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Phylogenetic Relationships in Earthworm *Megascolex* Species (*Oligochaeta: Megascolecidae*) with Addition of Two New Species

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Abstract: *Megascolex* (Oligochaeta: Megascolecidae) are endemic species to India and Sri Lanka, however, to date their molecular taxonomy and phylogenetic relationships have not been reported. We applied the first integrative approach using morpho-anatomical features and a COI dataset to unveil species delimitation (SD), molecular taxonomy, and phylogenetic relationships in *Megascolex* species. Our morpho-anatomical results revealed nine *Megascolex* species, namely, *M. auriculata*, *M. cochinensis cochinensis*, *M. filiciseta*, *M. ratus*, *M. travancorensis travancorensis*, *M. triangularis*, *M. konkanensis konkanensis*, *M. polytheca polytheca*, and *M. polytheca zonatus*. We also reported the occurrence of two new species, namely, *M. papparensis* sp. nov, and *M. vazhichlensis sp. nov.* Such findings were also supported by the analysed COI dataset, in which these new species appeared distinct on the phylogenetic trees with strong support. The studied Megascolex species appeared paraphyletic and formed three subclades on Bayesian inference (BI) and Maximum Likelihood (ML) phylogenetic trees. The first clade consisted of six species: *M. cochinensis cochinensis*, *M. polytheca polytheca*, *M. polytheca zonatus*, *M. konkanensis konkanensis*, *M. filiciseta*, and *M. auriculata* with strong posterior probability support. The second clade consisted of M. travancorensis travancorensis, *M. papparensis* sp. nov, and *M. vazhichlensis* sp. nov with strong support. The third clade consisted of M. ratus and M. triangularis with good support. In addition, the validation of species was confirmed by SD methods, in which the congruence among OTUs was observed with the clear barcode gap of 12–14% suggested by ABGD analysis. However, the species *M. ratus* and *M. travancorensis travancorensis* show deep intraspecific divergence and, therefore, require more sampling data. Such findings are essential to study the phylogenetics and evolution of the genus and, nonetheless, demand larger COI datasets to make concrete conclusions.

Keywords: COI; endemic species; *M. papparensis* sp. nov; *M. vazhichlensis* sp. nov; Phylogenetic relationships

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1. Introduction

Earthworms are the group of Oligochaeta worms that are known for their soil-dwelling properties and ecosystem functions that include soil turnover, soil fertility, and biomass [1,2]. Megascolecidae is the most diverse family of earthworms [3] comprising over 1000 species [3,4], most of them being native to Asia and Australia [4]. Out of many genera of the family *Megascolecidae*, the genus *Megascolex* was first described by Templeton [5] under the name *M. caeruleus* from the alpine regions of Ceylon (Sri Lanka), characterised by the presence of a row of small numerous spines or setae on each segment. Since then, 37 species and sub-species of *Megascolex* have been reported from Sri Lanka [6]. In India, the

genus is comprised of 33 species [7], out of which 24 species were presented in a checklist by Narayanan et al. [8]. However, with the addition of *M. lawsone* from the state [9], the list was updated to 25 species with the presence of *M. insignis* as the only near-endemic species which occurs both in India and Sri Lanka [6]. The distribution of *Megascolex* species in India has been reported from peninsular parts, particularly in the southern part of India [9–12]. Kerala is an important constituent part of the Western Ghats and houses 99 species of earthworms [9]; however, just a few molecular studies have been conducted on *Drawida* and *Moniligaster* species [13] and there are no molecular records for *Megascolex* species.

Since earthworm taxonomy can be quite challenging particularly due to conservative morphological features [14], the taxonomy and diversity assessments are unstable and in need of revision. With the availability of online databases such as the Barcode of Life Database (BOLD) and the National Centre for Biotechnology Information (NCBI) that hold thousands of DNA barcodes, there are opportunities to use these information resources to improve the study of the taxonomy, evolution, and ecology of many organisms [15–17]. Nonetheless, in earthworms, these data have only been used to clarify a few taxonomic groups and the application of DNA barcoding to most of the Indian earthworm species is still deficient. This limits inferences about their actual diversity and correct classification [18]. Progress in molecular taxonomy of some Indian genera/species complexes such as *Amynthas* [19], *Aporrectodea caliginosa* species complex [20], *Drawida* and *Moniligaster* [13], *Eutyphoeus* [21,22], *Kanchuria* [23], as well as the molecular diversity and genetic variability of some other earthworm species [24–27] has significantly added to work in the field over the past few years. Nevertheless, most of the species remained untouched by the molecular approach and have only been described by morpho-anatomical observations that require strong diagnostic features, extensive labour, and expertise to segregate complex species and cryptic species.

The present investigation aimed to provide the DNA barcodes as well as the morpho-anatomical descriptions using an integrative approach in *Megascolex species* collected from different sites of the Western Ghats in Kerala to unveil their phylogenetic relationships on the tree of life.

2. Material and Methods

2.1. Study Area and Collection of Earthworms

Kerala is a small state located on the western tip of peninsular India (Figure 1), occupying 38,863 sq. km and comprising approximately 1.18% of the landmass of India [28]. The state is blessed with diversified micro- and macro-environmental conditions contributing to diverse flora and fauna. This mega biodiversity hot-spot area of the Western Ghats hosts about 30%, 24%, and 35% of animal, plant, and fresh water fish species of India [28] in spite of its small size. Earthworms were collected from different sites using the digging and hand-sorting techniques following the method described by Lewis and Taylor [29].

2.2. Morpho-Anatomical Observation

The preserved earthworm specimens were screened for morphological observations following dissections under a stereomicroscope (Leica Model No. M60). The specimens were stored in the museum, at the Department of Zoology, Dr. Harisingh Gour Vishwavidyalaya (A Central University) Sagar, MP. The species' names with sample ID, process ID, collection sites, GPS coordinates, date of collection, name of collectors, and BOLD accession numbers are provided with the description of each species. The comparative account of morphological characters of *Megascolex* species is provided in Table 1.

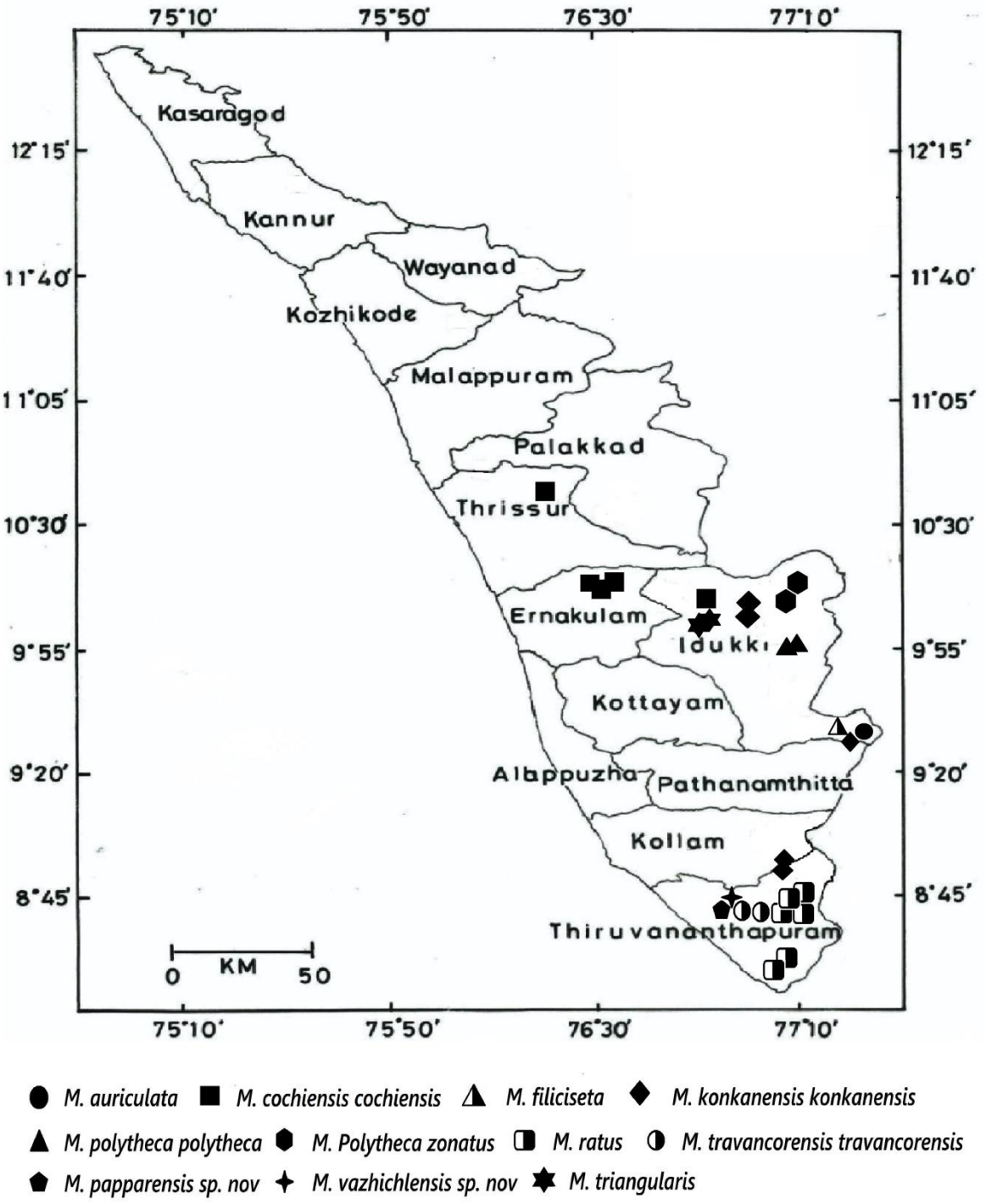


Figure 1. Collection of *Megascolex* species from various geographical spots of Kerala, Western Ghats, India.

Abbreviations used: sp., spermathecal pores; fp., female pore; mp., male pore; amp, ampulla; div., diverticulum; sp.d., spermathecal duct.

Table 1. Comparative account of morphological characters of selected *Megascolex* species which belong to different clades in phylogenetic trees.

Species /Characters	*M. papparensis sp. nov.*	*M. vazhichlensis sp. nov.*	*M. cochinensis cochinensis*	*M. ratus*	*M. triangularis*
Size mm	85	175	190–208	140–350	138–150
Pigmentation	Light grey	Mild grey	Darkly coloured	Dorsally violet brown, ventrally grey	Dark brownish
Diameter mm	3	3	3–4	7–8	5–6
No. of segments	196	256	125–180	160–180	190–210
Clitellum	Dark brown, xiii–xvii	$\frac{1}{2}$ xiii–xvi	$\frac{1}{2}$ xiii–$^{1/3}$ xvii	Saddle shaped, xiv–xviii	xiv–xvii
Setae	Absent in clitellar region	Irrugular post-clitellur with small dorsal break	Setae ring broken	More crowded ventrally, no break	?
Dorsal pore start	5/6	4/5	5/6	?	6/7
Spermathecal pores	*a* lines	*b* lines	*a* lines, sometimes on *a*/*b* or *b*	*f* lines	*ab* lines
Male pores	$\frac{1}{2}$ xviii	xviii, in lines *b* and *c*	xviii	xviii, on *h*	At *a*
Female pores	Paired, *aa*	Paired, *aa*	Single, *aa*	paired on *a*	Paired, *aa*
Gizzard	vi	v	v	vi	v
Testes and funnels	Free, xi and xii	Free, x and xi	Free, x and xi	Free, x and xi	Free, x and xi
Seminal vesicles	xi and xii	xi and xii	x and xii	?	xi and xiii
Calciferous glands	?	?	absent	?	absent
Spermatheca	Ampula oval-shaped, short diverticulum	Suasage-shaped without shining diverticulum	Ampula ovoid with shining diverticulum	Large sac-shaped ampula, tiny diverticulum	Ampula large, multiloculate diverticulum
Prostates	Large in xviii–xxiv	xvi–ix	Tightly packed in xviii	In xviii	xviii, no prostatic ducts seen
Penial setae	?	?	absent	?	?

2.3. DNA Extraction, Amplification, and Sequencing

For sequencing, 28 samples of collected *Megascolex* species were sent to Barcode of Life Data Systems (BOLD), Biodiversity Institute of Ontario, University of Guelph, Canada to obtain DNA barcodes and accession numbers. The COI dataset of various earthworm species used in sequence alignments and data analysis for the current study is given in Supplementary Materials Data S1.

2.4. Sequence Data Curation, Alignment, and Genetic Distances

The alignment of the final COI dataset was performed in MEGA X [30] using MUSCLE [31]. Additionally, sequence composition was calculated and the sequences with insertions, deletions, and stop codons were removed, and all sequences were checked for ambiguous nucleotide sites and saturation. The uncorrected pairwise genetic distances were estimated within and between species [32] in MEGA X. In addition, the cluster sequencing analysis was performed on BOLD to infer intraspecific distances amongst *Megascolex* species compared to the distances of their nearest neighbours (NN).

2.5. Phylogenetic Analysis

Maximum likelihood (ML) and Bayesian inference (BI) methods were applied to reconstruct phylogenetic trees. The final dataset for nucleotide substitution model fit for phylogenetic tree reconstruction was tested by using jModelTest 2 [33] with the GTR+I+G4 model, at seed 419195. The maximum likelihood analysis was undertaken using raxmlGUI [34]. The bootstrap resampling with 1000 replicates was performed to support the individual branches of the ML tree. The final tree was visualised and edited on iTol v5 (Interactive Tree of Life (https://itol.embl.de; assessed on 4 October 2022) [35]. For BI analysis, the program BEAST [36] was employed and the parameters were set to 1,000,000 MCMC (Markov chain Monte Carlo) steps with the tree sampled at every 10,000 generations while discarding the first 10% as burn-in. Using Tracer v1.6 [37], the output was examined for all parameters and the maximum clade credibility tree was accessed using Tree Annotator v1.5.3 [38]. The final tree was visualised and edited using Fig Tree v1.4.2 [39]. The posterior probabilities with >0.90 were set for each branch in BI analysis. The topological differences between ML and BI phylogenetic trees were compared at the level of resolution obtained by each taxon and its bootstrap support.

2.6. Species Delimitation (SD) Analysis

Species delimitation was performed using three standardised methods; Automated Barcode Gap Discovery (ABGD) [40], Assemble Species by Automatic Partitioning (ASAP) [41], and the Refined Single Linkage (RESL) algorithm, which forms the basis of the Barcode Index Number system (BIN) [42]. ABGD was implemented with the MUSCLE aligned matrix as the input file in the ABGD package of iTaxoTools [43], adopting Kimura (k80) model = 2.0, X (relative gap width) = 1.0, and keeping other parameters as default values (Pmin = 0.001, Pmax = 0.1; Steps 10; Nb bins = 20). For ASAP, the output file was used in the ASAP program with default settings of iTaxoTools. BIN analysis was conducted in data analysis option of the BOLD database.

3. Results

3.1. Megascolex auriculata Aiyer, 1929

Megascolex auriculata Aiyer, 1929: 64.

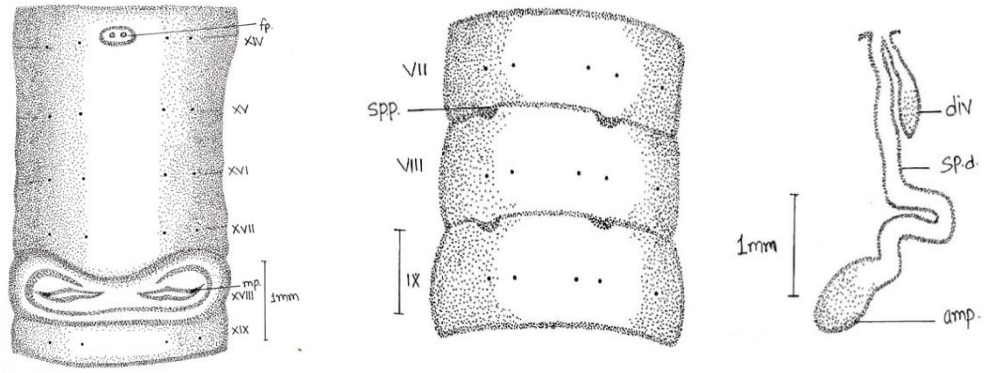
Material examined: Sample ID: KERL0272A4; Process ID: IEW414-17; towards south of Periyar National Park, submerged area, Kottayam (9°27′22.6″ N 77°13′53.8″ E), Kerala, India; 28 October 2015; Coll. Samuel W James and Shweta Yadav; BOLD accession No: ADH2012.

Description:

Length, 180 mm; diameter, 2 mm; segments, 318. *Prostomium epilobous*. Clitellum in xiv–1/2 xvii, dark-pigmented. First dorsal pore at 9/10. *Setae lumbricine* loses orientation posterior. Setae *ab* closely and *cd* widely paired; the distance between *ab* same throughout the body, while *cd* varied after lxi segment and *d* moved outwardly. At the extreme lower end, setae 9 or 10 in number and setae *e* very close to *d* without maintaining specific orientation. On xviii, two opposite-oriented separate trenches present. Male pores bounded with pit-like depression, encircled by circular lobe-like depression. Female pores paired in xiv between *aa* encircled by whitish oval circle. Spermathecal pores two pairs in 7/8 and 8/9 in lines *b* (Figure 2).

Septa 6/7–9/10 very thick and 10/11–11/12 less thickened. Large barrel-shaped gizzard in vi. Oesophageal swelling at vii-xiii with numerous villi-like structures on its inner surface. Testes and funnels single pair in segment xi. Seminal vesicles large follicular in xii. Intestine begins in xvi. Last heart in xiii. The ectal half of the duct is wider than ental. Penial setae absent. The spermathecae are in viii and ix, ampullae long club-shaped with cylindrical diverticulum. Nephridia tufts are found, with up to six segments with one-two pair tufts, 8–9 tufts with long coiled tubes in the clitellar area, and 5–6 small-sized tufts in the post-clitellar region. Prostates racemose, loosely lobed in xviii-xxii segments,

duct arises from the anterior part of the gland after 3–4 coils and opens outside. The duct emerges from the anterior portion of the gland after 3–4 coils and opens outside.



(**a**) (**b**) (**c**)

Figure 2. Camera lucida illustration of *Megascolex auriculata* (**a**) genital region; (**b**) spermathecal region; (**c**) spermathecae.

Distribution: The species is endemic to Kerala. Dist. Idukki, Kumili; Vandiperiyar Kottayam; Athirampuzha [8,10].

3.2. Megascolex cochinensis cochinensis Stephenson, 1915

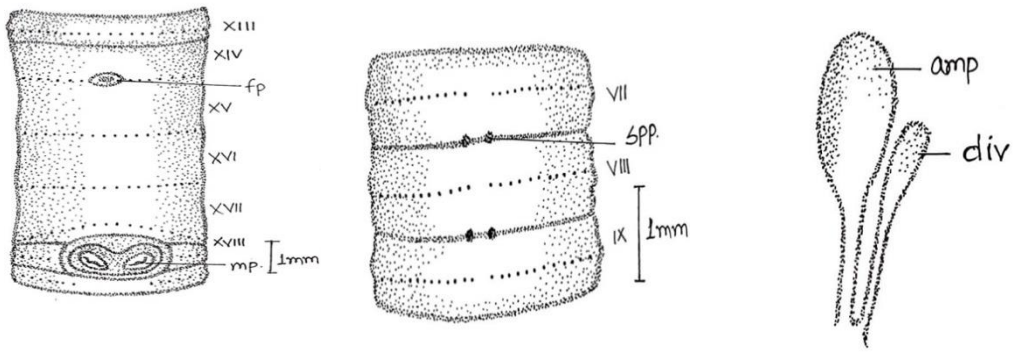
Megascolex cochinensis Stephenson, 1915: 96–97.

Megascolex cochinensis cochinensis Stephenson. Blakemore 2007: 33.

Material examined: Sample ID: KERL0265A4; Process ID: IEW378-17; near waterfall, Adimali (10°02′03.8″N 76°56′24.0″E), Idukki, Kerala, India; 15 September 2015; Coll. Samuel W James and Shweta Yadav; BOLD Accession No: ADH2818. Sample ID: KEL17-773-27A25; Process ID: IEW727-17; Range Mannamangalam Peechi-Vazhani Wildlife Sanctuary (10°31′40.8″N 76°22′26.0″E), Thrissur, Kerala, India; 1 September 2017; Coll. Shweta Yadav; BOLD Accession No: ADL2102. Sample ID: KEL17-881-33A17; Process ID: IEW835-17; Vavlla sector, Chimmini Wildlife Sanctuary (10°26′55.3″ N 76°27′32.8″ E), Thrissur, Kerala, India; 1 September 2017; Coll. Shweta Yadav; BOLD Accession No: ADL1460. Sample ID: KEL17-882-33A18; Process ID: IEW836-17; Vavlla sector, Chimmini Wildlife Sanctuary (10°26′55.3″ N 76°27′32.8″ E), Thrissur, Kerala, India; 1 September 2017; Coll. Shweta Yadav; BOLD Accession No ADL1460. Sample ID: KEL17-886-33A22; Process ID: IEW840 17; Vavlla sector, Chimmini Wildlife Sanctuary (10°26′55.3′ N 76°27′32.8″ E) Thrissur, Kerala, India; 1 September 2017; Coll. Shweta Yadav; BOLD Accession No ADL1460.

Description:

Large-sized worm; length, 190–208 mm; diameter 3–4 mm. Segments 125–180. Colour darkly pigmented in three specimens collected from same location and one non-pigmented collected from different location. Clitellum pale yellow in dark-coloured specimens extending over $\frac{1}{2}$ xiii–1/3 xvii, setae present in clitellar region. Prostomium closed epilobic, first dorsal pore 5/6. Setae perichaetine, ring broken, intersetal intervals irregular, pre-clitellar *aa* = 2 or 3 *ab*, clitellar *aa* = *ab*, post-clitellar *aa* = 3 *ab*, setal counts were 46/v, 50/ix 54/xii, 42/xx, 36/xl. Male pores wavy slit-like on 18 segment, wavy slits approaching each other posteriorly within a light-coloured oval swollen area. Female pores single (in one specimen seems paired) mid-ventrally in line of setal ring in an oval whitish patch, on 14 segment extends laterally beyond *aa*. Spermathecal pores two pairs in segments 7/8 and 8/9, at *a* and sometimes on *a*/*b* or *b* lines (Figure 3).



(**a**) (**b**) (**c**)

Figure 3. Camera lucida illustration of *Megascolex cochinensis cochinensis* (**a**) genital region; (**b**) spermathecal region; (**c**) spermathecae.

Septa 4/5 and 5/6 are thin; 6/7–11/12 moderately thickened; 12/13–13/14 thickened. Gizzard large barrel-shaped in v. Calciferous glands absent. Intestine begins in xiv. Last hearts in xiii. Excretory system is micronephridal; large thick tufts from oesophagus to gizzard; 5–6 pairs of bushy tufts in clitellar region. Testes and male funnels free in x and xi. Seminal vesicles large in xi and xii attached to anterior septum. Prostates' tightly packed lobules appear as a solid structure in xviii; duct thick, wide, and straight. Ovaries in xiii. Spermathecae two pairs in viii and ix, ampulla ovoid, duct long, ental part shiny. No penial setae.

Distribution: The species is near endemic and has been located in Thrissur: Forest Tramway (nr. Vazhachal); Dist. Kottayam, Athirampuzha [8].

3.3. Megascolex filiciseta Stephenson, 1915

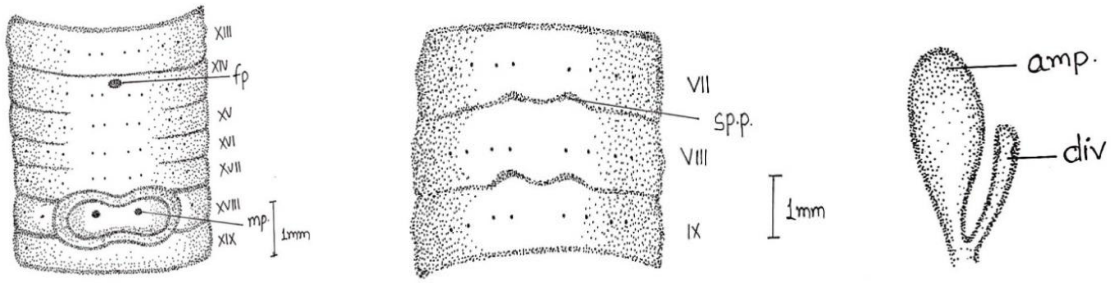
Megascolex filiciseta Stephenson, 1915: 94–96.

Material examined: Sample ID: KERL0272A2; Process ID: IEW412-17; towards south of Periyar National Park, submerged area, Kottayam (9°27′22.6″ N 77°13′53.76″ E), Kerala, India; 28 October 2015; Coll. Samuel W James and Shweta Yadav; BOLD accession No: ADH2018.

Description:

Length, 86 mm; diameter, 3 mm; segments, 140. Colour dorsally dark-bluish grey; extremely dark at posterior ends; 3–4 fine dark stripes present dorsally. Thick and stiff body wall. Clitellum not very clear; dorsally slight difference in colour observed in xiii-xvi. Prostomium epibolic marked by median groove, and major part hidden in transverse groove. First dorsal pore 5/6. In pre-clitellar region *aa* = 2 *ab*, while distance between all setae was much reduced from clitellar region, it was difficult to observe the accurate distance between the setae; the longitudinal lines of setae broke dorsally while being ventrally distinct, numbers 28/v, 30/ix, and 22/xxv. Male pores in xviii on small porophores between *a* and *b* lines. Female pore was difficult to observe; appears to be in a whitish presetal ovoid patch present on xiv between *aa*. Spermathecal pores minute; two pairs in 7/8 and 8/9 at *a* (Figure 4).

Septa 5/6–7/8 delicate; 8/9–13/14 relatively thickened. Gizzard large barrel-shaped in vi. Calciferous glands absent. Intestine begins in xvi. Last hearts in xiii. Excretory system micronephridial; bushy tufts present behind iv with numerous tubules emerging from the main stem; loops prominent behind xiv, not attached to septs. Testes and male funnels are free in x and xi; seminal vesicles small; lobed in ix and xii. Prostates small, flattened, and confined to xviii; duct not visible. Spermatheca two pairs in viii and ix close to ventral nerve cord; ampulla ovoid; duct short; cylindrical diverticulum almost half length of ampullae. Penial setae present; shaft bow-shaped; tapers towards the distal ends; tip slightly curved with stout teeth.



(**a**) (**b**) (**c**)

Figure 4. Camera lucida illustration of *Megascolex filiciseta* (**a**) genital region; (**b**) spermathecal pore; (**c**) spermathecae.

Distribution: Only known from the type locality, i.e., Palakkad: Parambikulam, Kerala [8].

3.4. Megascolex konkanensis konkanensis Fedarb, 1898

Megascolex konkanensis Fedarb, 1898: 434–436. Michaelsen 1910: 75; Stephenson 1916: 328.

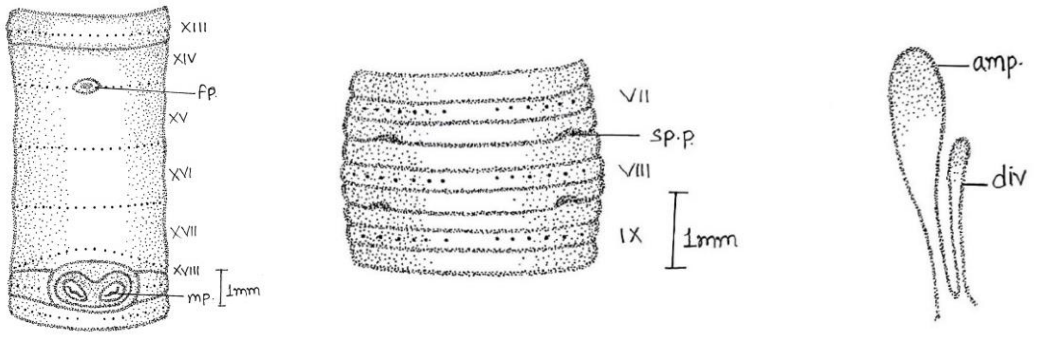
Megascolex konkanensis konkanensis Fedarb. Blakemore, 2007: 34.

Material examined: Sample ID: KERL0267A4; Process ID: IEW381-17; Grassland, Prambikulam Road, Muthalamada (10°23′34.4″ N 76°46′32.2″ E), Kerala, India; 17 September 2015; Coll. Samuel W James and Shweta Yadav; BOLD accession No: ACU5977. Sample ID: KERL0267A6; Process ID: IEW383-17; Grassland, Prambikulam Road, Muthalamada (10°23′34.4″ N 76°46′32.2″ E), Kerala, India; 17 September 2015; Coll. Samuel W James and Shweta Yadav; BOLD accession No: ACU5977. Sample ID: KERL0270A5; Process ID: IEW397-17; Thenmala Reservoir, Shendurney Wildlife Sanctuary (8°53′03.7″ N 77°10′03.4″ E), Kollam, Kerala, India; 26 October 2015; Coll. Shweta Yadav; BOLD accession No: ACS2283. Sample ID: KERL0270A7; Process ID: IEW399-17; Thenmala Reservoir, Shendurney Wildlife Sanctuary (8°53′03.7″ N 77°10′03.4″ E), Kollam, Kerala, India; 26 October 2015; Coll. Shweta Yadav; BOLD accession No: ACS2283. Sample ID: KERL0271A1; Process ID: IEW411-17; Mlappara, Periyar National Park (9°27′22.6″ N 77°13′53.8″ E), Kollam, Kerala, India; 27 October 2015; Coll. Shweta Yadav; BOLD accession No: ACU6070.

Description:

Large-sized worm; length, 170–316 mm; diameter, 4–5 mm; segments, 260–316. Colour grey with bluish irregular marks. Prostomium epilobic with two short longitudinal grooves on the dorsal surface of the first segment. First dorsal pore in 5/6. Dorsal setal gaps; before clitellum *zz* equal to two times larger than *yz*, while behind clitellum less than a gap *zz* = 1/2 *yz*. Ventrally in front of clitellum *aa* = 2 *ab* and behind *aa* = 3 *ab*/4 *ab*. Setae visible from ii. The distance interval between *ab* and *bc* is also irregular. Setal numbers were 36/vi, 32/ix, 38/xii, and 34/xxi. Clitellum 14–17. Male pores on xviii on transverse oval papillae placed towards lateral side of the segment (the actual pore unrecognizable); papillae enlarged dumbbell-shaped; surrounded by darker area of corresponding shape; occupy xvii–ix segments; anteriorly a little close towards the middle line. Female pores minute in oval white space between *aa* in xiv, while exact pore is not visible. Spermathecal pores tiny in 7/8 and 8/9 in line with setae *e* (Figure 5).

Septa 5/6–11/12 moderately thickened. Gizzard large in vi. Pharynx large with one pair mucous glands in v. Last heart in xiii. Intestine begins in xviii. Spermatheca two pairs in viii and ix; pear-shaped elongated ampullae with little small/equal-sized duct. In two specimens, the anterior part of duct as wide as ampullae. The club-shaped large diverticulum; length equal to or little larger than duct. Well-developed globular seminal vesicles in xi-xii approaching towards each other. Prostate large, mop-like, and bushy with numerous finger-shaped lobules. The duct was thick, glistening, and straight-inwardly directed, without muscular sac. Without calciferous glands and penial setae.



(**a**) (**b**) (**c**)

Figure 5. Camera lucida illustration of *Megascolex konkanensis konkanensis* (**a**) genital region; (**b**) spermathecal pores; (**c**) spermathecae.

Distribution: The species is native peregrine to Kerala and has been reported from different regions of the country: Dist. Kozhikode: Tiruvallur (Thiruvallur), Calicut (Kozhikode); Dist. Palakkad: Chitoor (Chittur); Dist. Malappuram: Tirur, Kanjikode, Palghat (Palakkad); Dist. Ernakulam: Kalady; Dist. Thrissur: Kavalai; Dist. Kottayam: Athirampuzha, Kottayam; Dist. Kollam: Kulattupuzha (Kulathupuzha), Maddathoray (Madathara), Pathanapuram, Quilon (Kollam), Shasthancottah (Sasthamkotta); Dist. Alappuzha: Kerumaadi; Dist. Thiruvananthapuram: Trivandrum (Thiruvananthapuram); Travancore [8].

3.5. Megascolex polytheca polytheca Stephenson, 1915

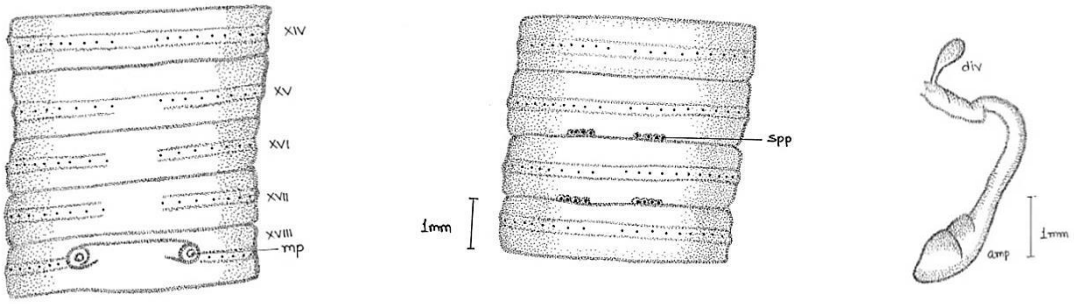
Megascolex polytheca Stephenson, 1915: 89–90.

Megascolex polytheca polytheca Stephenson. Blakemore 2007: 36.

Material examined: Sample ID: KERL0276A5; Process ID: IEW441-17; Reserve Forest, Kanan Devan Hills (10°06′39.2″ N 77°05′28″ E), Munnar Kerala, India; 27 October 2015, Coll. Shweta Yadav; BOLD accession No: ADH2014. Sample ID: KERL0264A5; Process ID: IEW373-17; (10°6′56.16″ N 77°5′13.56″ E), Eravikulam National Park, Munnar, Kerala, India; 14 September 2015, Coll. Shweta Yadav, BOLD accession No: ADH2014.

Description:

Length, 105–140 mm; diameter, 3 mm; segments, 159–194. Colour grey, slightly darker at anterior end. The anterior part of the body before clitellum relatively thick and stout with sharply demarcated segments, while post-clitellar region smoother and cylindrical. Setae on segment iii-xii are arranged on raised segmental equators. Clitellum not profoundly distinguishable; in 13–17 with visible setal rings. Prostomium epilobous with hinder end of open tongue, and a transverse groove at the front end of the tongue present. First dorsal pore at 4/5. Setae ring closed dorsally *zz* = 1/2 *yz* and ventrally *aa* = 3 *ab*, behind the male aperture *aa* = 4 *ab or* 5 *ab*, *ab* > *bc*. Setae *a* and *b* are relatively larger than other setae. Setae *a* to *e* are in regular longitudinal lines. Setal numbers counted were 51/v, 52/ix, 53/xiii, 48/xx, and 46/xxxi. Male pores on large circular raised papillae, which are enclosed within biconcave depressions in xviii. The papillae are confined to xviii in one specimen, while in the other partly in xvii. A dark-coloured oval spot present at *aa* in xiv, while female aperture not distinguished. Spermathecal pores 4–7 in numbers 7/8 and 8/9. On separating the lips of the groove, a row of 4–7 white dots visible on each side. These points are surrounded by a dark area and begin internally between the lines *b* or *c*(Figure 6).



(**a**) (**b**) (**c**)

Figure 6. Camera lucida illustration of *Megascolex polytheca polytheca* (**a**) genital region; (**b**) spermathecal pore; (**c**) spermathecae.

Septa 4/5 very delicate; 5/6 and 6/7 slightly thickened; 7/8–11/12 highly thickened. The large barrel-shaped gizzard in v. Calciferous glands absen;, oesophagus dilated deep yellow and vascular in xii. Intestine begins in xix. The last heart in xiii. Micronephridial excretory system. Spermathecae are small, circular, and 4–7 in number. Ampullae of spermathecae club-shaped organ with a long stalk with backwardly directed dilated end and more or less parallel to each other in a close set row. Male funnels in x and xi and seminal vesicles attached to anterior walls of xi and xii and made up of ovoid lobules. The ovaries are composed of finger-like lobes and break up at their free ends into strings of ova. Prostates large in xviii composed of small lobes and closely compacted together; duct short, muscular, stout, and widened near its termination.

Distribution: This species is endemic to Kerala. Thrissur: Kavalai in forest tramway [8].

3.6. Megascolex polytheca zonatus Stephenson, 1915

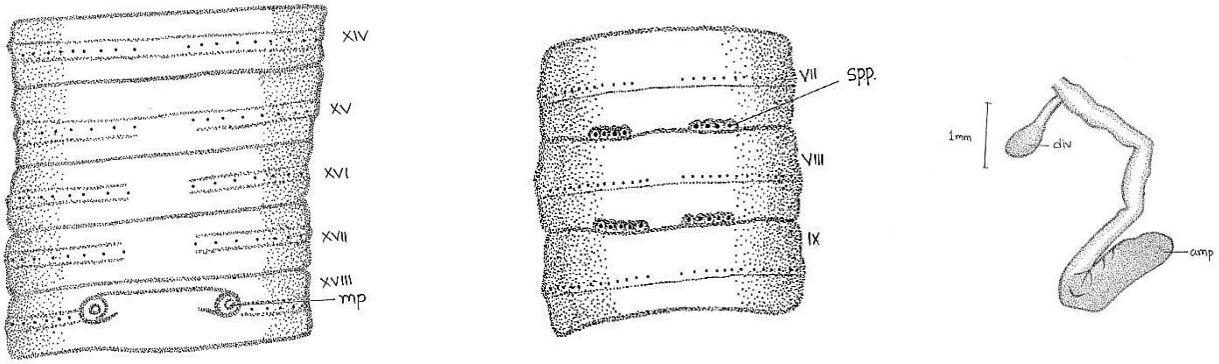
Megascolex polytheca var. *zonatus* Stephenson, 1915: 90–91.

Megascolex polytheca zonatus Stephenson. Blakemore 2007: 36.

Material examined: Sample ID: KERL0273A3; Process ID: IEW418-17; (10°6′56.88′′N 77°5′20.4′′E), Eravikulam National Park, Kannan Devan Hills, Kerala, India; 29 October 2015, Coll. Shweta Yadav; BOLD accession No: ADH2015. Sample ID: KERL0273A4; Process ID: IEW419-17; near Agraharam resort (10°6′56.88′′ N 77°5′20.4′′ E), Eravikulam National Park, Kannan Devan Hills, Kerala, India; 29 October 2015, Coll. Shweta Yadav; BOLD accession No: ADH2015.

Description:

Length, 112–118 mm; diameter, 2.5–2.75 mm; segments, 115–122. Colour grey. The anterior part of the body before clitellum relatively thick and stout with sharply demarcated segments, while post-clitellar region relatively constant in diameter. Setae perichaetine, clitellum browner than body in 13–17 with visible setal ring. Prostomium similar to *M.p. polytheca* epilobous with hinder end of the tongue open, and a transverse groove at the front end of the tongue present. First dorsal pore at 5/6. Setae ring closed dorsally *zz* = 1/2 *yz* and ventrally *aa* = 2 *ab*, behind the male aperture *aa* = 3 *ab*, *ab* > *bc*. Setae *a* and *b* are relatively larger than other setae. Setae *a* to *e* are in regular longitudinal lines. Setal numbers counted were 48/v, 50/ix, 52/xiii, 48/xx, and 48/xxxi. Male pores on large circular in lines with setae b; raised papillae which are enclosed within biconcave depressions in xviii segment. The male region quite similar to *M. polytheca polytheca* occupies xviii. A dark-coloured oval spot present at *aa* in xiv, while female aperture not distinguished. Spermathecal pores numerous in 7/8 and 8/9. On separating the lips of the groove, a row of 4–7 white dots visible on each side. These points are surrounded by a dark area and begin internally between the lines *b* or *c* (Figure 7).



(**a**) (**b**) (**c**)

Figure 7. Camera lucida illustration of *Megascolex polytheca zonatus* (**a**) genital region; (**b**) spermathecal pore; (**c**) spermathecae.

Spermathecae small, five in each side of spermathecal groove. Each is a club-shaped organ with a long stalk with backwardly directed dilated end and more or less parallel to each other in a close set row. Male funnels in x and xi and seminal vesicles attached to anterior walls of xi and xii and made up of ovoid lobules. The ovaries are composed of finger-like lobes and break up at their free ends into strings of ova. Prostates large in xviii composed of small lobes and closely compacted together; duct short, muscular, stout, and widened near its termination.

The internal anatomy agrees closely with *M. polytheca polytheca*, however, the spermathecae are relatively smaller than *M. polytheca polytheca*, with four spermathecae on each side of the spermathecal groove. The ampulla of the spermathecae is distinguishable from the duct, is ovoid, dark purple in colour, and can be easily differentiated from *M. p. polytheca*; the diverticulum is club-shaped and simple; duct is cylindrical.

Distribution: The species is endemic to Kerala; Dist. Palakkad: Parambikula [8].

3.7. Megascolex ratus Cognetti de Martiis, 1911

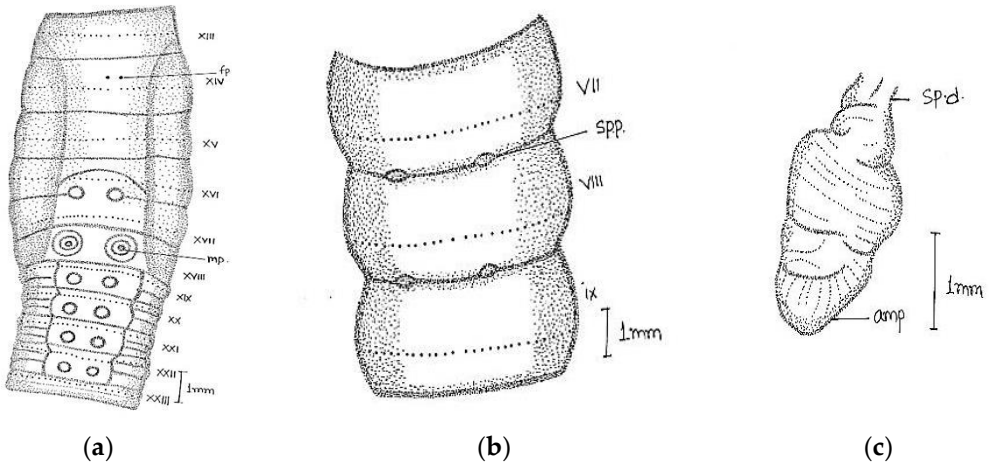
Megascolex ratus Cognetti, 1911: 500–502. Michaelsen 1913: 87; Stephenson 1916: 327; Aiyer 1929: 68.

Material examined: Sample ID: KERL0268A4; Process ID: IEW387-17; Neyyar Dam and Wildlife Sanctuary (8°32′00.4″N 77°08′53.5″E), Thiruvanathapuram, Kerala, India; 26 October 2015; Coll. Shweta Yadav; BOLD accession No: ADH2820. Sample ID: KERL0270A1; Process ID: IEW395-17; Rosemala, Shendurney Wildlife Sanctuary (8°53′03.7″ N 77°10′03.4″ E), Kollam, Kerala, India; 24 October 2015; Coll. Shweta Yadav; BOLD accession No: ADH2016. Sample ID: KERL0270A9; Process ID: IEW401-17; Rosemala, Shendurney Wildlife Sanctuary (8°53′03.7″ N 77°10′03.4″ E), Kollam, Kerala, India; 26 October 2015; Coll. Shweta Yadav; BOLD accession No: ADH2016. Sample ID: KERL0274A1; Process ID: IEW422-17; Mannoorkara, close to Peppara Dam Wildlife Sanctuary, (8°38′35.2″ N 77°10′50.5″ E), Thiruvananthapuram, Kerala, India; 26 November 2015; Coll. Shweta Yadav; BOLD accession No: ADH2819. Sample ID: KERL0274A9; Process ID: IEW427-17; Mannoorkara, close to Peppara Dam Wildlife Sanctuary (8°38′35.2″N 77°10′50.5″E), Thiruvananthapuram, Kerala, India; 26 November 2015; Coll. Shweta Yadav; BOLD accession No: ADH2019. Sample ID: KERL0275A1; Process ID: IEW430-17; Mannoorkara, close to Neyyar Wild Life Sanctuary (8°33′00.2″ N 77°14′33.0″ E), Vazhichal, Kerala, India; 27 November 2015; Coll. Shweta Yadav; BOLD accession No: ADH2820.

Description:

Length, 140–350 mm; diameter, 7–8 mm; segments, 160–180. Colour dorsally violet-brown, ventrally grey. Prostomium short, broad, tanylobous, dorsally with longitudinal furrows that do not reach the posterior margin. Segments x-xiv biannulate. Setae more

crowded in the ventral line than in the dorsal region, circles of setae are interrupted in the mid-ventral line of genital region where aa = 1/2 ab. There is no dorsal break, while in pre-clitellar *aa* = 2 *ab* and behind the clitellum *aa* = 3 *ab*. At x segment 108 setae and at xviii 130 setae. Clitellum prominent in one specimen out of seven collected specimens while setae present in entire clitellar region. Clitellum saddle-shaped occupying xiv- xviii and without intersegmental furrows. On xiv it is little extended towards xiii. Male pores on xviii, in the lines of setae *h*; between the male pores the setae absent. Male pores are placed on whitish tubercles supported by swollen papillae. Paired genital markings in 17, 19–22, sometimes also in 16 and 23. Female pores in xiv presetal on *a*, transversely extended. Spermathecal pores two pairs in intersegmental furrows 7/8 and 8/9 in the lines of the setae *f* (Figure 8).



(**a**) (**b**) (**c**)

Figure 8. Camera lucida illustration of *Megascolex ratus* (**a**) genital region; (**b**) spermathecal pore; (**c**) spermathecae.

Septa 7/8 and 8/9 thickened. Gizzard large in vi. Intestine begins in xiv. Last hearts in xiii. Two pair of testes in x and xi encapsulated in a large lobulated structure, which is compressed between strong septa. Prostates with strong, muscular cylindrical duct, which terminates into little folded lobular structure. Two pairs of Spermathecae in viii and ix with sac-shaped transversely striped ampullae, broader distally and rounded proximally. The duct opens into a tiny diverticulum, which is enclosed in the duct wall.

Distribution: The species is near endemic to Kerala and has been reported in Dist. Thiruvananthapuram: Bonaccord (Bonacaud, Bonakkad), Chimungi (Chemmunji), Coorloon, Mukkunni Reserve Forest, Trivandrum (Thiruvananthapuram) [8].

3.8. Megascolex travancorensis travancorensis Michaelsen, 1910

Megascolex travancorensis Michaelsen, 1910: 72–73.

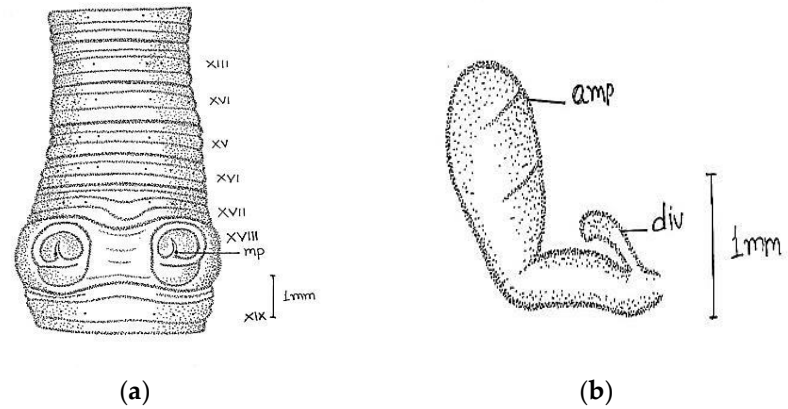
Megascolex travancorensis travancorensis Michaelsen. Blakemore 2007: 37.

Material examined: Sample ID: KERL0275A2; Process ID: IEW431-17; close to Neyyar Wild Life Sanctuary (8°33′0.216″ N 77°14′33″ E), Vazhichal, Kerala, India; 27 October 2017; Coll. Shweta Yadav; BOLD accession No: ADH2010. Sample ID: KERL0269A3; Process ID: IEW389-17; Peppara Wildlife Sanctuary (8°37′14.7″ N 77°10′3.36″ E), Thiruvananthapuram, Kerala, India; 25 October 2017; Coll. Shweta Yadav; BOLD accession No: ADH2017.

Description:

Length, 118–122 mm; diameter, 3.0–3.2 mm; segments, 182–198. Colour grey. Prostomium epilobic. Setae ii-viii enlarged and closely paired anteriorly, while onwards loses orientation, more or less in longitudinal lines. Setal counts were 12/v, 12/xiii, 16/xxii, 20 xl. Clitellum not distinct. Dorsal pores start at 4/5. Male pores in xviii on raised egg-shaped cushions in lines *b*, within longitudinal curved slits in each cushion, run posterior to anterior from the base of circular rings. Female pore tiny in xiv, very hard to recog-

nise. Spermathecal pores hard to recognise, apparently two pairs in 7/8 and 8/9 in lines *b* (Figure 9).



(**a**) (**b**)

Figure 9. Camera lucida illustration of *Megascolex travancorensis travancorensis* (**a**) genital region; (**b**) spermathecae.

Septa 6/7–12/13 thickened. Gizzard in vi. Last hearts in xiii. Micronephridial system. Two pairs of testis funnels free in x and xi. Seminal vesicles small; densely packed racemose in xi and xii. Prostates fairly large, elongated, rectangular, and deeply incised with a cracked uneven surface occupying xvi-xxii; duct fairly long, shiny, thick distal part goes straight forward, and proximal part goes back to the irregular curve. Proximal part of the duct longer and covered by the glandular part. Spermathecae large, ampullae pear-shaped, dark in colour, distally narrowed, and severely bent at the opening. The duct is narrow than ampullae. A slender club-shaped distally somewhat bent diverticulum present. No penial setae.

Distribution: The species is endemic to Kerala and reported so far from Dist. Kollam: Kottarakkara, Kulathupuzha, Dist.Thiruvananthapuram: Killipalam, Pallode (Palode) [8].

3.9. Megascolex papparensis sp. nov.

LSIDurn:lsid:zoobank.org:act:BC2E4221-2984-4697-BC73-EEF09894E051

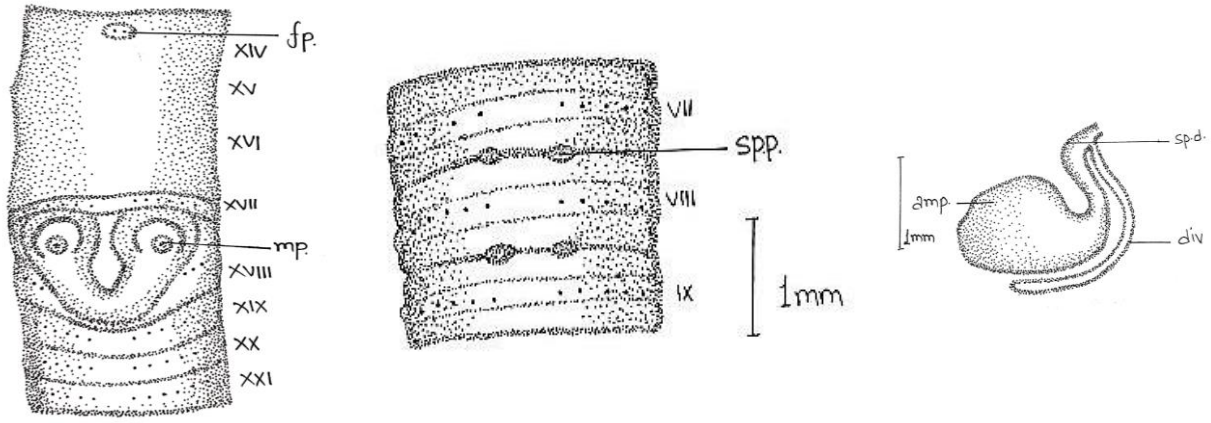
Holotype: Clitellate specimen (Sample ID: KERL0274A14; Process ID: IEW366-17); registration number: DHSGV-ZDM-H004; BOLD accession No: ADH2299, Mannoorkara, close to Peppara Dam and Wildlife Sanctuary (8°38′35.2″ N 77°10′50.5″ E), Thiruvananthapuram, Kerala, India; 26 November 2017, Coll. Shweta Yadav.

Paratypes: Clitellate specimen (Sample ID: KERL0274A14-1; Process ID: IEW366-17); registration number: DHSGV-ZDM-272015-013; BOLD accession number: ADH2299, Mannoorkara, close to Peppara Dam and Wildlife Sanctuary (8°38′35.2″ N 77°10′50.5″ E), Thiruvananthapuram, Kerala, India; 26 November 2017, Coll. Shweta Yadav.

Clitellate specimen (Sample ID: KERL0274A14-2; Process ID: IEW366-17); registration number: DHSGV-ZDM-272015-014; BOLD accession No: ADH2299; collection site similar to other paratypes.

Description:

Length, 85 mm; diameter, 3 mm; segments, 196. Prostomium epiobic with raised growth. Colour light grey. Setae paired in longitudinal lines *aa* = 2 *ab* = 3 *ab* in pre-clitellar region, *aa* = 1.5 *ab* in post-clitellar region, while setae absent in clitellar region. Setal counts were 12/v, 17/xii, 20/xx, and 21/xxx. Clitellum dark-brown coloured in 13–17. Dorsal pores start at 5/6. Male pores ca $\frac{1}{2}$ circumference apart in a groove in xviii on oval glandular papillae, both papillae united by v-shaped groove at the distal end. The male region is slightly extended, sometimes appears in xix, while on deep observation it remains in xviii and touches the boundary of xix. Female pores paired in light-coloured oval patch in *aa*. Spermathecal pores two pairs in 7/8 and 8/9 in *a* lines (Figure 10).



(**a**) (**b**) (**c**)

Figure 10. Camera lucida illustration of *Megascolex papparensis sp. nov.* (**a**) genital region; (**b**) spermathecal region; (**c**) spermathecae.

Septa 6/7–12/13 thickened. Gizzard large in vi. Last hearts in xiii. Funnels free in xi and xii. Seminal vesicles compact in xi and xii. Prostate large, occupies xviii-xxiv segments; duct proximally very thin and curved. Spermathecae ampulla oval and slightly bent at anterior end; duct long and slightly bent; diverticulum shorter than ampulla.

Etymology: "*papparensis*" is derived from its type of habitation, Peppara Dam, Kerala.

Remarks: Particularly in terms of the prostatic duct, this species is similar to *Megascolex travancorensis* var. *quilonensis* [44]. However, the structure of spermathecae varies, especially in diverticulum length.

Variations: The main variation observed between holotype and paratype was the size of the prostate. In the holotype, the prostate covered six segments (xviii–xxiv) and in the paratypes it occupied eight segments, xvii-xxiv and xviii-xxv, respectively. Further, the colour of the clitellum in the holotype was darker as compared to paratypes, and in the holotype the clitellum occupied xiii-xvii segments and in paratypes it occupied xiii 1/2, xvi 1/2, or xvii segments.

3.10. Megascolex triangularis Stephenson, 1925

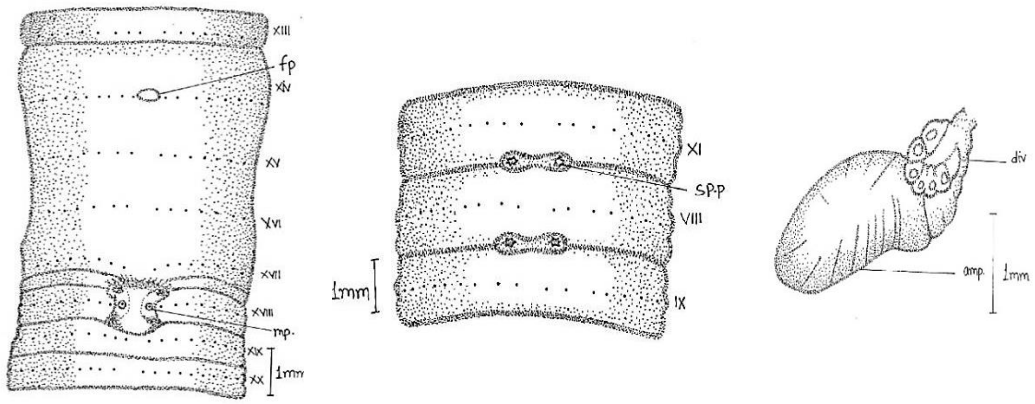
Megascolex triangularis Stephenson, 1925: 56–57.

Material examined: Sample ID: KERL0264A6; Process ID: IEW374-17; Reserve Forest (10°06′56.2″ N 77°05′13.6″ E), Kannan Devan Hills, Kerala, India; 29 October 2017; Coll. Shweta Yadav; BOLD accession No: ADH2301; Sample ID: KERL0273A1; Process ID: IEW416-17; Reserve Forest (10°06′56.9″ N 77°05′20.4″ E), Kannan Devan Hills, Kerala, India; 29 October 2017; Coll. Shweta Yadav; BOLD accession No: ADH2301.

Description:

Length, 138–150 mm; diameter, 5–6 mm; segments, 190–210. Dorsal pores start in 6/7. Clitellum 14–17. Prostomium? Colour dark brownish. Setae more than 50 per segment. Male pores paired, slit-like structure, situated at *a* in centre of the curved grooves, which are concave laterally and span 17/18 to 18/19. Female pores in 14th in whitish oval patch. Spermathecal pores two pairs in 7/8 and 8/9 at *ab* lines (Figure 11).

Septa 5/6–11/12 thickened. Gizzard large in v. Intestine begins in iv. No calciferous glands present. Male funnels in 10 and 11. Seminal vesicles in 11 and 13. Last heart in 13. Prostate racemose in 18; no prostatic duct seen. Spermathecae ampulla large ovate; duct short and wide without any demarcation; diverticula multiloculate. Penial setae absent.



(**a**) (**b**) (**c**)

Figure 11. Camera lucida illustration of *Megascolex triangularis* (**a**) genital region; (**b**) spermathecal region; (**c**) spermathecae.

Distribution: The species is known from the original description; Dist. Thrissur: Kavalai, Kerala [8].

3.11. Megascolex vazhichlensis sp. nov.

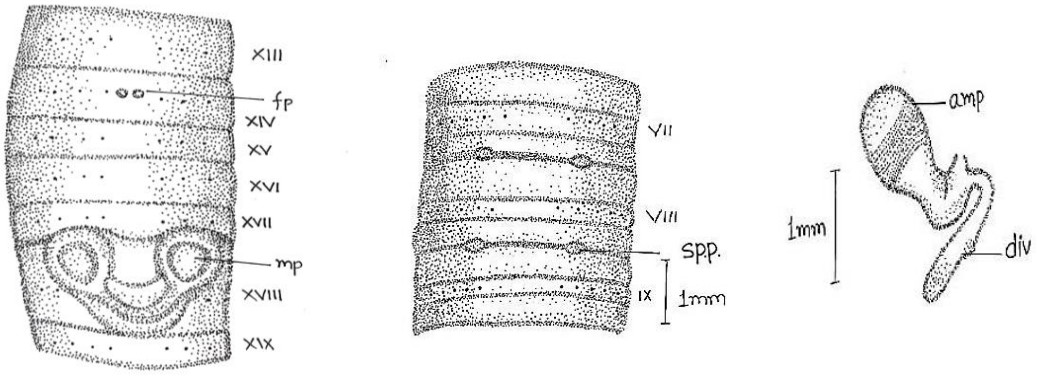
LSID. urn:lsid:zoobank.org:act:7C257761-1B02-4100-A738-9C39A2D5B857

Material examined: *Holotype* Clitellate specimen (Sample ID: KERL0275A3; Process ID: IEW449-17); registration number: DHSGV-ZDM-H005; BOLD accession No: ADH2821. Forest land (8°33′00.2″ N 77°14′33.0″ E), Vazhichal Kerala, India; 27 November 2017, Coll. Shweta Yadav.

Description:

Length, 175 mm; diameter, 3 mm; colour mild grey; segments, 256; secondary annulation in vii, viii, and ix. The anterior end is truncated (not tapering); prostomium is small and triangular, the pointed posterior angle directed upwards. First dorsal pore in 4/5. In pre-clitellar region *aa* > 2 *ab*; in clitellar region *aa* = 1/2 *ab* = 2 *ab*. The ventral setae are in definite longitudinal lines; setae of viii and ix are relatively small. The dorsal setae are in irregular intervals *zz* = 4–5 *yz*. The numbers were 16/v,18/xi, 21/xx, and 30/xxx. Setae at irregular intervals in post-clitellar region with smaller dorsal break in posterior region *ca zz* = 3 *yz*. Clitellum $\frac{1}{2}$ xiii–xvi. On segment xviii, two projected U-shaped grooves connecting two male pore papillae. The two grooves are united horizontally at the base. It is difficult to observe exact position of male aperture, apparently in lines of *b* and *c*. The female pores (?) paired represented by two separate white spots between *aa*. The spermathecal apertures are small in 7/8 and 8/9 in lines of setae *b*, while slits laterally extended (Figure 12).

Septa 5/6 very thin; 6/7–10/11 relatively thickened; from 13/14 considerably thickened. Gizzard large barrel-shaped in v. Intestine begins in xvi. The last hearts in segment xiii. The micronephridia are present as large tufts from v to ix; thick covering of micro nephridia present in clitellar region and behind the prostates. Testes and funnels are free in x and xi. Seminal vesicles composed of lobules, attached to the anterior walls of segments xi and xii. Those in xi are small and those in xii are moderate in size. Prostates are flat, occupy xvi-ix; duct long, thick, curved, shining, and backwardly directed. Ovaries are not visible. Spermathecae are sausage-shaped, bent towards their free end, and dilated at the extremity. The duct is short, moderately stout, half as thick as the ampulla, single posteriorly curved elongated and club-shaped; shining diverticulum present.



(**a**) (**b**) (**c**)

Figure 12. Camera lucida illustration of *Megascolex vazhichlensis sp. nov.* (**a**) genital region (**b**) spermathecal region; (**c**) spermathecae.

Etymology: "*vazhichlensis*" is derived from its type of habitation, the Vazhichal Kerala.

Remarks: The species resembles *Megascolex pepparensis sp. nov.*, particularly in the male region, where the prostate of *M. vazhichlensis* sp. nov occupies four segments (xvi–xix) with a consistently thickened duct. A large-sized prostate (xviii-xxiv) with a thin curved ental end are characteristic of *Megascolex pepparensis* sp. Further, spermathecae of *Megascolex pepparensis sp. nov.* have oval-shaped and slightly bent ampullae and *M. vazhichlensis sp. nov.* have club-shaped ampullae.

3.12. Intraspecific and Interspecific Genetic Distances

The mean nucleotide sequence composition was A = 28.6; T = 30.4; G = 18.6; and C = 22.4 with GC% = 40.04, GC% at codon 1 = 53.25, GC% at codon 2 = 42.26, and GC% at codon 3 = 24.80, which has been reported in many earthworm species [13,45]. The values of uncorrected pairwise genetic distances are provided in Figure 13. In *Megascolex* species, the mean intraspecific distance recorded was 1.232% and the minimum interspecific distance recorded was 15.18%. The minimum interspecific distance recorded was between *M. polytheca polytheca* and *M. polytheca zonatus* (14.3%) and between *M. polytheca polytheca* and *M. cochinensis cochinensis* (14.5%). Contrary to this, the maximum interspecific distance was found between *M. vazhichlensis* sp. nov and *M. auriculta* (20.3%) and between *M. vazhichlensis* sp. nov and *M. triangularis* (19.8%), respectively. Additionally, we recorded highest intraspecific genetic distance in *M. travancorensis travancorensis* (9.0%), followed by *M. ratus* (6.0%). The result of the cluster sequencing analysis indicated no overlapping interactions between genetic distances among *Megascolex* species (Table 2).

Table 2. Cluster Sequencing of COI barcodes of *Megascolex* species of Kerala depicting distances to nearest neighbour (NN).

OTUs	Process ID	Average Distance	Max Distance	Taxon	Count	Distance to NN
OTU-1	IEW366-17	0	0	*M. papparensis* sp. nov	1	14.9206
OTU-2	IEW373-17 IEW441-17	0	0	*M.polytheca polytheca*	2 2	13.7614
OTU-3	IEW374-17 IEW416-17	0	0	*M.triangularis*	2	17.4603
OTU-4	IEW378-17	0	0	*M. cochinensis cochinensis*	1	3.2051
OTU-5	IEW381-17 IEW383-17	0.3144	0.3144	*M.konkanensis konkanensis*	2	2.5396

Table 2. *Cont.*

OTUs	Process ID	Average Distance	Max Distance	Taxon	Count	Distance to NN
OTU-6	IEW387-17 IEW430-17	0.8012	0.8012	*M.ratus*	2	2.5641
OTU-7	IEW389-17	0	0	*M. travancorensis travancorensis*	1	9.0909
OTU-8	IEW395-17 IEW401-17	0.3205	0.3205	*M.ratus*	2	6.0897
OTU-9	IEW397-17 IEW399-17	0	0	*M.konkanensis konkanensis*	2	2.2222
OTU-10	IEW411-17	0	0	*M.konkanensis konkanensis*	1	2.2222
OTU-11	IEW412-17	0	0	*M.filiciseta*	1	15.0793
OUT-12	IEW414-17	0	0	*M. auriculata*	1	15.5555
OTU-13	IEW418-17 IEW419-17	0	0	*M. polytheca zonatus*	2	14.3540
OTU-14	IEW422-17	0	0	*M.ratus*	1	6.0897
OTU-15	IEW427-17	0	0	*M.ratus*	1	2.5641
OTU-16	IEW449-17	0	0	*M. vazhichlensis sp. nov.*	1	14.9371
OTU-17	IEW431-17	0	0	*M.travancorensis travancorensis*	1	9.0909
OTU-18	IEW835-17 IEW836-17 IEW840-17	0	0	*M. cochinensis cochinensis*	3	3.2051
OTU-19	IEW727-17	0	0	*M. cochinensis cochinensis*	1	4.6474

Species	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]
M. auriculata	**N/A**										
M. cochinensis cochinensis	0.163	**0.03**									
M. filiciseta	0.159	0.154	**N/A**								
M. konkanensis konkanensis	0.158	0.153	0.163	**0.02**							
***M. papparensis* sp. nov**	0.190	0.168	0.197	0.179	**N/A**						
M. polytheca polytheca	0.187	0.145	0.162	0.151	0.151	**0**					
M. polytheca zonatus	0.170	0.160	0.170	0.151	0.189	0.143	**0**				
M. ratus	0.176	0.175	0.176	0.161	0.175	0.178	0.155	**0.06**			
M. travancorensis travancorensis	0.194	0.191	0.184	0.176	0.171	0.175	0.187	0.164	**0.0**9		
M. triangularis	0.187	0.184	0.190	0.186	0.183	0.175	0.184	0.179	0.195	**0**	
***M. vizhichlensis* sp. nov**	0.203	0.172	0.197	0.168	0.176	0.168	0.178	0.171	0.152	0.198	**N/A**

Figure 13. Intraspecific (bold diagonally) and interspecific uncorrected pairwise genetic distances of *Megascolex* species. Species with single COI sequence are represented by N/A.

3.13. Phylogenetic Studies and Species Delimitation

The phylogenetic studies based on the COI dataset fell into distinct clusters in BI (Figure 14) and ML trees (Figure 15). Except for the species represented by single COI sequences, the phylogenetic analysis shows full support for each taxon as they were fully recovered on both BI and ML trees with strong supports. Moreover, there were minor differences in topologies of BI and ML trees. Furthermore, the three species delimitation methods produced different OTUs (Figure 16). The ABGD method found 15 OTUs, while ASAP analysis at 2.0 and 3.0 ASAP scores gave 18 and 13 OTUs, respectively. The BIN analysis also revealed 18 OTUs. Additionally, the ABGD depicted a clear barcode gap of 12–14% within *Megascolex* species (Figure 17).

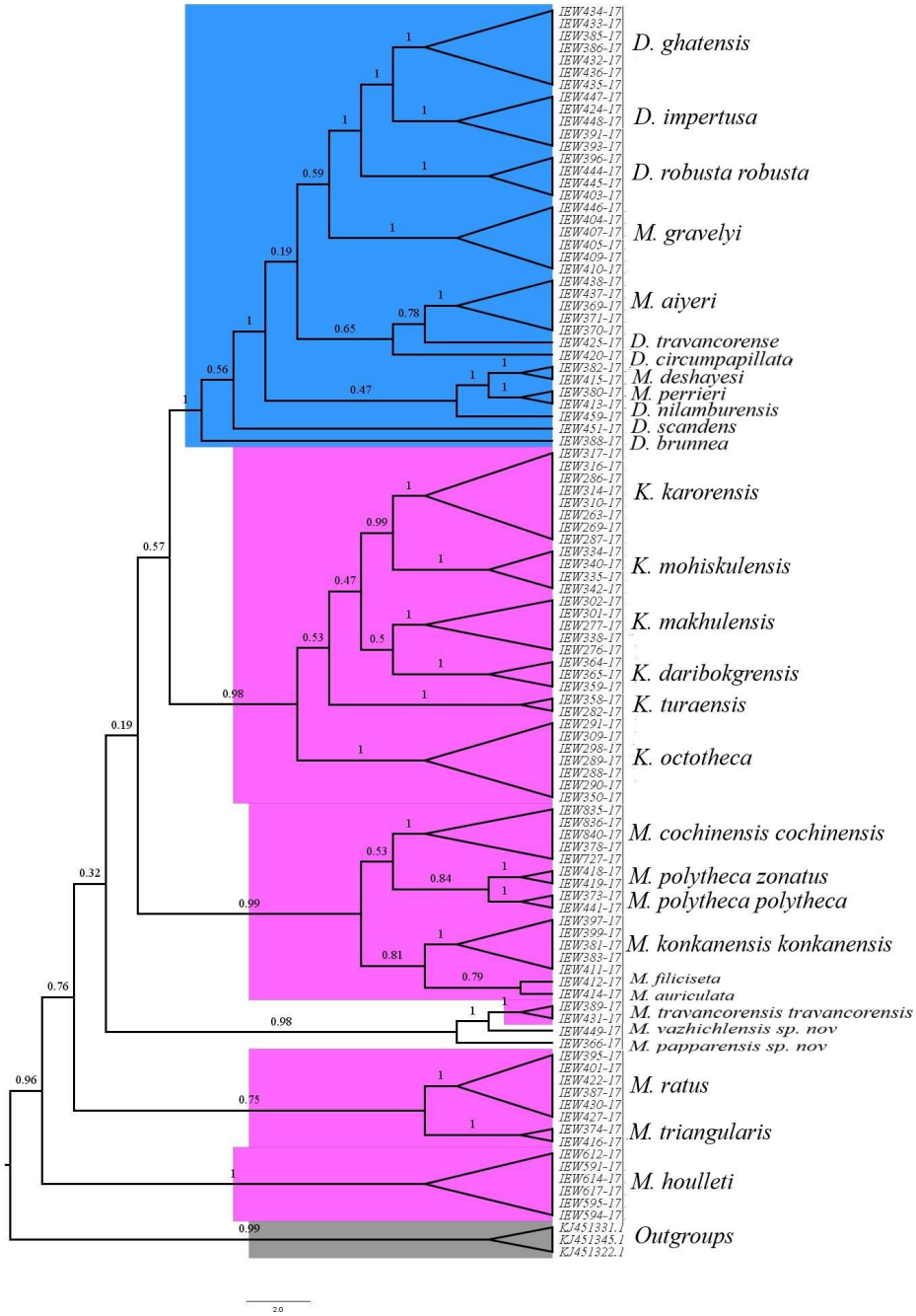


Figure 14. BI phylogenetic tree, depicting paraphyletic clades of *Megascolex* species with posterior probabilities node values at each node. The families Moniligastridae and Megascolecidae are represented in blue and pink colours; grey colour shows outgroups.

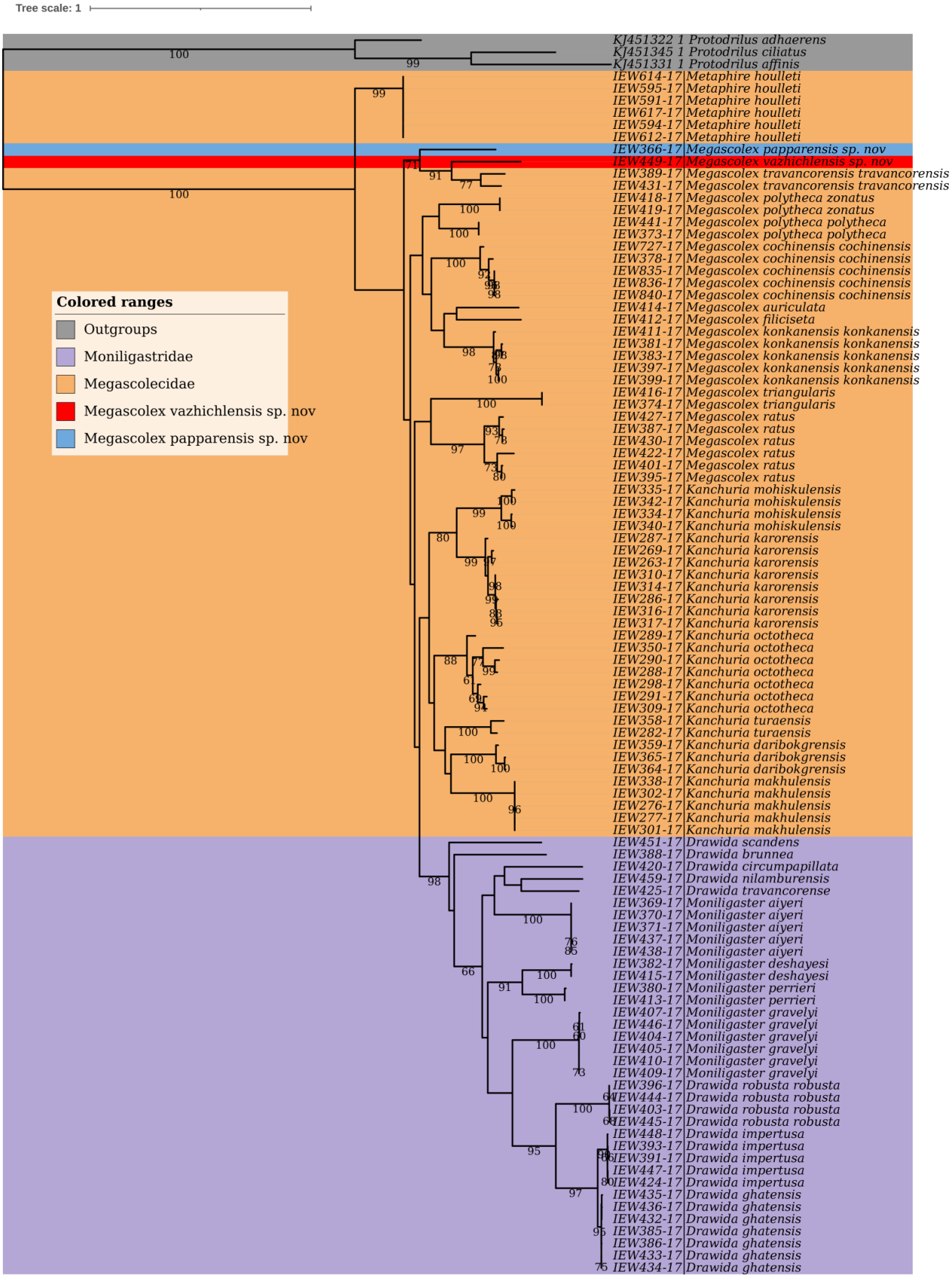


Figure 15. Maximum Likelihood (ML) phylogenetic tree with highest log likelihood (−4616.68), containing COI barcodes of earthworm species.

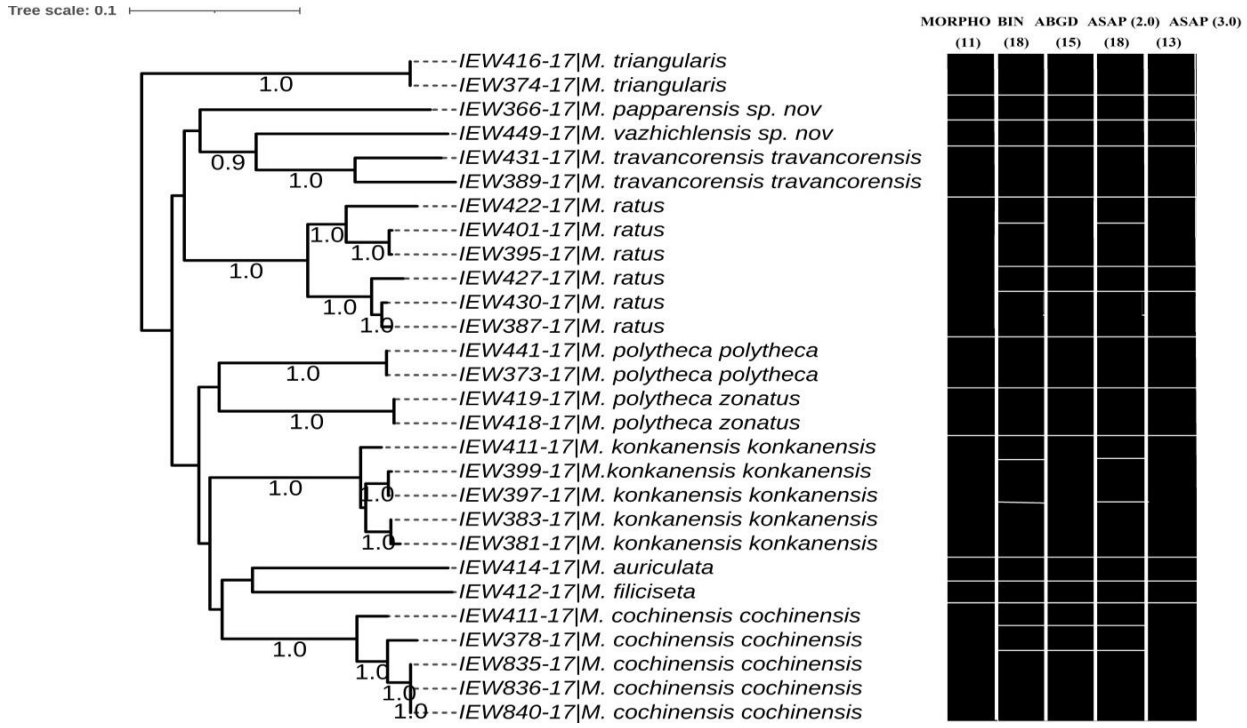


Figure 16. Maximum likelihood dendrogram showing species delimitation analyses of the COI dataset of *Megascolex* species of Kerala (black rectangles). MORPHO = Morphology, BIN = Barcode Gap Index Number, ABGD = Automatic Barcode Gap Discovery, and ASAP = Assemble Species by Automatic Partitioning at 2.0 and 3.0 ASAP scores.

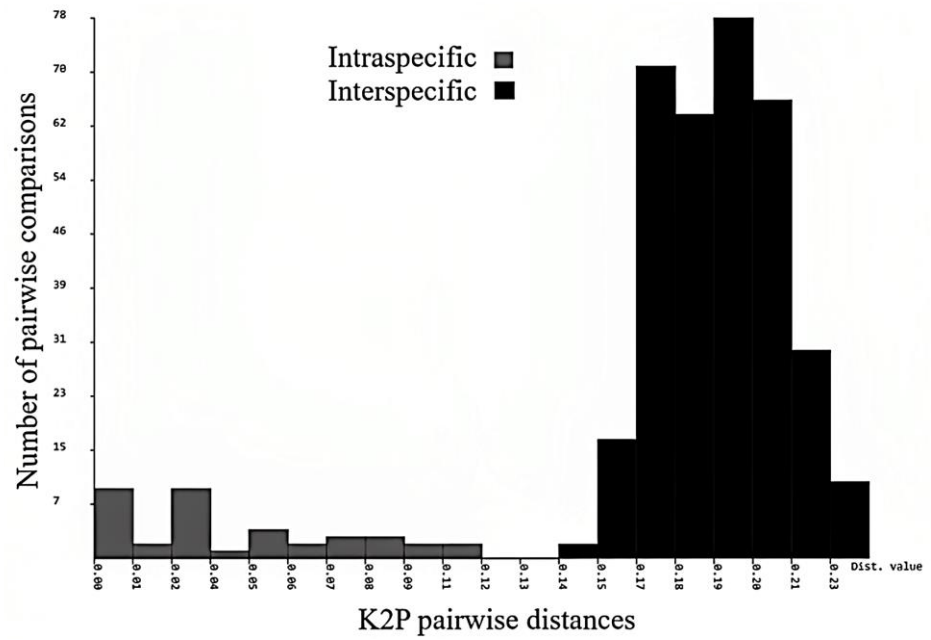


Figure 17. ABGD analysis showing a clear barcode gap in *Megascolex* species (X axis = genetic distances Y axis = frequency).

4. Discussion

In the present investigation, the collected species from the Kerala part of the Western Ghats were discriminated into nine species, namely, *Megascolex auriculata*, *M. cochinensis cochinensis*, *M. filiciseta*, *M. ratus*, *M. travancorensis travancorensis*, *M. triangularis*, *M. konkanensis konkanensis*, *M. polytheca polytheca*, and *M. polytheca zonatus*, based on their

morpho-anatomical observations. In addition, two new species were added to the genus, i.e., *M. papparensis sp. nov.* and *M. vazhichlensis sp. nov.* This segregation was further confirmed based on the DNA barcoding approach. In DNA barcoding, the reliability depends on a clear discontinuity between values of intraspecific and interspecific genetic divergences. In our report, the ABGD reflects the clear and no overlap barcode gap of 12–14%, which supports the validation and accuracy of DNA barcoding in the delimitation of *Megascolex* species. Moreover, in DNA barcoding, the species were considered distinct if their maximum intraspecific distances were less than the distances to their nearest neighbours (NN). In our cluster sequencing report, the maximum intraspecific genetic distance in each species was less than the distance to their nearest neighbour. Furthermore, based on such a limited COI dataset, certain conclusions were inferred. Firstly, looking at the family levels in both BI and ML phylogenetic trees, the species of the family Moniligastridae formed a distinct monophyletic clade in both phylogenetic trees (BI and ML), within a phylogenetic *Megascolecidae* (a monophyletic family of Crassiclitellata), to which the studied *Megascolex* species belongs. These results are not in accordance with the findings of James et al [46], which showed that Moniligastridae is the sister taxon to the Crassiclitellata. Secondly, the genus *Megascolex* appeared paraphyletic as revealed by BI and ML phylogenetic trees. On the BI tree, three clades were found with good posterior probability supports. Clade one with a high posterior probability of 0.98 was composed of *M. polytheca polytheca*, *M. polytheca zonatus*, *M. konkanensis konkanensis*, *M. cochinensis cochinensis*, *M. filiciseta*, and *M. auriculata*, respectively. Subsequently, the second clade with a good posterior probability value of 0.75 was formed by *M. travancorensis travancorensis*, *M. ratus*, and *M. triangularis* species. The last clade consisted of *M. travancorensis travancorensis*, *M. papparensis sp. nov.*, and *M. vazhichlensis* sp. nov with a higher posterior probability value of 0.99. Although similar clades were observed on the ML tree, only the clade formed by *M. travancorensis travancorensis*, *M. papparensis sp. nov.*, and *M. vazhichlensis* sp. nov show good clade support of 75, whereas the other two clades show weak supports. Additionally, in a phylogenetic tree study, a model of short internal and long external branches was detected in species, namely, *M. ratus* and *M. travancorensis travancorensis*, which is due to the high genetic divergence commonly found in some earthworm species. We followed three SD approaches to delimit species, and the results show incongruence in OTUs (BIN (18), ABGD (15), ASAP 2.0 (18), and ASAP 3.0 (13)). All the approaches show congruence in terms of the number of OTUs for species *M. triangularis*, *M. polytheca polytheca*, and *M. polytheca zonatus*, respectively. Contrary to this, *M. travancorensis travancorensis* was split by BIN, ASAP 2.0, and ASAP 3.0 into putative species, although it was merged by ABGD analysis. Similarly, in *M. cochinensis cochinensis*, *M. konkanensis konkanensis*, and *M. ratus*, the BIN, ASAP 2.0, and ASAP 3.0 split the individuals of these species into putative species. Generally, the incongruence in OTUs was seen in species of *M.cochinensis cochinensis*, *M. konkanensis konkanensis*, and *M. ratus* with high intraspecific genetic divergence. Moreover, the high intraspecific divergence in *M. travancorensis travancorensis*, *M. cochinensis cochinensis*, *M. konkanensis konkanensis*, and *M. ratus* was retained in each of them as single species due to their lesser intraspecific distance compared to their nearest neighbours (NN).

The high divergence in *M. cochinensis cochinensis* could be explained in that some of the individuals of this species were collected from Chimmini Wild Life Sanctuary, Kerala, and a few others were taken from Peechi-Vazhani Wildlife Sanctuary and Adimali Idukki Waterfall, respectively, with an average distance of 100 km and varied habitats. Such variations in habitats may perhaps induce selection [47], and, due to poor dispersal capability [48], they show large intraspecific divergence. A similar case is with *M. konkanensis konkanensis* and *M. ratus*, in which the individuals of these species differed in the habitats where they had been collected. Moreover, owing to a long evolutionary history [49], a direct effect of selection forces the soil-dwelling invertebrates to evolve to survive in specific habitats and stabilizing selection may perhaps select forms that diverge from the morphological optimum [47]. Conversely, the high genetic divergence found in earthworms can be explained by poor dispersal capability as they travel only limited distances/year [48], excluding the

occurrence of passive dispersal by waterways or vertebrate predators [50]. This low dispersal ability is generally reflected as isolation by distance flow, where the genetic segregation is extremely linked to the geographical distance of the species.

The taxa which occur naturally, occupy limited geographical ranges, and are restricted to a specific geographical region are termed endemic species [51]. India is known to host diverse endemic fauna and flora because of its two major hotspots, namely the Himalayas and the Western Ghats. The Western Ghats mountain range located in the southwestern portion of India along with Sri Lanka is known for its biodiversity hotspots [52,53] and contains several endemic species. The level of endemism in these regions is quite common among various taxa such as fishes, land snails, trees, amphibians, odonates, and reptiles [54,55]. This sort of endemism is also true for earthworms that exceptionally show a high level of endemism (71.6% in Sri Lanka and 77% in the Western Ghats) as reported by Narayanan et al. [6,56]. The *Megascolex* species with their origins in Gondwanaland are ancient lineages with 36 and 25 species [9] in Sri Lanka and Kerala, a part of Western Ghats, respectively. It is noteworthy that the earthworm fauna of Sri Lanka possesses a close relation to the Western Ghats mountains of India, especially in the Kerala state [8]. Given the Indian mainland and the present Sri Lanka island were together before the breakup of the Gondwana land [57], comparative studies may highlight an understanding of the evolutionary history of *Megascolex* species, which could be achieved with the supplementations of their molecular data. Since the high rate of endemism is of immense bio-geographical significance nonetheless, the endemism of species is predominantly influenced by poor regional survey and taxonomic impediments and, therefore, the status of given species may alter with its distribution range and expansion. In such cases, the present study serves as a barcode reference library to infer the study of their molecular taxonomy and phylogenetic relationships. Moreover, the integrative methods are essential to work on the species that are considered difficult to discriminate exclusively on taxonomical features alone. Therefore, involving integrative methods not only provides species delimitations but also deciphers their molecular phylogeny, evolution, and population ecology. Such studies have already begun in Southeast Asian countries [58,59], America [60,61], and Europe [62,63], which has opened new avenues in the fields of their ecology, conservation, and sustainable development. Therefore, to achieve such objectives, there is an urgent demand for molecular data on Indian earthworms that are not only diverse in the country but also endemic to the sub-continent.

5. Conclusions

In biological research, species play a central role in all its branches and are the fundamental unit for measuring biodiversity, particularly soil invertebrates, which are of immense importance. Nonetheless, precise estimation of species and their boundaries is challenging. With the emergence of integrative methods to delimit species both at the classical taxonomical platform, using morpho-anatomical screening as well as DNA barcoding, the true level of biodiversity at few interfamilial/generic levels and species complexes has been estimated. The current report provided the first barcode library of endemic *Megascolex* species of India that have been collected from the Kerala part of Western Ghats. Moreover, we reported two new *Megascolex* species, i.e., *papparensis sp. nov.* and *M. vazhichlensis sp. nov*., along with the occurrence of nine species, namely, *M. auriculata, M. cochinensis cochinensis*, *M. filiciseta*, *M. ratus*, *M. travancorensis travancorensis*, *M. triangularis*, *M. konkanensis konkanensis*, *M. polytheca polytheca*, and *M. polytheca zonatus* from the Kerala state of the Western Ghats. Additionally, the phylogenetic relationship of *Megascolex* species fell into three distinct clusters and appeared paraphyletic on the trees with strong branch supports. We also reported a clear barcode gap of 12–14% in studied *Megascolex* species, reflecting the potential of DNA barcoding in these species. The study may aid in unveiling a better understanding of the phylogenetic relationships and evolution of *Megascolex* species that are important endemic resources of India.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d14111006/s1, The COI dataset used in phylogenetic analysis is provided in Supplementary Materials Data S1.

Author Contributions: A.R.L. identified worms using integrated approach of taxonomy, performed phylogenetic analysis and drafted the manuscript; S.S.T. performed molecular analysis; P.T. drawn illustration; S.W.J. and S.Y. collected the samples, edited and approved the final draft of the manuscript. All authors have read and agreed to the published version of the manuscript.

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