



# The first multi-gene phylogeny of the Macrostomorpha sheds light on the evolution of sexual and asexual reproduction in basal Platyhelminthes<sup>☆</sup>



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## ABSTRACT

The Macrostomorpha—an early branching and species-rich clade of free-living flatworms—is attracting interest because it contains *Macrostomum lignano*, a versatile model organism increasingly used in evolutionary, developmental, and molecular biology. We elucidate the macrostomorph molecular phylogeny inferred from both nuclear (18S and 28S rDNA) and mitochondrial (16S rDNA and COI) marker genes from 40 representatives. Although our phylogeny does not recover the Macrostomorpha as a statistically supported monophyletic grouping, it (i) confirms many taxa previously proposed based on morphological evidence, (ii) permits the first placement of many families and genera, and (iii) reveals a number of unexpected placements. Specifically, *Myozona* and *Bradynectes* are outside the three classic families (Macrostomidae, Microstomidae and Dolichomacrostomidae) and the asexually fissioning *Myomacrostomum* belongs to a new subfamily, the Myozonariinae nov. subfam. (Dolichomacrostomidae), rather than diverging early. While this represents the first evidence for asexuality among the Dolichomacrostomidae, we show that fissioning also occurs in another Myozonariinae, *Myozonaria fissipara* nov. sp. Together with the placement of the (also fissioning) Microstomidae, namely as the sister taxon of Dolichomacrostomidae, this suggests that fissioning is not basal within the Macrostomorpha, but rather restricted to the new taxon Dolichomicrostomida (Dolichomacrostomidae + Microstomidae). Furthermore, our phylogeny allows new insights into the evolution of the reproductive system, as ancestral state reconstructions reveal convergent evolution of gonads, and male and female genitalia. Finally, the convergent evolution of sperm storage organs in the female genitalia appears to be linked to the widespread occurrence of hypodermic insemination among the Macrostomorpha.

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## Quotation

“...hüte dich aber vor Namen-Registern von Würmern, wovon flüchtige Kenntnis nichts nützt, und eine genaue in's Unendliche führt.” Georg Christoph Lichtenberg

## 1. Introduction

The free-living flatworms—formerly “Turbellaria”—are a highly diverse and paraphyletic group of early branching Platyhelminthes, which according to current molecular evidence belong to the Lophotrochozoa (Dunn et al., 2008; Philippe et al., 2011; Egger et al., 2015). The Platyhelminthes comprise (i) the Catenulida, the proposed earliest diverging flatworm lineage, and (ii) the Rhabditophora, having rhabdite-secreting glands that facilitate ciliary gliding and substrate adhesion (Martin, 1978), and a modified genetic code for mitochondrial protein translation (Telford et al., 2000). According to ribosomal sequence data and more recent phylogenomic analyses the Macrostomorpha are the sister group of all other rhabditophorans (Baguña and Riutort,

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2004; Larsson and Jondelius, 2008; Laumer and Giribet, 2014; Egger et al., 2015; Laumer et al., 2015). Surprisingly, their phylogeny has been largely neglected, despite their important phylogenetic position, species richness (currently about 250 species) and global distribution in aquatic environments (Rieger, 2001). Limited progress may be partly due to the highly variable quality of the taxonomic work, and to unresolved taxonomic issues resulting from an exquisite, but never fully published, taxonomic monograph by Rieger (1971a,b,c). The macrostomorphan *Macrostomum lignano* Ladurner, Schärer, Salvenmoser and Rieger 2005 has recently become a model organism for molecular, developmental and evolutionary biology (e.g. Ladurner et al., 2005, 2008; Salvenmoser et al., 2010; Schärer et al., 2011). To facilitate placing these advances in a comparative phylogenetic framework, an understanding of the phylogenetic interrelationships in this group has gained increased urgency.

The taxonomy of these small and fragile microturbellarians has been difficult to study, requiring drawings from live observations, permanent preparations of hard parts (thus losing most information on soft or non-sclerotized parts), and laborious anatomical studies from serial sections. Recently, a combination of extensive digital photomicrography (detailing both hard and soft parts) that can be made available online as a digital morphological voucher and molecular analyses from individually documented specimens, has been advocated and successfully implemented in this organismal group (Ladurner et al., 2005; Schärer et al., 2011). These advances greatly facilitate the analysis of microturbellarians, both with respect to direct identification by non-specialists under near-field conditions, and analysis of metagenetic data from environmental samples, some of which suggest that these organisms are abundant and diverse (Creer et al., 2010; Fonseca et al., 2010).

The Macrostomorpha Doe 1986 currently comprise two main taxa, the species-poor Haplopharyngida Karling 1974 and the highly diverse Macrostomida Karling 1940. The latter comprises three families, the Macrostomidae van Beneden 1870, the Microstomidae Luther 1907 and the Dolichomacrostomidae Rieger 1971. Additionally, there is a currently poorly placed genus in the Macrostomida, *Myomacrostomum* Rieger 1986, which was originally considered to take an early diverging position (Rieger, 1986), but currently has an uncertain phylogenetic position (Rieger, 2001). Like in most other free-living flatworms, the taxonomic classification of the Macrostomorpha is largely based on the morphology of their hermaphroditic reproductive system. However, recent studies show that these morphological traits are prone to convergent evolution (Schärer et al., 2011; Tessens et al., 2014), making a comparative molecular phylogenetic framework indispensable to understand the evolution of reproductive systems in this group. This convergent evolution of reproductive morphologies is further supported by our results, and, to better understand these, we therefore briefly introduce the main evolutionary forces driving the evolution of reproductive systems in simultaneous hermaphrodites.

Being simultaneously male and female, hermaphrodites experience conflicts over the outcome of mating interactions, as they may often prefer donating rather than receiving sperm, at least once individuals have received sufficient sperm to fertilize their own eggs (Charnov, 1979; Michiels, 1998; Anthes et al., 2010; Schärer et al., 2014). In unilaterally mating species this can result in struggles over who plays the male mating role, as in penis-fencing polyclad flatworms that try to hypodermically inseminate their partners without being inseminated themselves (Michiels and Newman, 1998; Lange et al., 2013). In reciprocally mating species, individuals mate simultaneously as both males and females and may engage in matings rather to donate than to receive sperm (Charnov, 1979; Schärer et al., 2014). This can lead to adaptations in the recipient to control the fate of received

ejaculates, such as genitointestinal ducts or copulatory bursae that digest or neutralize ejaculates (Medina et al., 1988; Sluys, 1989; Westheide, 1999), or behaviors like the intriguing ‘suck’ in *Macrostomum lignano* that may manipulate received ejaculates (Vizoso et al., 2010; Schärer et al., 2011). This in turn may lead to adaptations in the donor aimed at preventing the recipient from exercising control, such as the transfer of manipulating allohormones (Chase and Blanchard, 2006) or sperm morphologies that hinder their removal (Vizoso et al., 2010; Schärer et al., 2011). These contrasting interests sustain sexually antagonistic coevolution—a form of sexual selection driven by sexual conflict, which can lead to rapid selection on sexual persistence and resistance traits (Arnqvist and Rowe, 2005; Schärer et al., 2014). Moreover, donors may be selected to bypass the recipient-controlled genital system by resorting to hypodermic insemination, which in turn may relax selection on previously complex female genitalia, even leading to their loss, and eventually even to the evolution of novel female genitalia (Stutt and Siva-Jothy, 2001; Lange et al., 2013).

In this study we aim to: (i) create a comprehensive multi-gene phylogenetic framework for the Macrostomorpha, evaluating the proposed interrelationships of macrostomorph taxa and establishing the phylogenetic position of the model organism *Macrostomum lignano*; (ii) use this phylogeny to study reproductive character evolution and determine useful diagnostic traits; (iii) establish suitable mitochondrial 16S and COI markers for the Macrostomorpha; and (iv) document the usefulness of online repositories for digital morphological vouchers and DNA-based taxonomy.

## 2. Materials and methods

### 2.1. Selection, collection and documentation of specimens

We chose two Catenulida, *Stenostomum* sp. (Stenostomidae) and *Paracatenula* sp. (Retronectidae), as outgroup taxa, because the Catenulida are the earliest branching clade within the Platyhelminthes and the sister group of all Rhabditophora (Larsson and Jondelius, 2008; Egger et al., 2015; Laumer et al., 2015). Furthermore, we selected three Polycladida, *Hoploplana californica* and *Paraplanocera oligoglena* (Acotylea), and *Boninia divae* (Cotylea) (see Table 1); as additional outgroup taxa because the Polycladida are currently considered to be close relatives of the Macrostomorpha (Baguña and Riutort, 2004; Laumer and Giribet, 2014; Egger et al., 2015; Laumer et al., 2015). Additionally we tested whether adding additional Rhabditophora clades as outgroups had an influence on the tree topology.

Ingroup Macrostomorpha representatives (see Table 1) were collected in a range of countries, habitats and water bodies using different extraction techniques. We used MgCl<sub>2</sub>-decantation for marine samples and oxygen depletion for freshwater samples (Pfannkuche and Thiel, 1988; Schockaert, 1996). Specimens were documented as described in Ladurner et al. (2005) and Schärer et al. (2011). Briefly, the habitus and morphology of live specimens were extensively documented in squeeze preparations under bright-field, phase-contrast, and/or, preferably, differential interference contrast (DIC) illumination and at magnifications of 40× to 1000× using a compound microscope (Diaplan or DM2500, Leica Microsystems; BHT, Olympus) and a digital c-mount video camera (DFW-X700, SONY; DFK 41BF02, The Imaging Source). Digital morphological vouchers (pictures and videos) of each sequenced specimen, created with image-capture software (BTV Pro 6.0b1, available at <http://www.bensoftware.com/>), are deposited on the Macrostomorpha Taxonomy and Phylogeny website (<http://www.macrostomorpha.info/>) and the Dryad Digital Repository (Janssen et al., 2015, <http://dx.doi.org/10.5061/dryad.b5908>) (see

**Table 1**

The analyzed species, specimen vouchers (deposited on the Macrostromorpha Taxonomy and Phylogeny website, <http://macrostromorpha.info/> and Dryad Digital Repository Janssen et al., 2015, <http://dx.doi.org/10.5061/dryad.b5908>), lactophenol whole mount preparations (deposited at the Natural History Museum, London, NHMUK accession codes), and the sequenced gene fragments with their GenBank accession numbers. For details on ingroup species identification, sampling locations and taxonomic status see the "Taxonomic notes" section and Table C. Entries with an asterisk (\*) represent additional deposited voucher specimens (sensu Pleijel et al., 2008) that were not included in the phylogenetic reconstruction because their sequences were near identical to the main specimen (hologenophore vouchers) and/or because they stemmed from the same sample (paragenophore vouchers). Species in bold typeface are new species described here.

Species	Specimen	Whole mount (NHMUK)	Nuclear genes		Mitochondrial genes	
			18S	28S	16S	COI
<b>Outgroup species</b>						
<i>Stenostomum</i> sp.	<sup>a</sup> Concatenated		FJ384811	FJ384850	KP308283	KP308282
<i>Paracatenula</i> sp.	<sup>b</sup> IZ 29193		KC869782	KC869835	KC869743	–
<i>Hoploplana californica</i>	<sup>b</sup> DNA106152		KC869797	KC869850	KC869753	–
<i>Paraplanocera oligoglena</i>	<sup>b</sup> IZ 29194		KC869796	KC869849	KC869752	–
<i>Boninia divae</i>	<sup>b</sup> DNA105955		KC869793	KC869846	KC869751	–
<b>Ingroup species</b>						
<i>Haplopharynx rostratus</i>	MTP LS 591		KP730509	KP730527	KP730474	KP730579
<i>Haplopharynx papii</i>	MTP LS 720	2015.1.30.1	KP730502	KP730534	KP730457	–
* <i>Haplopharynx papii</i>	MTP LS 709	2015.1.30.2	–	–	–	–
* <i>Haplopharynx papii</i>	MTP LS 724	2015.1.30.3	–	–	–	–
<i>Haplopharynx</i> cf. <i>quadristimulus</i> sp. C	<sup>c</sup> DNA106018		KC869787	KC869840	–	–
<i>Myozona lutheri</i>	MTP LS 692		KP730491	KP730521	KP730466	KP730582
* <i>Myozona lutheri</i>	MTP LS 690		–	KP730528	–	–
<i>Myozona</i> sp.	<sup>c</sup> MTP LS 667		–	KP730538	–	–
<i>Myozona</i> sp.	<sup>d</sup> MTP LS 731		KP730501	–	–	–
<b><i>Bradynectes</i> cf. <i>robinhoodensis</i></b>	MTP LS 180	2015.1.30.4	FJ715298	FJ715318	–	–
<i>Bradynectes sterreri</i>	MTP LS 162	2015.1.30.5	KP730507	KP730535	KP730456	KP730564
* <i>Bradynectes sterreri</i>	MTP LS 165	2015.1.30.6	KP730515	–	–	–
* <i>Bradynectes sterreri</i>	MTP LS 164	2015.1.30.7	–	–	–	–
* <i>Bradynectes sterreri</i>	MTP LS 167	2015.1.30.8	–	–	–	–
<i>Paromalostomum minutum</i>	MTP LS 555	2015.1.30.9	KP730511	KP730532	KP730460	–
<i>Paromalostomum</i> cf. <i>minutum</i>	MTP LS 696		KP730512	KP730542	KP730469	KP730581
<i>Paromalostomum dubium</i>	MTP LS 124		KP730489	KP730548	KP730463	KP730563
<i>Paromalostomum massiliensis</i>	MTP LS 708	2015.1.30.10	KP730506	KP730533	KP730479	KP730578
<i>Paromalostomum fuscum</i>	MTP LS 119		KP730504	KP730517	–	–
<b><i>Austromacrostromum arumoidicornum</i></b>	MTP LS 638	2015.1.30.11	KP730495	KP730541	KP730472	KP730571
* <b><i>Austromacrostromum arumoidicornum</i></b>	MTP LS 639	2015.1.30.12	–	–	–	–
* <b><i>Austromacrostromum arumoidicornum</i></b>	MTP LS 640	2015.1.30.13	–	–	–	–
<i>Cylindromacrostromum riegeri</i>	MTP LS 546	2015.1.30.14	KP730485	KP730544	KP730465	KP730583
<i>Cylindromacrostromum</i> cf. <i>notandum</i>	MTP LS 250		KP730488	KP730536	KP730476	KP730560
<i>Dolichomacrostromum uniporum</i>	<sup>e</sup> MTP LS 222		FJ715295	FJ715315	KP730478	–
<i>Myozonaria bistylifera</i>	MTP LS 632	2015.1.30.15	KP730486	KP730518	–	KP730573
<i>Myozonaria bistylifera</i>	MTP LS 671	2015.1.30.16	KP730510	KP730546	–	KP730584
<b><i>Myozonaria fissipara</i></b>	MTP LS 678	2015.1.30.17	KP730497	KP730519	KP730464	KP730574
* <b><i>Myozonaria fissipara</i></b>	MTP LS 623		KP730498	KP730526	KP730451	KP730575
* <b><i>Myozonaria fissipara</i></b>	MTP LS 683		–	KP730531	KP730452	KP730562
* <b><i>Myozonaria fissipara</i></b>	MTP LS 653		KP730482	KP730553	KP730471	KP730566
* <b><i>Myozonaria fissipara</i></b>	MTP LS 651		KP730492	KP730522	KP730447	KP730577
<i>Myozonariinae</i> sp.	MTP LS 669		KP730503	KP730552	KP730473	KP730572
* <i>Myozonariinae</i> sp.	MTP LS 673		–	KP730525	KP730454	KP730569
<b><i>Myomacrostromum rubrioculum</i></b>	MTP LS 670		KP730499	KP730551	KP730450	–
* <b><i>Myomacrostromum rubrioculum</i></b>	MTP LS 689	2015.1.30.18	–	KP730529	–	–
<i>Karlingia lutheri</i>	MTP LS 806	2015.1.30.19	KP730490	KP730545	KP730455	KP730559
* <i>Karlingia lutheri</i>	MTP LS 780	2015.1.30.20	–	KP730516	KP730446	–
<i>Acanthomacrostromum</i> sp.	<sup>b</sup> DNA105907		KC869788	KC869841	KC869748	–
<i>Microstromum lineare</i>	MTP LS 394		KP730484	KP730557	–	KP730567
* <i>Microstromum lineare</i>	MTP LS 356		–	KP730520	–	–
<i>Microstromum lineare</i>	<sup>b</sup> DNA105906		KC869791	KC869844	KC869750	–
<i>Microstromum papillosum</i>	MTP LS 146		FJ715296	FJ715316	KP730453	KP730570
* <i>Microstromum papillosum</i>	MTP LS 159		–	–	–	–
* <i>Microstromum papillosum</i>	MTP LS 160a		–	–	–	–
* <i>Microstromum papillosum</i>	MTP LS 160b		–	–	–	–
<i>Microstromum</i> sp. A	MTP LS 700	2015.1.30.21	KP730494	KP730554	KP730470	–
<i>Microstromum</i> sp. B	MTP LS 524	2015.1.30.22	KP730505	KP730547	–	KP730580
<i>Microstromum</i> sp. C	MTP LS 660		KP730483	KP730523	KP730462	–
* <i>Microstromum</i> sp. C	MTP LS 635		KP730493	KP730556	KP730480	–
<i>Microstromum</i> sp. D	MTP LS 666		KP730487	KP730537	KP730448	KP730576
<i>Psammomacrostromum</i> nov. sp. 1	MTP LS 380		KP730508	KP730539	–	–
* <i>Psammomacrostromum</i> nov. sp. 1	MTP LS 384		–	–	–	–
* <i>Psammomacrostromum</i> nov. sp. 1	MTP LS 385		–	–	–	–
* <i>Psammomacrostromum</i> nov. sp. 1	MTP LS 422		–	–	–	–
* <i>Psammomacrostromum</i> nov. sp. 1	MTP LS 481		–	–	–	–
<i>Psammomacrostromum</i> nov. sp. 6	MTP LS 194		–	KP730524	–	–
<i>Psammomacrostromum</i> nov. sp. 2	MTP LS 309		KP730481	KP730543	KP730445	–
<i>Psammomacrostromum</i> nov. sp. 3	MTP LS 624		KP730500	KP730530	KP730458	–
<i>Psammomacrostromum</i> nov. sp. 4	MTP LS 633		KP730514	KP730555	KP730459	–

Table 1 (continued)

Species	Specimen	Whole mount (NHMUK)	Nuclear genes		Mitochondrial genes	
			18S	28S	16S	COI
<i>Psammomacrostomum</i> nov. sp. 5	MTP LS 719		KP730513	KP730540	KP730461	KP730585
<i>Macrostomum pusillum</i>	<sup>a</sup> MTP LS 112		FJ715313	FJ715333	KP730449	KP730558
<i>Macrostomum spirale</i>	<sup>a</sup> MTP LS 227		FJ715308	FJ715328	KP730475	KP730565
<i>Macrostomum tuba</i>	MTP LS 586	2015.1.30.23	KP730496	KP730549	KP730468	KP730586
<i>Macrostomum hystrix</i>	<sup>a</sup> MTP LS 68		FJ715303	FJ715323	KP730477	KP730561
<i>Macrostomum lignano</i>	<sup>a</sup> MTP LS 244		FJ715306	FJ715326	KP730467	KP730568

<sup>a</sup> To obtain a complete *Stenostomum* sp. dataset for all four genes a concatenation was made between the 18S and 28S rRNA genes of *Stenostomum* sp. 'island' (specimen K04:81) of Larsson and Jondelius (2008) and the COI and the 16S rRNA genes extracted from a mitochondrial genome of another *Stenostomum* species (T. Littlewood, unpublished data).

<sup>b</sup> From Laumer and Giribet (2014).

<sup>c</sup> From Laumer and Giribet (2014), who list this species as *Haplopharynx* sp.

<sup>d</sup> These two specimens of *Myozona* sp. were concatenated in the phylogenetic analysis.

<sup>e</sup> From Schärer et al. (2011).

Table 1 and the “Taxonomic notes” section for details on specimens). After documentation, most specimens were cut with a scalpel, their anterior portion preserved in absolute ethanol for DNA extraction, and their posterior portion, containing the genitalia, ruptured to document sperm morphology (Janicke and Schärer, 2010), and, whenever possible, fixed in lactophenol to serve as a permanent preparation of the sclerotized parts of the male genitalia (Schockaert, 1996), and deposited at the Natural History Museum London (Table 1).

## 2.2. Extraction, PCR amplification and sequencing

Genomic DNA was extracted from either the anterior portion or the complete specimen using the DNeasy tissue kit (QIAGEN). PCR reactions were performed in 25 µl with illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare) (Table A shows primers and PCR conditions). Quality and size of PCR products (2 µl) were verified on a GelRed™-stained 1% agarose gel. If multiple fragments were electrophoretically separated, the correctly sized band was cut out and purified using the QIAquick Gel Extraction Kit (QIAGEN). Sanger sequencing of purified PCR products was carried out by Microsynth AG (Balgach, Switzerland) using an ABI 3730XL sequencer in forward and reverse directions. Consensus sequences were assembled using CodonCode Aligner (3.7.1 CodonCode Corporation). All contigs were subjected to BLAST searches (<http://www.ncbi.nlm.nih.gov>) to check for possible contaminations.

We use a multi-gene approach combining nuclear ribosomal RNA genes and, using newly developed markers, mitochondrial genes (Table A). The complete 18S (~1700 bp) and partial 28S ribosomal RNA genes (~1100 bp) have been useful in resolving the phylogeny of these and other Platyhelminthes (e.g. Bagueña and Riutort, 2004; Tessens et al., 2014; Schärer et al., 2011). We further add the “Folmer” region of the mitochondrial cytochrome *c* oxidase subunit 1 gene, COI (709 bp) widely used in species barcoding (primer sequences modified from Folmer et al., 1994) and a second mitochondrial gene, partial 16S rRNA (463 bp) (primer sequences modified from Xiong and Kocher, 1991).

## 2.3. Molecular analysis

Multiple sequence alignments of 16S, 18S and 28S were made using the Q-INS-i algorithm in MAFFT 7.157 (Katoh and Standley, 2013), which accounts for rRNA secondary structure. COI sequences were translated using the TranslatorX web server (Abascal et al., 2010), using the rhabditophoran genetic code (Telford et al., 2000), and the nucleotides aligned according to an amino acid alignment constructed using MAFFT. Post alignment trimming was done with the parametric profiling method of

ALISCORE 2.2 (Misof and Misof, 2009). Gaps were treated as ambiguous characters to control for missing data and the default sliding window was used. The best fitting substitution model was estimated using the Akaike Information Criterion in jModelTest 2.1.2 (Darriba et al., 2012). Alignments were concatenated with Geneious R6 (Biomatters; <http://www.Geneious.com>).

Phylogenetic analyses were conducted using Bayesian and maximum likelihood methods. Bayesian analyses were carried out using the parallel version of MrBayes 3.2.1 (Ronquist and Huelsenbeck, 2003) on the STEVIN Supercomputer Infrastructure at Ghent University (Department EWI). Two analyses were done using the standard priors and the GTR + I + G model using three heated (temp = 0.2) and one cold chain per analysis. Gaps were treated as missing data and all genes were treated as different partitions. Model parameters were calculated independently for each gene partition. Analyses were run for 25 million generations, sampling trees every 500th generation. Run convergence was assessed using standard deviation of split frequencies and PSRF (Potential Scale Reduction Factors). Burn-in was arbitrarily chosen to be 25% of the results, and evaluated using a generation/Log-likelihood scatterplot.

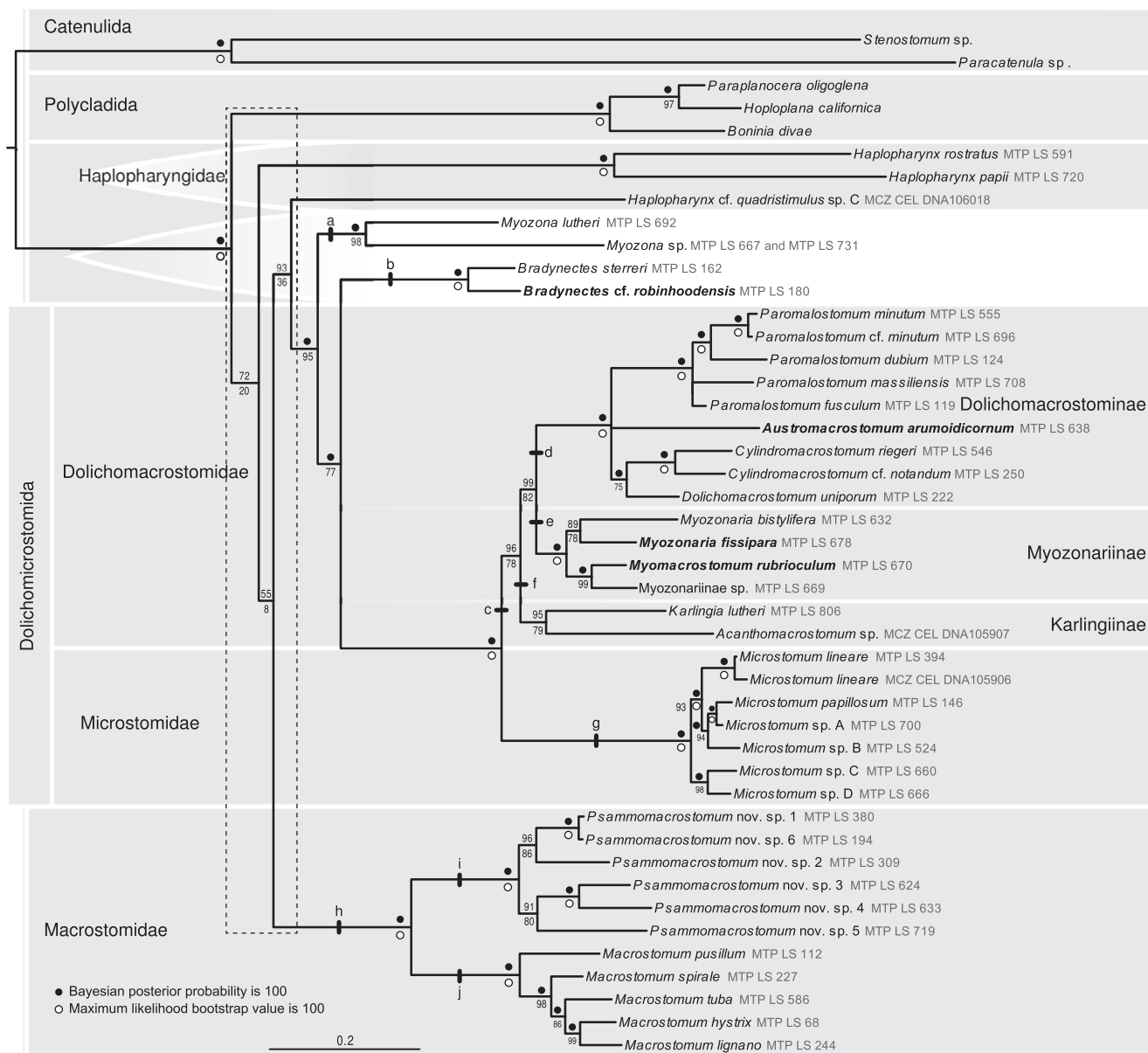
Maximum likelihood analyses were performed using RAXML 7.0.4 (Stamatakis, 2006) with 5000 bootstrap replicates under the GTR + I + G model, again treating every gene as a separate partition. Maximum likelihood based ancestral state reconstructions (ASRs) were performed in Mesquite (Maddison and Maddison, 2014) on the rooted bootstrap replicates of the maximum likelihood analysis.

## 3. Results and discussion

### 3.1. Dataset and phylogenetic inference

The concatenated alignment of the four genes included 45 specimens, had a total length of 4609 bp, and was reduced to 3894 bp after post-alignment trimming of ambiguously aligned positions. The GTR + I + G substitution model fitted best all gene partitions except for the 16S gene, where it was second after the TIM2 + I + G model. As the topologies of the Bayesian and maximum likelihood consensus trees were congruent, we summarized the bootstrap values from the maximum likelihood analysis and the posterior probabilities on the Bayesian majority rule consensus tree (Fig. 1). This topology is supported by both mitochondrial and ribosomal gene fragments, as revealed by their single gene trees (data not shown). Due to difficulties in COI amplification for certain macrostomorph clades (genera *Psammomacrostomum* and *Haplopharynx*), we used the partial 28S rDNA as a barcode gene. In doing so, we follow the argument of Vanhove et al. (2013) that





**Fig. 1.** Molecular phylogeny of the Macrostomorpha. Consensus tree based on the combined 18S and 28S nuclear sequences, and the 16S and COI mitochondrial sequences (for a total of 4609 bp) from 40 Macrostomorpha covering all major groups and most genera, and five outgroup species. Values above branches are Bayesian posterior probabilities (filled dots when 100) and values below branches are ML bootstrap values (open dots when 100). Note that the stippled box indicates an unresolved basal polytomy. The topologies of the trees derived from Bayesian and ML analyses are in agreement, as are the individual gene trees (not shown). The accession code after the species name identifies the morphological documentation of each sequenced specimen (a hologenophore voucher), which we have deposited as a digital morphological voucher at <http://macrostomorpha.info> and <http://dx.doi.org/10.5061/dryad.b5908> (MTP LS 'accession code') GenBank accession numbers are given in Table 1. For details on phylogenetic reconstruction see Materials and Methods. Taxa in bold typeface represent newly described species. The bars with letters indicate important characters: (a) sperm stored in muscular constriction at the end of the gut; (b) no vagina, large male atrium; (c) common genital pore, bursal organ (exception *Myomacrostomum* and *Myozonariinae* sp.); (d) no caudal gut, no muscle ring, typical fused penis and accessory stylet; (e) muscle ring and presence of caudal sensory organ; (f) no caudal gut, no eyes, no muscle ring; (g) ciliary pits, permanent preoral gut extension; (h) paired ovaries and paired testes; (i) copulatory cirrus; (j) posterior pointing penis stylet.

28S rDNA is equally useful in providing a first phylogenetic placement of flatworm species. Within the Macrostomorpha, both genes provide good phylogenetic resolution as 51% of the 28S and 62% of the COI regions are parsimony informative.

### 3.2. Macrostomorpha: monophyly and support of the main subgroups

While our phylogenetic analysis does not lend much statistical support for the Macrostomorpha as a monophyletic grouping, it confirms its close association with the Polycladida (Fig. 1; see also Baguña and Riutort, 2004; Larsson and Jondelius, 2008; Laumer and Giribet, 2014; Egger et al., 2015; Laumer et al., 2015). This tree

topology was not affected by adding additional Rhabditophora clades as outgroups (data not shown). The lack of phylogenetic resolution at the base of the tree reflects the difficulty of recovering the relationships at the base of the Platyhelminthes in other studies using a similar set of marker genes (Baguña and Riutort, 2004; Laumer and Giribet, 2014). And while a phylogenomic approach, as recently applied to a broader set of Platyhelminthes (Egger et al., 2015; Laumer et al., 2015), might potentially resolve these deep splits, this was beyond the scope of our study. We obtain support for several clades within the Macrostomorpha, namely the Macrostomidae (excluding *Myozona* and *Bradynectes*), the Microstomidae and the Dolichomacrostomidae. However, we

recover neither the Haplopharyngida nor the Macrostomida. In the following we discuss the findings for these clades in some depth.

### 3.2.1. Haplopharyngida

The Haplopharyngida currently include a single genus comprising three valid species, *Haplopharynx rostratus* Meixner 1938, *H. papii* Schockaert 2014 and *H. quadristimulus* Ax 1971. Our re-examination of the available data for the latter species suggests that it actually comprises four distinct species: *H. quadristimulus* sensu stricto Ax 1971, *H. cf. quadristimulus* sp. A (Rieger, 1977), *H. cf. quadristimulus* sp. B (Rieger, 1977; Doe, 1986a,b) and *H. cf. quadristimulus* sp. C (Laumer and Giribet, 2014) (see Taxonomic notes for details).

The genus *Haplopharynx* is characterized by several morphological apomorphies, namely a posteriorly located anus, a protrusible proboscis and a female gonopore posterior to the male pore. The genus displays a huge genetic variation, with *H. cf. quadristimulus* sp. C 16.4% and 16% divergent from *H. papii* and *H. rostratus*, respectively. Even the monophyletic *H. rostratus* and *H. papii* have a very deep split (15.5% divergence), considerably exceeding the observed variation in other macrostomid families. This genetic variation is reflected in considerable morphological differences in the copulatory organ between *H. rostratus* and the *H. quadristimulus* species group (Ax, 1971; Rieger, 1977) and in variation of the gland morphology associated with the proboscis between *H. papii* and *H. rostratus* (Ax, 1971; Schockaert, 2014). Due to this and the uncertain phylogenetic position of *H. cf. quadristimulus* sp. C, we think additional species and genetic markers are necessary to properly evaluate the monophyly of the genus *Haplopharynx* and its important early branching position within the Macrostomorpha.

### 3.2.2. Macrostomidae

We recover two well-supported sister clades within the Macrostomidae, the genera *Macrostomum* (always bearing a stylet) and *Psammomacrostomum* (bearing a muscular cirrus). The type species of the latter genus, *Psammomacrostomum equicaudum*, was described by Ax (1966). Since then similar genera have been proposed based solely on the presence of an unpaired ovary, *Antromacrostomum* Faubel 1974, and *Siccomacrostomum* Schmidt and Sopot-Ehlers 1976, or unpaired ovary and testis, *Dunwichia* Faubel, Bloome and Cannon 1994. As all our collected specimens had paired testes and ovaries, we consider the proposed six new species as belonging to *Psammomacrostomum* (a detailed taxonomic treatment of this genus will be published separately). Importantly, the gonads may be difficult to observe due to their often small size and sometimes incomplete development. For example, we found a specimen of *Psammomacrostomum* nov. sp. 3 with incomplete development of one testis. Moreover, our *Psammomacrostomum* nov. sp. 6. was collected near the type locality of *Antromacrostomum armatum* and matches its description except by clearly having paired ovaries (see also Taxonomic notes). We therefore think it necessary to re-evaluate the validity of these genera, especially since unpaired gonads could represent an ancestral trait of the Macrostomidae and potentially provide new insights into the evolution of their gonad morphology (see Section 3.3.2).

### 3.2.3. Myozona and Bradynectes

According to our phylogeny, the genera *Bradynectes* Rieger 1971 and the earlier branching *Myozona* Marcus 1949 are sister groups of the Dolichomicrostomida (i.e. Dolichomacrostomidae + Microstomidae; see below) instead of belonging to the Macrostomidae. The genus *Bradynectes* is represented in our analysis by two genetically divergent (3.3%) ‘forms’ of the species *Bradynectes sterreri* Rieger 1971, which we found on the same beach. These forms were erected by Rieger mainly because of

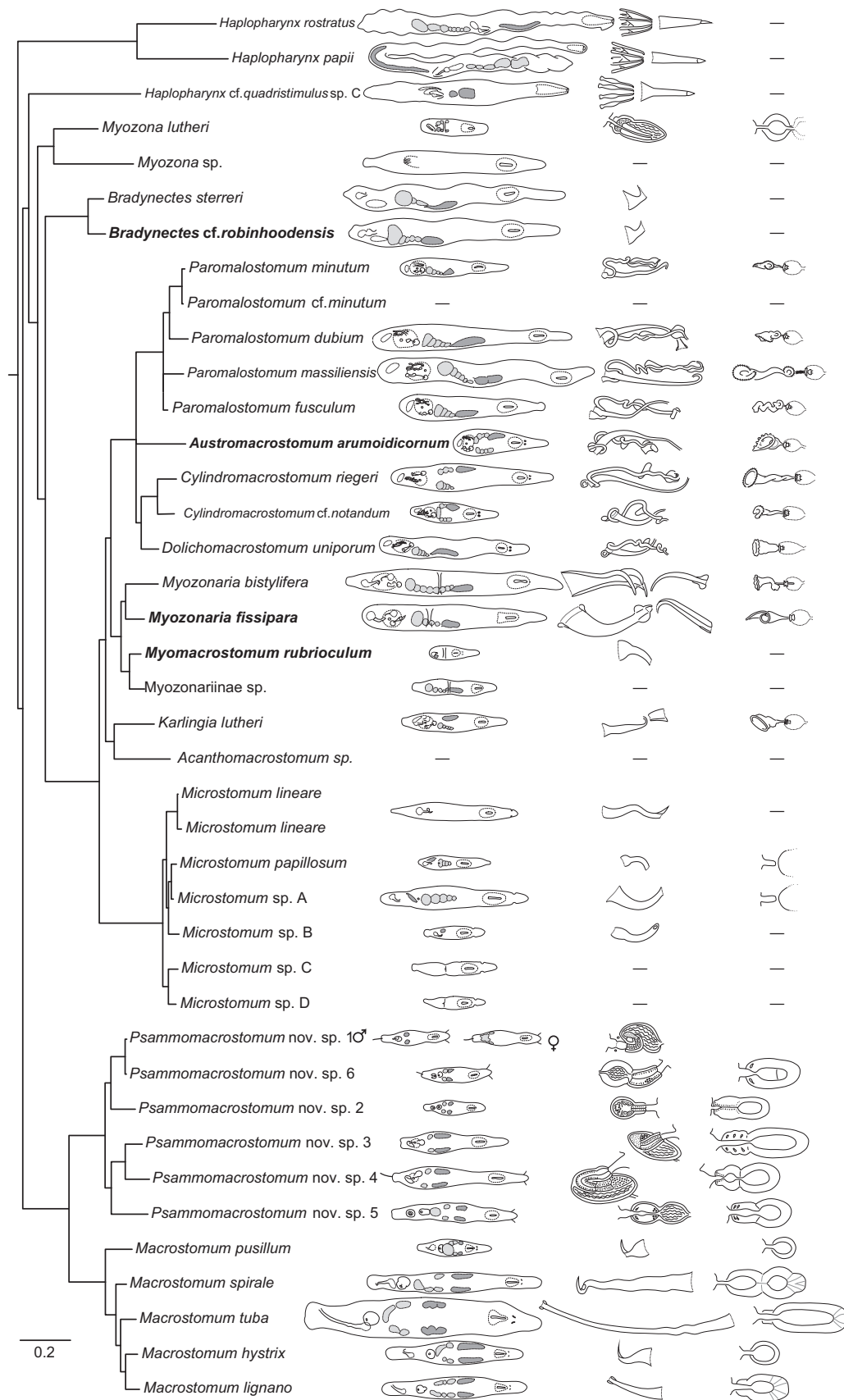
differences in the morphology of the sperm (Fig. 12), and our data confirm the idea that sperm morphology is a useful taxonomic trait in this group. We have therefore moved these different forms to species level, yielding: *Bradynectes sterreri* sensu stricto Kristineberg-form, *B. robinhoodensis* nov. sp. (Robin Hood’s Bay form) and *B. carolinaensis* nov. sp. (Carolina form) (see Taxonomic notes).

The genus *Myozona* is characterized by a genitointestinal connection and, although the two taxa in our analysis are genetically highly divergent (8.1%), it retains a well-supported monophyly. Due to difficulties in amplifying different genetic markers, we concatenated sequences from two specimens of *Myozona* sp., potentially causing an underestimation of the biodiversity of this genus in our study. This genus contains species bearing a cirrus, as our *M. lutheri*, but also stylet-bearing species such as *M. stylifera* Ax 1956, where the morphology of stylet and vesicula granulorum greatly resemble that of *Bradynectes*. Molecular and morphological information of stylet-bearing species may provide a link between these two genera. Furthermore, considering the notable diversity in this genus, which includes a species lacking a clear muscle ring (*Myozona* sp., see below), it is evident that more representatives will be needed before assigning a higher-level taxon to this clade.

### 3.2.4. Dolichomicrostomida

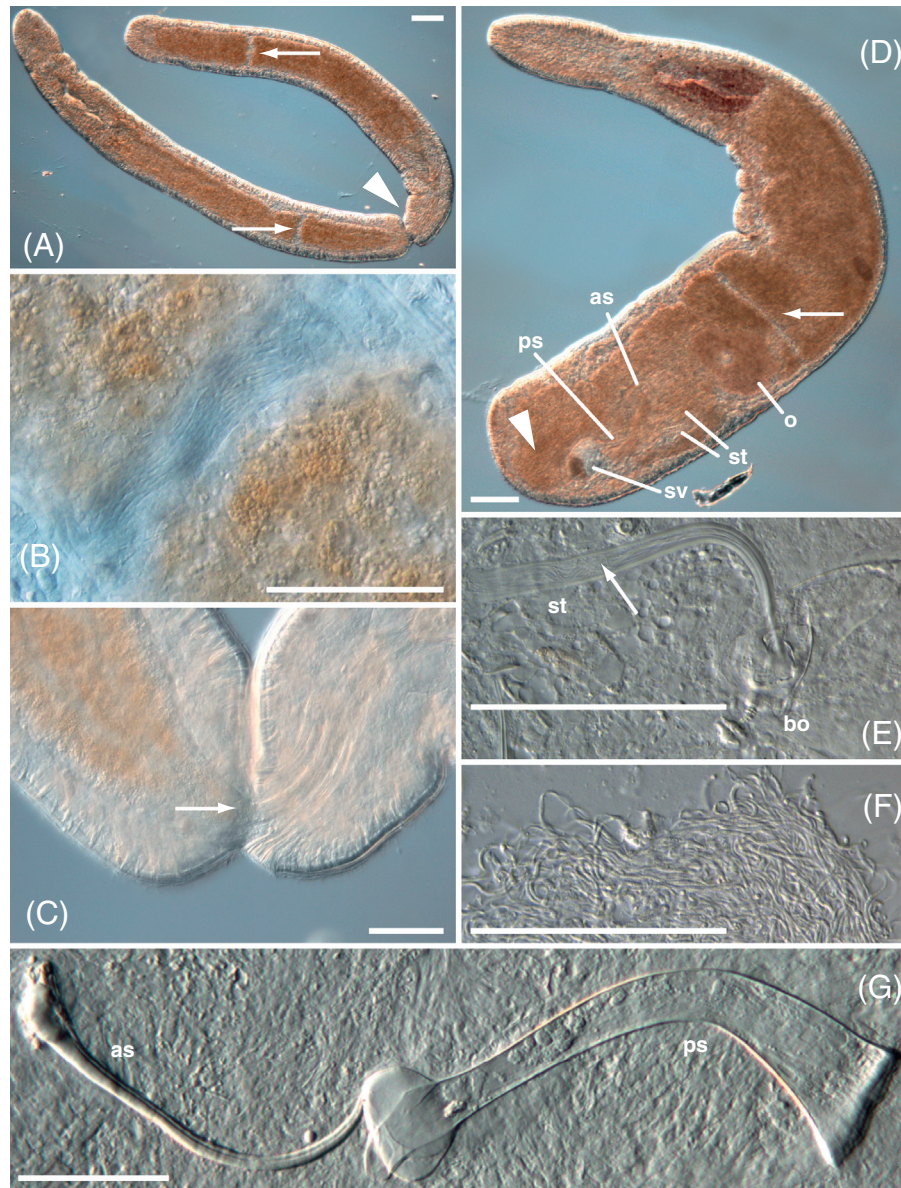
Our results strongly support a monophyletic grouping of the Microstomidae and the Dolichomacrostomidae—a clade we propose to name Dolichomicrostomida—and they do not support the traditional placement of the Microstomidae at the base of the Macrostomorpha. The presence of asexual fissioning was thought to be a basal trait, linking the Microstomidae with the Catenulida, a hypothesis clearly rejected by our ASR (see Section 3.3.1). Tyler (1976), in a comparative study of adhesive gland morphology, concluded, with cautious reservation, that the Microstomidae may be closer to the Macrostomidae, with which they share insunk anchor cells and long papillae, than to the Dolichomacrostomidae, which have a similar branching pattern of the releasing gland neck. He also suggested that the association between rhabdites and adhesive papillae indicated a closer association of Dolichomacrostomidae to *Bradynectes* than to the Microstomidae. Both hypotheses are rejected by our results. Indeed, Rieger (2001) argued that the insunk anchor cells could have evolved convergently, as the anchor cells in the Microstomidae are not insunk as deeply as those in *Macrostomum*, and that the association between rhabdite glands and adhesive papillae is debatable, suggesting a closer relationship between the adhesive glands of the Microstomidae and Dolichomacrostomidae. Likewise, Riedel (1932) grouped Microstomidae and Dolichomacrostomidae, based on similarities in the process of oogenesis. Finally, two rDNA based molecular phylogenies (Littlewood et al., 1999; Litvaitis and Rohde, 1999) also recovered this grouping, albeit with low taxonomic coverage of the Macrostomorpha. Despite the strong molecular evidence for the Dolichomicrostomida there are currently no morphological apomorphies to diagnose this clade.

The monophyletic Microstomidae are represented in our analyses by seven species of the species-rich genus *Microstomum*, which show the typical ciliary pits. Although we find some well-supported clades within the genus, our sampling only covers a small portion of the known biodiversity in this taxon. Also, given the scarcity of morphological characters in these often only asexually fissioning specimens, we have largely refrained from attaching species names to these specimens (see Taxonomic notes). We argue that extensive taxonomic and phylogenetic work, including the microstomid genera *Alaurina* and *Myozonella*, will be required to properly describe this clade’s biodiversity.



**Fig. 2.** Morphological diversity of the Macrostromorpha. Mapped onto the molecular phylogeny (first column) are the overall habitus (second column, anterior to the right) indicating the positions of testis (dark gray), ovary (light gray), male genitalia (shown enlarged in third column), female genitalia (shown enlarged in fourth column), and other characteristic morphologies, such as the proboscis organ (anterior, stippled in *Haplopharynx*), mouth and pharynx (anterior, solid and stippled), muscle ring (arched solid double line), and eyes. Note that these drawings are highly schematic and not intended as taxonomic illustrations. Taxa in bold typeface are newly described here (see Taxonomic notes for more detailed descriptions).





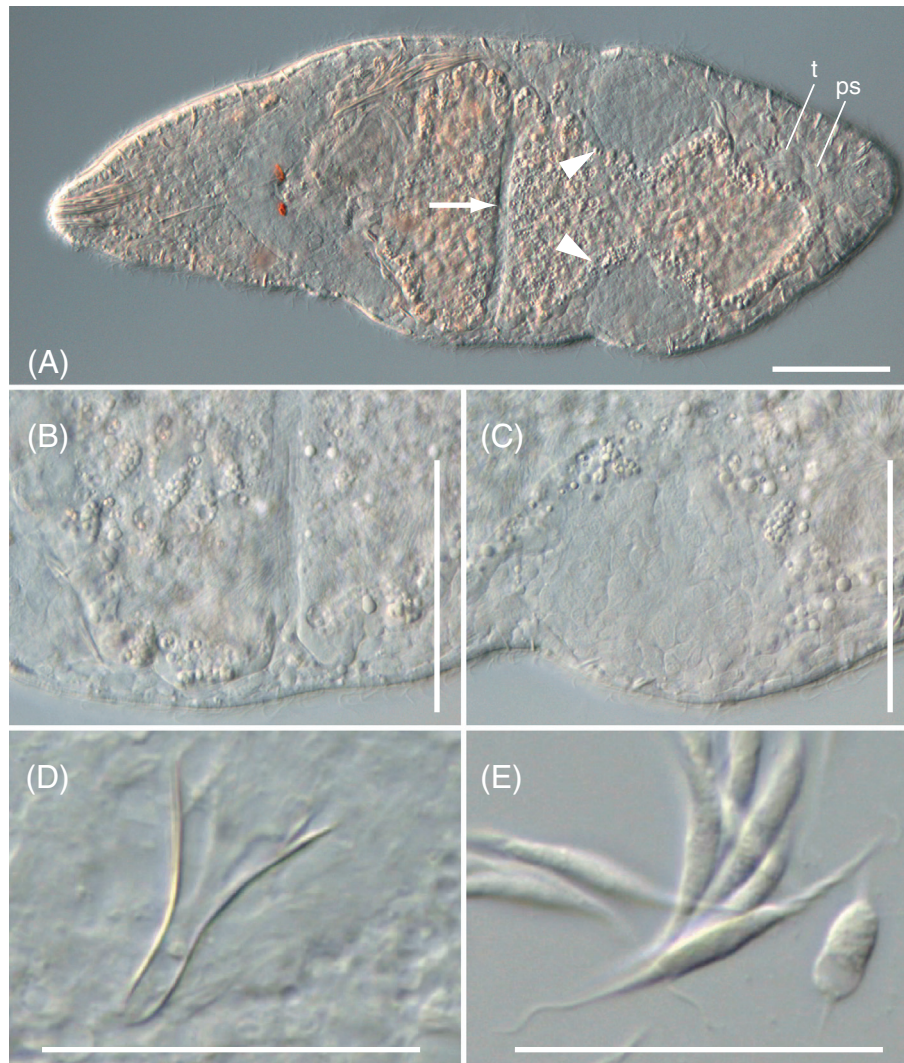
**Fig. 3.** Photomicrographs of *Myozonaria fissipara* nov. sp. (Myozonariinae) (A–C, MTP LS 651, paratype and hologenophore voucher; D–G, MTP LS 678, holotype and hologenophore voucher) (see Fig. 14 for taxonomic drawings). (A) The overall asexual habitus with two zooids in the process of fissioning at a clear fission plane (arrow head), both with clearly visible muscle rings (arrows) and pharynges. (B) Detail of the muscle ring of the anterior zooid showing circular muscle fibers. (C) Detail of the fission plane, clearly showing that the gut is still connected between the zooids (arrow). (D) The overall habitus of a sexual specimen with clearly visible muscle ring (arrow) and reproductive structures, including a mature oocyte (o), penis stylet (ps), accessory stylet (as), seminal vesicle (sv), and sperm tubes (st); note that the gut extends posteriorly (arrow head). (E) Bursal organ (bo) with attached sperm tube (st) filled with sperm (arrow). (F) Sperm cells released from the ruptured seminal vesicle; (G) accessory stylet (as) and penis stylet (ps). Note that panels E–G are from a highly squeezed preparation used to make the deposited permanent lactophenol preparation. Scale bars: 50 µm, except A, D 100 µm.

The sister group of the Microstomidae, the Dolichomacrostomidae, was originally characterized by the presence of a common genital atrium and a sclerotized bursal organ (but see Section 3.2.5). The bursal organ, first observed by de Beauchamp (1927), was defined by Rieger (1971b) as consisting of a mouth-piece (Mundstück), a mid-piece (Mittelstück), and sperm tubes (Spermatuben) often found connected to the mouth-piece through the mid-piece (e.g. Figs. 13 and 14). Rieger (1971b) split the Dolichomacrostomidae into the subfamilies Karlingiinae Rieger 1971 and Dolichomacrostominae Rieger 1971. In our results, the Dolichomacrostominae remains monophyletic, whereas the Karlingiinae (sensu Rieger, 1971) is clearly polyphyletic, with a clade around the genus *Karlingia*, and a second one around *Myozonaria*, for which we erect a new subfamily (Fig. 1; see also Taxonomic notes).

### 3.2.5. Karlingiinae and Myozonariinae nov. subfam

The first clade within the Karlingiinae sensu Rieger 1971, containing *Karlingia lutheri* (Marcus, 1948) and *Acanthomacrostomum* sp. (from Laumer and Giribet, 2014) branches at the base of the Dolichomacrostomidae (Fig. 1). The second clade encompasses Dolichomacrostomidae with a muscle ring, and clusters with the Dolichomacrostominae (Fig. 1). Due to this paraphyly we propose to create two subfamilies, the first clade retains the name Karlingiinae, as it was originally named after the genus *Karlingia* by Rieger (1971b). The Karlingiinae are now diagnosed as Dolichomacrostomidae with unpaired gonads bearing a penis stylet, an accessory stylet and a bursal organ. The gut does not extend caudally over the genitalia. Eyes, a muscle ring, and caudal sensory organs are absent.



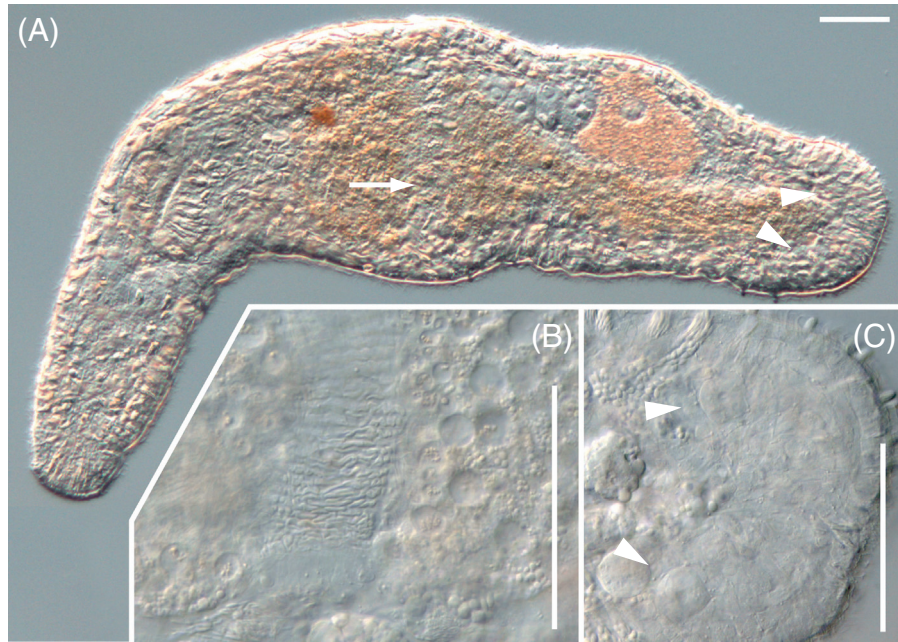


**Fig. 4.** Photomicrographs of *Myomacrostomum rubrioculum* nov. sp. (Myozonariinae) (A–C, MTP LS 670, holotype and hologenophore voucher; D–E, MTP LS 689, paratype and hologenophore voucher) (see Fig. 15 for taxonomic drawings). (A) The overall habitus with the characteristic red eyes, muscle ring (arrow), a forming fission plane (arrow heads), and the location of the testis (t) and penis stylet (ps); (B) detail of the muscle ring showing circular muscle fibers; (C) detail of the tissue during the fission plane formation; (D) simple funnel-shaped penis stylet; (E) spindle-shaped sperm cells. (A–C) Scale bars: 50 µm; (D–E) scale bars: 20 µm.

The second clade we name Myozonariinae nov. subfam., after the genus *Myozonaria*, and it encompasses the Dolichomacrostomidae with muscle rings. Within it we find one poorly resolved clade with two species of the genus *Myozonaria* Rieger 1968 (Fig. 1): *M. bistylifera* Rieger 1968, and a new species, *M. fissipara* nov. sp., which is the first instance of asexual fissioning within this genus (Fig. 3; see Taxonomic notes and Fig. 14). A second, well supported clade contains a new species of the genus *Myomacrostomum* Rieger 1986, *Myomacrostomum rubrioculum* nov. sp. (Fig. 4; see Taxonomic notes and Fig. 15), suggesting that this previously unplaced genus belongs to the Myozonariinae, and does not represent a basal form at the split between Dolichomacrostomidae and Macrostomidae, as previously suggested (Rieger, 1986). We infer the presence of asexual fissioning in this species from the prominent fission planes we observed (Figs. 4 and 15). The second taxon in this clade is an unidentified Myozonariinae sp. with a prominent muscle ring (Fig. 5). Although this species shows some affinities to the genus *Paramyozonaria* Rieger 1971, the lack of genitalia in our specimens precluded further identification. Based on its morphology, we think

*Paramyozonaria* probably belongs to the Myozonariinae, but a careful taxonomic reassessment and phylogenetic placement of this genus is needed to clarify the unclear boundaries between different genera of the Myozonariinae. Particular care should be taken when dealing with the morphology of single specimens, for which molecular identification is indispensable. For example, *Myozonaria* differs from *Paramyozonaria* mainly by having an accessory gland and an accessory stylet, but we found a specimen of *M. bistylifera* entirely lacking both (MTP LS 671), suggesting that such structures can be lost.

A potential apomorphy for the Myozonariinae is the presence of a caudal sensory organ (Fig. 5), which was described as being unpaired in *Myozonaria* and paired in *Paramyozonaria* (Rieger and Tyler, 1974). This hypothesis remains unconfirmed for *Myozonaria ascia* and *Paramyozonaria riegeri*, whose descriptions make no mention of the organ (Sopott-Ehlers and Schmidt, 1974a). Furthermore, the caudal sensory organ may not represent a genus-diagnostic trait, being variable within the genus *Myomacrostomum*. While *Myomacrostomum rubrioculum* and *M. bichaeta* Rieger 1986 have a paired caudal sensory organ, it is



**Fig. 5.** Photomicrographs of Myozonariinae sp. (Myozonariinae) (A, MTP LS 673, hologenophore voucher; B–C, MTP LS 669, hologenophore voucher). (A) Sexual individual with clearly visible muscle ring (arrow) and approximate location of the caudal sensory organs (arrow heads); (B) detail of the muscle ring showing muscle fibers; (C) caudal sensory organs (arrow heads). Scale bars: 50 µm.

unpaired in *M. unichaeta* Rieger 1986, suggesting either trait convergence or paraphyly of the genus *Myomacrostromum*. In species with dense rhabdite glands, such as our *M. bistylifera*, the caudal sensory organ can be difficult to observe *in vivo*, but long ciliary tufts that are often associated with it may be good indicators of its presence (Rieger and Tyler, 1974). In summary, our results suggest that a muscle ring, a caudal sensory organ, and the caudal extension of the gut are diagnostic traits of the Myozonariinae. This would make *Myozonaria arcassonensis* Rieger 1971 an unlikely member of this taxon, as it lacks all three traits. Although these morphological features suggest close ties to the genus *Karlingia*, a genetic characterization of this species and a denser taxon sampling of the Myozonariinae would be desirable to confirm this hypothesis.

### 3.2.6. Dolichomacrostominae

We recover two well-supported clades and one unresolved taxon within the Dolichomacrostominae (Fig. 1). The first clade includes the monotypic *Dolichomacrostromum uniporum* Luther 1947 and two species of *Cylindromacrostromum* nom. nud. Rieger 1971: *Cylindromacrostromum mediterraneum* (Ax, 1955) (originally described as *Paromalostomum mediterraneum*), and *Cylindromacrostromum* cf. *notandum* (which closely resembles the species description of *Paromalostomum notandum* Ax 1951). The second clade includes five representatives of the genus *Paromalostomum* Ax 1951 (Fig. 1). The phylogenetic position of *Austromacrostromum arumoidicorum* nov. sp. (see Taxonomic notes and Fig. 13), belonging to the genus *Austromacrostromum* nom. nud. Rieger 1971 (previously containing only a single species originally described as *Dolichomacrostromum mortenseni* Marcus 1950) remains unresolved. Our phylogeny lends support to the monophyly of *Cylindromacrostromum* and possibly a separate placement of *Austromacrostromum*, lending support to Rieger's intended classification. We provide updated diagnoses for these two and all other genera within the Dolichomacrostominae (Table B). Note that in doing so we have placed greater emphasis on traits that can be

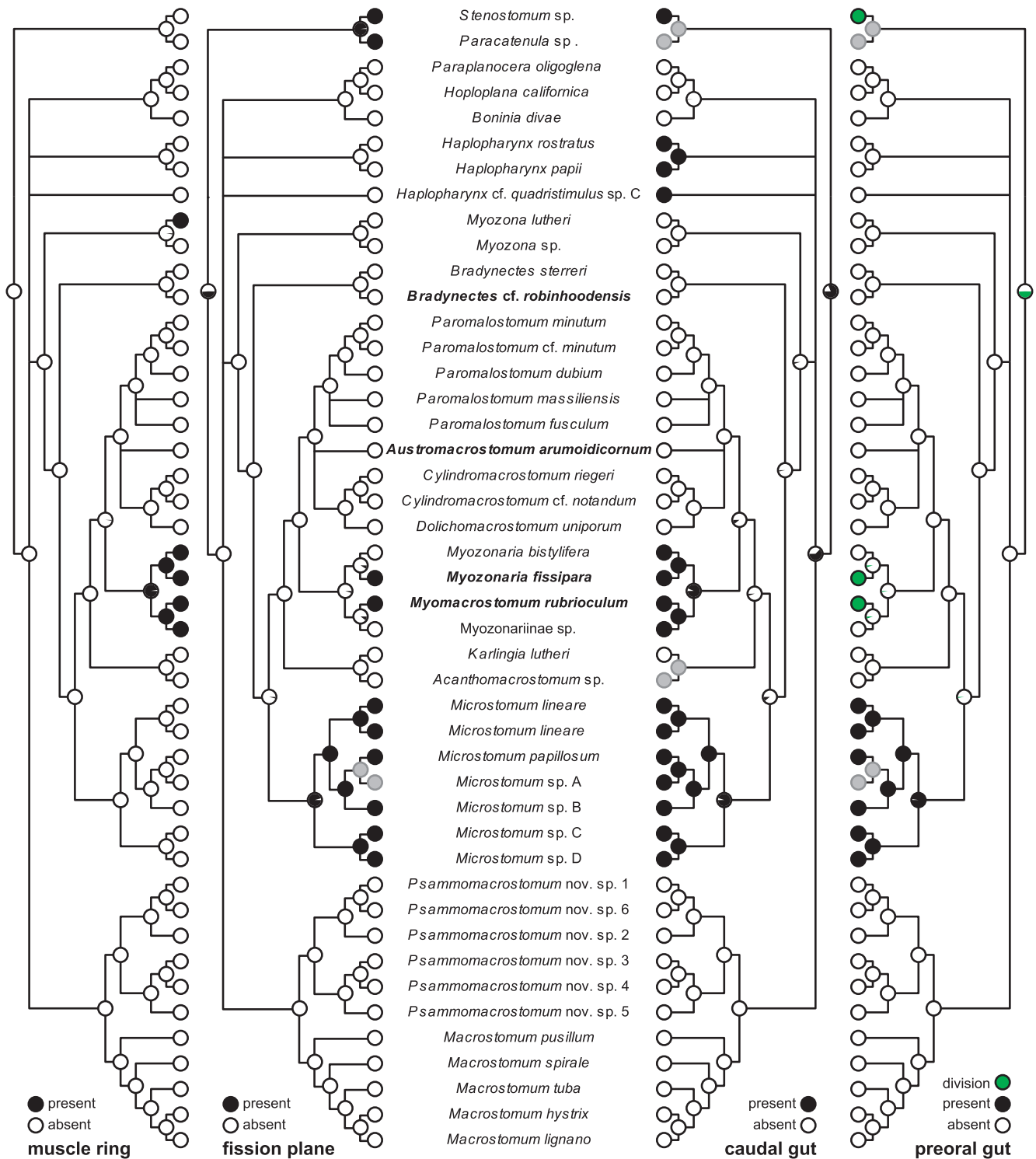
observed *in vivo*, in accordance to the stated aims of our article. An unpaired ovary, the typical stylet and the absence of eyes seem to be good characters to distinguish *Paromalostomum*. Although also included by Rieger (1971c) as a diagnostic trait for that genus, the absence of eyes may not have much diagnostic power, given its variable nature among other macrostomorphans, for example within the genus *Macrostromum* (L. Schärer, pers. obs.). Our analysis lacks representatives of the genera *Meiochaeta* Rieger 1971 (misspelled by Rieger 1971c as *Meiochaeta* in two occasions), *Megamorion* Rieger and Sterrer 1968, and *Paramacrostromum* Riedel 1932, which, based on morphology, belong to the Dolichomacrostominae (Rieger, 1971c), but whose phylogenetic placement still remains unclear.

### 3.3. Ancestral state reconstructions

#### 3.3.1. Evolution of asexual fissioning

Our ASR suggests at least two separate origins of asexual fissioning within the Macrostromorpha, based on the presence of fission planes (Fig. 6), namely at the base of the Microstomidae and in the Myozonariinae. Our ASRs also suggest that asexual fissioning might be linked to gut morphology (Fig. 6), as all observed Macrostromorpha with fission planes also show a gut that extends caudally over the genitalia (Fig. 6). This caudal gut is probably a remnant of the intestinal connection between adjoining zooids (e.g. Fig. 3), and could thus be an indicator of asexual fissioning. Accordingly, *Myozonaria bistylifera* (this study, and Rieger, 1968, p. 291) and Myozonariinae sp. (Fig. 5), which both have a caudal gut, may also be capable of asexual fissioning. The same may apply to *Myozonaria jenneri* Rieger and Tyler 1974, *Paramyozonaria riegeri* Sopott-Ehlers and Schmidt 1974a and *Paramyozonaria bermudensis* Rieger 1971, suggesting that asexual fissioning could be a common strategy among the Myozonariinae. Importantly, the absence of a caudal gut does not necessarily imply an incapability of asexual fissioning. For instance, Rieger (1986) draws the sexually mature zooid of the fissioning *Myomacrostromum unichaeta* without a



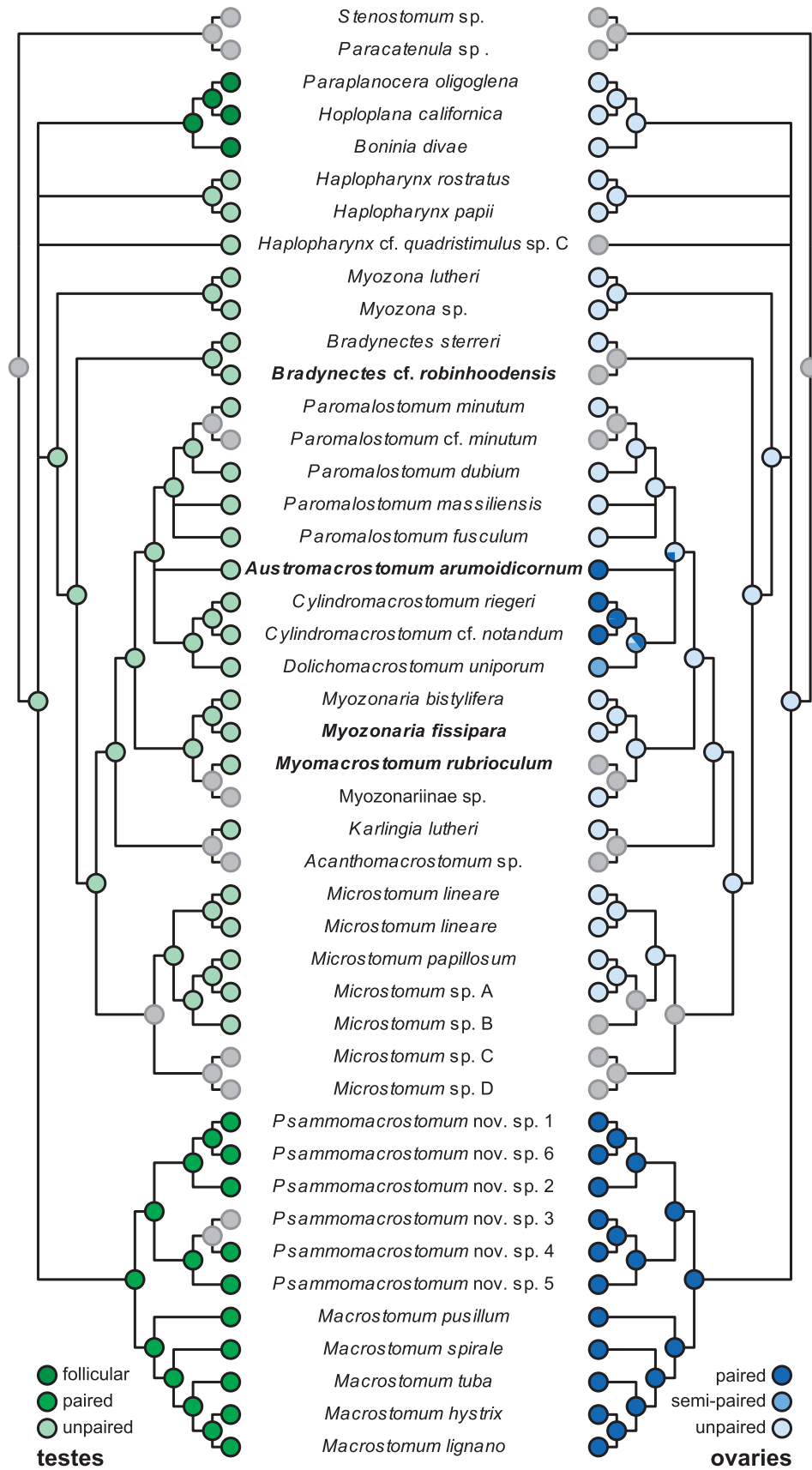


**Fig. 6.** Maximum likelihood based ancestral state reconstructions (ASRs) of four characters that have been linked to asexual fissioning. Pie charts on internal nodes indicate the likelihoods of the different character states at each node and gray nodes indicate equivocal or unknown character states. From left to right, prominent muscle ring on the gut (present/absent); evidence of a fission plane (present/absent); caudal gut (present/absent); preoral gut (present only during division/present/absent).

caudal gut, suggesting it could eventually disappear after fission. Note that the caudal gut in *Haplopharynx* (Fig. 6) is due to the posteriorly located anus (Karling, 1965). Equivalently, in the posterior zooids the gut connection is anterior to the brain (e.g. Fig. 3), potentially leading to a permanent intestinal extension in species with asexual fissioning, a trait we indeed find in most species of *Microstomum*, but not in the *Myozonariinae* (preoral gut, Fig. 6).

A third possible sign of asexual fissioning is the presence of a ring of muscle fibers around the gut, which may originate from, or even function as, a fission plane (Rieger, 1986, 2001). In our study, this muscle ring is present in all species of the *Myozonariinae*, some of which are fissioning, and our ASR suggests that it convergently evolved in the genus *Myozona*, where no evidence of fissioning has been observed (Fig. 6). We found





**Fig. 7.** Maximum likelihood based ancestral state reconstructions (ASRs) of the gonads. Pie charts on internal nodes indicate the likelihoods of the different character states at each node and gray nodes indicate equivocal or unknown character states. On the left side, testis (follicular/paired/unpaired); on the right side, ovary (paired/semipaired/unpaired). Note that follicular testes only occur outside the Macrostomorpha, and that all unpaired gonads are single gonads.

no muscle ring in our Microstomidae, although they fission (Fig. 6), which seems to be the case for most Microstomidae. An exception may be *Myozonella microstomoides* Beklemishev 1955, which combines a muscle ring with the typical microstomid ciliary pits and asexual fissioning. Exploring its phylogenetic position would thus be very interesting. Moreover, the formation of the fission plane does not appear to coincide with the position of the muscle ring (see e.g. Figs. 3 and 4), so a link between the muscle ring and asexual fissioning currently looks very unlikely. The muscle ring fibers could rather originate from the muscular sheath surrounding the gut, and, for example, be used mechanically to help the digestion of diatoms (Marcus, 1949). Furthermore, all muscle rings are likely not homologous, evolving twice within the Macrostromorpha (or three times, if *Myozonella* is actually a member of Microstomidae), and are also present in distantly related flatworms, e.g. the catenulids *Myostenostomum* Luther 1960 and *Myoretronectes* Noreña-Janssen and Faubel 1996.

The new phylogenetic placement of the Microstomidae and the genus *Myomacrostromum* in our phylogeny, together with the presence of asexual fissioning in *Myozonaria fissipara*, sheds new light on the evolution of asexual fissioning within the Macrostromorpha. The separate origins of asexual fissioning contradict the traditional assumption that asexual fissioning is a basal macrostromorphan reproductive strategy shared with the Catenulida (Rieger, 1986, 2001), and casts additional doubts on the proposed plesiomorphy of asexual reproduction in Platyhelminthes, and even in early divergent Bilateria (Ehlers, 1985; Rieger, 1986). Indeed, Jondelius et al. (2011) found asexual fissioning to be a derived feature in the Acoela, and also within the Tricladida there seem to be multiple origins of asexual fissioning (Riutort et al., 2012; Álvarez-Presas and Riutort, 2014). Interestingly, there seems to be a clear link between the presence of asexual fissioning and the capability of regeneration (Egger et al., 2007), suggesting that the presence of a totipotent neoblast stem cell system may play a crucial role in the evolution of asexual fissioning (Martín-Durán and Egger, 2012).

### 3.3.2. Evolution of testes and ovaries

Our ASRs suggest that the ancestral macrostromorphan reproductive system consisted of unpaired testes and ovaries (Fig. 7). We find that paired ovaries independently evolved twice, once at the base of the Macrostromidae and once within the Dolichomacrostrominae, where they are found in *Cylindromacrostromum* and *Austromacrostromum*, with *Dolichomacrostromum* in a possibly transitional semi-paired state. In contrast, paired testes seem to have evolved only once, at the base of the Macrostromidae (Fig. 7). This contradicts the earlier view of paired gonads being the basal condition (Luther, 1947; Ax, 1951; Rieger, 1971b), where unpaired testes were thought to have evolved via shrinking of one testis, and unpaired ovaries from a fusion between two ovaries (Rieger, 1971b). Our results could, however, be somewhat biased by incomplete taxon sampling, as several species with paired ovaries, likely belonging to the Dolichomacrostromidae, are not represented in our analysis, namely *Paramacrostromum tricladoide* Riedel 1932, *Paramyozonaria simplex*, *Megamorion brevicauda* Rieger and Sterrer 1968, and *Bathymacrostromum spirale* Faubel 1977. Depending on their phylogenetic position these taxa could represent yet another independent evolution of this trait. Likewise, paired testes have been documented for some Microstromidae (e.g. *Microstromum dermatophthalmum* Riedel 1932, *Microstromum bispiralis* Stirewalt 1937, *M. jenseni* Riedel 1932 and *M. hamatum* Westblad 1953), and, if their phylogenetic position can be confirmed, would mean the convergent evolution of

this trait in the Macrostromorpha. In some of these microstromids, one testis appears to develop before the other, making this character state dependent on the age or physiological condition of the worm, and possibly requiring a re-assessment of species described with one or few specimens (see the section Macrostromidae for a similar issue). In conclusion, although the inclusion of currently unrepresented taxa could somewhat alter our results, we think unpaired gonads is the plesiomorphic state in the Macrostromorpha, with convergent evolution of paired ovaries and potentially of paired testes, if the position of the aforementioned microstromids were to be confirmed.

### 3.3.3. Evolution of male genitalia and bursal organ

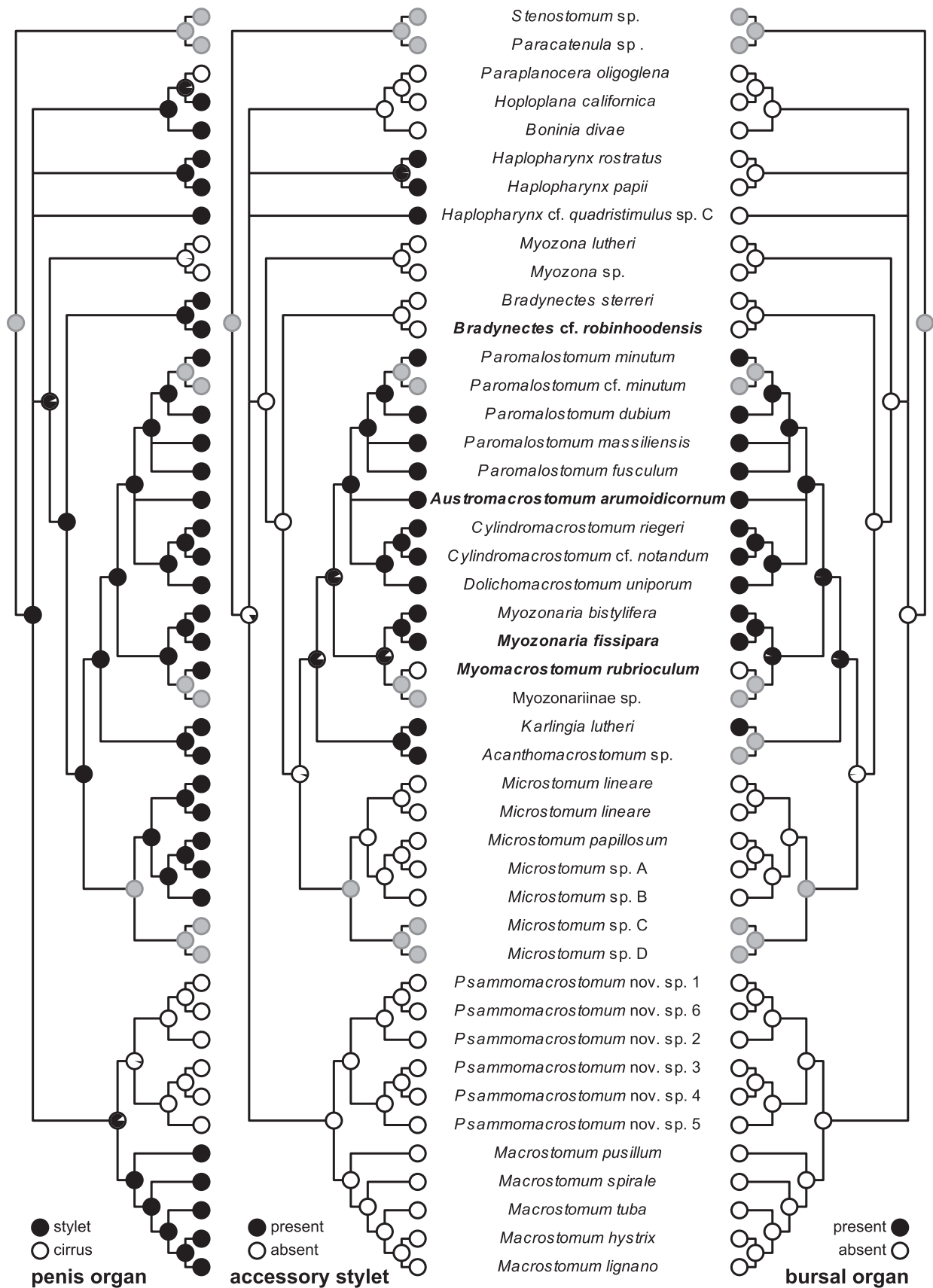
The penis stylet is present in most taxa in our ASR, which suggests it is a plesiomorphy in the Macrostromorpha. It seems to have been replaced by an unarmed muscular cirrus on two separate occasions: in the genus *Myozona* (but recall that *Myozona stylifera* carries a stylet), and at the root of the genus *Psammomacrostromum* (Fig. 8). Our ASR also suggests that penis stylet and cirrus are homologous in the Macrostromorpha. Evolutionary transitions from penis stylets to cirri may indeed occur frequently in other free-living flatworms (e.g. at least three times in the Polycystididae, Tessens et al., 2014), supporting the idea of homology. Interestingly, *Myozona aerumnosa* Sopott-Ehlers and Schmidt 1974b, has a cirrus with two small sclerotized hooks, which might possibly represent an intermediate state.

Despite its plesiomorphy, the location and orientation of the penis stylet diverge substantially between the different groups. It is located centrally in *Haplopharynx*, and, to varying degrees, posteriorly in all other represented taxa. The stylet in *Macrostromum* points posteriorly (Fig. 2), with the vasa deferentia connected directly and anteriorly, whereas in the remaining Macrostromorpha the stylet points anteriorly, with vasa deferentia turning around and connected posteriorly. Interestingly, penis orientation nicely supports the exclusion of *Bradynectes* and *Myozona* from the Macrostromidae (Fig. 2).

In contrast to the suggested homology of the penis, the presence of other male sclerotized structures, namely accessory spines within the Haplopharyngida and accessory glandular stylets in the Dolichomacrostromidae seems to be the result of convergent evolution (Fig. 7). In this context, our phylogeny may support the idea of Rieger (1971b, p. 254) that the large male atrium present in *Bradynectes*—which lacks female genitalia, possibly due to hypodermic insemination, see below—might be linked to the origin of the common genital atrium found in the Dolichomacrostromidae (although both the Microstromidae and *Myomacrostromum* lack a common genital atrium). Interestingly, the evolution of a common genital atrium seems to have led to drastic morphological changes in the genitalia (Fig. 8), namely the evolution of a (female) sclerotized bursal organ (probably a completely new female genital system, see next section) and a (male) accessory glandular stylet (which also appears to be completely new). The glandular stylet is either fused with the penis stylet (Dolichomacrostrominae, for more details see Table B), independent of it (*Myozonariinae* and *Karlingiinae*) or simply absent (*Paramyozonaria*, unfortunately missing from our phylogeny).

### 3.3.4. Evolution of the female reproductive system

The female reproductive system in the Macrostromorpha is even more variable than the male genitalia (Fig. 2). In the simplest case, both a gonopore and a sperm receptacle are missing, as in most, if not all, studied species of *Haplopharynx* and *Bradynectes* (Rieger, 2001). We propose that this simple arrangement could be linked to hypodermic insemination (see next section). In the remaining



**Fig. 8.** Maximum likelihood based ancestral state reconstructions (ASRs) of the male and female genitalia (see Fig. 9 for details on sperm transfer and storage). Pie charts on internal nodes indicate the likelihoods of the different character states at each node and gray nodes indicate equivocal or unknown character states. From left to right, penis organ (stylet/cirrus); accessory stylet (present/absent); bursal organ (present/absent).



taxa we recover the evolution of four different, possibly convergent, female genitalia with, in some cases, highly complex structures to receive, transport and store sperm.

A first type, present in the Macrostromidae, is a female antrum connected to a vagina. The antrum can contain a modified epithelium that serves as an anchoring site for sperm (the cellular valve or ‘Durchgangsapparat’, Luther, 1947) and variably complex muscular constrictions (Fig. 2), and has been shown to become simplified in species with hypodermic insemination (Schärer et al., 2011; see also next section). We suggest that the female systems of *Macrostromum* and *Psammomacrostromum* are likely homologous, both due to their close phylogenetic position and considerable morphological similarities.

A second type of female genitalia is found within *Myozona*, where a duct connects the lumen of the sperm receptacle and that of the gut, often with a muscular sphincter separating both regions (e.g. Marcus, 1949; Papi, 1953; Ax, 1956). In our mature specimen of *Myozona* sp., and in *M. stylifera*, this genitointestinal duct is very wide and the muscle sphincter appears lax and undifferentiated. Despite this sometimes permanent connection, the epithelia of the sperm receptacle and the gut are clearly different (e.g. Marcus, 1949; Papi, 1953; Ax, 1956). Interestingly, the lack of even these simple structures in the neighboring *Bradynectes* suggests the rapid evolution of different sexual strategies. Moreover, a genitointestinal duct has been proposed for *Promacrostromum paradoxum* An-der-Lan 1939, which, although still phylogenetically unplaced, is likely to be more closely related to *Macrostromum* than to *Myozona*, suggesting another example of convergent evolution in sexual traits.

A third type of female genitalia is present within the Microstromidae, consisting of a very simple connection between the ovaries and the female gonopore. This may on one hand be linked to the importance of asexual fissioning as a reproductive strategy in that family, which would reduce the importance of sexually antagonistic coevolution, but also to the presence of hypodermic insemination, at least in some *Macrostromum* species (see next section).

A fourth type is found within the Dolichomacrostromidae, where the ovary is connected to the common genital atrium through a bursal organ (consisting of the mouth- and mid-piece, e.g. Figs. 13C and 14C–E), which often carry structures containing sperm (sperm tubes, e.g. Figs. 13C, D and 14C). While the mouth- and mid-piece are clearly associated with the female reproductive system, the origin of the sperm tubes remains uncertain. Rieger (1971c) favors the idea that the sperm tubes are female-derived. However, the sperm tubes are only temporarily attached to the bursal organ, and can be found in various states of degradation within the genital atrium. This could suggest that a new sperm tube is formed during each copulation, and thus may equally likely be produced by the male system of the sperm donor, like a spermatophore, possibly through the accessory stylet, or involving secretions from the highly complex vesicula granulorum associated with the penis stylet. Until the origin is further clarified we suggest considering the sperm tubes to be a separate structure from the bursal organ. Interestingly, all of this complexity seems to have been secondarily lost in the asexually fissioning *Myomacrostromum* (this study, and Rieger, 2001).

Given this drastic morphological variation, it appears unlikely that these four different types of female genitalia are homologous. The reduction of female genitalia may also have evolved more than once, in *Bradynectes*, *Myozona* and *Myomacrostromum*. Moreover, additional female gonopores have evolved on several different

occasions within the Macrostromorpha, namely once in *Microstromum spiriferum* Westblad 1953, once in *Macrostromum gieysztori* Ferguson 1939 (originally described by Ferguson (1939), but sometimes considered as belonging to *Axia* or *Promacrostromum*; see Schärer et al. (2011) for a detailed discussion) and possibly on a third occasion in *Promacrostromum paradoxum* An-der-Lan 1939.

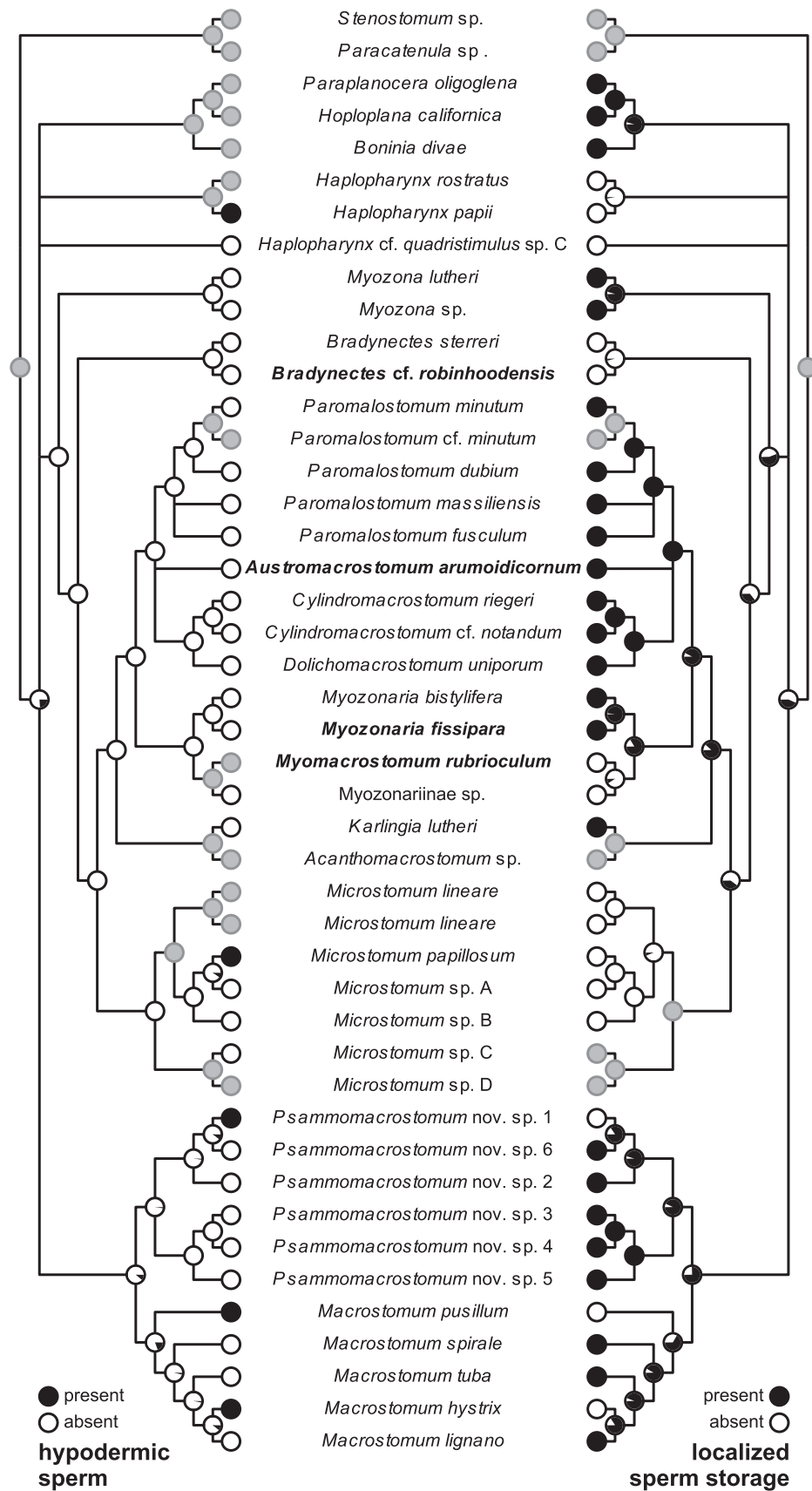
This high rate of evolutionary turnover might be linked to the occurrence of different copulation strategies, i.e. unilateral hypodermic mating vs. reciprocal copulation, and their respective effects on sexually antagonistic coevolution, as already demonstrated within the genus *Macrostromum* (Schärer et al., 2011). We therefore explore this link in some more detail in the next section.

### 3.3.5. Evolution of hypodermic insemination

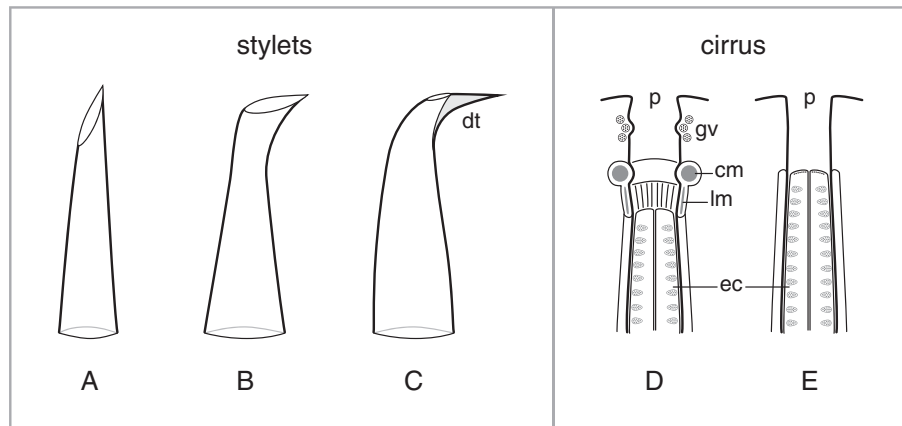
Although direct information on the mating behavior in the Macrostromorpha is currently restricted to *Macrostromum* (Schärer et al., 2004, 2011; Ramm et al., 2012; Marie-Orleach et al., 2013; L. Schärer, pers. obs.) and one *Psammomacrostromum* species (L. Schärer, pers. obs.), we can infer general insemination strategies from the observation of sperm in either localized sperm storage structures, i.e. indicative of copulation, or in the parenchyma, i.e. indicative of hypodermic insemination.

As with *Macrostromum* (Schärer et al., 2011; Ramm et al., 2012), we have found sperm cells in the parenchyma of *Psammomacrostromum* nov. sp. 1 (3 specimens), *Microstromum papillosum* (4 specimens) and *Haplopharynx papii* (3 specimens), suggesting hypodermic insemination in these taxa (see also Taxonomic notes). Consistent with earlier observations in *Macrostromum* (Schärer et al., 2011), the latter two taxa have needle-like stylets (Fig. 10). In *Psammomacrostromum* nov. sp. 1 the cirrus is delimited by an extra circular muscle and a circle of granular vesicles (Fig. 10, clearly visible in MTP LS 481), absent within the other species of *Psammomacrostromum* collected in this study (and for which we found sperm in the female antrum), possibly indicating that these structures might be adaptations related to hypodermic insemination (Fig. 10). Hypodermic insemination with a cirrus has been documented in the acoele *Archaphanostoma agile* (Apelt, 1969; Ax and Apelt, 1969).

Our ASR shows at least five independent origins of hypodermic insemination in the Macrostromorpha (Fig. 9). All species with hypodermic sperm also lack sperm storage organs and have simple female genitalia (Fig. 9), supporting the view that transitions between different copulation strategies play an important role in shaping female genitalia (Schärer et al., 2011). Interestingly, hypodermic insemination within the Macrostromorpha may be more widespread, as, although we have not yet found hypodermic sperm or seen inseminations, *Bradynectes*, *Haplopharynx* and *Myomacrostromum* also have needle-like stylets and lack localized sperm storage organs. More research on the copulation behavior of these and other Macrostromorpha is needed to better evaluate these hypotheses. Interestingly hypodermic mating strategies have evolved in several other clades of Platyhelminthes, including Polycladida (Michiels and Newman, 1998; Lange et al., 2013), Prorhynchida (Laumer, 2015 and references therein), and Monogenea (Macdonald and Caley, 1975; Llewellyn, 1983) suggesting additional origins of this mating strategy. The widespread occurrence of hypodermic mating among Platyhelminthes can be partly explained by the hermaphroditic nature of many Platyhelminthes, as traumatic mating has been proposed to evolve more easily in hermaphroditic taxa (Lange et al., 2013).



**Fig. 9.** Maximum likelihood based ancestral state reconstructions (ASRs) of the nature of sperm transfer and sperm storage. Pie charts on internal nodes indicate the likelihoods of the different character states at each node and gray nodes indicate equivocal or unknown character states. On the left side hypodermic sperm (present/absent); on the right side localized sperm storage (present/absent).



**Fig. 10.** Characteristics of the male penis organs involved in hypodermic insemination in the species studied. The left panel shows general types of hypodermic stylets, all variations of a needle-like design. (A) Straight tube with oblique terminal bevel (e.g. *Haplopharynx*); (B) curved tube with flattened terminal bevel that can either be broadened with respect to the tube (e.g. *Bradynectes*) or of similar diameter (e.g. some *Microstomum* species); (C) curved tube with flattened terminal or subterminal bevel flanked by a sharp distal thickening (dt) (e.g. some *Macrostomum* species, *Microstomum lineare*, *Microstomum papillosum*). The right panel shows (D) a diagram of the cirrus organ found in *Psammomacrostomum* nov. sp. 1., showing the sphincter-like structure of the sheath at the tip of the eversible cirrus (ec), formed by a ring of longitudinal muscles (lm), and crowned by a circular muscle (cm). Close to the gonopore (p), we observe a ring of granular vesicles (gv). Contrast this with (E) the general cirrus organ found in *Psammomacrostomum* nov. sp. 2, which lacks both structures, as do the other species of *Psammomacrostomum* observed in this study.

#### 4. Outlook

In this study we provide the first comprehensive molecular phylogeny of the Macrostomorpha. While we cover a relatively small fraction of the enormous biodiversity within this taxon (i.e. 40 of the about 250 described species, about 15%), the analyzed species include representatives of all major taxa and most genera. As with other microturbellarians, the Macrostomorpha have classically been extremely underrepresented in ecological and biodiversity studies, in part due to the unsuitable fixation methods commonly used in benthic ecology, and because they have been notoriously hard to study, requiring drawings from living specimens and laborious investigations of serially sectioned material. Here we extensively documented field-collected specimens with digital photomicrographic images and videos, a method recently proposed (Ladurner et al., 2005; Schärer et al., 2011), and which we clearly show to be a highly suitable and efficient approach to study intricate anatomical details of this group of free-living flatworms.

Moreover, digital morphological vouchers of each sequenced specimen, available online, provide a permanent link between morphology and molecules. The lack of clearly documented specimens of deposited sequences is a severe shortcoming in current molecular phylogenetic practice (e.g. Pleijel et al., 2008; Astrin et al., 2013). Pleijel et al. (2008) suggests the term 'hologenophore' voucher for "a sample or preparation of the same individual organism as the study organism. Parts are used for the molecular study, while other parts of the same organism are deposited as voucher". For the small and fragile microturbellarians, where a substantial portion or the entire worm is needed to obtain sufficient DNA for molecular analyses, images taken before DNA extraction are the only viable way of producing an informative hologenophore voucher. Whenever multiple specimens from the same physical sample can be collected, one can probably produce 'paragenophore' vouchers, i.e. "an individual organism collected at the same time and place as the study organism, and identified by the author as belonging to the same operational taxonomic unit. The voucher in this case is another individual than the one used for the molecular study". In this framework, classical type material such as serial sections or whole-mount permanent preparations, can be at best 'paragenophore' vouchers, as these specimens cannot also be sequenced. Here we used both types of vouchers, often depositing more than one voucher of each type.

We think that this approach represents a valuable alternative, or at least addition, to traditionally conserved museum specimens (which are often hard to consult and unsuitable for genetic analysis). Although we also deposited permanent lactophenol preparations of the genital hard structures of the newly described species (i.e. true physical holotype and hologenophore vouchers, see Table 1), we think that the level of insight that photomicrographic documentation of the living worm provides is far superior to the static and often distorted view that a permanent preparation can offer. Moreover, the availability of near-live images of field-collected worms allows the extraction of additional unplanned data, for example, on the feeding ecology based on the detailed observation of the gut contents.

We think our approach will greatly facilitate the future exploration of the biodiversity and phylogenetic relationships within this important early branching group of Platyhelminthes (and indeed a whole range of other small organisms). This is necessary to answer many important questions related to, amongst other fields, the evolution of reproductive systems and sexuality. In combination with the increasing knowledge on, and experimental prowess of, the model organism *Macrostomum lignano*, the Macrostomorpha offer tremendous opportunities in this context.

#### 5. Taxonomic notes: species identification, sampling locations, and taxonomic status of the studied specimens

The importance of a strong link between DNA sequences and morphological vouchers has recently been highlighted (Pleijel et al., 2008; Astrin et al., 2013). With this aim we have deposited digital morphological vouchers for all the sequenced Macrostomorpha specimens, not only at Dryad Digital Repository (Janssen et al., 2015, <http://dx.doi.org/10.5061/dryad.b5908>), but also on the Macrostomorpha Taxonomy and Phylogeny database (<http://macrostomorpha.info>), including images, videos, and collection data (see above, also Schärer et al., 2011). Each specimen carries a unique accession code (e.g., MTP LS 123, short for Macrostomorpha Taxonomy and Phylogeny, Lukas Schärer, specimen ID 123) and all media are named with the date and time of acquisition (i.e. YYYY-MM-DD\_hh-mm-ss; e.g. 2014-07-31\_08-23-45), providing an unambiguous identifier for each media item. In Table C we provide notes on the taxonomic status and the sampling of all specimens we used in this study.



Below we describe the new species introduced in this study and discuss the taxonomic status of some of the species and specimens studied (see Table 1 for details on obtained sequences).

### 5.1. *Haplopharyngida*

*Haplopharynx papii* Schockaert 2014. While this species matches the overall morphology of *H. rostratus* quite well, the stylet carries only six accessory spines (sometime called needles) rather than 7–9 in *H. rostratus* (Karling, 1965; Pawlak, 1969). The stylet is positioned off-center from the accessory spines in *H. rostratus* and in our specimens of *H. papii*, but Schockaert (2014) does not observe this in his *H. papii*, possibly because he studied stylets from lactophenol preparations. In all our deposited specimens we observe evidence for hypodermically inseminated sperm in the posterior parenchyma.

#### 5.1.1. *Haplopharynx quadristimulus* species group

*Haplopharynx* cf. *quadristimulus* sp. C. The material suggests that this species is similar, but not identical, in overall morphology to *Haplopharynx quadristimulus* Ax 1971 from the French Mediterranean Coast, and also to two different ‘forms’ from North Carolina, USA (Rieger, 1977; Doe, 1986a,b). A re-examination of the available data suggests that there are currently four different species in the *Haplopharynx quadristimulus* species group (Fig. 11), all of which are characterized by having paired seminal vesicles, a central funnel-shaped stylet (i.e. consisting of a conical mouth and a narrow nearly parallel stem) and four (or sometimes five, see Doe, 1986b) accessory spines: (i) *Haplopharynx quadristimulus* Ax 1971 (Fig. 11A) (body 4–5 mm, stylet 80–82  $\mu$ m and with a wide mouth and a 50  $\mu$ m stem, accessory spines 65–67  $\mu$ m with proximal ends strongly spatulate); (ii) *Haplopharynx* cf. *quadristimulus* sp. A (Fig. 11B) corresponding to the specimen from Swansboro Coast Guard Station depicted in Fig. 7a of Rieger (1977) (body 1 mm, stylet 50  $\mu$ m and with a narrow mouth and a 25  $\mu$ m stem, accessory spines 40–50  $\mu$ m, thus similar in size to stylet); (iii) *Haplopharynx* cf. *quadristimulus* sp. B (Fig. 11C), corresponding to the specimen from Bogue Banks depicted in Fig. 7b of Rieger (1977) and also the specimens studied in detail by Doe (1986a,b) (2–3 mm long, stylet 60  $\mu$ m with narrow

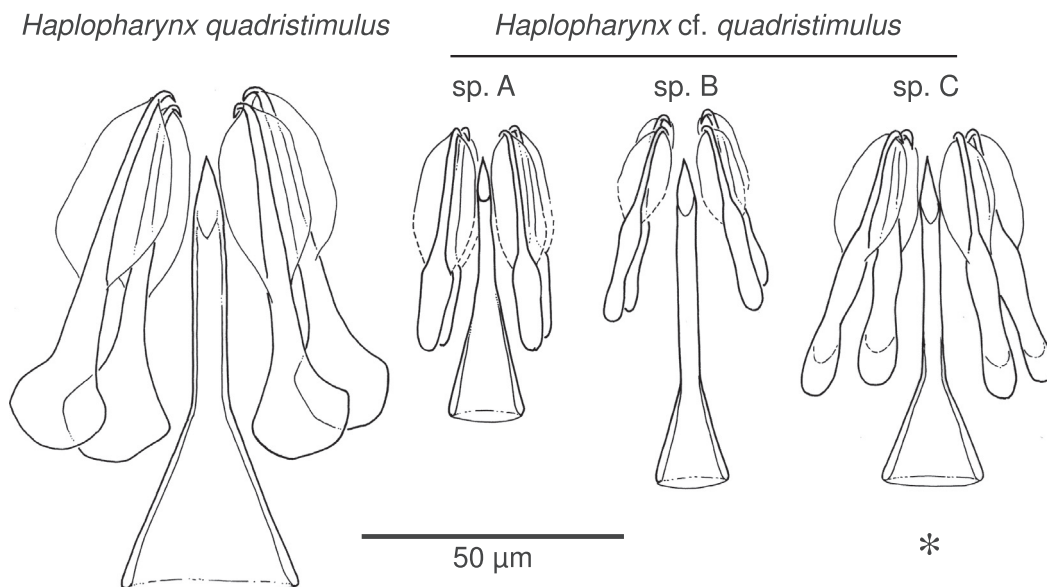
mouth and a 40–45  $\mu$ m stem, accessory spines 40–50  $\mu$ m, thus clearly shorter than stylet); and finally, (iv) *Haplopharynx* cf. *quadristimulus* sp. C (Fig. 11D), corresponding to the specimen of Laumer and Giribet (2014) (size unclear but the deposited movie was taken with 10 $\times$ , 20 $\times$ , and 40 $\times$  objectives, stylet with wide mouth and a long stem, about 70% of the stylet length, accessory spines distally very slender and slightly longer than the stem). *H. cf. quadristimulus* sp. C is the species we included in our phylogenetic analyses, and is only distantly related to *H. rostratus* and *H. papii*. No molecular information is available for the other species. Despite the considerable morphological differences of the male copulatory organ (see Fig. 11), we have refrained from attaching names to these species, because the currently available material does not suffice for a formal description of these taxa.

### 5.2. *Myozona*

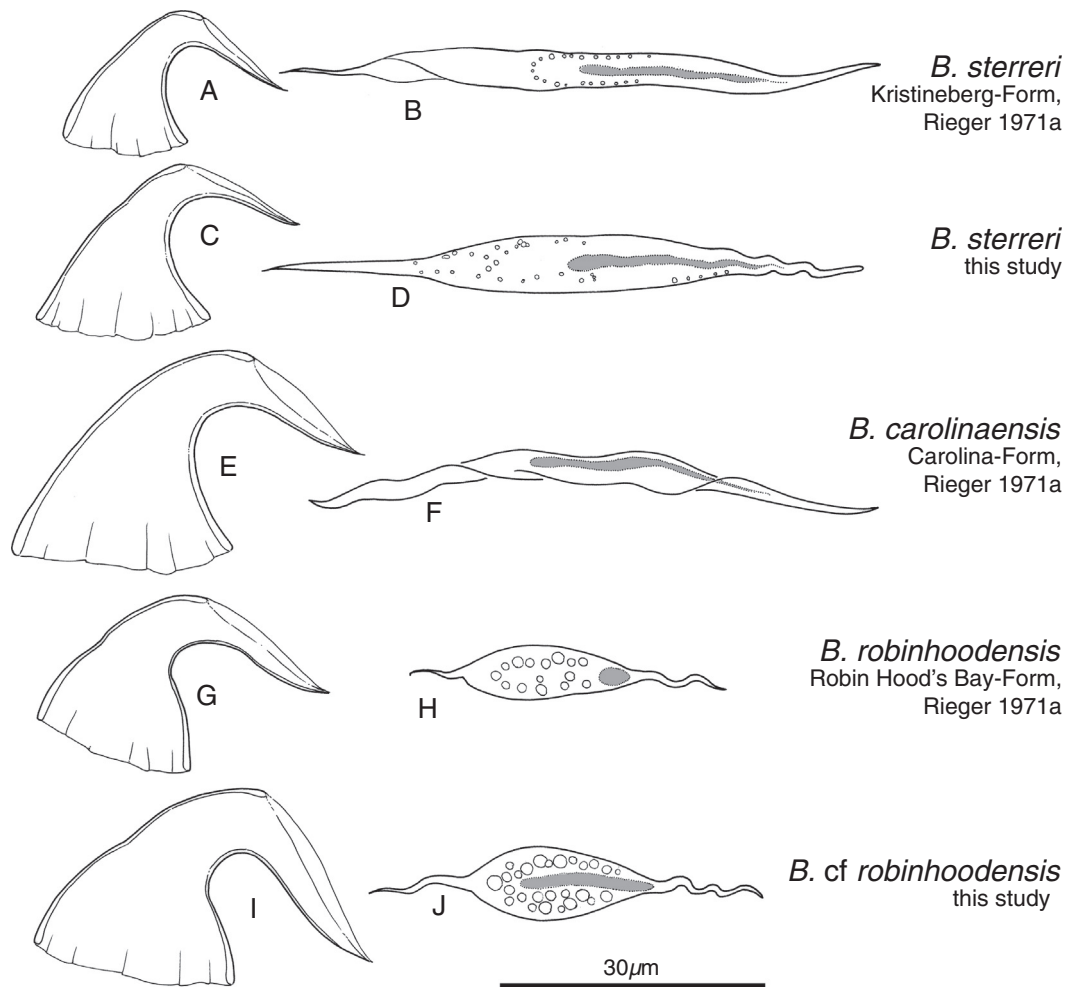
Our *Myozona* sp. resembles *Myozona stylifera* Ax 1956 in general appearance and in the wide luminal genitointestinal connection between the bursal organ (in our specimen clearly containing sperm) and the gut. Our specimen also had one ovary and one testis running along each side of the gut. However, we did not observe a stylet (but we did find a seminal vesicle), and, although the gut is highly muscular throughout, (see deposited movies), we observed no clear muscle ring, a trait considered typical for *Myozona* (note that the constriction in the mid-body seen in the pictures is the result of wounding during preparation). Because we only obtained a 28S sequence for this specimen we supplemented the molecular data with an 18S sequence of a juvenile *Myozona* sp. (MTP LS 731), which is most likely the same or a very similar species, as it has the characteristic lacunae in the pharynx region and lacks a clear muscle ring, and its sequences also cluster in the same clade in single-gene trees (not shown). Given these taxonomic and molecular uncertainties, additional specimens are needed for a complete identification.

### 5.3. *Bradynectes*

*Bradynectes sterreri* Rieger 1971 was originally described as comprising three different ‘forms’, one from the Swedish West



**Fig. 11.** A comparison of the male copulatory organs of four species in the *Haplopharynx quadristimulus* group. *Haplopharynx quadristimulus*, *Haplopharynx* cf. *quadristimulus* sp. A, *Haplopharynx* cf. *quadristimulus* sp. B, and *Haplopharynx* cf. *quadristimulus* sp. C. Scale bar refers to the first three species, and it is only approximate for *H. cf. quadristimulus* sp. C (\*).



**Fig. 12.** A comparison of the male copulatory organs (left) and sperm (right) of *Bradynectes sterreri* (A, B), *Bradynectes carolinaensis* nov. sp. (E, F), and *Bradynectes robinhoodensis* nov. sp. (G, H), with the specimens collected in our study (C, D, specimen MTP LS 165; and I, J, specimen MTP LS 180). (A, E, F, G, are drawn from micrographs and B, H are composites of drawings and description in Rieger (1971a)).

Coast (Kristineberg form), one from US East Coast (Carolina form) and one from the English East Coast (Robin Hood's Bay form), differing in, among other traits, the morphology of the sperm (Rieger, 1971a). Our phylogeny and previous results (Schärer et al., 2011) support the idea of Rieger that sperm morphology is a useful taxonomic trait. We re-examined the available data and conclude that at least some of the proposed forms of *Bradynectes* represent different species. These are (i) *Bradynectes sterreri* Rieger 1971, corresponding to the 'Kristineberg form' (Fig. 12A and B). This species from Klubban, Sweden (close to the Kristineberg marine station), was originally collected by Wolfgang Sterrer from fine sublittoral sand close to the low water line (body 2 mm, stylet: opening  $o = 13 \mu\text{m}$ , concave side  $k = 21 \mu\text{m}$ , convex side  $x = 19 \mu\text{m}$ , sperm: long and slender, length  $sl = 64 \mu\text{m}$ , nucleus length  $snl = 20 \mu\text{m}$ . (ii) *Bradynectes carolinaensis* nov. sp., corresponding to the 'Carolina form' (Fig. 12E and F), from Beaufort, NC, USA, was collected in coarse sublittoral sand at 28 m water depth (body 1.1 mm, stylet  $o = 16 \mu\text{m}$ ,  $k = 32 \mu\text{m}$ ,  $x = 31 \mu\text{m}$ , sperm long and slender,  $sl = 68 \mu\text{m}$ ,  $snl = 30 \mu\text{m}$ ), and (iii) *Bradynectes robinhoodensis* nov. sp., corresponding to the 'Robin Hood's Bay form' (Fig. 12G and H), from Yorkshire, England, was collected in medium and fine intertidal surface sand (body 1.1 mm, stylet  $o = 16 \mu\text{m}$ ,  $k = 25 \mu\text{m}$ ,  $x = 22 \mu\text{m}$ , sperm short and stout,  $sl = 41 \mu\text{m}$ ,  $snl = 2.5 \mu\text{m}$ ). For the diagnoses of these three new *Bradynectes* species we refer the reader to the original diagnoses of the 'forms' (Rieger, 1971; p. 232).

Two more forms and one species have since been described, all of which were raised to species level by Faubel and Warwick (2005) (see also comments in Schärer et al. (2011)). Unfortunately, despite its taxonomic importance within this genus, no information on the sperm morphology was reported for these species. They are: (iv) *Bradynectes syltensis* Faubel and Warwick 2005 (therein listed as *Bradynectes syltensis* Faubel 1974, although this name first appeared in Faubel and Warwick (2005), without being marked as a new species), corresponding to the 'Sylt form' of Faubel (1974) from List, Sylt, Germany, collected in intertidal sand 0.15 m inside the sediment on the same beach our specimens were collected (body 1.2 mm, stylet  $o = 20 \mu\text{m}$ ,  $k = 41 \mu\text{m}$ ,  $x = 45 \mu\text{m}$ ). Along the stylet morphology Faubel used a sphincter on the anterior end of the seminal vesicle and the ovary's position between testis and gut as diagnostic characters (see below). (v) *Bradynectes scheldtensis* Faubel and Warwick 2005 (therein listed as *Bradynectes scheldtensis* Martens and Schockaert 1981, although this name first appeared in Faubel and Warwick (2005), without being marked as a new species), corresponding to the 'Scheldt form' of Martens and Schockaert (1981) from Eastern Scheldt, Belgium, collected in fine sublittoral sand at 3–4 m water depth (body 2.5 mm, stylet  $o = 10 \mu\text{m}$ ,  $k = 26 \mu\text{m}$ ,  $x = 24 \mu\text{m}$ ). The stylet morphology, absence of sphincters and the ovary's position are the diagnostic characters (see below). And finally (vi) *Bradynectes scilliensis* Faubel and Warwick 2005, presumably from medium eulittoral sand in Lawrence's Bay, Scilly Isles, UK (material

described from sites 2a and 2b at Lawrence's Bay; but in the discussion at least one of the two specimens declared from site 7, a rock pool on White Island) (body 2.1 mm, stylet  $\sigma = 32 \mu\text{m}$ ,  $k = 57 \mu\text{m}$ ,  $x = 45 \mu\text{m}$ ). Stylet morphology, sphincters at both ends of the seminal vesicle and the ovary placed between the testis and the gut are the diagnostic features (see below).

Before attempting to place our own specimens we need to discuss the usefulness of some of the different diagnostic characters. The exquisite reconstructions of Rieger (1971a) do not suggest any sphincters in any of his species, nor are sphincters observed by Martens and Schockaert (1981) or in our specimens (see below). Instead Rieger (1971a) shows that the whole seminal vesicle is surrounded by a strong muscular sheath, which also matches the drawing of Martens and Schockaert (1981) and our own observations. This may put into question the diagnoses of Faubel, especially as we were unable to observe a sphincter in the movie deposited together with the holotype of *Bradynectes scilliensis*. Given these considerations we argue that a detailed histological documentation of the nature of the sphincters is necessary before considering this as a valid diagnostic trait.

Regarding the ovary's position, Rieger (1971a) clearly states that it is highly variable, depending on the size of the oocytes and the vas deferens, and on the position of the testis (left or right). Moreover, he never claims the ovary's position is not between the testis and the gut, and even draws it that way for all three forms (Rieger, 1971a, Fig. 4C, E and F). We therefore think that the ovary being between the testis and the gut is not a valid diagnostic for this genus. Given these considerations on the sphincter morphology and the ovary position we argue that a reassessment of validity of *B. scheldtensis*, *B. syltensis* and *B. scilliensis* is necessary as the diagnosis of these species is now only based on the variable stylet morphology. Preferably this reassessment should include the sperm morphology and a molecular phylogenetic placement of these species.

We have previously reported on a *Bradynectes* specimen (MTP LS 180, Schärer et al., 2011; Fig. 12I and J) (body 2.3 mm, stylet opening; concave side; convex side is  $21 \mu\text{m}$ ;  $35 \mu\text{m}$ ;  $29 \mu\text{m}$ , sperm short and stout, sperm length  $40 \mu\text{m}$ , sperm nucleus length  $11 \mu\text{m}$ ). We see no sphincters (but a clear muscular sheath), and the position of the ovary is unfortunately unclear. This specimen matches *Bradynectes robinhoodensis* quite well, namely in terms of geography (North Sea) and habitat (intertidal sand), and although our specimen is somewhat larger both in body and stylet size, it matches quite well in overall stylet and sperm morphology (including the presence of prominent granules on both sides). The biggest discrepancy is with respect to the size and shape of the sperm nucleus (compare Fig. 12H and J), which in *Bradynectes robinhoodensis* is much smaller and round (a shape that is similar to some spermiogenesis stages). Note, however, that Rieger (1971a) points out that for this species he only observed mature sperm from formol-glycerol total preparations, which he acknowledges could affect his interpretations. We will therefore name our specimen *Bradynectes* cf. *robinhoodensis* until the nature of the sperm morphology of specimens from the type locality can be further clarified.

Remarkably, a further *Bradynectes* specimen (MTP LS 162, Fig. 12C and D), from the same location, but at the surface instead of below ground, clearly differs from our former specimen genetically (Fig. 1), and, among other traits, in the size and morphology of the sperm cells. This is more evident in an additional barcoded specimen (MTP LS 165) and two additional non-barcoded specimens (MTP LS 164 and 167) that we deposit from the same sample (means of 3–4 specimens, body 2.5 mm, stylet  $15 \mu\text{m}$ ;  $27 \mu\text{m}$ ;  $25 \mu\text{m}$ , sperm long and slender, sperm length  $68 \mu\text{m}$ , sperm nucleus length  $23 \mu\text{m}$ ). We see no sphincters in any of the specimens (but always a clear muscular sheath) and the position of

the ovary is at the posterior end of the testis, as reported by Rieger (1971a). These specimens closely match *Bradynectes sterreri* (as defined above), namely in term of geography (North Sea) and habitat (fine sand around the low water line), body size, sperm and stylet size and morphology. We therefore consider these specimens to be *Bradynectes sterreri*.

#### 5.4. Dolichomacrostominae

##### 5.4.1. Paromalostomum

We report a juvenile *Paromalostomum* cf. *minutum* (MTP LS 696) from the Ligurian Sea, which closely clusters with our *P. minutum* from the Adriatic Sea (MTP LS 555, see Table C) and shares the characteristically large caudal glands, but which differs in 33 bp (~1%). Further determination is currently not possible without investigating additional adult specimens from the Ligurian locality.

##### 5.4.2. Austromacrostomum

***Austromacrostomum arumoidicornum* nov. sp.** (Fig. 13).

**Holotype.** A digital morphological voucher (MTP LS 638) and a permanent lactophenol preparation from the same specimen (Table 1), collected on 29. April 2010 in coarse shell sand at 2–7 m water depth, Sant' Andrea Bay, Elba, Italy; 42.8087, 10.1418 (type locality). From the type specimen we also provide sequences of the 18S rDNA, 28S rDNA, 16S and COI gene regions (Table 1).

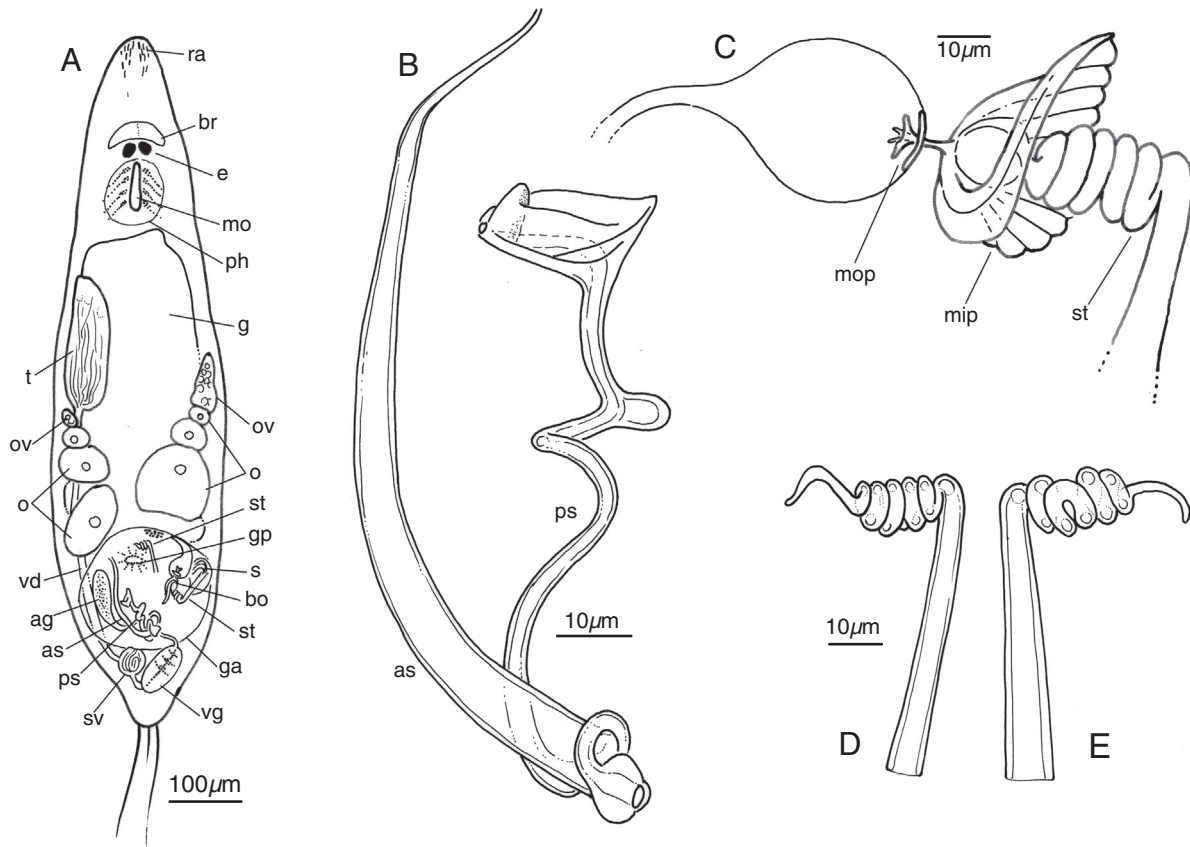
**Other material and localities.** Two additional extensively documented specimens (MTP LS 639 and MTP LS 640), collected from the same sample as the holotype and deposited as permanent lactophenol preparations (Table 1, paratypes).

**Etymology.** The species name refers to the resemblance of the end of the stylet ("cornum") to the spathe and spadix of the arum lily ("arumoidi", Arum-like), a plant of the genus *Zantedeschia* (Araceae).

**Diagnosis.** *Austromacrostomum arumoidicornum* nov. sp. matches the updated diagnosis of the genus *Austromacrostomum* very well in having paired eyes, paired ovaries and the characteristic spiral sperm tubes (see also Table B). Our species differs from *Austromacrostomum mortenseni* (Marcus, 1950), originally described from Ilha de São Sebastião, Brazil as *Dolichomacrostomum mortenseni*, in having a different penis stylet tip.

**Description.** The body size of *Austromacrostomum arumoidicornum* nov. sp. varies between 0.97 and 1.17 mm, with prominent paired eyes associated with the brain (Fig. 13A). The gut is straight and simple and ends before the genitalia. The large unpaired testis is lateral, between the ovary and the pharynx. The testis is connected to a vas deferens that broadens into a round seminal vesicle. A male gonoduct extends caudally and turns around before ending into the large oval vesicula granulorum, which distally contains prominent granules and is surrounded by a pronounced muscular sheath. The vesicula granulorum is connected to the penis stylet, while a large accessory gland is connected to the accessory stylet. The slightly bent accessory stylet is  $135 \mu\text{m}$  long and proximally connected to the penis stylet, and considerably shorter than the accessory stylet in live specimens (Fig. 13B). When measured in squeezed lactophenol preparation, however, the curved penis stylet is  $164 \mu\text{m}$  long and distally bears a characteristic and complex tip (Fig. 13B). The paired ovaries are positioned laterally below the testis and connected to the common genital atrium (positioned around U 75–80) through a bursal organ consisting of a mid-piece, bearing a ridged flap and a mouth-piece. The complete bursal organ is  $50\text{--}56 \mu\text{m}$  long measured from the tip of the mouth-piece along the ridge to the end of the flap (Fig. 13C), and is morphologically similar to the one of *Austromacrostomum mortenseni*. In MTP LS 638 we observe that the bursal organ is connected to a sperm tube. The shaft of the sperm tubes is  $50\text{--}60 \mu\text{m}$





**Fig. 13.** Taxonomic description of *Austromacrostromum arumoidicorum* nov. sp., drawings from micrographs of live specimens. (A) Habitus and internal organization; (B) accessory and penis stylets; (C) bursal organ showing attached distal end of sperm tube; (D, E) sperm tubes. B, E, from MTP LS 638; C, from MTP LS 640; D, from MTP LS 639; A, from various specimens. ag, accessory gland; as, accessory stylet; bo, bursal organ; br, brain; e, eyes; g, gut; ga, genital atrium; gp, gonopore; mip, mid-piece; mo, mouth opening; mop, mouth-piece; o, oocytes; ov, ovary; ph, pharynx; ps, penis stylet; ra, rhammites; s, sperm; st, sperm tube; t, testis; vd, vas deferens; vg, vesicula granulorum; sv, seminal vesicle.

long while the sperm tubes including the characteristic terminal spiral typical for the genus *Austromacrostromum* are 81–86  $\mu\text{m}$  long (Fig. 13D and E).

#### 5.4.3. *Cylindromacrostromum*

Our *Cylindromacrostromum* cf. *notandum* (MTP LS 250) matches *Cylindromacrostromum notandum* (Ax, 1951) from the Kieler Bucht, Germany. However, our specimen appears to be smaller in size (1 mm vs. 2–2.5 mm). Due to the lack of details on the morphology of the bursal organ in the original description we cannot compare these structures more precisely. The final placement as a separate species or as *C. notandum* would ideally require sequencing the latter species.

#### 5.5. *Myozonariinae* nov. subfam

**Diagnosis.** *Myozonariinae* are diagnosed as *Dolichomacrostromidae* with a muscle ring, a caudal sensory organ, and a caudal gut extension (except *Myozonaria arcassonensis* Rieger 1971, see Section 3.2.5).

##### 5.5.1. *Myozonaria*

###### *Myozonaria fissipara* nov. sp. (Figs. 3 and 14).

**Holotype.** A digital morphological voucher (MTP LS 678) and a permanent lactophenol preparation from the same specimen (Table 1), collected on 30. April 2010 from sand between two *Posidonia oceanica* patches at 6 m water depth, Cala della Ruta, Pianosa, Italy; 42.5747, 10.0665 (type locality). From the type

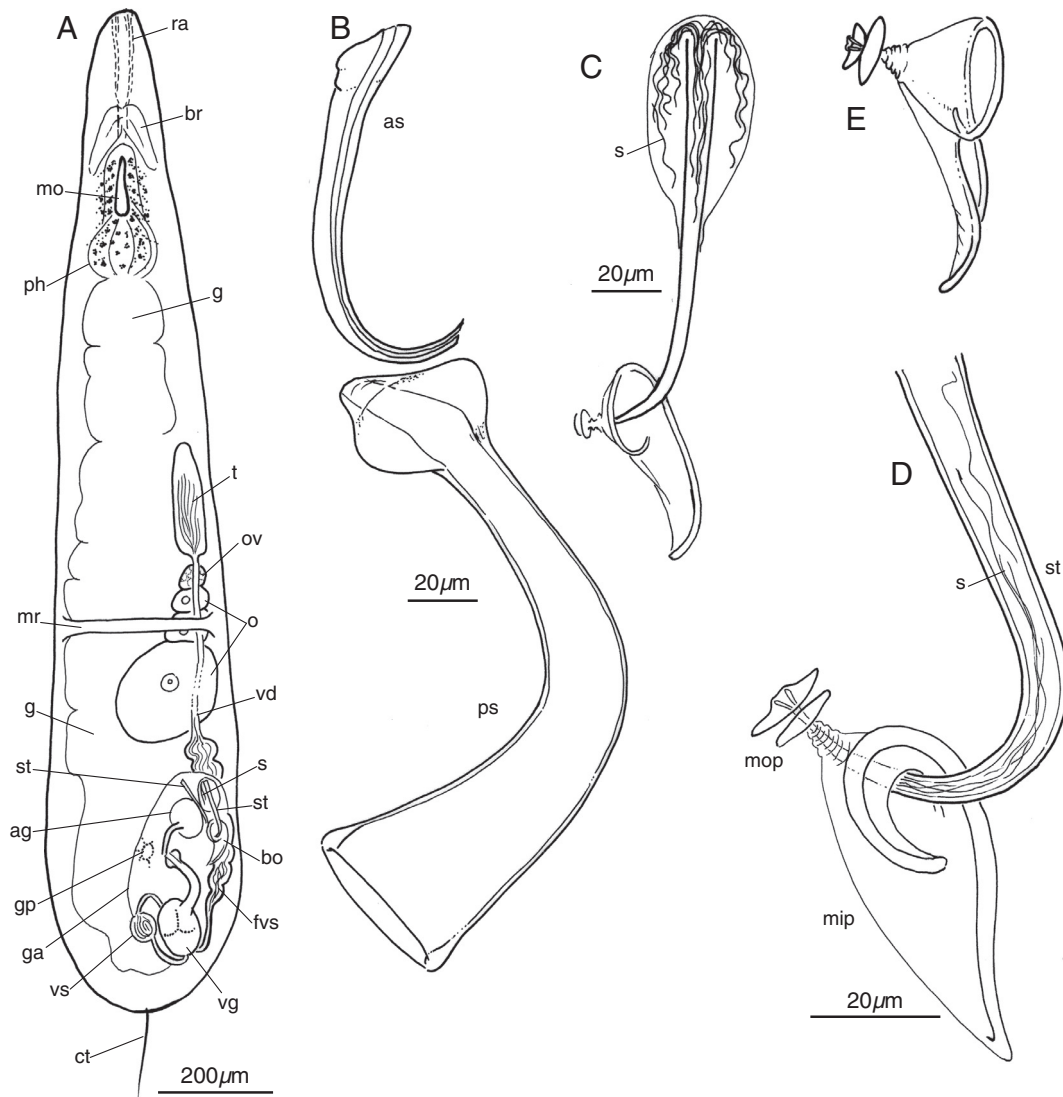
specimen we also provide sequences of the 18S rDNA, 28S rDNA, 16S and COI gene regions (Table 1).

**Other material and localities.** Four additional extensively documented specimens (paratypes). MTP LS 651 and MTP LS 683 are both in a state of asexual fissioning, and both specimens consist of two zooids. MTP LS 653 and MTP LS 623 are immature specimens representing individual zooids. MTP LS 651, MTP LS 683 and MTP LS 623 are identical in all sequences studied, while the COI region of MTP LS 678 and MTP LS 653 differs in 1 bp and 2 bp, respectively, suggesting that we are dealing with a single species here (see Table 1 for sequence information). All specimens were collected in Sant' Andrea Bay, Elba, Italy (42.8087, 10.1418), at 2–7 m water depth, on 29. April 2010, and 26. April 2010 (MTP LS 623). The habitats were coarse shell sand (MTP LS 651 and MTP LS 653), the valley of a sand ripple (MTP LS 683), and a brownish crest on top of a sand ripple (MTP LS 623).

**Etymology.** The species name refers to the ability of asexual fissioning.

**Diagnosis.** This species matches the genus diagnosis of *Myozonaria* Rieger 1968 (see also Rieger, 1971b), namely a separate penis stylet and accessory stylet in combination with an unpaired caudal sensory organ and a muscle ring. This species is further characterized by the presence of asexual fissioning, which contrasts with all other described species in the genus.

**Description.** Sexually mature specimens of *Myozonaria fissipara* are approximately 1.73 mm long (Fig. 14A). The animals have a brown coloration, distinctly reddish-brown on the pharynx region ('chocolate mouth'). A fine muscle ring is located at U66 and an unpaired caudal sensory organ bearing a single ciliary tuft is



**Fig. 14.** Taxonomic description of *Myozonaria fissipara* nov. sp. (Myozonariinae), drawings from micrographs of live specimens. (A) Habitus and internal organization; (B) accessory and penis stylets; (C) bursal organ with sperm tube attached, sperm and vesicle; (D) bursal organ with sperm tube attached; (E) bursal organ, schematic reconstruction. A–D, from MTP LS 678; E, from various specimens. ag, accessory gland; as, accessory stylet; bo, bursal organ; br, brain; ct, ciliary tuft; e, eyes; fvs, false seminal vesicle; g, gut; ga, genital atrium; gp, gonopore; mip, mid-piece; mo, mouth opening; mop, mouth-piece; mr, muscle ring; o, oocytes; ov, ovary; ph, pharynx; ps, penis stylet; ra, rhammities; s, sperm; st, sperm tube; t, testis; vd, vas deferens; vg, vesicula granulorum; sv, seminal vesicle.

present. Even in sexually mature specimens, the gut extends caudally over the genitalia, almost reaching the posterior end of the animal. The unpaired testis is located anterior to the muscle ring and connects to a vas deferens that broadens posterior to the muscle ring into a false seminal vesicle, and, after narrowing and turning around, it empties into the seminal vesicle. The seminal vesicle is connected to the vesicula granulorum (diameter 104 µm), which is attached to the penis stylet. The penis stylet is slightly bent and 193 µm long, with a characteristic distal thickening (Fig. 14B). The posteriorly bent and slender accessory stylet is 146 µm long (Fig. 14B) and connected to an accessory gland. The female system consists of an unpaired lateral ovary posterior to the muscle ring. The sclerotized bursal organ consists of a slightly rounded and extended mid-piece that bears a flap and a mouth-piece (Fig. 14E). The complete bursal organ is 67 µm long and in our holotype specimen connected to a 102 µm long sperm tube (Fig. 14C and D). Around the common genital atrium two more sperm tubes in different states of degradation were observed, of which one is slightly longer (114 µm).

#### 5.5.2. *Myomacrostromum*

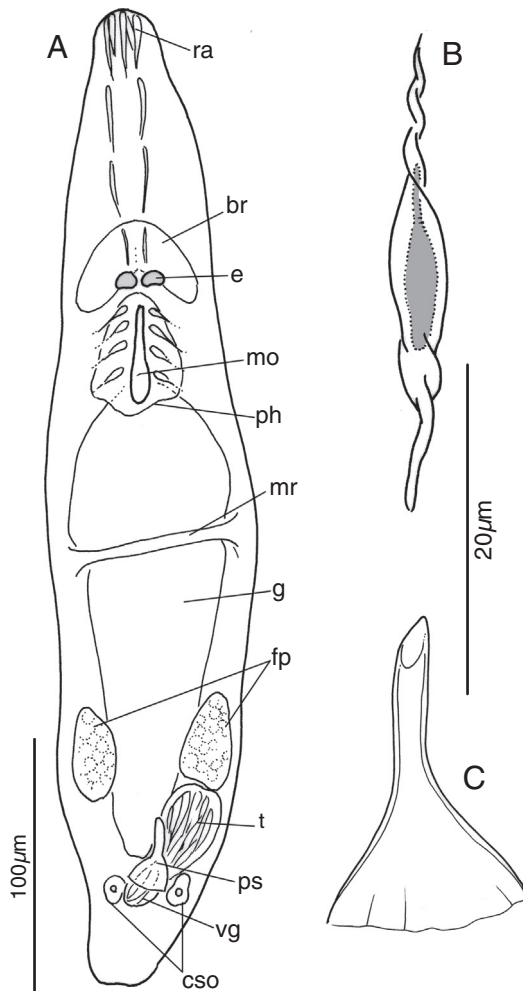
##### *Myomacrostromum rubrioculum* nov. sp. (Figs. 4 and 15).

**Holotype.** A digital morphological voucher (MTP LS 670) in the process of forming a fission plane, collected on 30. April 2010 from sand between two *Posidonia oceanica* patches at 6 m water depth, Cala della Ruta, Pianosa, Italy; 42.5747, 10.0665 (type locality). We provide sequences from 18S and 28S rDNA, 16S and COI regions for this species (see Table 1).

**Other material and localities.** An additional extensively documented specimen (MTP LS 689) also deposited as a permanent lactophenol preparation (Table 1, paratype), and matching perfectly in 28S rDNA sequence to the holotype. That specimen was collected on 3. May 2010 from an open sand flat at 11 m water depth (Sant' Andrea Bay, Elba, Italy; 42.8087, 10.1418).

**Etymology.** The species name refers to the presence of red eyes.

**Diagnosis.** This species closely matches the diagnosis of the genus *Myomacrostromum* Rieger 1986 (i.e. small unilateral testis, simple anteriorly pointing stylet and a caudal sensory organ). *Myomacrostromum rubrioculum* nov. sp. clearly differs from the



**Fig. 15.** Taxonomic description of *Myomacrostomum rubrioculum* nov. sp. (Myozonariinae), drawings from micrographs of live specimen MTP LS 689. (A) Habitus and internal organization; (B) sperm (nucleus in gray); (C) ps, penis stylet; br, brain; cso, caudal sensory organ; e, eyes; fp, formation of the fission plane; g, gut; mo, mouth opening; mr, muscle ring; ph, pharynx; ps, penis stylet; rhammites; t, testis; vg, vesicula granulorum.

existing species, *Myomacrostomum unichaeta* Rieger 1986 and *Myomacrostomum bichaeta* Rieger 1986 from the US East Coast by having red eyes and by geographic location. Moreover, it is clearly different from *M. unichaeta* in that it has a paired caudal sensory organ similar to *M. bichaeta*.

**Description.** Both specimens are comparable in size (MTP LS 670 is 369 μm; MTP LS 689 is 363 μm; Fig. 15A). The animal has paired bright red colored eyes on top of the brain. This species bears a fine muscle ring around the gut at U 50. In MTP LS 670 we observe a fission plane being formed out of two bowl-shaped undifferentiated parenchyma regions, a situation strongly resembling the formation of a fission plane as described by Rieger (1986). These regions superficially resemble paired gonads, but they are clearly not. The gut slightly extends caudally over the genitalia, and a paired caudal sensory organ is present. The unpaired small testis is located posterior of the muscle ring in close proximity to the penis stylet and the vesicula granulorum, which slightly protrudes into the anteriorly pointing penis stylet. The funnel-shaped penis stylet is 18.5 μm long measured along the middle of the stylet (Fig. 15C). The sperm cells are approximately 26–32 μm long and spindle shaped (Figs. 15B and 4). Unfortunately, both collected specimens were immature in their female function, therefore we have coded the characters related

to the female reproductive system and the presence of hypodermic sperm as unknown in our ASR.

### 5.5.3. Other Myozonariinae

*Myozonariinae* sp. The phylogenetic position and the presence of a muscle ring and a paired caudal sensory organ suggest that this species belongs to the Myozonariinae. The general morphology, paired caudal sensory organ (Fig. 5) and the position of the ovary suggest that this species could be a *Paramyozonaria* Rieger 1971, or *Myomacrostomum bichaeta* Rieger 1986. Complete identification of this species will require information about the male genitalia. Interestingly the prominent muscle ring appears to have additional transverse muscle fibers or a sclerotized structure.

### 5.6. Karlingiinae

**Diagnosis.** Karlingiinae are diagnosed as Dolichomacrostomidae with unpaired gonads bearing a penis stylet, an accessory stylet and a bursal organ. The gut does not extend caudally over the genitalia. Eyes, a muscle ring, and caudal sensory organs are absent.

#### 5.6.1. *Acanthomacrostomum*

An *Acanthomacrostomum* sp. from Laumer and Giribet (2014) (their accession number DNA105907) was collected near the Bocas del Toro Research Station, Panama (for details see <http://mczbase.mcz.harvard.edu/>). A movie of the live specimen was kindly provided to us by Christopher Laumer and we deposit this movie and several informative movie frames extracted from it (as MCZ CEL DNA105907). This material clearly suggests this specimen is an *Acanthomacrostomum* Papi and Swedmark 1959, but it offers too little detail to compare it to the other species in this genus.

### 5.7. Microstomidae

*Microstomum lineare* Müller 1773 was originally described from an unknown location and can clearly be considered a problematic taxon. Our specimen (MTP LS 394) carries the characteristic red eyespots, and its 28S sequence is identical to a previously deposited 28S sequence (AJ270172; Littlewood et al., 2000). We deposit a barcoded specimen (MTP LS 356) with an identical 28S sequence. Our specimen MTP LS 394, however, does differ by 1.7% from the deposited 18S sequence of another specimen (D85092, Katayama et al., 1996), suggesting that there is some cryptic diversity within this species.

A further *Microstomum lineare* from Connecticut, USA was collected, identified and sequenced by Laumer and Giribet (2014), with accession number DNA105906. (see <http://mczbase.mcz.harvard.edu/>). Unfortunately, no images exist of the actually sequenced specimen, but images of live specimens from the same population were kindly provided to us by Christopher Laumer and are accessible online from the specimen page. This specimen differs, respectively, in 8 bp (0.5%) and 20 bp (1.9%) from the 18S and 28S sequences of our specimen (MTP LS 394), suggesting that this may be a similar species. Its 18S region, however, differs from the aforementioned D85092 in 35 bp (2%), again suggesting considerable cryptic diversity in this taxon.

We have collected four additional *Microstomum* spp. that either present all or at least some of the diagnostic traits of the genus, e.g. gut extends pre-orally (3/4), presence of ciliary pits (4/4), asexual fission plane (3/4), adhesive glands on the head (3/4), presence of nematocysts (2/4). But given the scarcity of diagnostic traits in this genus we at this stage refrain from attempting any further species identification, and we distinguish them as species A, B, C, and D.



### 5.8. *Psammomacrostomum*

We have collected six different species that all show paired ovaries, paired testes and a cirrus. Based on these morphological traits we place these species in the genus *Psammomacrostomum* Ax 1966 (see discussion in Macrostromidae section). As all our species appear to be new representatives of the genus, we have decided to formally describe them in a separate publication, which will also include an updated diagnosis of the genus.

*Psammomacrostomum* nov. sp. 1 shows some similarities to *Psammomacrostomum equicaudum* Ax 1966 from Arcachon, France (mistakenly called *P. equicaudatum* in Schärer et al. (2011)), but it differs clearly in the shape of the copulatory cirrus. Moreover, while in our species the vasa deferentia are medially joined at the level of the testis, the more detailed description provided by Ax and Faubel (1974) is ambiguous about that character. Finally, our species is clearly different from *Psammomacrostomum turbanelloides* Karling 1974 from the Swedish South Coast, in the structure of the copulatory organ and in having paired rather than unpaired gonads. Interestingly, we found no evidence of simultaneous presence of male and female gonads in the numerous studied specimens from several different locations, suggesting that this species is not a simultaneous hermaphrodite. So far this phenomenon has not been observed in other species of the genus.

*Psammomacrostomum* nov. sp. 6 is considerably similar to *Antromacrostomum armatum* Faubel 1974 from Sylt, Germany, a species that was described in close proximity and in a similar habitat. In his diagnosis Faubel (1974) states that there are four proliferation centers in the medially joined testes, which we clearly do not observe in our specimen. Moreover, our specimen has a paired rather than unpaired ovary, and no spicule-like organs in the pharynx. This, and the molecular results suggest our species is a *Psammomacrostomum*.

*Psammomacrostomum* nov. sp. 2 was previously considered a new genus (called Gen. nov., sp. nov. 1 in Schärer et al. (2011), see also specimens MTP LS 55 and MTP LS 59 therein) because of the presence of a vagina and a female antrum, thus contrasting with the original diagnosis of *Psammomacrostomum equicaudum*, which lacks these structures. But the likely presence of hypodermic insemination in *Psammomacrostomum* nov. sp. 1 makes it plausible that the complexity of the female genitalia depends on the mating mode, as has been shown for the genus *Macrostromum* (Schärer et al., 2011) and also in this study. Therefore we have decided against the erection of a new genus and place this species in the genus *Psammomacrostomum*.

The only collected specimen of *Psammomacrostomum* nov. sp. 3 has only one properly developed testis, plus a mass of undifferentiated cells clearly visible in the appropriate region. Our own detailed observations in both field-caught and laboratory-reared species of the genus *Macrostromum* suggest that failed development of a gonad occurs with appreciable frequency. Given the phylogenetic clustering, and morphological similarity to *Psammomacrostomum* nov. sp. 4, we are therefore still placing this species into the genus *Psammomacrostomum*.

*Psammomacrostomum* nov. sp. 4 differs from *Psammomacrostomum* nov. sp. 3 both genetically and by having a longer and more strongly curved cirrus.

*Psammomacrostomum* nov. sp. 5 has considerable similarities with *Dunwichia arenosa* Faubel, Bloome and Cannon 1994 from Dunwich, Australia, in terms of the morphology of the male and female genitalia. In their diagnosis, however, Faubel et al. (1994) state that *D. arenosa* has unpaired gonads, which is clearly not the case in our species. We note that our specimens had an unusual parenchyma, which gives the gonads an unusual appearance, potentially affecting the diagnosis of the original description.

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### Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ympev.2015.06.004>.

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