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A phylogenomic framework of Gastrotricha evolutionary relationships

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ABSTRACT

Gastrotricha are microscopic acoelomate worms that play key ecological roles in marine and freshwater meiobenthic communities. For a long time, their relationships have been inferred primarily from morphology, or from molecular studies based on only a few genes, due to the scarcity of genomic resources. Here we analyzed 221 nuclear loci from 31 species spanning nine families (including 28 newly assembled genomes), providing the first phylogenomic framework for the phylum. Our results support the deep split between the two main orders, Macrodasyida and Chaetonotida, and clarify the long-debated position of *Neodasys*, which is consistently recovered as sister to Macrodasyida. Within Chaetonotida, our analyses confirm the monophyly of the recently established clade Oiorpata. Phylogenetic mapping indicates a single, primary marine-to-freshwater transition within Oiorpata. Since all oiorpatans are parthenogenetic, this suggests that the pre-existing reproductive mode may have facilitated the colonization of freshwater environments. Comparative genomic analyses reveal that Chaetonotida genomes have lost a defined set of conserved BUSCO genes, 9.6% in Muselliferidae + Xenotrichulidae and up to 11.2% in Oiorpata, indicating a broader genomic reorganization within the order. Together, these results provide both confirmation and new insights into the evolutionary history of Gastrotricha, demonstrating how expanding genomic datasets in understudied meiofaunal lineages can uncover hidden dimensions of metazoan genome evolution and diversification.

1. Introduction

Despite the increasing amount of published genomic data collected over the last decades, many lineages of invertebrates still lack genomic resources and thus a reliable reference molecular phylogeny. This issue is often nested in historical and technical reasons and it is particularly true for certain groups, such as the phylum Gastrotricha (Araújo et al., 2024; Martínez et al., 2025). Their microscopic nature created an impassable sequencing challenge for a long time, primarily due to the difficulty of obtaining sufficient DNA. Only recently has this barrier been overcome, thanks to advances in whole-genome amplification (Roberts et al., 2024).

These tiny, acoelomate worms are considered the closest relatives of the Platyhelminthes flat worms, together constituting the clade Rouphozoa (Struck et al., 2014; Laumer et al., 2015; Brusca et al., 2023). Comprised by over 900 species (WoRMS Editorial Board, 2025), the phylum Gastrotricha is one of the numerically smallest phyla among Spiralia. Despite that, gastrotrichs show an incredible variety of shapes, sizes (0.08–3.8 mm body length), reproductive strategies and lifestyles (Cesaretti et al., 2024, 2026; Kosakyan et al., 2026). Furthermore, they play a key role within the meiofaunal community, constituting an important player in the aquatic food webs of both marine and freshwater

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habitats, where they eat microalgae, bacteria and small protists by aspiration and in turn are preyed upon by small macrofauna, carnivorous ciliates, and free-living flatworms (Balsamo and Todaro, 2002; Todaro and Luporini, 2022). Ubiquitous in the stricter meaning of the term, gastrotrichs inhabit a wide range of aquatic environments, from clean littoral and shallow-sublittoral marine sands to clear or eutrophic freshwater systems, both lentic and, occasionally, lotic (Todaro et al., 2019a). They can also occur in extreme habitats, such as abyssal depths (Trokhymchuk and Kieneke, 2024), in caves (Todaro et al., 2006a), near hydrothermal vents (Kieneke and Zekely, 2007), in hot springs (Guerne, 1888), in the Arctic and Antarctica (Todaro et al., 2005; Kolicka et al., 2016, Kolicka et al., 2020; Sørensen et al., 2025).

All gastrotrichs are covered by a transparent, bilayered cuticle that can be smooth or form scales or spines. The outermost layer of the cuticle, known as the exocuticle, covers all locomotory and sensory cilia, a condition apomorphic to the phylum (Ruppert, 1991). Locomotory cilia are primarily found on the ventral surface, while sensory cilia that are involved in touch, mechanoreception, photoreception, and chemoreception are usually concentrated in the head region but can also be found along the length of the body. Additionally, some species may have other sensory structures, such as eye spots and flap-like chemoreceptors (Todaro et al., 2019a; Balsamo et al., 2020). The cuticle also forms tube-like extensions along the body, which contain the outlets of a duo-gland adhesive system, which help an animal maintain its position in sediments or on submerged vegetation (Tyler and Riger, 1980). Internally, gastrotrichs possess a straight digestive tract that includes a myoepithelial pharynx and monolayered, cellular intestine that ends with a ventral anus. Protonephridia function in the removal of metabolic wastes and, in freshwater species, serve also in osmoregulation (Kieneke and Schmidt-Rhaesa, 2015). Muscles in various orientations generally parallel the digestive tract; helicoidal (spiral) muscles around the digestive tract are one of the most intriguing and distinctive features of the gastrotrich muscular system, and a possible apomorphy for the phylum (Hochberg and Litwaitis, 2001a, b; Leasi et al., 2006; Leasi and Todaro 2008, Cesaretti et al., 2023). The nervous system consists of multiple nerve cords that extend posteriorly from the clusters of neuronal cell bodies located laterally in the brain (Rothe et al., 2011; Todaro et al., 2015).

Gastrotrichs were among the first small animals examined and described with early microscopes (Müller, 1773). Initially, they were categorized alongside other tiny animals and protozoans as "infusorians." However, Ehrenberg, (1830) distinguished gastrotrichs and rotifers from protozoans. Later, Mečhnikov (1865) introduced the term Gastrotricha, acknowledging their differences from rotifers. Consequently, for many decades, the study of gastrotrich systematics and phylogenetics largely depended on morphology-based methods (e.g., Remane, 1936; Hummon, 1974; Hochberg and Litvaitis, 2000, Kieneke et al., 2008).

Since the early 2000s, molecular phylogenetic studies have often been fragmented, typically depending on a limited number of genes, such as 18S rDNA, 28S rDNA, and mtCOI. These studies also frequently focus on a restricted set of taxa, usually at the family or order level (e.g., Todaro et al., 2003, 2006b, 2011; Kånneby et al., 2013; Bekkouche and Worsaae, 2016). The most taxonomically comprehensive molecular phylogeny of the phylum to date was proposed by Paps and Riutort (2012), although it was based solely on 18S rDNA sequences.

Gastrotrichs are divided into two significantly diverse orders. Macrodasyida is comprised by nearly 380 species (38 genera and 12 families; WoRMS Editorial Board, 2025; Cesaretti et al., 2026), almost all marine (Todaro et al., 2015), typically hermaphrodites with paired gonads and internal cross-fertilization (Guidi et al., 2022). In general, macrodasyidans lack a distinct head, the body ranges from short and broad to long and strap-like, the posterior body end is forked, bilobed, rounded, or drawn out into a tail. The sides of the body are parallel and bear from few to numerous adhesive tubes, with well-defined sensorial organs (Hochberg and Litvaitis, 2000). On the other hand, the order Chaetonotida is comprised by 520 species (belonging to 8 families and 37 genera; WoRMS Editorial Board, 2025). Chaetonotids are recognizable by the well-defined head lobe and by a tenpin-shaped body with the iconic bifurcated posterior end bearing two, but in some taxa zero or four adhesive tubes (Todaro et al., 2019b). Recent phylogenetic analyses have identified a distinct assemblage within Chaetonotida, now recognized as the Oiorpata clade, which encompasses the families Chaetonotidae, Dasydytidae, and Neogosseidae. Oiorpata forms a monophyletic group composed of primarily parthenogenetic species, which may be found in both marine and freshwater ecosystems (Gammuto et al., 2024). In several Oiorpata species, one or two bilateral rudimentary testes, located next to the midgut, have been documented by various researchers (Weiss and Levy, 1979; Kisielewska, 1981; Balsamo and Todaro, 1987, 1988; Weiss, 2001). These testes are actually morphologically abnormal sperm packets. Research using transmission electron microscopy (TEM) has focused on the rod-shaped spermatozoa of *Lepidodermella squamata* and *Chaetonotus maximus*. These spermatozoa primarily consist of condensed chromatin rods enclosed within a cell membrane (Hummon, 1984; Balsamo, 1992). The unusual structure of these gametes, along with the absence of sperm ducts, raises questions about their potential for fertilization.

A major advance in understanding gastrotrich phylogeny has come from the recent sequencing of 21 complete mitochondrial genomes (Kosakyan et al., 2026). These data strongly reinforce the deep divergence between the two main orders, Macrodasyida and Chaetonotida, which is particularly striking at the mitochondrial level and reflects substantial genomic divergence separating the two lineages, extending well beyond their morphological differences (Kosakyan et al., 2026). The striking variability in mitochondrial genomes may thus reflect broader and yet undetected genomic differences between the two orders. Nevertheless, some internal relationships remain unresolved. For instance, the position of the genus *Neodasys* (Remane, 1927) continues to challenge gastrotrich systematics. Its ambiguous morphological traits have led to its association with both orders multiple times over the past century (Remane, 1927; Paps and Riutort, 2012).

Here, we assembled 28 new genomes and collected 221 nuclear genes from 31 gastrotrich species spanning nine families. This dataset provides the first comprehensive phylogenomic framework based on nuclear loci for the phylum.

2. Methods

2.1. Taxonomic identification and DNA sequencing

We sampled 28 gastrotrich species during multiple field surveys (Table S1). Freshwater specimens were extracted by gently stirring sediment samples and decanting the suspension into Petri dishes for screening under a stereomicroscope. Marine specimens were isolated from sandy sediments treated with a 7% $MgCl_2$ solution following Todaro and colleagues (2019b). Individual gastrotrichs were picked with a glass micropipette and whole-mount in 1% (freshwater) or 7% (marine) $MgCl_2$ on microscope slides for morphological examination under differential interference contrast (DIC) optics. Voucher images were taken using a Nikon Eclipse 90i or a Leitz Dialux 20 microscopes equipped with digital cameras. After imaging, single individuals were recovered from slides and preserved in 96% ethanol at −20 °C for molecular work.

For DNA extraction, ethanol-preserved individuals were air-dried to remove residual ethanol, resuspended in PBS, and subjected to whole genome amplification (WGA) using the REPLI-g Single Cell Kit (QIAGEN®) according to the manufacturer's instructions. Amplified DNA was validated by PCR amplification and Sanger sequencing of the 18S rDNA gene using standard primers and conditions (Cesaretti et al., 2026). The resulting sequences were compared to GenBank references using BLAST to confirm taxonomic identity. Verified WGA products were used for library preparation with the TruSeq DNA PCR-Free Kit and

sequenced on an Illumina NovaSeq 6000 platform (2 × 150 bp), generating approximately 40 million paired-end reads per sample.

2.2. Genome and transcriptome assemblies

We assembled 28 new genomes from Illumina short reads (150 bp). The quality of raw reads was assessed with FastQC v0.12.1 (Andrews, 2010); Illumina adapters and low-quality reads were removed with Trimmomatic v0.39 (LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36) (Bolger et al., 2014). We also downloaded and assembled transcriptomes of two species from NCBI (see Table S1). Genomes and transcriptomes were assembled using SPAdes v4.1.0 (default settings; Bankevich et al., 2012) and Trinity v2.1.1 (default settings; Grabherr et al., 2011), respectively. Contigs were taxonomically annotated using DIAMOND blastx v2.1.11.165 (Buchfink et al., 2021) against the NCBI non-redundant (nr) protein database, applying the default DIAMOND search sensitivity. Contaminant filtering was then performed using BlobTools v1.0 (Challis et al., 2020), removing all the contigs without metazoan hits. Successively, assembly continuity was improved using Redundans v2.0.1 (Pryszcz and Gabaldón, 2016) (Fig. S1).

Assembly statistics were retrieved through QUAST v5.3.0 (Gurevich et al., 2013). Transcriptomes were filtered for contaminants using the same pipeline, retaining only transcripts that had a metazoan ID in their lineage through a custom-made python script (see the Data Availability section). Finally, we added the published genome of *Lepidodermella squamata* (Dujardin, 1841) (Roberts et al., 2024) to the species dataset, that was also used as reference for the overall quality of our assemblies. Our final dataset comprised a total of 31 species.

2.3. Gene dataset

In order to extract a conserved set of nuclear genes, we performed BUSCO v5.8.2 (Simão et al., 2015; Manni et al., 2021) on genome assemblies using the Metazoa_odb10 dataset. BUSCO was executed in genome mode with AUGUSTUS v3.5.0 (Stanke et al., 2008) as gene predictor. As no Gastrotricha-specific AUGUSTUS model was available, the *Schistosoma* (Platyhelminthes) species model was chosen as the initial parameter set. Then, automatic retraining (–long mode) was applied to optimize gene predictions. Similarly, we performed BUSCO on RNA-seq assemblies (transcriptome mode). In this case, transcripts were clustered beforehand with MMseqs2 v17.b804f (Steinegger and Söding, 2017) using the easy-cluster mode, retaining only one representative per cluster. We kept sequences with at least 95% identity and 80% reciprocal coverage to reduce redundancy and thereby avoid an artificially high number of complete, but duplicated genes in the BUSCO output.

We performed BUSCO after running both Blobtools (Challis et al., 2020) and Redundans (Pryszcz and Gabaldón, 2016) steps, and for each assembly we retained the BUSCO output showing the higher number of complete single-copy genes (Fig. S1). Additionally, we re-blasted genes against the non-redundant protein database (nr) (Goldfarb et al., 2025) using very-sensitive settings, and inspected the taxon of the top hits to confirm that no contaminants had escaped the previous filtering steps.

The free-living flatworm *Macrostomum cliftonense* was designated as the outgroup, due to the close relationship between Gastrotricha and Platyhelminthes, with Macrostomida representing a basal lineage within the latter (Egger et al., 2015). We created two matrices: one composed of single-copy genes shared by 90% of species (M90), and another by those shared by 80% of species (M80). There was not a single gene common to all the species.

2.4. Locus filtering

For both matrices, multiple sequence alignments were generated using MAFFT v7.525 (Katoh and Standley, 2013) with –maxiterate 1000 –localpair settings, while poorly aligned regions were trimmed using TrimAl v1.5 with the −automated1 option (Capella-Gutiérrez et al., 2009). Alignment statistics were calculated using AMAS (Borowiec, 2016). Gene alignments with more than 15% missing data were removed. To minimize compositional bias, we removed genes containing amino acids with a Z-score < -3 or > 3 in relative amino acid frequency. Individual misaligned sequences were detected and pruned using SpruceUp v2022.3.3 (Borowiec, 2019), excluding alignment windows exceeding the 97th percentile of a fitted Weibull distribution. Loci were further filtered based on Relative Composition Variability (RCV), excluding those with RCV > 0.35, using PhyKIT 2.0.1 (Steenwyk et al., 2021). Final site-based trimming was performed with ClipKIT v2.3.0 (Steenwyk et al., 2020) maintaining only parsimony-informative and constant sites, maximizing phylogenetic signal, while reducing noise. Loci passing all filters were concatenated using AMAS (Borowiec, 2016).

2.5. Tree search and Topological Tests

IQTREE v2.2.6 (Minh et al., 2020) was used to perform maximum likelihood tree inferences. ModelFinderPlus optimized the model selection and partition merging (−m TESTNEWMERGE) and we selected 100 bootstrap replicates for branch support estimation. Moreover, IQTREE2 was used for tree inferences with mixture models (C60+G+F). Gene trees (ModelFinderPlus and 1,000 UltraFast bootstrap) were used as input for the coalescent-based tree, performed using ASTRAL-IV (Zhang et al., 2025).

Phylogenetic inference under the CAT-GTR model was performed with PhyloBayes-MPI (Lartillot et al., 2013). Two independent Markov chain Monte Carlo (MCMC) chains were run in parallel. Convergence between chains was assessed with the bpcomp command in PhyloBayes, based on the maximum discrepancy in bipartition frequencies (maxdiff < 0.1).

The analysis of quartets was performed to unveil the phylogenetic signal of the ambiguous *Neodasys* sp. towards macrodasyidans or chaetonotidans. We used the M80 concatenated alignment as input for the Likelihood Mapping Analysis (LMA), wrapped in IQTREE2 (−lmap 10,000 −m TEST).

Robinson-Foulds distances (Robinson and Foulds, 1981) were calculated between each gene tree of the M80 matrix and the concatenated-based ML tree of M80, using the phylogenetic toolkit PhyKIT 2.0.1 (Steenwyk et al., 2021). Gene and Sites Concordance Factor (gCF and sCF; –scf 100) were calculated with IQTREE2 on the M80 concatenated alignment and the respective ML tree. gCF represented the percentage of genes that support a single branch from a species tree. Similarly, sCF evidenced the number of sites supporting a specific topology. High bootstrap values may not coincide with high concordance factors. Thus, calculating gCF and sCF represented important step to disentangle the phylogenetic support across the tree. Since Incomplete Lineage Sorting (ILS) is one of the most common causes for low sCF and gCF, to identify branches likely affected by this phenomenon, we tested the distribution of the support for alternative topologies using a $\chi 2$ test. If ILS is the reason of weak concordances, we should expect that the number of genes or sites supporting the two alternative trees be equal (Lanfear and Hahn, 2024).

2.6. Ancestral state reconstruction

Ancestral state reconstruction was performed using stochastic character mapping as implemented in the *phytools* 2.0 package (Revell, 2024) in R (R Core Team, 2025). We manually defined ecological (Marine/Freshwater) and reproductive (Hermaphrodite/Parthenogenetic) traits. Taxa with missing or ambiguous character states were excluded from the analysis (Table S6).

Trait evolution was modeled using a continuous-time Markov model of discrete character evolution (*fitMk*), and equal rate (ER) model. Stochastic character mapping was then performed using the *make.simmap*

function under the selected model, with 1,000 simulations to generate posterior distributions of ancestral states and transition counts. Since no fossil calibration points are available for Gastrotricha, we used an ultrametric molecular phylogeny without time calibration.

2.7. Loss of Universal metazoan genes

We explored lineage-specific gene loss using a custom R script to list and compare missing BUSCOs across species and clades (Macrodasyida, Chaetonotida, and Oiorpata). Missing genes in Chaetonotida and Oiorpata were used as foreground sets for Gene Ontology (GO) enrichment analysis (TopGO; Alexa et al., 2006), with a background consisting of BUSCO genes present in at least one gastrotrich species and with functional annotation available from InterProScan (803 genes). Enriched GO terms were summarized with ReviGO (Supek et al., 2011) and plotted with Cytoscape (Shannon et al., 2003).

In addition, to test the putative partial loss of nuclear genes involved in oxidative phosphorylation (OXPHOS), we retrieved 77 nuclear-encoded OXPHOS proteins from 12 representative metazoan species (Table S12) from OrthoDB v10 (Kriventseva et al., 2019) and searched for homologs in gastrotrich genomes using tblastn (e-value = 1e−5; Camacho et al., 2009).

3. Results

3.1. Species dataset

The final dataset was composed of 28 new whole-genome assemblies (WGS), two transcriptomes and the recently published genome of *Lepidodermella squamata* (Roberts et al., 2024). Globally, it consisted of 31 species (9 families, 26 genera; Table S1).

The contaminants-free assembly sizes ranged from 45.6 Mb to 187.5 Mb (median = 73.3 Mb, considering contigs longer than 500 bp; Table S2, Fig. S2). There were no significative assembly length differences between Macrodasyida and Chaetonotida (Fig. S2). Among genome assemblies, BUSCO completeness score (Single + Duplicated Genes) ranged from 41.5% (*Neodasys* sp.) to 78.7% (*Lepidodermella* sp.), while the most complete set of genes was retrieved in the transcriptome of *Diuronotus aspetos* (81.8%; Fig. 1, S3; Table S3).

The median length of aligned and trimmed proteins in the two matrices was 158.0 sites in M90 and 204.5 sites in M80. Due to the high number of short proteins, we did not exert a length cutoff, while we favored a more stringent control over other parameters. The median percentage of missing sites was 4.6% in M90 alignments and 3.9% in M80. After the quality filters, M90 decreased from 55 genes to 44, encompassing 6,098 amino acid sites, while the more permissive M80 passed from 243 genes to 221 (38,790 sites; Table S4).

3.2. Phylogenetic inference

Independently of the phylogenetic approach or matrix, we found the two orders Macrodasyida and Chaetonotida to be distinct (Fig. 1, S5). The topologies inferred using M80 matrix showed higher support compared to those from the M90, but remained largely congruent. Thus, we considered M80 as the reference matrix for the subsequent analyses and the discussion. Trees retrieved from M90 are shown in Fig. S5. The historically controversial *Neodasys* sp. was consistently placed close to Macrodasyida, receiving moderate support by maximum likelihood analysis (using both partition models and mixture models). The Bayesian inference (posterior probability, PP = 1.0) and the coalescence-based tree (PP = 0.98) also fully support this relationship (Fig. 1, S5), as well as the likelihood mapping analysis (77.2% of quartets; Fig. S6). Regardless of the inference approach, macrodasyidans showed the same topology (Fig. S5).

The genera *Turbanella* and *Paraturbanella* constituted the monophyletic family Turbanellidae. The same holds for the family Redudasyidae (genera *Redudasys* and *Anandrodasys*). On the other hand, the family Macrodasyidae (represented by *Macrodasys, Thaidasys,* and *Urodasys* herein) was not monophyletic. Our trees support *Crasiella* sp. (Planodasyidae) in sister relationship with *Mesodasys* sp. (Cephalodasyidae, family not monophyletic). However, this relationship is influenced by taxonomic sampling, as the genus *Megadasys*, which has been recovered as sister to *Crasiella* in previous phylogenetic studies (Paps and Riutort, 2012; Cesaretti et al., 2026), was not included in our dataset. M80 gave conflicting topologies within Oiorpata depending on methods: partitioned ML and the coalescent-based species tree recovered the two marine species *Chaetonotus neptuni* and *Aspidiophorus tentaculatus* as early branches, whereas mixture-model ML (C60+F+G) and the Bayesian analysis recovered an alternative topology (Fig. S5).

The numerically dominant genus *Chaetonotus* showed a polyphyletic origin, as well as the genera *Aspidiophorus and Lepidodermella*. Our results confirmed the paraphyletic nature of the Chaetonotidae. The Dasydytidae family, represented by the genera *Dasydytes*, *Setopus* and *Stylochaeta* in our dataset, is recovered as paraphyletic in our analysis.

3.3. Tree support

Despite the overall high nodal support across the phylogenetic trees, concordance factors (genes and sites) revealed conflict among many resolved nodes (Fig. 2, Table S5a). The distribution of concordance factor's support between alternative topologies showed significant differences in 12 nodes (χ^2 test; Table S5b), rejecting the expected nearly equal support for alternative topologies in presence of incomplete lineage sorting (Lanfear and Hahn, 2024). Out of these 12 nodes, 11 concerned chaetonotidans, as highlighted in Fig. 2. Longer branches showed higher concordance for both sites and genes (gCF: Spearman's rho = 0.83, p-value = $0.3 \cdot 10^{-7}$; sCF: Spearman's rho = 0.58, p-value = $0.1 \cdot 10^{-4}$).

3.4. Ancestral state reconstruction

The discrete character "habitat" (marine vs. freshwater) was coded as binary for the 31 species: 20 species were annotated as marine (Table S6). Based on 1,000 stochastic simulations under the ER model, an average of 3.77 transitions between states was inferred. Transitions from marine to freshwater occurred more frequently (mean = 2.24) than the reverse (mean = 1.53). The common ancestor of the Oiorpata clade was reconstructed as marine with high probability (PP = 0.82). On the other hand, all the freshwater oiorpatans share a freshwater ancestor, suggesting an early ecological shift in this group (except *C. apolemmus*, which represents a lineage that likely re-invaded the marine habitat). Conversely, ancestors within Macrodasyida and non-Oiorpata chaetonotidans retained a marine state with strong support (0.97 < PP < 0.99; Fig. 3; Table S7).

The discrete character "sexual strategy" (hermaphrodite vs. parthenogenetic) was coded as binary for 29 species present in the phylogenetic tree, with 14 annotated as hermaphroditic (Table S6). In fact, because of uncertainties about the sexual strategy of *Paradasys* sp. (lack of muscles associated with the reproductive organs; Leasi et al., 2006) and *Thaidasys tongiorgii* (copulatory organ present but testes absent; Todaro et al., 2015), they were excluded from the analysis. Under the ER model, we obtained an average of 2.35 transitions between states. Node pie charts across the tree show that most internal nodes leading to clades of Macrodasyida were reconstructed as hermaphroditic with high confidence (PP > 0.99; exception for the common ancestor of *R. fornerise* and *A. agadasys*), while all Oiorpata ancestors were retrieved as parthenogenetic (Fig. 3; Table S8).

3.5. Gene loss

The Chaetonotida order had a higher median BUSCO gene completeness per species (Single + Duplicated: 75.1%) than

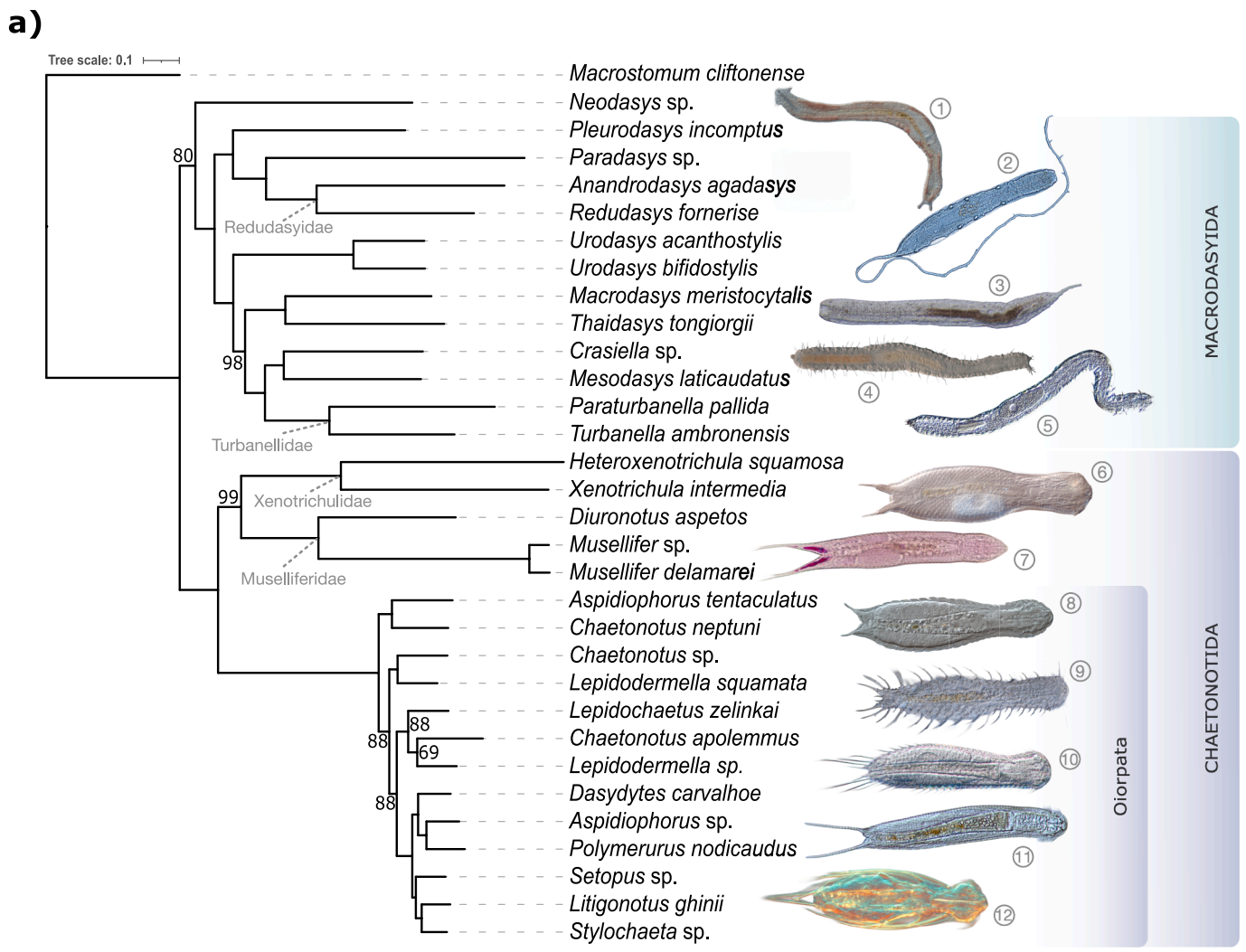

Fig. 1. **a)** Maximum Likelihood tree based on 221 proteins (38,790 amino acids) and partition models. When not indicated, the node was fully supported (bootstrap = 100). Monophyletic families are labeled across the phylogeny. On the right side, some representatives of both orders: (1) *Neodasys* sp., (2) *Urodasys acanthostylis*, (3) *Macrodasys meristocytalis* (4) *Crasiella* sp., (5) *Thaidasys tongiorgii*, (6) *Xenotrichula intermedia*, (7) *Musellifer* sp., (8) *Lepidodermella squamata*, (9) *Chaetonotus neptuni*, (10) *Lepidochaetus zelinkai*, (11) *Polymerurus nodicaudus*, (12) *Dasydytes carvalhoe*. **b)** Distribution of BUSCO completeness scores, divided by order (median Macrodasyida = 59.3%, median Chaetonotida = 75.1%. **c)** Negative linear relationship between relative Robinson-Foulds distances calculated on M80 gene trees and gene lengths (Pearson's $r = -0.42$; p-value $= 1.1 \cdot 10^{-10}$).

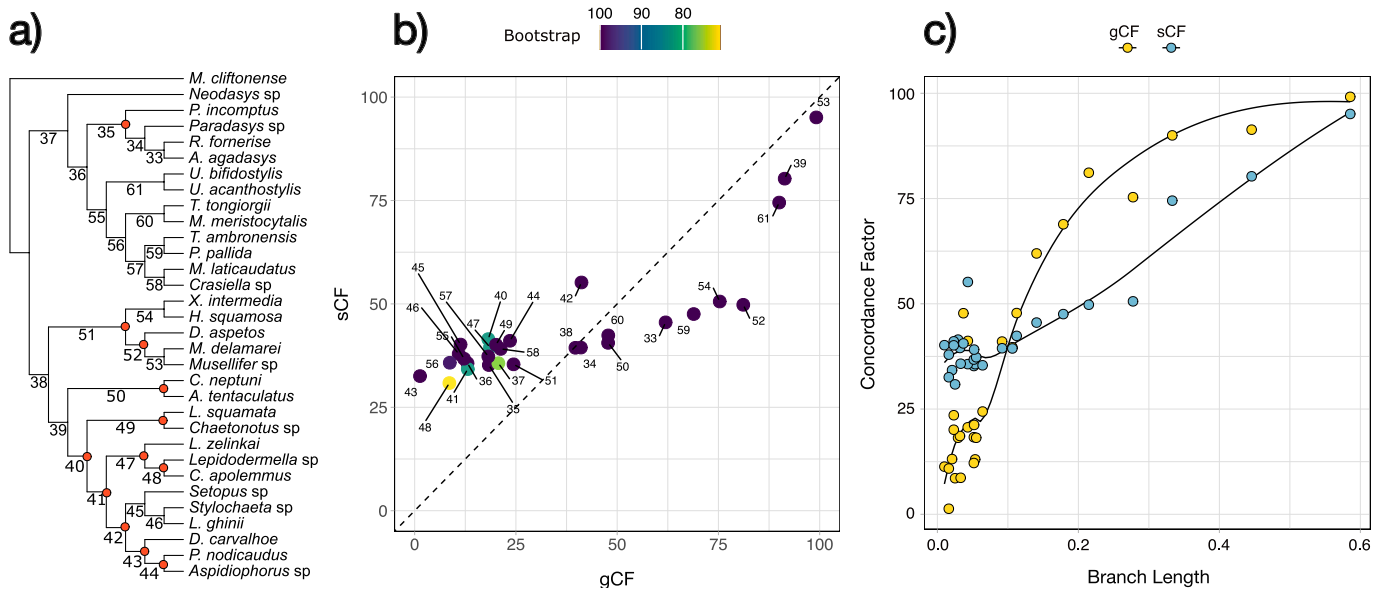

Fig. 2. gCF and sCF indicates concordance factors. While gCF means the percentage of gene trees showing a particular branch from a species tree, sCF represents the number of sites supporting that branch. **a)** M80 topology with numbers referring to the two subsequent figures. Red points indicate branches that failed $\chi 2$ test for gCF and/or sCF. **b)** gCF/sCF ratio; **c)** covariation between branch lengths and concordance factors, highlighted by a LOESS smoothing curve.

Macrodasyida (59.3%; Fig. 1). However, when considering presence across each clade, Macrodasyida retained at least one representative for 98.2% of the total BUSCO gene set, meaning that nearly all genes were present in at least one species on the clade. In comparison, this percentage was lower in Chaetonotida (93.6%), specifically in Oiorpata (88.8%) (Fig. 3, S7; Table S9).

Out of the 954 BUSCOs included in the Metazoa_odb10 dataset, 13 were missing in all gastrotrich species. In contrast, 48 BUSCOs were absent from the common ancestor of all chaetonotidans. Within Chaetonotida, Oiorpata and the Muselliferidae + Xenotrichulidae clade showed distinct set of missing genes, with 44 and 33 missing BUSCOs, respectively. Only two BUSCOs were exclusively absent in Macrodasyida (Fig. 3, S7; Table S9). The 48 genes missing in all chaetonotidans were predominantly associated with broad biological processes such as cell communication and signaling, regulation of cellular processes, organelle organization and mitosis (Fig. S8). In the Cellular Component category, most of the overrepresented terms were associated with cytoskeletal structures, such as cilia and flagella (Table S10).

The set of 44 genes lost in Oiorpata was significantly enriched for functions related to translation, peroxisomes and metabolic processes (Table S10). Conversely, the 33 genes missing in Muselliferidae + Xenotrichulidae were mainly associated with autophagy, starvation and the regulation of catabolic processes (Table S10).

We then specifically examined genes involved in oxidative phosphorylation (OXPHOS) to assess whether this metabolism shows lineage-specific signatures. Out of the 77 nuclear OXPHOS genes, 68 were detected in at least one species. Two genes, *ndufa1* and *qcr10*, were found in only a single species. Additionally, *cox7c* was restricted to Chaetonotida, while *cox6c* occurred only in Oiorpata. No other lineage-specific patterns were observed. The slightly lower number of nuclear OXPHOS genes in Macrodasyida is driven by *U. bifidostylis*, which has only 15 genes, likely reflecting a suboptimal quality assembly (Table S13, S14).

4. Discussion

The limited availability of extensive molecular datasets for many understudied invertebrate clades represents a critical gap in our understanding of their diversity and evolutionary history (Martínez et al., 2025). Gastrotricha are no exception: the first genome of the phylum was published only very recently (Roberts et al., 2024). To date, comprehensive studies on gastrotrich phylogeny have focused on internal relationships, traditionally relying on morphological characters alone (e.g., Hochberg and Litvaitis, 2000; Kieneke et al., 2008). Despite the growing application of molecular approaches (Paps and Riutort, 2012; Kånneby and Todaro, 2015; Kolicka et al., 2020; Gammuto et al., 2024; Cesaretti et al., 2026), the use of only a few markers in previous studies has left many questions about in-group phylogenetic relationships unresolved. In this context, our results provide a major step forward, clarifying relationships that were previously inconsistent or discordant. Our analyses confirmed the current subdivision of Gastrotricha into two distinct orders (Kolicka et al., 2016, 2020). Because of the debated position of the genus *Neodasys*, which is currently classified among Chaetonotida, we included the genome of *Neodasys* sp. in the analysis despite its relatively low BUSCO completeness. The hypothesis of a close relationship between *Neodasys* and Macrodasyida has been discussed by several authors (Todaro et al., 2003, 2006; Petrov et al., 2007; Kieneke et al., 2008; Paps and Riutort, 2012; Kolicka et al., 2020). *Neodasys* remains of particular interest due to persistent phylogenetic uncertainty, which has been attributed to conflicting morphological traits (e.g., pharyngeal ciliation, adhesive glands, pharyngeal lumen shape, and reproductive structures; Kieneke et al., 2009). Even the availability of complete mitochondrial genomes has not clarified its placement (Kosakyan et al., 2026). In fact, although mitochondrial genomes are valuable for phylogenetic inference, their reliability on some nodes can be affected when lineages experience unusual evolutionary pressures or structural changes (e.g., rearrangements, duplications, or transfers) (DeSalle and Tessler, 2025; Wallnoefer et al., 2025). Such deviations do not preclude the use of mitochondrial genes as markers, but they can increase noise, reduce resolution, and require careful interpretation in phylogenetic analyses (e.g., Formaggioni et al., 2022; Larson et al., 2025). Gastrotrich mitogenomes seem affected by deep lineage-specific differences: macrodasyidans show highly variable gene orders and even evidence of mitochondrial gene transfers to the nuclear genome, whereas chaetonotidans are more conserved. Using mitogenomes alone, no nodal support or synteny evidence supported the *Neodasys* + Macrodasyida relationship (Kosakyan et al., 2026). In contrast, our analyses consistently recovered *Neodasys* sp. as sister to Macrodasyida, regardless of dataset or phylogenetic method employed. The non-monophyletic status of the family Macrodasyidae confirmed

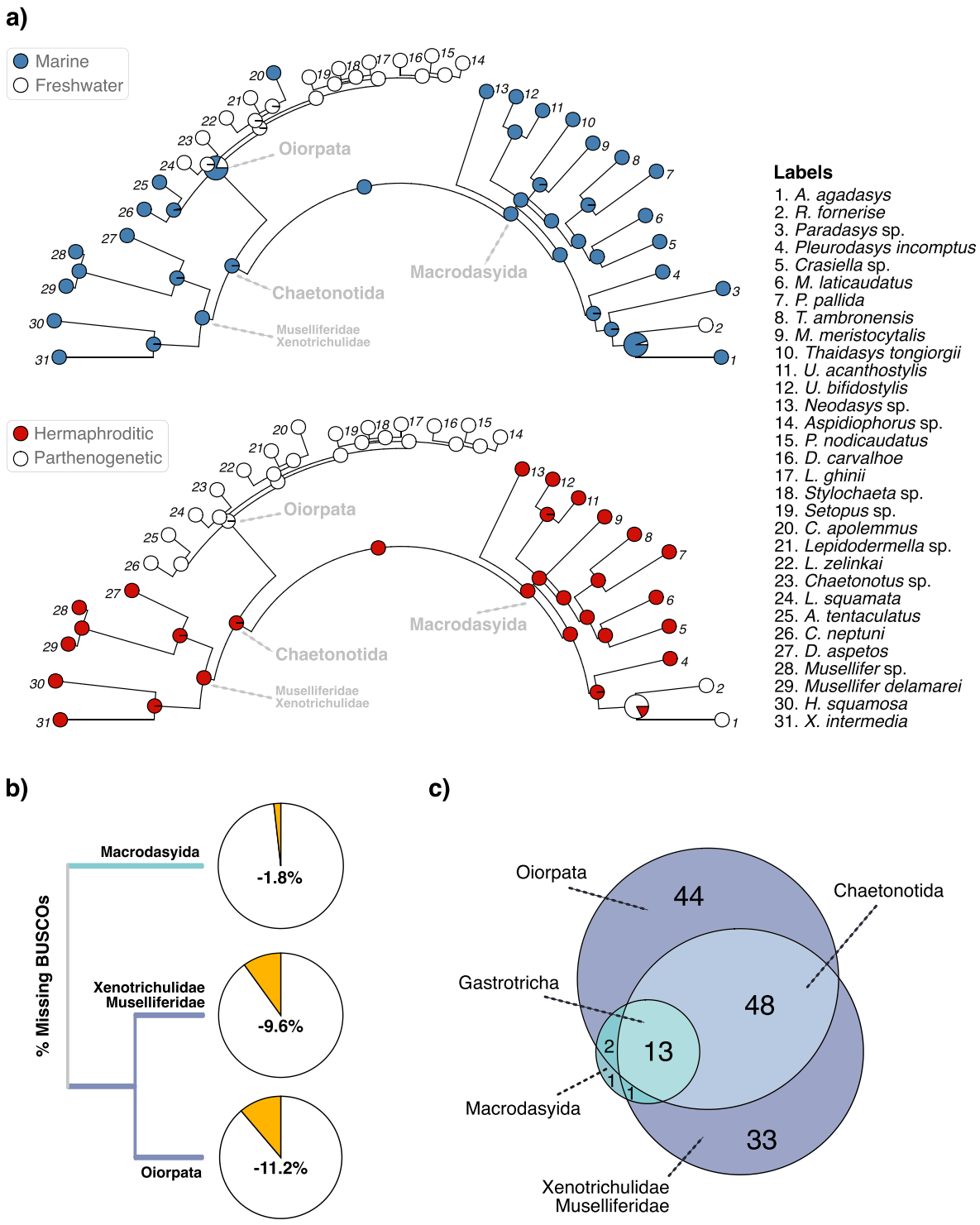

Fig. 3. **a)** Ancestral State Reconstruction of habitat transition (above) and sexual strategies (below). Characters are mapped on the same maximum likelihood topology (M80; partition models). **b)** Percentage of missing BUSCOs on the total number of 954 (Metazoa_odb10). **c)** Venn plot showing the number of shared missing genes by groups.

the result of previous studies (e.g., Paps and Riutort, 2012; Kieneke and Todaro, 2021).

We confirmed the monophyly of Turbanellidae (Todaro et al., 2015; Campos et al., 2025; Cesaretti et al., 2026) and Redudasyidae (Todaro et al., 2012, 2019a; Cesaretti et al., 2026), as well as the alliances of (polyphyletic) Macrodasyidae and Cephalodasyidae as highlighted in Cesaretti et al. (2026).

The monophyly of Muselliferidae + Xenotrichulidae is consistent with their reproductive condition as functional hermaphrodites (Ferraguti et al., 1995; Todaro et al., 2005; Balsamo et al., 2010), as well as with their neuromuscular similarities (Leasi and Todaro, 2008; Bekkouche and Worsaae, 2016) and contrasts with the alternative

hypothesis of Xenotrichulidae + Oiorpata proposed by Kolicka and colleagues (2020).

Within Oiorpata, the polyphyletic nature of the genera *Aspidiophorus*, *Chaetonotus*, and *Lepidodermella* has also been recovered by other authors and is likely the result of historical taxonomic misidentifications arising from misleading morphological homoplasy in the traits used for classification within the group (Kolicka et al., 2020; Rataj Križanová and Vďačný, 2024; Gammuto et al., 2024; Minowa et al., 2025). Furthermore, extremely short branches complicated the reconstruction of relationships within Oiorpata and hindered the resolution of its deeper nodes, which in some cases failed to reach full bootstrap support. Concordance analyses often revealed low concordance values for both genes and sites. In most cases, we identified incomplete lineage sorting as the main source of conflict, whereas in others, mainly concentrated within Oiorpata, we attributed the discordances to a combination of short alignments and reduced branch lengths (Shen et al., 2016). It is important to notice that high bootstrap supports for the comprehensive tree often do not implicate high supports from individual loci (Lanfear and Hahn, 2024) and there are many examples of that in literature (Fontaine et al., 2015; Copetti et al., 2017; Wu et al., 2018; Edelman et al., 2019; Hime et al., 2021; Roberts et al., 2023).

Macrodasyidans are predominantly marine, with a few known exceptions, such as *R. fornerise*, whereas chaetonotidans show a more heterogeneous and balanced pattern, including both marine and freshwater species (Saponi and Todaro, 2023; Saponi et al., 2024). Habitat transitions have occurred repeatedly across metazoan lineages, including shifts between marine and freshwater environments (Lee and Bell, 1999; Vermeij and Dudley, 2000; Logares et al., 2009). Such transitions are generally rare, but, when they do occur, freshwater invaders often originate from habitats with broad salinity ranges, which favor the evolution of wide salinity tolerances (Lee and Bell, 1999). In this sense, gastrotrichs represent a suitable model, since many species are known to inhabit euryhaline environments such as estuaries and brackish waters (Balsamo and Todaro, 1988; Todaro et al., 2006a, 2019b; Kånneby et al., 2013). These ecological conditions may have provided the necessary physiological plasticity to facilitate the colonization of freshwater by a common ancestor of most chaetonotidans. Most species of the group Oiorpata included in our analyses, inhabit freshwater environments and share a common ancestor (coherently with the topology based on mitochondrial protein-coding genes; Kosakyan et al., 2026), while the two marine species *A. tentaculatus* and *C. neptuni* form a clade, which is resolved as sister group to all remaining taxa (Figs. 1, 3). Within this framework, the marine species *C. apolemmus* clearly represents a case of secondary colonization of the marine environment. From a broader perspective, we reject the hypothesis proposed by Kolicka and colleagues (2020) that all extant Oiorpata originated from freshwater environments. Our data suggest that the ancestor of Oiorpata was actually a saltwater dweller, supporting the scenario of a single marine-to-freshwater transition (Kånneby et al., 2013). However, due to the limited taxonomic sampling in our study, our results do not contradict the possibility of multiple independent marine re-invasions within Oiorpata, as demonstrated by our findings related to *C. apolemmus*. Conversely, Bayesian inference recovered a topology supporting multiple independent freshwater invasions. However, considering that habitat transitions are generally considered rare events, the single-transition hypothesis results in the most parsimonious explanation (Maddison, 2006; Maddison et al., 2007; Logares et al., 2009; Jurdzinski et al., 2022). Nevertheless, some caution is needed when interpreting these results. Our taxon sampling is limited, which makes it difficult to draw firm conclusions on whether a single freshwater invasion is truly the most likely scenario. In addition, the proposed topology (Fig. 1) shows a high level of hidden discordance among loci, a factor that can easily generate spurious relationships within Oiorpata, which may also apply to the planktonic Dasyidytidae (Hibbins et al., 2023; Lanfear and Hahn 2024).

Habitat transitions are known to profoundly influence speciation and extinction rates (Yoder et al., 2010), and this principle may help explain the rapid diversification of the Oiorpata suborder. The invasion of freshwater habitats by Oiorpata likely accelerated speciation, since such transitions often impose strong ecological pressures requiring both morphological and genetic adaptations (Holterman et al., 2019). Freshwater environments are typically fragmented and heterogeneous, generating additional constraints on population connectivity (Puebla, 2009; Bloom et al., 2013). Physical barriers further restrict gene flow among populations, while fluctuating salinity, variable food resources, and limited nutrient availability create additional selective pressures (Bloom et al., 2013; Jamy et al., 2022). Together, these factors can drive rapid divergence, shaping both the morphological and genetic traits observed in Oiorpata. As described in detail by Kolicka and colleagues (2020), the freshwater invasion by Oiorpata led to several synapomorphies, including cuticular modifications and reductions of testes and spermatozoa. In this context, the shift from hermaphroditism to obligate parthenogenesis in the common ancestor of Oiorpata may have facilitated colonization of freshwater habitats. Interestingly, the only freshwater species among Macrodasyida included in our dataset, *R. fornerise*, is parthenogenetic. Its sister taxon, the marine *Anandrodasys agadasys*, is also parthenogenetic, suggesting that their common ancestor was marine and parthenogenetic (Todaro et al., 2012). This reinforces the hypothesis that parthenogenesis in Oiorpata evolved prior to the colonization of freshwater habitats. Parthenogenetic species are expected to gradually lose genes associated with sexual reproduction, including those required for male functions (e.g., acrosome biogenesis and copulatory organs) and female traits involved in mating and fertilization (e.g., pheromone signaling or sperm storage structures) (van der Kooi and Schwander, 2014). When pleiotropic constraints are absent, such losses may occur through the accumulation of mutations under relaxed purifying selection, or through directional selection reducing energetically costly sexual traits (Fong et al., 1995; Schwander et al., 2013). In order to definitely unravel the relationships between the colonization of freshwater environments and the use of parthenogenesis as reproductive strategy (or their unrelatedness), genes related to sexual strategy should be investigated in further studies.

We detected a phylogenetic distribution of missing BUSCOs, with special reference to Chaetonotida. The observed reduction in BUSCO completeness along the branch leading to Chaetonotida (−6.4% of total BUSCOs), and the more pronounced decrease in Oiorpata (−11.2%) and in Muselliferidae and Xenotrichulidae (−9.6%) reinforces the deep subdivision between Macrodasyida and Chaetonotida, which may be deeper than previously recognized. Because BUSCOs represent highly conserved genes involved in essential cellular processes across Metazoa (Simão et al., 2015; Alam et al., 2025), their systematic absence in a clade warrants attention.

This pattern is unlikely to reflect lower genome quality, as incompleteness would produce random losses across assemblies (Rödelsperger, 2021; Cunha et al., 2023). Moreover, median BUSCO completeness was higher in Chaetonotida than in Macrodasyida.

There are three alternative (and not mutually exclusive) explanations for a non-random distribution of missing BUSCOs.

The first hypothesis is that some missing BUSCOs may result from the high sequence variability in gastrotrichs compared to the metazoan BUSCO background. As shown in Cunha and colleagues (2023), some divergent metazoan phyla that are not represented in the BUSCO database display much lower proportions of missing genes. Gastrotricha, however, is a long-branched phylum (Struck et al., 2014; Roberts et al., 2024), and technical failure in ortholog detection due to accelerated sequence evolution or divergence in gene structure relative to the Metazoa_odb10 core set could explain, at least partially, some of the observed missing BUSCOs.

The second hypothesis is that some missing genes may be located in genomic regions that are difficult to sequence or assemble with short reads, such as GC-rich or highly repetitive regions (Kim et al., 2022). This technical limitation can lead to systematic absences in specific

lineages, which could be resolved with improved genome assemblies.

The third hypothesis consists of genuine losses in some lineages: the observed BUSCO absences may represent genuine gene loss events, potentially linked to broader genomic reorganization within the Chaetonotida lineage. Under this light, the missing genes associated with mitosis may reflect the eutely of gastrotrichs, particularly Chaetonotida (Oiorpata), which have a fixed number of somatic cells in the adult body (Manylov, 1995). Notably, missing genes in Oiorpata include those involved in mitochondrial translation and peroxisomal processes, while species in this clade retain an extremely conserved mitochondrial gene number and order (Kosakyan et al., 2026). This contrast may reflect lineage-specific evolutionary constraints or compensatory mechanisms that preserve genome organization despite gene loss, illustrating how genomic architecture can evolve under selective pressures while maintaining essential functions (Adams and Palmer, 2003; Albalat and Cañestro, 2016).

Concluding, the present results underscore the value of investigating poorly characterized meiofaunal lineages, such as gastrotrichs, through large-scale molecular datasets. Broadening genomic and taxonomic sampling in these groups can illuminate hidden evolutionary and genomic dynamics, revealing aspects of biodiversity and metazoan evolution that remain obscure when relying on few markers only.

CRediT authorship contribution statement

Oscar Wallnoefer: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Anush Kosakyan:** Writing – review & editing, Resources, Investigation, Conceptualization. **Agata Cesaretti:** Resources, Investigation. **Francesco Saponi:** Resources, Investigation. **Leandro Gammuto:** Resources, Investigation. **Valentina Serra:** Resources, Investigation. **Giulio Petroni:** Resources, Investigation. **Federico Plazzi:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Conceptualization. **M. Antonio Todaro:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2026.108601.

Data availability

We have shared the link to data in the “Data Availability Statement” section.

The newly generated sequencing reads have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1425028.

All the original data, including genome assemblies, and scripts necessary to reproduce the analyses reported in this study can be accessed through FigShare (https://doi.org/10.6084/m9.figshare.30686585), and at the GitHub repository (https://github.com/oscarwallnoefer/Gastrotricha_Phylogenomics.git).

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