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# A new model organism among the lower Bilateria and the use of digital microscopy in taxonomy of meiobenthic Platyhelminthes: *Macrostomum lignano*, n. sp. (Rhabditophora, Macrostomorpha)

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## Abstract

*Macrostomum lignano* n. sp. is a member of the Macrostomorpha, the basal-most subtaxon of the Platyhelminthes–Rhabditophora. This new species can be easily cultured in the laboratory and has been already the subject of several developmental/evolutionary studies. The small size, with only about 25 000 cells constituting the major bilaterian organ systems, makes this simultaneous hermaphrodite a possible candidate for a new model organism that is phylogenetically more basal than any of the model organisms currently used in such studies within the Bilateria. *M. lignano* belongs to the largest genus of the Macrostomorpha. Over 100 marine, fresh water and brackish water species are contained in the genus *Macrostomum*, some of them with worldwide distribution pattern. Within it, *M. lignano* is a member of the *M. tuba*-species group, which we have summarized here. In the species description, we have used a novel approach to document such small soft-bodied meiobenthic organisms: we provide extensive digital micrographical documentation, which are deposited as a CD together with the type material.

**Key words:** Development – evolution – hermaphroditism – reproduction – systematics – Turbellaria

## Introduction

An understanding of the developmental biology of basal Bilateria has been hindered by the lack of a suitable laboratory model system. Since *Caenorhabditis elegans* and *Drosophila melanogaster* appear derived in several aspects, a more basal organism would be preferable for many fundamental questions. Such organisms could be among the Acoelomorpha (i.e. the Nemertodermatida and Acoela), the Catenulida, and the Rhabditophora (i.e. a taxon including the main groups of the Platyhelminthes, Ehlers 1985). There is considerable recent debate over the phylogenetic position of the Platyhelminthes (Baguña and Riutort 2004b, and references therein). For example, a recent review argued that the Acoelomorpha are at the base of the Bilateria, and the Catenulida and the Rhabditophora at the base of the Lophotrochozoa (Baguña and Riutort 2004a). Given this ongoing debate we believe that it is more important than ever to add suitable model organisms to these understudied clades, in order to more fully understand their evolution and development (Rieger et al. 1994; Ladurner et al. 1997, 2000; Gschwentner et al. 2001; Rieger and Ladurner 2003; Morris et al. 2004).

Only among the Rhabditophora are there established laboratory model systems, such as the planarians (e.g. Baguña 1998; Sánchez Alvarado and Newmark 1999; Newmark and Sánchez Alvarado 2002), which due to their derived embryonic development, and to their large size and dispersed organ systems, are not ideal for addressing certain questions. In addition, molecular evidence confirms that this group is not basal within the Rhabditophora (Jondelius et al. 2002). The microturbellaria would be more suitable in this regard, and they have clearly defined organ systems. Moreover, they are small and transparent, a feature that allows to study most questions on whole-mounts, instead of tissue samples.

Suitable model organisms that can be cultured easily and reliably have been searched for among the Acoela (Apelt 1969) and the basal rhabditophoran taxon Macrostomida (Tyler 1981; Rieger et al. 1988). Culturing has so far been successful only with diatom feeding species. Best results were obtained with the cosmopolitan genus *Macrostomum*, and particularly with *M. hystricinum marinum* and *M. pusillum* from the Northern Atlantic (Gelhen and Lochs 1990; Rieger et al. 1991a, 1994, 1999; Mair et al. 1996; Reiter et al. 1996; Ladurner et al. 1997).

In the Northern Adriatic, Italy, we have now found an ideal model organism, which is well suited for studying development and evolution of lower Bilateria. The main aim of this paper is to formally describe this new species, which at present is being cultured in several laboratories. Recent publications on the developmental and reproductive biology of this species (e.g. Ladurner et al. 2000, 2005; Peter et al. 2001, 2004; Salvenmoser et al. 2001; Nimeth et al. 2002, 2004; Schärer and Ladurner 2003; Bebenek et al. 2004; Morris et al. 2004; Schärer et al. 2004a,b, 2005) were to this date referring to a brief, preliminary description of it in Ladurner et al. (2000), which includes a line drawing where the majority of organs and structures are identified (a schematic sagittal section depicting the main tissue types can be found in Ladurner et al. 2005). In addition, we aim at presenting a novel approach for the taxonomy of meiobenthic Platyhelminthes, namely the use of digital video microscopy. This technique allows to produce high quality pictures of freshly extracted specimens, which can then be shared with other researchers.

## Materials and Methods

### Collection methods

We used standard collection and extraction techniques (Cannon and Faubel 1988). Sediment samples were collected with spoons and brought to the laboratory in small vials. Subsampling was facilitated by suspending the sediment in a seawater and 7% $MgCl_2$-solution to anaesthetize the organisms which were then extracted via sieving through 63 or 100 μm nylon nets. Nets were placed in petri dishes with fresh seawater, and animals collected from above or below the nets

under a dissecting microscope. Recovered animals were squeezed between a microscope slide and cover slip, and observed under Leitz Diaplan or Reichert Polyvar compound microscopes.

### Digital documentation

We have used a novel approach to describe freshly extracted microturbellaria. Classical descriptions of microturbellaria primarily rely on schematic drawings, whole mount preparations, and reconstructions from serial sections (for Macrostomida see e.g. Ferguson 1939a–e, 1940a–c; Luther 1947, 1960; Ax 1951a,b, 1956, 1959; Papi 1951, 1959; Rieger and Sterrer 1968; Rieger 1971a–c; Schmidt and Sopott-Ehlers 1976). All of these approaches do not describe the living organism well, and extensive photomicrographic documentation used to be unpractical and resulting pictures used to be difficult to share with other researchers because of high printing costs. We have solved this by taking an interference contrast compound microscope, a digital microscope video camera, and a computer in to the field, which allowed us to take detailed digital micrographs of freshly extracted microturbellaria. These pictures can be used in print (as we do below), deposited with the type material in digital form (which we did here), made available via the Internet (which we are planning to do), and shared with other researchers in an unprecedented way. Due to the fragile nature of most microturbellaria there is a long tradition of work with life animals (see references above). The new technology allows to acquire and store accurate digital micrographs of such observations for other investigators.

Freshly squeezed worms were observed with a Leitz Diaplan compound microscope. We then took digital micrographs of at magnifications of 40× to 1000×. The set-up included (1) a c-mount connector to the microscope; (2) a digital c-mount video camera (Sony DFW-X700) which was connected to the (3) Apple PowerMacintosh G4/450 (running MacOS 9.2) or Apple PowerBook G4 (running MacOS 10.2) via the built-in FireWire connection (Apple, Cupertino, CA, USA). In order to acquire the images sent by the camera to the computer we used the shareware image capture software BTV Pro (available on the Internet at http://www.bensoftware.com). Under this set-up the camera delivers colour (24-bit) or greyscale (8-bit) images at a resolution of $1024 \times 768$ pixels, which is sufficient to resolve structures in detail. This set-up is preferable to higher resolution cameras, as it delivers a full resolution preview on the computer screen at 15 frames per second, which greatly facilitates focusing, and allows capturing good pictures even of moving specimens.

### Localities

*Macrostomum lignano* was first found in 1995 in samples that had been collected near a pumping station, which pumps rainwater from the drainage canal system behind the lagoonal dike into the Laguna di Marano, Lignano, Italy, Adriatic Sea (Fig. 1, site P1, 45.69180°N, 13.13123°E). Collections had been taken between high-tide and mid-tide level in the upper few millimetres of a thinly oxidized surface layer of the sediment of the highly sheltered beach. The first specimens were observed in laboratory cultures of *Macrostomum pusillum* started from these collections. Soon all cultures of *M. pusillum* were overgrown by *M. lignano*. Within a few month we could establish this species as the so far best growing *Macrostomum*-species in our laboratory, and cultures have been successfully kept since then and have in the meantime been established in a number of other laboratories.

In spring and summer 2002, we could collect a few individuals of the same species near the original collection site (i.e. site P1) and also in relatively clean sand just below mid-tide level of the outer, seaward beach of Isola di Martignano (locally known as Isola delle Conchiglie, Fig. 1, site X, 45.62469°N, 13.15916°E). The species thus appears to occur at salinities between full strength seawater (in the northern Adriatic about 32‰) and rarely at lower salinities of about 20‰.

In spring 2003, we finally found two sites with large quantities of specimens: first on Isola di Martignano close to a sea wall 50 m from the beach and probably only covered during very high tides (Fig. 1, site PS, 45.62233°N, 13.15444°E, see photographs on the Digital Type Material CD submitted with the type material); a second near Bibione at the edge of a lagoon that probably also only fills during spring tides (Fig. 1, site UV, 45.69042°N, 13.08197°E). Finally, based on the stylet morphology only, the same or a very similar species was found in 2002 at two sites on the Sithonia Peninsula, Greece, by Tom Artois and Ernest Schockaert, which they made available to us as lactophenol embedded whole-mount preparation (see Fig. 8b).

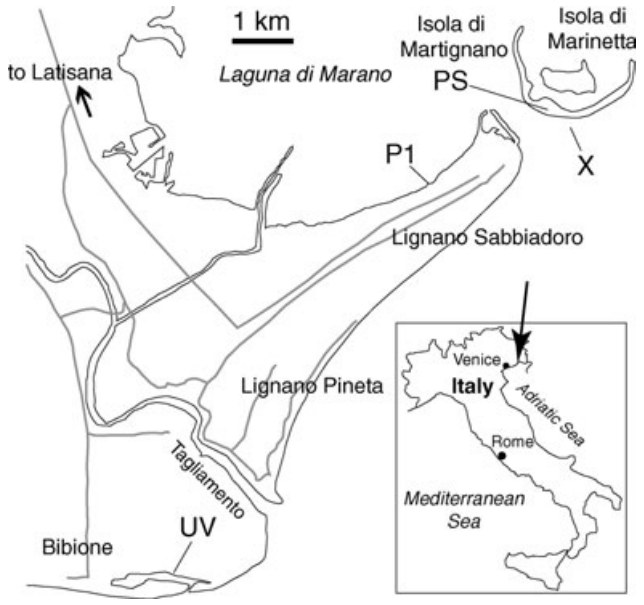


Fig. 1. Map of the type localities of *Macrostomum lignano*. Photographs of the sites P1, PS, and UV are included on the Digital Type Material CD submitted together with the other type material

### Serial sections

Worms were anaesthetized, and the following preparation procedures were used: (1) fixation in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) containing 10% sucrose. After rinsing in 0.1 M PBS, worms were postfixed in 1% osmium tetroxide in 0.05 M PBS. After rinsing again, they were dehydrated in a standard ethanol series and embedded in Spurr's low viscosity resin (Spurr 1969). Resin blocks were serially sectioned on a Leica Autocut 2040 with Ralph knives (section thickness was 0.5 or 1.0 μm). A glue (Pattex, Henkel, Germany) was used to obtain banding of sections. Serial sections were stained according to Richardson (Richardson et al. 1960) and mounted in Cedarwood Oil; (2) fixation in hot Bouin's solution without Osmium postfixation, subsequent treatment as above; (3) following a standard glutaraldehyde-osmium tetroxide double fixation, animals were embedded in Epon/Araldite (after Mollenhauer) and sections were stained with Haidenhain's iron haematoxylin according to the protocol of Smith and Tyler (1984).

## Results

### Species description – Genus *Macrostomum* Beneden E, 1870 – *Macrostomum lignano* n. sp.

*Holotype*

One serial sagittal section deposited at the Natural History Museum Vienna (inventory number 4578) (Figs 2–8; Table 1).

*Paratypes*

(1) Two sets of serial sagittal sections (inventory numbers 4579 and 4580); (2) five whole mounted animals fixed with formalin-glycerol and embedded in glycerine (inventory numbers 4581–4585); (3) 13 specimens fixed after Eisenmann and

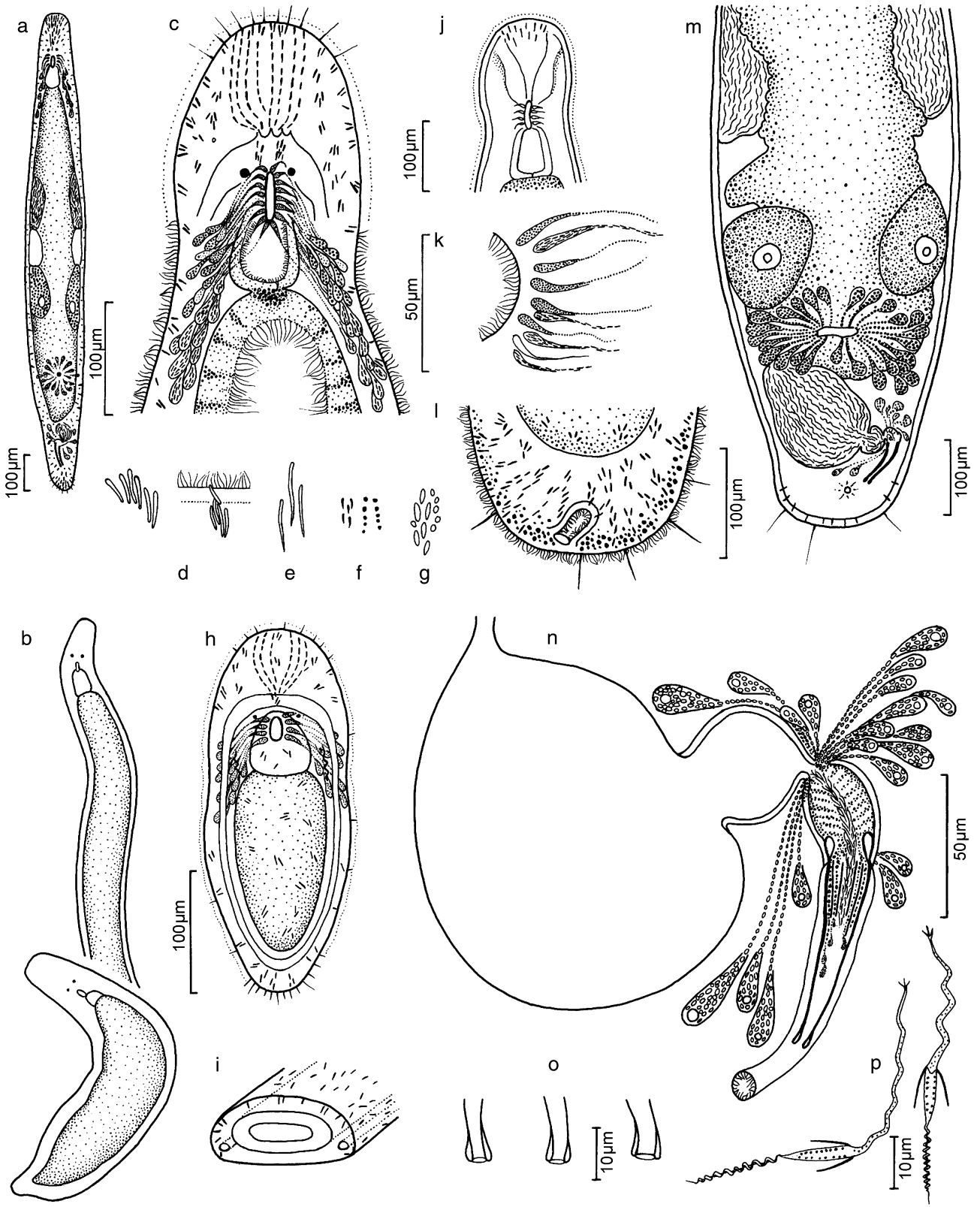


Fig. 2. Line drawings of living specimens of *Macrostomum lignano* from cultures examined between 1997 and 2001. (a) Habitus of the animal (ventral view, unsqueezed); (b) animal swimming off after having adhered with the tail plate; (c) anterior portion of the animal (dorsal view, slightly squeezed); (d) rhabdites (detail and situation in epidermis); (e) rhammites; (f) two kinds of granules in the glands in the pharyngeal gland ring; (g) shell and cement gland granules; (h) hatchling; (i) diagram of the mid body of the hatchling shown in (h) to illustrate the predominantly dorsal distribution of the Rhabdites; (j) rostrum and pharynx, with ventral furrows on rostrum for channelling food towards mouth opening (ventral view, unsqueezed); (k) gland ring of Pharynx around mouth opening (ventral view, squeezed); (l) caudal end with not well separated tail plate of subadult specimen; (m) caudal portion of mature animal (dorsal view, slightly squeezed); (n) male copulatory apparatus (dorsal view, squeezed), spermatozoa in the seminal vesicles not shown; (o) variation in the distal tips of the copulatory stylet; (p) spermatozoa

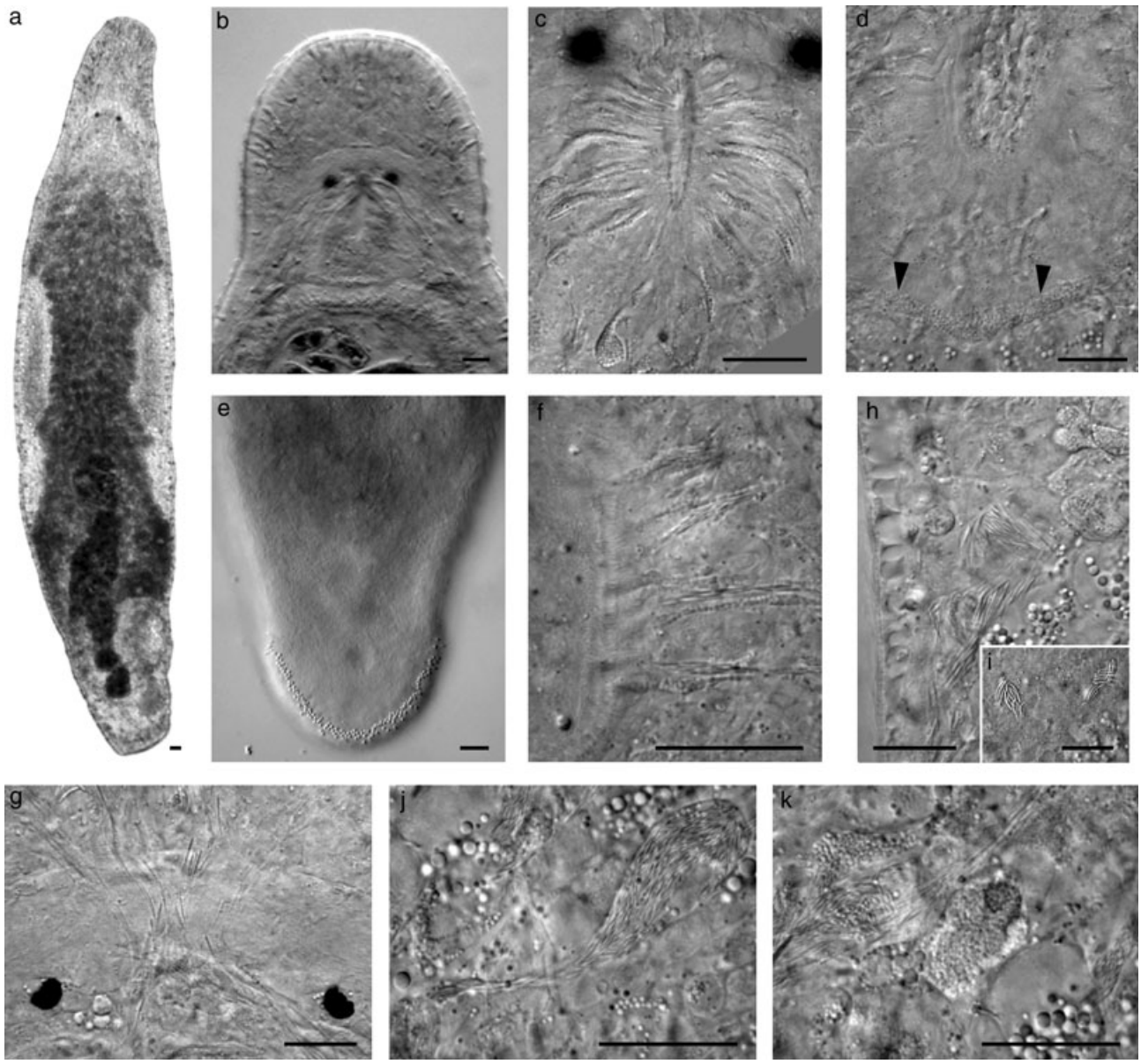


Fig. 3. Digital interference contrast micrographs of the somatic structures of living *Macrostomum lignano* from the cultures (the names in the brackets denote the deposited file names on the Digital Type Material CD submitted together with the other type material). (a) Overview of an adult worm (Mlig A 1). Note the somewhat darker appearance of the testis due forming sperm; (b) rostrum with brain, eyes and pharynx (Mlig G 9); (c) mouth with pharynx glands (Mlig G 31); (d) glandular girdle connecting pharynx and gut (Mlig H 19), see arrowheads; (e) tail plate with adhesive glands (Mlig H 11); (f) pharyngeal glands with two kinds of granules (Mlig H 21); (g) rhammites through brain (Mlig F 35); (h) rhammite gland bodies (Mlig F 36); (i) rhabdites (Mlig F 38); (j) pharyngeal gland body with long granules (Mlig H 22); (k) pharyngeal gland body with round granules (Mlig H 23). Scale bar 20 μm

Alfert (1982) and embedded in Spurr for TEM (inventory number 4586); (4) DNA samples of 100 whole individuals in 100% ethanol in 10 batches of 10 (inventory numbers 19895–19904); (5) three copies of the Digital Type Material CD containing series of digital microphotographs of eight individuals and detailed information on type locality (inventory number 4587).

*Etymology*

The name refers to the type locality.

*Diagnosis*

A *Macrostomum* species that belongs to the *M. tuba* species group (Fig. 2). Body length between 1 and 2 mm, body width about 0.3 mm, length of rostrum (region in front the eyes) 100–150 μm. Sensory hairs (visible under low power IC) primarily at the rostral and caudal end of the animal. Mouth opening about 150 μm from tip of rostrum. With two cerebral pigment cup eyes (diameter 8–10 μm), which are very rarely malformed, even in cultures of more than 8 years. The colourless animals (transmitted light) may reach up to 2 mm length in cultures and may appear brownish/greenish in reflected light because of the diatoms in the gut. A glandular girdle at the transition of pharynx (80–100 μm long) and gut is particularly well developed (Figs 3d and 5b). The gut extends caudally to the level of the false seminal vesicle. Tail plate of adult animals not very distinct, with an average 120 adhesive organs ($n = 15$) in a U-shaped pattern (Fig. 3e).

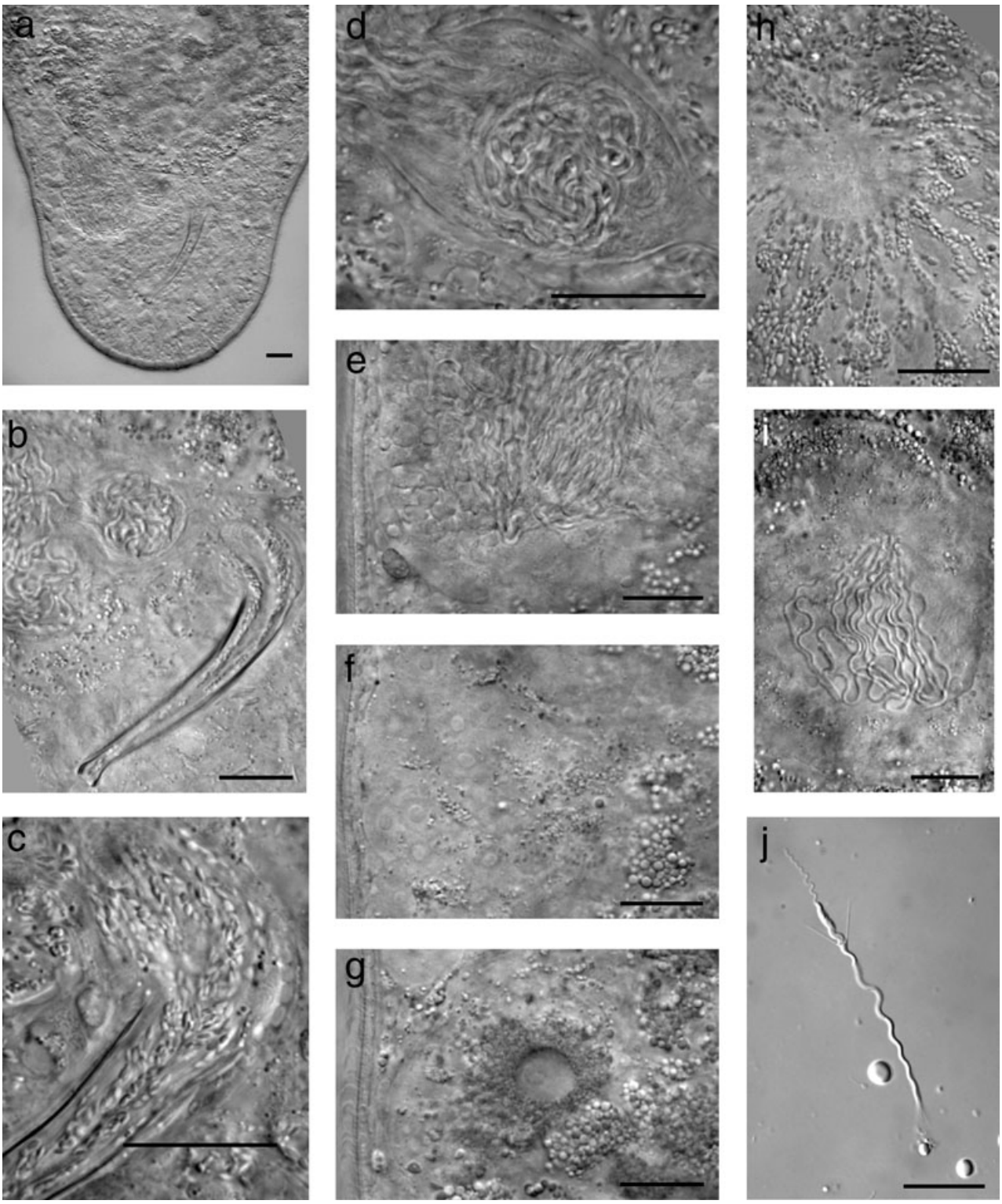


Fig. 4. Digital interference contrast micrographs of the genital structures of living *Macrostomum lignano* from the cultures (the names in the brackets denote the deposited file names on the Digital Type Material CD submitted together with the other type material). (a) tail plate with seminal vesicle and stylet (Mlig H 18); (b) stylet with vesicula granulorum and muscular seminal vesicle (Mlig G 34); (c) vesicula granulorum with prostate secretion granules (Mlig H 26); (d) seminal vesicle connecting to the false seminal vesicle on the left and to the vesicula granulorum on the right (Mlig H 25); (e) caudal part of the testis with developing sperm (Mlig F 25); (f) ovary with oocytes (Mlig F 26); (g) yolk formation in oocyte (Mlig F 27); (h) vagina surrounded by cement/shell glands (Mlig G 25); (i) received sperm in the female antrum, note the polarized sperm that stick in the cellular valve (Mlig G 29); (j) individual spermatozoa. Scale bar 20 μm

Stylet slightly curved, its distal opening with slightly asymmetric thickening. Mean stylet length 69 μm ($n = 20$). Mean proximal opening 14 μm ($n = 18$). Distal glandular swellings of the prostatic glands in the prostatic vesicle reach also far into the stylet (Figs 4b,c and 8a). Ovaries and testes form two separate clusters at about mid-body. Both visible in

the dissecting microscope. Position of female pore: 270 μm from caudal end of animal ($n = 9$). Often with large, false seminal vesicle in addition to the actual seminal vesicle ($35 \times 25$ μm) and the prostatic vesicle (vesicula granulorum) ($30 \times 25$ μm).

In contrast to the usual chromosome number of other species of *Macrostomum*, which is $2n = 6$, the new species has a chromosome number of $2n = 8$ (Egger and Ishida 2005).

## Description

*Habitus and behaviour*

Worms are very active and strongly negatively phototactic (Fig. 2a,2b and 3a). When not swimming away from light, they often attach to the substrate with their adhesive glands on the tail plate, and perform a kind of scanning behaviour. Another stereotypical behaviour is a contraction of the whole body, while the worms remain attached with the tail plate (Fig. 2b). Food uptake, regurgitation of undigested food pellets (in characteristic, sausage-like form) and reproductive behaviour can readily be observed (Schärer et al. 2004a). A first illustration of this species and the stylet was published earlier (Ladurner et al. 2000, Fig. 1).

In addition to the verbal description of the species we include a table with detailed quantitative measurements of a number of morphological traits (Table 1).

*Epidermis and body wall*

In light microscopical section, the epidermis is 5–8 μm high and shows typical characteristics of the epidermis of the genus *Macrostomum* (Rieger et al. 1991a,b; for details). Nuclei of epidermal cells are highly lobate. Epidermal cilia are 5–7 μm long. A completely continuous basal matrix is lacking as in most investigated *Macrostomum*-species. Rhabdite glands (Fig. 2c,d,h,l,i) are scattered over the dorsolateral portions of the body, but particularly well developed on the dorsal side of the tail plate (Fig. 2i). Fewer may occur also on the ventral side. Unpublished results (C. Seifarth, personal communication) show that the body wall musculature corresponds to the pattern described for *M. hystricinum* by Rieger et al. (1994). About 40 dorsoventral muscles can be observed in the tail plate of adult animals. The epidermal nuclei in the anterior portion of the rostrum are found below the epithelial level (Fig. 5a).

In the tail plate and near the brain urn-shaped invaginations of the epidermis may occur (Fig. 2l). These may be found in freshly collected animals as well as in animals cultured for several years in the laboratory. Similar phenomena have been noticed in freshly collected specimens of certain dolichomacrostomids (Rieger 1971b). Further details have been described for the species under the name of *Macrostomum* sp.: light microscopical images of epidermal surface and isolated epidermal cells (see Ladurner et al. 2000, Figs 2d, 6 and 7a), phalloidin staining of body wall musculature during regeneration (Salvenmoser et al. 2001), immunocytochemical staining of longitudinal muscles (Rieger and Ladurner 2003, Fig. 4), epidermal ultrastructure (see Nimeth et al. 2002, Fig. 4), and body wall musculature of adult and freshly hatched animals (Ladurner et al. 2005).

*'Body cavity', parenchyma and neoblast system*

The body cavity is acoelomate. As is the case in all species in the genus *Macrostomum*, the space between gut and epidermis

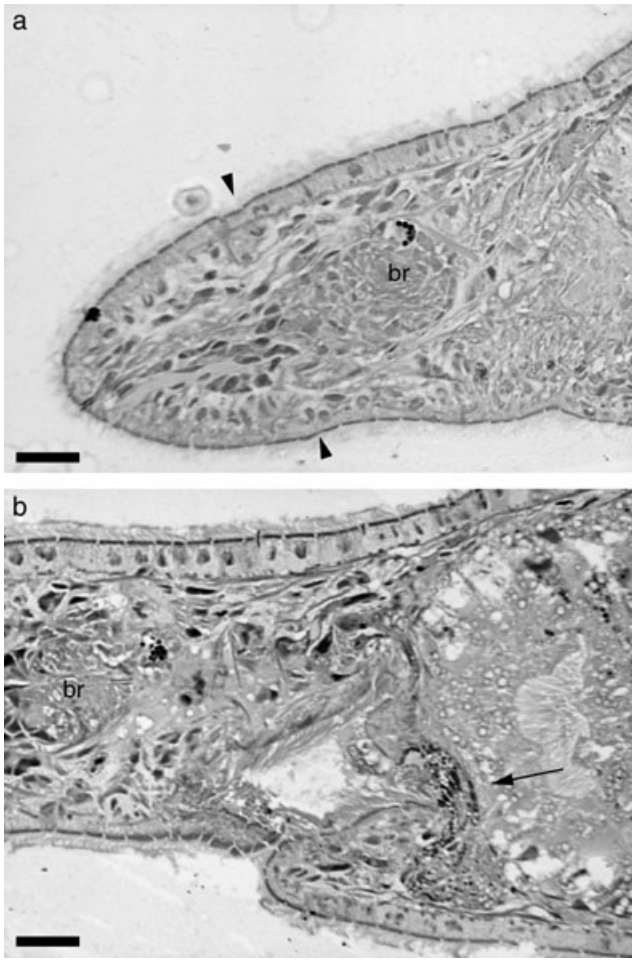


Fig. 5. Digital micrograph of a parasagittal section through the rostrum (a) and a sagittal section through the pharynx (b) of *Macrostomum lignano* (glutaraldehyde-osmium double fixation stained with Haidenhein's Hematoxylin). Note the arrowheads, which indicate the range where the epidermal nuclei are found below the epithelial level, and the arrow, which indicates the position of the glandular girdle (br marks the brain). Scale bar 20 μm

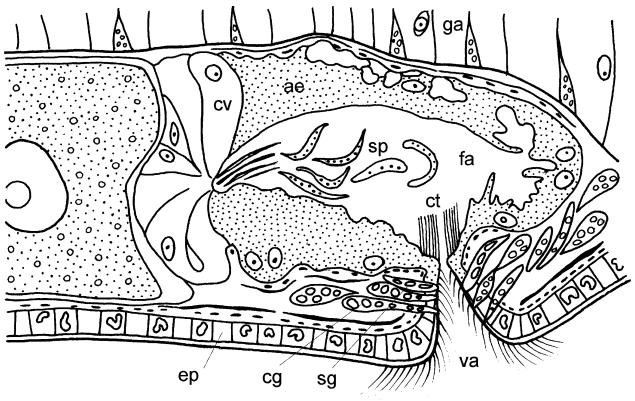


Fig. 6. Vagina and female antrum of *Macrostomum lignano*. Reconstruction from sagittal sections (glutaraldehyde-osmium double fixation stained with Haidenhein's Hematoxylin). ae, antral epithelium; cg, cement glands; ct, ciliary tuft; cv, cellular valve; ep, epidermis; fa, female antrum; ga, gastrodermis; sg, possible shell glands; sp, sperm; va, vagina

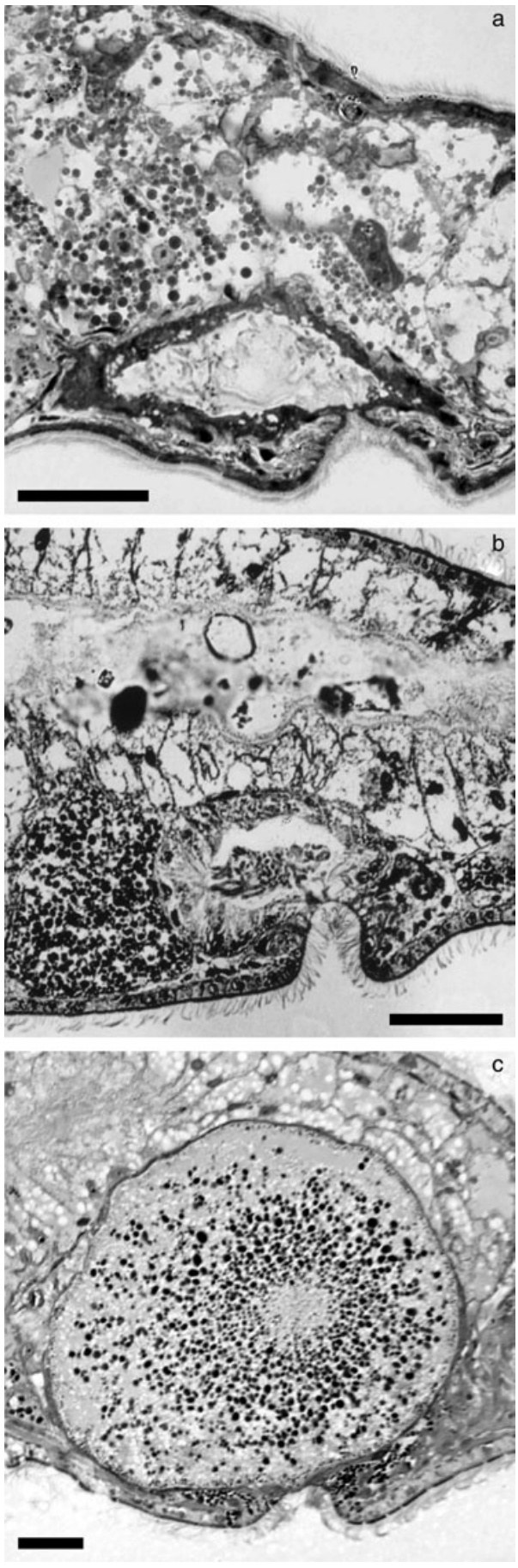


Fig. 7. Digital micrograph of sagittal sections through the vagina and female antrum of *Macrostomum lignano*. (a) Empty female antrum (fixed with 4% paraformaldehyde); (b) female antrum containing received sperm (fixed with hot Bouin's and stained with Richard's Methylin Blue); (c) female antrum containing a mature egg (fixed with glutaraldehyde-osium double fixation and stained with Heidenhein's Hematoyxlin). Scale bar 20 μm

is narrow. It contains the main parts of the nervous system and the protonephridia. While the rostrum is filled mostly with nerve cells and necks of the rhammite glands that are located on the dorsolateral side in front of the testes, the tail plate and the region behind the gut is filled with the male genital organs (in particular with the many prostatic glands cells) and the gland cells and the insunken anchor cells of the 120 adhesive papillae). Further details have been described for the species under the name of *Macrostomum* sp.: observations of neoblasts in living animals (Rieger et al. 1999) bromodeoxyuridine labelling of neoblasts (Ladurner et al. 2000; Nimeth et al. 2004; Schärer et al. 2004b).

*Nervous system*

The brain (Figs 2c,h, 3b,g and 5a) and the main longitudinal nerve cords, as well as the presence of a postpharyngeal commissure (character not shown) correspond to the general pattern of the genus. In addition, a ventral and dorsal pair of nerve cords is present. The main longitudinal nerve cords unite in the tail plate to form a small caudal ganglion (Fig. 2h), which is well visible also in live animals. Further details have been described for the species under the name of *Macrostomum* sp.: embryonic development of nervous system (Morris et al. 2004), ventral nerve cords, immunocytochemistry (Ladurner et al. 2005), serotonergic and FMRFamide nervous system (P. Ladurner, unpublished data).

*Protonephridia*

Protonephridia occur as a pair of longitudinal canals, dorsal to the main nerve cords, and numerous attached flame bulb cells. We have not yet identified the excretory pores. The protonephridial canal system extends above the brain into the rostrum. The caudal extension of the system is not yet clarified. Further details have been described for the species under the name of *Macrostomum* sp.: embryonic origin of protonephridia (Morris et al. 2004).

*Digestive system*

The pharynx (Figs 2c,h, 3c,f and 5b) is 100 μm long and is a typical 'pharynx simplex coronatus' as described for the genus *Macrostomum* by Doe (1981). We have not yet determined the number of different gland cells in its gland ring, which can vary significantly between species in this genus. A glandular girdle, i.e. glandular cells with granular contents, is found ventrally, at the transition of pharynx epithelium and gut epithelium. They are most characteristic for this species and are visible also in live animals (Figs 3d and 5b). Gastrodermal cells are very tall, and they feature large nuclei and very long cilia. One can observe the function of the long cilia in the gut lumen of live specimens: by their movement they form a functional 'trophic membrane' that separates the food from the surface of the gastrodermal cells. Granular clubs are dispersed between gastrodermal cells. Further details have been described for the species under the name of *Macrostomum* sp.: granular club cells and gastrodermal

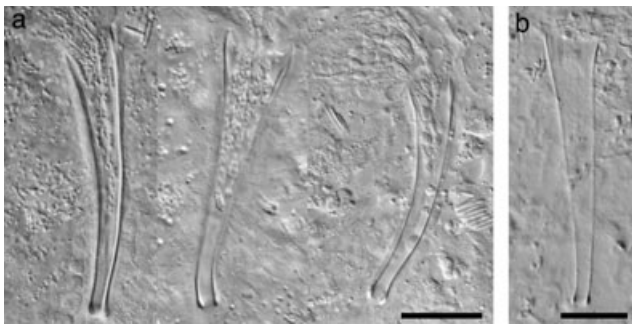


Fig. 8. Digital micrographs of stylets. (a) *Macrostomum lignano*, three specimens from the cultures between 1997 and 2001; (b) from a whole mount specimen, probably belonging to *Macrostomum lignano*, found in Greece (D). Scale bar 20 μm

Table 1. Morphometric measurements based on live material from the type locality and the cultures

| Parameter | Mean (μm) | SD | *n* |
|---|---|---|---|
| Body length | 1250 | 330 | 15 |
| Body width | 290 | 80 | 15 |
| Rostrum length | 125.5 | 34.1 | 15 |
| Eye Ø | 9.8 | 1.8 | 6 |
| Mouth from tip | 149.4 | 43.4 | 5 |
| Pharynx length | 87.5 | 13.6 | 10 |
| End of gut from end | 130.5 | 43.6 | 11 |
| Testis length | 151.6 | 57.1 | 14 |
| Testis width | 56.8 | 22.2 | 14 |
| False seminal vesicle Ø | 109.7 | 42.8 | 15 |
| Seminal vesicle Ø | 33.3 | 14.7 | 12 |
| Prostatic vesicle Ø | 29.9 | 8.9 | 13 |
| Stylet length | 68.7 | 6.6 | 20 |
| Ovary length | 116.1 | 47.7 | 14 |
| Ovary width | 46.8 | 23.6 | 14 |
| Female pore from end | 268.4 | 99.1 | 9 |

phagocytes in immunocytochemical staining (Ladurner et al. 2005).

**Reproductive organs**

*Testes*

The paired testes (Figs 2a,m, 3a and 4e) occupy together about 5.5% (range 3–9%) of the body and are located in the central region of the animal (Schärer and Ladurner 2003). In culture dishes they can often be seen to contain forming sperm, which appear as a dark shadow in the centre of the testis. The vas deferens may be seen in squeezed specimens. The two ducts merge shortly before the false seminal vesicle into which they empty.

*Spermatozoa*

Spermatozoa (Figs 2p and 4j) are complex and decorated with the typical bristles as ultrastructurally described for example by Rohde and Watson (1991). The function of these bristles remains unclear.

*Male genitalia*

The copulatory organ (Figs 2n,o, 4a–c and 8) consists of a false seminal vesicle, a muscular seminal vesicle, prostate gland cells, a copulatory bulb at the proximal end of the stylet, and a long, slightly curved stylet with a slightly asymmetric distal thickening (see also Fig. 8). The size of the false seminal vesicle strongly depends on the mating history of the worms, being much smaller after high copulatory activity (Schärer and Ladurner 2003). Similarly, the prostate gland cells are much denser when worms have been prevented from mating for some time (L. Schärer, unpublished data). The necks of the prostate gland cells enter into the central region of the stylet (Figs 2n and 4b), where they form the intra-stylet portion of the prostatic vesicle. Thin rods, most likely long ciliary rootlets (sensu Doe 1982), are also visible. The stylet exits through a ciliated male antrum in the tail plate, and is inserted into the female antrum of the mating partner during copulation (L. Schärer, personal observation).

*Ovaries*

The paired ovaries (Figs 2a and 4f) are located behind the testes, and together they occupy about 3.5% (range 2–6%) of the body (Schärer and Ladurner 2003). They are weakly structured, and often gradually change into a growth zone, where oocytes are provisioned with yolk (Fig. 4g). Yolk usually forms first around the well visible nucleus, and eventually makes the eggs an optically rather dense structure. Throughout egg formation the nucleus is clearly discernable.

*Female genitalia*

The growth zone is connected to the female antrum through the specialized tissue, referred to as cellular valve ('Durchgangsapparat' or 'Verschlussapparat') (Figs 4i, 6 and 7). The female antrum (also called female atrium) opens towards the outside through a very short, ciliated vagina, which is associated with a dense set of cement and shell glands. Usually only one gland cell type has been noted in this genus (Rieger et al. 1991b). In some serial sections proximal glands with naviculate granules (surmised as shell granules) can be distinguished from presumed cement glands with more roundish granules (Fig. 6). This distinction is difficult to make in live preparations, but already Luther (1905) noted for *M. tuba* different granule types around the female pore. The antral epithelium is ciliated only in a small region at the opening of the vagina into the antrum (Fig. 6). Cilia of this ciliary tuft are much longer than the cilia in the vagina and – together with the antral musculature (inner longitudinal and outer circular muscles) – may play an important role when a ripe egg is situated in the antrum. Special kneading movements of the egg can be observed before egg laying (L. Schärer, personal observation). Different fixations – and perhaps also different stages in the life cycle yield rather different pictures of the antrum epithelium (see Fig. 7) When the mature egg with the characteristic distribution of the cortical shell granules and the more central yolk granules is situated in the antrum its epithelium is extremely stretched out into a thin tissue layer around the egg, except for the cellular valve that remains distinct (Fig. 7).

The following additional details have been described for the species under the name of *Macrostomum* sp.: shape of the copulatory organ and photomicrograph of stylet (Ladurner et al. 2000), socially induced changes in the allocation to male reproductive function (Schärer and Ladurner 2003; Schärer et al. 2004b, 2005), mating behaviour (Schärer et al. 2004a).

## Discussion

### Taxonomic position of the new species

More than 100 species living in freshwater, brackish and marine environments are presently recognized in the genus *Macrostomum* (also see Tyler and Bush 2003). All species have a highly stereotypic body organization, with a caudal adhesive disc, and a typical location and structure of male and female organs (see references in Introduction). Beklemischev (1951) has introduced the concept of artificial 'species groups' in this large genus. He discerns three such groups, one each around *M. tuba*, *M. hystricinum* and *M. orthostylum* (see also Rixen 1961). As a first step to establish a more natural classification, Rieger (1977) has restricted the *M. hystricinum* species group (later called '*M. hystricinum* clade') to fewer species. Based on stylet morphology and other anatomical features the clade represents very likely a monophyletic grouping in this form. Two subclades (the *M. hystricinum* subclade and the *M. pusillum* subclade) have been distinguished in this clade by Ladurner et al. (1997). Because of its elongate, narrow stylet with distal thickenings in its wall, *M. lignano* is associated with the species group around *M. tuba* (Graff 1882).

According to Beklemischev (1951) the *M. tuba* species group consisted of: *M. tuba* (Graff, 1882), *M. minutum* (Luther, 1947), *M. infundibuliferum* Plotnikov, 1905, *M. rhabdophorum* Beklemischev, 1927, *M. pseudoobtusum* Beklemischev, 1927 (nom. dub.), *M. lutheri* (Beklemischev, 1927), and *M. clavistylum* Beklemischev, 1951. However, we think that the latter species might not belong to the group because the shape and the distal opening of its stylet is distinctly different.

From the species described by Luther (1947), *M. tenuicauda* and *M. curvituba*, and another species lacking eyes, can be added to the above list of species. Rixen (1961) has added *M. curvituba* Luther 1947 (eyes lacking) and *M. subterraneum* Rixen, 1961 (eyes weakly developed, stylet bent) to the list. He considered *M. tenuicauda* to be a link between the *M. orthostylum* and the *M. tuba* group. From our re-examination of *M. tenuicauda* from salt marshes in Sylt (courtesy Dr K. Reise and Dr W. Armonies) we conclude that this species is closely related to *M. lignano* described here, and should also be included in the *M. tuba* species group (see below).

Considering the characters of the stylet, some 25 additional species of the more than 100 valid ones in the genus *Macrostomum* in the turbellarian taxonomic database of Tyler and Bush (2003) may belong to the *Macrostomum tuba* species group. They all have an elongate, narrow stylet (sometimes bent in one or more directions), with blunt distal thickenings in its wall. This brings the number of species in the *M. tuba* species group to over 30 (Table 2). In addition, about 35

Table 2. Preliminary list of species belonging to the *Mactostomum tuba* species group, based on characteristics of the stylet, i.e. stylet a long, narrow tube, with symmetrical or asymmetrical (asym) distal wall thickenings

| Species | Stylet length (μm) | Comments |
|---|---|---|
| *M. aegypticum* Beltagi, 1972 | 394 | Proximally bent in a sharp angle |
| *M. amaniense* Young, 1976 | 160 | Unique asym thickening in distal wall |
| **M. brevituba* Armonies & Hellwig, 1987 | 60–70 | |
| *M. cairoense*, Beltagi, 1972 | 490 | |
| *M. christinae* Young, 1976 | 72 | |
| *M. coxi* Young, 1976 | 200 | |
| **M. curvituba* Luther, 1947 | 80 | |
| *M. deltanasis* Beltagi & El-Said, 2002 | 321 | Distal wall thickening slight |
| *M. extraculum* Ax & Amonies, 1990 | 68–72 | Stylet bent in two directions |
| *M. galloprovinciale* Schmidt and Sopott-Ehlers, 1976 | 30 | Stylet short |
| *M. graffi* Ferguson, 1939 | NA | |
| *M. greenwoodi* Faubel and Cameron, 2001 | 97 | With distal wall thickening (re-examination of holotype, this paper) |
| **M. ideficis* Schmidt and Sopott-Ehlers, 1976 | 17–21 | Stylet short |
| *M. infundibuliferum* Plontikov, 1905 | NA | |
| *M. ismailiensis* Beltagi, Ibrahim & Moustafa, 2001 | 180 | |
| *M. lewisi* Ferguson, 1939 | 104 | Stylet bent twice |
| *M. longituba* Papi, 1953 | Up to 143 | Stylet opening not terminal |
| **M. lutheri* Beklemischev, 1927 | Up to 210 | Stylet measurement after Papi 1951 |
| **M. magnacurvituba* Ax, 1994 | 140–175 | |
| *M. mediterraneum* Ax, 1956 | 88–90 | |
| **M. minutum* (Luther, 1947) | 75–82 | |
| *M. niloticum* Beltagi, 1972 | 130 | Distal wall thickening slight |
| *M. pseudoobtusum* Beklemischev, 1927 | NA | nom. dub. stylet proximally bent, distal opening on one side |
| *M. purpureum* Reisinger & Kelbetz, 1964 | NA | Stylet bent |
| *M. poznaniense* Kolasa, 1973 | 45 | Short stylet, distal stylet wall thickening slight |
| *M. prognosticis* Schmidt and Sopott-Ehlers, 1976 | 25–30 | Short stylet, bent |
| *M. quiritium* (Beklemishev, 1951) | 140 | With distal thickened stylet wall, see Kolasa 1973 |
| *M. rhabdophorum*, Beklemischev, 1927 | NA | Stylet distally bent |
| *M. reynoldsoni* Young, 1976 | 147 | Stylet distally bent |
| *M. subterraneum* Rixen, 1961 | 90 | Stylet bent |
| *M. tennesenesis*, Ferguson, 1940 | 170 | Slight distal wall thickenings |
| *M. tenuicauda* Luther, 1947 | 66 | Measurement after Luther 1960 |
| *M. tuba* (Graff, 1882) | 160–480 | Measurement after Luther 1960 |
| *M. timavi* Graff, 1905 | NA | Unique asym thickening in distal wall |

NA, not available.
*Species lacking eyes.

species are known to have rather long, narrow stylets (some are not straight) but they all lack distal wall thickenings. Such species may be related to the *M. tuba* species group, or they may represent links to the *M. orthostylum* species group characterized by a straight stylet tube with sharp edges at its distal opening.

Even though stylet morphology is a most significant feature for the taxonomy of Macrostomida (discussion in Rieger 1977), a thorough revision of the genus *Macrostomum* and of the *M. tuba* species group will have to include other features as well, and should be complemented with a molecular phylogeny.

Moreover, Schärer et al. (2004a) have recently described the copulatory behaviour of our study species. Additional observations on the copulatory behaviour of a number of other *Macrostomum* species suggest that stylet morphology may not necessarily lead to natural species groups: the copulatory behaviour of one blunt-tipped species and two species with sharp stylet tips was more similar to the pattern in *M. lignano* than to the copulatory behaviour of *M. tuba* (L. Schärer, personal observation). This suggests that such behavioural traits may also be considered in taxonomical work.

The combination of the similarities in (1) stylet length and (2) stylet shape (straight to slightly curved, narrow tube with slightly wider proximal opening, and with blunt distal ending, with distinct thickenings of this wall around the distal opening), (3) the presence of eyes, and particularly (4) similar habitats (high up in the intertidal, invading salt marshes) make *M. tenuicauda* Luther 1947 the closest relative of the new species. However, a reinvestigation of *M. tenuicauda* from salt marshes on Sylt has demonstrated that the two species clearly are distinct (see also description in Luther 1947, 1960; Karling 1974; Ax and Armonies 1990).

*Macrostomum lignano* shares certain traits also with *M. mediterraneum* Ax, 1956 and with *M. greenwoodi* Faubel and Cameron, 2001. Although the stylet is distinctly longer (80–100 μm) in *M. mediterraneum*, and the asymmetrical distal wall thickening is a distinguishing feature, the general shape of the stylet as well as the female antrum/vagina complex are similar to the situation in *M. lignano*. However, *M. mediterraneum* lacks the ventral ciliary pad at the opening of the vagina into the antrum, and the cellular valve at the transition of the oviduct into the antrum is less well developed.

No distal thickenings of the stylet are described in *M. greenwoodi* by Faubel and Cameron (2001). However, our re-examination of the type material has established the presence of such thickenings. Also, *M. greenwoodi* occurs in similar habitats (in salt marshes that are also high in the intertidal) and thus represents another species closely related to *M. lignano* and *M. tenuicauda*. As a distinguishing feature the stylet in *M. greenwoodi* is more than 25% longer than the mean stylet length in *M. lignano* (reported to be close to 100 μm by Faubel and Cameron 2001), and 90–100 μm according to our re-examination of their holotype.

*M. tenuicauda* and *M. greenwoodi* feature similarities and differences in structures of the female antrum: both in *M. lignano* and *M. tenuicauda* the ciliation of the antrum is restricted to long cilia near the opening of the vagina (less well visible in Bouin's fixed specimens, see Fig. 7b). But *M. lignano* lacks the long cilia and the wide opening of the antrum to the oviduct seen in *M. tenuicauda* (Luther 1947). The funnel-shaped short vagina is similar in *M. lignano* and in *M. greenwoodi*. In the holotype of *M. greenwoodi* a distinct ciliation at the opening of the vagina into the antrum could not be found.

Restriction of long cilia to the ventral wall of the female antrum has been described for a number of *Macrostomum*-species that appear unrelated in stylet morphology (e.g. *M. pithecusae* Papi, 1959, *M. ermini* Ax, 1959, *M. karlingi* Papi, 1953, *M. tenuicauda* Luther, 1947, this species). Its value as a taxonomical character is therefore questionable. As mentioned above, this feature may be related to moving the egg in the antrum shortly before egg deposition occurs, and convergence in this feature appears possible.

Stylets with similar features (see above) are also seen in certain species from freshwater habitats: in *M. christinae* Young, 1976, *M. poznaniense* Kolasa, 1973, and perhaps *M. infundibuliferum* Plotnikov, 1905.

The combination of characters renders *M. lignano* distinct from all described species in the species group. In addition, we have found a special feature of the digestive tract (glandular girdle at the pharynx/gut transition). Re-examinations of other *Macrostomum*-species (*M. hystricinum*-species group: e.g. *M. hystricinum marinum*, and *M. pusillum*-populations from Grado, Northern Adriatic and Maine, USA) and of the holotype of *M. greenwoodi* suggest that such modifications at the pharynx/gut transition occur more often in the genus. However, the distinctness of this feature in our new species appears to be unique.

In summary, *M. tenuicauda* (Luther, 1947), *M. greenwoodi*, (Faubel and Cameron, 2001) and our new species *M. lignano* represent a closely related, predominately diatom-feeding clade of the *M. tuba* species group. All three species live in similar habitats (higher up in the intertidal, close to or in salt marshes), and the clade shows worldwide distribution. The three species may be related more closely to the marine species *M. mediterraneum* Ax, 1956 and the fresh water species as *M. christinae* Young, 1976 and *M. poznaniense* Kolasa 1973.

### The use of video microscopy to enhance systematics of microturbellaria and meiofauna

The systematics of free living Platyhelminthes has greatly benefited from photomicrography of living specimens since the 1970s (for Acoela, e.g. Fegley et al. 1984; Gschwentner et al. 2002; for Macrostomida,. e.g. Schmidt and Sopott-Ehlers 1976; Ax and Armonies 1990); for Catenulida, e.g. Sterrer and Rieger 1974; for Rhabditophora, e.g. Karling 1978; Schockaert and Martens 1985; Ax and Armonies 1990; Ax 1995). However, space restrictions in journals and printing costs have limited the extent to which such photomicrographs were included in species descriptions. Today digital microscopy allows for intensified photographic documentation of new and rediscovered species by an order of magnitude. We want to demonstrate the use of this technology with the Digital Type Material CD provided with the type-material of the new species. This new information technology will improve comparisons of small, difficult to preserve meiobenthic organisms significantly.

### *Macrostomum lignano*, a model organism for studying the evolution and development of the lower Bilateria

Molecular and genetic evidence revealed that developmental genes are highly conserved throughout the animal kingdom

(Wolpert et al. 2002; Gilbert 2003; Pires-daSilva and Sommer 2003). Established model organisms such as *Drosophila melanogaster* or *Caenorhabditis elegans* have provided insight into molecular mechanisms involved in development, pattern formation or cell differentiation (Thieffry and Sanchez 2003). Among Platyhelminthes planarian flatworms have been used as models to study regeneration and pattern formation (Saló and Baguña 2002; Agata 2003; Sánchez Alvarado et al. 2003). Several characteristics qualify *M. lignano* as a suitable model organism in a more general sense in developmental and evolutionary biology (see publications dealing with this new species in result section): ease of culture maintenance, small size, transparency, short generation time of 3 weeks, the phylogenetic position at the base of the Bilateria, embryonic development with spiral cleavage, world wide distribution of closely related species, hermaphroditism with non-selfing fertilization, the total cell number of about 25 000 cells, regenerative capacity, the extraordinary cell renewal system with stem cells (neoblasts) representing the only dividing cell type, tissue plasticity, and the availability of monoclonal antibodies and ESTs. Genetic and molecular techniques including RNA-interference and methods to produce transgenic animals are currently adapted for *M. lignano*. Future directions towards microarrays and proteomic approaches will provide additional data for comparative developmental biology of established model organisms and a basal bilaterian.

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## Zusammenfassung

*Ein neuer Modellorganismus für die basalen Bilaterier und die Verwendung von digitaler Mikroskopie für die Taxonomie meiobenthischer Platyhhelminthen: Macrostomum lignano n. sp. (Rhabditophora, Macrostomorpha)*

*Macrostomum lignano* n. sp. gehört zu den Macrostomorpha, dem ursprünglichsten Subtaxon der Plathelminthes-Rhabditophora. An der neuen Art, die sich im Labor besonders einfach züchten lässt, wurden bereits zahlreiche entwicklungsbiologische und evolutionsbiologische Studien durchgeführt. Die kleinen simultan-hermaphroditischen Tiere, bei denen nur etwa 25'000 Zellen alle wichtigen Organsysteme der Bilateria ausbilden, sind für derartige Untersuchungen ein möglicher Modellorganismus, der phylogenetisch weit ursprünglicher ist als die üblichen Modellorganismen. *Macrostomum* ist die größte Gattung der Macrostomorpha. Sie umfasst mehr als 100 Meer-, Süss- und Brackwasser-Arten, einige davon mit weltweiter Verbreitung. Innerhalb der Gattung ist *M. lignano* der *M. tuba*-Artengruppe zuzurechnen, die zusammenfassend dargestellt wird. Zur Charakterisierung dieser meiobenthischen neuen Art wird als neuartige Dokumentationsform digitales Bildmaterial verwendet, das als CD zusammen mit dem Typenmaterial hinterlegt wird.

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