Supporting Information

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SI Materials and Methods

PCR and Sequencing. For each species we sequenced (in one to three specimens) the complete small subunit ribosomal DNA (ssrDNA) and part (D1–D3) of the large subunit ribosomal DNA (lsrDNA) resulting in a total of ~2,850 base pairs. Total genomic DNA (gDNA) was extracted from the DNA samples using the DNeasy tissue kit (QIAGEN) following the manufacturer's instructions; gDNA was eluted in 2× 100-μL volumes. PCR reactions were carried out in 25-μL volumes using Illustra puReTaq Ready-To-Go PCR beads (GE Healthcare), 1 μL of 10 μM of each primer (a list of primers is given in Table S2), and 1–2 μL gDNA extract. Partial lsrDNA (1,142–1,189 base pairs) was amplified using ZX-1 (1) + 1500R (2); difficult templates were amplified with nested PCR using ZX-1 + 1200R and 300F + 1500R. ssrDNA (1,706–1,711 base pairs) was amplified using WormA + WormB; difficult templates were amplified with nested PCR using Macro_18S_200F + Macro_18S_1640R. Cycling conditions for lsrDNA were as follows: denaturation for 5 min at 95 °C followed by 40 cycles of 30 s at 95 °C, 30 s at 55 °C, 2 min at 72 °C, and 7 min extension at 72 °C. Cycling conditions for ssrDNA were as follows: denaturation for 2 min at 94 °C followed by 40 cycles of 30 s at 94 °C, 30 s at 54 °C, 2 min at 72 °C, and 7 min extension at 72 °C. PCR amplicons were gel-excised using the QIAquick Gel Extraction Kit (QIAGEN) or purified directly using the QIAquick PCR Purification Kit (QIAGEN) following the manufacturer's instructions. Cycle-sequencing from both strands was carried out on an ABI 3730 DNA Analyzer, Big Dye version 1.1 using ABI BigDye chemistry. Contiguous sequences were assembled and edited using Sequencher version 4.6 (GeneCodes Corp.), and sequence identity was checked using BLAST (http://www.ncbi.nih.gov/BLAST/). New sequences have been deposited with GenBank under accessions FJ715295–FJ715334 inclusive (Table S3).

Phylogenetic Analysis. Alignments were performed in ClustalX (3) using default settings and were improved by eye in MacClade (4). Regions that could not be aligned unambiguously were excluded from the analysis. The full alignments for lsrDNA and ssrDNA gene partitions (with an indication of exclusion sets) are available upon request. MODELTEST version 3.7maxX (5) was used to select a model of evolution using the Akaike Information Criterion. Phylogenetic trees were constructed using Bayesian inference (BI) with MrBayes version 3.1 (6) and using maximum likelihood (ML) with PAUP* version 4.0b10 (7). For BI, likelihood settings were set to number of substitution types (nst) = 6, rates = invgamma, ngammacat = 4 [equivalent to the general time-reversible plus proportion of invariant sites plus gamma-distributed rate variation across sites (i.e., GTR+I+G) model of nucleotide evolution]; parameters were estimated separately for each gene. Four chains (temp = 0.2) were run for 5×10^6 generations and sampled every 10^3 generations; 5×10^5 generations were discarded as burn-in. ML analyses were performed using successive approximation: Model parameters were estimated based on a starting tree determined by neighbor joining. A heuristic search was performed implementing the estimated model parameters using nearest-neighbor–interchange branch swapping. Model parameters were estimated on the best tree, and a heuristic search was performed using subtree-pruning-regrafting branch swapping. After model parameters were estimated, heuristic searches using tree-bisection-reconnection (TBR) branch swapping were performed until the topology remained unchanged. In addition to posterior probability values from BI analyses, nodal support was estimated using ML bootstrapping (100 replicates) as implemented in GARLI version 0.942 (8) using default settings, except setting Genthreshfortopoterm to 10^4 generations. Clades were considered to have high nodal support if BI posterior probability was ≥95% and ML bootstrap resampling was ≥70%.

Constraint Analysis. We performed a constraint analysis that tested whether the data support the a priori hypothesis of a single origin of the hypodermic mating syndrome. A constrained tree holding the five taxa with this feature as monophyletic was loaded as a backbone constraint before ML analysis under the same model as the unconstrained tree (Fig. S2). Log likelihood scores of constrained and unconstrained trees were used in a Shimodaira–Hasegawa test (9), as implemented in PAUP*, with 10^3 RELL bootstrap replicates.

Ancestral State Reconstruction. We performed ancestral state reconstructions to infer the character states at the base of the genus *Macrostomum* and the base of clade 2 (both nodes are well supported in the ML and BI analyses). We used Mesquite version 2.5 (10) to estimate ancestral states of characters illustrated in Fig. 2 and listed in Table S1, under a ML continuous-time Markov model (Mk1) on the ML tree shown in Fig. S1. Ancestral states were reported as proportional likelihoods at each node for both character states. Missing data resulted in some ancestral character states being reported as equivocal (Fig. S3).

Analysis of Correlated Evolution. We used BayesDiscrete in BayesTraits (available at http://www.evolution.reading.ac.uk/BayesTraits.html) to test for correlated evolution between pairs of discrete binary character states (11). BayesTraits uses a reversible-jump Markov chain Monte Carlo (RJ MCMC) approach to search among possible models of character state evolution while sampling from a set of trees derived from a Bayesian phylogenetic analysis, thus taking phylogenetic uncertainty into account (11). The analysis is run twice for each pair of character states, once allowing for dependent (or correlated) evolution (dep), and once restricting the models to the null hypothesis of independent evolution (indep). The rationale is that under independent evolution the transition rate of character 1 from one state to the other should be independent of the state of character 2 (11). The statistical inference compares the two analyses by the harmonic means (H) of the resulting likelihoods using the test statistic $2\times(\log H_{\text{dep}} - \log H_{\text{indep}})$. By convention, values for this test statistic >2 are taken as positive evidence that the dependent model is favored, and values >5 and >10 represent strong and very strong evidence, respectively (11).

We performed the analyses on a reduced taxon set containing only the genus *Macrostomum*, the main focus of our study. Specifically, we tested if the character states for copulation behavior (0, hypodermic; 1, reciprocal) correlate with those for sperm bristles (0, absent; 1, present), or female antrum morphology (0, simple; 1, thickened). (Fig. 2, Fig. S3, and Table S1 give details on character states.) Note that the character states for sperm bristles and stylet morphology (0, needle-like; 1, not needle-like) are fully congruent among the available *Macrostomum* species, so the results we present for sperm bristles also are valid for stylet morphology. Moreover, the character states for the copulation behavior and the sucking behavior (0, never observed; 1, present) are nearly congruent, but the latter character has more uncertainty about its states (Table S1); here we focus only on the

copulation behavior. Using this method we performed analysis A (sperm bristles vs. copulation behavior) and analysis B (female antrum morphology vs. copulation behavior), each with a separate run for dependent and independent models.

Based on initial ML runs, we used a RJ hyperprior with a gamma distribution (rjhp gamma 0 1 0 1) for the RJ MCMC analyses (11). Next we optimized the rate deviation (ratedev) parameters to achieve acceptance rates between 20–40% (11) and settled for 0.18 and 0.12, respectively, for the dependent and independent runs of analysis A and 0.3 and 0.25, respectively, for the dependent and independent runs of analysis B. We ran the analyses with a sample of 500 best trees taken from our Bayesian phylogenetic analysis (performed as outlined above but with the reduced taxon set) for 505×10^6 iterations with a burn-in of 5×10^6 iterations and sampling period of 10^3 for the independent models and for $1{,}020 \times 10^6$ iterations with a burn-in of 2×10^7 iterations and sampling period of 4×10^3 for the dependent models (to achieve reliable convergence stability).

Notes on Species Identification, Sampling Locations, and Taxonomic Status of the Studied Specimens

We have deposited extensive digital reference material for all specimens that we have sequenced to construct the molecular phylogeny on the online Macrostomorpha Taxonomy and Phylogeny database (available at http://macrostomorpha.info), an EDIT scratchpad (12), including images, videos, and maps. Each specimen carries a unique accession number (e.g., MTP LS 200, short for Macrostomorpha Taxonomy and Phylogeny, Lukas Schärer, specimen ID 200). In the following section we give detailed notes on species identification, sampling locations, and taxonomic status of the species and specimens studied.

Dolichomacrostomum uniporum Luther 1947 was described from Tvärminne, Finland (13). Rieger (14) and Ax (15) list the Baltic Sea, the east and west coasts of Sweden, and the Irish Sea as the distribution. Our specimen (MTP LS 222, not documented) was taken from a laboratory culture that we established from specimens collected on March 13, 2007, at low tide on an intertidal sand flat in the Königshafen, Sylt, Germany (55°02′51.0′′N, 8°25′12.5′′E). It matches the descriptions by Luther (13, 16) and Rieger (14) in every detail studied. Because the sequenced specimen was not documented, we deposited an additional specimen (MTP LS 200) taken from the same sample location from which we founded the laboratory culture.

Microstomum papillosum von Graff 1882 was described by Claparède (17) as a larva of a dendrocoel flatworm from the coast of Norway but was recognized as a *Microstomum* and named by von Graff (18). Our identification is based on the description of Faubel (19) from Sylt, Germany. Our specimen (MTP LS 146) was collected on March 8, 2007, at low tide in the top layer of a sandy intertidal mud flat in the Königshafen, Sylt, Germany (55°02′23.9′′N, 8°23′53.1′′E). It matches the description of Faubel (19) in every detail studied.

Bradynectes sterreri Rieger 1971 was described with three different forms from Kristineberg (west coast of Sweden), Beaufort, NC (east coast of the United States), and Robin Hood's Bay (east coast of England), respectively (20). In addition, Faubel (21) and Martens and Schockaert (22) published additional forms from Sylt, Germany, and the Eastern Scheldt, The Netherlands, respectively, which Faubel and Warwick (23) later referred to as "*Bradynectes syltensis*" and "*Bradynectes scheldtensis*," respectively. However, these species were never formally named. Finally, Faubel and Warwick (23) named a species, *Bradynectes scilliensis* Faubel and Warwick 2005, from the Scilly Isles, England. For use as an outgroup species, the exact species identity of our specimen is not critical. Our specimen (MTP LS 180) was collected on a sandy beach in front of the old Litoralstation, List, Sylt, Germany (55°00′55.5′′N, 8°26′18.0′′E), about 15 cm deep in the sediment.

Gen. nov. 1, sp. nov. 1 (Macrostomidae) has been collected repeatedly by our group from a single location in Lignano, Italy (45°41′28.5′′N, 13°07′54.0′′E). The location is on the shore of the Laguna di Marano below dense vegetation and consists of relatively clean sand with some humus content This genus probably belongs to a diffuse group of Macrostomidae that lack a sclerotized stylet, although currently it is unclear if these taxa form a monophyletic clade (24). These taxa might include *Myozona* Marcus 1949, *Siccomacrostomum* Schmidt and Sopott-Ehlers 1976, *Dunwichia* Faubel, Bloome and Cannon 1994, *Psammomacrostomum* Ax 1966, and *Antromacrostomum* Faubel 1974. Our species is clearly distinct from *Myozona* by the absence of a muscular gizzard in the gut, from *Siccomacrostomum* by the absence of a common genital opening, from *Dunwichia* by the presence of paired testes and ovaries, from *Psammomacrostomum* by the presence of a vagina and female antrum, and from *Antromacrostomum* by the absence of an armed pharynx and the presence of a paired ovary. However, the copulatory organ in our species is similar to that of *Psammomacrostomum equicaudatum*, and the close proximity of the male and female genital openings is similar to *Antromacrostomum armatum*. Thus, these three genera may be closely related. Our specimen (MTP LS 309) was collected at low tide from the typical location on July 22, 2007. From two additional specimens (MTP LS 55 and MTP LS 59), collected from the same location on April 9, 2006, we obtained partial sequences of 18S, which were identical to that of the main specimen. We plan to name this genus in honor of the late Reinhard M. Rieger, and a detailed taxonomic description will be presented elsewhere.

Macrostomum sp. nov. 1 has been collected repeatedly by our group from a single location in Lignano, Italy (45°41′28.7′′N, 13°07′54.3′′E). The sample location is among coarse algae-covered gravel, which lies on a strongly anoxic base of finer sediment. The species belongs to a group of *Macrostomum* species that are not easy to distinguish based on morphology alone, and therefore we cannot exclude the possibility that it corresponds to a previously described species. However, we can state clearly that it differs from the other species with a similar morphology that we have collected in Europe. It differs from *Macrostomum pusillum* Ax 1951 by the absence of long sensory cilia and droplets in the stylet, from *Macrostomum hystricinum marinum* Rieger 1977 by the absence of a separated tail plate, and from *Macrostomum hystrix* Ørsted 1843 sensu Luther 1905 (see below) by the absence of large testes. One characteristic of this *Macrostomum* species is that it can swim very fast through open water. Our specimen (MTP LS 302) was collected at low tide from the typical location on July 16, 2007.

Macrostomum hystricinum marinum Rieger 1977 was described from the west coast of the United States and the Mediterranean from sheltered beaches and shallow subtidal fine sand flats with salinity above 25‰ (25). It belongs to a group of *Macrostomum* species that are not easy to distinguish based on morphology alone. Our specimen (MTP LS 278) was collected on July 16, 2007, at low tide on an intertidal sand flat near Grado, Italy (45°42′51.7′′N, 13°23′06.0′′E), where this species was collected previously together with R. M. Rieger. The published 18S sequence of *Macrostomum hystricinum* (GenBank AF051329 from ref. 26) stemmed from a laboratory culture of *Macrostomum hystricinum marinum* from a population collected by R. M. Rieger on the west coast of the United States. That sequence, however, is somewhat distinct from that of our Mediterranean form.

Macrostomum pusillum Ax 1951 was described from fine sands in the intertidal zone of the North Sea coast of Germany (27). Ax and Armonies (28) list the distribution as North Sea, Baltic Sea, Atlantic Coast of Norway, Mediterranean, Black Sea, southeast Canada, and Alaska. Although it belongs to a group of *Macrostomum* species that are not easy to distinguish based on morphology alone, the identity of *Macrostomum pusillum* is

thought to be clear because of its long sensory cilia and droplets in the stylet. Our specimens were collected from two localities. One specimen (MTP LS 112, not documented) was taken from a laboratory culture that we are maintaining from specimens collected on April 8, 2006, at low tide on an intertidal sand flat in Lignano, Italy (around 45°41′30″N, 13°07′52″E). From an additional specimen (MTP LS 53) taken from the same sample location from which we founded the laboratory culture, we obtained a partial sequence of 18S, which was identical to that of the main specimen. Another specimen (MTP LS 132) was collected on March 7, 2007, at low tide on an intertidal sand flat near Rantum, Sylt, Germany (54°50′53.8″N, 8°17′54.4″E). Because the stylet of this sequenced specimen was not documented in much detail, we deposited an additional specimen (MTP LS 136) collected in the same sample. The specimens from Lignano and Sylt clearly are genetically distinct but currently cannot be distinguished based on their morphology. Because the type specimen of this species is from the North Sea, the Mediterranean form probably should be renamed.

Macrostomum balticum Luther 1947 was described from Tvärminne, Finland (13) based on material from Tor Karling. Luther (16) lists the Baltic Sea, the west coast of Sweden, and Sylt, Germany, as the distribution. Our specimen (MTP LS 144) was collected on March 7, 2007, at low tide on an intertidal sand flat near Rantum, Sylt, Germany (54°50′54.1″N, 8°17′55.0″E). It matches the descriptions of Luther (13, 16) in every detail studied.

Macrostomum spirale Ax 1956 was discovered by Schulz (29) from a sandy mudflat in Amrum, Germany. It was supposed to be described by Meixner, but his original account was never published because of the Second World War. The species later was described formally by Ax (30) from the Etang de Canet, near Perpignan, France. Ax (15) lists the distribution as North Sea, Baltic Sea, Channel Coast of England, Mediterranean, Black Sea, and Alaska. Our specimen (MTP LS 227, not documented) was taken from a laboratory culture that we established from specimens collected on March 7, 2007, from a water-covered salt marsh near Rantum, Sylt, Germany (54°50′50″N, 8°17′53″E). Because MTP LS 227 was not documented, we deposited an additional specimen (MTP LS 138) taken from the same sample location from which we founded the laboratory culture. We also obtained a partial 18S sequence from one additional specimen (MTP LS 1B) collected in Bibione, Italy (45°38′02″N, 13°04′32″E), which was identical to that of the main specimen.

Macrostomum longituba Papi 1953 was described from a small ditch near the sea in the San Rossore Park near Pisa, Italy (31). Our specimen (MTP LS 274) was collected on July 15, 2007, from a small drainage ditch in an agricultural area near Bibione, Italy (45°38′50.1″N, 13°01′10.7″E). These ditches are close to the mouth of the Tagliamento River and thus are quite variable in salinity. Our specimen matches the original description in every detail studied, except in the exact position of the opening in the tip of the stylet, which was drawn as a lateral hole, whereas our specimen suggests a sharp turn at the end of the stylet tip.

Macrostomum clavituba Ax 2008 was described from the brackish Etang de Salses, north of Perpignan, France (15). Our specimen (MTP LS 301) was collected on July 15, 2007, from the surface layer of relatively coarse sand at the high-tide level of a beach in the Laguna di Marano, Marano, Italy (45°45′23.0″N, 13°09′53.4″E). The sample location is unusual in that it consists of relatively clean sand in a highly protected area where wave action stems primarily from passing boats and fishing vessels. Because the stylet of this specimen was not documented in much detail, we deposited an additional specimen (MTP LS 514) collected from the same sample location on May 3, 2004. Moreover, from an additional specimen (MTP LS 1A, not documented), which also was collected from that sample, we obtained a partial sequence of 18S, which was identical to that of the main specimen. Our specimens match the original description in every detail studied.

Macrostomum gieysztori Ferguson 1939 was described originally by Ferguson (32) based solely on a drawing of the tip of a stylet of a freshwater species collected in rice fields in the La Albufera wetlands south of Valencia, Spain, by Gieysztor (33). This drawing matches the drawing of Ferguson (32) and our specimens very well. Gieysztor, without any justification, considered this species to correspond to *Macrostomum gracile* Pereyaslawzewa 1982, which is a marine species from the Black Sea and whose stylet bears no resemblance to the one depicted by Gieysztor. However, von Graff (34) describes a species from the same area that he calls "*Macrostomum gracile* von Graff 1905" which has a stylet somewhat more similar to that of Gieysztor's specimen but which does not match Pereyaslawzewa's specimen well. We therefore agree with Ferguson (21) that *Macrostomum gieysztori* is a separate species. Papi (35), based on a detailed study of the female antrum, moved *Macrostomum gieysztori* to the genus *Promacrostomum*, which is characterized by two female genital openings (36). Finally, Ferguson (37) moved the species to the new genus, *Axia*, to distinguish it from *Promacrostomum paradoxum* An-der-Lan 1939, which has a connection between the female antrum and the gut, a feature that is absent in this species. Given that our specimen clusters well within the genus *Macrostomum*, we suggest that the old name *Macrostomum gieysztori* be reinstated. Moreover, we note that the genus name *Axia* has been occupied by a genus of Lepidoptera since 1821 (38). Our specimen (MTP LS 264) was collected on July 1, 2007, from a captured source of the Rio Genal in Juzcar, Andalusia, Spain (36°37′37″N, 5°10′27″W). Because the female antrum of the genotyped specimen was not documented well and probably was in formation, we deposited an additional specimen (MTP LS 344) collected from the same site on March 30, 2008.

Macrostomum quiritium Kolasa 1973 originally was described from a basin in the Poznan palm house, in Poznan, Poland (39). In that paper Kolasa attributes the name *Macrostomum quiritium* to Beklemischev (40), who, however, never named such a species. Instead Beklemischev (40) described a variety of *Macrostomum japonicum* Okugawa 1930 from an aquarium of a Russian malaria research facility, which he called "*Macrostomum japonicum* var. *quiritium* Beklemischev 1951." However, neither *Macrostomum japonicum* (by the shape of the stylet) nor *Macrostomum japonicum* var. *quiritium* (by the arrangement of the seminal vesicles and the vesicula granulorum with respect to the stylet) matches the species described by Kolasa. *Macrostomum quiritium* has been collected repeatedly by our group from a small pond in the Tropenhaus of the Botanical Garden of the University of Basel, Switzerland (47°33′31.0″N, 7°34′54.5″E). This pond contains a great diversity of aquatic plants of worldwide tropical origin. It appears likely that this species was introduced with the plants. Our specimen (MTP LS 102) was collected from this pond on November 6, 2006. Our specimens match *Macrostomum quiritium* in many aspects studied, including the type of the collection site, but because of the lack of collections in the natural habitat and the resulting wide range of possible origins, our species identification should be regarded with caution.

Macrostomum tuba von Graff 1882 was described from a pond in the Botanical Garden of Munich, Germany (18). Moreover, there are worldwide reports of this or similar species, mostly from artificial ponds or aquaria. The different published 18S sequences of *Macrostomum tuba* (GenBank U70080 from ref. 41, called "U70081" in their paper, and D85091 from ref. 42) and *Macrostomum* sp. (GenBank L41127 from ref. 43) are all somewhat distinct from that of our specimen. No reference material for these specimens is available, and in some cases it is not clear where they were collected. The taxon *Macrostomum tuba* therefore is best regarded currently as an assemblage of dorsoventrally flattened fresh-water *Macrostomum* species with very long and

slender stylets about 200–300 μm in length and a worldwide distribution, and it probably also includes the species described as *Macrostomum tuba gigas* Okugawa 1930, *Macrostomum gigas* Okugawa 1930, and *Macrostomum bulbostylum* Ferguson 1939. *Macrostomum tuba* has been collected repeatedly by our group from small ponds in the Victoriahaus of the Botanical Garden of the University of Basel, Switzerland (47°33′32.7″N, 7°34′54.1″ E). Our specimen (MTP LS 261) was collected at this locality on June 26, 2007. Our specimen matches *Macrostomum tuba* in many aspects studied, including the type of the collection site. However, because of the lack of collections in the natural habitat, and because these ponds contain a great diversity of aquatic plants of worldwide origin, it is difficult to judge if the collected specimens match the type species.

Macrostomum finlandense (Ferguson 1940) was described originally as "*Macrostomum viride* Luther 1905" from freshwater in Lohja (Lojo), Southern Finland (44). It later was transferred to *Macrostomum ruebushi finlandensis* by Ferguson (45), then to *Macrostomum appendiculatum finlandensis* by Luther (pp. 11–14 in ref. 13), and finally to its current designation by Luther (pp. 72–73 in ref. 15). Moreover, the (sub)species name has been referred to variably as "*finlandense*," "*finnlandense*," or "*finlandensis*." Luther (16) lists Finland and Italy as the distribution, but other authors have reported it from Holland and Germany (46–48) and from Romania (49). Our specimen (MTP LS 91) was collected by Peter Ladurner from the Schwarzsee near Kitzbühel, Austria (47°27′22.9″N, 12°21′58.7″), on July 4, 2006. Because this specimen was not documented in much detail, we deposited an additional specimen (MTP LS 515) collected in the same sample. The specimens match the description by Luther (13, 16) in every detail studied.

Macrostomum kepneri (Ferguson and Jones 1940) was described originally by Ferguson and Jones (50) as "*Macrostomum ruebushi* var. *kepneri*" from brackish water in Norfolk, VA, and later was transferred to its current designation by Ferguson (37). Our specimen (MTP LS 285) was collected on July 15, 2007, from a small drainage ditch in an agricultural area near Bibione, Italy (45°38′34.5″N, 12°58′52.5″E), which is close to the Adriatic Sea and thus is quite variable in salinity. The specimen matches the original description in every detail studied. However, given the large distance between the type locality and our collection site, the species identity needs to be regarded with some caution.

Macrostomum lignano Ladurner, Schärer, Salvenmoser and Rieger 2005 was described from clean intertidal sand of the northern Adriatic Sea around Lignano, Italy (51). Our specimen (MTP LS 244, not documented) was taken from a laboratory culture from specimens collected on May 3, 2003, from the PS (45°42′14.2″N, 13°09′28.7″E) and on May 5, 2003, from the UV (45°38′2.6″N, 13°04′34.0″E) type localities respectively (see ref. 51 for a description of the PS and UV sample locations). Because the sequenced specimen was not documented, we deposited an additional specimen (MTP LS 517) taken from the PS location.

Macrostomum hystrix Ørsted 1843 sensu Luther 1905 was described by Ørsted (52) from the Baltic Sea and studied in detail by Luther (44) from samples collected in Tvärminne, Finland. It belongs to a group of *Macrostomum* species that are not easy to distinguish based on morphology alone, and as a result the species boundaries within this group often are unclear. Our molecular phylogeny, however, clearly reveals that *M. hystrix* is not closely related to the other species in clade 1 (e.g., *M. pusillum* and *M. hystricinum marinum*), despite its striking similarity in both male and female traits, which has caused even an expert like Luther to synonymize this species variably with *Macrostomum appendiculatum* (13) and *Macrostomum hystricinum* (16). However, Luther (44) gives an exquisite drawing of the stylet of his specimen (ref. 33, plate 4, fig. 1) that matches ours in every detail, and both the above names are taxonomically problematic. We therefore prefer to refer to our specimens as *Macrostomum hystrix* Ørsted 1843 sensu Luther 1905. Convergent evolution evidently can lead to very similar outcomes and trait simplifications, and we thus argue strongly against the future use of the genera *Inframacrostomum* and *Archimacrostomum*, as has been stressed repeatedly (53, 54), or the unwarranted erection of new genera based on minor morphological differences (see also *Macrostomum gieysztori*). Our specimen (MTP LS 68) was collected on April 10, 2006, from a drainage canal in an agricultural area near Bibione, Italy (45°39′16.2″N, 13°04′ 10.2″E). This canal is close to the mouth of the Tagliamento River and therefore is highly variable in salinity. From an additional specimen (MTP LS 1G, not documented), collected on April 1, 2005, from a nearby sample location (45°38′33.5″N, 12°58′52″E), we obtained a partial sequence of 18S, which was identical to that of the main specimen. Because the main specimen was not documented in much detail, we deposited an additional specimen (MTP LS 292), collected from the second site on July 15, 2007.

Macrostomum mystrophorum Meixner 1926 was described briefly by Meixner (55) from moss in a fresh-water spring in the Steiermark, Austria, based primarily on the morphology of the stylet. A more detailed description was given by Papi (31) from a flooded zone near the sea in the San Rossore park near Pisa, Italy (variable salinity, ~5‰). Our specimen (MTP LS 64) was collected on April 10, 2006, from a small drainage ditch in an agricultural area near Bibione, Italy (45°39′16.5″N, 13°04′11.9″ E). This ditch is close to the mouth of the Tagliamento River and therefore is highly variable in salinity. At the time of collection the salinity was about 12‰. Because the main specimen was not documented in much detail, we deposited an additional specimen (MTP LS 516) collected in the same sample. Our specimens match the description by Papi (31) in every detail studied.

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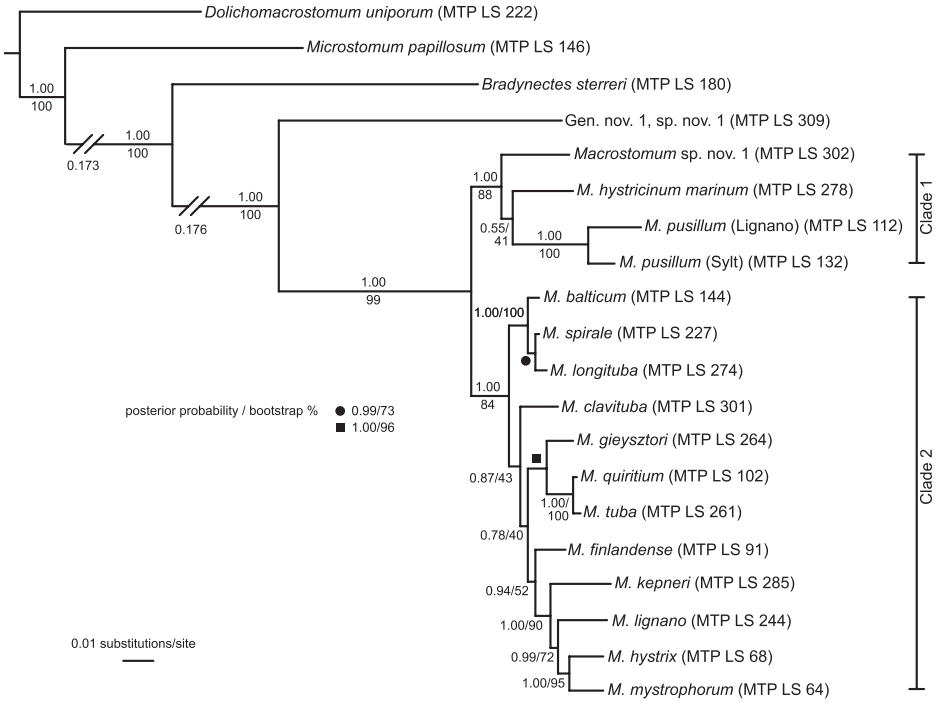


Fig. S1. Molecular phylogeny of 16 *Macrostomum* and four outgroup species. ML tree based on combined partial lsrDNA and complete ssrDNA sequences (for a total of ~2,850 base pairs) from 16 *Macrostomum* and four outgroup species, covering members of all three families in the order Macrostomida (Platyhelminthes: Macrostomorpha). Values above branches are Bayesian posterior probabilities, and values below branches are ML bootstrap values. The topologies of trees derived from Bayesian and ML analyses are in broad agreement. Final ML model settings were as follows: nucleotide frequencies [π (A) = 0.2334; π (C) = 0.2233; π (G) = 0.2894; π (T) = 0.2538]; rate matrix [(A,C) = 0.6007; (A,G) = 3.6492; (A,T) = 2.1686; (C,G) = 0.4266; (C,T) = 7.9278; (G,T) = 1.0000]; invariable sites = 0.5247; γ shape parameter = 0.4968; log likelihood = −11679.817. The accession code identifies the morphological documentation of each sequenced specimen, which we have deposited as digital reference material at http://macrostomorpha.info (GenBank accession numbers are given in Table S3). Details on phylogenetic reconstruction are given in *SI Materials and Methods*.

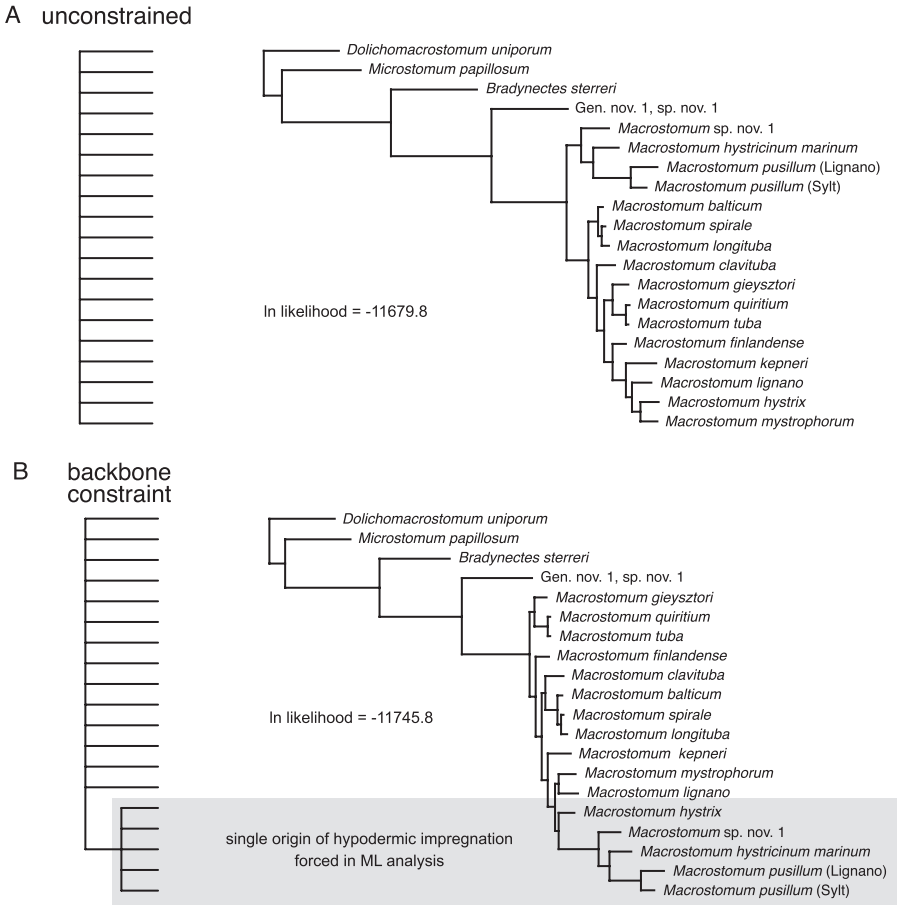
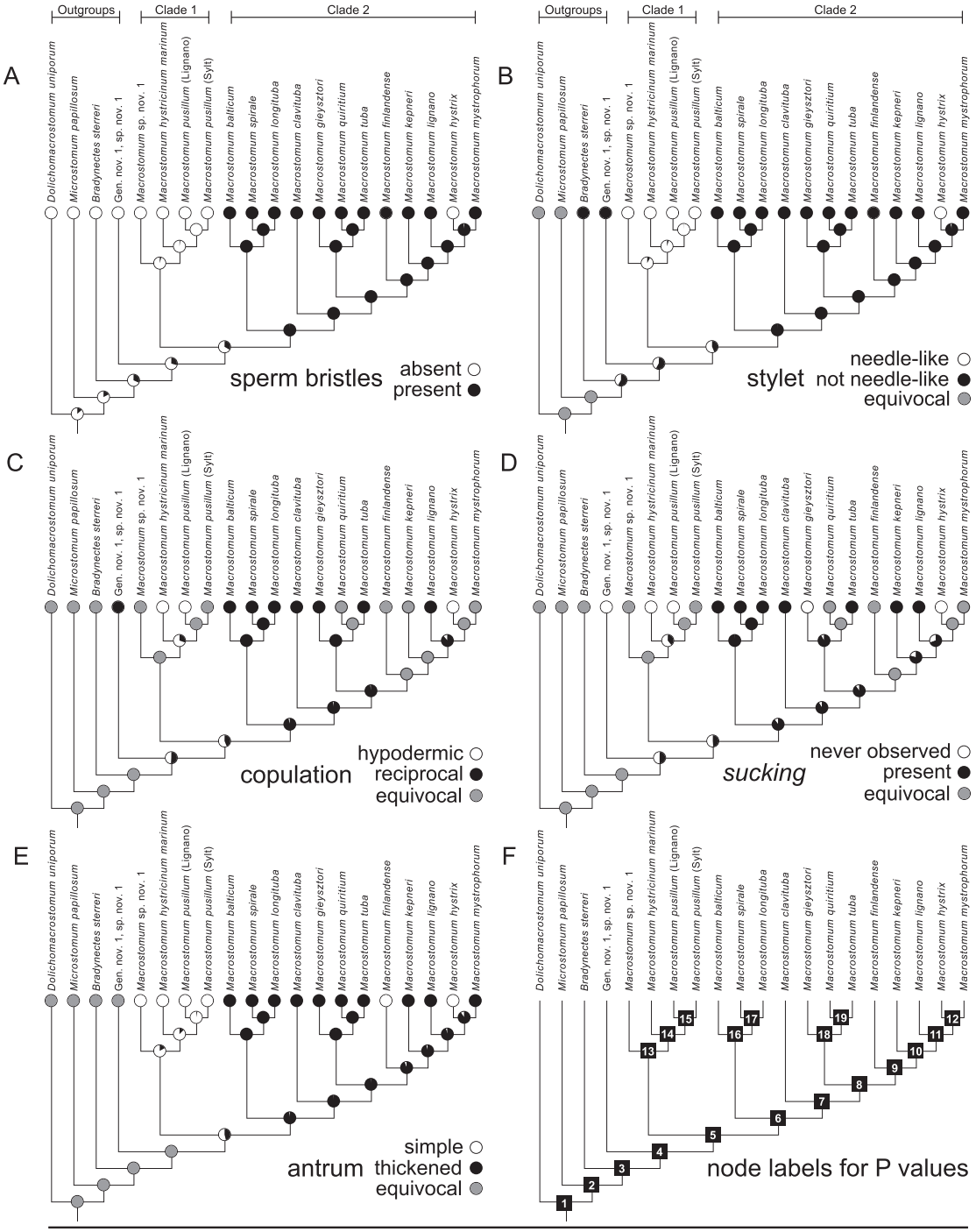


Fig. S2. Unconstrained and constrained tree used for the Shimodaira–Hasegawa test. The test shows that the unconstrained tree (*A*) fits the data significantly better (Δ −ln likelihood = 66.0; $P < 0.001$) than the constrained tree (*B*). Details of analysis are given in *SI Materials and Methods*.



G

	Probability of the black character state at each node in (f); e - equivocal																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
bristles	0.13	0.17	0.31	0.29	0.33	0.99	1.00	1.00	1.00	1.00	1.00	1.00	0.04	0.02	0.00	1.00	1.00	1.00	1.00
stylet	e	e	0.58	0.57	0.43	0.99	1.00	1.00	1.00	1.00	1.00	0.96	0.08	0.05	0.00	1.00	1.00	1.00	1.00
copulation	e	e	e	0.51	0.44	0.96	0.97	0.97	e	e	0.88	e	e	0.30	e	1.00	1.00	1.00	e
sucking	e	e	e	0.50	0.49	0.90	0.91	0.90	e	0.79	0.69	e	e	0.39	e	0.99	1.00	0.91	e
antrum	e	e	e	e	0.46	0.97	0.99	0.99	0.94	0.96	0.95	0.90	0.02	0.12	0.03	1.00	1.00	0.99	1.00

Fig. S3. ML ancestral state reconstruction of character states. The small pie charts indicate the likelihoods of the black vs. white character states at each node, and gray nodes indicate equivocal character states. (*A*) Sperm bristles. (*B*) Stylet morphology. (*C*) Copulation behavior. (*D*) Sucking behavior. (*E*) Female antrum morphology. (*F*) Phylogeny with ancestral nodes numbered. (*G*) Probabilities (*P* values) for the black character state at each node. Details on analysis are provided in *SI Materials and Methods*.

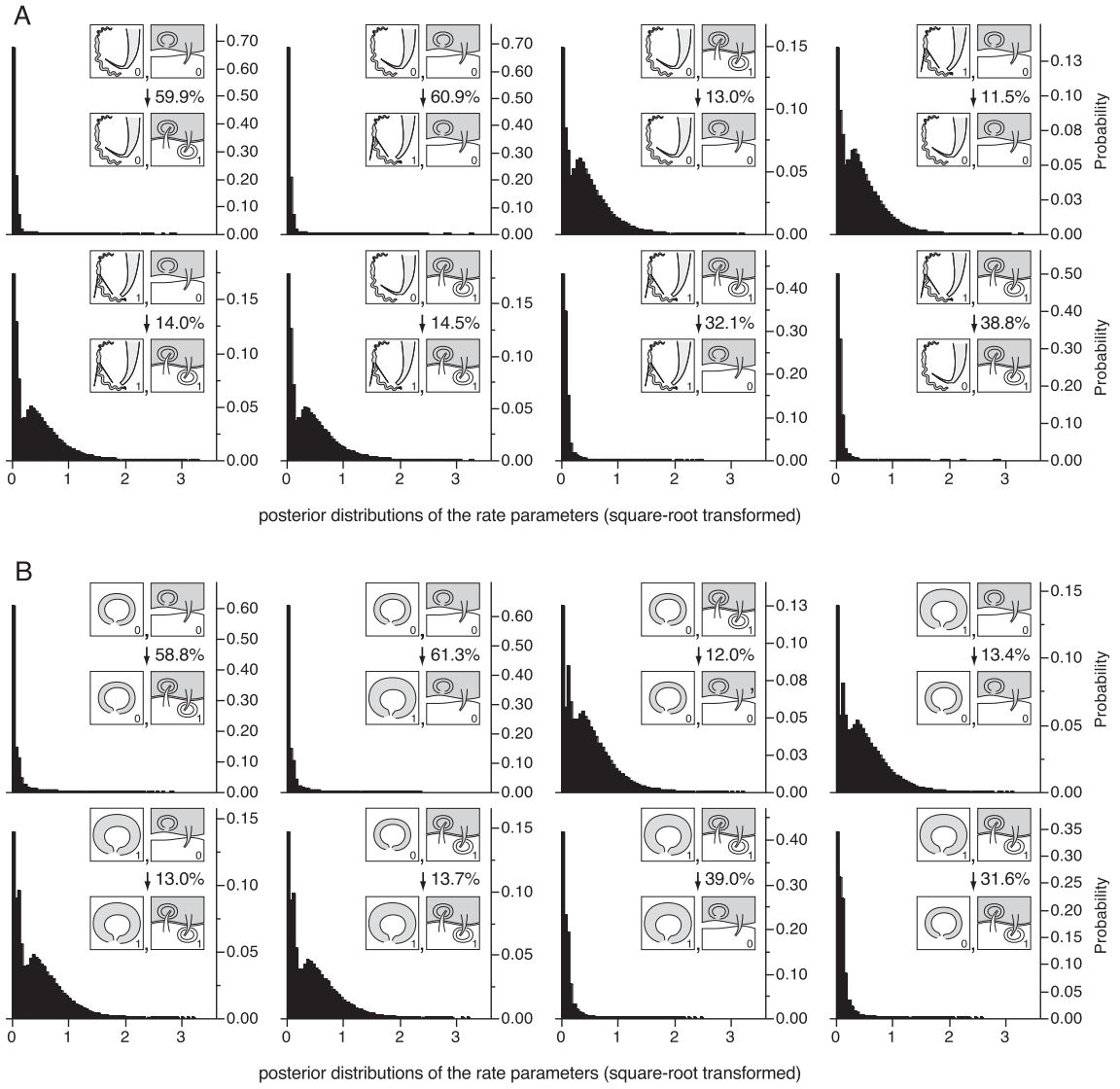


Fig. S4. Posterior distributions of the rate parameters of the models of character state evolution. Graphs are based on 2.5×10^5 observations drawn from 10^9 iterations of the Markov chain. (*A*) Correlated evolution between the sperm/stylet morphology and the copulation behavior. (*B*) Correlated evolution between the female antrum morphology and the copulation behavior. The percentages indicate the proportion of the rate parameter estimates that are zero, with higher values indicating less likely transitions. The panels are arranged so that vertical pairs correspond to rates that would be expected to be the same if the independent model of character evolution were true, which is never the case in our data. For example, in the first pair of analysis A, the transition from hypodermic to reciprocal mating is more likely when bristles are present than when they are not (i.e., the rate parameter estimate is zero in 14% and 59.9% of the iterations, respectively). Details on analysis are provided in *SI Materials and Methods*.

Table S1. Character states of all species studied

Species	Bristles	Stylet	Copulation	Sucking	Antrum
Dolichomacrostomum uniporum	0[a]	ND[f]	ND[l]	ND[l]	ND[r]
Microstomum papillosum	0[b]	ND[g]	ND[m]	ND[m]	ND[s]
Bradynectes sterreri	0[c]	1[h]	ND[n]	ND[n]	ND[t]
Gen. nov. 1, sp. nov. 1	0	1[i]	1	0	ND[u]
Macrostomum sp. nov. 1	0	0	ND	ND	0
Macrostomum hystricinum marinum	0	0	0[o]	0	0
Macrostomum pusillum (Lignano)	0	0	0	0	0
Macrostomum pusillum (Sylt)	0[d]	0	ND	ND	0
Macrostomum balticum	1	1	1	1	1
Macrostomum spirale	1	1	1	1	1
Macrostomum longituba	1	1	1	1	1
Macrostomum clavituba	1	1	1	1	1
Macrostomum gieysztori	1	1	1	0[p]	1
Macrostomum quiritium	1	1	ND	ND	1
Macrostomum tuba	1	1	1	1[q]	1
Macrostomum finlandense	1[e]	1[j]	ND	ND	0
Macrostomum kepneri	1	1[k]	ND	1	1
Macrostomum lignano	1	1	1	1	1
Macrostomum hystrix	0	0	0	0	0
Macrostomum mystrophorum	1	1	ND	ND	1

Sperm bristles (0, absent; 1, present), stylet morphology (0, needle-like; 1, not needle-like), copulation behavior (0, hypodermic; 1, reciprocal), sucking behavior (0, never observed; 1, present); female antrum morphology (0, simple; 1, thickened); ND, no data.

[a]ND for *D. uniporum*; character state based on data for other Dolichomacrostomidae, namely *Paromalostomum fusculum* (1, 2) and *P. atratum* (3).

[b]ND for *M. papillosum*; character state based on data for other Microstomidae, namely *M. spiculifer* (4) and Microstomidae (5).

[c]Sopott-Ehlers and Ehlers (2) state, "[T]he two lateral ledges found in spermatozoa of *B. sterreri* are discussed to correspond to the pair of 'lateral bristles' known from *Macrostomum* species," but even if they were homologous, these structures do not protrude outside of the sperm and thus cannot have a sperm anchoring function.

[d]Sperm ultrastructure suggests putative rudimentary bristles (6).

[e]Bristles are small but clearly visible.

[f]Homology is unclear; many Dolichomacrostomidae have two stylets, a penis stylet and a gland stylet, the latter of which can be needle-like.

[g]Stylet shape very variable within the Microstomidae.

[h]Stylet tip opening is oblique, not needle-like.

[i]Has no stylet but has a fleshy cirrus, which is representative of a number of presumably related genera (*SI Text*).

[j]Stylet tip opening is oblique, not subterminal; lacks distal thickening.

[k]stylet tip has a tapering, flexible flap.

[l]No data on mating behavior exist for any Dolichomacrostomidae.

[m]No data on mating behavior exist for any Microstomidae.

[n]No data on mating behavior exist for any *Bradynectes* species.

[o]Not observed directly but inferred from the presence of sperm in the parenchyma.

[p]*M. gieysztori* has two female genital openings, perhaps explaining the absence of the postcopulatory sucking behavior.

[q]*M. tuba* is the largest *Macrostomum* species in our dataset and shows a behavior that may correspond to the sucking behavior but which looks somewhat different because of the large size of the worms.

[r]Homology is unclear; the Dolichomacrostomidae have a common (male and female) genital opening and atrium genitale (7).

[s]Homology is somewhat unclear (8).

[t]Lacks a vagina and female antrum (8, 9); mechanism of sperm transfer is unclear.

[u]Structure of female antrum is unclear.

1. Rohde K, Faubel A (1997) Spermatogenesis in *Macrostomum pusillum* (Platyhelminthes, Macrostomida). *Invertebr Reprod Dev* 32:209–215.
2. Rieger RM (1971) Die Turbellarienfamilie Dolichomacrostomidae Rieger: II. Teil. Dolichomacrostominae 1. *Zool Jb Syst* 98:598–703.
3. Faubel A (1974) Macrostomida (Turbellaria) von einem Sandstrand der Nordseeinsel Sylt. *Mikrofauna Meeresboden* 45:1–32.
4. von Graff L (1882) *Monographie der Turbellarien. I. Rhabdocoelida* (Willhelm Engelmann, Leipzig, Germany). 420 plates.
5. Rohde K, Faubel A (1997) Spermatogenesis in *Macrostomum pusillum* (Platyhelminthes, Macrostomida). *Inv Rep Dev* 32:209–215.
6. Rieger RM (1971) Die Turbellarienfamilie Dolichomacrostomidae nov. fam. (Macrostomida): I. Teil, Vorbemerkungen und Karlingiinae nov. subfam. 1. *Zool Jb Syst* 98:236–314.
7. Rieger RM (2001) Phylogenetic systematics of the Macrostomorpha. *Interrelationships of the Platyhelminthes*, eds Littlewood DTJ, Bray RA (Taylor & Francis, New York), pp 28–38.
8. Rieger RM (1971) *Bradynectes sterreri* gen nov., spec. nov., eine psammobionte Macrostomide (Turbellaria). *Zool Jb Syst* 98:205–235.

Table S2. Primers used for amplification and sequencing

lsrDNA primers		
PCR and sequencing primers		
ZX-1[a]	F	ACCCGCTGAATTTAAGCATAT
1200R	R	GCATAGTTCACCATCTTTCGG
1500R	R	GCTATCCTGAGGGAAACTTCG
Additional sequencing primers		
300F	F	CAAGTACCGTGAGGGAAAGTTG
ECD2	R	CTTGGTCCGTGTTTCAAGACGGG
1090F	F	TGAAACACGGACCAAGG
ssrDNA primers		
PCR and sequencing primers		
WormA	F	GCGAATGGCTCATTAAATCAG
WormB	R	CTTGTTACGACTTTTACTTCC
Macro_18S_200F	F	GGCGCATTTATTAGATCAAAACCA
Macro_18S_1640R	R	GCAAGCCCCGATCCCTGTC
Additional sequencing primers		
300F	F	AGGGTTCGATTCCGGAG
600R	R	ACCGCGGCKGCTGGCACC
1270F	F	ACTTAAAGGAATTGACGG
1270R	R	CCGTCAATTCCTTTAAGT
1200F	F	CAGGTCTGTGATGCCC

All primers are 5′–3′. F, forward; R, reverse.
[a]Modified from the original ZX-1 (1): ACCCGCTGAAYTTAAGCATAT; Y replaced with T.

1. Van der Auwera G, Chapelle S, De Wachter R (1994) Structure of the large ribosomal subunit RNA of *Phytophthora megasperma*, and phylogeny of the oomycetes. *FEBS Lett* 338: 133–136.

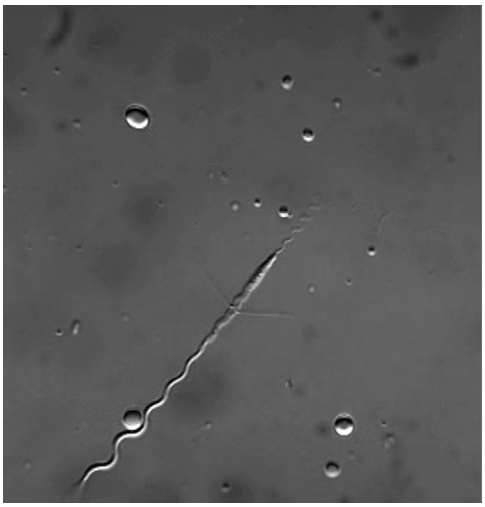
Table S3. GenBank accession numbers for each taxon and gene

	GenBank accession	
Taxon	lsrDNA	ssrDNA
Dolichomacrostomum uniporum (MTP LS 222)	FJ715315	FJ715295
Microstomum papillosum (MTP LS 146)	FJ715316	FJ715296
Bradynectes sterreri (MTP LS 180)	FJ715318	FJ715298
Gen. nov. 1, sp. nov. 1 (MTP LS 309)	FJ715317	FJ715297
Macrostomum sp. nov. 1 (MTP LS 302)	FJ715332	FJ715312
Macrostomum hystricinum marinum (MTP LS 278)	FJ715331	FJ715311
Macrostomum pusillum (Lignano) (MTP LS 112)	FJ715333	FJ715313
Macrostomum pusillum (Sylt) (MTP LS 132)	FJ715334	FJ715314
Macrostomum balticum (MTP LS 144)	FJ715330	FJ715310
Macrostomum spirale (MTP LS 227)	FJ715328	FJ715308
Macrostomum longituba (MTP LS 274)	FJ715329	FJ715309
Macrostomum clavituba (MTP LS 301)	FJ715324	FJ715304
Macrostomum gieysztori (MTP LS 264)	FJ715321	FJ715301
Macrostomum quiritium (MTP LS 102)	FJ715319	FJ715299
Macrostomum tuba (MTP LS 261)	FJ715320	FJ715300
Macrostomum finlandense (MTP LS 91)	FJ715322	FJ715302
Macrostomum kepneri (MTP LS 285)	FJ715327	FJ715307
Macrostomum lignano (MTP LS 244)	FJ715326	FJ715306
Macrostomum hystrix (MTP LS 68)	FJ715323	FJ715303
Macrostomum mystrophorum (MTP LS 64)	FJ715325	FJ715305

Species are listed in the order in which they appear in the tree. The MTP accession code identifies the morphological documentation of each sequenced specimen (http://macrostomorpha.info). All sequences are new for this study.

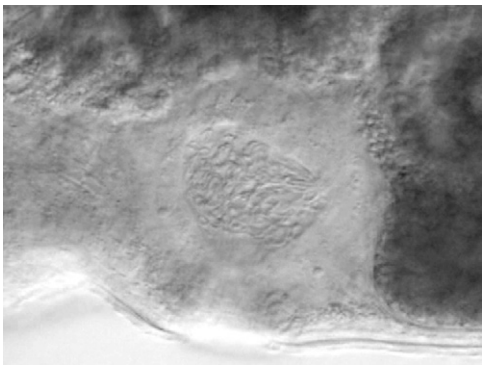
Movie S1. A copulating pair of the flatworm *Macrostomum lignano*. Note that one individual performs the postcopulatory sucking behavior, after which a bundle of sperm shafts can be seen sticking out of the female genital opening.

Movie S1



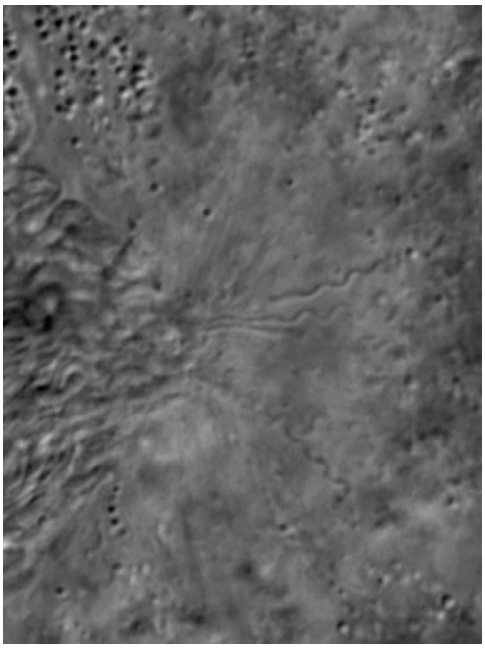
Movie S2. A single sperm of the flatworm *Macrostomum lignano*. Note the highly motile feeler and shaft, which allow the sperm to perform complex movements.

Movie S2



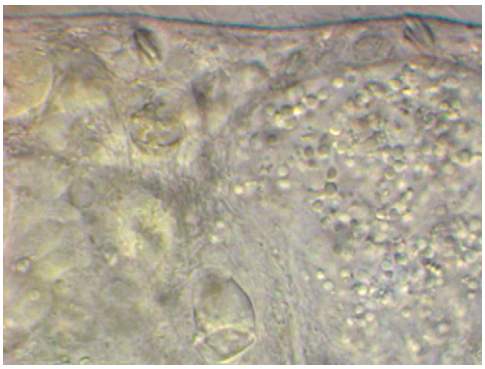
Movie S3. Anchored received sperm in a live specimen of the flatworm *Macrostomum lignano*. Note the thickened epithelium of the female antrum (i.e., the translucent rim around the sperm) and the polarized nature of the sperm, most of which are anchored in the cellular valve (i.e., the part of the antrum epithelium closest to the forming oocyte, which is the dark area on the right).

Movie S3



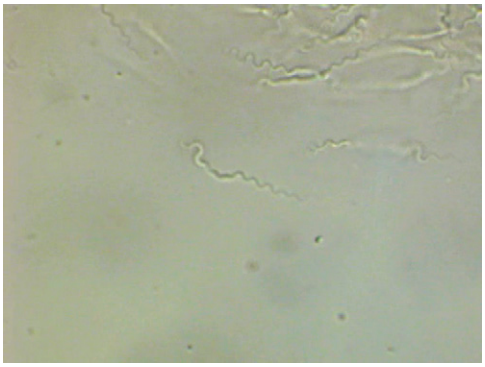
Movie S4. Detail of the anchored sperm of the specimen in Movie S3. Note the undulating sperm feelers, which are deeply embedded in the cellular valve.

Movie S4



Movie S5. Focusing through the parenchyma of a live specimen of the flatworm *Macrostomum hystrix*. Note the abundant hypodermically inseminated and highly motile sperm.

Movie S5



Movie S6. Sperm of the flatworm *Macrostomum hystrix*. Note the highly motile feeler and shaft, and the lack of bristles and brush.

Movie S6