

Still waters run deep: Large scale genome rearrangements in the evolution of morphologically conservative Polyplacophora

Reviewed Preprint

v1 • November 28, 2024

Not revised

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eLife Assessment

This **fundamental** study explores how genotypic changes relate to phenotypic stasis or variation within chitons, a molluscan group. Chitons are significant because their ancient body plan has remained largely unchanged for millions of years, yet the paper reveals rapid and large-scale genomic changes. This **compelling** study is a splendid advance in approximately doubling the number of sequenced chiton genomes, providing what appears to be among the best genome annotations for chiton genomes available to date. The study's key focus is on the genomic rearrangements across five reference-quality genomes of chitons and their implications for understanding evolutionary mechanisms, particularly in comparison to other molluscan clades.

<https://doi.org/10.7554/eLife.102542.1.sa2>

Abstract

The phylum Mollusca is bifurcated into the hyperdiverse Conchifera (Gastropoda, Bivalvia, Cephalopoda, et al.) and the spiny Aculifera (Polyplacophora and Aplacophora). High quality genomic data for the deeply divergent, morphologically constrained chitons, would be expected to offer an opportunity to explore ancient genetic traits and evolutionary mechanisms preserved across the long span of animal evolution. We present a comparative analysis of five reference quality genomes, including four *de novo* assemblies, covering all major chiton clades: Lepidopleurida (*Deshayesiella sirenkoi*), Callochitonida (*Callochiton septemvalvis*) and Chitonida (*Acanthochitona discrepans* and *A. rubrolineata*), and an updated phylogeny for the phylum. Comparison with conchiferan genomes recovers 20 ancient molluscan linkage groups (MLGs) that are relatively conserved in bivalve karyotypes, but subject to re-ordering, rearrangement, fusion, and early occurring duplication (or fission) in chitons and conchiferan genomes. Two congeners each have 8 haploid chromosomes, results of two independent fusion events from the karyotype of their most recent common ancestor.

The largest number of novel fusions is in the supposedly most plesiomorphic clade Lepidopleurida, and the chitonid *Liolophura japonica* has a partial genome duplication, extending the known occurrence of large-scale gene duplication within Mollusca. The extreme and dynamic genome rearrangements in this class stands in contrast to most other animals and is not explained by accumulated change since the deep divergence time of this clade. The apparently conservative phenome of chitons is not reflected in a conservative genome.

Introduction

The genomic mechanisms that enable or limit the evolution of major changes remains one of the great questions of evolutionary biology. Living members of Mollusca represent the broadest morphological disparity of any animal phylum: mollusc body plans encompass squid, worms, living rocks and candy-coloured tree snails, as well as diverse intermediate and additional novel forms known from the fossil record. Early work on chromosome numbers in meiotic division used molluscs, and interest in understanding patterns in chromosome numbers across species also led to fundamental insights in animal polyploidy (1 [link](#)). Despite playing a key role in early advances, this important phylum lags in terms of quality and taxonomic coverage of whole genome sequence data (2 [link](#)). Reconstructing the evolution of genome architecture through deep time in diverse molluscs remains critical to understand genome evolution in animals.

Prior work has reasonably assumed that rates of intra-chromosomal gene translocation are constant within major groups(3 [link](#)). If this is true, syntenic rearrangement could be a clocklike indicator of divergence times. But rates of genomic rearrangement are not well known in molluscs, nor how they might vary across this vast clade, and higher rates of rearrangement confound reconstruction of ancestral states. In molluscs, an excellent fossil record allows an independent record of divergence times that will provide more insight into the variability (and utility) of rates of rearrangement as a measure of divergence time.

Chitons have long been considered the key taxon to understanding the ancestral molluscan body plan(4 [link](#)). Around 1000 living chiton species worldwide all possess an eight-part shell armour that has remained superficially unchanged with relatively little variation since the Carboniferous, over 300 Million years ago(5 [link](#)). Chitons are increasingly important for bio-inspired design, which will benefit from genomic tools to understand genetic control of their flexible armour, unique sensory systems, and iron mineralised radula(6 [link](#)). Chitons have separate sexes and most reproduce by broadcast spawning, the animals are grazers and most are not highly ecologically specialised(7 [link](#)). Chitons are generally conserved, yet the known species richness in chitons is higher than for the more morphologically and behaviourally diverse cephalopods. The adaptations that drive speciation in this group remain an open question.

Here, we sequenced four new reference-quality genomes that cover the three taxonomic orders of living chitons: *Deshayesiella sirenkoi* (Lepidopleurida), *Callochiton septemvalvis* (Callochitonida), *Acanthochitona discrepans* and *A. rubrolineata* (Chitonida). We use these genomes together with previously published genomic data to reconstruct a phylogeny for the phylum. This allows us to confidently reconstruct ancestral chromosome arrangement of total-group Mollusca, and at different transitions within Polyplacophora.

Results & discussion

Chiton genomes show frequent and extreme rearrangement

Four new chiton genomes represent the most complete genomes sequenced for the class, adding to several previously published partial or complete genomes (6, 8–10) (SI Appendix, Tables S1–S3). We sequenced chromosome-level genomes of *Deshayesiella sirenkoi* (Lepidopleurida) from the Daikoku vent field, Western Pacific Ocean, *Callochiton septemvalvis* (Callochitonida) and *Acanthochitona discrepans* (Chitonida) both from the intertidal of Strangford Lough, N. Ireland, and the congener *A. rubrolineata* from the intertidal of Qingdao, China. These were sequenced with PacBio HiFi and scaffolded using Hi-C, resulting in high quality assemblies with over 97% BUSCO completeness (94% for *Callochiton septemvalvis*) and with numbers of chromosomes that differed among genera from 8–13 (Fig. 1, SI Appendix, Fig. S1, Table S1). These species show high levels of heterozygosity, ranging from almost 1% in *Deshayesiella* to 4.12% in *Callochiton* (Fig. 1, SI Appendix, Fig. S2).

Our phylogenetic results confirm the placement of chitons as sister to a monophyletic Conchifera, and we recover the expected topology within Polyplacophora consistent with other recent work using genomic and morphological characters (Fig. 2). Within Conchifera, we confirm the topology of other recent studies (11); however, our supplementary analyses recovered Scaphopoda sister to Gastropoda but with lower support (SI Appendix, Fig. S3). Comparison with genomes from other molluscan classes shows the molluscan ancestor had a genome composed of 20 linkage groups (Fig. 2), we refer to these as the Molluscan Linkage Groups (MLG) 1–20. This confirms previous studies that also predicted a haploid karyotype of 20 for the molluscan ancestor based on other metazoans (12). Three important fusion events are apparent synapomorphies for Polyplacophora, present in all living chitons and no Conchifera: MLG 4+16+18, MLG 7+10, and MLG 8+9 (Fig. 3, Fig. 4, SI Appendix, Figures S4–S7). There are additional fusions and intra-chromosomal rearrangements that are different in every species sampled.

Previous work demonstrated the variability in chiton karyotypes; species in the clade Chitonida have reported haploid numbers ranging maximally from 6 to 16 (13) with a mode of 11 (1). Our data give the first indication for expected chromosome count in a species in Lepidopleurida ($1n=11$) within this established range. Using five chromosome-level genome assemblies for chitons, we reconstructed the ancestral karyotype for Polyplacophora (more strictly the taxonomic order Neoloricata), and all intermediate phylogenetic nodes to demonstrate the stepwise fusion and rearrangement of gene linkage groups during chiton evolution (Fig. 3).

Chitons demonstrate extreme genome rearrangement, even within a single genus. This represents not only gene order differences but syntenic (co-occurrence) changes in genomic architecture. Species of the genus *Acanthochitona* have a relatively short divergence time of maximally ~23 My based on the fossil record (14). *Acanthochitona discrepans* and *A. rubrolineata* each have 8 haploid chromosomes but these result from two different fusions compared to the reconstructed ancestral karyotype of *Acanthochitona* (Fig. 1, Fig. 4C). Previous karyotype data for *A. discrepans* were actually based on specimens of *A. crinita*; the species are very similar but the correct identification can be determined based on the geographic distributions (15). *Acanthochitona crinita* has 9 haploid chromosomes (16): this is yet another syntenic arrangement within the same genus. There are major changes between congeners in different ocean basins (the Pacific *A. rubrolineata*) but also between two species in the NE Atlantic (*A. discrepans* and *A. crinita*) that are morphologically and ecologically almost indistinguishable.

Living species of Lepidopleurida retain more plesiomorphic morphology and this clade has a deep fossil record extending to the lower Carboniferous (5). The largest number of novel fusions among chiton genomes is three, in the lepidopleuran *Deshayesiella* (Fig. 4A). The remaining living chitons form two sister clades recognised as separate orders: Chitonida and Callochitonida (17). *Callochiton* also has two additional fusion events, and the chitonid *Liolophura japonica* has a partial genome duplication, with two linkage groups fused apparently at

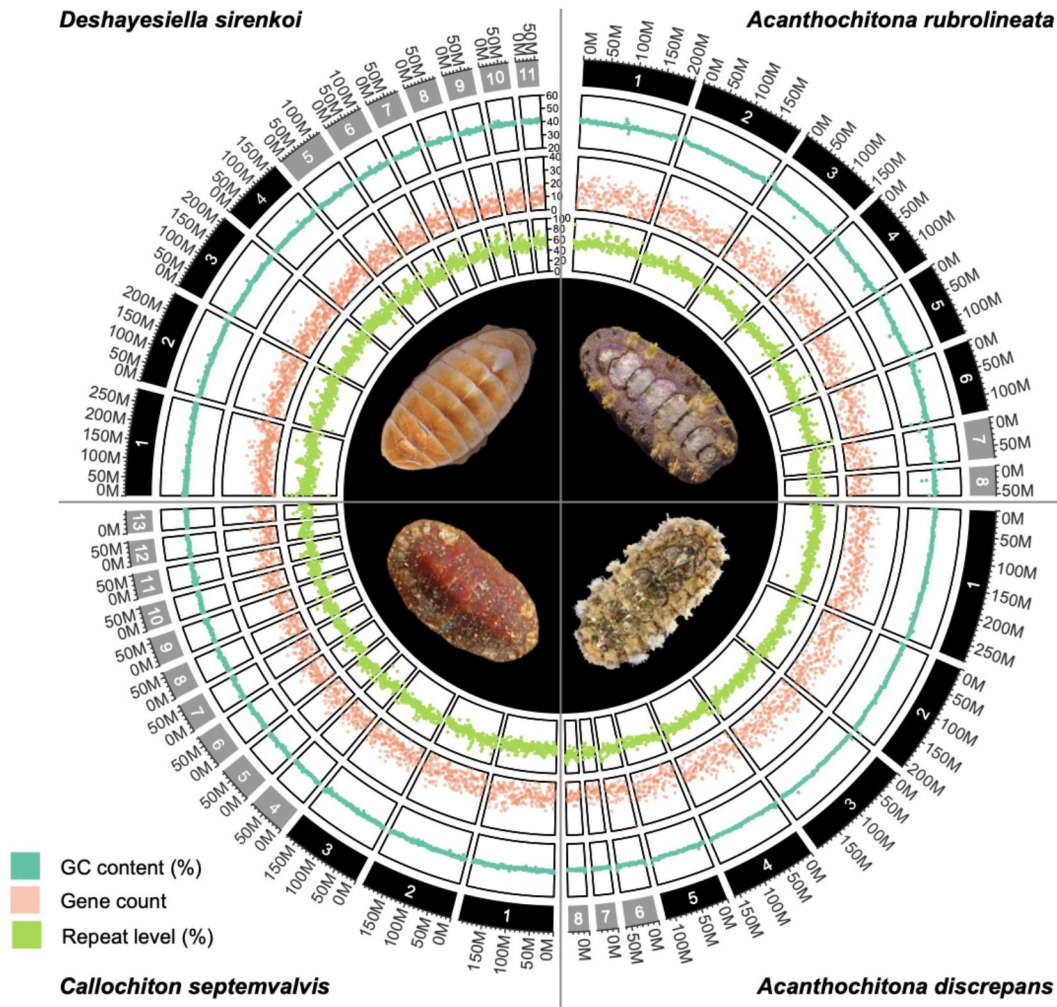


Fig. 1.

CIRCOS plots for 4 new chitons genome assemblies, clockwise from top left: one species in the order Lepidopleurida *Deshayesiella sirenkoi*, and three species in the clade Chitonida *sensu lato*: *Acanthochiton rubrolineata*, *A. discrepans*, and *Callochiton septemvalvis*. Each quarter circle shows the pseudo-chromosome content for each species, in order of size, with concentric rings indicating GC content, gene count, percent repeat content, and a photograph of the respective species.

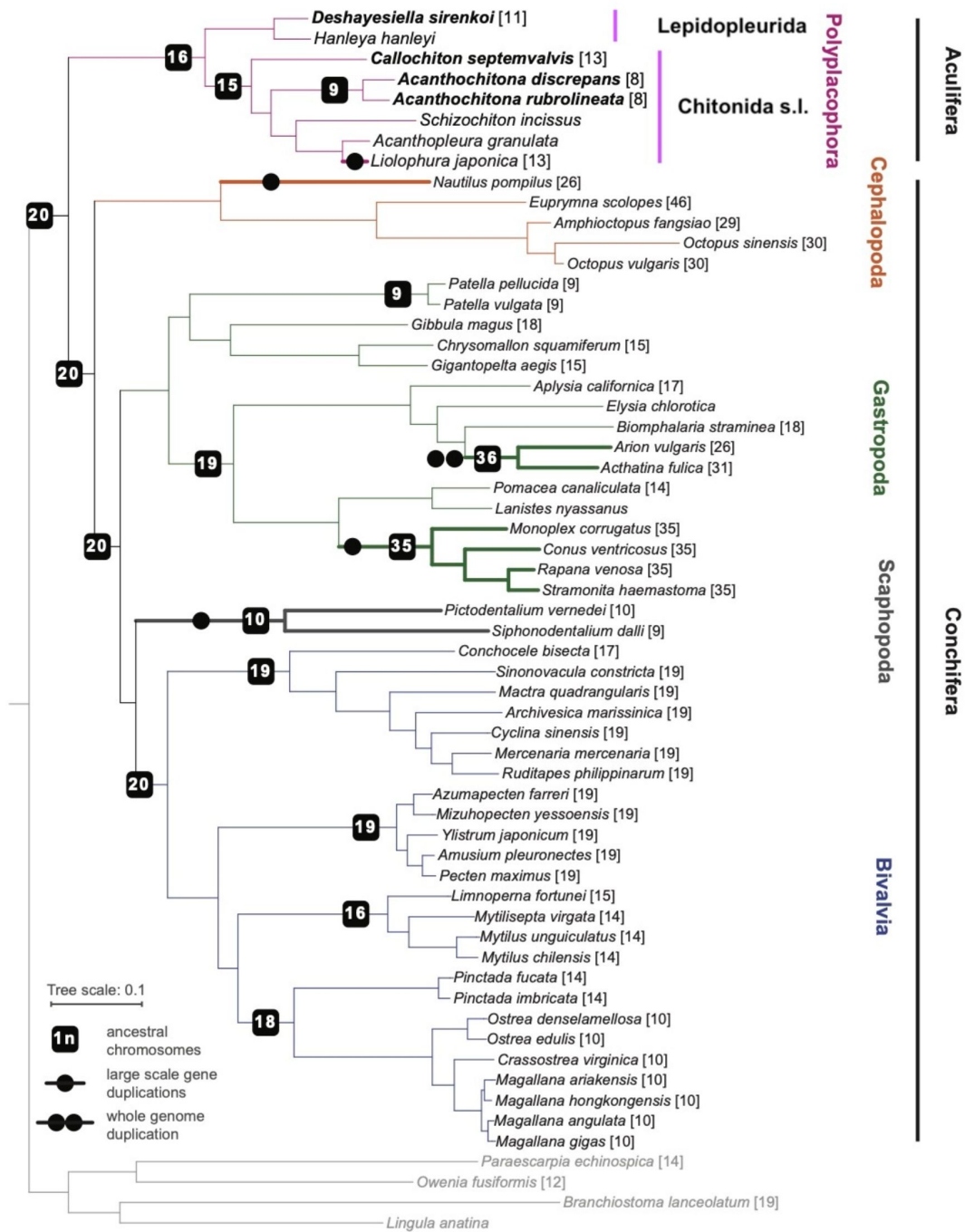


Fig. 2.

Phylogeny of Mollusca, with new genomes noted in bold type, and the chromosome number in square brackets for each species where known. Lineages with known whole (double circle) or partial genome (single circle) duplication are in thicker lines for emphasis; boxes on branches show the reconstructed ancestral 1n chromosome number for the respective clade.

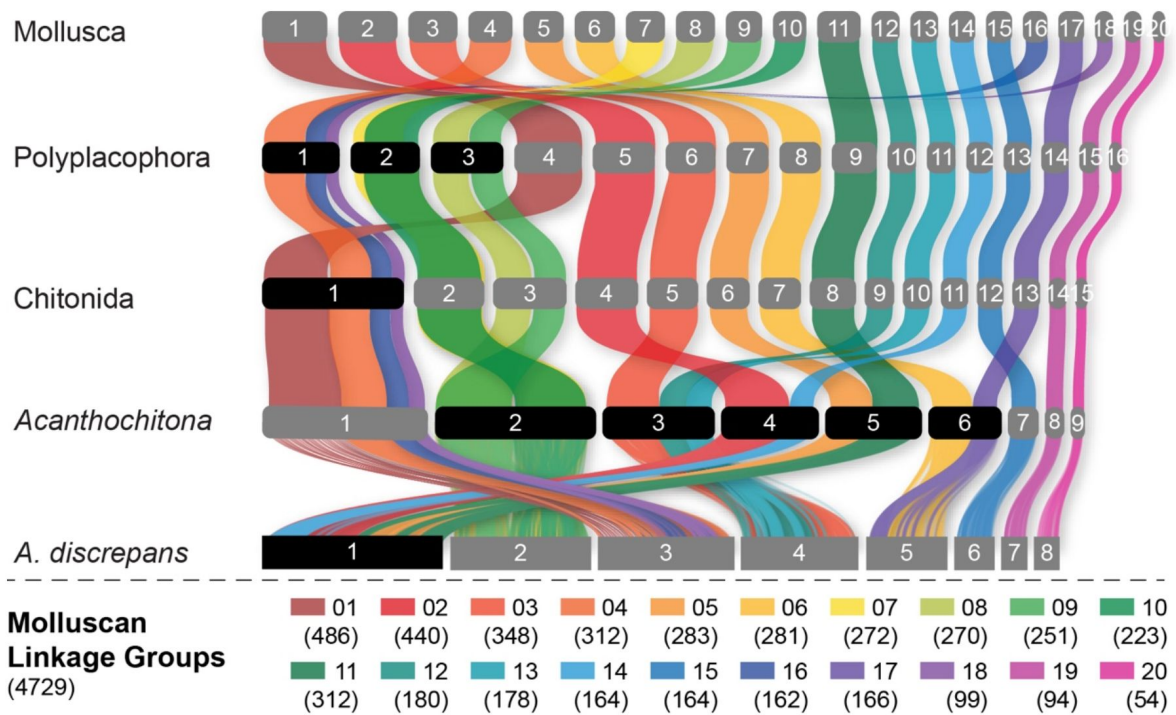


Fig. 3.

Evolution of the ancestral molluscan linkage groups (MLGs) within Polyplacophoran using the lineage leading to *Acanthochitona discrepans* as an example. MLGs are distinguished by colours, at the top of the diagram and in the key at bottom showing the number of orthologs. Each row is the reconstructed karyotype of the ancestor of living Polyplacophora, the order Chitonida *sensu lato*, and the genus *Acanthochitona*.

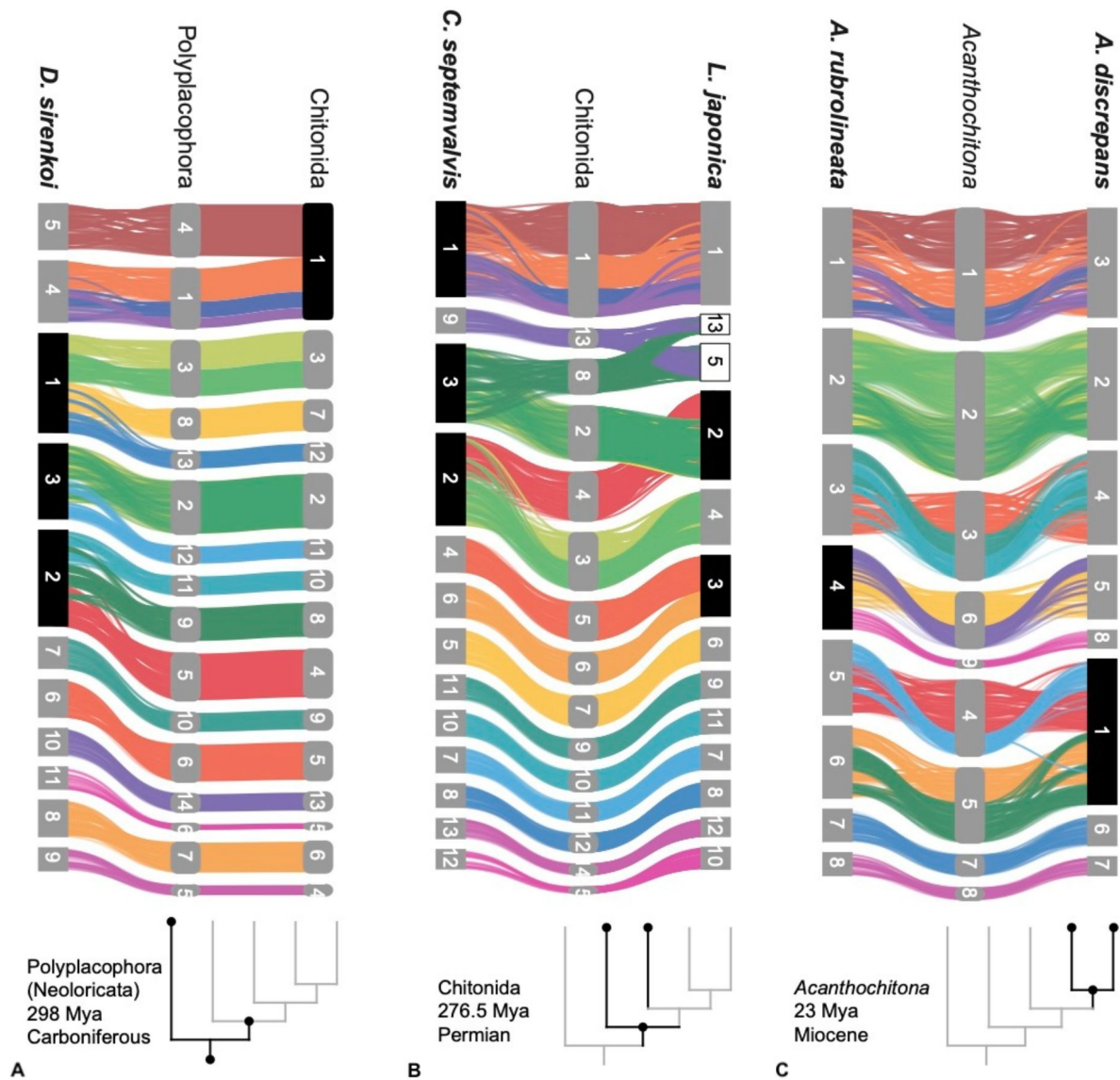


Fig. 4.

Syntenic rearrangements of MLGs within the evolution of Polyplacophora. Each part shows the reconstructed karyotypes of an ancestor (middle) and two descendent lineages, with a schematic cladogram for orientation. From left to right, the divergence of A) ancestor of Polyplacophora, leading to the lepidopleuran species *Deshayesiella sirenkoi* (left) and ancestor of Chitonida (right), B), ancestor of Chitonida leading to the callochitonid *Callochitona septemvalvis* (left) and chitonid *Liolophura japonica* (right) and C) ancestor of the genus *Acanthochitona* leading to the two congeneric species *A. rubrolineata* (left) and *A. discrepans* (right). Colours and presentation are as in **Figure 3**. Chromosome fusions are highlighted with chromosome numbers in black boxes, and duplications in white boxes.

first and then duplicated (Fig. 4B). In our reconstruction of ancestral karyotypes, there is no differences in arrangement between the ancestor of Chitonida or the ancestor of (Chitonida + Callochitonida). The four species in Chitonida (*sensu lato*, including *Callochiton*) share a large, fused chromosome (MLG 01+04+16+18) that is notably not well-mixed in *Liolophura japonica* (i.e., Chr01 in *Liolophura japonica*, Chr01 in *Callochiton septemvalvis*, Chr01 in *Acanthochitona rubrolineata*, and Chr03 in *A. discrepans*). Part of this pattern has its origin in the ancestral chiton karyotype and is retained in the lepidopleuran *Deshayesiella* (i.e., MLG 04+16+18). This implies a variable rate of intra-chromosomal rearrangement, with several of the MLGs conserved.

Ancestral karyotypes for Mollusca

Chromosome numbers are not strongly conserved in animals. Changes in chromosome numbers are hypothesized to be an important driver of the diversification of Lepidoptera, with a strong correlation shown between rates of chromosome number changes and speciation(18). The instability in lepidopteran chromosome numbers has mainly been studied from karyotype data but recently confirmed in genomic data(19), and changes within genera are known to encompass both neutral and adaptive evolution(20, 21). However, the level of rearrangement may be less than that in Polyplacophora.

Even within a single species, chromosome fusions are not uncommon, with Robertsonian translocations occurring in roughly 1 out of every 1000 live human births(22). A previous study speculated that chromosome loss in related clades of chitons may be the result of Robertsonian translocation(13). Although we still lack information on the telomere, this simple mechanism is not a sufficient explanation for several more complex intrachromosomal rearrangements (e.g. MLG 7+10: Fig. 4). Chromosome fusion does not present any immediate reproductive barrier, yet chromosome rearrangements between sister species can act as Dobzhansky-Muller incompatibilities and generate reproductive isolation(18). Chitons are broadcast spawners, so such barriers may be speculatively advantageous, but so too are bivalves where these syntenic rearrangements are not found.

In order to compare syntenic changes within Polyplacophora to other mollusc clades, we re-analysed all available molluscan genomes in context of the newly identified MLGs. For example, the divergence of the bivalve clade Imparadentia (Lucinida + Venerida) is estimated in the Silurian, ca. 430 Mya(23), or more than 150 My deeper than the divergence of crown group Polyplacophora, yet species within Imparadentia have almost no syntenic rearrangement (SI Appendix, Fig. S7, S8). Bivalves in Pteriomorphia, which have an even deeper origin in the lower Ordovician, ca. 485 Mya, show some rearrangement but members of two orders (Arcida and Pectinida) are highly similar, while two species in Ostreida that are intensively cultivated differ from these others and from each other (SI Appendix, Fig. S8).

Potential adaptive roles may be connected to contrasting mobility of different MLGs. The smallest two MLGs are the most conserved across the five chiton genomes; MLG20 is the only group that remains separate in all bivalve and chiton taxa. This region is also not duplicated in partial genome duplications in Neogastropoda, but is duplicated in whole genome duplication in two hermaphroditic terrestrial gastropods (*Achatina fulica* and *Arion vulgaris*) (Fig. 5, SI Appendix, Fig. S5).

Earlier models based on karyotype data predicted three whole genome duplication events in the evolutionary history of living molluscs: in Neogastropoda, Stylommatophora, and coleoid cephalopods (1). Available genomes of Stylommatophora confirm whole genome duplication (24), as well as large scale gene duplications in Neogastropoda (SI Appendix, Fig. S6). In coleoid cephalopod genomes, the co-occurrence of loci from MLGs and intensive fusions (SI Appendix, Fig. S6) might not be easily resolved but may be more likely from the chromosome-disrupted processes reported in previous work (25). By contrast, in *Nautilus* the linkage group that contains the conserved *hox* gene sets (Chr11, MLG16) (26) shows no signal of co-occurrence with other MLGs

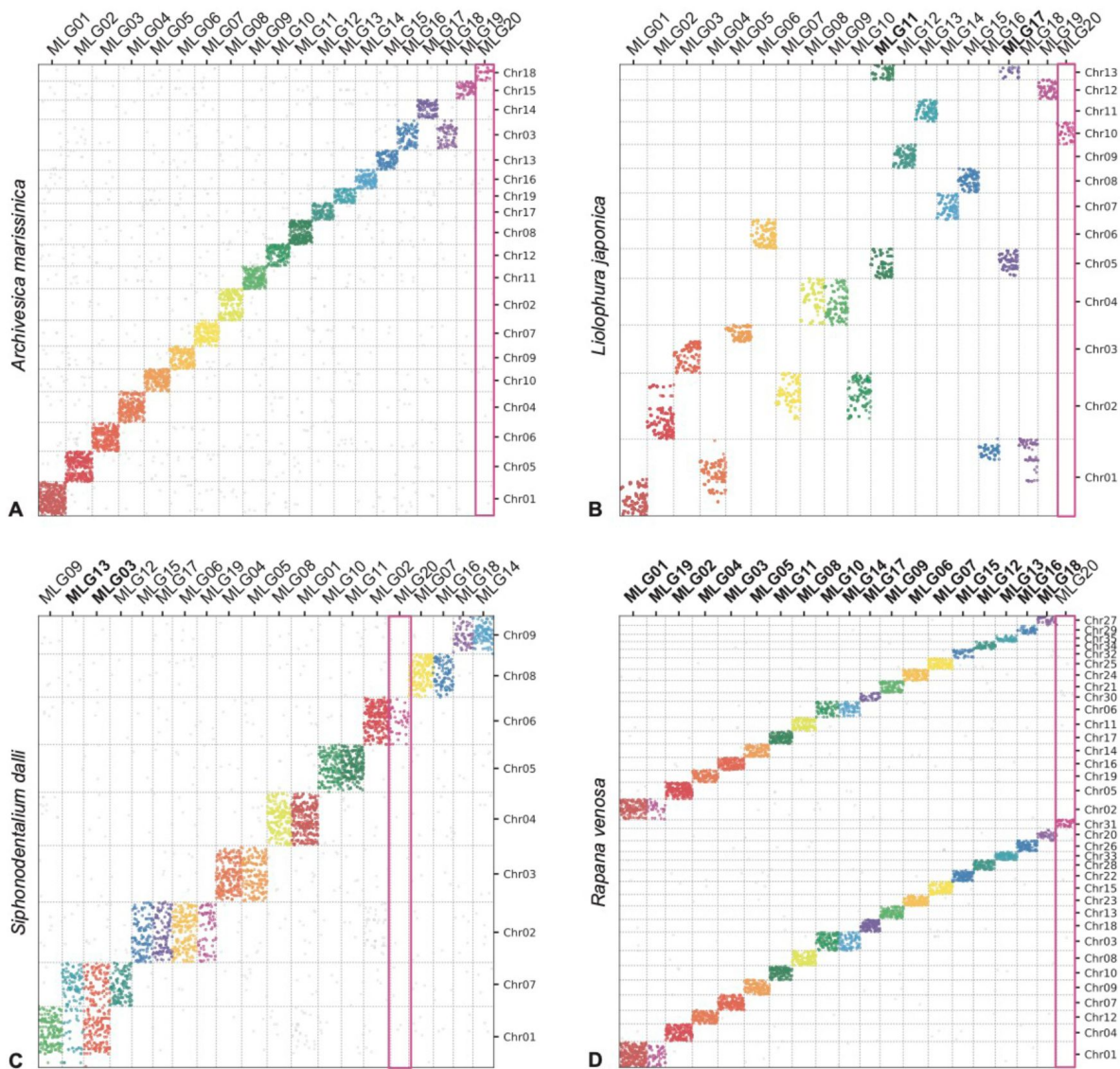


Fig. 5.

Oxford plots comparing gene occupancy of species from four different classes of molluscs (vertical) to the ancestral molluscan linkage groups (MLG, horizontal): A) the bivalve *Archivesica marissinica* retains a plesiomorphic karyotype reflecting the 20 MLGs, B) the chiton *Liolophura japonica* has large scale gene duplication in MLG11 and MLG17 on separate pseudochromosomes, C) the scaphopod *Siphonodetalium dalli* has large scale gene duplication of MLG13 and MLG03 on separate pseudochromosomes, D) the gastropod *Rapana venosa* demonstrates a nearly whole genome duplication with MLG20 not duplicated. Colours follow the presentation in [Figure 3](#).

or on other chromosomes (SI Appendix, Fig. S6). New results also show a partial and ancient genome duplication in the chiton *Liolophura* (Fig. 2, Fig. 4). Given that the MLGs are well conserved in all chitons if not their order (SI Appendix, Fig. S4), the event in *Liolophura japonica* is most likely a duplication instead of fission. Whole or partial genome duplication is now known from four molluscan classes, with contrasting patterns of ploidy (e.g. gastropods) or tandem (e.g. scaphopods) duplications (Fig. 5).

Reconstructing genome evolution is naturally more difficult for groups with high rates of intra-chromosomal rearrangement (27, 28). These patterns of co-occurrence of loci on the same chromosomes (synteny) should also persist for longer in evolutionary time, compared to faster rates of change in gene order (12, 29). Syntenic changes do not necessarily follow overall rates of translocation, which do not differ obviously among molluscs (SI Appendix, Table S4, S5). Confounding effects of rapid gene order or linkage group changes are not limited to issues of phylogenetic distance, when even closely related species have significant syntenic differences. Nonetheless a focus on linkage group arrangements is a promising direction for the many unresolved questions of deep molluscan phylogeny. Recent studies have championed the importance of synteny-based studies on genome evolution, as a basis to understand deep divergences and also the general mechanisms that underlie genomic architecture, fusion, fission, and translocation (3). A general trend in insect and vertebrates is for increasing chromosome numbers in derived lineages (29, 30). Fusion events may be more common in more recently derived chitons or connected to specific adaptations.

Understanding diversification requires diverse clades

Chitons are broadcast spawners, are not migratory, cannot reasonably be subject to strong sexual selection, and many similar species co-occur in parapatric or sympatric radiations (31). Chromosome rearrangement is an attractive speculative explanation that could support maintaining species boundaries in this clade, for example *Acanthochitona discrepans* (1n=8) and *A. crinita* (1n=9) (16). The relatively small number of reference quality genomes for most molluscan groups is a temporary limitation to these analyses. High heterozygosity seems to be very common in molluscs, and is clearly a feature of chitons, which causes difficulty for high quality genome assembly. The heterozygosity for *Callochiton* at 4.12% far exceeds the 2.95% genomic heterozygosity reported as “one of the highest” for Lepidoptera (32). Large scale analyses based on genomics are equivocal about the drivers of genetic diversity (33, 34), and further data from diverse clades must be included.

One key issue is whether polyplacophoran molluscs are conservative *per se* or whether their diagnostic body plan adaptations are so distinctive and strange that this has overshadowed full understanding of additional adaptive traits: fully innervated shells, iron mineralised radula, living at almost all depths and latitudes of the world ocean. While the body plan of chitons has persisted for over 300 My, this is a template for remarkable adaptations that have only recently begun to be appreciated.

The concept of “diverse groups” is mostly based on perceived variability, typically manifested via morphological, ecological, or genetic differences. Lepidopterans are commonly regarded as super-diverse, but their striking colours and different wing shapes are variations within a clearly constrained theme. Chitons, despite exceeding 1000 species in diverse ecological niches, with a wide range of morphological adaptations, are considered a “minor” and “neglected” clade. The group is taxonomically challenging – on the one hand, because of the supposed morphological stasis as a group, on the other, because of their high interspecific variability (14). Chitons exhibit chromosome rearrangements, even at the genus level within *Acanthochitona*, at an almost unprecedented level. This is clear in the first comparative genomic study, compared to orders of magnitude more data for better studied groups. All this poses a more general question on how we define variability, how we perceive it, and also, if we actually understand it at all? While lepidopteran variability is conspicuous, chitons are equally variable, clearly demonstrated

through their genetic rearrangement. Recognition of variability in the genome and phenome is crucial in understanding and re-evaluating unbiased measures of diversity in overlooked groups of organisms.

Materials and methods

Specimens were collected alive and flash frozen in liquid nitrogen (*A. rubrolineata*) or frozen at -80°C and stored at -80°C. High molecular weight genomic DNA was extracted from the foot of an individual specimen of each species, following the guidelines of the SDS method, and used for PacBio high fidelity (HiFi) sequencing and Hi-C library preparation. The PacBio Sequel II / Iie instrument was used for sequencing in CCS mode. The qualified HiFi reads (i.e., adapter-free) were processed for de novo genome assembly using hifiasm v0.16.1-r375 (35). For transcriptome sequencing, five separate tissues were dissected from the same specimen as DNA extraction, and preserved in RNAlater: foot (F), perinotum (P), radula sac (R), shell edge (S), and visceral mass (V).

Adapters in the reads were checked and removed using HiFiAdapterFilt version 2.0. The genome survey was finished using jellyfish v2.2.10 and Genomescope v2.0 (36), implemented 21- and 23-mer, which produced the estimated genome size and heterozygosity level for each species (SI Appendix, Fig. S2). The four genomes were assembled de novo based on qualified HiFi reads using hifiasm v0.16.1 (35), with some additional curation steps for *C. septemvalvis* because of the high heterozygosity (SI Appendix). Potential contamination in the genome was detected and removed using blobtools v1.1.1 (37). Duplicated haplotigs and overlaps were removed for *D. sirenkoi*, *A. discrepans* and *A. rubrolineata* using Purge_Dups v1.2.6 (38).


The aligner STAR version 2.7.10a or STAR v2.7.3a (39) was employed to map RNA-seq data into the working genome data. The resulting alignment file served as an important support in prediction methods. *Ab initio* gene prediction was performed on the repeat-masked assembly in comparison with other published genomes (SI Appendix). Summary information on assembly, gene model, and annotation is provided in Table S2.

We identified putative orthologous sequences shared among genomes or transcriptomes for 58 molluscs and 4 additional metazoans to generate an alignment for phylogenetic analysis. The alignments were removed if the overlap among them was less than 20 amino acids and there were less than 75% of taxa sampled, then, each alignment was used to construct “approximately maximum likelihood” tree using FastTree version 2.1.11 (40). Phylogenetic relationships were investigated using IQ-TREE version 2.1.3 (41) with the “-MFP” model to compute the best-fit model of each partition and 1000 ultrabootstraps to test the topological support. To test the impact on conchiferan topology we ran a second analysis excluding Solemyida, the earliest diverging clade in Bivalvia (SI Appendix, Fig. S3).


The method for identifying ancient linkage groups followed previously published works (12, 42, 43). We reconstructed the linkages of the orthologues in molluscs, with the demonstrated commands, and conserved and representative proteins and their presumptive locations (<https://github.com/ylify/MLGs>). In detail, the ancient and conserved linkage groups were inferred from 7 chromosome-level mollusk assemblies, including 1 gastropod (*Gibbula magus*), 1 bivalve (*Mizuhopecten yessoensis*), and the 5 chitons (*A. rubrolineata*, *A. discrepans*, *C. septemvalvis*, *D. sirenkoi*, and *Liolophura japonica*). The obtained gene set consisted of 4729 homologs, which could be detected in all 7 assemblies and located in 20 linkage groups. The ancestral states of nodes were predicted to trace the karyotype evolutionary route in Polyplacophora, from the common ancestor of Mollusca to the genus *Acanthochitona*. Similarly, the nodes with more than two chromosome-level genomes within Mollusca were also investigated for the chromosome number of their common ancestor. We also compared the translocation rates across Mollusca, based on the non-

syntenic rate of change divided by estimated divergence time. In this study, 25 genomes from four classes were selected to calculate the translocation rate at the inter-chromosome level, including 2 scaphopods, 5 chitons, 8 bivalves, and 10 gastropods.

Data availability

The four chiton genomes projects have been deposited with the NCBI BioProject, with *Acanthochitona discrepans* in PRJNA1114954, *A. rubrolineata* in PRJNA1114370, *Callochiton septemvalvis* in PRJNA1114372, *Deshayesiella sirenkoi* in PRJNA1114373. In each project, the whole-genome sequencing data, Hi-C data, RNA-seq data have been affiliated. The genome assembly and gene-model predictions are also available on GitHub, as well as the constructed Molluscan Linkage Groups (MLGs) is also available, with the conserved and representative sequences and their predictive corresponding locations (<https://github.com/ylify/MLGs> )

Code availability

The commands used in this study have been deposited on GitHub (<https://github.com/ylify/MLGs> )

, including the genome assembly, repeats and coding regions prediction, phylogenomic inference, and mutual best hits and ancient linkage groups detections.

Acknowledgements

We thank Carola Greve, Damian Baranski, Alexander Ben Hamadou, and Charlotte Gerheim of the Translational Biodiversity Genomics (TBG) project based in the Senckenberg Research Institute and Museum, Frankfurt, for support with lab work and sequencing. We thank the staff of the Queen's University Marine Laboratory, Portaferry, N Ireland, and Chong Chen (JAMSTEC) for support with field work and specimen collection. This is contribution number 28 of the Senckenberg Ocean Species Alliance.

This study was supported by the Natural Science Foundation of Shandong Province (ZR2023IQ014) and the Fundamental Research Funds for the Central Universities (202172002 and 202241002). This research was further supported by a generous philanthropic donation to the Senckenberg Gesellschaft für Naturforschung that funds the Senckenberg Ocean Species Alliance, and by Leibniz Association project PHENOME (P123/2021).

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Reviewer #1 (Public review):

Summary of Key Findings:

The authors identified 20 ancient molluscan linkage groups (MLGs) that are largely conserved in other molluscan groups but highly dynamic and rearranged in chitons. This contrasts with the stability seen in other animal groups.

Significant chromosome rearrangements, fusions, and duplications were observed in chitons, particularly in the most basal clades like Lepidopleurida, indicating that chitons undergo more extensive genomic changes than expected.

Chitons exhibit extremely high levels of genomic heterozygosity, exceeding that of other molluscan species and even Lepidoptera. This presents challenges for assembling high-quality genomes but also points to genetic diversity as a driver of evolutionary processes.

Partial genome duplications, particularly in *Liolophura japonica*, extend the knowledge of gene duplication events within the broader Mollusca clade.

The paper speculates that these genomic rearrangements may contribute to maintaining species boundaries in sympatric and parapatric radiations, as observed in certain *Acanthochitona* species.

Strengths:

The use of high-quality genomic data, including four de novo genome assemblies, provides robust evidence for the conclusions.

The research challenges the common assumption that chitons are evolutionarily conservative, showing that their genomes are highly dynamic despite their morphological stasis.

The study adds to the understanding of how chromosomal rearrangements might contribute to speciation, a concept that can be applied to other taxa.

Limitations:

The paper acknowledges that the limited availability of high-quality genomes across molluscs may restrict the scope of comparative analyses. More genomic data from other molluscan groups could strengthen the conclusions.

The role of high heterozygosity in chitons is highlighted, but more information is needed to clarify how this affects genome assembly and evolutionary outcomes.

Implications for Future Research:

The research raises important questions about the relationship between genomic instability and phenotypic stasis, which can inform studies in other animal groups.

The findings call for a re-evaluation of how we define and measure biodiversity, particularly in "neglected" clades like chitons. Further studies could focus on linking the observed genomic changes to specific adaptive traits or ecological niches.

<https://doi.org/10.7554/eLife.102542.1.sa1>

Reviewer #2 (Public review):

Summary:

The authors provide four new annotated genomes for an important taxon within Mollusca known as Polyplacophora (chitons). They provide an impressive analysis showing syntenic relationships between the chromosomes of these four genomes but also other available chiton genome sequences and analysis of 20 molluscan linkage groups to expand this analysis across Mollusca.

Strengths:

The authors have selected particular chiton species for genome sequencing and annotation that expand what is known about genomes across portions of chiton phylogenetic diversity lacking genome sequences. The manuscript is well-written and illustrated in a concise manner. The figures are mostly clear, allowing a reader to visually compare the syntenic relationships of chromosomes, especially within chitons. Their phylogenetic analysis provides a simple manner to map important events in molluscan genome evolution. This study greatly expands what is known about molluscan and chiton comparative genomics.

Weaknesses:

I am not especially convinced that chitons have experienced more substantial genomic rearrangements or other genomic events than other molluscan classes, and for this reason, I did not personally find the title compelling: "Still waters run deep: Large scale genome rearrangements in the evolution of morphologically conservative Polyplacophora." Are the documented events "large scale genomic rearrangements"? It seems that mostly they found

two cases of chromosome fusion, plus one apparent case of whole genome duplication. What do they mean by "Still waters run deep"? I have no idea. I guess they consider chitons to be morphologically conservative in their appearance and lifestyle so they are calling attention to this apparent paradox. However, most chiton genomes seem to be relatively conserved, but there are unexpected chromosome fusion events within a particular genus, *Acanthochitona*. Likewise, they found a large-scale gene duplication event in *Acanthopleurinae*, a different subfamily of chitons, which is quite interesting but these seem to be geologically recent events that do not especially represent the general pattern of genome evolution across this ancient molluscan taxon.

<https://doi.org/10.7554/eLife.102542.1.sa0>