

MOLLUSK GENETICS

A genome-based phylogeny for Mollusca is concordant with fossils and morphology

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Extreme morphological disparity within Mollusca has long confounded efforts to reconstruct a stable backbone phylogeny for the phylum. Familiar molluscan groups—gastropods, bivalves, and cephalopods—each represent a diverse radiation with myriad morphological, ecological, and behavioral adaptations. The phylum further encompasses many more unfamiliar experiments in animal body-plan evolution. In this work, we reconstructed the phylogeny for living Mollusca on the basis of metazoan BUSCO (Benchmarking Universal Single-Copy Orthologs) genes extracted from 77 (13 new) genomes, including multiple members of all eight classes with two high-quality genome assemblies for monoplacophorans. Our analyses confirm a phylogeny proposed from morphology and show widespread genomic variation. The flexibility of the molluscan genome likely explains both historic challenges with their genomes and their evolutionary success.

Phylum Mollusca exhibits the widest disparity of body plans in metazoan evolution, and the interrelationships of the living lineages have been a subject of contentious debate for centuries (1–3). The eight classes of extant Mollusca are each unambiguously monophyletic, but their diverse morphologies allow many plausible sister-group combinations. The three most diverse groups are more familiar—bivalves, gastropods, and cephalopods—and the other five include worm-mollusks (Solenogastres, Caudofoveata), eight-shelled chitons (Polyplacophora), tubular infaunal predators (Scaphopoda), and headless limpets (Monoplacophora). More than 500 million years of morphological evolution have produced even more transitional forms in the fossil record, many with unclear affinities (4). Most studies have focused on deep phylo-

genetic nodes, resolving the clades Conchifera (Bivalvia, Cephalopoda, Gastropoda, Monoplacophora, Scaphopoda) and Aculifera (Caudofoveata, Polyplacophora, Solenogastres) (2, 5, 6). Resolving the topological positions of all eight clades is critical to understanding molluscan evolution (7), but genomic resources were limited or lacking for most classes.

Early molecular phylogenetic studies of Mollusca sometimes completely conflicted with previous morphology-based hypotheses (8). Phylogenomic studies using transcriptome data covered all classes but had low coverage, outdated orthology inference methods, and uncertain topological support (2, 6). Results from standard genetic markers, mitochondrial genomes, transcriptomes, morphology, and the fossil record have at times seemed to be all mutually contradictory but increasingly converged toward a potential consensus (7, 9). Beyond the question of resolving molluscan phylogeny, a larger issue is understanding the fundamental evolutionary factors that made it so difficult and controversial to resolve.

We present a total group phylogeny for Mollusca based on genome-wide markers comprising all metazoan Benchmarking Universal Single-Copy Orthologs (BUSCO) genes in a phylogenomic analysis that includes all major clades and multiple representatives from all eight living classes. We assembled two near-complete genomes of the enigmatic deep-sea class Monoplacophora and generated genomes for five additional taxonomic classes: Caudofoveata, Scaphopoda, Gastropoda, Bivalvia, and Polyplacophora. This dataset, combined with previously published genome assemblies, underpins our new phylogenetic analysis, which supports the origin of the phylum in the Cambrian and a rapid split into the major clades Aculifera and Conchifera (Fig. 1). Congruent with the extensive fossil record of Mol-

lusca and phylogenetic hypotheses proposed from morphology (Fig. 2), we found that Monoplacophora is sister to the remaining Conchifera, and Cephalopoda is sister to the clade Gastropoda + Diasoma (Scaphopoda + Bivalvia).

Selecting representative data

Genome-wide data have played a pivotal role in reconstructing complex histories of the tree of life; however, genomic resources for Mollusca remain markedly underrepresented and unevenly distributed, with only 300 genomes available for more than 100,000 living species (Fig. 3A). The quality of these assemblies varies widely, in large part because of intrinsic technical difficulties that begin with challenges for sample acquisition, preservation, and DNA extraction and sequencing from animals that produce copious mucopolysaccharides (10). Assembly quality is further hampered by variability in genome size, heterozygosity, and repeat content, even within particular clades (Fig. 3, B to D). We generated 13 de novo genomes that fill gaps for key classes (two monoplacophorans, one caudofoveate, four chitons, one scaphopod), deeply divergent or contentious lineages (*Solemya*, *Verpa*, *Tectura*), and diverse morphologies (*Scintilla*, *Concholepas*) (fig. S1 and tables S1 and S2). We considered genome quality and phylogenetic diversity, retaining species in controversial branches even where genome quality was slightly lower (fig. S2 and table S3). This totals 77 species covering all eight classes, including two Caudofoveata, two Solenogastres, eight Polyplacophora, two Monoplacophora, 11 Cephalopoda, 22 Gastropoda, three Scaphopoda, and 27 Bivalvia.

Our phylogenomic inference relied on metazoan BUSCO genes (11). BUSCO represents a universal gene set that provides effective comparative power across the phylum. Because BUSCO genes are conserved regions, they are suitable for reconstructing deep divergences. Strict quality control in the selected genomes allowed us to identify a sufficient number and more even distribution of loci across species (figs. S2 and S3): Each species had an average of 897 BUSCO genes (94%) identified in the genome assemblies (table S4). To test the impact of gene occupancy on topology, we compared the metazoan BUSCO set and a reduced 96-gene dataset with 100% occupancy (figs. S3 and S4).

The balance of taxon sampling and character (gene) occupancy is a persistent dilemma, especially in deeply divergent organismal groups (12). High coverage of lineages, high-quality molecular markers, and sufficient molecular loci enabled us to construct the phylogeny within extant Mollusca. Coupling these factors with a more even distribution of molecular markers across species also allowed us to recover the major branches within each class and estimate the divergence times within the molluscan tree of life (Fig. 1).

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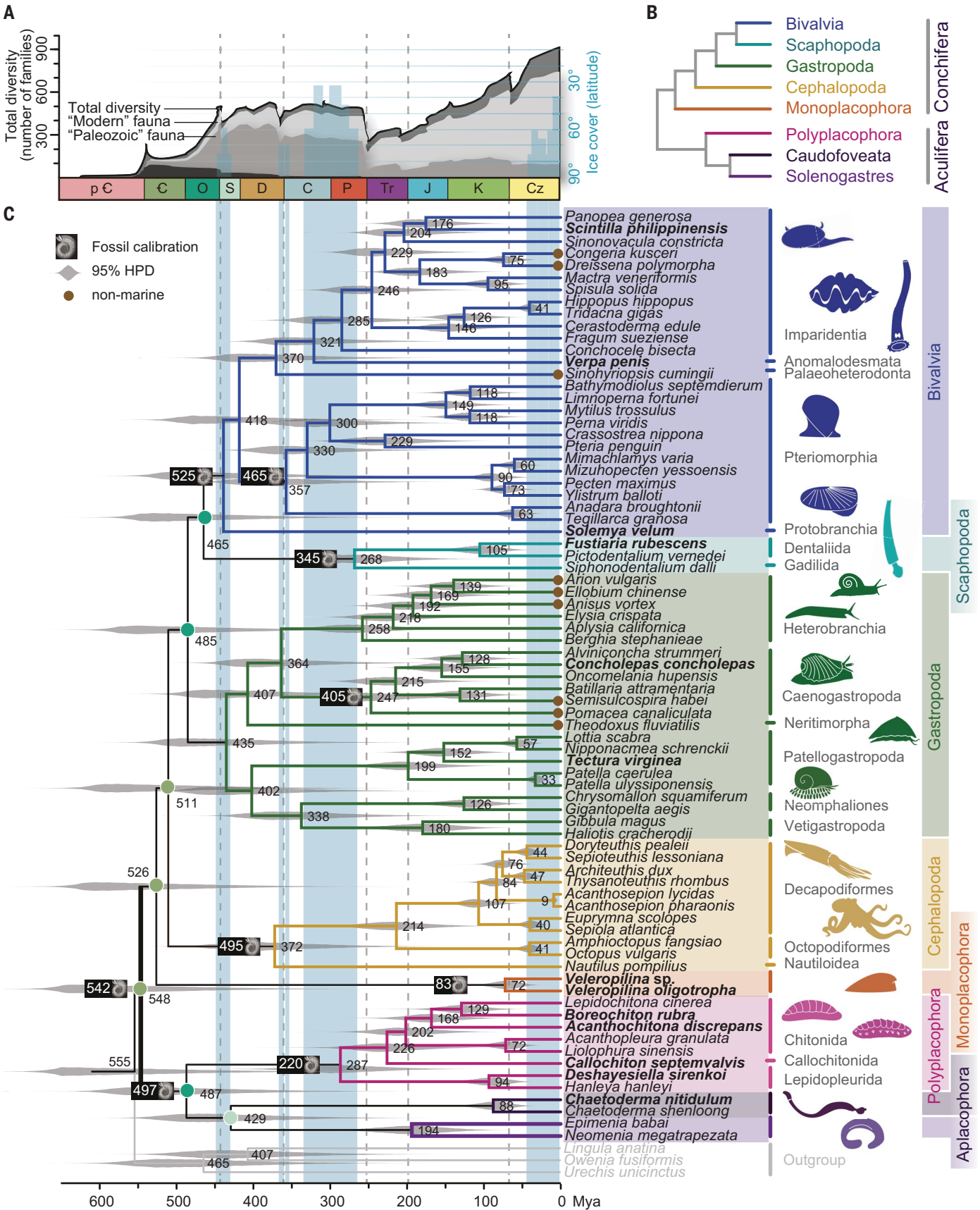


Fig. 1. Mollusca timetree. (A) The timeline of global biodiversity is based mainly on the diversity of the molluscan fossil record (55), which is influenced by mass extinctions (gray dashed lines) and climate change with periods of extensive ice cover (56) (blue bars, right y axis). Marine invertebrate fossils, dominated by Mollusca, are divided into three successive faunas in the Sepkoski curve shown here: Cambrian, Paleozoic, and Modern (55). (B) Schematic showing the overview phylogeny of the eight living classes of Mollusca. (C) Phylogenetic topology was computed by ASTRAL-Pro using a set of 954 BUSCO family trees from 77 species and three outgroups. Key clades are illustrated with icons (table S7). Species

The resulting tree recovered a topology that harmonizes key elements from former hypotheses into one topology: Aculifera, including a monophyletic Aplacophora (2, 6); Conchifera with basal branching Monoplacophora; and the Gastropoda-Bivalvia-Scaphopoda (2, 6, 13, 14) group, including Diasoma (13) (Fig. 2 and fig. S5).

A molluscan backbone

Morphological arguments about molluscan phylogeny historically focused on two alternative topologies, one with the clades Aculifera and Conchifera and the other with basal branching Aplacophora (vermiform mollusks) and a shelled clade “Testaria” (9) (Fig. 2). Early studies based on several loci recovered controversial alternative topologies with a putative sister relationship between Polyplacophora and Monoplacophora, termed Serialia, and other previously uncharacterized combinations (8). Later phylogenomic studies, despite low taxon sampling and data quality limitations, have mostly recovered Aculifera and Conchifera, but the relationships within Conchifera remained unresolved.

The fossil record of Mollusca provides important evidence to complement genomic data (Fig. 1A and table S5), and vice versa: In our topology, the origin of cephalopods predates Bivalvia and Gastropoda, although both appear earlier than cephalopods in the fossil record (3). Long-standing confusion over the affinities of

univalved and bivalved small shelly fossils from the early Palaeozoic may be reinterpreted when considering a stable backbone phylogeny.

The first transcriptome-based phylogeny that included all eight classes resolved Monoplacophora as the sister to Cephalopoda (6), as did another recent study (14). This hypothesis is problematic because it is not supported by morphology or the fossil record (9). Adding partial genome data for a monoplacophoran resolved Monoplacophora as the sister to the remaining Conchifera, as predicted by morphological studies (2). Our subset analysis of a smaller gene set reproduced the Cephalopoda + Monoplacophora relationship; however, it also failed to recover a plausible ingroup topology among some bivalves (fig. S4). Cephalopoda + Monoplacophora is an anomalous result that likely arises from limited datasets.

Among Conchifera, Rostroconchia represents the ninth, extinct class of Mollusca, which appears before both gastropods and bivalves in the fossil record (3). Rostroconchs exhibit character combinations from bivalves and scaphopods and are important evidence supporting Diasoma (3, 13). Evidence for Diasoma derives from morphology and fossils, but molecular data alone are not unequivocal. All phylogenomic studies have consistently supported a Gastropoda-Bivalvia-Scaphopoda clade. Early analyses recovered a clade [Bivalvia + (Gas-

tropoda + Scaphopoda)], but often with relatively low support for interclass relationships (2, 6, 13, 14). The first phylogenomic analysis that included scaphopods recovered Diasoma, which was also found here (Fig. 1). Analyses of scaphopod genomes support interpretations of incomplete lineage sorting (13). Molecular data show higher support for a Gastropoda-Bivalvia-Scaphopoda polytomy than either bifurcation (Fig. 2 and fig. S5). These groups likely represent the descendants of a complex and rapid radiation from a common conchiferan ancestor. This clade, here named Megalopodifera (big foot-bearing), is united by the veliger larva (although the larvae of monoplacophorans are entirely unknown), a body that can retract into the shell, a reduction in the number of foot-retractor muscles, and a large foot extending beyond the shell.

The molluscan tree of life

Internal topologies and timing within each class in the reconstructed phylogeny correspond well with established phylogenetic consensus and resolve some points of ongoing debate [see supplementary text (15)]. The monophyly of Aculifera is not controversial and is supported by fossil (5), anatomical (16), and molecular studies [see supplementary text (15)]. A deep split between Solenogastres and Caudofoveata in the Silurian corresponds to a

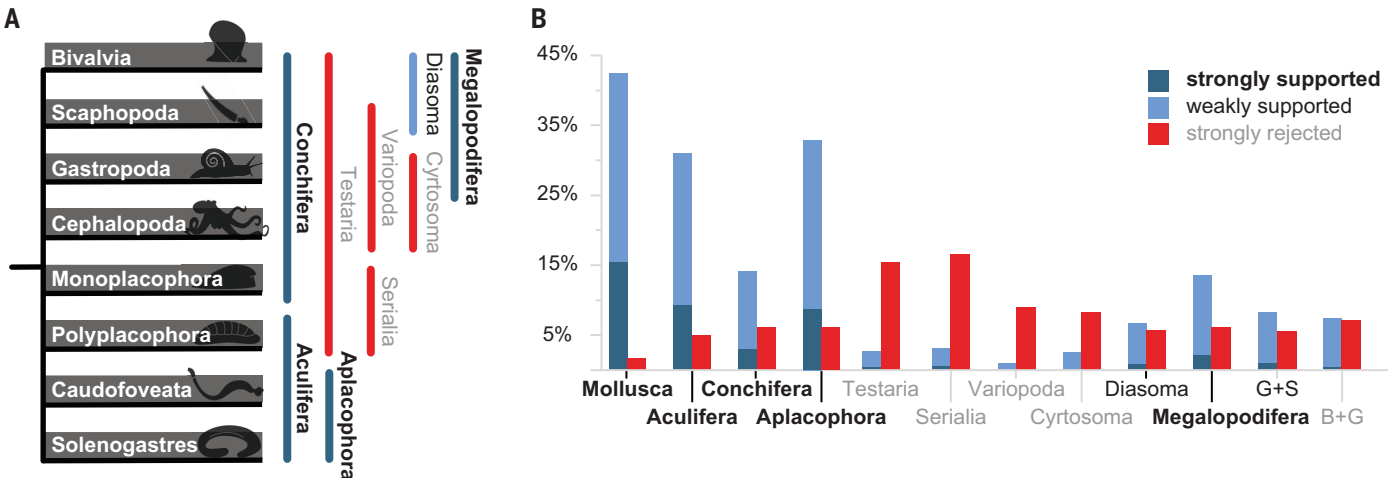


Fig. 2. Support for competing hypotheses in Molluscan relationships. (A) Schematic polytomy illustrating a subset of proposed molluscan ingroup relationships; colors illustrate the support. (B) Bar chart (57) comparing the proportion among 945 gene trees that support (blue) and reject (red) different proposed clades within Mollusca.

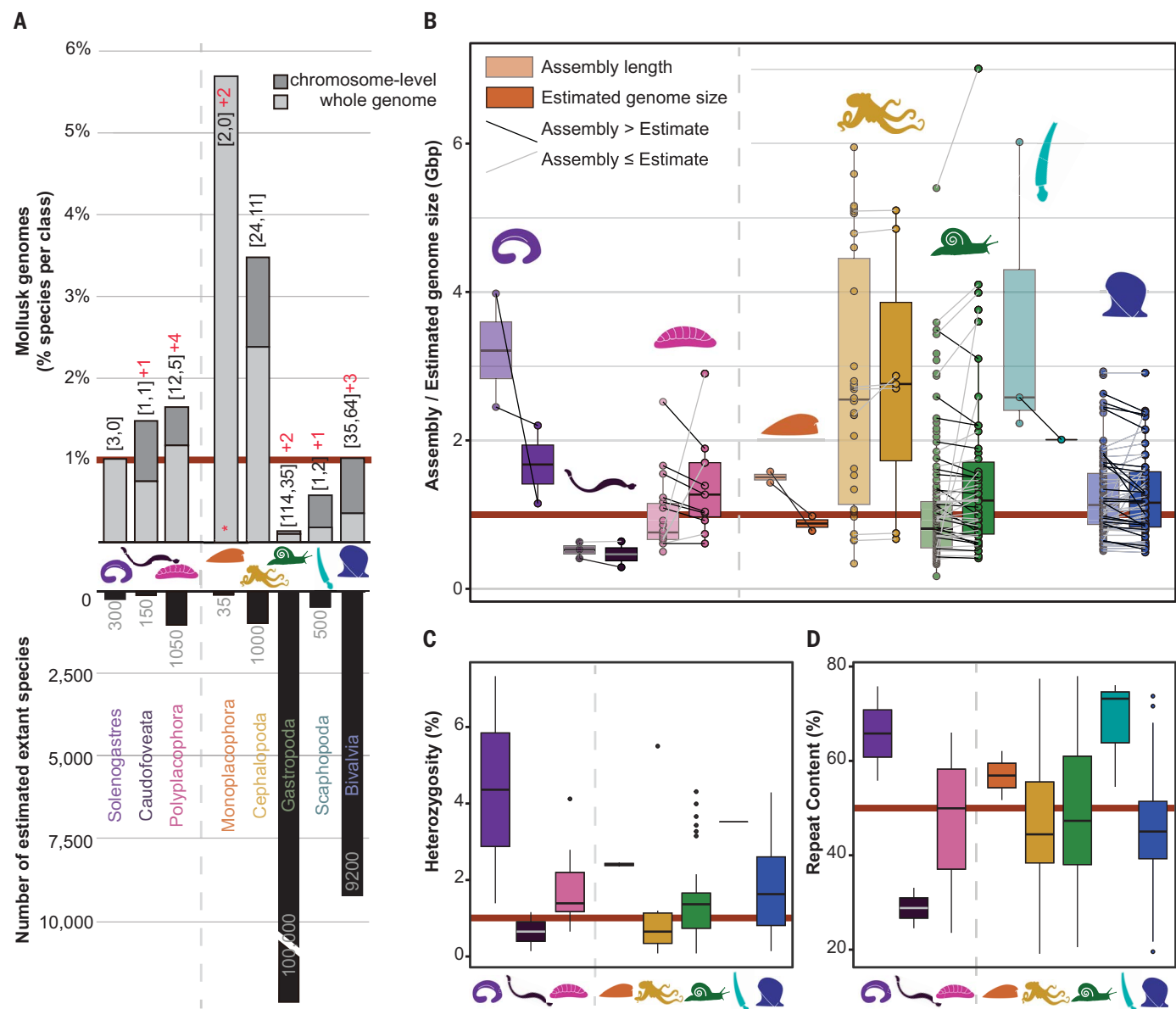


Fig. 3. Status and features of presently published molluscan genomes per class. (A) Variation in taxonomic richness and genome- or chromosome-level availability. The numbers in brackets show nonchromosomal assemblies (first) and chromosomal-level assemblies (second), including those in this study and all those published in National Center for Biotechnology Information (NCBI) GenBank (20 May 2024). The numbers of new genomes are indicated in red (the asterisk in monoplacophorans indicates that all available data are from the present study). A dashed line separates classes in Aculifera (left) and Conchifera (right). (B) Box plot summarizing reported assembly length (light colored) and estimated genome size (dark colored) median and inner quartile values for

each class. Individual dots (without connecting lines) are values that are missing predicted genome-size data. Darker lines highlight the common phenomenon of assembly size being higher than genome size because of very high heterozygosity. This phenomenon is not observed in cephalopods because the high percentage of repetitive sequences makes genome assemblies incomplete, combined with redundancy caused by high heterozygosity. (C) Estimated genomic heterozygosity per class. (D) Estimated repeat content per class. For box plots, the center line represents the median, box limits are upper and lower quartiles, and whiskers are minimum and maximum values.

radiation of disparate fossils attributed to stem aplacophorans (5). Diversification within Aplacophora is difficult to calibrate because of the poor fossil record of shell-less forms; the Mesozoic splits between sampled species in each class here may indicate that biodiversity recovery after the Triassic-Jurassic extinc-

tion included a radiation in Solenogastres. The topology of sampled chitons is consistent with morphological and paleontological evidence as well as previous genetic and genomic work (17, 18).

The two species used for the first full genomes of Monoplacophora include *Veleroipilina*

oligotropa from the Clarion Clipperton Zone and central Pacific as well as an undescribed *Veleroipilina* species collected from the Aleutian Trench that was morphologically indistinguishable from *V. oligotropa* but geographically distant (table S1). The divergence time between them was calibrated using an earlier

divergence time estimate for the living monoplacophorans, which estimated the first split among living monoplacophorans in the Cretaceous (19). Genome data from additional monoplacophoran groups and regions will likely influence this result.

Within Cephalopoda, the phylogeny of Decapodiformes (squid and cuttlefish) has been much debated, with conflicting hypotheses from mitochondrial genomes (20) or nuclear transcriptome data (21). Our phylogeny confirms a topology proposed from a large-scale five-gene analysis (22). Sepiolina has a basal branching position within Decapodiformes, which also agrees with results from transcriptomes (21, 23); we recognize Sepiolina as a taxonomic order, Sepiida, on the basis of this topology. Sepiida resolved as sister to the squids (Oegopsida + Myopsida). This scenario is more parsimonious than the previous omics-based phylogenetic analyses because it suggests only a single loss of the calcareous phragmocone in the evolution of the clade. Our time-calibrated phylogeny indicates a relatively very recent radiation of Decapodiformes in the Cretaceous, in agreement with earlier work showing accelerating diversification rates in the Cenozoic (22). The diversification of extant coleoid cephalopods has been rapid.

Within living scaphopods, the new genome for *Fustiaria* revealed a much shallower divergence than previously predicted within Dentaliida (24) and for the origin of crown group Scaphopoda. Scaphopods have a long and complex fossil record with many stem lineages, and the biology of extant species remains poorly understood (3, 25).

Gastropoda is divided into two fundamental clades: Psilogastropoda, which unites Patellogastropoda as sister to Vetigastropoda + Neomphaliones, and Adenogonogastropoda, which comprises Neritimorpha as sister to Apogastropoda (Caenogastropoda + Heterobranchia) [see supplementary text (15)]. This structure corroborates transcriptome-based studies that excluded the deep-sea subclass Neomphaliones (26, 27). A subsequent transcriptome study with expanded taxon sampling recovered contradictory topologies but supported Adenogonogastropoda (28), first identified in an early molecular study (29) and later named on the basis of morphocladistic analysis (30). The early Cretaceous internal split in Neomphaliones also matches the scant fossil record for the group because it postdates the earliest neomphaline fossil from the Late Jurassic (31).

In contrast to most morphological analyses, our results indicate that Patellogastropoda is not the earliest branching clade within gastropods. A secondarily derived Patellogastropoda is recovered in most omics-based reconstructions (27, 32). One recent transcriptome tree (28) could recover basal-branching Patellogas-

tropoda when rapidly evolving sites representing more than half of the dataset were excluded. Incongruity between morphology and molecular results leaves the position of Patellogastropoda uncertain, or awaiting further explanation. The limpet form has evolved convergently more than 50 times among gastropods but has its largest radiation in the patellogastropods (33). It is well established that patellogastropods are split into two clades, Patellicida + Nacellida (3), but divergence-time estimates in other studies vary wildly depending on the calibration points used within the clade. Our results are concordant with the fossil record, because the earliest known crown-group patellogastropod fossil is from the Triassic of Italy (34).

Our Caenogastropoda topology recovered Neogastropoda (*Concholepas*) nested within a paraphyletic Littorinimorpha (*Oncomelania* + *Alviniconcha*), consistent with previous findings (35). Our tree also supports Cerithioidea (*Batillaria* + *Semisulcospira*) clustering with hypsogastropods, which does not contradict the morphologically supported Sorbeoconcha concept (3). Some other studies (36), by contrast, unexpectedly recovered Cerithioidea as the sister to the architaenioglossan superfamily Ampullarioidea (represented here by *Pomacea*). In Heterobranchia, we recovered a monophyletic Tectipleura as sister to Ringipleura (*Berghia*). Within Tectipleura, the observed sequence of branching in Panpulmonata first gives rise to Sacoglossa (*Elysia*), then Hygrophila (*Anisus*), and finally Ellobioidea (*Ellobium*) and Stylomatophora (*Arion*), consistent with the recent consensus (3, 37, 38).

We recovered the consensus backbone phylogeny for bivalves (39), with Protobranchia (*Solemya*) in the earliest derived position, followed by Pteriomorpha, then Palaeoheterodonta (*Sinohyriopsis*), and Anomalodesmata (*Verpa*), sister to Imparidentia. Relationships within Pteriomorpha and Imparidentia also mostly align with other molecular and morphological results (40, 41) [see supplementary text (15)]. One notable deviation is that the order Adapedonta (*Panopea*, *Sinonovacula*) is paraphyletic; the split between Adapedonta + Galeommatida (represented here by *Scintilla*) formed a conflicted node noted in earlier work (39, 42). The reduced shells of many galeommatidans led to a poor fossil record and taxonomic uncertainty. The rich fossil record of bivalves provides a well-resolved chronology for the origin of major lineages; however, our time-calibrated tree does not account for the impact of mass extinctions and rapid bursts that shaped bivalve diversification (43). Within Imparidentia, all orders except Lucinida first appear in the fossil record after the end-Permian mass extinction event (40, 42), as also found here. The common ancestor of crown-group Imparidentia necessarily had an origin

before the end of the Permian, and the clade extends deeper in the stratigraphic record, which is not accounted for in our timetree.

Evolution of molluscan forms

The animal biomineralization toolkit predates the evolution of Mollusca (44). Mollusks are effective at repurposing ancient gene families in new ways (45). Phylogenetic relationships of phyla within Lophotrochozoa are not well resolved, and alternative hypotheses have different implications for the evolution of mineralized skeletons. Early small shelly fossils with molluscan affinity were used to calibrate the origin of Mollusca (table S5). Some putative early mollusks lack a solid shell, such as *Odontogriphus* and *Shisanian* (4); however, the ancestor of Aculifera + Conchifera did possess a shell, and fossils lacking solid shells represent further diversity and not the condition of the common ancestor leading to Aculifera + Conchifera.

Fossil Mollusca present additional character mosaics that are not found in any living groups. The early Cambrian *Pelagiella exigua*, which had a coiled shell but flexible chaetae, was interpreted as a stem gastropod (46), but our timetree suggests that it is more likely a stem conchiferan. The deep divergence between Aplacophora + Polyplacophora (around 440 million years ago) is congruent with mosaic aculiferan forms in the fossil record (*Phthipodochiton*, *Kulindoplaux*) from the Ordovician to Silurian (453 million to 422.9 million years ago) and much greater disparity in fossil forms in the aculiferan stem group. Character combinations underscore the flexibility of the molluscan genome to repeatedly redeploy morphological adaptations.

Body plans in living Mollusca include evidence of extensive convergence and character shifts on shorter timescales. For example, Juliidae, gastropods with bivalved shells, were once thought to be a transitional form between gastropods and bivalves but instead represent a recently derived adaptation to a specialized ecological niche (47). Endolithic bivalves occur in 11 separate families; *Verpa*, included here, has morphological modifications so extreme that it cannot be accommodated in morphometric comparisons (48), but this family has a Cenozoic origin (49).

Important evolutionary transitions often require the transformation of shell mineralization and associated physiology, as seen in the repeated colonization of terrestrial and freshwater environments among bivalves, gastropods, and one estuarine cephalopod. Even the few nonmarine lineages included in our study show that terrestrialization events were time independent and not directly connected to global patterns (Fig. 1). Morphological and ecological plasticity has been a constant throughout the long history of the phylum.

Evolution of molluscan genomes

Broad taxon sampling now reveals that many molluscan genomes have extremely high heterozygosity and a high proportion of repetitive sequences (Fig. 3). These factors can result in highly fragmented assemblies, with mixed diploid sequences that are difficult to separate clearly, and multiple steps are needed for each new genome (1) (table S2). In Mollusca, concurrent high heterozygosity results in most assembly sizes being larger than the estimated genome size owing to the inclusion of another haplotype (Fig. 3 and tables S2 and S6). Estimates of heterozygosity for six of the eight classes are above 1%, with some species above 4%; these rates easily surpass those of other animal groups, such as Lepidoptera (butterflies and moths), where heterozygosity is considered high (50).

Genome features are not linearly correlated to morphological or ecological adaptations. The morphologically conserved Polyplacophora show very high rates of chromosome rearrangement (17). Well-conserved syntenic regions in more recent radiations of animals, such as mammals or birds, result in relatively similar genome structures, facilitating whole-genome comparisons (51, 52). A new understanding of the variation in molluscan genomes provides a foundation for further work on fundamental questions of genome evolution, including the drivers of heterozygosity. Adding chromosome-level genomes for molluscan groups (e.g., Monoplacophora) is needed for comprehensive syntenic comparisons and to understand the role of chromosome rearrangements in speciation and cladogenesis. Several major lineages still have no genomes available: the bivalve clade Archiheterodonta and, at a finer scale, the bivalve orders Nuculida and Trigoniida (*Neotrigonia* is the only living member of an order known otherwise from fossils). Adding genomes for the gastropod order Cocculinida may alter, or confirm, the position of Patellogastropoda. Increased taxon sampling, especially in scaphopods, is important to fully understand molluscan topology.

Conclusions

Extensive economic and research interests have consistently held Mollusca at the forefront of diverse disciplines, including fisheries, neurobiology, ecotoxicology, and biomimetic design. However, their extraordinary diversity fundamentally hinders a confident assessment of their evolutionary history. The question of why early genetic studies struggled to recover reliable evolutionary patterns is potentially answered by the extreme variability among genomes that are now available for all classes and their major clades within the phylum. Large-scale genomic analyses across the tree of life have provided inconclusive evidence regarding the drivers of genetic diversity (53, 54). These analyses are

presently limited by incomplete sampling; to establish general principles of organismal phenotype and genome, they must also include Mollusca, the most extensive experiment in animal body plans.

A stable topology for the total group Mollusca is fundamental for understanding the evolution of body plans and the fossil record. Based on this new phylogeny, we infer that the molluscan ancestor had a solid dorsal shell, a foot, multiple dorsal-ventral and oblique foot-retractor muscles, a radula, and no eyes. Throughout the long evolutionary history of Mollusca and continuing today, aspects of a flexible genome led to a flexible phenotype: Endless forms of mollusks showcase the power of animal evolution.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Supplementary Text
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