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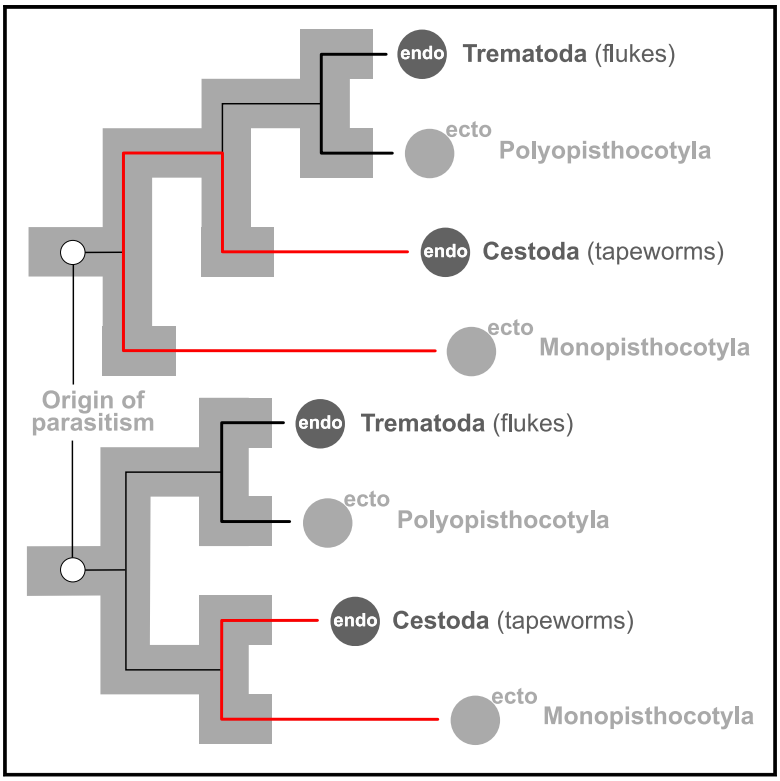
The evolution of endoparasitism and complex life cycles in parasitic platyhelminths

Graphical abstract



Highlights

- Transcriptomics reveal novel evolutionary scenarios for parasitic flatworms
- Endoparasitic life strategies evolved independently in Neodermata
- Complex life cycles characteristic of tapeworms and flukes evolved separately
- Monopisthocotyla new class and Polyopisthocotyla new class are promoted

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In brief

Brabec et al. present phylogenomic evidence of the evolution of hallmark life strategies within parasitic flatworms, including the independent adoption of endoparasitism and complex life cycles typical of tapeworms and flukes.

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The evolution of endoparasitism and complex life cycles in parasitic platyhelminths

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SUMMARY

Within flatworms, the vast majority of parasitism is innate to Neodermata, the most derived and diversified group of the phylum Platyhelminthes.[1,2] The four major lineages of Neodermata maintain various combinations of life strategies.[3] They include both externally (ecto-) and internally feeding (endo-) parasites. Some lineages complete their life cycles directly by infecting a single host, whereas others succeed only through serial infections of multiple hosts of various vertebrate and invertebrate groups. Food sources and modes of digestion add further combinatorial layers to the often incompletely understood mosaic of neodermatan life histories. Their evolutionary trajectories have remained molecularly unresolved because of conflicting evolutionary inferences and a lack of genomic data.[4] Here, we generated transcriptomes for nine early branching neodermatan representatives and performed detailed phylogenomic analyses to address these critical gaps. Polyopisthocotylea, mostly hematophagous ectoparasites, form a group with the mostly hematophagous but endoparasitic trematodes (Trematoda), rather than sharing a common ancestor with Monopisthocotylea, ectoparasitic epithelial feeders. Phylogenetic placement of the highly specialized endoparasitic Cestoda alters depending on the model. Regardless of this uncertainty, this study brings an unconventional perspective on the evolution of platyhelminth parasitism, rejecting a common origin for the endoparasitic lifestyle intrinsic to cestodes and trematodes. Instead, our data indicate that complex life cycles and invasion of vertebrates' gut lumen, the hallmark features of these parasites, evolved independently within Neodermata. We propose the demise of the traditionally recognized class Monogenea and the promotion of its two subclasses to the class level as Monopisthocotyla new class and Polyopisthocotyla new class.

RESULTS AND DISCUSSION

We sequenced seven transcriptomes from early diverging representatives of five cestode orders, including three unsegmented (monozoic) groups, a previously omitted representative of a principal trematode lineage Aspidogastrea, and a representative of Polyopisthocotylea (Data S1). These parasitic lineages have never been investigated in phylogenomic analyses or were represented by the most derived representatives, especially in the case of tapeworms. Our supermatrix included 83 platyhelminth taxa (51 of which were parasites), containing 225 genes (77,824 amino acid sites) selected based on >60% representation of the included platyhelminths after manual curation of orthologs (see details in Data S1). Maximum likelihood (ML) and Bayesian inference phylogenies estimated using the profile mixture models LG + C60 + G_4 + F (hereafter referred to as LG-C60) in IQ-TREE and CAT + GTR + G_4 (hereafter referred to as CAT-GTR) in Phylobayes, respectively, yielded two alternative topologies that contradict each other with respect to the phylogenetic position of the cestodes (Figures 1A and 1B). Although the LG-C60 model resolved the cestodes as sister to the group formed by Polyopisthocotylea + Trematoda, the CAT-GTR model resolved them as sister to Monopisthocotylea. In addition to Phylobayes, an ML analysis ran under CAT-GTR with posterior mean site frequencies model (CAT-PMSF[5]) in IQ-TREE (Figure S1) has recovered the same topology as Phylobayes. It is important to note that the internal topology between the four major neodermatan lineages remained unchanged across the analyses; the difference between the estimates is limited to the position of the root: the LG-C60 topology places root between Monopisthocotylea and other lineages, whereas the CAT-GTR topology roots Neodermata between Monopisthocotylea + Cestoda and Polyopisthocotylea + Trematoda. As it is impossible to objectively decide which of the two topologies is more likely correct, we discuss the evolution of Neodermata in the frame of both topologies.

Comprehensive understanding of the phylogeny of Neodermata

Neodermata is a group characterized morphologically by the presence of a ciliated larva (with the exception of a few derived taxa) whose epidermis is replaced with a new syncytial layer (tegument or neodermis) at all later developmental stages. Our analyses confirm the status of the parasitic Neodermata as the

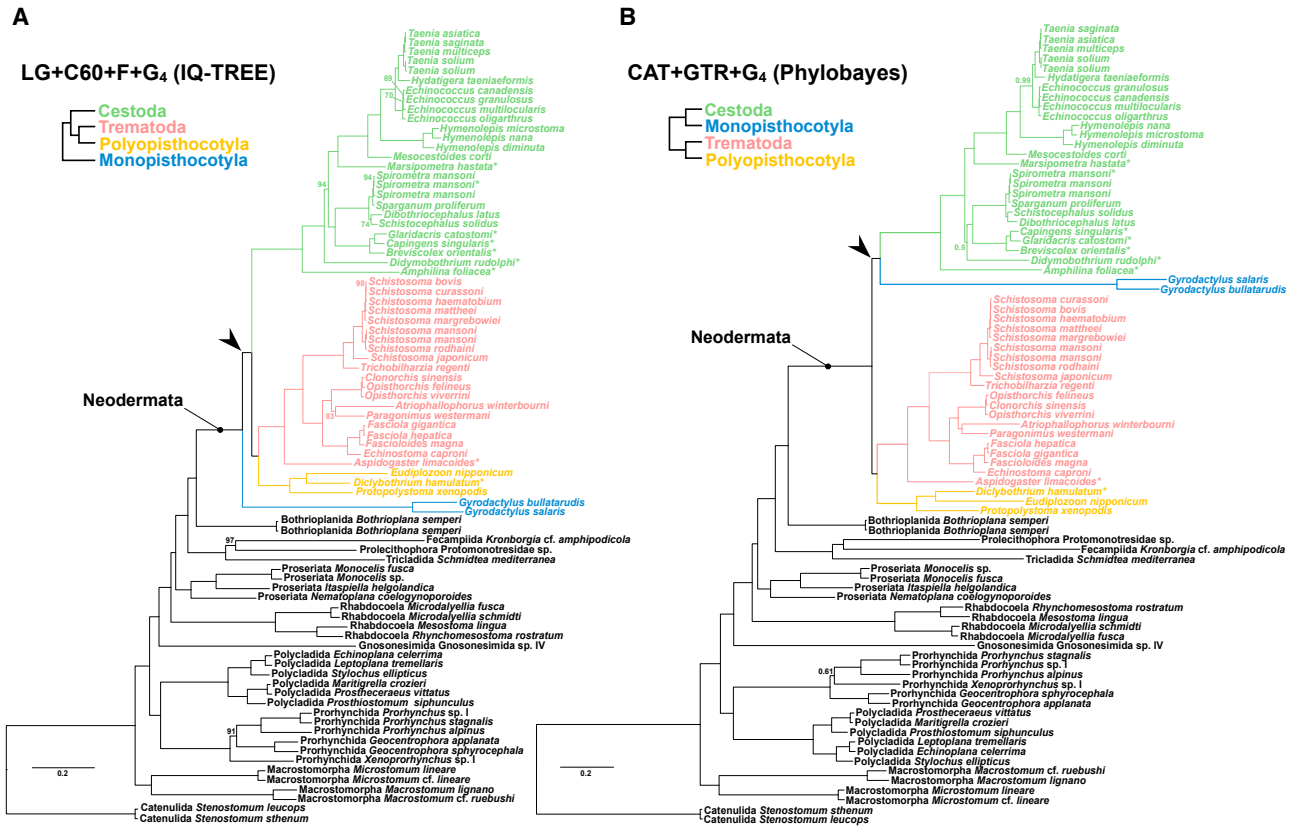


Figure 1. Interrelationships of Neodermata

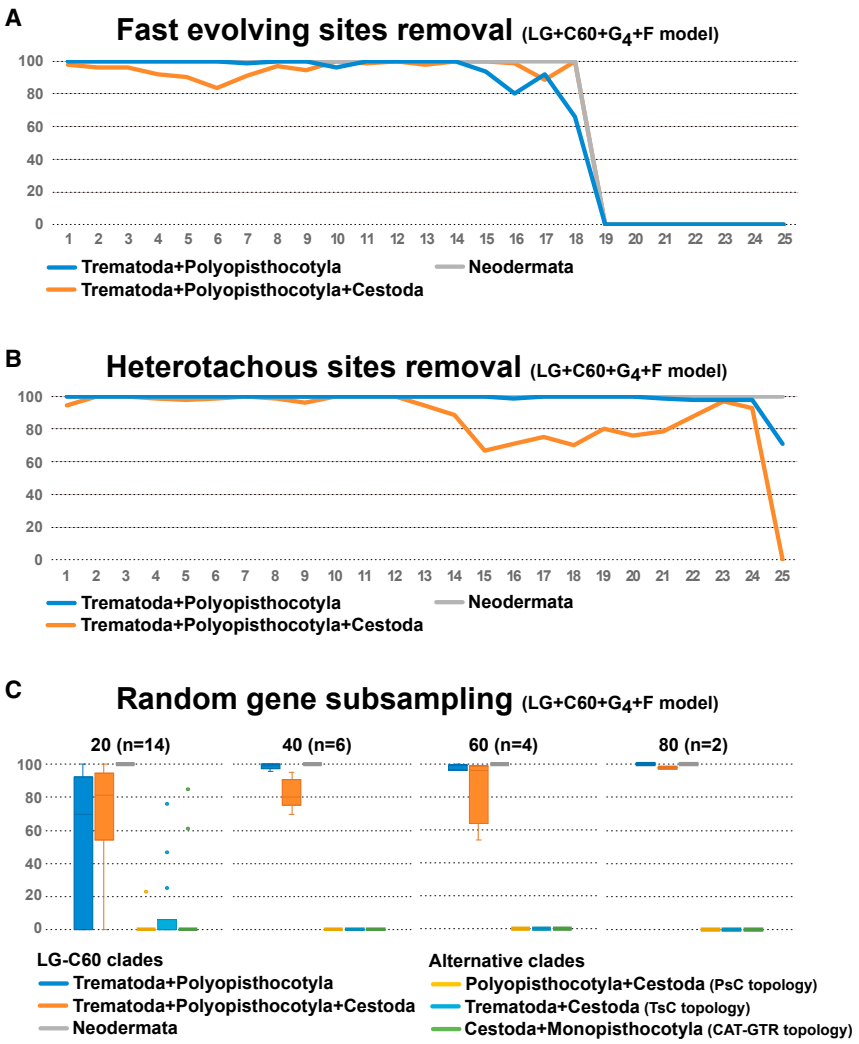
Phylograms are based on 225 genes (77,824 amino acid sites) and rooted with Catenulida. Four parasitic groups of Neodermata are highlighted in group-specific colors. Newly characterized transcriptomes marked with an asterisk. Black arrow marks the conflict between the two phylogenies. The branch length scale bar indicates number of substitutions per site.

(A) Maximum likelihood phylogeny estimated using the LG + C60 + F + G_4 model in IQ-TREE. Nonparametric posterior mean site frequencies (PMSF) standard bootstrap support (1,000 repetitions) shown only when less than 100.

(B) Bayesian inference consensus of four independent Markov chains run for 19,000 generations in Phylobayes under the CAT + GTR + G_4 model (burn-in 3,000 trees). Posterior probabilities shown only when less than 1.0. See also Figures S1 and S2 for CAT-PMSF and unrooted phylogenies, respectively.

most derived group of Platyhelminthes and the free-living *Bothrioplana* as their closest living relatives, as previously suggested.[1,2,6] However, our phylogenetic estimates reveal an unorthodox evolutionary scenario for the relationships within the major Neodermata groups, in which polyopisthocotylean monogeneans are sister to Trematoda (Figures 1A, 1B, and S1). This scenario has never been hypothesized by flatworm systematists and has virtually never been recovered with molecular data, except for a single analysis based on a reduced 28S ribosomal DNA (rDNA) dataset (D3 domain)[7] and a more recent phylogenomic analysis based on automatically predicted groups of orthologous genes.[8] Conflicting phylogenetic signals have also emerged from whole genome comparisons that included a polyopisthocotylean representative *Protopolystoma xenopodis*, in addition to the medically relevant cestodes and trematodes[9]: Coghlan and colleagues' analysis of a dataset of 202 single-copy genes (21,649 amino acids) found trematodes as sister to *P. xenopodis*, but at the same time, they also found that trematodes shared nearly twice as many gene families with cestodes (1,329) than with the polyopisthocotylean representative (725), and the number of shared genes showed a similar pattern.[9]

Earlier molecular phylogenies inferred from a single locus, typically parts of the nuclear ribosomal operon or mitochondrial genomes (reviewed in Olson and Tkach[10]), proposed two other competing hypotheses for the evolution of Neodermata. Most studies tended to consider trematodes as sister to cestodes (Trematoda sister to Cestoda [TsC] topology, Figure 2D), whereas others placed trematodes as the earliest-branching neodermatans (Trematoda basal [Tb] topology, Figure 2D). The Tb scenario is particularly notable because it substantiates the "cercomer" theory[11,12] that assumed a common evolutionary origin for cestodes and the two monogenean lineages (Monopisthocotylea + Polyopisthocotylea) based on the presumed homology of the oncospheral (larval) hooks of cestodes and the haptoral (posterior attachment organ) hooks of monogeneans. The cercomer theory was supported by some rDNA data[6,13–15] but less often with phylogenomic approaches,[2,9,16] although there were some exceptions.[1] Another frequently debated topological scenario considers the monophyly of Monogenea, a long-held morphology-based hypothesis that Monopisthocotylea and Polyopisthocotylea share a common ancestor (Monopisthocotylea + Polyopisthocotylea monophyly [MPm] topology, Figure 2D). Our analyses, using the



LG-C60 model AU test

topology	logL	deltaL	p-AU	
LG-C60	-2497906.9	0.03	0.841	+
CAT-GTR	-2497963.4	56.48	0.039	-
TsC	-2498009.7	102.78	0.001	-
MPm	-2498126.8	219.93	0.000	-
PsC	-2498050.5	143.62	0.000	-
Tb	-2498105.9	198.99	0.001	-
Pb	-2498095.2	188.25	0.002	-
NsK	-2499259.3	1,352.40	0.000	-

CAT-GTR model AU test

topology	logL	deltaL	p-AU	
LG-C60	-2090312.0	37.07	0.000	-
CAT-GTR	-2090275.0	0.03	0.455	+
TsC	-2090295.7	20.78	0.036	-
MPm	-2090340.8	65.88	0.000	-
PsC	-2090337.3	62.33	0.000	-
Tb	-2090291.7	16.76	0.093	+
Pb	-2090332.7	57.73	0.000	-

Figure 2. Stability of selected internal nodes and alternative topologies

(A and B) Effects of removing (A) fast evolving and (B) heterotachous sites on the phylogenetic inference of the basal nodes of Neodermata.

(C) Test of gene sampling bias. Distribution of nodal supports for monophyly of selected groups after random gene subsampling of the 225-gene dataset.

(D) Schematic depiction of the alternative topological scenarios tested with the AU test: TsC, Trematoda sister to Cestoda; MPm, Monopisthocotylea + Polyopisthocotylea monophyly; Tb, Trematoda basal; Pb, Polyopisthocotylea basal; PsC, Polyopisthocotylea sister to Cestoda; NK, *Kronborgia* (Fecampiida) sister to Neodermata. See Data S1 for more details. See also Figure S3 for phylogenies inferred after the removal of the highest gene-wise log-likelihood score genes.

Systematic error does not influence inferred topologies

We performed several analyses to investigate whether systematic errors influenced our phylogenetic inferences. Due to computational intensity, we performed these analyses using the LG-C60 model only. First, we removed outgroup taxa that tend to form long branches relative to ingroup taxa, which can cause a long-branch attraction (LBA) artifact. This removal did not affect the resulting ingroup topology (Figure S2). Second, we independently performed iterative removal of the fastest-evolving sites and most heterotachous sites, neither of which showed strong effects on the topology or statistical support (Figures 2A and 2B; Data S1). In these dataset manipulations, none of the competing topologies received any statistical support. For the fast-evolving site removal, the topology collapsed only after nearly 70% of the sites had been removed (step 18, Figure 2A). For the heterotachous site removal, the topology did not fall apart until 92.5% of the amino acids had been removed (step 24, Figure 2B). Thus, we did not find any evidence that long-branch attraction and other systematic errors affect the topology inferred in the ML analysis.

To test for gene sampling bias, genes were randomly subsampled into sets of 20% (n = 14 datasets), 40% (n = 6), 60% (n = 4), and 80% (n = 2) of the full dataset, under a 95% confidence interval that all genes were included in one of the sub-datasets, as in Salomaki et al.[17] Each of these randomly subsampled datasets was subjected to phylogenetic reconstruction and bootstrapping to

most comprehensive gene- and taxon-rich dataset to date, reject all these scenarios and suggest a new interpretation of platyhelminth evolution.

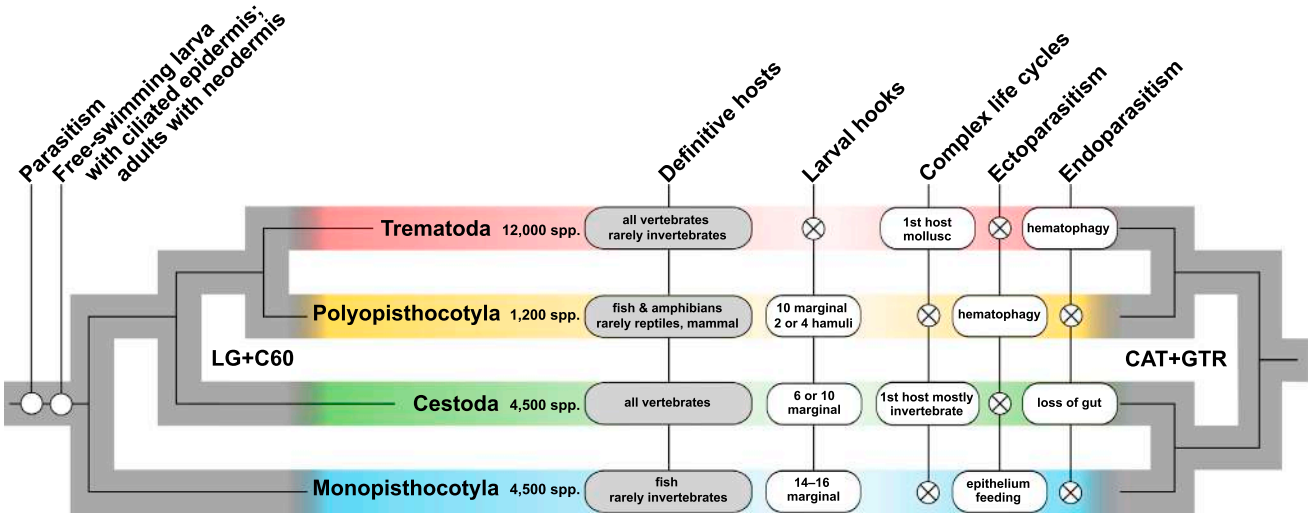


Figure 3. Evolution of the parasitic flatworms (Neodermata) and the distribution of conspicuous biological traits characteristic of individual groups

The maximum likelihood (LG + C60 + F + G_4) scenario is shown on the left, and the Bayesian inference under the CAT + GTR + G_4 model is shown on the right. Presence of individual traits is depicted with white circles that can contain taxon-specific details relevant for a given trait. Absence of a trait is depicted with ⊗. See also Data S2 for an overview of basic characteristics of the four classes.

examine support for the alternative topologies (Figure 2C; Data S1). As expected, the datasets based on 20% of the genes showed the highest variability, with the clade Trematoda + Polyopisthocotylea receiving mixed support. More importantly, the competing topologies remained unsupported in all cases of subsampling, except for a few outlying datasets based on only 20% of the genes.

To further investigate whether the topology found was influenced by a few highly biased loci, we identified genes with the strongest influence on the conflicting topologies (n.b., the LG-C60, CAT-GTR, TsC, and MPm topologies) as recovered by the difference in gene-wise log-likelihood scores (ΔGLS) approach of Shen et al.[18] Removal of five genes with the greatest impact on the ΔGLS supporting either of the hypothesized alternative topologies (Data S1) also did not alter the LG-C60 topology (Figure S3). Finally, we performed the approximately unbiased (AU) topology test on a set of alternative topologies constrained to the CAT-GTR, TsC, MPm, and Tb, as well as three other topologies (Figure 2D). The AU test with LG-C60 model clearly favored the LG-C60 topology and rejected all seven alternative topologies tested, whereas the CAT-GTR model favored the CAT-GTR topology and rejected all but the Tb alternative topology (rejection probability at 0.05; Figure 2D; Data S1).

Endoparasitic versus ectoparasitic strategies

Parasitism is a commonly encountered life strategy among eukaryotes, but the intimacy and intricacy of the relationships between neodermatans and their numerous hosts have few analogies elsewhere. Both trematodes and cestodes were able to establish themselves as strict endoparasites (hypothetically to escape predators by moving into their hosts' bodies). They evolved complicated life cycles colonizing all vertebrate groups and diversified into a large number of species (Figure 3). Until now, the origin of endoparasitism has been considered a single evolutionary event (the isolated endoparasites within Monopisthocotylea and Polyopisthocotylea are thought as secondary lineage-specific adaptations) with two possible scenarios: endoparasitism either (1) evolved from ectoparasitism (which itself represented the first step away from a free-living lifestyle) or (2) represents the original life strategy of all neodermatans, which was later superseded by monogeneans' switch to ectoparasitism.[3] It was primarily molecular data that supported the hypothesis that endoparasitism was the derived strategy novel solely to the last common ancestor of trematodes and cestodes, two of the most "sophisticated" lineages of flatworms.[19]

Both LG-C60 and CAT-GTR phylogenies suggest that endoparasitism arose twice independently from ectoparasitism in Neodermata or, alternatively and equally likely, one or two reversals to ectoparasitism occurred in Polyopisthocotylea and Monopisthocotylea, depending on the phylogenetic position of the cestodes (Figure 3). Examples of secondary life strategy switches in certain small taxa within Neodermata support neither of these versions unanimously. For example, there are more than a hundred endoparasitic (~2.5% of known) species among the otherwise ectoparasitic Monopisthocotylea (mostly within the genera *Enterogyrus* and *Calicotyle*). About 8.3% of all Polyopisthocotylea species are also endoparasitic, found primarily within Polystomatidae, one of the major families formerly considered a separate monogenean lineage.[20,21] There is no reversal of life strategy toward ectoparasitism in cestodes, but trematodes house a few exceptions: adults of the relatively basal family Transversotrematidae[22] live under the scales of fish, and some representatives of Accacoeliidae live on fish gills.[23] Both modes of parasitism also arose several times in non-neodermatan flatworm lineages. The group Fecampiida (represented by *Kronborgia* in our dataset), consists of several small, clearly delineated families, all of which are obligate endoparasites of various invertebrate hosts, including crustaceans, molluscs, polychaetes or, less often, fish.[24–26] Other lesser-known parasitic lineages include Genostomatidae, ectoparasites of crustaceans; Acholadidae, endosymbionts of sea stars that have even lost their guts; and

Temnocephalida, ectocommensals of crustaceans.[27,28] None of the above-mentioned non-neodermatan parasitic lineages is mutually interrelated according to molecular phylogenies,[6,28,29] and all display only relatively simple life cycles.[30]

Origins of complex life cycles in Neodermata

Our phylogenomic analyses refute the most parsimonious explanation for the common origin of complex life cycles of trematodes and cestodes. Both groups begin life as free-living larvae that hatch from an egg, which is always armed with hooks in cestodes but never in trematodes (Figure 3). Larvae usually develop in aquatic environments before entering either one or a chain of several intermediate hosts, often (but not exclusively) linked by predator-prey relationships. However, the first intermediate hosts of trematodes and cestodes belong to different taxonomic groups. Trematodes begin their parasitic life almost exclusively in molluscs (except Aporocotylidae found in polychaetes), where they reproduce clonally (except Aspidogastrea), whereas the first intermediate hosts of cestodes span several groups across arthropods and annelids or, exceptionally, vertebrates. Given the lack of reliably resolved relationships within Neodermata, previous authors have tended to consider a single switch from direct (single-host) to complex (multiple-host) life cycles as the most plausible scenario,[3,19] but our data challenge this scenario.

Parasitism pervades the tree of life and is found within at least 223 metazoan lineages and 15 phyla.[31] Some sub-lineages of these parasites have also evolved complex life cycles[32–34] in a manner similar to Neodermata—the nematode families Ascarididae and Anisakidae[35] or Pentastomida within crustaceans,[36] for example. Although cartilaginous and bony fishes serve as definitive hosts for many early-branching cestode and trematode lineages, they are less prevalent among trematodes.[23] Trematodes of elasmobranchs are also less abundant and probably represent secondary host switches from teleosts.[37] It is becoming increasingly clear that the complexity of life cycles has not evolved in concert across the individual lineages during the evolutionary history of Neodermata. Although complex life cycles are believed to have helped cestodes and trematodes colonize a wide range of tetrapod hosts (along with the adoption of asexual reproduction by trematodes in molluscs), the increasing complexity of their life cycles was sometimes accompanied by simplification through progenesis, a seemingly contradictory developmental adaptation thought to further increase transmission success.[38]

Suppression of Monogenea Carus, 1863 and proposal of two new classes

Our two phylogenies based on genomic data provide compelling evidence for the non-monophyly of Monogenea, which has been widely accepted based on previous phylogenies based on mitochondrial genome[39] and a larger rDNA fragment.[6] According to our analyses, Monopisthocotylea are either the earliest-diverging lineage of Neodermata or a sister group of Cestoda, whereas Polyopisthocotylea forms a sister lineage to Trematoda according to both models (Figures 1A and 1B). The idea of independent origins for the constituent groups of Monogenea based on the putative homoplasy of several morphological, ultrastructural, and ecological characters[40–43] has long been debated. However, the relative phylogenetic positions of the two groups remained unclear, and the paraphyletic monogeneans were thought to form either the earliest-diverging lineages of Neodermata or a relatively derived group to trematodes. Previously, monopisthocotyleans had been reconstructed as the earliest-branching neodermatan lineage by some single-gene analyses,[7,44] mitogenomics,[39] and phylogenomic analyses in which monopisthocotyleans were the sole representatives of Monogenea.[2] In sharp contrast to that, recent phylogenetic inferences based on orthologous groups of proteins have reconstructed monopisthocotyleans as sister to cestodes, although without convincing statistical support.[8] Our analyses complement these recent studies by reliably resolving the position of Polyopisthocotylea using transcriptomic data, a suggestion that has been called for previously.[4] Although our analyses do not fully clarify the position of cestodes within Neodermata, the signals for the non-monophyly of the two monogenean lineages are strong and consistent, justifying the suppression of the class Monogenea.

Consequently, we propose to elevate the two subclasses proposed already by Odhner[45] to the class level as Monopisthocotyla new class and Polyopisthocotyla new class. A summary of the major synapomorphies and taxonomic diagnoses of all classes of Neodermata is provided in Data S2. Several platyhelminth morphologists independently argued for mutual non-homology of numerous characters in monogeneans—from ciliated larval (oncomiracidial) morphology, including attachment adaptations in the haptor and development of the glandular system that supports attachment of the parasite to the host, to a number of adult body characters, including the organization of the haptor, presence or absence of the genitointestinal canal, and sperm ultrastructure (summarized in Euzet and Combes[42] and Data S2). The differences in feeding strategies and the spatial organization of digestion are probably most striking to non-specialists: monopisthocotylans feed on the epithelia and mucus of their fish hosts, whereas polyopisthocotylans feed almost exclusively on host blood. Thus, the dogma of monophyly of monogeneans, which lasted for more than 150 years, was based on erroneous assumptions about the homologies shared by the two groups and an underestimation of the numerous differences that exist.[42,43,46]

Our interpretation of the evolution of parasitic flatworms (supported by our transcriptomic data, in combination with publicly available genomic data) clearly refutes the common origin of endoparasitism in cestodes and trematodes. Our phylogenomic analyses show that the ectoparasitic polyopisthocotyleans form a sister lineage of the endoparasitic trematodes, whereas monopisthocotyleans, the second ectoparasitic group, are either sister to cestodes or the earliest-diverging lineage of Neodermata. In view of the concordance of the phylogenies and the additional tests performed here, we propose suppression of the paraphyletic class Monogenea and elevation of its two original subclasses to Monopisthocotyla new class and Polyopisthocotyla new class.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2023.08.064.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.B., E.D.S., M.K., T.S., and R.K.; methodology, E.D.S. and M.K.; formal analysis, J.B. and E.D.S.; investigation, J.B., E.D.S., and R.K.; resources, M.K. and R.K.; data curation, J.B. and E.D.S.; writing – original draft, J.B.; writing – review & editing, J.B., E.D.S., M.K., T.S., and R.K.; visualization, J.B. and E.D.S.; supervision, M.K., T.S., and R.K.; project administration, T.S.; funding acquisition, T.S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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REFERENCES

1. Laumer, C.E., Hejnol, A., and Giribet, G. (2015). Nuclear genomic signals of the 'microturbellarian' roots of platyhelminth evolutionary innovation. eLife *4*, e05503. https://doi.org/10.7554/eLife.05503.
2. Egger, B., Lapraz, F., Tomiczek, B., Müller, S., Dessimoz, C., Girstmair, J., Škunca, N., Rawlinson, K.A., Cameron, C.B., Beli, E., et al. (2015). A transcriptomic-phylogenomic analysis of the evolutionary relationships of flatworms. Curr. Biol. *25*, 1347–1353. https://doi.org/10.1016/j.cub.2015.03.034.
3. Littlewood, D. (2006). The evolution of parasitism in flatworms. In Parasitic Flatworms: Molecular Biology, Biochemistry, Immunology and Physiology, A.G. Maule, and N.J. Marks, eds. (CABI Publishing), pp. 1–36.
4. Littlewood, D.T.J., and Waeschenbach, A. (2015). Evolution: A turn up for the worms. Curr. Biol. *25*, R457–R460. https://doi.org/10.1016/j.cub.2015.04.012.
5. Szánthó, L.L., Lartillot, N., Szöllősi, G.J., and Schrempf, D. (2023). Compositionally constrained sites drive long-branch attraction. Syst. Biol. *72*, 767–780. https://doi.org/10.1093/sysbio/syad013.
6. Laumer, C.E., and Giribet, G. (2014). Inclusive taxon sampling suggests a single, stepwise origin of Ectolecithality in Platyhelminthes. Biol. J. Linn. Soc. Lond. *111*, 570–588. https://doi.org/10.1111/bij.12236.
7. Litvaitis, M.K., and Rohde, K. (1999). A molecular test of platyhelminth phylogeny: inferences from partial 28S rDNA sequences. Invertebr. Biol. *118*, 42. https://doi.org/10.2307/3226911.
8. Caña-Bozada, V., Robinson, M.W., Hernández-Mena, D.I., and Morales-Serna, F.N. (2023). Exploring evolutionary relationships within Neodermata using putative orthologous groups of proteins, with emphasis on peptidases. Trop. Med. Infect. Dis. *8*, 59. https://doi.org/10.3390/tropicalmed8010059.
9. Coghlan, A., Tyagi, R., Cotton, J.A., Holroyd, N., Rosa, B.A., Tsai, I.J., Laetsch, D.R., Beech, R.N., Day, T.A., and Hallsworth-Pepin, K. (2019). Comparative genomics of the major parasitic worms. Nat. Genet. *51*, 163–174. https://doi.org/10.1038/s41588-018-0262-1.
10. Olson, P.D., and Tkach, V.V. (2005). Advances and trends in the molecular systematics of the parasitic Platyhelminthes. Adv. Parasitol. *60*, 165–243. https://doi.org/10.1016/S0065-308X(05)60003-6.
11. Bychowsky, B. (1937). Ontogenesis and phylogenetic interrelationships of parasitic flatworms. News of the Academy of Sciences, USSR Department of Mathematics and Natural Sciences *4*, pp. 1353–1383.
12. Ehlers, U. (1985). Das phylogenetische System der Plathelminthes (Gustav Fischer).
13. Campos, A., Cummings, M.P., Reyes, J.L., and Laclette, J.P. (1998). Phylogenetic relationships of Platyhelminthes Based on 18S ribosomal gene sequences. Mol. Phylogenet. Evol. *10*, 1–10. https://doi.org/10.1006/mpev.1997.0483.
14. Littlewood, D.T.J., Rohde, K., and Clough, K.A. (1999). The interrelationships of all major groups of Platyhelminthes: phylogenetic evidence from morphology and molecules. Biol. J. Linn. Soc. *66*, 75–114.
15. Littlewood, D.T.J., and Olson, P.D. (2001). Small subunit rDNA and the Platyhelminthes: signal, noise, conflict and compromise. In Interrelationships of the Platyhelminthes, D.T.J. Littlewood, and R.A. Bray, eds. (Taylor & Francis), pp. 262–278.
16. Hahn, C., Fromm, B., and Bachmann, L. (2014). Comparative genomics of flatworms (Platyhelminthes) reveals shared genomic features of Ecto- and Endoparastic Neodermata. Genome Biol. Evol. *6*, 1105–1117. https://doi.org/10.1093/gbe/evu078.
17. Salomaki, E.D., Terpis, K.X., Rueckert, S., Kotyk, M., Varadínová, Z.K., Čepička, I., Lane, C.E., and Kolisko, M. (2021). Gregarine single-cell transcriptomics reveals differential mitochondrial remodeling and adaptation in apicomplexans. BMC Biol. *19*, 77. https://doi.org/10.1186/s12915-021-01007-2.
18. Shen, X.-X., Hittinger, C.T., and Rokas, A. (2017). Contentious relationships in phylogenomic studies can be driven by a handful of genes. Nat. Ecol. Evol. *1*, 126. https://doi.org/10.1038/s41559-017-0126.
19. Park, J.-K., Kim, K.-H., Kang, S., Kim, W., Eom, K.S., and Littlewood, D.T. (2007). A common origin of complex life cycles in parasitic flatworms: evidence from the complete mitochondrial genome of Microcotyle sebastis (Monogenea: Platyhelminthes). BMC Evol. Biol. *7*, 11. https://doi.org/10.1186/1471-2148-7-11.
20. Euzet, L., and Combes, C. (1998). The selection of habitats among the Monogenea. Int. J. Parasitol. *28*, 1645–1652. https://doi.org/10.1016/s0020-7519(98)00062-9.
21. Kearn, G.C. (1994). Evolutionary expansion of the Monogenea. Int. J. Parasitol. *24*, 1227–1271. https://doi.org/10.1016/0020-7519(94)90193-7.
22. de Pérez-Ponce de León, G.P.-P., and Hernández-Mena, D.I. (2019). Testing the higher-level phylogenetic classification of Digenea (Platyhelminthes, Trematoda) based on nuclear rDNA sequences before entering the age of the 'next-generation' tree of life. J. Helminthol. *93*, 260–276. https://doi.org/10.1017/S0022149X19000191.

23. Cribb, T.H., Bray, R.A., Olson, P.D., and Littlewood, D.T.J. (2003). Life cycle evolution in the digenea: a new perspective from phylogeny. Adv. Parasitol. *54*, 197–254.

24. Jennings, J.B. (1971). Parasitism and commensalism in the Turbellaria. Adv. Parasitol. *9*, 1–32. https://doi.org/10.1016/S0065-308X(08)60158-X.

25. Jennings, J.B. (1997). Nutritional and respiratory pathways to parasitism exemplified in the Turbellaria. Int. J. Parasitol. *27*, 679–691. https://doi.org/10.1016/s0020-7519(97)00010-6.

26. Cannon, L., Hayward, C., Whittington, I., Rohde, K., Cribb, T., Xylander, W., Caira, J., Reyda, F., McClelland, G., and Taraschewski, H. (2005). Helminth parasites. In Marine Parasitology, K. Rohde, ed. (CABI Publishing), pp. 47–54.

27. Rohde, K. (1994). The origins of parasitism in the Platyhelminthes. Int. J. Parasitol. *24*, 1099–1115. https://doi.org/10.1016/0020-7519(94)90185-6.

28. Laumer, C.E., and Giribet, G. (2017). Phylogenetic relationships within Adiaphanida (phylum Platyhelminthes) and the status of the crustacean-parasitic genus Genostoma. Invertebr. Biol. *136*, 184–198. https://doi.org/10.1111/ivb.12169.

29. Gordeev, I., Biserova, N., Zhukova, K., and Ekimova, I. (2022). The first report of a parasitic 'turbellarian' from a cephalopod mollusc, with description of Octopoxenus antarcticus gen. nov., sp. nov. (Platyhelminthes: Fecampiida: Notenteridae). J. Helminthol. *96*, e73. https://doi.org/10.1017/S0022149X22000657.

30. Willems, W.R., Wallberg, A., Jondelius, U., Littlewood, D.T.J., Backeljau, T., Schockaert, E.R., and Artois, T.J. (2006). Filling a gap in the phylogeny of flatworms: relationships within the Rhabdocoela (Platyhelminthes), inferred from 18S ribosomal DNA sequences. Zool. Scr. *35*, 1–17. https://doi.org/10.1111/j.1463-6409.2005.00216.x.

31. Weinstein, S.B., and Kuris, A.M. (2016). Independent origins of parasitism in Animalia. Biol. Lett. *12*, 20160324. https://doi.org/10.1098/rsbl.2016.0324.

32. Parker, G.A., Ball, M.A., and Chubb, J.C. (2015). Evolution of complex life cycles in trophically transmitted helminths. I. Host incorporation and trophic ascent. J. Evol. Biol. *28*, 267–291. https://doi.org/10.1111/jeb.12575.

33. Parker, G.A., Ball, M.A., and Chubb, J.C. (2015). Evolution of complex life cycles in trophically transmitted helminths. II. How do life-history stages adapt to their hosts? J. Evol. Biol. *28*, 292–304. https://doi.org/10.1111/jeb.12576.

34. Benesh, D.P., Chubb, J.C., Lafferty, K.D., and Parker, G.A. (2022). Complex life-cycles in trophically transmitted helminths: do the benefits of increased growth and transmission outweigh generalism and complexity costs? Curr. Res. Parasitol. Vector. Borne Dis. *2*, 100085. https://doi.org/10.1016/j.crpvbd.2022.100085.

35. Li, L., Lü, L., Nadler, S.A., Gibson, D.I., Zhang, L.-P., Chen, H.-X., Zhao, W.-T., and Guo, Y.-N. (2018). Molecular phylogeny and dating reveal a terrestrial origin in the Early Carboniferous for ascaridoid nematodes. Syst. Biol. *67*, 888–900. https://doi.org/10.1093/sysbio/syy018.

36. N.J. Smit, N.L. Bruce, and K.A. Hadfield, eds. (2019). Parasitic Crustacea, State of Knowledge and Future Trends (Springer). https://doi.org/10.1007/978-3-030-17385-2.

37. Cribb, T.H., Bray, R.A., and Littlewood, D.T. (2001). The nature and evolution of the association among digeneans, molluscs and fishes. Int. J. Parasitol. *31*, 997–1011.

38. Poulin, R. (2003). Helminth life cycles: evolutionary increases and decreases in complexity. In Taxonomy, Ecology and Evolution of Metazoan Parasites. Livre-hommage à Louis Euzet, C. Combes, and J. Jourdane, eds. (Presses Universitaire de Perpignan), pp. 167–183.

39. Perkins, E.M., Donnellan, S.C., Bertozzi, T., and Whittington, I.D. (2010). Closing the mitochondrial circle on paraphyly of the Monogenea (Platyhelminthes) infers evolution in the diet of parasitic flatworms. Int. J. Parasitol. *40*, 1237–1245. https://doi.org/10.1016/j.ijpara.2010.02.017.

40. Justine, J.-L., Lambert, A., and Mattei, X. (1985). Spermatozoon ultrastructure and phylogenetic relationships in the monogeneans (Platyhelminthes). Int. J. Parasitol. *15*, 601–608. https://doi.org/10.1016/0020-7519(85)90004-9.

41. Justine, J.-L. (2001). Spermatozoa as phylogenetic characters for the Platyhelminthes. In Interrelationships of the Platyhelminthes, D.T.J. Littlewood, and R.A. Bray, eds. (Taylor & Francis), pp. 231–238.

42. Euzet, L., and Combes, C. (2003). Some controversial questions regarding Monogenea. In Taxonomy, Ecology and Evolution of Metazoan Parasites. Livre-Hommage à Louis Euzet, C. Combes, and J. Jourdane, eds. (Presses Universitaires de Perpignan), pp. 303–320.

43. Justine, J.-L., and Poddubnaya, L.G. (2018). Spermiogenesis and spermatozoon ultrastructure in basal polyopisthocotylean monogeneans, Hexabothriidae and Chimaericolidae, and their significance for the phylogeny of the Monogenea. Parasite *25*, 7. https://doi.org/10.1051/parasite/2018007.

44. Mollaret, I., Jamieson, B.G.M., Adlard, R.D., Hugall, A., Lecointre, G., Chombard, C., and Justine, J.-L. (1997). Phylogenetic analysis of the Monogenea and their relationships with Digenea and Eucestoda inferred from 28S rDNA sequences. Mol. Biochem. Parasitol. *90*, 433–438. https://doi.org/10.1016/s0166-6851(97)00176-x.

45. Odhner, T. (1912). Homologien der weiblichen Genitalwege bei den Trematoden und Cestoden. Nebst Bemerkungen zum natürlichen System der monogenen Trematoden. Zool. Anz. *39*, 337–351.

46. Justine, J.-L. (1998). Non-monophyly of the monogeneans? Int. J. Parasitol. *28*, 1653–1657. https://doi.org/10.1016/s0020-7519(98)00060-5.

47. Bolger, A.M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics *30*, 2114–2120. https://doi.org/10.1093/bioinformatics/btu170.

48. Bushmanova, E., Antipov, D., Lapidus, A., and Prjibelski, A.D. (2019). rnaSPAdes: a de novo transcriptome assembler and its application to RNA-Seq data. GigaScience *8*, giz100. https://doi.org/10.1093/gigascience/giz100.

49. Lartillot, N., Rodrigue, N., Stubbs, D., and Richer, J. (2013). PhyloBayes MPI: phylogenetic reconstruction with infinite mixtures of profiles in a parallel environment. Syst. Biol. *62*, 611–615. https://doi.org/10.1093/sysbio/syt022.

50. Tice, A.K., Žihala, D., Pánek, T., Jones, R.E., Salomaki, E.D., Nenarokov, S., Burki, F., Eliáš, M., Eme, L., Roger, A.J., et al. (2021). PhyloFisher: A phylogenomic package for resolving eukaryotic relationships. PLoS Biol. *19*, e3001365. https://doi.org/10.1371/journal.pbio.3001365.

51. Minh, B.Q., Schmidt, H.A., Chernomor, O., Schrempf, D., Woodhams, M.D., von Haeseler, A., and Lanfear, R. (2020). IQ-TREE 2: New models and efficient methods for phylogenetic inference in the genomic era. Mol. Biol. Evol. *37*, 1530–1534. https://doi.org/10.1093/molbev/msaa015.

52. Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics *30*, 1312–1313. https://doi.org/10.1093/bioinformatics/btu033.

53. Howe, K.L., Bolt, B.J., Shafie, M., Kersey, P., and Berriman, M. (2017). WormBase ParaSite − a comprehensive resource for helminth genomics. Mol. Biochem. Parasitol. *215*, 2–10. https://doi.org/10.1016/j.molbiopara.2016.11.005.

54. Whelan, S., Irisarri, I., and Burki, F. (2018). PREQUAL: detecting non-homologous characters in sets of unaligned homologous sequences. Bioinformatics *34*, 3929–3930. https://doi.org/10.1093/bioinformatics/bty448.

55. Katoh, K., and Standley, D.M. (2013). MAFFT multiple sequence alignment, software version 7: improvements in performance and usability. Mol. Biol. Evol. *30*, 772–780. https://doi.org/10.1093/molbev/mst010.

56. Ali, R.H., Bogusz, M., and Whelan, S. (2019). Identifying clusters of high confidence homologies in multiple sequence alignments. Mol. Biol. Evol. *36*, 2340–2351. https://doi.org/10.1093/molbev/msz142.

57. Capella-Gutiérrez, S., Silla-Martínez, J.M., and Gabaldón, T. (2009). trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. Bioinformatics *25*, 1972–1973. https://doi.org/10.1093/bioinformatics/btp348.

58. Wang, H.-C., Minh, B.Q., Susko, E., and Roger, A.J. (2018). Modeling site heterogeneity with posterior mean site frequency profiles accelerates accurate phylogenomic estimation. Syst. Biol. *67*, 216–235. https://doi.org/10.1093/sysbio/syx068.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
RNAlater	Invitrogen	Cat#AM7020
Critical commercial assays		
Monarch Total RNA Miniprep kit	New England Biolabs	Cat#T2010S
Deposited data		
Phylogenetic tree files and alignments	Zenodo	https://doi.org/10.5281/zenodo.7562084
Aspidogaster limacoides (transcriptome)	NCBI BioProject ID PRJNA925671	Assembly available at Zenodo: https://doi.org/10.5281/zenodo.7562084
Amphilina foliacea (transcriptome)	NCBI BioProject ID PRJNA925671	Assembly available at Zenodo: https://doi.org/10.5281/zenodo.7562084
Breviscolex orientalis (transcriptome)	NCBI BioProject ID PRJNA925671	Assembly available at Zenodo: https://doi.org/10.5281/zenodo.7562084
Capingens singularis (transcriptome)	NCBI BioProject ID PRJNA925671	Assembly available at Zenodo: https://doi.org/10.5281/zenodo.7562084
Glaridacris catostomi (transcriptome)	NCBI BioProject ID PRJNA925671	Assembly available at Zenodo: https://doi.org/10.5281/zenodo.7562084
Didymobothrium rudolphii (transcriptome)	NCBI BioProject ID PRJNA925671	Assembly available at Zenodo: https://doi.org/10.5281/zenodo.7562084
Spirometra mansoni (transcriptome)	NCBI BioProject ID PRJNA925671	Assembly available at Zenodo: https://doi.org/10.5281/zenodo.7562084
Marsipometra hastata (transcriptome)	NCBI BioProject ID PRJNA925671	Assembly available at Zenodo: https://doi.org/10.5281/zenodo.7562084
Diclybothrium hamulatum (transcriptome)	NCBI BioProject ID PRJNA925671	Assembly available at Zenodo: https://doi.org/10.5281/zenodo.7562084
Software and algorithms		
Trimmomatic 0.39	Bolger et al.[47]	RRID:SCR_011848; https://github.com/usadellab/Trimmomatic
rnaSPAdes	Bushmanova et al.[48]	RRID:SCR_016992; http://cab.spbu.ru/software/spades/
TransDecoder	N/A	RRID:SCR_017647; https://github.com/TransDecoder/TransDecoder
Phylobayes	Lartillot et al.[49]	RRID:SCR_006402; https://github.com/bayesiancook/phylobayes
PhyloFisher	Tice et al.[50]	https://github.com/TheBrownLab/PhyloFisher
IQ-TREE 1.6.12	Minh et al.[51]	RRID:SCR_017254; http://www.iqtree.org/
RAxML 8.2.12	Stamatakis[52]	RRID:SCR_006086; https://github.com/stamatak/standard-RAxML

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jan Brabec (brabcak@paru.cas.cz).

Materials availability

No new reagents were generated in this study.

Data and code availability

- All new raw RNA sequencing read data have been deposited into NCBI's short-read archive (SRA) under BioProject PRJNA925671 (SRA accessions SRX19281359-SRX19281367). Transcriptome assemblies, orthology/paralogy single gene assignments, alignments and resulting tree files are publicly available at Zenodo (https://doi.org/10.5281/zenodo.7562084).
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Specimen details

Newly characterised specimens (Data S1) were collected opportunistically by the authors or our collaborators, fixed alive in RNAlater (Invitrogen) and long-term stored in -80 °C until further processing. *Aspidogaster limacoides* was collected from white bream (*Blicca bjoerkna*) in the delta of the river Danube, Romania. *Amphilina foliacea* was collected from sterlet (*Acipenser ruthenus*) in river Danube, Hungary. *Breviscolex orientalis* was collected from barbel steed (*Hemibarbus barbus*) in Makino, Tahashima-shi, Japan. *Capingens singularis* was collected from black buffalo (*Ictiobus niger*) in Pascagoula River, USA. *Glaridacris catostomi* was collected from white sucker (*Catostomus commersonii*) in Connecticut River, USA. *Didymobothrium rudolphii* was collected from sand sole (*Pegusa lascaris*) in the Atlantic, Portugal. *Spirometra mansoni* was collected from edible frog (*Pelophylax esculentus*) in the delta of the river Danube, Romania. Both *Marsipometra hastata* and *Diclybothrium hamulatum* were collected from American paddlefish (*Polyodon spathula*) in Bluff Creek, USA.

METHOD DETAILS

RNA extraction, sequencing, and assembly

Total RNA was extracted from single worm individuals using the Monarch Total RNA Miniprep kit (New England Biolabs) following the manufacturer's v3.0 protocol and submitted to Macrogen Europe for library construction using the TruSeq Stranded mRNA LT preparation kit and sequencing to generate 40 million 150 bp pair-end reads per library on the Illumina NovaSeq6000 platform (Data S1). Quality of the resulting reads was checked with FastQC, low-quality bases and Illumina adapters were trimmed and reads shorter than 100 nucleotides excluded using Trimmomatic 0.39[47] and re-checked with FastQC. Surviving reads were assembled using rnaSPAdes,[48] followed by ORF prediction and translation in TransDecoder. Previously characterised transcriptomes of both parasitic and free-living Platyhelminthes were downloaded either as raw sequence data from the Sequence Read Archive (SRA) repository or as proteins from the WormBase ParaSite[53] or NCBI's Sequence sets databases (Data S1). Raw sequence data from the SRA archive were checked with FastQC, followed by a technical sequence and low-quality reads removal as described above.

Dataset construction

Phylogenomic dataset construction was carried out using tools of the software package PhyloFisher[50] with the following steps: (i) predicted protein sequences from TransDecoder were searched for the presence of homologs of each of the 240 protein-coding genes provided with the curated PhyloFisher eukaryotic database, using the fisher.py script and the script's phylogenetically informed route of identifying up to five candidate homologs; (ii) the identified sequences were added to the corresponding single-protein homologs in the PhyloFisher's eukaryotic database using PhyloFisher's working_dataset_constructor.py script; (iii) the resulting fasta files were filtered for non-homologous sites and sequencing errors using PREQUAL,[54] and aligned using MAFFT's G-INS-I algorithm.[55] DIVVIER[56] (-partial -mincol 4 -divvygap) was run to filter out alignment uncertainty and errors, and finally the filtered alignments were trimmed of sites comprised of >90 % gaps using trimAL[57] (-gt 0.1). For each resulting alignment, phylogenies were constructed using IQ-TREE 1.6.12[51] under the LG+C20+F+G_4 profile mixture model selected to balance between analysis' speed and performance. Nodal support values were estimated in RAxML 8.2.12[52] using 100 replicates of rapid bootstraps under the PROTGAMMALG4X model. Bootstrap support was mapped to each tree and the resulting trees were manually inspected to identify orthologs, paralogs, and contamination for each taxon using the ParaSorter graphical user interface of PhyloFisher. We followed the same criteria for paralog/ortholog identification described in length by Salomaki et al.[17]; (iv) the final phylogenomic dataset of 225 genes was based on 60% taxon occupancy of ingroup taxa. After taxon and ortholog selection, the final supermatrix composed of the newly added taxa plus *Schistosoma mansoni* and *Taenia solium* (the only platyhelminth representatives from the starting dataset of PhyloFisher) was created using the PhyloFisher script matrix_constructor.py according to the default settings.

Phylogenetic analyses

The final supermatrix comprised 83 taxa and 77,824 sites. The phylogenomic analyses were run under maximum likelihood criterion in IQ-TREE, using the profile mixture model LG+C60+F+G_4. Nodal support was estimated using 1,000 nonparametric bootstraps computed under the posterior mean site frequencies (PMSF) model[58] (Figure 1A). Outgroup selection of Catenulida was informed by previously inferred phylogenies. Bayesian phylogenetic analysis was run in Phylobayes[49] as four independent Markov chains under the CAT+GTR+G_4 model for 19,016 to 19,160 generations (Figure 1B). Convergence of only two of the four chains was achieved, using a burn-in of 3,000 trees. Since three of the four chains of Phylobayes remained divergent, the CAT-PMSF pipeline[5] was utilised to perform a tree search using the CAT+GTR+G_4 model-estimated parameters out of the Bayesian framework (Figure S1). This was achieved through the following steps: (i) the ML topology estimated under the LG+C60+F+G_4 model in IQ-TREE was fixed as a guide topology for Phylobayes analysis with a CAT+GTR+G_4 model. Two chains have been run until the effective sample size of all parameters reached at least 100; (ii) the posterior mean-site specific stationary distributions of amino acids were extracted and subsequently used to fix the site-specific stationary distributions in IQ-TREE phylogeny inference; (iii) the PMSF[58] method was used to estimate 1,000 nonparametric bootstraps under the PMSF model. In addition, an unrooted ML phylogeny was also inferred from the supermatrix dataset with two representatives of Catenulida removed to evaluate the effect of the removal of the relatively long branch on the ingroup topology (Figure S2).

Constrained topologies test

The approximately unbiased (AU) topology tests were performed in order to assess if the CAT-GTR topology and six other historically relevant evolutionary scenarios (depicted schematically on Figure 2D) could be statistically rejected on the basis of the 225-gene dataset. The constrained maximum likelihood estimates were calculated in IQ-TREE using the identical models (LG-C60 and CAT-PMSF) as in the initial analyses, and analysed by the AU test along with the original topologies and further 92 (LG-C60 model) or 93 (CAT-GTR model) local topologies saved during the LG-C60 and CAT-PMSF analyses in IQ-TREE, respectively, in order to provide statistical significance for the AU test.

Systematic error tests and random gene subsampling

The following PhyloFisher utilities were employed to examine the supermatrix for sources of systematic error: the fast_site_remover.py script was used to iteratively remove 3,000 of the fastest evolving sites per step within the supermatrix resulting in 25 iteratively smaller datasets; the heterotachy.py script was used in an analogous manner to produce 25 reduced datasets by iteratively removing the most heterotachous sites (i.e. sites of the highest within-site rate variation). Genes within the 225-gene supermatrix were randomly subsampled using the random_resampler.py script, resulting in 14, 6, 4, and 2 alternative datasets in which 20, 40, 60 and 80 percent of the original dataset was randomly subsampled, respectively. The number of replicates was derived as the minimum number necessary for a 95% probability of sampling every gene when subsampling a given proportion of the original gene set as in Salomaki et al.[17] All the generated alternative datasets were then analysed phylogenetically: ML tree search was run in IQ-TREE under the LG+C20+G_4 model, nodal support values were estimated in RAxML using 100 replicates of rapid bootstraps under the PROTGAMMALG4X model.

Identification of genes with strong topology signal

We defined the strength of the topology signal as the difference in the log-likelihood scores for the unconstrained LG-C60 topology against the ML tree constrained to a conflicting topology, as done by Shen et al.[18] The 225-gene supermatrix was analysed individually as single-gene ML analyses under the LG+C20+G_4 model in IQ-TREE, constrained to: (1) the original LG-C60 topology resulting from the full supermatrix or, (2) an alternative topology in conflict with the LG-C60 topology. The alternative topologies tested included the following: (i) the CAT-GTR topology estimated in Phylobayes in which cestodes were constrained as sister to monopisthocotyleans; (ii) cestodes constrained as sister to trematodes (TsC topology); (iii) monogeneans constrained as a monophyletic group (MPm topology). The site-wise log-likelihood values (SLV) for both the LG-C60 and the alternative topologies were saved and used to calculate the difference in SLV between the LG-C60 and the alternative topology. The difference in gene-wise log-likelihood scores (ΔGLS) under the LG-C60 and the alternative topology was then calculated through summarising the differences of the SLVs of all sites within a given gene. The influence of the removal of a few genes on the phylogenetic inference was tested by excluding 5 genes with the highest absolute ΔGLS values from the supermatrix and estimating an ML tree from the reduced datasets using the LG+C60+F+G_4 model. Nodal supports were estimated using 10,000 repetitions of ultrafast bootstrap in IQ-TREE.