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Description of two new species of the earthworm genus, *Metaphire* (Clitellata: Megascolecidae), from Kedah, Peninsular Malaysia

Beewah Ng[1], Ueangfa Bantaowong[2], Piyoros Tongkerd[1], Somsak Panha[1*]

Abstract. A field survey of terrestrial earthworms was conducted in the states of Penang, Kedah, and Perak, in Peninsular Malaysia, and revealed two new species of terrestrial earthworm belonging to the genus *Metaphire* Sims & Easton, 1972, which are described herein. *Metaphire sedimensis* new species, belongs to the octothecal group, characterised by having small circular of genital markings on post-setal of VI, VII, VIII, XVII, and XVIII. *Metaphire hijauensis* new species, has three pairs of spermathecae at VII–IX, typical of the sexthecal *houlleti* species group but differs from these other members by its body size, position of the intestine origin and the number and shape of genital marking glands. Evolutionary divergence between the new species and their most closely related species revealed a high interspecific genetic distance of 16.4–22.9%, while the pairwise distance between *M. sedimensis* new species, and *M. hijauensis* new species was 21.3%. The detailed descriptions and illustrations of the two new species are supplemented with their DNA barcodes in order to facilitate future species identifications.

Key words. taxonomy, DNA barcode, terrestrial earthworm, Malay Peninsula

INTRODUCTION

The first account on the terrestrial earthworms of the Malay Peninsula was provided by Frank E. Beddard, based on the collection of Richard Evans during the Skeat Expedition of 1899 (Beddard, 1900a, b), in which he listed 14 species, 10 of which were new. Stephenson (1932) re-examined the Skeat Expedition material and corrected the identities of three species from Beddard's list including a new described species, *Metaphire baruana* (Stephenson, 1932) (as *Amyntas bosschae* Horst, 1893 in Beddard [1900b]). A subsequent survey conducted by Gordon E. Gates significantly increased the number of earthworm species known from the Malay Peninsula since 1935 (Gates, 1935, 1936a, b, 1938, 1949). To date, approximately 91 species of earthworm have been identified from Malaysia, and these are classified among 22 genera and eight families, namely: Moniligastridae Claus, 1880; Almidae Duboscq, 1902; Lumbricidae Rafinesque-Schmaltz, 1815; Glossoscolecidae Michaelsen, 1900; Ocnerodrilidae Beddard, 1891; Octochaetidae Michaelsen, 1900; Exxidae Blakemore, 2000; and Megascolecidae Rosa, 1891. Of these, 31 species are recorded mainly from the Malay Peninsula (Horst, 1893a, b; Beddard, 1900a, b, 1902; Rosa, 1901; Michaelsen, 1902, 1907, 1928; Stephenson, 1923, 1932; Gates, 1935, 1936a, b, 1938, 1949), while 38 species are known from Sabah and Sarawak, on the island of Borneo. Out of the 91 identified species, 22 are cosmopolitan species.

Metaphire Sims & Easton, 1972, from the earthworm family Megascolecidae Rosa, 1891, is one of the largest genera and is widely distributed in East and Southeast Asia (Gates, 1935, 1936a, b, 1938; Bantaowong et al., 2011, 2016; Zhang et al., 2016). It is one of the dominant terrestrial earthworm genera throughout Malaysia, with approximately 19 species having been reported (Perrier, 1872; Beddard, 1900a, b; Rosa, 1901; Michaelsen, 1902; Gates, 1926, 1938, 1949; Stephenson, 1932), of which eight are known to occur in the Malaysian state of Kedah, namely: *M. peguana* (Rosa, 1890); *M. pulauensis* (Beddard, 1900); *M. balingensis* (Gates, 1949); *M. bipora* (Beddard, 1900); *M. flocellana* (Gates, 1949); *M. malayana* (Beddard, 1900); *M. mamillana* (Gates, 1931); and *M. strellana* (Gates, 1949).

Species of *Metaphire* are claimed to have similar morphological characteristics with those of *Amynthas* Kinberg, 1867, the only difference among them being either the presence (in *Metaphire*) or absence (in *Amynthas*) of the copulatory pouch in the male pore areas (Sims & Easton, 1972; Bantaowong et al., 2011, 2016; Zhang et al., 2016). Their classification to the species level is often confusing mainly due to the similar morphological characteristics. Taxonomic identification based on classical morphological analyses can be challenging due to either the convergence in phenotype among distinct species or the failure to identify cryptic species. These taxonomic bottlenecks can potentially be eliminated using the ever-expanding available genetic databases. For instance, Jeratthitikul et al. (2017) used a combination of morphological and DNA-based (CO1) characters to distinguish the species of *Amynthas*

[1]Animal Systematics Research Unit, Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand; Email: somsak.pan@chula.ac.th (*corresponding author)

[2]Division of Biology, Faculty of Science and Technology, Rajamangala University of Technology Thanyaburi, Pathum Thani 12110, Thailand.

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and *Metaphire* from Thailand. A major bottleneck in the identification processes – the lack of taxonomic expertise – could be largely eliminated by using CO1 DNA barcoding (Herbert et al., 2003). The combined linkage between the molecular data (molecular operational taxonomic units) and the respective morphological characters could then provide a better and more robust circumscription of species and a key solution for identification and discovery of new species (Decaëns et al., 2013).

Recent earthworm collections in Peninsular Malaysia have yielded specimens of two morphotypes that are distinct from other species of *Metaphire* previously reported from northern Malaysia and southern Thailand. These proposed two new species are described in this paper, and the 658 bp sequences of their CO1 gene fragment, as used in DNA barcodes (Hebert et al., 2003; Huang et al., 2007), are also provided.

MATERIALS & METHODS

Earthworm specimens were collected from tropical forest in the states of Penang, Perak, and Kedah, in Peninsular Malaysia. The topsoil was carefully dug under the leaf litter or near visible casts and sorted by washing with clean water in order to remove the unwanted debris. The worms were then killed in 30% (v/v) ethanol, photographed, transferred to 5% (w/v) formalin fixation for approximately 12 h, and then transferred to 70% (v/v) ethanol for longer-term preservation and subsequent morphological studies. Duplicate specimens and/or tissue samples were preserved in 95% (v/v) ethanol for molecular analysis.

The examination (and subsequent description) of each species was made using an Olympus SXZ7 stereoscopic light microscope. The external and internal morphological characters were observed and recorded, and drawings were made using a camera lucida. Detailed information, such as the number of segments and the body length and width were counted or measured. The data obtained was then used for identification and classification of the earthworms using the keys provided by Beddard (1892a, b, 1893, 1900a, b); Beddard & Fedarb (1895); Gates (1926, 1931, 1935, 1936a, b, 1937, 1938, 1949, 1972); Horst (1883, 1893a, b); Kinberg (1867); Michaelsen (1891, 1892, 1896, 1899, 1922, 1928, 1934a, b); Perrier (1872, 1875); Rosa (1890, 1896); Ude (1925); Sims & Easton (1972); Stephenson (1917, 1932); Vaillant (1868); Fletcher (1886); and Bantaowong et al. (2011, 2016). Holotype and paratype specimens of the newly described species have been deposited in the Chulalongkorn University, Museum of Zoology, Bangkok, Thailand (CUMZ) and paratypes at the Lee Kong Chian Natural History Museum, Singapore.

The anatomical abbreviations used follow Bantaowong et al. (2016): fp, female pores; gm, genital marking; gmg, genital marking gland; ic, intestinal caeca; mp, male pores; pg, prostate gland; sc, spermathecae; sp, spermathecal pores; sv, seminal vesicles; coll, collector.

DNA extraction and amplification. The muscular tissue (approximately 0.5 cm^2) near the posterior end of the type specimens (N = 1) were detached from the worm and used for the molecular analyses. The detached muscular tissue was homogenised with a cutter and then the total genomic DNA was extracted according to the standard protocol of the NucleoSpin® Tissue kit (Macherey-Nagel, Germany).

The universal COI primers, LCO 1490 and HCO 2198 (Folmer et al., 1994), were used to amplify a 650–700 bp fragment of the highly conserved regions of the mitochondrial CO1 gene. The PCR amplifications were performed in a reaction volume of 50 µL in a thermal cycler at 94°C for 2 min followed by 35 cycles of 94°C for 60 s, 42°C for 60 s and 72°C for 90 s, and then finally followed by 72°C for 5 min to ensure that the remaining single stranded DNA was fully extended before a hold at 20°C. The gel resolved and extracted amplicons were then sent for commercial sequencing at Bioneer Corp., Korea. The consensus COI fragment sequence for the two new earthworm species, *M. sedimensis* new species and *M. hijauensis* new species, were submitted to GenBank (NCBI) with accession codes MF479444 and MF479445, respectively.

Sequence alignment and analysis. Sequence alignment and editing was performed using the MEGA 6 software (Tamura et al., 2013), and the consensus sequences were used to search for homologues using the BLASTn algorithm to the NCBI GenBank and Bold (version 4) databases. All sequences were then aligned, edited and used to construct a phylogenetic tree based on the Maximum Likelihood (ML) analysis, using the Kimura 2-Parameter model (Kimura, 1980) with 1000 bootstrap replicates. Comparative sequences of *M. khaoluangensis* (KU565276.1), *M. trangensis* (KU565319.1) and *M. songkhlaensis* (KU565294.1) were taken from Jeratthitikul et al. (2017). In addition, material of *P. litoralis* (MF 488722 and MF488736) was extracted by Mr. Teerapong Seesamut from the CUMZ collection (unpublished data).

TAXONOMY

Megascolecidae Rosa, 1891
Metaphire Sims & Easton, 1972

Metaphire sedimensis Ng & Panha, new species
(Fig. 1; Table 1)

Material examined. Holotype: One adult (CUMZ 3672), Sedim, Kedah, Malaysia, 5°24′48.6″N, 100°46′51.3″E, coll. B.W. Ng, J.Y. Sam & S.P. Yeap, 23 July 2016. 6 paratypes: 6 adults (CUMZ 3673), 2 adult paratypes deposited at LKCNHM (ZRC.ANN.0377), same collection data as for the holotype.

Diagnosis. Medium size, length 140–177 mm, diameter 4.67–5.72 mm at the clitellum, segments 102–111. Prostomium epilobic. First dorsal pore in 12/13. Spermathecal pores small, latero-ventrally paired at intersegment 5/6/7/8/9. Male pores paired in copulatory pouch in XVIII. Genital markings small

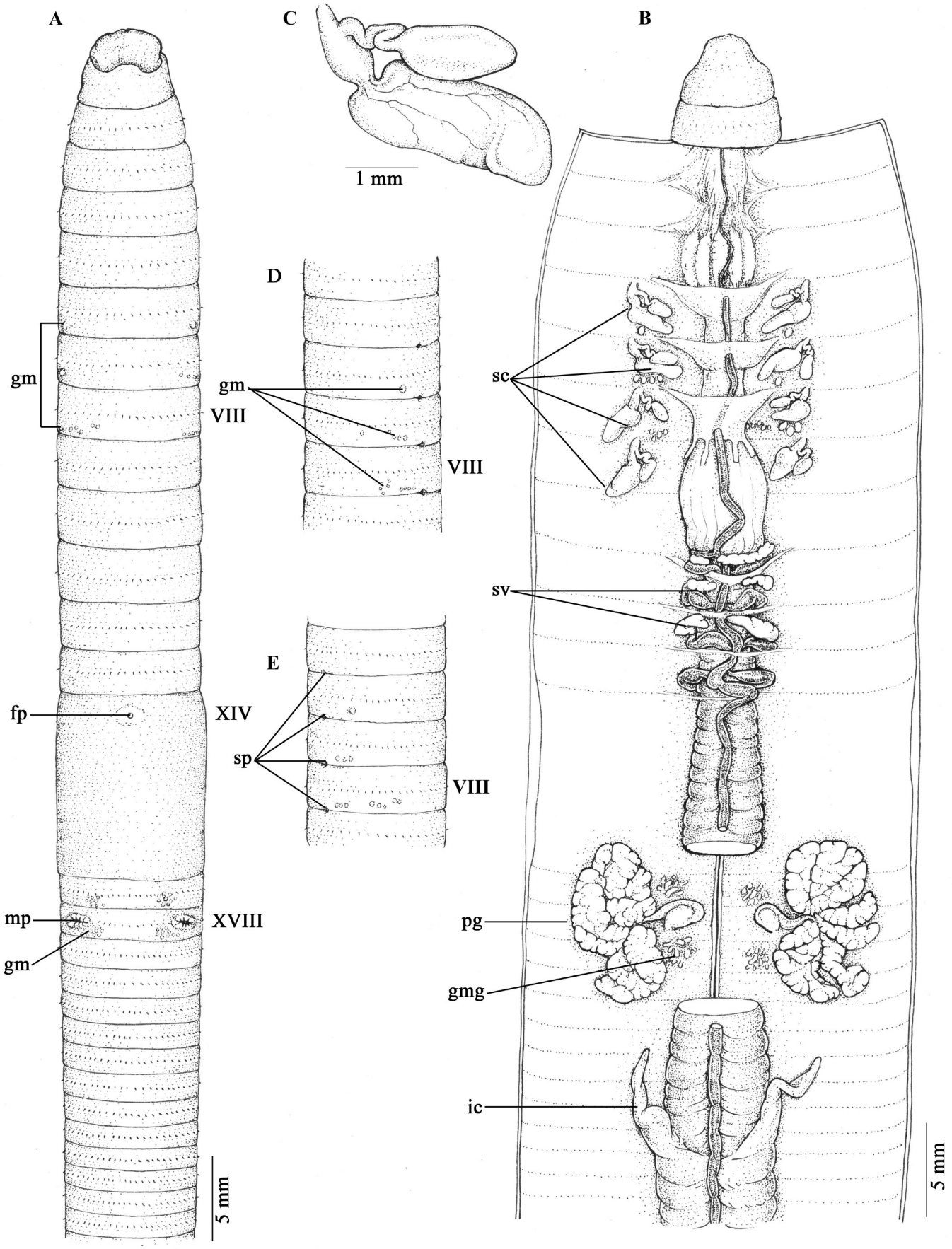

Fig. 1. *Metaphire sedimensis* new species (holotype). A, external ventral view; B, internal dorsal view; C, enlarged viewed of spermathecae; D, external view, ventrolateral view of left-side of genital marking; E, external view, ventrolateral view of right-side of spermathecae pores.

Table 1. Comparison of the morphological characters in the octothecal group of *Metaphire* species from southern Thailand and northern Peninsular Malaysia.

Morphology characters	***M. sedimensis* new species (N = 7)**	***M. songkhlaensis*[a]**	***M. pulauensis*[a]**	***M. malayana*[b]**	***M. fovella*[c]**	***M. balingensis*[c]**	***M. strellana*[c]**
Body length (mm)	140–177	165–300	143–165	93–110	42–73	63–120	46–62
Body width (mm)	4.67–5.72	9	6	4	2–4	4–5	2–3
Segment number	102–111	89–116	104–110	nil	nil	nil	nil
First dorsal pore	12/13	12/13	12/13	11/12	12/13 or 13/14	11/12 or 12/13	11/12 or 12/13
Setae between male pore	14	20–27	11	9	9–12	10–12	13–14
Spermathecae shape	Sessile sac-shape	Elongate sac	Pear shaped	–	–	–	–
Genital marking[d]	VI–VIII, XVII–XVIII	VIII–IX, XIX–XXII	VIII–IX, XVII–XVIII	VI–IX, XVII–XVIII	VI–VIII, XVII–XIX	VII–VIII, XVI–XIX	VII–XI, XIX–XXIV
Type locality	Sedim, Kedah	Songkhla, Hat Yai	Pulau Bidang, Kedah	Aring, Kelantan	Kaki Bukit, perlis	Baling, Kedah	Baling, Kedah
Accession number (GenBank, museum)	MF479444, CUMZ 3672	KU565294.1, CUMZ 3374A	–	–	–	–	–

Data for [a]*M. songkhlaensis* and *M. pulauensis* are from Bantaowong et al. (2016), [b]*M. malayana* from Beddard (1900b), [c]*M. fovella*, *M. balingensis* and *M. strellana* are from Gates (1949). – = nil. [d]Commas separate pre- and post-clitellar genital markings.

circular on both spermathecal (on VI, VII, and VIII) and male pore (on XVII and XVIII) regions. Spermathecae four pairs in segments VI–IX, ampulla elongated, club-shaped, with duct shorter than ampulla. Diverticulum with elongated tubular and twisted shape, with oval bulb terminal. Holandric, intestinal caeca simple prostate glands large. Small sessile genital marking glands corresponding to external genital markings.

Description of holotype. *External characters.* Body length 177 mm, diameter 5.72 mm at clitellum, 102 segments. Prostomium epilobic. First dorsal pore at 12/13. Clitellum annular at XIV–XVI, with no setae. Setal numbering 14–17 in VII, 27 in VIII, 30–40 in XX, 33–38 in X, and 14 between male pores in XVIII. Single female pore at XIV.

Male pores in copulatory pouches, transversely slit-like, conspicuous opening, in XVIII. 0.33 circumference apart ventrally, distance between male pores 5 mm. A pointed penis-like projection within copulatory pouches or everted. Four pairs spermathecal pores, intersegment 5/6–8/9, slit like, 0.67 body circumference ventrally apart, distance between spermathecal pores 10 mm. 1–9 small circular genital markings, post-setal of VI, VII,VIII, XVII and XVIII. Unpaired genital markings, mid-ventral, post-setal of VII and VIII.

Internal characters. Septa, 5/6–7/8 and 12/13/14 thickened, 8/9 and 9/10 aborted. Gizzard large within IX–X. Last heart in XIII. Intestinal origin at XIV. Intestinal caeca originating in XXVI, simple, extending to XXI.

Ovaries paired in XIII. Four pairs spermathecae in VI–IX. Ampulla elongated, club-shaped, with duct shorter than the ampulla. Diverticulum with elongated and twisted slender, oval bulb ampulla. Several genital marking glands, elongated bulb shaped, near spermathecae.

Holandric. Testes sacs paired in X and XI. Seminal vesicles paired in X, XI, and XII. Prostate glands paired in XVIII, racemose, large, occupying segments XVII–XIX. Prostatic duct C-shaped, numerous genital marking glands next to copulatory pouch, sessile corresponding to each external genital markings.

Variation. The holotype length measured 177 mm body length with 102 segments; the 6 paratypes ranged in size from 140–160 mm body length and with 111–118 segments. There is variation in the number of genital markings in the post-setal of VI, VII, VIII, XVII, and XVIII (Table 2).

Etymology. This new species was named after the type locality (Sedim).

Distribution. The new species is known only from the type locality.

Table 2. Variation in the occurrence of genital markings in post-setal of VI, VII, VII, XVII and XVIII in *Metaphire sedimensis* new species.

Specimen[a]	Paired					Unpaired		
	VI	VII	VIII	XVII	XVIII	VI	VII	VIII
1	+	+	+	+	+	–	+	+
2	+	+	–	+	+	–	+	+
3	–	+	+	+	+	–	+	–
4	–	+	+	+	+	–	+	+
5	?	?	?	+	+	–	?	?
6	–	+	+	+	+	+	+	+
7	+	+	+	+	+	–	+	+

[a]Specimen no. 1 is the holotype, the others are paratypes. Presence is denoted by a plus sign (+), absence is shown with a minus sign (–) and missing data are denoted with a question mark (?).

Habitat. The new species was found inside a ditch covered with sandy loam soil (pH 5.70), at a depth of about 10 cm. The soil surface was covered with thick leaf litter in a forest or near an agricultural area.

Remarks. *Metaphire sedimensis* new species, belongs to the octothecal group, with spermathecal pores in 5/6–8/9, and with pre- and post-clitellar (postsetal) genital markings in VI–IX and XVII–XVIII. There are six species from Thailand and Peninsular Malaysia that appear to be close to the new species, namely: *M. songkhlaensis* Bantaowong & Panha, 2016 from Songkhla, Hat Yai; *M. pulauensis* (Beddard, 1900) from Pulau Bidan, Kedah; *M. malayana* (Beddard, 1900) from Aring, Kelantan; *M. fovella* (Gates, 1949) from Kaki Bukit, Perlis; *M. balingensis* (Gates, 1949) and *M. strellana* (Gates, 1949) from Baling, Kedah. However, the new species (Fig. 1) is basically smaller in size compared to *M. songkhlaensis* (Bantaowong et al., 2016: p. 65, Fig. 2) from Hat Yai. Moreover, differences in the number of setae between male pores strongly distinguish these two species. Apparently, the number of setae between male pores is generally lower in *M. sedimensis*, new species, than in *M. songkhlaensis* (Table 1). *Metaphire sedimensis*, new species, is fairly similar to *M. pulauensis* (Bantaowong et al., 2016: p. 67, Fig. 3) in terms of its body size and number of segments, but can be distinguished by the pattern of genital markings. *Metaphire pulauensis* has sieve-like genital markings which are formed by 40–50 pore-like papillae, and are unpaired, mid-ventral, and presetal on VII, VIII, IX, XVII, and XVIII. *Metaphire sedimensis*, new species, on the other hand, not only has unpaired genital markings, mid-ventral, and post-setal genital markings on VIII, but also has small, circular papillae, and paired postsetal genital markings (c. 1–9) on VI, VII,VIII, XVII, and XVIII.

The external anatomy of *M. sedimensis*, new species, is very close to *M. balingensis* (Gates, 1949: p. 10), *M. malayana* (Beddard, 1900: p. 893, Fig. 1), *M. fovella* (Gates, 1949: p. 21), and *M. strellana* (Gates, 1949: p. 34) but these five species are clearly distinguished from each other by the position of the genital markings. *Metaphire sedimensis*, new species, differs from *M. malayana*, *M. forella*, and *M. strellana* in the absence of presetal genital markings (present in the latter three species). Postsetal genital markings are observed in *M. sedimensis*, new species, and *M. balingensis*. However, the genital markings of *M. balingensis* are unpaired and located at the median of VII–VIII, XVI–XIX, whereas the genital markings of the new species are paired on VI–VIII, XVII–XVIII and unpaired on VII, VIII.

Metaphire hijauensis Ng & Panha, new species

(Fig. 2; Table 3)

Material examined. Holotype: One adult (CUMZ 3674), Bukit Hijau, Kedah, Malaysia, 5°30′04.5″N, 100°46′19.1″E, coll. B.W. Ng, J.Y. Sam & S.P. Yeap, 23 July 2016, Paratypes: 6 adults (CUMZ 3675), 2 adult paratypes deposited at LKCNHM (ZRC.ANN.0376), same collection data as for holotype.

Other material examined. One adult (CUMZ 3676), Sedim, Kedah, Malaysia, 5°24′48.6″N, 100°46′51.3″E, coll. B.W. Ng, J.Y. Sam & S.P. Yeap, 23 July 2016.

Diagnosis. Small size, length 66–87 mm, diameter 3.05 mm at the clitellum, segments 101–120. Prostomium epilobic. First dorsal pore in 10/11. Spermathecal pores minute paired in intersegment 6/7/8/9. Male pores paired in XVIII, within copulatory pouches. Spermathecae three pairs, in segments VII–IX, large sac-shaped ampulla with long duct. Diverticulum with elongated tubular and twisted shape attached to duct. Holandric, intestinal caeca simple. Prostate glands large, duct C-shaped.

Description of holotype. *External characters*. Body length 87 mm, diameter 3.05 mm at clitellum, 118 segments. Prostomium epilobic. First dorsal pore at 10/11. Clitellum annular at XIV–XVI, with setae. Setal numbering 34 in VI, 34 at VII, 48 in VIII, 55 in XX, 56 in XXX and 8 between male pores in XVIII. Single female pore at XIV.

Male pores paired in XVIII, 0.244 circumference ventrally apart, each in copulatory pouches, rounded-shaped opening, distance between male pores 3 mm. Genital markings absent. Three pairs spermathecal pores, in furrows 6/7–8/9, slit like, 0.455 body circumference apart, distance between spermathecal pores 5 mm. No genital markings observed at spermathecal pore area.

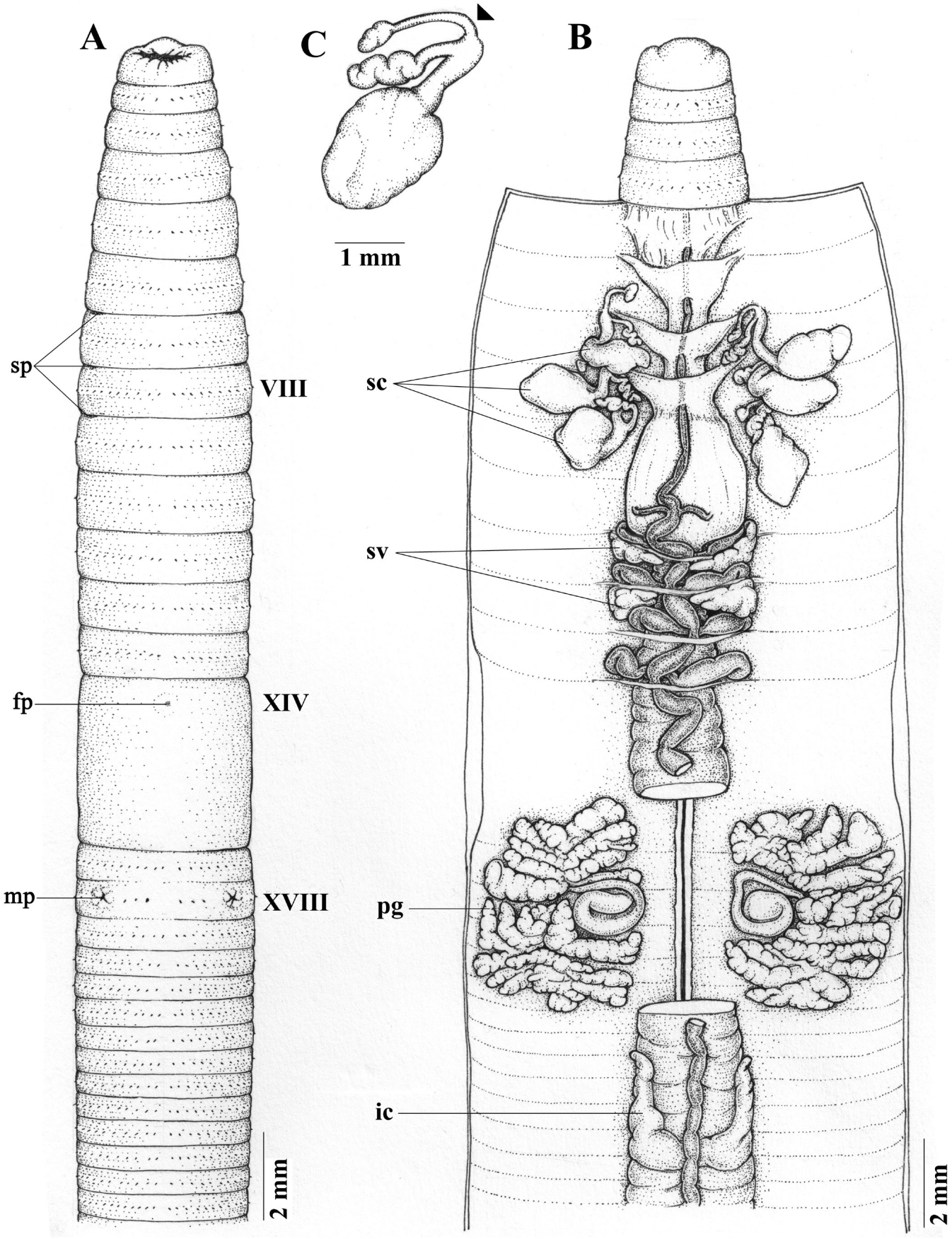

Fig. 2. *Metaphire hijauensis* new species (holotype). A, external ventral view; B, internal dorsal view; C, enlarged viewed of spermatheca.

Table 3. Comparison of the morphological characters in the sexthecal *Metaphire houlleti* species group from southern Thailand and northern Peninsular Malaysia.

Morphological characters	***M. hijauensis* new species (N = 7)**	***M. mamillana*[a]**	***M. khaoluangensis*[b]**	***M. perichaeta*[c]**	***M. virgo*[d]**	***M. khaochamao*[b]**	***M. houlleti*[c]**
Body length (mm)	66–87	52–85	130–265	160	152–157	166	82–193
Body width (mm)	2.8–3.1	2.5–4	10	5	5	6	3.3–5.4
Segment number	101–120	–	113–132	118	129	118	89–138
First dorsal pore	10/11	12/13	12/13	12/13	11/12	12/13	11/12 or 12/13
Setae between male pore	8	6–9	10–14	12	12	–	8–17
Spermathecae shape	Sessile elongate sac-shaped	–	Elongate	Pear shaped	Small	Elliptic	Large oval shaped
Type locality	Bukit Hijau, Kedah	Myanmar	Khaoluang moutain, Thailand	Patthalung, Thailand	Songkhla, Thailand	Rayong, Thailand	Calcutta
Accession number (GenBank, museum)	MF479445, CUMZ 3674	nil	KU565276.1, CUMZ 3466	NHMUK 1924.3.1.231	–	–, CUMZ3382	KU565270.1, CUMZ 3460

Data for [a]*M. mamillana* are from Gates (1931), [b]*M. khaoluangensis* and *M. khaochamao* from Bantaowong et al. (2016), [c]*M. perichaeta* from Beddard (1900b), [d]*M. virgo* from Stephenson (1972) and [e]*M. houlleti* from Gates (1972). – = nil.

Internal characters. Septa, 5/6–7/8 and 10/11–13/14 thickened, 8/9 and 9/10 aborted. Gizzard large within IX–X. Last heart in XIII. Intestinal origin at XV. Intestinal caeca originating in XXVII, simple, extending to XXIV.

Ovaries in XIII. Three pairs spermathecae in VII–IX. Big sac-shaped ampulla with long duct. Diverticulum with elongated tubular, twisted-shaped stalk attached to duct. Pair of elongated thin sac-shaped genital marking glands next to the spermathecae.

Holandric. Testes sacs paired in X and XI. Seminal vesicles paired in XI and XII. Prostate glands paired in XVIII, racemose, large, occupying segments XVI–XIX. Prostatic duct C-shaped.

Variation. The holotype measures 87 mm in body length with 118 segments; the paratypes ranged in size from 66–80 mm body length with 101–120 segments.

Etymology. This new species was named after the type locality (Bukit Hijau).

Distribution. This new species are found from the type locality and Sedim, Kedah. The distance between the two localities was about 24 km.

Habitat. Found inside a ditch covered with sandy loam soil (pH 5.88), at about 10 cm depth and very close to a garbage bin. Casts were observed on the top of the soil.

Remarks. *Metaphire hijauensis*, new species, belongs to the sexthecal group, with spermathecal pores in 6/7–8/9, without any genital markings. This species keys out with the *houlleti* group, to which Sims & Easton (1972) assigned 40 species, and 11 of which were recorded from adjacent countries in Southeast Asia, namely: *Metaphire mamillana* (Gates, 1931), *M. umbraticola* (Gates, 1932), and *M. quadrigemina* (Gates, 1932) from Myanmar; *M. amplectens* (Michaelsen, 1934) and *M. dawydovi* (Michaelsen, 1934) from Vietnam; *M. bindjeynsis* (Michaelsen, 1899) from Sumatra, Indonesia; *M. khaoluangensis* Bantaowong & Panha, 2016, *M. perichaeta* (Beddard, 1900), *M. virgo* (Beddard, 1900), and *M. khaochamao* Bantaowong & Panha, 2016 from Thailand; and *M. houlleti* (Perrier, 1872), which is widespread in East and South-East Asia. *Metaphire houlleti*, *M. mamillana*, *M. perichaeta* and *M. virgo* are also found in Malaysia.

Metaphire hijauensis, new species, is fairly similar to *M. mamillana* and *M. quadrigemina* in body size, but it is easily distinguished from *M. mamillana* and *M. quadrigemina* by the position of the first dorsal pore and the position of intestine origin. The first dorsal pore is on 10/11 for *M. hijauensis* (Fig. 2), new species, while that of *M. mamillana* (Gates, 1931: p. 400, Fig. 33) and *M. quadrigemina* (Gates, 1932: p. 486) are on 12/13. As for the intestine origin, it originates at XV for *M. hijauensis*, new species, whereas the intestine of *M. quadrigemina* begins in XVI. Posterior portion of XV or anteriorly in XVI are the intestine origin for *M. mamillana*. In addition, *M. hijauensis*, new species, can be distinguished from these two species by the intestinal caeca originating

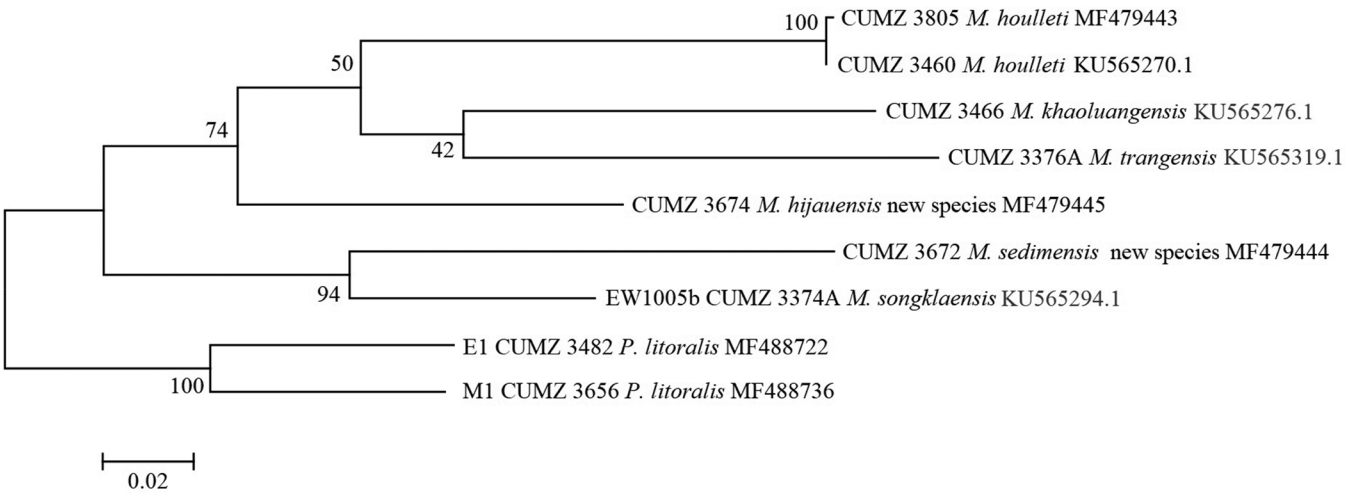

Fig. 3. Molecular affiliation of *M. sedimensis* new species, and *M. hijauensis* new species, based on ML analysis of the CO1 DNA barcode sequence, with *Pontodrilus litoralis* as the out-group. Bar represents the number of nucleotide substitutions per site. Codes before the species name are the museum reference number, whilst those afterwards are the GenBank sequence accession numbers, Jeratthitikul et al. (2017).

Table 4. Interspecific variation in the newly described *Metaphire* species and some closely related *Metaphire* species from COI partial gene sequence analysis (K2P model); lower left distance collected from the CO1 partial gene pair-wise comparison and upper right are the standard deviations.

	***M. houlleti*[a]**	***M. houlleti*[b]**	***M. khaoluangensis*[c]**	***M. songkhlaensis*[d]**	***M. trangensis*[e]**	***M. sedimensis* new species[f]**	***M. hijauensis* new species[g]**
1	–	0.002	0.02	0.023	0.021	0.021	0.019
2	0.002	–	0.019	0.023	0.02	0.021	0.019
3	0.199	0.197	–	0.021	0.02	0.02	0.02
4	0.238	0.236	0.206	–	0.02	0.018	0.019
5	0.205	0.202	0.189	0.205	–	0.022	0.02
6	0.223	0.221	0.213	0.164	0.229	–	0.02
7	0.186	0.183	0.194	0.186	0.2	0.213	–

[a] *M. houlleti* collected during this study (MF479443, CUMZ 3805).
[b]*M. houlleti* (KU565270.1, CUMZ 3460), [c]*M. khaoluangensis* (KU565276.1, CUMZ 3466), [d]*M. songkhlaensis* (KU565294.1, CUMZ 3374A) and [e]*M. trangensis* (KU565319.1, CIMZ 3376A) from Jeratthitikul et al. (2017).
[f]*M. sedimensis* (MF479444, CUMZ 3672) and [g]*M. hijauensis* (MF479445, CUMZ 3674) from this study.

in XXVII and extending to XXIV, while in the latter two species it is found in XXVII–XX.

Metaphire hijauensis, new species, can be distinguished from the regional species recorded from Vietnam (*M. amplectens* and *M. dawydovi*), Myanmar (*M. umbraticola*), Sumatra (*M. bindjeynsis*), and Thailand (*M. khaoluangensis*, *M. perichaeta*, *M. khaochamao*, and *M. virgo*), and from the cosmopolitan species (*M. houlleti*), by the aspects of body size, number of segments and the location of the first dorsal pore. The shape of spermathecal significantly separates *M. hijauensis* (Fig. 2), new species, from *M. amplectens* (Michaelsen, 1934: p. 501) and *M. dawydovi* (Michaelsen, 1934: p. 517). *Metaphire dawydovi* has flask-shaped ampullae with small diverticulum and *M. amplectens* has obclavate ampullae with zigzag diverticulum, while *M. hijauensis*, new species, has big sac-shaped ampullae with elongated tubular and twisted diverticulum. The new species is very close to *M. houlleti*, but differs in the form of male pores. The male pores of *M. hijauensis* are round while in *M. houlleti* they are C-shaped slits (Bantaowong et al., 2011: p. 59, Fig. 3). Additionally, numerous long-stalked genital marking glands, with a round or slightly lobed head are present near the spermathecae in *M. houlleti*, whereas *M. hijauensis*, new species, has only one genital marking gland next to each spermatheca. The character comparisons between *M. hijauensis*, new species, and the other closely species are presented in Table 3.

MOLECULAR ANALYSIS

The systematic positions of *M. sedimensis* new species and *M. hijauensis* new species were inferred based on the partial CO1 sequence (658 bp). The ML phylogenetic tree (Fig. 3) was clearly divided into two well-separated clades, one of which contained of *M. sedimensis*, new species, and *M. songkhlaensis*, and had good support (ML bootstrap value, 94%), while the other clade contained the *houlleti* group, including *M. hijauensis*, new species, with moderate support (ML boostrap value, 74%). Genetic distances were calculated using Kimura-2 parameter model for DNA sequence alignment. Interspecific variation in each partial

sampling of genes was 0.2–23.8% (Table 4). The mean interspecific variation between *M. sedimensis* new species and *M. songkhlaensis* was 16.4%; and between *M. hijauensis* new species and the other *houlleti* group species was 18% (*M. houlleti*) and 19.4% (*M. khaoluangensis*).

DISCUSSION

Previous records indicated that there are 30 species of *Metaphire* species distributed in Malaysia, described between 1868 to 1949, with eight of these being found in the state of Kedah. From 1949 to the present, these numbers have remained unchanged. The two new species reported in this study, *M. sedimensis* and *M. hijauensis*, are the first terrestrial earthworms to be described from Malaysia in this century. The present sampling locations, Sedim and Bukit Hijau, were situated in the dipterocarp forest of Gunung Inas, Kedah, and the distance between the two localities was about 24 km.

The two new species of *Metaphire* show a significant difference in size: *M. sedimensis* new species is double the size (140–177 mm) of *M. hijauensis* new species (66–87 mm). Their respective microhabitats showed a slight difference: *M. sedimensis* new species was mainly found in soil covered with a very thick leaf litter with no visible casts. In contrast, *M. hijauensis* new species was found in a ditch that was fully covered with soil, but without any leaf litter and a high density of casts was easily observed on top of the soil in which the worms were found.

Four related species with homologous COI sequences were accessed in the BOLD and NCBI databases. All of them were reported from Thailand. These were *M. songkhlaensis* Bantaowong & Panha, 2016, *M. houlleti* (Perrier, 1872), *M. khaoluangensis* Bantaowong & Panha, 2016, and *M. trangensis* Bantaowong & Panha, 2016. *Metaphire sedimensis* new species, and *M. songkhlaensis* were grouped into a clade with a relatively high bootstrap support of 94%. These two earthworms are very similar in their morphological characters (e.g., presence of preclitellar and postclittelar genital markings), but the K2P distance (16.4%) between them is sufficient to discriminate them as two different species (James et al., 2010; Decaëns et al., 2013).

The other new species, *M. hijauensis* new species, which was placed into the *houlleti* group in the ML phylogenetic tree, had 541 out of 658 (82.22%) nucleotides identical to that of the *M. houlleti* collected from Bukit Hijau. Morphologically, the latter species is larger, with numerous genital marking glands around the spermathecae, while the new species is small in size and possesses only one genital marking gland near each spermatheca. With a high genetic divergence (22.3%) between them, these two species of earthworms can be unambiguously recognised as two different species.

In conclusion, the evolutionary divergence between those four species and the two new species revealed high interspecific genetic distances, viz. 16.4–22.9% for *M. sedimensis* new species, and 18.3–20.0% for *M. hijauensis* new species (Table 4). Also, the pairwise distance between the two new species was 21.3%. With the combination of the molecular and morphological data, there is sufficient evidence to support the recognition of *M. sedimensis* and *M. hijauensis* as new species.

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