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Cover: Long-tailed Shrike *Lanius schach* resting on a dry branch after courtship. Digital illustration on Procreate. © Aakanksha Komanduri.

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COMMUNICATION

Unveiling genital specializations in *Megascolex travancorensis* (Oligochaeta: Megascolecidae) through scanning electron microscopy

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Abstract: When an earthworm reaches reproductive age, its clitellum, also referred to as the genital collar, observably enlarges and becomes more glandular. Genital features in Megascolecidae, including male and female pores, papillae, grooves, and glandular thickenings, show species-specific patterns linked to reproduction, thereby serving as important characters in taxonomy and systematics. This work uses comprehensive scanning electron microscopy (SEM) images to investigate the male reproductive anatomy of *Megascolex travancorensis* Michaelsen, 1910. A slit-shaped male pore located on a raised genital papilla, paired prostatic ducts, a seminal groove, retracted penial setae, and eye-like genital marks on segment 18 are among the distinctive morphological features highlighted in the investigation. Fine-scale structural adaptations have been shown by SEM, which has also helped to classify accurately the genus *Megascolex* taxonomically and provide important information about reproductive morphology.

Keywords: Clitellum, earthworms, genital papilla, penial setae, prostatic ducts, reproduction, seminal groove, taxonomy, tubercula pubertatis.

സംഗ്രഹം: ഒരു മണ്ണിര പ്രത്യുൽപാദന പ്രായത്തിലെത്തുമ്പോൾ, ജനനേന്ദ്രിയ കോളർ (clitellum) ശ്രദ്ധേയമായി വലുതാകുകയും കൂടുതൽ ഗ്രന്ഥികളുള്ളതായി മാറുകയും ചെയ്യുന്നു. മെഗാസ്കോലെസിഡേയിലെ ജനനേന്ദ്രിയ സവിശേഷതകൾ, ആൺ-പെൺ സുഷിരങ്ങൾ, പാപ്പില്ലകൾ, നാളങ്ങൾ, എന്നിവ പ്രത്യുൽപാദനവുമായി ബന്ധപ്പെട്ട സ്പീഷീസ്-നിർദ്ദിഷ്ട പാറ്റേണുകൾ കാണിക്കുന്നു, അത് ടാക്സോണമിയിലും സിസ്റ്റമാറ്റിക്സിലും പ്രധാന സവിശേഷതകളായി പ്രവർത്തിക്കുന്നുണ്ട്. *Megascolex travancorensis* Michaelsen 1910 ന്റെ പുരുഷ പ്രത്യുത്പാദന ശരീരഘടനയെക്കുറിച്ച് അന്വേഷിക്കാൻ ഈ ലേഖനം സമഗ്രമായ സ്കാനിംഗ് ഇലക്ട്രോൺ മൈക്രോസ്കോപ്പി (SEM) ചിത്രങ്ങൾ ആണ് ഉപയോഗിച്ചിരിക്കുന്നത്. ജനനേന്ദ്രിയ പാപ്പില്ലയിൽ സ്ഥിതി ചെയ്യുന്ന ഒരു സ്ലിറ്റ് ആകൃതിയിലുള്ള പുരുഷ സുഷിരം,രണ്ട് പ്രോസ്റ്റേറ്റ് നാളങ്ങൾ, ഒരു സെമിനൽ നാളി, പിന്നോട്ട് ഉൾവലിഞ്ഞ് സ്ഥിതി ചെയ്യുന്ന penial seta, segment 18 ലെ കണ്ണ് പോലുള്ള ജനനേന്ദ്രിയ അടയാളങ്ങൾ എന്നിവ അന്വേഷണത്തിൽ എടുത്തുകാണിച്ചിരിക്കുന്ന വ്യതിരിക്തമായ രൂപഘടന സവിശേഷതകളാണ്. Megascolex genus നെ വർഗ്ഗീകരണപരമായി കൃത്യമായി തരംതിരിക്കാനും പ്രത്യുൽപാദന രൂപഘടനയെക്കുറിച്ചുള്ള പ്രധാന വിവരങ്ങൾ നൽകാനും SEM മുഖേന സഹായിച്ചിട്ടുണ്ട്.

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Author contribution: Jaya Manazhy and Aja Manazhy conceptualized the study and contributed to the review and editing of the manuscript. Sona Sajeev conducted the field study, performed SEM analysis, curated the data, prepared visualizations, and wrote the original draft. John Warren Reynolds provided resources and contributed to the review and editing. Santhosh Punnakattu Parambil contributed to data analysis and manuscript editing

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INTRODUCTION

Hermaphroditism and the presence of a clitellum, a specialized reproductive structure, are the main characteristics that set oligochaetes apart from polychaetes (Reynolds 1974). With more than 5,000 species of terrestrial earthworms, the oligochaetes make up about one-third of the phylum and are the second largest group within Annelida (Reynolds & Wetzel 2025). Their wide range of terrestrial environments highlights their astounding adaptability and ecological significance. The clitellum, a noticeable glandular growth near the front end of their reproductive system, is a crucial component that emerges at sexual maturity. Earthworms produce eggs for a large portion of the year and are regarded as either continuous or semi-continuous breeders (Olive & Clark 1978). Their reproductive activity is greatly influenced by environmental conditions, especially soil temperature and moisture content (Reynolds 2022).

The ventral or ventrolateral surface contains pairs of genital markings, which are external apertures for reproductive exchange, indicating their hermaphrodite characteristics. These apertures and related features display morphological characteristics unique to each species, which are useful in systematics and taxonomy. Genital features that are frequently connected to glands, such as discs, pits, grooves, or pads, are useful for identifying species (Blakemore 2006). Male genital area configuration and sperm transfer requirements are frequently correlated with the shape and location of these characteristics. Male pore arrangements vary; the megascolecine type has both male and prostatic pores on segment 18, but the acanthodriline, microscolecine, and balantine forms have them separated or relocated to segments 17 or 19, occasionally connected by seminal grooves or changed by segmental displacement. In Megascolecidae, the male pores may be associated with prostatic pores, both of them may appear on raised papillae or ridges, and posing tubular or racemose prostates which in turn associated with the posterior ends of vasa defentia. The megascolecids often possess various markings at sexual maturity in the form of tubercles which are the glandular thickenings on the ventral surface and situated both sides or on the clitellum (Figure 1).

There are studies available to identify oligochaetes morphologically across various regions of the world (Gates & Reynolds 2017; Khan et al. 2022; Kamal et al. 2023; Rathour & Julka 2024; Narayanan et al. 2025). There is still a dearth of ultrastructural research on earthworm reproductive architecture in the body of literature, which indicates a lack of thorough microscopic morphological characterization. In-depth ultrastructural examination of genital characteristics, especially with scanning electron microscopy (SEM), allows for accurate characterization of minute surface details such glandular areas, pore shape, and related textures, these are the aspects that conventional light microscopy frequently fails to discern. Proper sample preparation is essential to ensure accurate SEM imaging, minimizing artifacts and enhancing structural clarity. SEM is, therefore, an essential tool for researching the reproductive morphology of earthworms and enhancing species-level identification. The present study aims to characterize the ultrastructural morphology of the selected species, thereby providing insights into their taxonomic and functional significance.

Image 1. An aclitellate adult of *Megascolex travancorensis* on a *Macaranga tanarius* (L.) Müll. Arg. leaf. © Sona.

MATERIALS AND METHODS

Earthworms with well-developed clitellum were obtained from the soil by digging and hand sorting methods (Reynolds 1977, 2022), and also searching in organic micro habitats like leaf litter. Out of the 30 samples collected, ten well-developed clitellate adult specimens were preserved in 10% formaldehyde for further identification using a light microscope and each sample was assigned a code . The type specimens were deposited in Zoology Museum, Sree Kerala Varma College, Thrissur, Kerala with deposition number

THS_{20}1. For SEM analysis, two specimens were fixed in 5% glutaraldehyde ($C_5H_8O_2$) for 30 minutes to preserve ultrastructural details. Following fixation, the dissected clitellum was washed thoroughly and dehydrated using an ascending ethanol series. Specimens are mounted on to SEM stubs using conductive adhesive like carbon taps and coated with a thin layer of conductive material (gold/palladium) using a sputter coater to enhance image quality and reduce charging effects. Prepared samples are placed in the SEM chamber, where they are scanned by the electron beam to generate high-resolution images. The secondary soil data from various localities were obtained from the data provided by the Soil Survey Organisation Agriculture Department (Premachandran 2007). Observations were carried using Carl Zeiss EVO 18 Research SEM (Central laboratory for Instrumentation and facilitation, University of Kerala) and magnified the sample from 10X to 5K X.

RESULTS

Morphological identification by Light microscopy

Family Megascolecidae Rosa, 1891

(Diagnostic features from: descriptions of Stephenson 1923; Gates 1972; Blakemore 2006; Reynolds 2022 and also present observation)

Diagnostic Features

Dorsal pores: present

Male pores: male pores may be united with prostatic pores, paired or unpaired, commonly on 18, rarely on 19 or 20, behind female pores.

Setae: lumbricine or perichaetine.

Clitellum: annular, multilayered.

Nephridia: holoic or meroic.

Intestinal origin: behind ovarian segment.

Gizzard: Oesophageal gizzard(s) present; intestinal gizzard(s): present, or absent.

Last hearts: behind segment 11.

Calciferous glands: present or absent.

Ovaries: in 13, mostly fan shaped and with numerous egg strings.

Spermathecae: single, paired, or multiple; diverticulate (or rarely with intramural sperm chambers),

Prostates: present, tubular to racemose.

Remarks: The position of male pore and nature of prostate are the diagnostic features.

Genus *Megascolex* Templeton, 1844

(Diagnostic Features from: descriptions of Stephenson 1923; Aiyer 1929 and also present observation)

1844. *Megascolex* –Templeton, Proc. Zool. Soc. London, 12(1844): 89.

1895. *Megascolex* – Beddard, Monogr. Oligochaeta, p. 370.

1900. *Megascolex* – Michaelsen, Oligochaeta, Das Tierrich, p. 212.

1907. *Megascolex* – Michaelsen, Fauna S W Australia 1: 168.

1909. *Megascolex + Lampito* – Michaelsen, Mem. Indian Mus. 1: 178.

1916. *Megascolex* – Michaelsen, Mjobeg s Australia Exp. 52: 57.

2009. *Megascolex* – Ramasamy, Doctor of Philosophy in Zoology, Tamilnadu, India, p. 99.

Diagnostic features

Setae: perichaetine.

Spermathecal pores: 1–5 pairs between segments 4–9.

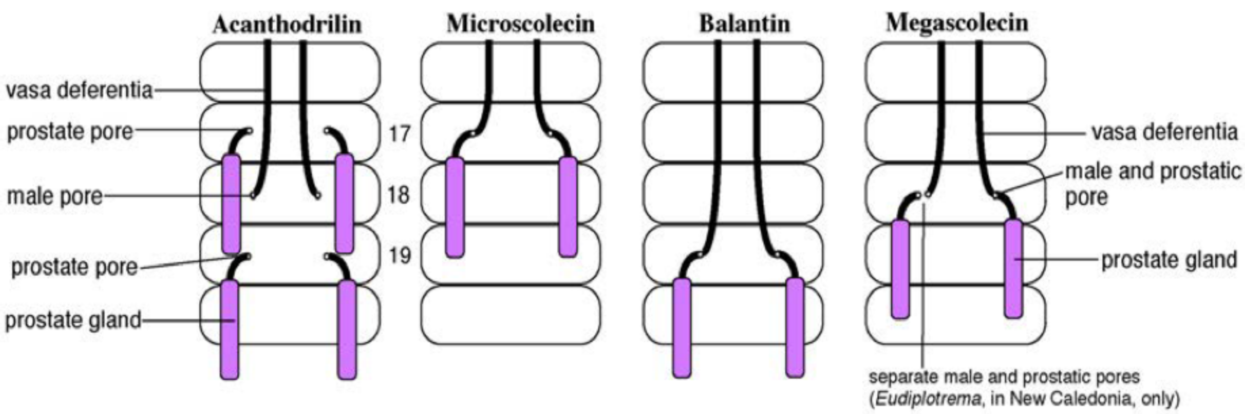

Figure 1. The chief arrangements of prostatic and male pores in the Megascolecidae, Megascolecinae, and Acanthodrilinae (Jamieson 2001).

Gizzard: one in 5, 6 or 7.

Nephridia: meroic.

Prostate: racemose to tubulo-racemose.

Previous records: India (Southern India, Bombay, eastern Himalaya), Australia, Ceylon, Tasmania, northern Island of New Zealand, southwestern Asia, and the Malay Archipelago.

Megascolex travancorensis Michaelsen, 1910

Redescription

1910. *Megascolex travancorensis* f. *typica* Michaelsen, Abh. Ver. Hamburg 19 (5): 72.

1913. *Megascolex travancorensis* var. *typicus*-Michaelsen, Mt. Mus. Hamburg 30(2): 85.

Collectors' name: Sona Sajeev.

Date of collection: 29.iv.2024.

General habitat: coconut plantation (*Cocos* sp.), cultivated area.

Soil texture: gravelly sandy clay, sandy clay loam, clay loam, gravelly clay and sand.

Physiography: low land, mid upland, mid land, mountainous region (classification based on Soil Survey Organisation Agricultural Department, Premachandran 2007).

(Diagnostic Features from: descriptions of Stephenson 1923 and also present observation)

External Features

Size: 80–140 mm; preclitellar region: 2 mm; clitellar region: 3 mm; post clitellar region: (1–2 mm).

Number of segments: 120–280, segment 2–5 biannular; 5–13 triannular.

Pigmentation: dark grey before preservation, unpigmented (Image 1).

Prostomium: epi-tanylobous.

Setae: perichaetine, 16 setae per segment in the middle of the body, However, anterior segments (= up to the segment 25) the setae in lumbricine arrangement (Figure 2).

First dorsal pore: 4/5 (3/4 in one specimen).

Clitellum: well, developed in segments 13–18 (= 6); in some specimens overlapping the male genital markings.

Male pores: in 18 on slightly elevated cushions extended to segment 19, in *ab*, *bc* and extends to ¾ of *cd* on each side which are ellipsoidal, their inner borders approximated and parallel, both cushions together almost fill up a somewhat depressed median area, which is bounded laterally and in front by a slight wall.

Female pores: paired.

Spermathecal pores: two pairs, between a and b, in 7/8 and 8/9, about ½ of the circumference apart.

Genital marking: pair of elevated markings in segments 17, 18 and 19 at more or less at cd in which it is highly ornamented with folding and wrinkles forming the shape of an eye at the base (= 19).

Internal Anatomy

Septa: 6/7 – 12/13 highly thickened, 13/14 and 14/15 moderately thickened.

Gizzard: large in 6.

Calciferous glands: absent.

Testis and funnels: free in 10 and 11.

Seminal vesicle: compactly racemose in 11 and 12.

Spermathecae: spermathecal ampulla large, pear-shaped and usually much bent at its ectal end; duct still thinner, very short, mostly concealed in the body-wall; diverticulum enters the ectal end of ampulla; is narrowly club-shaped and somewhat bent at its ectal end; a mass at the ectal end of ampulla seems to represent an incompletely formed spermatophore.

Prostates: racemose in the 17 and part of the following segments with three to five incisions to form lobes.

Ovary: two pairs in 13 and 14.

Penial setae: absent.

Remarks: The spermathecae similar to *Megascolex konkanensis.* The form of tubercula pubertatis is also a notable feature of the type species *M. travancorensis*. In some specimens, we noticed a digitiform projection in the segments 15/16/17/18. The actual function of these structures is not known and may be a genital marking or an artifact.

Previous records: Southern India.

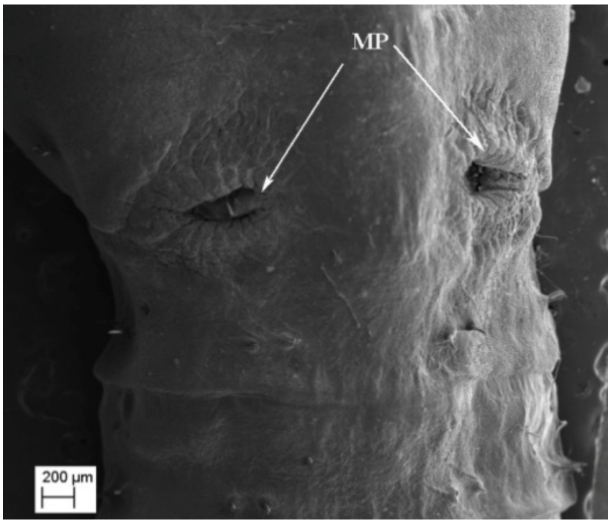

Image 2. Male pore of *Megascolex travancorensis*; MP—Male pore. © Sona.

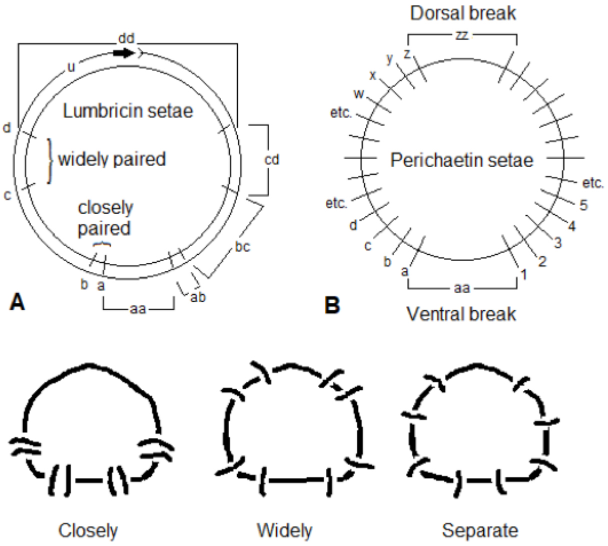

Figure 2. Arrangement of setae: Setae may be closely paired, widely paired, or separate (A, left). Perichaetine arrangement (B, right). The pheretimoid earthworms have numerous setae (as many as 150) per segment. Perichaetin setae are arranged in a ring, circumventing all segments of the specimen (with the exception of the first and last segments). The numbers of setae vary slightly from one segment to another and from one specimen to another. Within specimen variations are usually largest in the pre-clitellar region. The closest seta to the mV is a and they increase to the mL; the seta closest to the mD is z and they decrease to the mL. Sourнce: Reynolds, 2022.

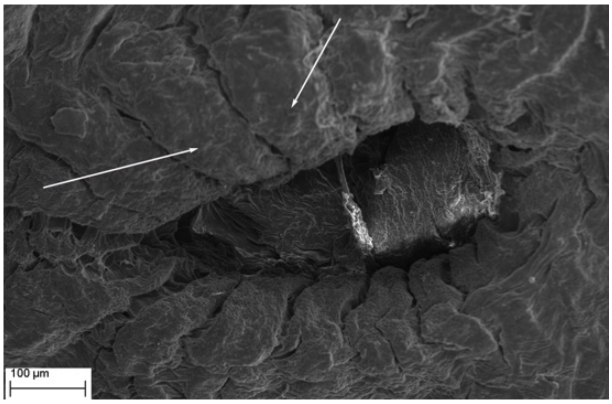

Image 3A. Genital papilla of *Megascolex travancorensis*. © Sona.

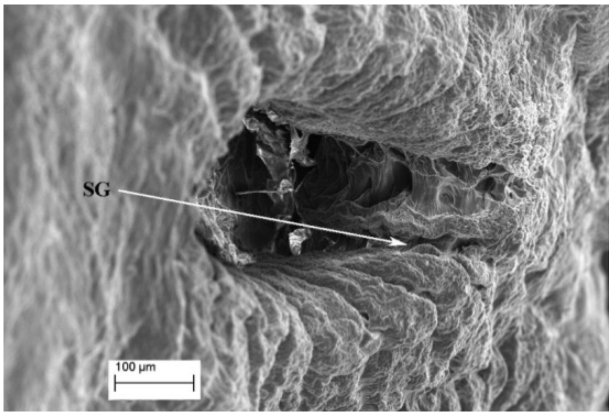

Image 3B. Seminal groove on segment 18 of *Megascolex travancorensis*; SG—Seminal groove. © Sona.

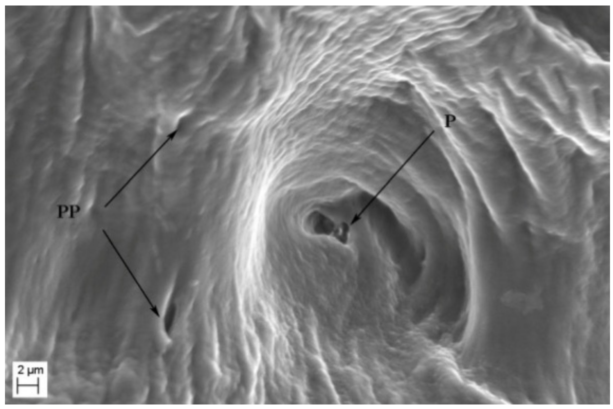

Image 4. Prostatic pore and Penial pore of *Megascolex travancorensis;* PP—Prostatic pore | P—Penial groove. © Sona.

Present collection: Thiruvananthapuram, Kollam, Pathanamthitta (south), Thrissur, Palakad (central), Kerala, southern India.

Ultramorphological analysis of genital pore using SEM

Male genital area is highly ornamented with folding, wrinkles, and grooves associated with more pronounced genital marking (Image 2). Although the eye shaped marking is visible by light microscopic analysis, the associated pores and furrows are inconspicuous. The exact segmental location, shape, pattern, and associated structures furnish useful taxonomic characteristics. The extension of clitellar glandular tissue in segments 13–18 overlapping the male genital markings on segment 18 suggests a close structural association between the clitellum and reproductive pores reflecting the genital specialization especially in the genus *Megascolex*. By ultrastructural analysis, the male pore is clearly visible on segment 18 as a small slit with a magnification of 60 X and slightly raised on a male genital papilla that slightly extended to segment 19 (Image 3A,B). Here, tubercula pubertatis appears as a pair of glandular thickened ridges arises on the segments 17–19. Seminal groove seen as a shallow channel that starts from the male pore and runs anteriorly towards the clitellum (Image 3B, 370 X) and that helps in transferring sperm during copulation. Prostatic ducts appear as two lateral grooves one above the other from the paired prostatic lobes (Image 4). A deep pore is seen near to the prostatic pore may be the

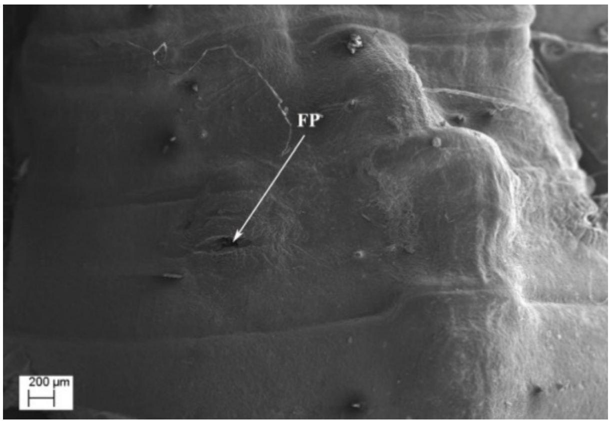

Image 5A. Female pore on segment 13 of Megascolex travancorensis; FP—Female pore. © Sona.

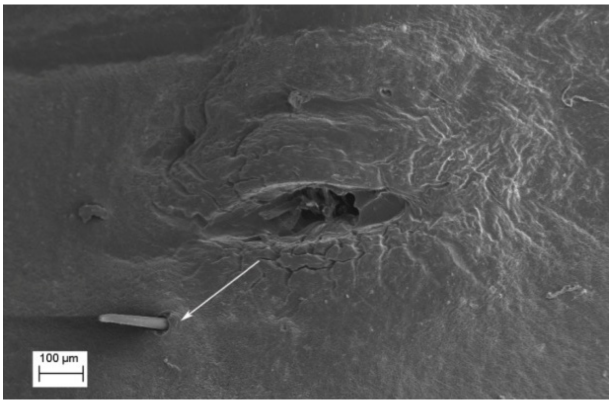

Image 5B. Female pore on segment 13 of *Megascolex travancorensis* at 'ab.' © Sona.

indication of the retracted penial setae (Image 4, 5KX).

DISCUSSION

Similar possibility discussed by Gates (1972) in 'Burmese earthworms' that male chambers may be eversible into characteristic porophores and may contain protrusible penes. Erséus (1981) discussed that *Bathydrilus rohdei* is provided with an X-shaped mid-ventral slit that, corresponds to the location of the tips of the penial setae of the mate. There are previous reports available for specialized genital setae and related glands that make up the sexual papillae may be used to inject sperms into the mate (Cosín et al. 2011). Ambreen et al. (2004) reported that the variations in genital papilla in the genus *Pheretima* (sensu lato) may contribute to species identification. Similar to our study, Yáñez et al. (2006) and Caramelo & Martínez-Ansemil (2012) used scanning electron microscopy to study particular mating structures and sperm transmission processes in aquatic microdriles, exposing copulation-related ultrastructural adaptations and specialized attachment features.

It should be noted that the families Ocnerodrilidae, Acanthodilidae, Octochaetidae, and Megascolecidae, the male pores are associated with the prostatic pores (Julka 1988). In megascolecids with the 'megascolecine' arrangement showed each type of gland connects to the male pores by a muscular prostatic duct that receives sperm from the testis via the vasa deferentia that often joins the duct near the junction with the gland and one pair of male and prostatic pores combined on segment 18 (Blakemore 2006). Here we can observe that, although *M. travancorensis*, there are separate pores of prostatic ducts and later joins with vasa deferentia to the exterior as male pore (see Image 2 and Image 4). Lone et al. (2022) reported high intra specific divergence for *M. travancorensis*. Female pores also clearly demarcated on 13 (Image 5A), however, not much ornamented and associated setae at ab (Image 5B). Because of its less physical diversity across species and relatively conserved position, the female pore is often regarded as having minimal taxonomic relevance. This work provides important taxonomic and systematic insights into *M. travancorensis*'s distinctive reproductive morphology by highlighting the genital markings and related structures in and around the clitellum.

CONCLUSION

Highly specialized and decorated genital characteristics that are important for species identification and reproductive function are revealed by the ultrastructural investigation of *M. travancorensis*. The male genital region is complex, as indicated by the existence of paired prostatic ducts, a seminal groove, and eye-shaped genital marks. Small structural adaptations such as retracted penial setae, lateral prostatic pores, seminal grooves, and glandular papillae can be found with SEM imaging. These discoveries provide useful taxonomic markers within the genus *Megascolex* and advance our knowledge of earthworm reproductive characteristics. Moreover, the distinct genital specializations observed may represent adaptations for efficient sperm transfer, mate recognition, and reproductive isolation, thereby offering insights into phylogenetic relationships.

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