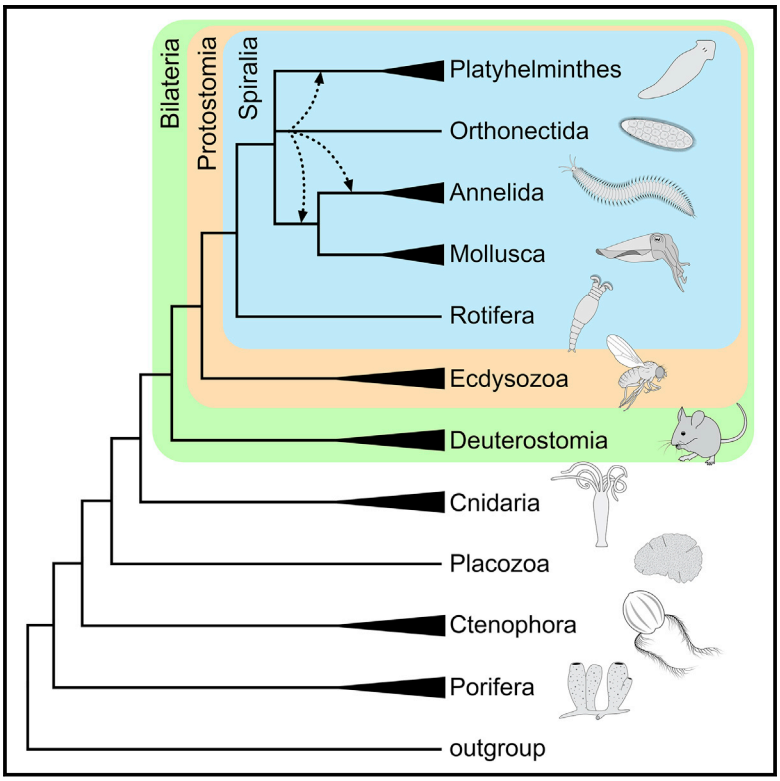
Report

Current Biology

The Genome of *Intoshia linei* Affirms Orthonectids as Highly Simplified Spiralians

Graphical Abstract



Highlights

- The orthonectid *Intoshia linei* has a small genome with only around 9,000 genes
- The phylogenomic analysis affirms orthonectids as highly simplified spiralians
- The orthonectid simplification is associated with reduction of developmental genes
- They have a compact genetic toolkit for the nervous system development and activity

Authors

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In Brief

The genomic data presented by Mikhailov et.al show that orthonectids, a group of highly simplified parasitic animals, are true bilaterians related to Lophotrochozoa and possess a reduced complement of genes implicated in the metazoan development and nervous system activity.

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The Genome of *Intoshia linei* Affirms Orthonectids as Highly Simplified Spiralians

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SUMMARY

Orthonectids are rare parasites of marine invertebrates [1] that are commonly treated in textbooks as a taxon of uncertain affinity [2]. Trophic forms of orthonectids reside in the tissues of their hosts as multinucleated plasmodia, generating short-lived, worm-like ciliated female and male organisms that exit into the environment for copulation [3]. These ephemeral males and females are composed of just several hundred somatic cells and are deprived of digestive, circulatory, or excretory systems. Since their discovery in the 19th century, the orthonectids were described as organisms with no differentiated cell types and considered as part of Mesozoa, a putative link between multicellular animals and their unicellular relatives. More recently, this view was challenged as the new data suggested that orthonectids are animals that became simplified due to their parasitic way of life [3, 4]. Here, we report the genomic sequence of *Intoshia linei*, one of about 20 known species of orthonectids. The genomic data confirm recent morphological analysis asserting that orthonectids are members of Spiralia and possess muscular and nervous systems [5]. The 43-Mbp genome of *I. linei* encodes about 9,000 genes and retains those essential for the development and activity of muscular and nervous systems. The simplification of orthonectid body plan is associated with considerable reduction of metazoan developmental genes, leaving what might be viewed as the minimal gene set necessary to retain critical bilaterian features.

RESULTS AND DISCUSSION

The Genome of *Intoshia linei* Is One of the Smallest among Metazoans

The genome of *Intoshia linei* was sequenced using the Illumina platform and assembled into a draft totaling 43.2 Mbp. The miniature by metazoan standards genome is predicted to encode about 9,000 genes—one of the lowest reported gene counts among metazoans, exceeding only the recently sequenced genomes of a myxozoan [6] and a plant-parasitic nematode [7]. The predicted genes are fairly intron rich and span an average of six exons, which in sum account for 23.1% of the genome. *I. linei* has some of the shortest introns seen in metazoans: the distribution of intron lengths peaks at 37 bp, and nearly a half of all introns are within a 30–50 bp size range (Figure S1). The genome compaction in *I. linei* is reflected in both lower gene count and higher average gene density in comparison with other metazoan genomes. However, it is not the most gene dense among metazoans: the average gene density in the genome of *I. linei* is around 200 genes per Mb, on par with that of *Caenorhabditis elegans* [8] but noticeably lower than the gene density in the highly compact genome of a parasitic nematode *Trichinella spiralis* [9] or a pelagic tunicate *Oikopleura dioica* [10, 11] (Figure S1). Despite its comparatively small size, the genome of *I. linei* carries a considerable amount of repetitive elements—more than a quarter of the total assembly size, with the largest contribution to the repetitive element repertoire provided by unclassified repeats (Table S1).

The Orthonectids Are Highly Simplified Spiralians

The orthonectid genes display an exceptionally high rate of sequence divergence, which is known to have a confounding effect on the phylogenetic inference [12, 13]. Previous studies using rRNA-based phylogenies placed Orthonectida within Bilateria but failed to determine their affiliation with any specific bilaterian taxon due to their highly divergent sequences [14, 15]. We investigated the phylogenetic position of *I. linei* using a 500-gene dataset and the tree inference methods of RAxML [16] and PhyloBayes [17]. To counteract the impact of systematic biases stemming from uneven evolutionary rates in the dataset, we performed tree reconstructions following the removal of homoplasy-prone fast-evolving sites and employing site-heterogeneous substitution model [18], which was shown to be more robust in dealing with reconstruction artifacts [19]. The obtained phylogenies agree on placing the orthonectid within Spiralia, a result supported by the morphological study [3], but nevertheless show discord in its exact phylogenetic placement. The maximum likelihood analysis or the Bayesian inference with a site-heterogeneous substitution model and a general time reversible exchange rate matrix (CAT-GTR) group *I. linei* with flatworms, while the analyses using the CAT model with flat exchange rates place it sister to annelids (Figures 1A, 1B, S2, and S3). To examine the phylogenetic relationship of *I. linei* within Spiralia in greater detail, we used the dataset of Struck et al.

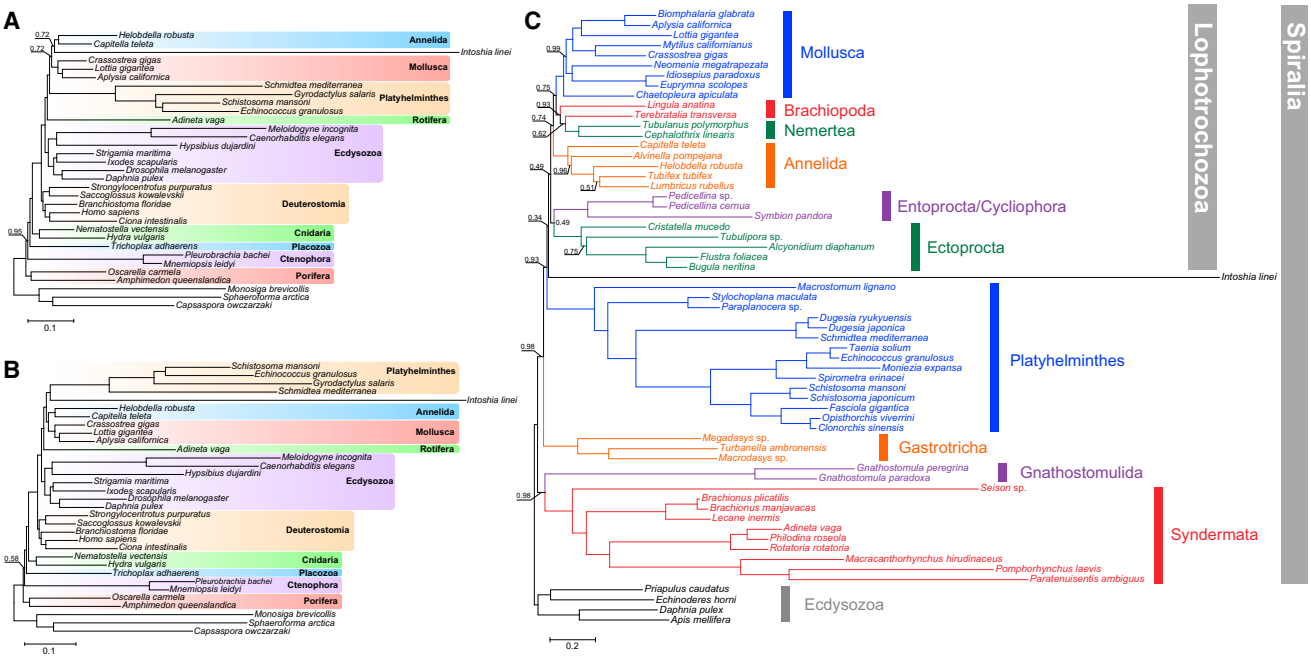

Figure 1. The Contentious Position of *Intoshi linei* in the Metazoan Phylogeny

(A and B) The trees in (A) and (B) were reconstructed by PhyloBayes with a dataset of slow-evolving sites (47,548 amino acid [aa] sites), assembled from 500 orthologous groups utilizing genomic data. The Bayesian inference was performed using the CAT + Γ4 model (A) and the CAT + GTR + Γ4 model (B).

(C) The spiralian phylogeny reconstructed on the basis of the dataset assembled by Struck et al. [20], relying primarily on transcriptomic data. The tree was reconstructed by PhyloBayes under the GTR + CAT + Γ4 model with a 22,909 aa site alignment that excludes positions with over 60% missing data. Support indexes for nodes with 1.00 posterior probability are omitted.

See also Figures S2 and S3.

[20] that focuses on taxonomic sampling of the spiralian taxa. The spiralian tree reconstructed by PhyloBayes (Figure 1C) is largely congruent with the previously reported multigene phylogenies [20, 21] but differs in some aspects, particularly in the position of gastrotrichs and the branching of the lophotrochozoan taxa. In the spiralian phylogeny, the orthonectid forms a lineage intercalating the branch that separates the classical Lophotrochozoa [22] from the rest of spiralians, including flatworms and rotifers. The latter result argues for an isolated position of orthonectids among the spiralian taxa, proposing a third alternative for the contested position within the group.

The spiralian ancestry of orthonectids implies a derived condition for the apparent simplicity of their organization and attributes meager gene complement of *I. linei* to extensive loss. Nearly 75% of orthologous groups inferred to be present in the spiralian ancestor are lost by the orthonectid (Figure S3D). The genome of *I. linei* retains only around 4,000 conserved ancestral orthologous groups—a thousand less than the corresponding number in the parasitic flatworms. A third of all predictions in the genome of *I. linei* find no hits in the NCBI's non-redundant database and are seen as orthonectid innovations. Among the domain families not associated with mobile genetic elements, the only family that appears to have expanded is a family of lecithin:cholesterol acyltransferase domains, which participate in lipid metabolism. Mapping the retained genes to molecular pathways shows that *I. linei* has a functional complement of components for glycolysis, pentose phosphate pathway, tricarboxylic acid cycle, and oxidative phosphorylation, but a range of metabolic pathways are likely impaired, including de novo synthesis of purine nucleotides and most amino acids (Figure 2). Almost all components of pathways for steroid biosynthesis and sphingolipid metabolism are missing. Similarly to the parasitic flatworms [23], *I. linei* lost most of the peroxisome components and may be devoid of the organelle itself (see Supplemental Information for details). The signaling pathways of NF-κB and STAT, implicated in immunity, growth, and development, also appear to be completely lost. The developmental signaling pathways of Wnt, Notch, and TGF-β seem to be intact, but the key elements of the Hedgehog pathway are missing (Table 1). It is notable that while most pathways that experience reduction in the parasitic flatworms also appear to be impaired in the orthonectid, the components of pathways for fatty acid metabolism and branched-chain amino acid degradation are preserved in *I. linei* but are absent from many of the parasitic flatworms [24].

The Orthonectid Simplification Is Associated with Reduction of Metazoan Developmental Genes

The repertoire of transcription factors in metazoan genomes is one of the properties that correlate with organismal complexity [25]. It is therefore unsurprising that the genome of *I. linei* has the minimal count of recognized transcription factors among bilaterian animals (Table 1). The families of C2H2 type zinc-finger proteins experience drastic contraction in *I. linei*, while the p53 homologs are absent from its genome altogether. The number of homeobox genes in the orthonectid is close to the number seen in parasitic flatworms—another group with extensively

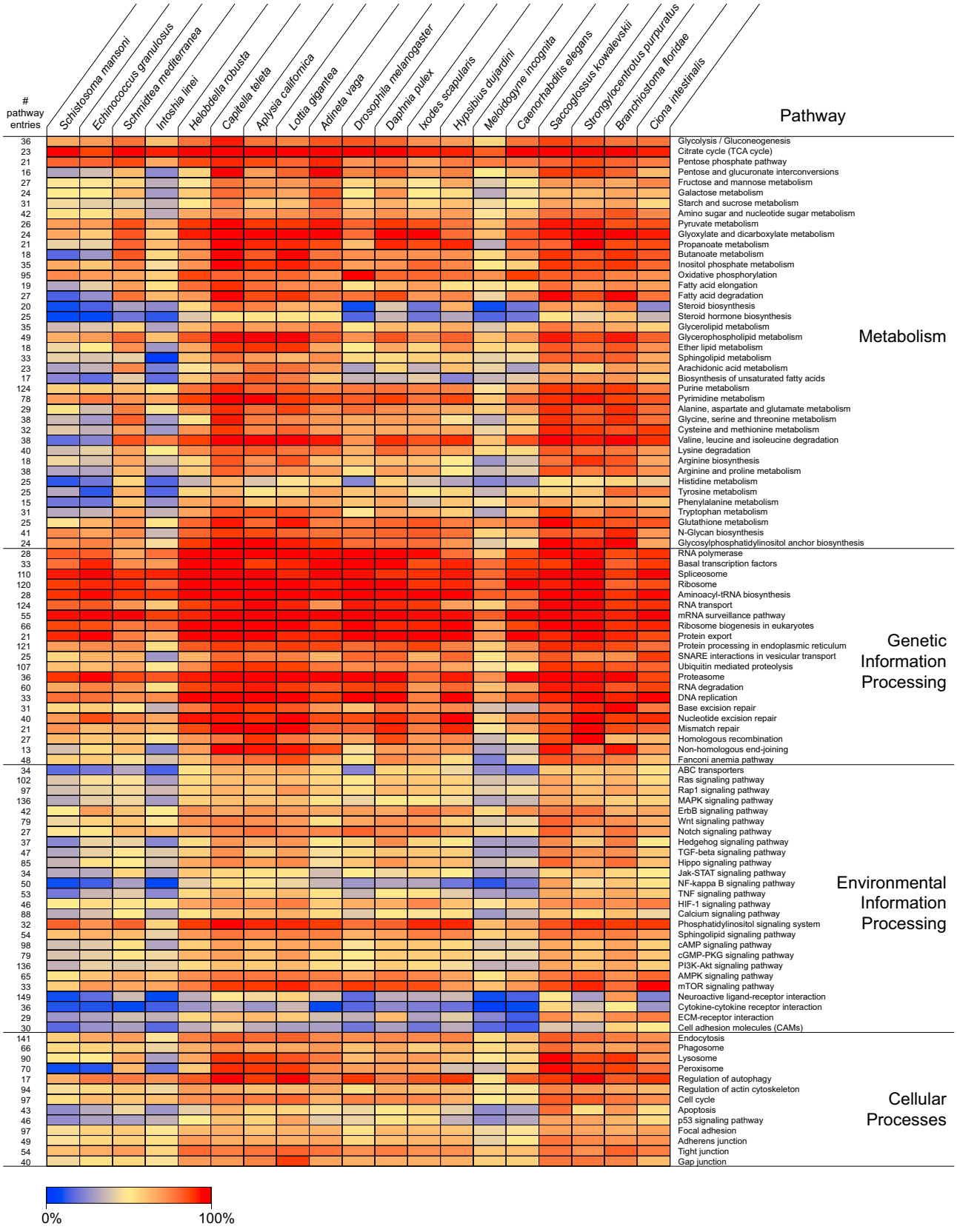

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Table 1. Transcription Factor Families and Signaling Pathway Ligands Implicated in the Metazoan Development

	I. linei	*E. granulosus*	*C. elegans*	*D. melanogaster*	*C. teleta*	*H. sapiens*
Homeobox	61	65	94	101	158	245
Forkhead box	20	15	15	18	42	50
High mobility group box	12	17	16	22	24	51
T-box	7	7	21	8	8	17
MADS box	2	4	2	2	2	5
Basic helix-loop-helix	24	25	38	53	82	105
Basic leucine zipper	8	19	22	16	26	41
C2H2 type zinc finger	75	153	186	296	443	803
ETS	3	9	10	8	13	29
Nuclear receptor	9	10	260	18	32	47
Rel/NF-κB	0	0	0	3	2	5
NFAT	0	1	0	1	1	5
SMAD	4	5	7	4	4	8
STAT	0	0	1	1	6	7
Wnt	3	6	5	7	12	19
TGF-β	4	3	5	7	14	37
Hedgehog	0	1	0	1	1	3
DSL ligand	1	3	10	2	6	5
Fibroblast growth factor	1	0	2	3	1	22

The gene counts are based on the analyses of protein domains, which were performed identically for the listed genomes. The majority of proteins were detected using the Pfam domain annotation with a gathering cutoff threshold; the C2H2 type zinc-finger proteins were detected using the InterPro domains (IPR007087 and IPR015880). Although the absence of the canonical Hedgehog signaling protein is not indicative of the whole pathway status, the genome of *I. linei* also lacks the Hint domain and orthologs of Patched, Smoothened, and Ci/GLI, which are integral to the Hedgehog pathway. *I. linei*, orthonectid; *E. granulosus*, flatworm; *C. elegans*, nematode; *D. melanogaster*, insect; *C. teleta*, annelid; *H. sapiens*, chordate. See also Figure S4.

reduced homeobox gene complement [23]. A closer look at their homeobox genes reveals that roughly a half of the retained families overlap between the orthonectid and parasitic flatworms (Figure S4). The genome of *I. linei* shows conservation of at least 37 homeobox gene families, which among others include *pax6*, *engrailed*, *sine oculis*, and *otx* orthologs. Notably, it encodes only three Hox type genes, which are known to play a pivotal role in regulating differentiation along the main body axis in bilaterians [26]. The Hox genes in *I. linei* represent the anterior and central Hox2, Hox4, and Hox6-8 families. The orthonectid Hox genes are located in different contigs and are neighbored by unrelated genes, which implies that unlike many of their bilaterian orthologs, they are not organized in a cluster. We found no posterior Hox genes in *I. linei*, which are usually conserved in bilaterians with one exception of a rotifer *A. vaga* [27]. A single ParaHox type posterior gene, *caudal*/*Cdx* ortholog, is present in *I. linei* genome. Aside from the transcription factors, an important part in regulation of gene expression in many metazoans is mediated by microRNAs [28]. The genome of *I. linei* encodes key elements of the microRNA pathway, including the Argonaute, Piwi, and Dicer orthologs, and components of the microprocessor complex, Drosha and Pasha orthologs. Presence of these genes might be an indication of a functional microRNA regulation system, although experimental data are needed to confirm its existence in the orthonectid.

Intoshia linei Retains a Compact Gene Set for the Nervous System Activity and Development

While the name Orthonectida suggests that they swim in a straightforward manner, in reality, *I. linei* exhibits complex movements, including spinning and bending (Figure 3; Movie S1). The presence of muscular and nervous systems, involved in coordinating these movements in Orthonectida, was only recently recognized [5, 29, 30]. The excitable tissues, however simply organized, imply the existence of action potential generating ion channels, neurotransmitter receptors, and electrical synapses. We searched the genome of *I. linei* to document the conserved genes involved in the development and functioning of these systems.

Most types of ion channels, including the voltage-gated channels necessary for the generation of action potential, are present in *I. linei*, yet their number is reduced in comparison to other metazoans [31]. There are 42 predicted proteins of tetrameric sodium, potassium, and calcium ion channels in *I. linei*.

Figure 2. Heatmap of Pathway Conservation in Bilaterians

The reference pathways were selected from the KEGG pathways collection. The number of pathway entries corresponds to the total amount of non-redundant pathway elements found in the surveyed bilaterian genomes. The color in each cell depicts the percentage of these non-redundant entries found in a given genome. See also Figure S3D.

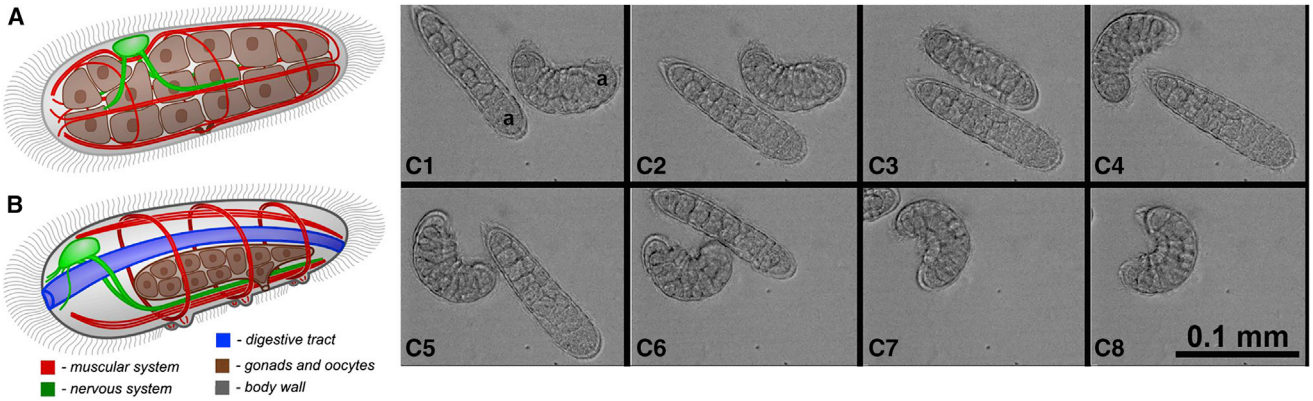

Figure 3. Orthonectid Body Plan and Locomotion

(A and B) Simplified scheme for the orthonectid body plan (A); the adult protostome bilaterian body plan (B). Orthonectids display typical bilaterian features such as the dorsal ganglion (brain) and have a muscular system and a single layer of ciliated epithelium cells (the only means for locomotion in Orthonectida). At the same time, they lack the digestive and excretory systems. The reproductive system of Orthonectida is also unusual: the germ cells are located in the body cavity without any gonad barrier.

(C1–C8) Two individuals of *I. linei* (adult females), which recently left their host, perform forward and reverse locomotion, propelled by means of numerous epithelial cilia. The organisms are seen turning and bending—the motions driven by coordinated muscle contractions. Time-lapse frames (1.25 s apart) are from Movie S1 (a, anterior part of the animal).

According to the Pfam analysis and reciprocal BLAST searches, one voltage-gated sodium ion channel and one voltage-gated calcium ion channel are present among the predicted proteins. *I. linei* has six predicted voltage-gated potassium channel proteins (one of them also had animal-specific KCNQ_channel signature and three proteins containing K+ channel tetramerisation domain BTB_2). One predicted *I. linei* protein is related both to the voltage-gated EAG K+ channels and to the cyclic nucleotide-gated cation channels. Three more proteins that encode cyclic nucleotide-binding domains and Ion_trans or Ion_trans_2 were predicted. One of them appears to have two copies of the cNMP_binding domain coexisting with the Ion_trans domains. The two-pore-domain inward rectifier potassium channels contribute to the resting potential and are known as "leak channels." Three predicted proteins of this family were found in *I. linei*. Among other potassium channels, two calcium-activated BK potassium channel alpha subunit proteins and one calcium-activated SK potassium channel protein domains were identified. Notably, the SK protein family is present only in bilaterian animals. Two inward rectifier potassium channels (IRK) according to the reciprocal BLAST hits have the best similarity to the G protein-activated inward rectifier potassium channels that act in the seven transmembrane G protein-coupled receptor (GPCR) pathway.

The BLAST and Pfam searches detected 11 hits with the innexin/pannexin-specific Pfam domain (PF00876) in *I. linei*. Although gap junctions are found in many tissues, they often take part in the cell-to-cell communications and function as electrical synapses in excitable tissues.

An array of ionotropic and metabotropic receptor genes is present in the *I. linei*. We found 14 genes for neurotransmitter-gated ion-channels in *I. linei*, identified by the specific transmembrane region domain (PF02932) and the ligand binding domain (PF02931). Two of these receptors are predicted to be ionotropic glycine receptors, and the rest are identified as the nicotinic acetylcholine receptors (Table S2). The Pfam and BLAST searches and the Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology assignments resulted in no hits for ionotropic glutamate receptors. Among the GPCRs, some hits indicate the presence of metabotropic glutamate receptors: five proteins with the domain of class 3 GPCRs (PF00003). Interestingly, the ionotropic neurotransmitter receptor proteins in the Orthonectida and Ctenophora, which are in some sense competing for the simplest nervous system in Metazoa, have very different profiles: while the orthonectid has lost the entire glutamate receptor family (iGluR), the ctenophores lack all ligand-gated Cys-loop receptors [32].

The locomotion in orthonectids relies on motile epithelial cilia, which are commonly activated by serotonin. Six of the *I. linei* neurons are also stained by the anti-serotonin antibodies [5]. Therefore, we expected to find elements of the serotonin (5-hydroxytryptamine [5-HT]) signaling in *I. linei*. We found no 5-HT-specific receptors of the ligand-gated ion channel family (PF02932), but two sequences for the 5-HT-specific GPCRs were detected and confirmed by bi-directional BLAST hits. Serotonergic neurons usually express serotonin symporter from the sodium:neurotransmitter symporter family (PF00209). This family of transporters is responsible for the synaptic recycling of neurotransmitters. The number of proteins from this family in *I. linei* is 27, which is comparable to the number seen in the genomes of animals with a developed nervous system. We were not able to reliably verify whether any of these transporters are specific for 5-HT. In summary, we found genes for ionotropic receptors to acetylcholine and glycine and genes for metabotropic receptors to acetylcholine, serotonin, histamine, dopamine, adrenalin, and glutamate, but no genes for receptors to GABA. The entire family of ionotropic glutamate receptors appears to be missing in the orthonectid.

The simplicity of *I. linei* nervous system is associated not only with the decrease in the receptor diversity but also with molecular mechanisms responsible for the nervous system development, axon guidance, and synapse formation. Semaphorins, important neuronal pathfinding signaling molecules, and their

receptors (plexins) are absent from the genome. This is also true for fasciclin domain involved in axonal guidance. At the same time, other players potentially involved in the nervous system development such as Netrin, Ephrins, Ephrin receptors, IgSF-CAMs, Cadherins, and Integrins are present.

While the core set of muscle proteins was already present before the emergence of animals, the troponin complex and titin appear to be an innovation specific to Bilateria and characteristic of bilaterian striated muscles [33]. Troponin is a complex of three proteins (troponin C, troponin I, and troponin T). These proteins are not detected in *I. linei* by BLAST search, and the troponin domain is not found by the Pfam search. The troponin complex in chordates is characteristic for skeletal and cardiac muscles, but not for smooth muscles. Morphological data suggest that *I. linei* muscles are similar to smooth muscles, so the troponin was likely lost in *I. linei*, and its absence is not a plesiomorphic trait. At the same time, another bilaterian hallmark, the myogenic regulatory factor, is present in the genome.

The Orthonectid Genome Retains Elements of the Metazoan Sensory Systems

Not much is known about the sensory systems in the orthonectids. The aquatic-stage *I. linei* females have a putative subepithelial receptor, which consists of three ciliated cells [3]. Additionally, ciliated and non-ciliated epithelial cells as well as neuronal processes may have a receptor function. We searched the genome of *I. linei* for clues on the putative sensory systems. Two predicted Piezo type proteins, ten Amiloride-sensitive sodium channels, two transient receptor potential (TRP) family proteins, and three TREK-1/TRAAK channel homologs could be potentially involved in mechanotransduction in *I. linei* by analogy to their use in *C. elegans*, *D. melanogaster*, and vertebrates. Some predicted *I. linei* proteins resemble TRPV and TRPM family members and may participate in temperature sensing. The presence or absence of photoreception in *I. linei* is not clearly confirmed by the genomic data. There are two distinct types of photoreceptive molecules in animals: 7-TM GPCRs class members (using retinal as chromophore) and flavoproteins cryptochromes. We found no proteins of the photolyase/cryptochrome family or flavin adenine dinucleotide (FAD) binding domain of DNA photolyase in *I. linei*, which excludes the common pathway for light sensing. The class of 7-TM GPCRs is extremely reduced in *I. linei* (comprising only 37 proteins), and photosensitive opsins are not easily recognized by bioinformatic approaches [34].

The morphological and genomic data clearly indicate that the simple organization of orthonectids is a derived trait associated with transition to obligate parasitism. Apart from the parasitic organisms comprising another enigmatic group, Rhombozoa, orthonectids represent an extreme case of simplification in Bilateria, which is reflected by the genome of *I. linei* in a remarkable extent of gene loss. The highly divergent sequences of *I. linei* remain a hindrance for phylogenetic inference, although it is worth noting that the affiliation of orthonectids with annelids obtained in some of our analyses was argued earlier on the basis of microvillar cuticle similarity and circular muscle metamery [3]. The orthonectid genome retains elements of the genetic toolkit for bilaterian development, which makes it a valuable object for evolutionary developmental biology as potentially the simplest model for the development of core bilaterian features, including muscular and nervous systems.

ACCESSION NUMBERS

The accession numbers for new data reported in this study are NCBI GenBank: Assembly GCA_001642005.1; NCBI BioProject: PRJNA316116; and NCBI BioSample: IDSAMN04576116.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, two tables, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2016.05.007.

AUTHOR CONTRIBUTIONS

Conceptualization, K.V.M., V.V.A., and Y.V.P.; Methodology, K.V.M., V.V.A., and Y.V.P.; Investigation, K.V.M., V.V.A., M.A.N., and Y.V.P.; Writing – Original Draft, K.V.M, V.V.A., G.S.S., and Y.V.P.; Writing – Review & Editing, K.V.M, V.V.A., and Y.V.P.; Resources, G.S.S., M.A.N., M.D.L., and A.A.P.

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