. 2001; Grande et al. 2002; Boore et al. 2004; Yokobori et al. 2004). However, the gene orders and phylogenetic position of the newly determined chiton mt genomes suggest that the coxl-trnD-cox2 gene arrangement found in K. tunicata is derived from the putatively ancestral coxl-cox2-trnD order (Figure 1). Not only do the closely related Cryptochiton stelleri, other Mopalioida (Cyanoplax caverna, Nuttallina californica) and Chitonida (Sypharochiton; Veale et al. Forthcoming
This gene order is also found in Cephalopoda (e.g., *Octopus* sp., *Vampyroteuthis* sp.; Yokobori et al. 2004; Allcock et al. 2011) and several Gastropoda (e.g., *Nerita* sp., *Lunella* sp.; Castro and Colgan 2010; Williams et al. 2014; see Stöger and Schrödl 2013). In addition, *cox1* and *cox2* are adjacent in some Bivalvia (e.g., *Solemya velum* Say: Plazzi, Ribani and Passamonti unpublished, RefSeq NC_017612).

Another derived feature of the mt genome of *K. tunicata* is the 424 bp-long, AT-rich non-coding sequence between *trnD* and *cox2*. This intergenic region is absent or very short in all three other chiton mt genomes reported here. Likewise, the change in coding strand of the 5′-*MCYWQGE*-3′ tRNA gene cluster found in the mt genomes of *C. caverna* and *N. californica* is derived from the putative ancestral condition found in *K. tunicata*, *C. stelleri*, and other molluscs including the above-mentioned aplacophorans, bivalve, gastropods and cephalopods (Figure 1). In fact, the 5′-*MCYWQGE*-3′ tRNA gene cluster could be a synapomorphy for molluscs, which evolved from a shorter 5′-*MCYWQ*-3′ cluster that is present in most lophotrochozoans (Stöger and Schrödl 2013). Interestingly, the derived gene order of *C. caverna* and *N. californica* could represent a molecular synapomorphy uniting at least *Cyanoplax* and *Nuttallina* plus associated genera, and possibly other members of Lepidochitonidae (see discussion below). However, more data from other genera in Mopaliodea are needed in order to pinpoint any clade associated with this mt genome rearrangement event. In particular, whether or not this rearrangement is also present in *Schizoplax* would provide another line of evidence in favour of the close relationships of *Schizoplax* and *Cyanoplax* within Lepidochitonidae (Eernisse 2009; Sirenko et al. 2013, see below). For example, discovering the rearrangement found in *Cyanoplax* and *Nuttallina* in other members of Lepidochitonidae represented here (e.g., *Lepidochitona cinerea*, *L. caprearum*, ‘*L.’ beanii’, *Schizoplax brandtii*), but not in species of *Tonicella*, *Boreochiton*, or *Dendrochiton*, would support our new concepts of Lepidochitonidae and Mopaliidae (see discussion below). The absence of this rearrangement in *L. cinerea* and *L. caprearum* would not invalidate our new concept of Lepidochitonidae, but it would imply that this gene rearrangement occurred in the ancestor of a particular subgroup nested within Lepidochitonidae. In that case, more robust support or rejection of the monophyly of Lepidochitonidae would need to come from other data sources. This rearrangement calls for further exploration of mt gene rearrangements in additional chiton lineages, including also Lepidopleurida and Chitonina. New mt gene rearrangements could not only provide additional phylogenetic synapomorphies with implications in higher-level chiton phylogeny, but should also lead to a better understanding of the origin and evolution of mt gene orders in this molluscan group.

The origin of the *cox1-trnD-cox2* gene rearrangement found in *K. tunicata* could be explained by the ‘tandem duplication–random loss’ (TDR) model, which is produced by slipped-strand mispairing or asynchrony in the points of initiation and termination of replication (Boore 2000). This is considered the main mechanism responsible for gene rearrangements in vertebrates (e.g., San Mauro et al. 2006; Irisarri et al. 2012), although evidence supporting this model in non-vertebrates is scarce (see Grande et al. 2008). In this case, a duplication of at least *cox2* and *trnD* would be required, which would be followed by the loss of redundant copies.
(upstream copy of \textit{cox2} and downstream copy of \textit{trnD}). Alternatively, the \textit{trnD} gene might have been moved upstream of the \textit{cox2} gene by a transposition mechanism mediated by direct repeats (Macey et al. 1997). Structures that form stems and loops such as tRNAs or replication origins have been suggested to mediate in gene duplications (Macey et al. 1997; San Mauro et al. 2006; Irisarri et al. 2010). In contrast, the change in coding strand of the tRNA gene cluster found in \textit{C. caverna} and \textit{N. californica} cannot be explained by the tandem duplication of mt regions. Traditionally, mt genomes have been considered to be free of recombination, and thus mechanisms such as the TDRL model have been proposed to explain gene rearrangements (Boore et al. 2005). However, an increasing number of rearrangements have been shown to be irreconcilable with the above mechanism and alternative models have been invoked. Some studies found evidence of recombination of mt genomes (Thyagarajan et al. 1996; Tsaousis et al. 2005), and illegitimate recombination via minicircle has been suggested to be responsible for some mt gene rearrangements (Dowton and Campbell 2001; Mueller and Boore 2005; Kurabayashi et al. 2008). In the model of illegitimate recombination, a region of the mt genome might be excised from the molecule to form a minicircle that has the potential of inserting into another location, resulting in non-tandem duplications. This illegitimate recombination mechanism could explain gene rearrangements found in \textit{K. tunicata C. caverna} and \textit{N. californica} (Figure 1).

Besides gene rearrangements, the presence of long AT-rich intergenic regions between genes encoded by opposite strands is particularly interesting. Similar regions have also been reported in the mt genomes of cephalopods (Boore 2006) and gastropods (Grande et al. 2008). It has been suggested that these regions might be involved in the replication process, similar to the two intergenic regions that contain the origins of replication and signals of transcriptions in vertebrate mt genomes (the control region or D-loop and a short intergenic region within a tRNA gene cluster; Boore 1999). Arthropods and possibly nematodes (Bernt, Braband, et al. 2013) also have similar AT-rich intergenic regions that have been suggested to contain the origins of replication of both strands (Zhang and Hewitt 1997; Saito et al. 2005). Nevertheless, any such role of these AT-rich regions in replication has not yet been demonstrated and their absence in other mt genomes raises the question of whether or not they are involved in replication.

\textbf{Molecular phylogeny of Acanthochitonina}

Although taxon sampling was not designed to address this issue, our analyses support a deep split between Lepidopleurida and Chitonida. The monophyly of Chitonida is acknowledged and uncontroversial (Buckland-Nicks 1995; Sirenko 1997, 2006; Sigwart et al. 2011, 2013), but the position of \textit{Callochiton} (representing Callochitonidae) has remained unsettled. In our analysis, \textit{Callochiton} is recovered basal in Chitonida with strong support, in agreement with some previous molecular studies (Wilson et al. 2010; Sigwart et al. 2011), but contrasting with others that favoured \textit{Callochiton} to be sister to Acanthochitonina (Sigwart et al. 2013), or Lepidopleurida (Okusu et al. 2003). Remarkably, only Wilson et al. (2010) and our study provide strong molecular support for the position of \textit{Callochiton} being basal in Chitonida, a hypothesis that is further supported by the observations that egg hull, sperm and fertilization process are intermediate between Lepidopleurida and
Callochiton shares with other Chitonida not only derived sperm and egg hull characters but also has typical Chitonida slits in its valve insertion plates, and shares with other Chitonida lateral gill placement, not posterior as in Lepidopleurida (Eernisse 1985; Sirenko 1993; Eernisse and Reynolds 1994; Sigwart 2008). To date, sperm and egg hull morphology apparently intermediate between Chitonida and Lepidopleurida provides intriguing evidence that Callochiton lacks what are presumed to be synapomorphies of an unnamed clade comprised of the remaining Chitonida besides Callochitonidae (Buckland-Nicks and Hodgson 2000). This hypothesis contrasts with the classification of Sirenko (2006), who included Callochitonidae within Chitonina. With Callochitonidae excluded, other Chitonida are united by synapomorphies of asymmetrical sperm mitochondria (Buckland-Nicks 1995) and the possession of elaborate egg hull projections of two contrasting types (Risbec 1946; Eernisse 1982, 1984; Sirenko 1993). These correspond nearly to the currently recognized Chitonida suborders: Acanthochitonina sensu Sirenko 2006 have cup- or cone-like hull projections whereas Chitonina sensu Sirenko 2006 (without Callochitonidae) have more numerous spiny hull projections.

Members of Acanthochitonina have abanal lateral gill rows, not adanal (posterior or lateral) as in all other chitons (Eernisse 1984; Sirenko 1993; reviewed in Eernisse and Reynolds 1994). The presumed plesiomorphic adanal condition (Lepidopleurida, Callochitonidae) corresponds to adding gills both anterior and posterior of the first-formed gill pair in ontogeny. In contrast, members of Acanthochitonina add gills only to the anterior of the first (and later largest) gill pair (Sirenko 1993).

Within Acanthochitonina, our analyses recovered two main lineages that only approximately coincide with the superfamilies Mopalioidea and Cryptoplacoidea sensu Sirenko 2006 (Figure 2 and Table 3). Our results are in agreement with those of Sigwart et al. (2013) in the taxonomic position of certain key genera. Both Plaxiphora and Nuttalallochiton were classified in Mopaliidae sensu Sirenko 2006, but they are resolved well apart from Mopalioida in our analyses (Figure 2) and previous molecular studies (Okusu et al. 2003; Sigwart et al. 2013). The basal split of Plaxiphora in Acanthochitonina received high Bayesian posterior probabilities from the multilocus data set (0.99), but had relatively low (<70%) bootstrap support in ML analyses (Figure 2 and Supplemental File 2). Previous studies favoured Plaxiphora as sister group to the remaining Cryptoplacoidea, but were not conclusive (Okusu et al. 2003; Sigwart et al. 2013). Neither hypothesis is yet well supported. Despite discrepancies between previous and our phylogenetic analyses in the specific phylogenetic positions of Nuttalallochiton and Plaxiphora, these genera are clearly resolved well apart from Mopalioida. The inclusion of Nuttalallochiton and Plaxiphora within Mopaliidae sensu Sirenko 2006 was rejected by topology tests, but they could not reject an alternative placement of Plaxiphora within Cryptoplacoidea (Table 2). Moreover, because our results support a fundamentally different position of Cryptochiton, which is instead associated with Mopaliidae, our topology test rejects the monophyly of Acanthochitonidae sensu Sirenko 2006 (Table 2). The placement of Plaxiphora outside Mopaliidae is further supported by aesthete morphology, which has been shown to be more similar for Plaxiphora and species within Acanthochitonidae than any of these are to Mopaliidae (Vendrasco et al. 2008). Our support for Plaxiphora as the sister lineage of all other Acanthochitonina would support the Mopaliidae aesthete pattern as derived and
the *Plaxiphora* or Acanthochitonidae condition as plesiomorphic for Acanthochitonina (Vendrasco et al. 2008). The distant phylogenetic relationship between *Plaxiphora* and *Mopalia* is especially noteworthy considering the strikingly similar external appearance frequently seen in members of these two genera, with an often broad body outline and girdles covered with spicules (*Plaxiphora*) or setae (*Mopalia* and certain genera within Mopaliidae, as herein restricted). Our results suggest that this superficial resemblance is due to convergent evolution, opening a new opportunity to explore the adaptive value of such morphology.

*Hemiarthrum setulosum* is an enigmatic species whose phylogenetic (and taxonomic) position has been long debated. For a long time, it was classified within Lepidopleurida (Pilsbry 1892–1894; Thiele 1909–1910; Bergenhayn 1955; Van Belle 1983; Sirenko 1993), but Sirenko (2006) reassigned it to Cryptoplacoidea, a result confirmed by the first molecular study including *Hemiarthrum* (Sigwart et al. 2013), and our analyses. As has long been noted, *Hemiarthrum* has tufts of spicules in its girdle, as in other Cryptoplacoidea. The position of *Hemiarthrum* outside Lepidopleurida is further supported by gill features. Even though it has relatively few gills and short gill rows (only about eight gills per row), there is an ‘interspace’ between the last gill in each row (Eernisse 1984) giving it a typical Chitonida lateral placement of its gills (see above), in contrast to the posterior condition ‘without interspace’ found in Lepidopleurida. Also, it has an abanal condition of its gill rows, supporting its placement within Acanthochitonina (Sirenko 2006). In Cryptoplacoidea Sirenko (2006) argued for a general evolutionary trend towards the reduction of the tegument that is most strongly pronounced in *Cryptochiton* and *Cryptoconchus* sp. However, our phylogenetic hypothesis strongly points out that this reduction of the tegument evolved convergently in the above-mentioned genera, in agreement with Sigwart et al. (2013), who showed the independent reduction of the tegument also in *Choriplax*.

Within Mopalioidea, our analyses recover two main clades with strong support, which we here refer to by the familiar names Lepidochitonidae and Mopaliidae, but with a revised composition that reflects our phylogenetic results (Table 3). Within Lepidochitonidae, we include *Lepidochitona*, *Cyanoplax sensu* Eernisse et al. 2007, *Nuttallina*, and *Schizoplax*. Besides *Schizoplax*, which has been classified in its own family (see below), all other above-mentioned genera have been previously assigned to either Lepidochitonidae *sensu* Van Belle 1983 or Tonicellidae *sensu* Sirenko 2006. Nevertheless, Tonicellidae is not an appropriate name for this grouping because the type genus (*Tonicella*) instead nests deeply within Mopaliidae Dall, 1889 (Figure 2), so we consider Tonicellidae a synonym of the latter. Following Stebbins and Eernisse (2009), our preference for Lepidochitonidae Iredale, 1914 avoids this problem, especially because Lepidochitonidae has been more widely used than the approximately equivalent Tonicellidae. Thus, we exclude *Tonicella*, *Boreochiton* and *Dendrochiton* from these families because they appear nested within Mopaliidae in our analyses (see below). Our proposition of Lepidochitonidae avoids the problem of including *Cyanoplax*, *Lepidochitona* and *Tonicella* into a polyphyletic Tonicellidae (Sigwart et al. 2013). The composition of both Tonicellidae and Mopaliidae *sensu* Sirenko 2006 was rejected with high significance by the AU tests based on the multilocus nucleotide data set (Table 2). AU tests on the multilocus amino acid data set also rejected Mopaliidae, but could not exclude Tonicellidae, both *sensu* Sirenko 2006 (Supplemental File 1). *Lepidochitona cinerea* + *L. caprearum* are congruently found in a basal position within Lepidochitonidae, receiving high Bayesian posterior
probabilities from both multilocus nucleotide and amino acid data sets. In contrast, these relationships received low bootstrap support from the ML analysis of the multilocus nucleotide data set and the two Lepidochitona species were grouped together as sister taxon of Mopaliiidae in the ML analysis of the multilocus amino acid data, receiving low support (Supplemental File 2). Previous molecular analyses (Okusu et al. 2003; Sigwart et al. 2013) agreed with our preferred hypothesis (L. cinerea basal in Lepidochitonidae) and no other evidence would support the alternative topology found by the ML analysis of the multilocus amino acid data set.

The clade formed by Cyanoplax, ‘Lepidochitona’ beanii (see below), Nuttallina, and Schizoplax is strongly supported by both ML and BI analyses. Until recently, Schizoplax had been accorded its own family, Schizoplacidae, due to its highly distinctive ‘hinged’ intermediate valves, which are divided into right and left halves and connected by a ligament (Kaas and Van Belle 1985b; Sirenko 2006). However, Eernisse (2009) and Sirenko et al. (2013) have noted close affinities with species referred to as Cyanoplax by Eernisse et al. (2007), including the Cyanoplax caverna exemplar represented here. Sirenko et al. (2013) considered Schizoplacidae to be a junior synonym of Tonicellidae sensu Sirenko 2006 but here we reassign the monotypic Schizoplacidae Bergenhayn, 1955 as a junior synonym of Lepidochitonidae Iredale, 1914, with the latter defined as in Eernisse et al. (2007) and Stebbins and Eernisse (2009), to exclude Tonicella, Boreochiton and Dendrochiton (Table 3). Kaas and Van Belle (1981) first relegated Cyanoplax to be a junior synonym of Lepidochitona and their decision was followed for some years (e.g., Ferreira 1982; Van Belle 1983; Eernisse 1986; Kaas and Van Belle 1998). Cyanoplax was revived for exclusively northeastern Pacific species (Eernisse 2004; see also Eernisse et al. 2007; Stebbins and Eernisse 2009). Our results are consistent with this usage because they support Cyanoplax caverna to be a separate lineage from Lepidochitona, the latter here represented by its type species, L. cinerea from the northeastern Atlantic and Mediterranean, and the Mediterranean L. caprearum. Cyanoplax caverna is basal to Schizoplax, Nuttallina californica + ‘Lepidochitona’ beanii (Figure 2).

The relative position of Schizoplax and Cyanoplax in our analyses is surprising considering the striking similarities between these genera (Sirenko et al. 2013). In fact, Schizoplax has been observed to group consistently as sister taxon of Cyanoplax species (and not with Nuttallina + ‘L.’ beanii) when a more complete representation of Cyanoplax and Nuttallina species are included for rrnL (D.J. Eernisse, unpublished). Therefore, these relationships need to be further investigated. In addition, our phylogenetic analyses suggest that ‘L.’ beanii is also inappropriately assigned to Lepidochitona, even though topology tests were unable to reject the monophyly of the genus (Cyanoplax was not included in this constraint) (Table 2). Current patterns of distribution, with L. cinerea and L. caprearum distributed in the northern Atlantic or Mediterranean and the remaining members of Lepidochitonidae included here in the North Pacific (Kaas and Van Belle 1985b; Eernisse unpublished data), further supports the placement of ‘L.’ beanii outside the genus, Lepidochitona; however, a more extensive biogeographic analysis of Lepidochitonidae is needed to examine the many other members assigned to this genus from the Atlantic, Caribbean, and tropical eastern Pacific (Kaas and Van Belle 1985b).

A second lineage within Mopalioidea is represented by members of Mopaliiidae sensu Sirenko 2006 but without Plaxiphora or Nuttalchochiton (see above) and additionally including the following genera: Tonicella, Boreochiton (both within
Tonicellidae *sensu* Sirenko 2006, *Dendrochiton* (not considered by Sirenko 2006, and considered a subgenus of *Lepidochitona* by Kaas and Van Belle, 1985b), and *Cryptochiton* (Figure 2 and Table 3). *Cryptochiton* was found to be the sister group of *Amicula* and nested within Mopaliidae with strong support (Figure 2). The position of *Cryptochiton* within Mopaliidae has already been noted by recent molecular analyses (Kelly and Eernisse 2008, Sigwart et al. 2013), and updated classifications that include *Cryptochiton* within Mopaliidae (Eernisse et al. 2007; Stebbins and Eernisse 2009; Sigwart et al. 2013). This is further evidenced by topology tests, which highly significantly rejected the historical position of *Cryptochiton* in Acanthochitonidae and the monophyly of Mopaliidae, both *sensu* Sirenko 2006 (Table 2). The close relationship between *Cryptochiton* and *Amicula* strongly suggest that the morphological affinity between these two genera is due to common descent, and not the product of evolutionary convergence, as suggested by Sirenko (2006; fig 1D—E).

*Dendrochiton* was considered a subgenus of *Lepidochitona* in previous classifications (Van Belle 1983; Kaas and Van Belle 1985b), but due to its phylogenetic position apart from other *Lepidochitona*, the shared presence of girdle setae with

| Table 3. Proposed systematic arrangement of selected Acanthochitonina. The systematic arrangement for the species sampled in this study is shown, as derived from the phylogenetic analyses. Proposed systematic changes with respect to current literature are noted with an asterisk (*) on the corresponding species; see main text for details. |
|---------------------------------------------------------------|
| **ACANTHOCHITONINA** Bergenhayn, 1930                        |
| **Cryptoplacoidea H. & A. Adams, 1858**                      |
| *Acanthochitonidae* Pilsbry, 1893                           |
| *Acanthochitona avicula*                                    |
| *Acanthochitona crinita*                                    |
| *Choneplax lata*                                            |
| *Cryptoconchus floridanus*                                  |
| *Cryptoplacidae H. & A. Adams, 1858*                        |
| *Cryptoplax iredalei*                                       |
| *Cryptoplax japonica*                                       |
| **Hemiarthridae** Sirenko, 1977                             |
| *Hemiarthurum setulosum*                                    |
| **No specific family assignment**                           |
| *Nutallochiton mirandus*                                    |
| **No specific superfamily assignment**                      |
| **Mopalioida Dall, 1889**                                   |
| *Lepidochitonidae* Iredale, 1914                            |
| *Cyanoplax cf. caverna*                                     |
| ‘*Lepidochitona’ beanii*                                    |
| *Lepidochitona caprearum*                                   |
| *Lepidochitona cinerea*                                     |
| *Nuttallina californica*                                    |
| *Schizoplax brandtii*                                       |
| **Mopaliidae Dall, 1889 (sensu Stebbins & Eernisse 2009)**  |
| *Amicula vestita*                                           |
| *Boreochiton beringensis*                                   |
| *Cryptochiton stelleri*                                     |
| *Dendrochiton flectens*                                     |
| *Dendrochiton gothicus*                                     |
| *Katharina tunicata*                                        |
| *Mopalia muscosa*                                           |
| *Placiphorella sp. A*                                       |
| *Placiphorella velata*                                      |
| *Tonicella lineata*                                         |
| *Tonicella lokii*                                           |
| **No specific superfamily assignment**                      |
| *Plaxiphora albida*                                         |
| *Plaxiphora biramosa*                                       |
Mopaliidae (Stebbins and Eernisse 2009) and similarities of aesthete channels to other members of the family (Vendrasco et al. 2008), it is appropriate to revive its originally proposed status as a separate genus *Dendrochiton* included in Mopaliidae (Kelly et al. 2007; Kelly and Eernisse 2008; Stebbins and Eernisse 2009). The phylogenetic relationships within Mopaliidae (as here redefined) are generally well resolved with strong support and consistency among methods of phylogenetic reconstruction and data sets. However, the position of *Katharina tunicata* received relatively low support in both BI and ML analyses (Figure 2 and Supplemental File 2). Because *Katharina* does not group as a sister lineage to other genera within Mopaliidae (as reconstituted here), we found no support for the concept of Katharininae, including only *Katharina*, as advocated by Kaas and Van Belle (1994). Previous phylogenetic analyses (Okusu et al. 2003; Kelly and Eernisse 2008; Sigwart et al. 2013) differed from ours both in taxon sampling and in the phylogenetic relationships recovered within this second mopalioid clade, as further evidenced by the highly significant results of topology tests (Table 2). Some of the topological differences likely arise from better taxon representation in our analysis. For example, the constraint in Kelly and Eernisse (2008) only conflicted with our result (Figure 1) by their weakly supported grouping of two outgroup species (*Katharina tunicata* and *Tonicella lineata*), whereas we were able to add *Boreochiton* and *Amicula* in our analyses. The resulting topologies from all of these studies imply the need for a new concept of Mopaliidae (type genus *Mopalia*) that can include these additional genera and that does not depend on the conventional morphological diagnosis of a posterior sinus in the tail valve (see Stebbins and Eernisse 2009) (Table 3). Our analyses strongly support that the sinus in the posterior valve has in fact multiple independent (approximately five) origins within Acanthochitonina, depending on how the positions of genera without this sinus are explained within the family. If preference is given to not re-evolving a more typical tail valve without a sinus, as in the deeply nested *Dendrochiton*, then a sinus could have arisen four separate times within Mopaliidae (present in *Cryptochiton + Amicula, Mopalia, Katharina, and Placiphorella*) and twice outside of Mopaliidae for *Plaxiphora* and *Nuttallochiton*. Eernisse (2004) noted that a terminal sinus often coincides with a slit girdle that can be arranged like a chimney to assist respiratory flow vertically away from the body. Despite its emphasis in classification systems (e.g., Van Belle 1983; Sirenko 2006), such tail valve sinuses have likely evolved convergently, and we speculate this might be related to increasing body size and escalating demands for oxygen.

From a biogeographic point of view, most members of the clade Mopaliidae *sensu* Stebbins and Eernisse 2009 share a North Pacific distribution (Kelly and Eernisse 2008; Vendrasco et al. 2012), whereas *Nuttallochiton* and *Plaxiphora* (now removed from Mopaliidae, see above) are mostly distributed in the Southern Hemisphere (Kaas and Van Belle 1987, 1994). Exceptions to the North Pacific distribution of mopaliids include the North Atlantic *Boreochiton ruber* and *Tonicella marmorea*, whose conspecific or closely related counterparts in the North Pacific (Kaas and Van Belle 1985b) indicate contemporary or (geologically) recent connectivity. The members of an apparent *Placiphorella atlantica* species complex (Kaas and Van Belle 1994; Schwabe and Sellanes 2010) are an example of poorly known deep-water chitons that collectively have a widespread distribution between the North Atlantic and southeastern Pacific. Even these noted exceptions are for species-rich genera whose members are predominantly North Pacific, and whose fossil history coincides
with the late Miocene cooling of this ocean basin (Vermeij 2005; Sirenko 2006; Kelly and Ernisse 2008; Vendrasco et al. 2012).

Overall, our phylogenetic analyses reveal important patterns on the higher-level systematics of Acanthochitonina and generally support the recent classification of Sirenko (2006), while also identifying several remaining flaws in the placement of particular genera. Table 3 summarizes the systematic results of our phylogenetic analyses. Our results show instances of morphological convergence, particularly in shell and other external characters such as girdle ornamentation. Here we also support a likely homology of external characters, which were previously postulated to be convergent (Sirenko 2006), while also newly supporting a convergent origin of a tail valve sinus in multiple lineages. These results illustrate the importance of incorporating independent data sources (e.g., from molecules or morphology) to understand the origin and evolution of particular morphological characters, as well as to assess their phylogenetic and taxonomic utility. The remaining puzzles we have highlighted point to the continued need for more extensive molecular sampling of molecular markers and taxa and the combination of available molecular and morphological data sets in order to disentangle the complex systematics, biology, and biogeography of Acanthochitonina, as well as other chitons.

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**Supplemental material**

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