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A new limnic species of *Macrostomum* (Platyhelminthes: Macrostomida) from Argentina and its muscle arrangement labeled with phalloidin

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ABSTRACT

A new species of *Macrostomum* from limnic environments in Argentina is described. *Macrostomum platensis* n. sp. differs from its congeners by the shape and length of its stylet. Moreover, the arrangement of the muscle system is described using rhodamine-labeled phalloidin. This method provides detailed information on the complex arrangement of muscles of the body wall, pharynx, head and reproductive organs. The resulting description is compared with previously published ones using the same methodological approach. The muscle arrangement may be considered for future taxonomic studies within the genus *Macrostomum* and might provide phylogenetically useful characters.

1. Introduction

The family Macrostomidae van Beneden, 1870 (Platyhelminthes: Macrostomida) is a group of cosmopolitan free-living flatworms occurring in a wide range of habitats, including limnic, brackish and marine environments. Species of the Macrostomidae, especially those of *Macrostomum* O. Schmidt, 1848, exhibit a typical spatulated shape. The anterior end of the body is frontally rounded while the middle portion is the broadest. The posterior region is more or less deeply waisted at the level of the reproductive organs or behind them. The end is truncated or pointed, and provided with a rim of adhesive glands for sticking to surfaces.

Out of approximately 140 known species of *Macrostomum* (Tyler et al., 2010), only 18 have been reported from South America (Marcus, 1946, 1949, 1954; Schmidt and Sopott-Ehlers, 1976; Noreña et al., 2003, 2006; Gamo and Leal-Zanchet, 2004; Brusa, 2006), half of them being limnic species. The first report of species of *Macrostomum* from Argentina regards two new species (i.e., *M. velastylum* Brusa, 2006 and *M. puntapiedrensis* Brusa, 2006), discovered in limnic and brackish environments, and also includes specimens that are similar to *Macrostomum vejdovskyi* Ferguson, 1940 and *Macrostomum viride* van Beneden, 1870, both from limnic environments (Brusa, 2006). However, detailed morphological studies of species of this genus in this geographical area are still scarce.

Despite the fact that studies on macrostomid require morphological analysis of particular body structures, such as the stylet – which is the most significant feature for the taxonomy of *Macrostomum* species (Ferguson, 1954; Rieger, 1977; Rieger et al., 1994) – other features such as the sperm morphology, which strongly varies between species, also provides relevant taxonomic information (Schärer et al., 2011). Furthermore, the muscle arrangement, which has emerged as a taxonomic feature in acoels (Hooge, 2001; Hooge and Tyler, 2005) and for which we have some data on macrostomids (e.g. Rieger et al., 1994; Reiter et al., 1996; Morris et al., 2007), might be also of interest.

In the present study, we describe a new species of the genus *Macrostomum*, *M. platensis* n. sp. from La Plata, Buenos Aires, Argentina. Furthermore, by using of phalloidin as a muscle marker, we also present a detailed description of the body-muscle arrangement, the musculature associated with the sexual organs, and the muscles at the level of the brain (including their association with the pharynx). This might provide a new set of morphological characters for species of *Macrostomum* that could be useful in future taxonomic studies.

2. Materials and methods

The specimens were collected in two different rainwater ponds, one of them in the Pereyra Iraola Park (34°50′48.11″S; 58°06′04.68″W) and the other in Berisso (34°57′50.93″S;

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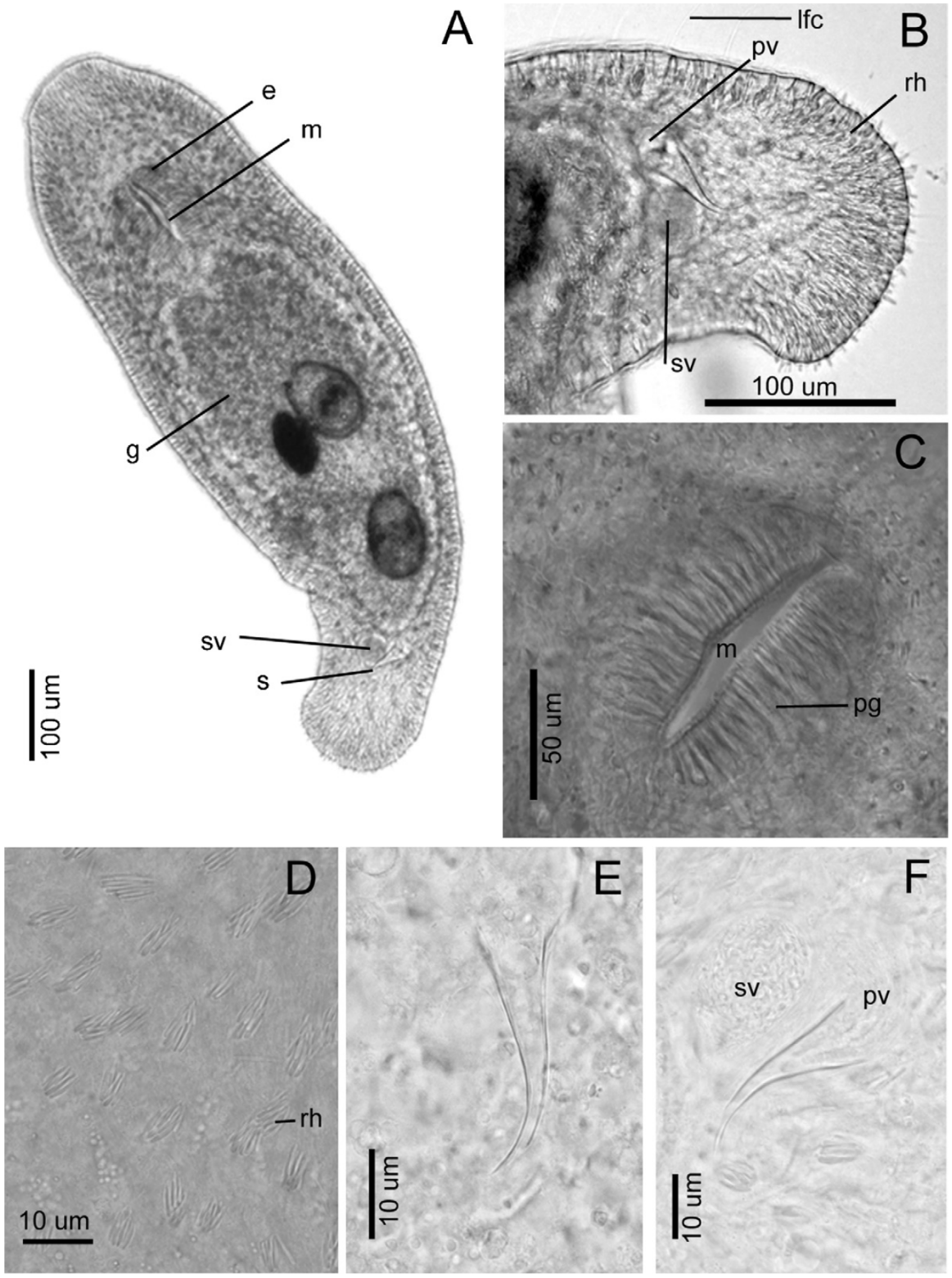

Fig. 1. *Macrostomum platensis* n. sp. (A) General view *in vivo*. (B) Caudal region of the same specimen, showing long flexible cilia on the lateral side of the body, rhabdites, and genital system. (C) Detail showing the mouth. (D) Detailed view of rhabdites at the dorsal surface. (E) Image with higher magnification showing the stylet. (F) Stylet, seminal vesicle and prostatic vesicle. (C)–(F) are interference contrast micrographs. e, eyes; g, gut; lfc, long flexible cilia; m, mouth; pg, pharyngeal glands; pv, prostatic vesicle; rh, rhabdites; s, stylet; sv, seminal vesicle.

57°48′01.01″W), both located near La Plata (Buenos Aires Province, Argentina). Samples were taken during 2010 (April to September) and 2011 (January) from vegetated areas, using a 125 μm mesh net. The material was transported to the laboratory and observed alive under a dissecting microscope. All the specimens used were maintained in the water of original location at a 12:12 h light/dark period until they were processed. Macrostomids were separated, observed and photographed *in vivo* as whole squash mounts

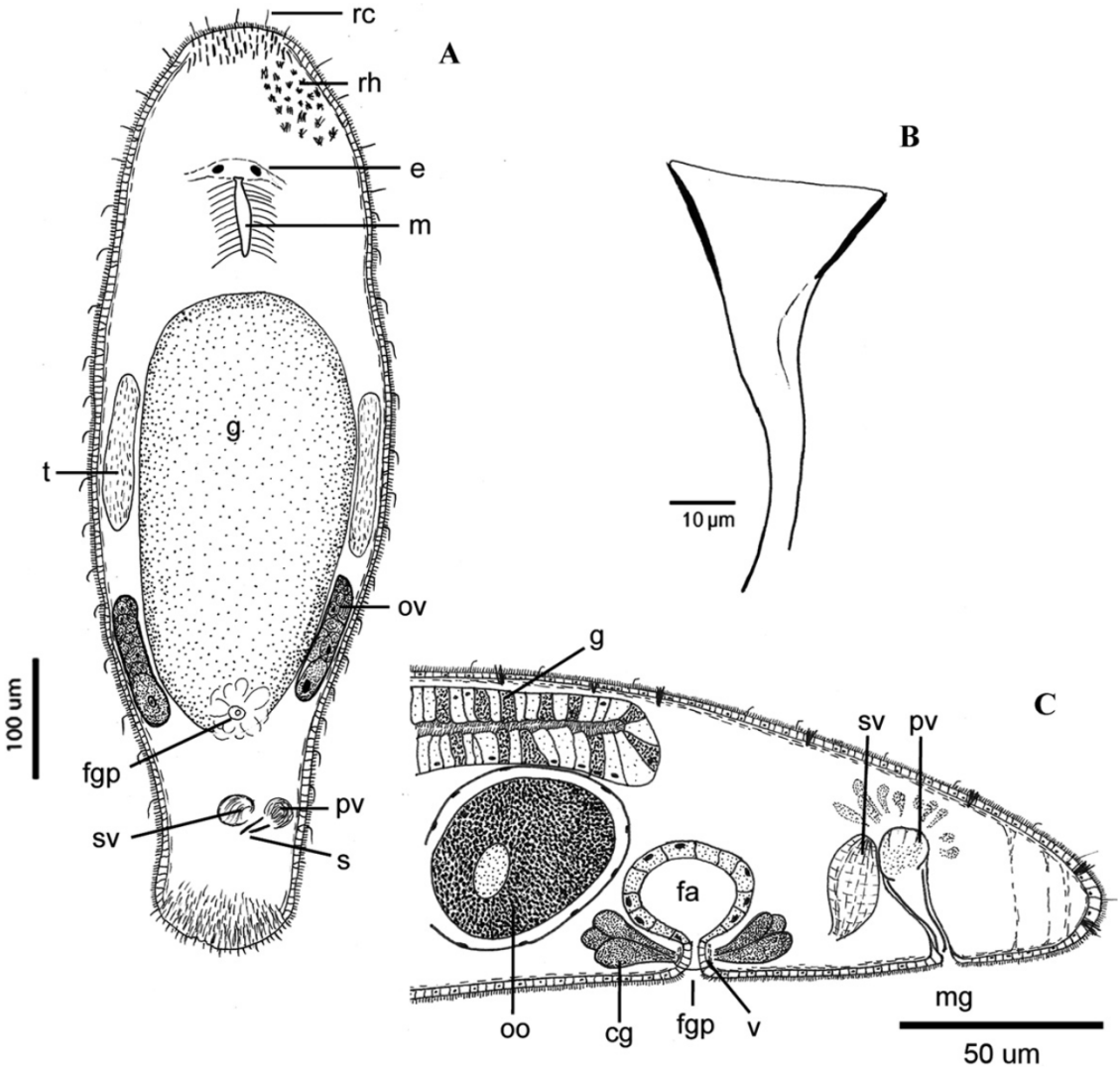

Fig. 2. *Macrostomum platensis* n. sp. Schematic drawings showing the internal morphology (A) Ventral view of a whole specimen. (B) Stylet from a squash preparation. (C) Reconstruction of the posterior region showing the genital system. cg, cement glands; e, eye; fa, female antrum; fgp, female gonopore; g, gut; m, mouth; mg, male gonopore; oo, oocyte; ov, ovary; pv, prostatic vesicle; rc, rigid cilia; rh, rhabdites; s, stylet; sv, seminal vesicle; t, testes; v, vagina.

under a compound microscope. Interference contrast illumination was used for photomicrographs of the stylet, rhabdites and pharynx.

Some specimens were then fixed in Bouin's solution for histological studies. They were embedded in Paraplast, serially sectioned (4 μm thick) in a frontal plane, stained with hematoxylin-eosin and mounted in synthetic Canada balsam. Polyvinyl–lactophenol whole mounts were prepared for studies of the stylet morphology. Measurements of the stylet were performed following Gelhen and Lochs (1990).

To analyze the muscle arrangement, specimens were fixed in formaldehyde-phosphate buffered saline (PBS) (4%) for 12 h, washed in PBS-Tween (0.05%) (PBS-T) and permeabilized in Triton X-100 (1%) for 24 h at 4 °C. The flatworms were then incubated overnight at 4 °C with rhodamine-phalloidin solution (Sigma–Aldrich) (1/1000). Finally, the specimens were mounted in Vectashield mounting medium. The resulting material was observed with a Zeiss LSM 510 Meta confocal laser scanning microscope. The images were analyzed by the use of the Zeiss LSM Image Examiner software. For counting fibers, the *orthogonal display mode* was used.

Whole mounts and sectioned specimens were deposited in the Invertebrate Collection at the Museo de La Plata, Argentina (MLP 6315–6321).

3. Systematics

Macrostomidae van Beneden 1870
Macrostomum O. Schmidt, 1848
Macrostomum platensis n. sp.

3.1. Holotype

Specimen *in toto*, collected on July 16, 2010 from a rainwater pond in the Pereyra Iraola Park (34°50′48.11″S; 58°06′04.68″W), La Plata, Buenos Aires, Argentina, mounted in polyvinyl–lactophenol, deposited at MLP under accession number MLP 6315.

3.2. Paratypes

Five specimens *in toto*; collected on June 16, 2010 (MLP 6316, two specimens) and June 24, 2010 (MLP 6317) from the type

locality; and on April 27, 2010 (MLP 6318) and May 12, 2010 (MLP 6319) from Berisso (34°57′50.93″S; 57°48′01.01″W); all specimens mounted in polyvinyl–lactophenol.

Two specimens fixed in Bouin's solution and two fixed in ethanol (100%), Pereyra Iraola Park, January 20, 2011 (MLP 6320).

3.3. Other material

One specimen frontally sectioned; Pereyra Iraola Park, same locality as holotype, September 10, 2010 (MLP 6321). Five specimens stained with rhodamine-labeled phalloidin.

3.4. Diagnosis

Body length about 1100 μm and maximum width 460 μm. Two black eyes in the cerebral region (diameter 10 μm). Rigid cilia at the anterior and posterior ends; long and flexible cilia on lateral sides of body. Stylet funnel-shaped and curved of a length of 47 μm. Proximal end broad (21 μm), narrowing towards the distal beveled opening end (10 μm). Paired, lateral testes in the central region of the body. Paired, lateral ovaries, posterior to testes.

3.5. Etymology

Name refers to the geographic area where the species lives.

3.6. Description

The body length of live specimens is about 1100 μm and the maximum width is 460 μm (Figs. 1A and 2A). The length of fixed specimens ranges between 430 and 680 μm; and the width between 190 and 460 μm.

The front is rounded (Figs. 1A and 2A). The body is wide at the level of the brain and then narrows towards the back, behind the female gonopore (Fig. 2A). Two black eyes about 10 μm in diameter.

The thickness of the epidermis is between 6 and 10 μm, including epidermal cilia of 4 μm long, covering the entire surface. It also presents rigid cilia at the anterior and posterior end of the body, which are longer than the other cilia (Fig. 2A). Very long (about 50 μm) and flexible cilia on lateral sides of the body can be also observed (Fig. 1B). A basal membrane is not evident. Rhabdites are dispersed along the body surface, being particularly abundant on the dorsal surface, especially on the tail plate (Figs. 1B and 2A). Rhabdites are 8–9 μm long, forming bundles of about four to nine (Fig. 1D).

The mouth (Fig. 1C), being about 100 μm long, and the pharynx are typical for the genus. Several pharyngeal glands enter into the pharynx forming a ring (Fig. 1C). Tall and vacuolated gastrodermal cells with basal and large nuclei are also found (Fig. 2C).

Paired ovoid ovaries are situated laterally, behind the testes (Fig. 2A), including big light-brown oocytes, which frequently contain abundant yolk. The female antrum connects to a short vagina surrounded by a dense set of cement glands, which are indistinguishable from the shell glands (Figs. 2C and 6A, C). No cellular valve around the antrum is present.

The testes are paired and elongated, located beside the intestine, in the central region of the body (Fig. 2A). Vasa deferentia were not observed. The seminal vesicle is spherical, presenting a thick muscular wall (Figs. 1B, 2C and 6D). The prostatic vesicle (vesicula granulorum) is also muscular (Figs. 1B, 2C and 6E). The stylet is funnel-shaped and curved, with an average length of 47 μm ($N = 3$; maximum and minimum length: 52 and 37 μm). The proximal end is broad and gradually narrows towards the distal beveled end (Figs. 1B, F, G and 2B). The average width of the proximal end is 21 μm ($N = 7$); the first proximal fourth of stylet is strongly thickened and the distal opening is about 10 μm, beveled, and located on convex side (Fig. 2B).

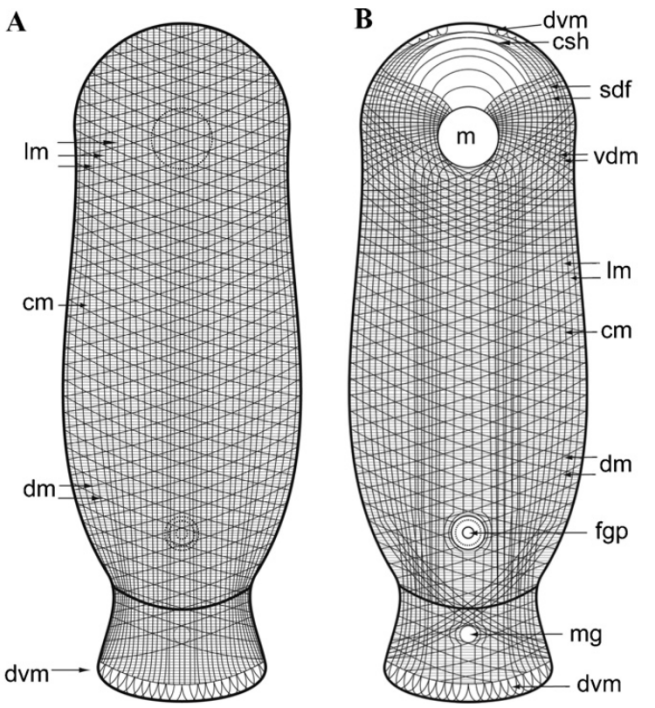

Fig. 3. *Macrostomum platensis* n. sp. Scheme of the arrangements of dorsal (A) and ventral (B) body wall musculature. cm, circular muscles; csh, crescent-shape muscles; dm, diagonal muscles running in two directions; dvm, dorsoventral muscles; fgp, female gonopore; lm, longitudinal muscles; m, mouth; mg, male gonopore; sdf, diagonal fibers associated to pharynx and body wall; vdm, ventral diagonal muscles.

3.7. Muscle arrangement

The fluorescence technique using rhodamine-labeled phalloidin allowed visualization of the muscle system in the whole body of *M. platensis* n. sp. The body musculature consists mainly of three layers: outer with circular fibers (cm); inner with longitudinal fibers (lm); and a third, median layer consisting of two layers of diagonally oriented fibers crossing each other (dm). All the layers together form a complex grid around body (Figs. 3 and 4A, C) (see Supplementary content 1).

Dorsoventral fibers (dvm) are present in the adhesive caudal plate and also in the anterior zone (Figs. 4C, E and 5A, B, E, F). The longitudinal fibers appeared thicker than the circular ones (Fig. 6F), showing forks, especially around the mouth (Fig. 5D). The number of dorsal longitudinal muscle fibers counted at the middle region of the body was around 38 (Fig. 3A). The diagonal muscles form a conspicuous set of fibers on both ventral and dorsal sides of the body (Figs. 3 and 4A, C). In the ventral plane – unlike the dorsal one – diagonal fibers (vdm) appear distinguishable only from mouth to caudal adhesive plate behind female gonopore (Figs. 4F and 5B). In the same plane, a subset of diagonal fibers connects the pharynx with body wall musculature (Fig. 5B and C).

In the head region, the muscle fibers form a complex assemblage, running into the interior of the worm around the brain and the pharynx, respectively. The ventral view of the head shows about 20 pairs of diagonal muscle fibers. These fibers enter into the mouth opening (sdf), to become part of the longitudinal musculature of the pharynx (Fig. 5B and C, Supplementary content 2). Some of these diagonal fibers (located close to the sagittal plane

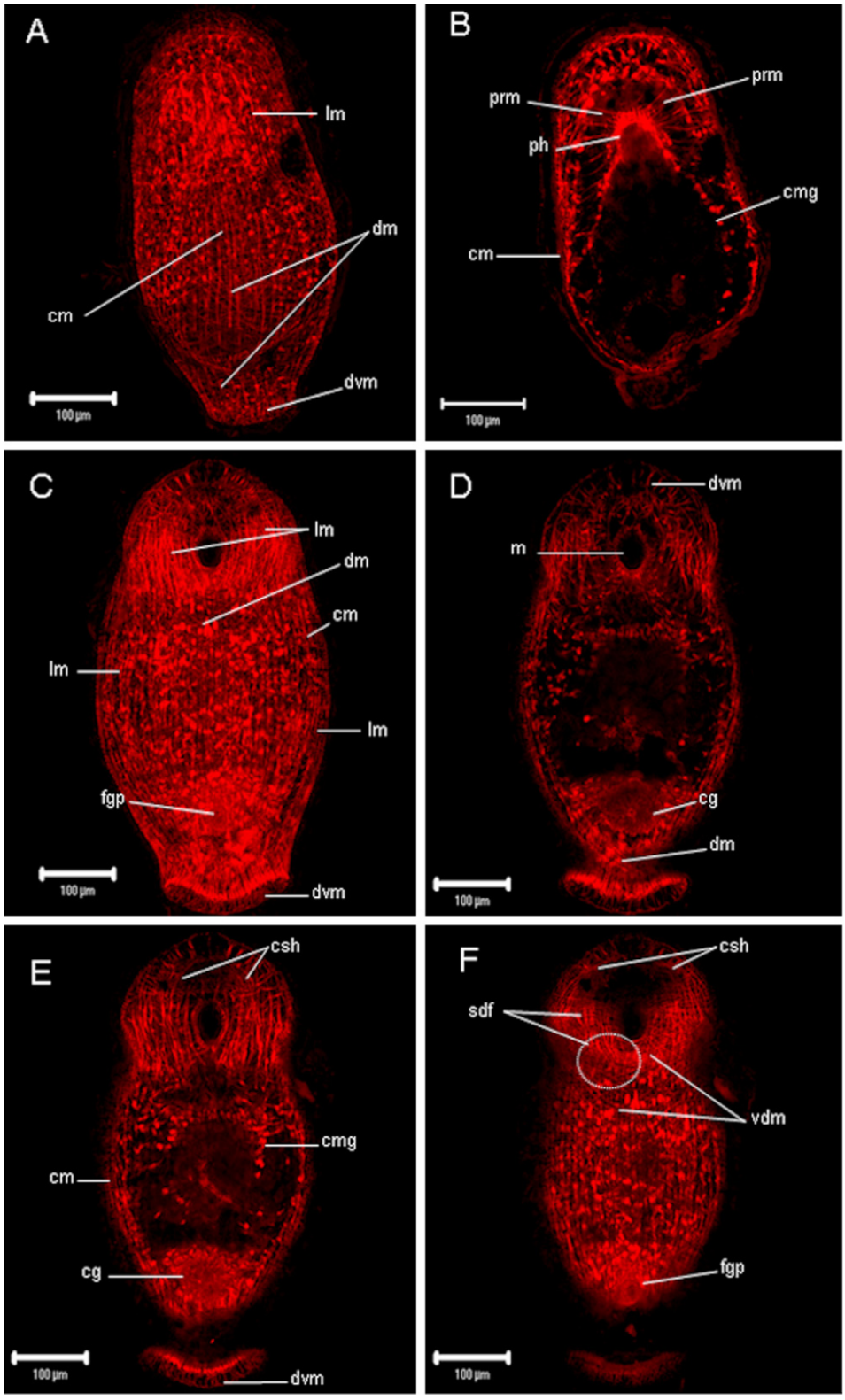

Fig. 4. *Macrostomum platensis* n. sp. CLSM images of whole-mounts labeled with rhodamine-phalloidin showing the muscle arrangement. (A) Z-projection showing distinguishable sets of body wall muscles on the dorsal side. (B) Single optical plane of the same specimen, showing the radial muscles associating pharynx with the body wall. (C) Z-projection showing the pattern of distinct sets of muscles on the ventral side. (D) Single optical plane close to dorsal side, showing the position of diagonal muscles and cement glands in the caudal region, and dorsoventral muscles in anterior region. (E) Single optical plane close to the ventral side, showing the circular muscle arrangement of the gut, circular muscle of the body wall, dorsoventral muscles (caudal region), and the crescent-shape muscles in the anterior region. (F) Ventral view showing both, the diagonal fibers associated to the pharynx and diagonal muscles behind to mouth. Dashed circle shows the diagonal fibers, which originate from the ventral longitudinal fibers. The images A–B and C–F belong to two different specimens. cg, cement gland; cm, circular muscles; cmg, circular muscles of gut; csh, crescent-shape muscles; dm, diagonal muscles running in two directions, and crossing in midbody; dvm, dorsoventral muscles; fgp, female gonopore; lm, longitudinal muscles; m, mouth; ph, pharynx; prm, pharyngeal radial muscles; sdf, diagonal fibers entering mouth associated to pharynx and body wall; vdm, ventral diagonal muscles.

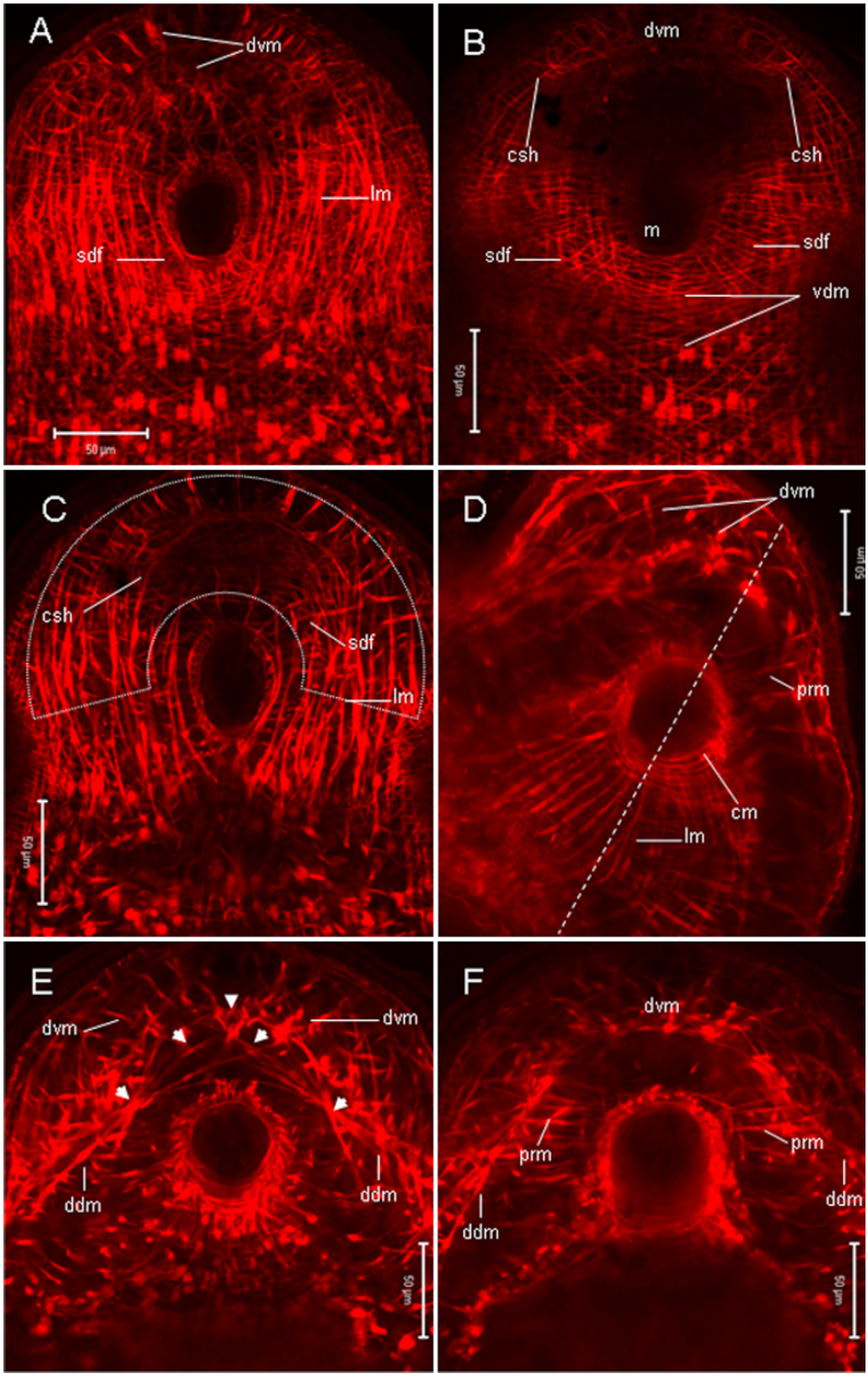

Fig. 5. *Macrostomum platensis* n. sp. CLSM images of rhodamine-phalloidin labeled muscles at the level of the head region. (A) Z-projection showing dorsoventral muscles in front of the pharynx. Longitudinal and diagonal fibers are evident. (B) Set of muscles in a ventral view, showing the diagonal fibers entering to the mouth, one additional set of diagonal fibers posterior to the mouth, and an assemblage of ventral cerebral muscles. (C) Sets of muscles in a deep section of the anterior region, showing the longitudinal fibers, the crescent-shaped muscles around the brain, and some diagonal fibers associated with the pharynx. The dashed crescent-shape indicates the brain region. (D) Detail of longitudinal and circular muscles forming the pharynx. The dashed line indicates the sagital axis of the body. (E) Dorsal view of muscle associated with the brain. Arrowheads indicate the crossing of different sets of fibers. (F) Single optical plane near the dorsal surface showing details of the radial and dorsal diagonal muscles at the level of the pharynx. cm, circular muscles; csh, crescent-shape muscles; ddm, dorsal diagonal muscles; dvm, dorsoventral muscles; lm, longitudinal muscles; m, mouth; prm, pharyngeal radial muscle; sdf, diagonal fibers entering mouth associated with the pharynx and the body wall; vdm, ventral diagonal muscles.

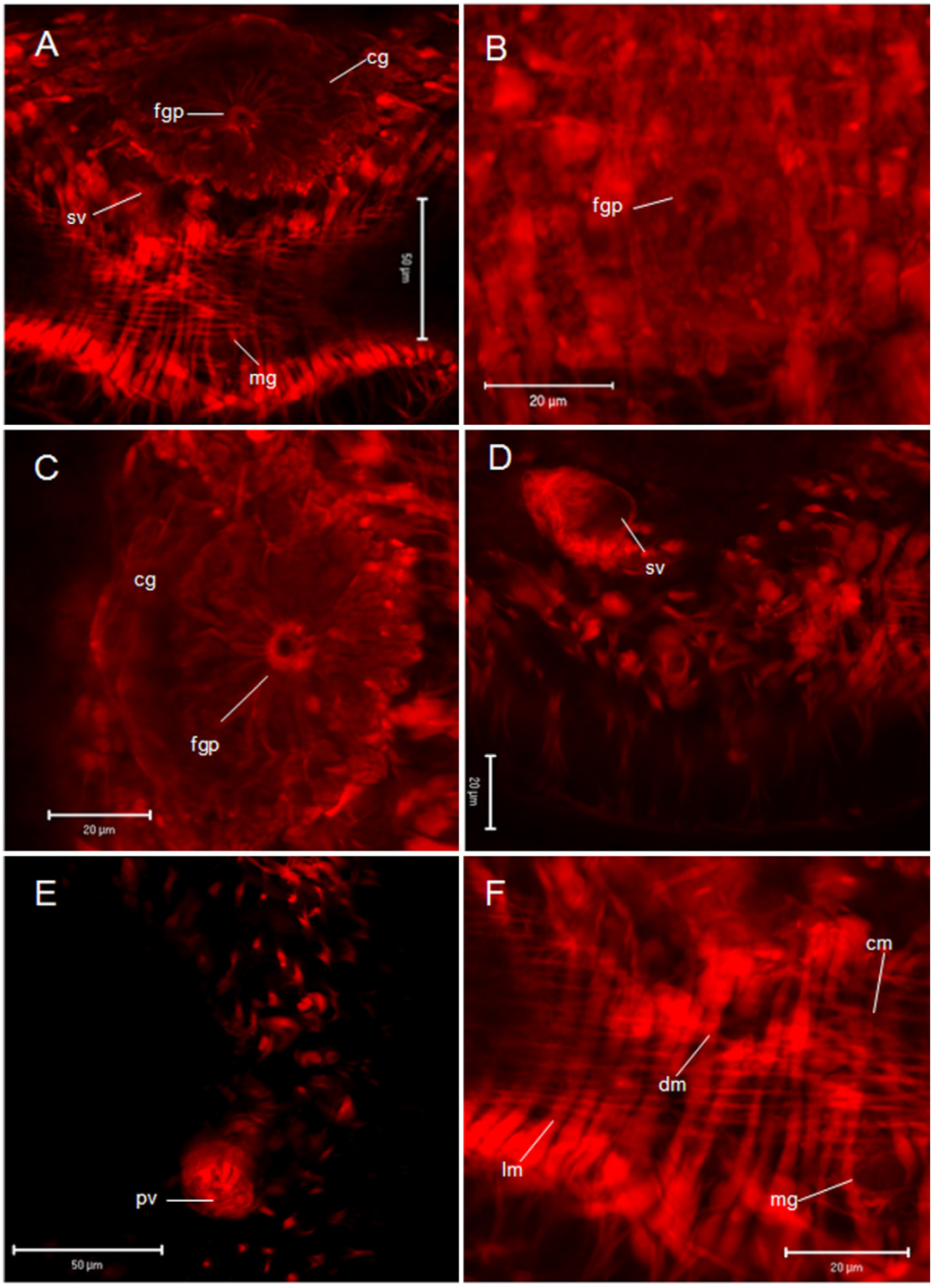

Fig. 6. *Macrostomum platensis* n. sp. CLSM images of the caudal adhesive plate showing the muscle arrangement associated with the genital system. (A) Seminal vesicle, male and female gonopore and cement glands. (B) Z-projection showing the musculature in the female gonopore region. (C) Detail of the female gonopore and cement glands. (D) Detail of the seminal vesicle musculature. (E) A dorsal plane of the same stack showing the muscles of the prostatic vesicle. (F) Detail of muscle arrangement in the caudal plate. cg, cement gland; cm, circular muscles; dm, diagonal muscles; fgp, female gonopore; lm, longitudinal muscles; mg, male gonopore; pv, prostatic vesicle; sv, seminal vesicle.

of the body) are forked, and seem to be related with the ventral longitudinal muscle fibers of the body wall (Figs. 4F and 5B). The ventral longitudinal muscles (lm) of the body wall in this region comprise around 16 pairs of fibers running lateral to the mouth (Figs. 4C and 5C) and then continuing backwards (Fig. 4C and F). The dorsal view shows a set of 10 pairs of short muscles, the pharyngeal radial (prm), connecting the net of pharyngeal fibers with muscles of the body wall (Figs. 4B and 5D, F, see Supplementary content 3).

The fiber assemblage around the brain is formed by crescent-shaped (csh), dorsoventral (dvm), and diagonal fibers (ddm) (Figs. 4D–F and 5B, C, F). Both dorsal (Figs. 4D and 5D–F) and ventral (Figs. 4E, F and 5B, C) cerebral fibers are located around the outer surface of the brain. Two pairs of coarser diagonal fibers arise from the lateral side (Fig. 5E), crossing each other and appearing forked (ddm). These fibers run from both sides, crossing numerous dorsoventral fibers at the body midline (Fig. 5E). Some crescent-shape (csh) muscle fibers are present around the brain region, being

visible only on the ventral side. These fibers are linked to dorsoventral muscle fibers (dvm) (Fig. 5B and C).

The female gonopore (fgp) is evident in the caudal portion of the body in front of the male pore (Fig. 6A). At this level, circular and longitudinal muscles of the body wall diverge around the sides of the gonopore. Longitudinal fibers run from the base of the antrum to the pore, some of them forking and forming a ring around the pore (Fig. 6B). Several cement glands (cg) are located at different levels around the vagina (Fig. 6A and C) reaching together a thickness of 16 μm.

Regarding the male reproductive system, the seminal (sv) and prostatic vesicles (pv) are connected by an intervesicular duct. The musculature of the seminal vesicle is thicker than that of the prostatic vesicle and consists of circular fibers (inner) and longitudinal fibers (outer) converging radially at the distal end (Fig. 6D and E). The muscle layer of the prostatic vesicle is less conspicuous, mostly developed at the proximal region, with a group of fibers converging in a radial fashion (Fig. 6E, see Supplementary content 4).

4. Discussion

4.1. Taxonomy remarks

Traditionally, the stylet morphology stands out as the most important taxonomic feature among Macrostomidae (Rieger, 1977; Ladurner et al., 2005a), and for many species this is the only diagnostic character currently used. However, recent studies have shown convergence between certain features of the reproductive system (Schärer et al., 2011), demonstrating the necessity of considering of additional traits.

Regarding stylet characteristics, the species most similar to *Macrostomum platensis* n. sp. are *M. curvata* Papi, 1951 (synonym *M. orthostylum* var. *curvata* Papi, 1951, after Ferguson, 1954), *M. karlingi* Papi, 1953 and *M. obtusum* Vejdovsky, 1895 *sensu* Papi, 1951. These species have a curved stylet, and a truncated terminal opening as in the new species.

The stylet of *M. curvata* is approximately 60 μm long, with a beveled end, but the diameter of the typically funnel-shaped proximal end is larger in the new species. This funnel-shape of the proximal end is similar to the condition of *M. obtusum* and *M. karlingi*, but in both species the distal half is thinner. Stylet length in *M. obtusum* is approximately 80–90 μm, while the longest stylet observed for the new species was 52 μm long. The distal opening is similar in both species.

The shape and length (60–64 μm and 18 μm diameter of the proximal end after Papi, 1953; 40–50 μm and 14–24 μm diameter of the proximal end after Kolasa, 1973) of the stylet of *M. karlingi*, are similar to those in the new species. The differences are in the distal end, which is not thickened in the new species, and in the distal half of the stylet with parallel sides in *M. karlingi*. The stylet in *M. platensis* n. sp. narrows gradually, and does not show a long distal area with parallel sides.

Two additional *Macrostomum* species, with less curved stylets, but also comparable with *M. platensis* n. sp., are *M. sensitivum* (Silliman, 1884) and *M. vejdovsky* Ferguson, 1940 (see Brusa, 2006). *Macrostomum sensitivum* has a straight stylet. Its length, between 35 μm (Kolasa, 1973) and 55 μm (Papi, 1951), is within the range observed in the new species. However, the diameter of the proximal end of *M. sensitivum*, between 10–15 μm (Kolasa, 1973) and 13–14 μm (Papi, 1951), is approximately one third of the diameter recorded for the new species, and the walls of its stylet are quite parallel, opposed to the funnel-shaped stylet in *M. platensis* n. sp.

The whole appearance and length of the stylet of *M. vejdovsky*, are similar to the new species. It is a simple, funnel-like tube, between 35 μm (Ferguson, 1940) and 52 μm (for *M.* cf. *vejdovsky*; Brusa, 2006) long. However, the stylet in *M. platensis* is curved and lacks the crenulations of the proximal end described for *M. vejdovsky* (Ferguson, 1940; Brusa, 2006).

A conspicuous feature that is constant in all specimens observed, and not described for the species mentioned above, is the thickened wall of the proximal stylet area.

4.2. Muscle arrangement

The body wall, gut, and genital system musculature are well-developed in free-living Platyhelminthes (Rieger et al., 1994). In our study, the specimens of *Macrostomum platensis* n. sp. labeled with rhodamine-phalloidin allowed visualization of the three-dimensional arrangement of their muscle system (see Supplementary content).

The first study using phalloidin methodology to visualize the macrostomid musculature was done on *Macrostomum hystricinum marinum* Rieger, 1977 (Rieger et al., 1994). Other reports providing detailed morphological information include studies on *M. lignano* Ladurner et al., 2005, highlighting the muscle arrangement (Ladurner et al., 2005b; Morris et al., 2006, 2007).

The muscle arrangement in *M. platensis* n. sp. showed a common body-wall muscle pattern in three-layered fibers, i.e., outer circular muscle fibers, inner longitudinal muscle fibers and intermediate diagonally oriented muscle fibers. The similarities that we found between the muscle arrangement of *M. platensis* n. sp., *M. lignano* (Ladurner et al., 2005a; Morris et al., 2007), and *M. hystricinum marinum* (Rieger et al., 1994) are based on the general arrangement of the three sets along the body of the worm. However, a detailed analysis showed that there were differences in the number of fibers that comprise each different set of muscles between the new species and those mentioned above. Future studies from additional species of the genus will most likely add to the taxonomic value of the muscular system.

The muscle arrangement of *M. platensis* shows a distribution pattern similar to that previously described for other *Macrostomum* species (Rieger et al., 1991, 1994; Morris et al., 2007). Despite that the individuals analyzed were only adults, and that the number of fibers may vary between different developmental stages (Rieger et al., 1994), in contrast to the observations by Rieger et al. (1994), who found a great variation in the number of fibers of the specimens analyzed, and in concordance with the observations of Morris et al. (2007), the number of the muscle fibers in the new species was quite constant in the specimens analyzed. Indeed, our results show that the number of body wall fibers in *M. platensis* n. sp. differs from *M. lignano* (Ladurner et al., 2005b).

(a) *Macrostomum platensis* n. sp. has circular muscle fibers being thinner than the longitudinal ones, in concordance with the description by Morris et al. (2007) for *M. lignano*.
(b) *M. hystricinum marinum* shows longitudinal forked fibers in the caudal plate (Rieger et al., 1994). In *M. platensis* n. sp. forked longitudinal fibers are mainly associated with the pharyngeal musculature.
(c) *M. platensis* n. sp. shows numerous diagonal fibers (20 pairs) associated with the mouth opening, apparently more than those described from *M. hystricinum marinum* (see Fig. 10 in Rieger et al., 1994).
(d) Dorsoventral fibers are present in the head and tail regions, as described from *M. hystricinum marinum* by Rieger et al. (1994). Morris et al. (2007) also described this kind of fibers for *M. lignano* as vertical fibers.

Our observations on the muscle arrangement of the female genital system are concordant with those reported by Rieger et al.

(1994): the circular, longitudinal and diagonal muscles of the body-wall are not involved in the musculature of the female gonopore.

5. Conclusions

Confocal laser scanning microscopy of phalloidin labeled specimens of *M. platensis* n. sp. shows that the musculature in the new species differs from previously described species. The stylet morphology was traditionally considered as a key character for species identification. However, new studies in which this character was analyzed together with sperm and other morphological features of the reproductive system, suggest the existence of convergent evolution (Schärer et al., 2011). Regarding this fact, the comparison of the muscle arrangement between the few species of this group seems to show inter-specific differences. In this way, this kind of analysis might constitute a new approach for taxonomic studies in the future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jcz.2011.08.006.

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