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Six new species of the genus *Metaphire* Sims & Easton, 1972 (Annelida: Oligochaeta: Megascolecidae) from southeastern Vietnam

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Abstract. Six new species of the earthworm genus *Metaphire* Sims & Easton, 1972, are described from southeastern Vietnam (provinces of Binh Duong, Binh Phuoc, and Ba Ria-Vung Tau), namely *M. haui*, *M. bariaensis*, *M. planatoides*, *M. songbeensis*, *M. setosa*, and *M. houlletoides*. The COI (cytochrome c oxidase subunit I) fragment sequence is also provided as an additional character for the recognition of the new species. Interspecific K2P distances among *Metaphire* species is from 14.2% to 23.3%, with a mean distance of 17.7%. In addition, an identification key to the species of *Metaphire* of southeastern Vietnam is also provided.

Key words. earthworms, diversity, taxonomy, genetic distance, Vietnam

INTRODUCTION

There are 212 recorded species of earthworm in Vietnam, distributed among 24 genera and eight families (Nguyen et al., 2016a). More new species have been recently discovered, including *Amynthas munglongoides* Nguyen, Lam & Nguyen, 2016; *A. antoanensis* Nguyen, Lam & Nguyen, 2016; *A. konkakinh* Nguyen, Lam & Nguyen, 2016; *Metaphire peguana laisonensis* Nguyen, Trinh, Nguyen & Nguyen, 2017; *M. malayanoides* Nguyen & Lam, 2017; *M. grandiverticulata* Nguyen & Lam, 2017; *M. xuanlocensis* Nguyen & Lam, 2017; *Polypheretima dorsotheca* Nguyen, Trinh, Nguyen & Nguyen, 2017; *P. insularis* Nguyen, Trinh, Nguyen & Nguyen, 2017; *P. medialis* Lam, Nguyen & Nguyen in Lam, Nguyen, Hoang & Nguyen, 2018; and *P. tabhinhensis* Lam, Nguyen & Nguyen in Lam, Nguyen, Hoang & Nguyen, 2018 (Nguyen et al., 2016b; Nguyen et al., 2017; Nguyen & Lam, 2017; Lam et al., 2018).

The diversity of earthworms has been well studied in almost all regions of Vietnam except the southeastern region, which is considered to be a bridge connecting the Highlands of Vietnam and the Cuu Long River delta. It is considered to be a transition zone between a hilly/mountainous area in the north and a plain area in the south. This region has a total area of 23,654 km^2, with six component provinces, namely Ho Chi Minh City, Ba Ria-Vung Tau, Binh Duong, Binh Phuoc, Dong Nai, and Tay Ninh. As the transition region, the southeastern Vietnam harbours diverse fauna and flora, although the earthworm diversity in this region is not well known. To date, only 27 species belonging to nine genera and four families have been documented in this region (Perrier, 1872; Omodeo, 1957; Nguyen, 2016; Nguyen et al., 2016a; Nguyen & Lam, 2017).

The earthworm genus *Metaphire* Sims & Easton, 1972, has 55 species widely distributed in Vietnam (Nguyen et al., 2016a), but only 12 have been recorded in southeastern Vietnam (Table 1). As part of our continuing research on Vietnamese earthworms, this paper aims to contribute to our understanding of the earthworm diversity through descriptions of six new species of *Metaphire* from southeastern Vietnam.

MATERIAL AND METHODS

Specimen collection, imaging, and identification. Earthworms were collected by manually digging soil in natural forests, regenerated forests, and plantations (Fig. 1). Earthworm specimens were cleaned with tap water, then killed in 2% formalin, and transferred to 4% formalin for fixation for approximately 10–12 hours. After that, they were transferred to new 4% formalin for long-term preservation and morphological studies, and to 96% ethanol for molecular analysis. Earthworms were externally observed under a microscope (Motic DM143-FBGG-C) and then dissected dorsally for internal observation. Transverse body sections were processed using the classical haematoxylin and eosin staining method (Feldman & Wolfe, 2014). The detailed protocol follows Nguyen et al. (2017). Colour images were taken using a camera attached to the microscope. Line drawings were redrawn based on the colour photographs.

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Table 1. *Metaphire* species previously recorded in the southeastern Vietnam.

No	Species	Localities	Source
1	*Metaphire anomala* (Michaelsen, 1907)	Tay Ninh, Dong Nai Provinces	Nguyen et al. (2016a)
2	*Metaphire bahli* (Gates, 1945)	Ba Ria-Vung Tau, Ho Chi Minh, Dong Nai, Tay Ninh Provinces	Nguyen et al. (2016a)
3	*Metaphire californica* (Kinberg, 1867)	Dong Nai Province	Nguyen et al. (2016a)
4	*Metaphire guillelmi* (Michaelsen, 1895)	Dong Nai Province	Nguyen et al. (2016a)
5	*Metaphire houlleti* (Perrier, 1872)	Dong Nai, Tay Ninh Provinces	Nguyen et al. (2016a)
6	*Metaphire pacseana* (Thai & Samphon, 1988)	Tay Ninh Province	Nguyen et al. (2016a)
7	*Metaphire peguana* (Rosa, 1890)	Dong Nai, Tay Ninh Provinces	Nguyen et al. (2016a)
8	*Metaphire posthuma* (Vaillant, 1868)	widely distributed	Nguyen et al. (2016a)
9	*Metaphire saigonensis* (Omodeo, 1957)	Ho Chi Minh city	Nguyen et al. (2016a)
10	*Metaphire xuanlocensis* Nguyen & Lam, 2017	Dong Nai Province	Nguyen & Nguyen (2017)
11	*Metaphire malayanoides* Nguyen & Lam, 2017	Dong Nai Province	Nguyen & Nguyen (2017)
12	*Metaphire grandiverticulata* Nguyen & Lam, 2017	Dong Nai Province	Nguyen & Nguyen (2017)

All images and line drawings were assembled using Adobe Photoshop CS6.

Material examined (including types) are stored in the Laboratory of Zoology, Department of Biology, Can Tho University (CTU), Can Tho City, Vietnam. The following abbreviations were used: CTU, Can Tho University; ag, accessory glands; amp, ampulla; dv, diverticulum; gm, genital markings; ocp, the openings of copulatory pouches; sp, spermathecal pore; ts, testis sacs; sv, seminal vesicles; ov, ovaries.

DNA extraction and sequencing. Total genomic DNA was extracted from body walls of earthworms using the DNAeasy Blood & Tissue Kit (QiagenTM). The 680bp fragment of the mitochondrial gene, cytochrome c oxidase subunit I (COI), was amplified using polymerase chain reaction (PCR) with the universal primer set (HCO-2190/COI-E and LCO-1498/LCO-1498m) (Folmer et al., 1994; Bely & Wray, 2004). The PCR condition for amplification was: an initial denaturation at 95°C for 2 min followed by 36 cycles of 95°C for 20 s, 42°C for 45 s, and 72°C for 1 min, and a final extension at 72°C for 5 min. PCR products were checked for potentially successful amplification of a fragment of each gene using electrophoresis in 1% Agarose-TBE 1X. Successfully amplified PCR products were purified and sequenced at Macrogen, Inc. (Korea) on an Applied Biosystems automatic sequencer (ABI3130 XL). Each successful sequence was manually checked using BioEdit ver.7.1 (Hall, 1999), and confirmed by BLAST searches (Altschul et al., 1990). All confirmed sequences were aligned with MUSCLE (Edgar, 2004). All nucleotide sequences are deposited at GenBank.

The dataset contained COI sequences from four new species and 23 other *Metaphire* sequences (6 species: *M. anomala*, *M. bahli*, *M. californica*, *M. guillelmi*, *M. houlleti*, and *M. peguana*). These 6 species were chosen due to their occurrence in Vietnam. *Amynthas aspergillus* was selected as an outgroup, as *Amynthas* is a sister genus of *Metaphire* (see Sims & Easton, 1972). All sequences were obtained from GenBank. The genetic distance was calculated using the Kimura 2 parameter (K2P) model performed in MEGA 7.0 with bootstrap of 1,000 (Kumar et al., 2016).

TAXONOMIC ACCOUNT

Family Megascolecidae Rosa, 1891

Metaphire Sims & Easton, 1972

Metaphire Sims & Easton, 1972: 215.

Type species. *Rhodopis javanica* Kinberg, 1867, by monotypy.

Distribution. The genus is widely distributed in the Oriental region, from Japan through to the Indo-Australian archipelago (Sims & Easton, 1972: 215).

Remarks. According to Sims & Easton (1972), the genus *Metaphire* is distinguished from *Amynthas* Kinberg, 1867, mainly by the presence of copulatory pouches, from *Pheretima* Kinberg, 1867, by the absence of micronephridia on the spermathecal ducts, and from *Pithemera* Sims & Easton, 1972, by the presence of intestinal caeca at or near xxvii.

Morphologically, *Metaphire* and *Amynthas* are clearly separated by the presence or absence of copulatory pouches.

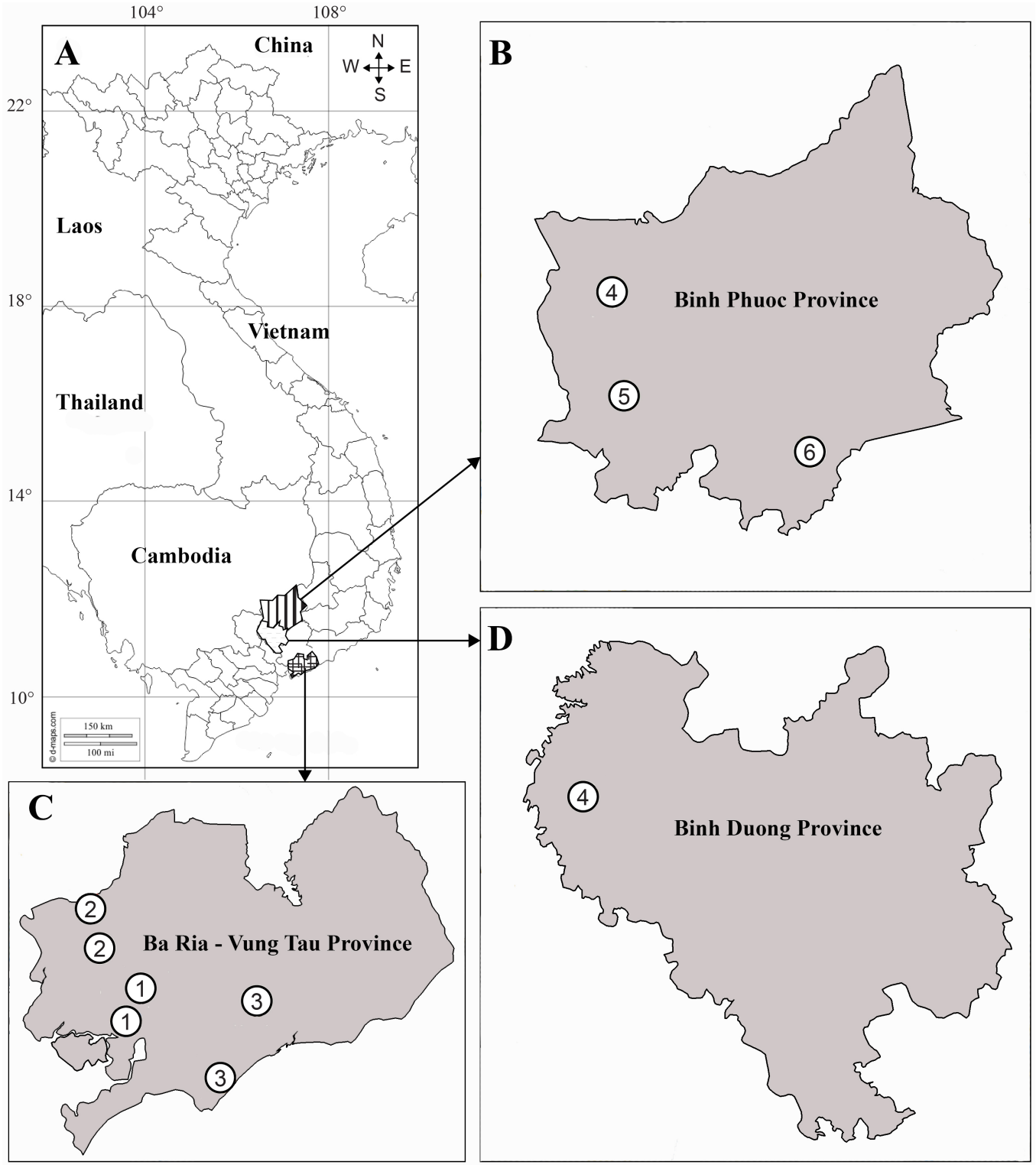

Fig. 1. Collecting sites in southeastern Vietnam. A, map of Vietnam and component provinces; B, Binh Phuoc Province; C, Ba Ria-Vung Tau Province; D, Binh Duong Province. 1, *Metaphire haui*, new species; 2, *Metaphire bariaensis*, new species; 3, *Metaphire planatoides*, new species; 4, *Metaphire songbeensis*, new species; 5, *Metaphire setosa*, new species; 6, *Metaphire houlletoides*, new species.

However, recent molecular studies show the polyphyletic relationship between *Metaphire* and *Amynthas* (Zhao et al., 2015; Jeratthitikul et al., 2017). Thus, more data is needed for better understanding of the relationship between these two genera.

***Metaphire haui*, new species**
(Figs. 1, 2)

Material examined. Holotype: 1 mature (CTU-EW.172.h01), regenerated forests (10°31′33.0″N, 107°09′44.8″E), Dinh Mts, Tan Hung District, Ba Ria city, Ba Ria-Vung Tau Province, 25 m asl, 26 October 2016, coll. Nguyen Phuc Hau. Paratypes: 4 matures (CTU-EW.172.p02) same data as holotype; 2 matures (CTU-EW.172.p03), bushes near the road (10°29′58.0″N, 107°08′31.0″E), Kim Dinh commune, Ba Ria city, Ba Ria-Vung Tau Province, 27 October 2016, coll. Nguyen Phuc Hau.

Non-types: 4 immatures (CTU-EW.172.p04), *Acacia* plantations (10°31′33.0″N, 107°09′44.8″E), Hac Dich commune, Tan Thanh District, Ba Ria-Vung Tau Province, 25 m asl, 26 October 2016, coll. Nguyen Phuc Hau.

Diagnosis. Medium-sized worm, length 107–165 mm, diameter 5.3–5.9 mm, unpigmented except light yellow clitellum. Prostomium epilobous. First dorsal pore in 7/8. Three pairs of spermathecal pores in dorsally 6/7/8/9. Male porophore disc-shaped, slightly elevated; male pores located inside copulatory pouches in the setal ring of xviii. Two pairs of genital markings in xvii and xix, in line with the openings of copulatory pouches. Holandric. Intestinal caeca simple. Septum 8/9 thick, but 9/10 absent.

Description. Body cylindrical, medium size; length 107–165 mm, average diameter 5.3–5.9 mm, weight 2.4–3.5 g, segments 101–205. Body transparent, uniformly unpigmented except light yellow clitellum. Prostomium ⅓ epilobous. First dorsal pore in 7/8. Setae perichaetine, pre-clitellar setae stouter and sparser than post-clitellar ones; setal number 65–87 in viii, 108–130 in xxx, 20–25 between male porophores in xviii; setal distance: aa = ab, zz=zy. Clitellum close, xiv–xvi, without dorsal pores and setae. Female pore single, in midventral xiv.

Three pairs of spermathecal pores located dorsally in intersegments 6/7/8/9. No genital markings in the spermathecal region.

Male porophore disc-shaped, slightly elevated from body wall; male pores located inside the copulatory pouches on the setal ring xviii; ventral distance between two openings of copulatory pouches about 0.3× body circumference. Two pairs of slightly elevated, disc-shaped genital markings on setal rings of xvii and xix, in line with the openings of copulatory pouches.

Septa 5/6/7/8/9 thick, 9/10 thin, 10/11/12/13 slightly thick. Oesophageal gizzard within viii–x. Intestinal origin at xv; caeca simple, originating at xxvii and extending anteriorly to xxiv or xxv. Last hearts in xiii. Pharyngeal micronephridia developed 5/6/7. Typhlosole simple, lamelliform. Lymph glands absent.

Three pairs of spermathecae in vii–ix. Ampulla mushroom-shaped, ducts robust, somewhat longer than ampulla, and enlarged basally. Diverticula twice longer than ampulla, directly attached to the base of ampulla duct; seminal chamber poorly developed. Spermathecal ducts without nephridia. No accessory glands.

Holandric. Testis sacs developed, connected in x–xi. Seminal vesicles well developed in xi–xii. Ovaries well developed in 12/13. Ovisac invisible. Prostate glands deeply lobuled, paired in xvii–xx; ducts long, coiled and attached to copulatory pouches which are highly elevated from body wall. Two pairs of large accessory glands in xvii and xix.

DNA barcode. A partial sequence of the mitochondrial gene cytochrome oxidase subunit I (COI) was uploaded to GenBank with the accession number MK902663.

Variation. One paratype specimen lacks spermathecal pore in the left side of intersegment 6/7.

Etymology. The new species, *Metaphire haui*, is named in honour of Mr. Nguyen Phuc Hau, who collected the type specimens.

Remarks. *Metaphire haui*, new species, is classified into the *Metaphire peguana* group characterised by having spermathecal pores in 6/7/8/9 and two pairs of genital markings in xvii and xix, in line with the openings of copulatory pouches (Sims & Easton, 1972). However, it differs from other known species in spermathecal pores located dorsally, first dorsal pore in 7/8, presence of thin septum 8/9/10, genital markings on xvii and xix, whereas other *peguana* species have spermathecal pores on ventral or lateroventral side, first dorsal pore in 12/13, absence of septum 8/9, and genital markings located in segmental furrows 17/18 and 18/19.

The new species is also similar to *Metaphire posthuma* (Vaillant, 1868) in having septum 8/9, two pairs of genital markings in xvii and xix, absence of genital markings in spermathecal region, and simple intestinal caeca. *Metaphire haui*, new species, is distinguished from *M. posthuma* in having three pairs of spermathecal pores in dorsal intersegments 6/7/8/9 (vs. four pairs in ventral intersegments 5/6/7/8/9), first dorsal pore in 7/8 (vs. 12/13), connected testis sacs (vs. separated testis sacs), larger size (107–165 mm vs. 67–101 mm in length; 5.3–5.9 mm vs. 4.0–4.8 mm in diameter), and being more segmented (101–205 vs. 98–119 segments).

Metaphire haui, new species, is also similar to *Metaphire nhuongi* Nguyen, 2016, in having two pairs of genital markings in xvii and xix, no genital markings in spermathecal region, simple intestinal caeca. However, the new species has three pairs of spermathecal pores in dorsal intersegments

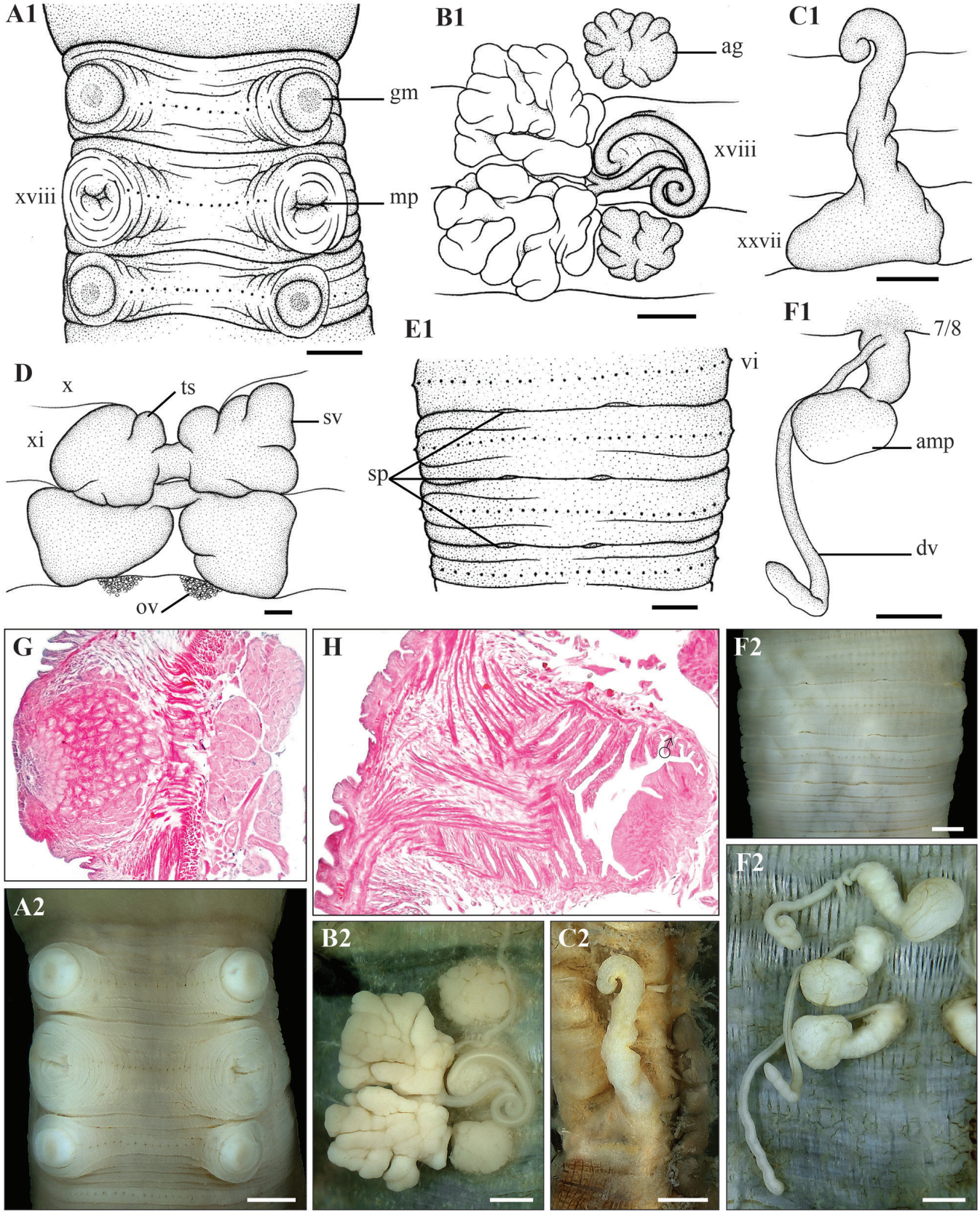

Fig. 2. *Metaphire haui*, new species, holotype (CTU-EW.172.h01). A1, A2, ventral side of the male region; B1, B2, left prostate gland; C1, C2, intestinal caecum; D, testis sacs and seminal vesicle; E1, E2, dorsal side of spermathecal region; F1, F2, left spermathecae; G, genital markings, transverse body section; H, male copulatory pouches, transverse body section. Scale bar = 1 mm.

6/7/8/9, first dorsal pore in 7/8, pharyngeal micronephridia in 5/6/7, presence of septum 8/9/10, connected testis sacs, and absence of lymph glands, whereas *M. nhuongi* has four pairs of spermathecal pores in ventrolateral intersegments 5/6/7/8/9, first dorsal pore in 12/13, pharyngeal micronephridia in 4/5/6, absence of septum 8/9, separated testis sacs, and lymph glands in 15/16.

Three species previously recorded in Vietnam, *M. amplectens* (Michaelsen, 1934), *M. scitula* (Gates, 1936), and *M. dorsobitheca* (Thai & Huynh, 1992), also have three pairs of spermathecal pores in dorsal intersegments 6/7/8/9. However, *M. haui*, new species, is distinguished from those species by the presence of two pairs of genital markings in xvii and xix, first dorsal pore in 7/8, thick septum 8/9. On the contrary, those species do not have genital markings in the male region, first dorsal pore in 10/11, and absence of septa 8/9/10. In addition, the new species is similar in size to *M. scitula* (length 107–165 mm, diameter 5.3–5.9 mm vs. length 100–120 mm, diameter 5 mm), but it is larger than *M. amplectens* and *M. dorsobitheca* (107–165 mm vs. 44 mm and 54 mm in length; 5.3–5.9 mm vs. 2–3 mm in diameter, respectively).

Metaphire bariaensis, new species
(Figs. 1, 3)

Material examined. Holotype: 1 mature (CTU-EW.169.h01), natural forests, (10°35′36.0″N, 107°06′50.0″E), Bao Quang Mts, Chau Pha commune, Tan Thanh District, Ba Ria-Vung Tau Province, 81.6 m asl, 24 October 2016, coll. Nguyen Phuc Hau. Paratypes: 2 matures (CTU-EW.169.p02), same data as holotype; 3 matures (CTU-EW.169.p03), regenerated forests (10°38′21.0″N, 107°05′08.0″E), Dinh Mts, Tan Hung commune, Ba Ria city, Ba Ria-Vung Tau Province, 72–75 m asl, 27 October 2016, coll. Nguyen Phuc Hau.

Diagnosis. Large-medium size, length 141–170 mm, average diameter 6.3–7.6 mm. Prostomium epilobous. First dorsal pore in 11/12. Four pairs of spermathecal pores in ventrolateral intersegments 5/6/7/8/9. Male pores located inside copulatory pouches in the setal ring xviii. Genital markings totally absent. Holandric. Intestinal caeca manicate. Septa 8/9/10 absent.

Description. Body cylindrical, large-medium size; length 141–170 mm, average diameter 6.3–7.6 mm, weight 3.4–6.0 g, segments 89–112. Dorsa light pink and ventra paler, clitellum reddish brown. Prostomium 1/2 epilobous. First dorsal pore in 11/12. Pre-clitellar setae stouter and sparser than post-clitellar ones; setal number 46–62 in viii, 60–81 in xxx, and 6–12 between two openings of copulatory pouches in xviii; setal distance aa > ab, zz > zy. Clitellum xiv–xvi, without setae and dorsal pores. Female pore single, in midventral xiv.

Four pairs of spermathecal pores in ventrolateral intersegments 5/6/7/8/9. No genital markings in spermathecal region. Male pores deeply located inside copulatory pouches in the setal ring xviii; ventral distance between two openings of copulatory pouches ca. 0.3× body circumference. No genital markings in male region.

Septa 5/6/7/8 thick, 8/9/10 absent, 10/11/12/13 slightly thick. Oesophageal gizzard within viii–x. Intestinal origin at xv; caeca manicate, paired within xxvii–xxiv, distal part somewhat rolling up. Last hearts in xiii. Pharyngeal micronephridia developed in 5/6/7. Typhlosole simple, lamelliform. Lymph glands absent.

Four pairs of spermathecae in vi–ix. Ampulla heart-shaped, scabrous; ducts shorter than ampulla (about ½ length), cylindrically slender. Diverticula much shorter than ampulla, directly attached to the base of ampulla ducts; seminal chamber oval-shaped. Spermathecal ducts without nephridia. No accessory glands.

Holandric. Testis sacs developed, connected in x–xi. Seminal vesicles well developed in xi–xii. Ovaries well developed on septum 12/13, ovisacs in 12/13 and more developed in 13/14. Prostate glands deeply lobuled, paired in xvi–xx; ducts very short, slightly curved. Accessory glands invisible.

DNA barcode. A partial sequence of the mitochondrial gene cytochrome oxidase subunit I (COI) was uploaded to GenBank with the accession number MK902662.

Etymology. The new species, *Metaphire bariaensis*, is named after the Ba Ria-Vung Tau Province where the types were found.

Remarks. The new species can be classified in the *Metaphire ignobilis* group characterised by having four pairs of spermathecal pores in 5/6/7/8/9, absence of additional copulatory pouches in xix and xx, absence of genital markings in male region, and being holandric (Sims & Easton, 1972). The *ignobilis* group currently contains *M. feuerborni* (Michaelsen, 1932), *M. ignobilis* (Gates, 1935), *M. tosaensis* (Ohfuchi, 1938), and *M. riukiuensis* (Ohfuchi, 1957) from Japan, *M. dalatana* (Michaelsen, 1934), *M. langbiangi* (Michaelsen, 1934), and *M. truongsonensis* (Thai, 1984) from Vietnam, *M. seponensis* (Thai & Samphon, 1989) from Laos, and *M. trangensis* Bantaowong & Panha, 2016, from Thailand.

Within the *ignobilis* species group, *Metaphire bariaensis*, new species, is slightly similar to *Metaphire truongsonensis* (Thai, 1984), *M. ignobilis* (Gates, 1935), and *M. trangensis* Bantaowong & Panha, 2016, in having epilobous prostomium, absence of genital markings in spermathecal region, last hearts in xiii, and intestinal origin at xv. However, the new species has manicate intestinal caeca while others have simple caeca. The new species also differs from *M. dalatana*, *M. truongsonensis*, and *M. seponensis* in having spermathecal pores located laterally, first dorsal pore in 11/12, and lobuled intestinal caeca. On the contrary, *M. dalatana*, *M. seponensis*, and *M. truongsonensis* have the first dorsal pore in 12/13, simple intestinal caeca, spermathecal pores located ventrally (*M. dalatana* and *M. seponensis*) or mediodorsally (*M. truongsonensis*). The new species is also larger than

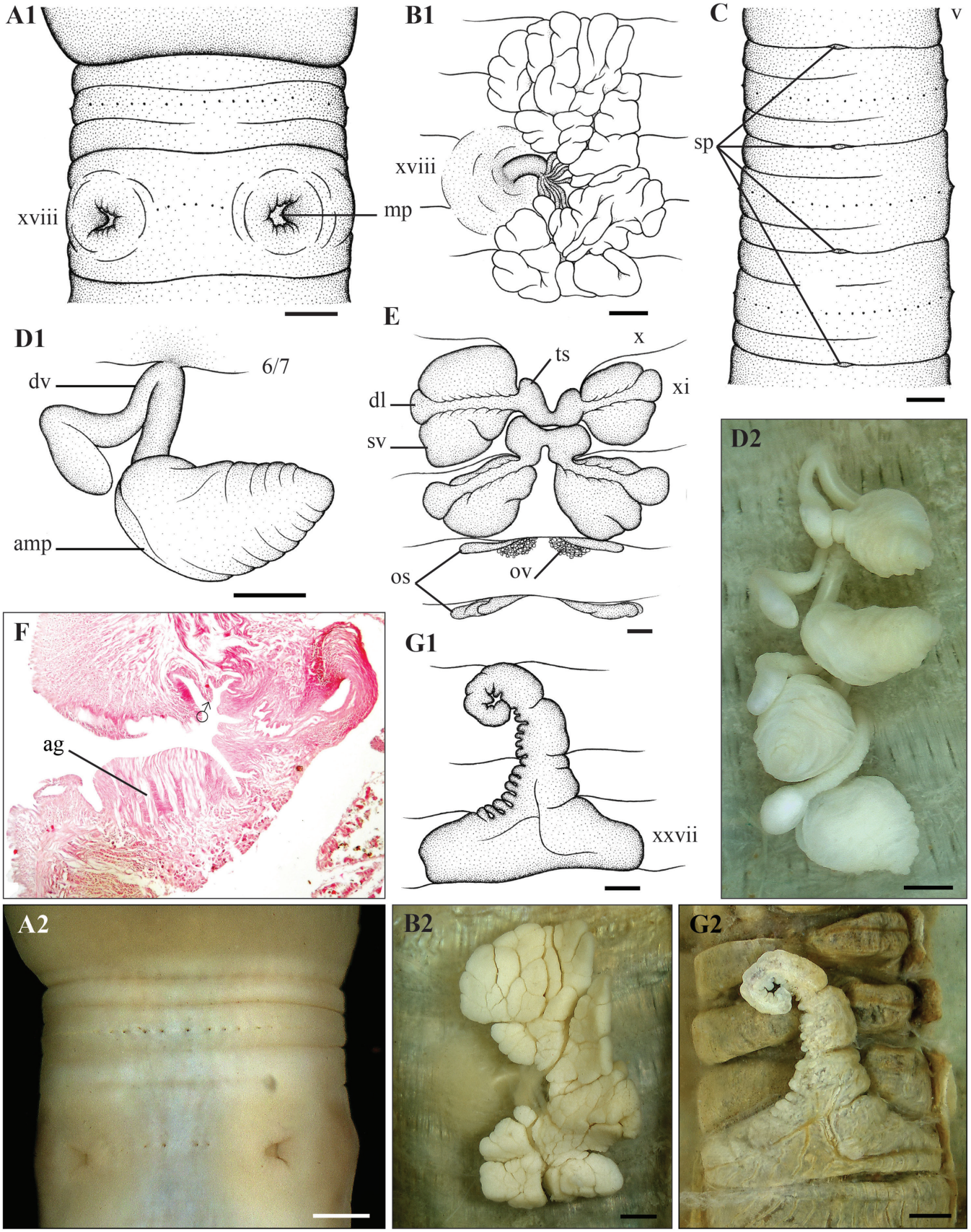

Fig. 3. *Metaphire bariaensis*, new species, holotype (CTU-EW.169.h01). A1, A2, ventral side of the male region; B1, B2, right prostate gland; C, lateral side of spermathecal region; D1, D2, spermathecae; E, testis sacs and seminal vesicles; F, male copulatory pouch, transverse body section; G1, G2, intestinal caecum. Scale bar = 1 mm.

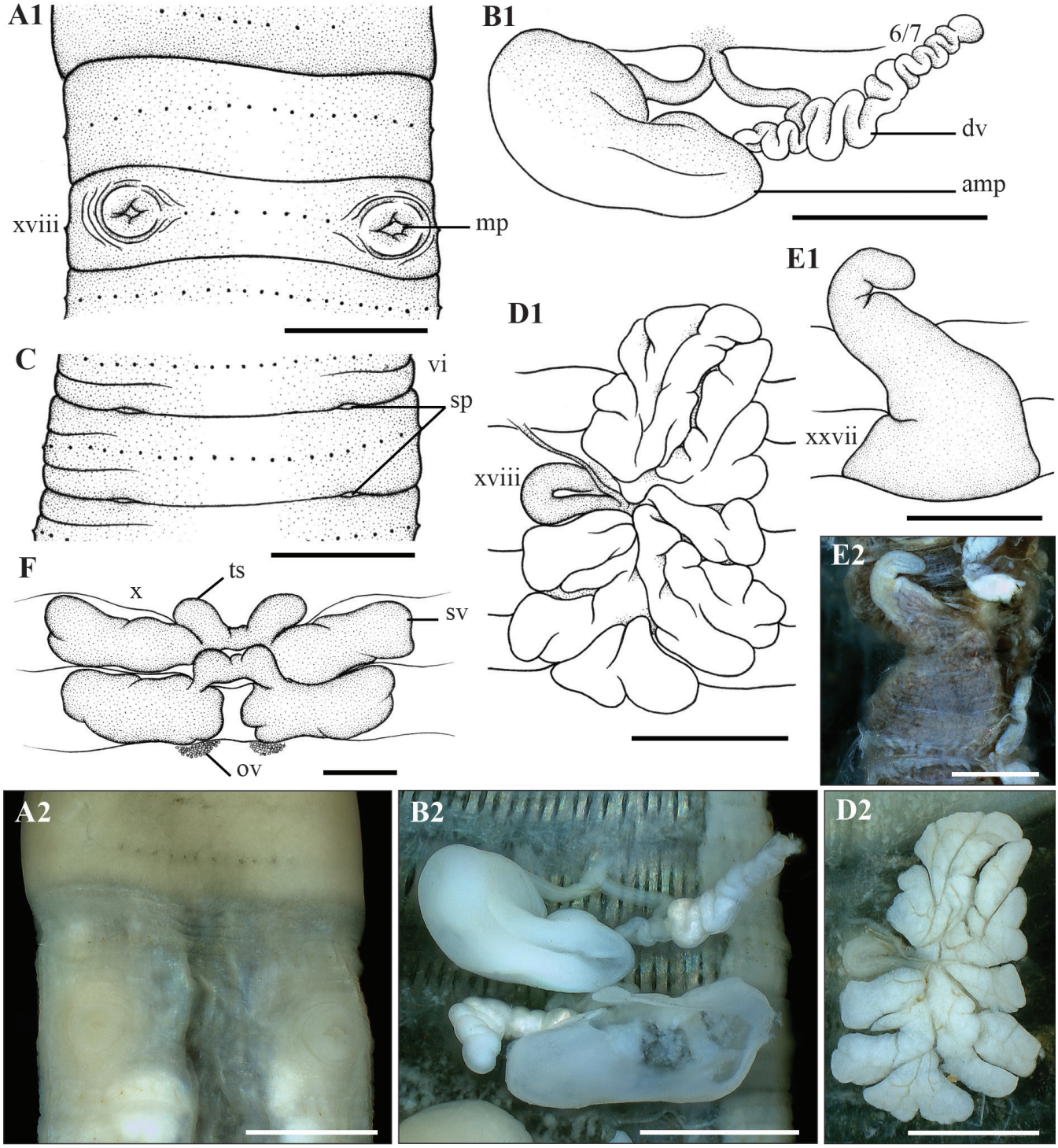

Fig. 4. *Metaphire planatoides*, new species, holotype (CTU-EW.171.h01). A, G, ventral side of the male region; B, ventral side of spermathecal region; C, H, spermathecae; D, I, right prostate gland; E, F, intestinal caecum. Scale bar = 1 mm.

M. dalatana and *M. truongsonensis* (141–170 mm vs. 52 mm and 80–93 mm in length; 6.3–7.6 mm vs. 2.5 mm and 4.7–4.8 mm in diameter, respectively). It is less segmented than *M. truongsonensis* (89–112 vs. 110–150 segments), but not different from *M. dalatana* in number of segments (89–112 vs. 96).

Similarly, *Metaphire bariaensis*, new species also differs from *M. langbiangi* in the position of the first dorsal pore (11/12 vs. 12/13), intestinal caeca (ventrally lobuled vs. deeply manicate), body size (141–170 mm in length, 6.3–7.6 mm diameter vs. 67 mm in length, 3 mm diameter). The new species is also distinguished from *M. trangensis* by having spermathecal pores located lateroventrally, lobuled intestinal caeca, and connected testis sacs, whereas *M. trangensis* has spermathecal pores on the ventral side, simple intestinal caeca, and separated testis sacs. However, the new species and *M. trangensis* are not different in body size (141–170 mm in length, 6.3–7.6 mm diameter vs. 120–169 mm in length, 7.3–7.6 mm diameter).

Metaphire planatoides, new species
(Figs. 1, 4)

Material examined. Holotype: 1 mature (CTU-EW.171.h01), natural forests (10°24′21.7″N, 107°16′18.2″E), Long Hai town, Long Dien District, Ba Ria-Vung Tau Province, 33 m asl, 25 October 2016, coll. Nguyen Phuc Hau. Paratypes: 2 matures (CTU-EW.171.p02), same data as holotype.

Diagnosis. Small-sized worm, length 64–67 mm, average diameter 2.4–2.8 mm. Body colourless, pale, except light brown clitellum. Prostomium epilobous. First dorsal pore in 11/12. Two pairs of spermathecal pores in ventral intersegments 6/7/8. Male pores located deeply inside copulatory pouches in the setal ring xviii. Genital markings totally absent. Holandric. Intestinal caeca simple. Septa 8/9/10 absent.

Description. Body cylindrical, small size, length 64–67 mm, average diameter 2.4–2.8 mm, weight 0.18–0.32 g, segments 89–96. Body colourless, pale except light brown clitellum. Prostomium 1/2 epilobous. First dorsal pore in 11/12. Pre-clitellar setae stouter and sparser than post-clitellar ones; setal number 39–41 in viii, 51–55 in xxx, 7–9 between two openings of copulatory pouches in xviii; setal distance aa=ab, zz=zy. Clitellum close, xiv–xvi, with only ventral setae, without dorsal pores. Female pore single, in midventral xiv.

Two pairs of spermathecal pores in ventral intersegments 6/7/8. No genital markings in spermathecal region. Male pores located deeply inside copulatory pouches in the setal ring xviii. Ventral distance between two openings of copulatory pouches ca. 0.35× body circumference. No genital markings in male region.

Septa 5/6/7/8 thick, 8/9/10 absent, 10/11/12/13 thin. Oesophageal gizzard within viii–ix. Intestinal origin at xv; caeca simple, paired in xxvii–xxv. Last hearts in xiii. Pharyngeal micronephridia developed in 5/6/7. Typhlosole simple, lamelliform. Lymph glands absent.

Two pairs of spermathecae in vii and viii. Ampulla mango-shaped; duct small, about ⅓ ampulla length. Diverticula long, but waved and folded, directly attached to the base of ampulla duct; seminal chamber tiny, oval-shaped. Spermathecal ducts without nephridia. Accessory glands absent.

Holandric. Testis sacs developed in x–xi, connected. Seminal vesicles developed in xi–xii. Ovaries developed in 12/13. Ovisacs invisible. Prostate glands deeply lobuled, paired in xvii–xx; ducts short, C-shaped. No accessory glands.

Etymology. The epithet "*planatoides*" is used to emphasise its similarity to *Metaphire planata* (Gates, 1926).

Remarks. *Metaphire planatoides*, new species, is assigned to the *Metaphire planata* group characterised by having two pairs of spermathecal pores in 6/7/8 and simple intestinal caeca (Sims & Easton, 1972). The *planata* group currently consists of at least six species, *M. planata* (Gates, 1926), *M. decipiens* (Beddard, 1912), *M. dunckeri* (Michaelsen, 1902), *M. ferdinandi* (Michaelsen, 1891), *M. parvula* (Ohfuchi, 1956), and *M. sintangi* (Michaelsen, 1922). The new species differs from these species except *M. planata* in the absence of genital markings in both spermathecal and male regions.

Metaphire planatoides, new species, is somewhat similar to *M. planata*, in having the first dorsal pore in 11/12, the absence of genital markings in both spermathecal and male regions, and the shape of the openings of copulatory pouches. However, it is distinguished by the absence of accessory glands in the spermathecal region, spermathecae with thin ducts, strongly waved diverticula, connected seminal vesicles, and its smaller size (length = 67 mm, diameter = 2.4–2.8 mm). On the contrary, *M. planata* has several accessory glands with long ducts, spermathecae with stout ducts, diverticulum straightly cylindrical, somewhat slightly expanded distally, separated seminal vesicles, and a larger size (length = 125 mm, diameter = 4.8 mm).

Metaphire songbeensis, new species
(Figs. 1, 5)

Material examined. Holotype: 1 mature (CTU-EW.176.h01), banana garden (11°45′39.0″N, 106°34′38.8″E), Loc Ninh commune, Loc Ninh District, Binh Phuoc Province, 85 m asl, 26 October 2017, coll. Luong Thi Huynh Tien. Paratypes: 9 matures (CTU-EW.176.p02), same data as holotype.

Non-types: 13 matures (CTU-EW.176.03), same data as holotype; 8 matures (CTU-EW.176.04), bushes (11°17′41.2″N, 106°24′22.1″E), Dau Tieng town, Dau Tieng District, Binh Duong Province, 53.4 m asl, 27 October 2017, coll. Dinh So Na.

Diagnosis. Small-sized worm, length 91–133 mm, average diameter 3.4–6.4 mm. Prostomium epilobous. First dorsal pore in 9/10. Four pairs of spermathecal pores in dorsal intersegments 5/6/7/8/9. No genital markings in spermathecal region. Male pores deeply located inside copulatory pouches in the setal ring xviii. Four to eight pairs of genital markings in xvii, xix, and subsequent segments, but in line with the openings of copulatory pouches. Holandric. Intestinal caeca simple. Septum 8/9 thick, but 9/10 thin.

Description. Body cylindrical, medium size, length 91–133 mm, average diameter 3.4–6.4 mm, weight 1.4–2.1 g, segments 206–236. Body transparent, uniformly unpigmented except orange yellow clitellum. Prostomium epilobous. First dorsal pore in 9/10. Pre-clitellar setae stouter and sparser than post-clitellar ones; setal number 87–156 in viii, 77–124 in xxx, 8–11 between two openings of copulatory pouches; setal distance aa=ab, zz=zy. Clitellum close, xiv–xvi, without setae and dorsal pores. Female single, midventral xiv.

Four pairs of spermathecal pores in dorsal intersegments 5/6/7/8/9; dorsal distance between two spermathecal pores about ⅓ body circumference. No genital markings in spermathecal region.

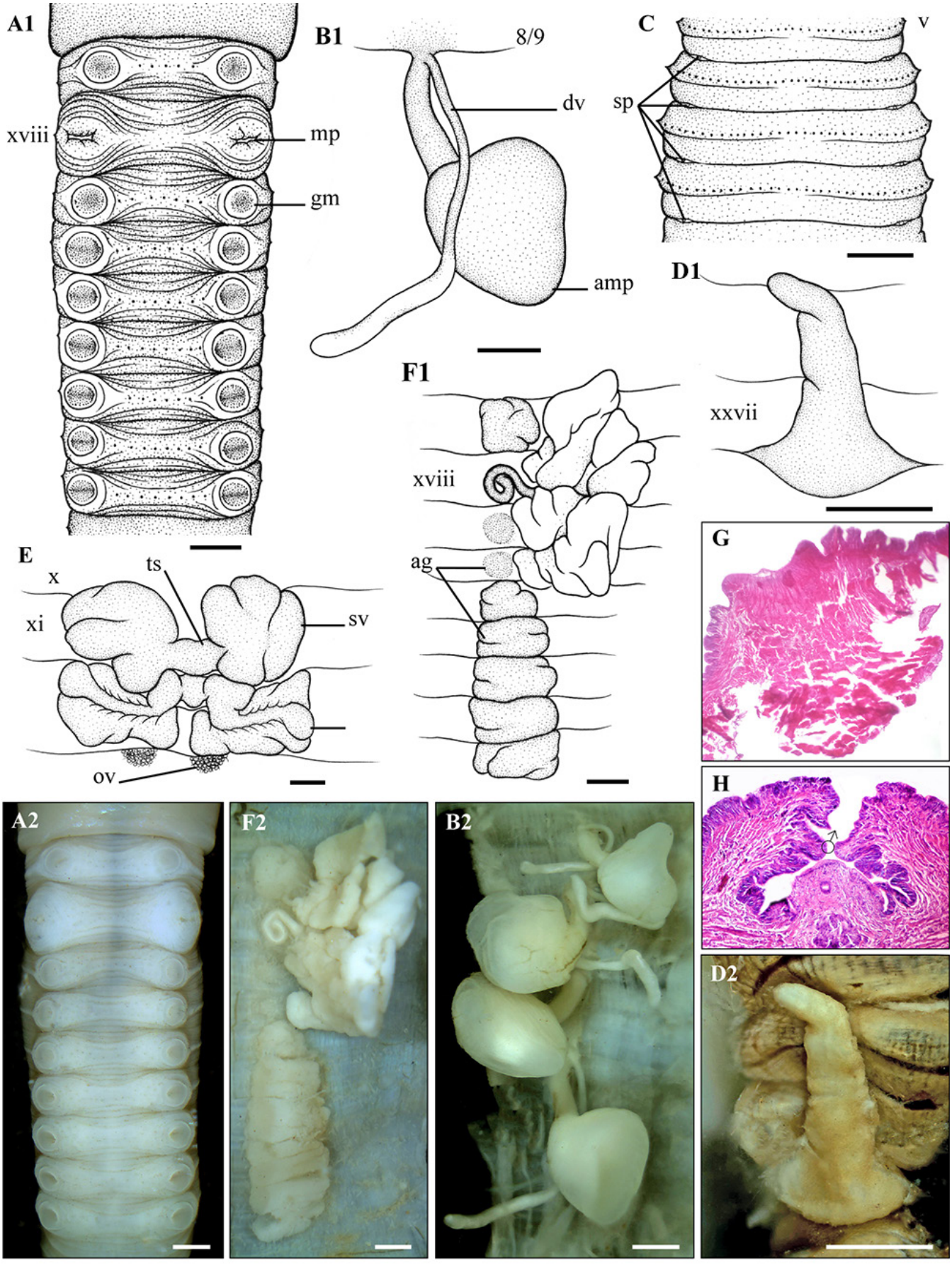

Fig. 5. *Metaphire songbeensis*, new species, holotype (CTU-EW.176.h01). A1, A2, ventral side of the male region; B1, B2, left spermathecae; C, dorsal side of spermathecal region; D1, D2, intestinal caecum; E, seminal vesicles; F1, F2, right prostate gland. G, genital markings, transverse body section; H, male copulatory pouch, transverse body section. Scale bar = 1 mm.

Male porophores slightly elevated from body wall, almost occupying xviii. Male pores located deeply inside copulatory pouches in the setal ring xviii. Ventral distance between two openings of copulatory pouches about 0.35× body circumference. Genital markings about 4–8 pairs, arranged in xvii, xix, and subsequent segments.

Septa 5/6/7/8/9 thick, 9/10/11/12/13 thin. Oesophageal gizzards after viii. Intestinal origin at xv; caeca simple, paired in xxvii–xxiv. Last hearts in xiii. Pharyngeal micronephridia developed in 5/6/7. Typhlosole simple, lamelliform. Lymph glands absent.

Four pairs of spermathecae in vi–ix. Ampulla large, oval or heart-shaped; duct about ½ ampulla length. Diverticula straight, longer than ampulla, directly attached to the base of ampulla duct; seminal chamber about diverticula length. Spermathecal ducts without nephridia. No accessory glands.

Holandric. Testis sacs well developed, connected in x and xi. Seminal vesicles developed in xi and xii. Ovaries in 12/13, ovisacs invisible. Prostate glands deeply lobuled, paired in xvii–xx; ducts slender and coiled. Accessory glands large, connected to genital markings.

Variations. Specimens collected from Binh Phuoc Province have spermathecal pores located dorsally and 5–8 pairs of genital markings, whereas specimens collected from Binh Duong Province have spermathecal pores located middorsally and only 4 pairs of genital markings.

DNA barcode. A partial sequence of the mitochondrial gene cytochrome oxidase subunit I (COI) was uploaded to GenBank with the accession number MN514952.

Etymology. The new species, *Metaphire songbeensis*, is named after the old name, Song Be province, which previously consisted of both Binh Phuoc and Binh Duong provinces.

Remarks. *Metaphire songbeensis*, new species, is similar to several species of the *Metaphire malayana* group, such as *M. strellana* (Gates, 1949), *M. fovella* (Gates, 1949), *M. malayana* (Beddard, 1900), *M. malayanoides* Nguyen & Lam, 2017, by having four spermathecal pores in 5/6/7/8/9, presence of genital markings in xix and subsequent segments, being holandric, and by having simple caeca. However, the new species is distinguished from these species by the absence of genital markings in spermathecal region, the presence of genital markings in xvii, spermathecal pores located dorsally, and thick septum 8/9, whereas the above species have genital markings in spermathecal region and in xvii (instead of xviii), spermathecal pores located ventrally or lateroventrally, and the absence of septum 8/9.

The new species is also similar to *M. posthuma* (Vaillant, 1868) by having genital markings in xvii and xix, and thick septum 8/9. However, it differs from *M. posthuma* in having spermathecal pores located dorsally (vs. ventrally), first dorsal pore in 9/10 (vs. 12/13), epilobous prostomium (vs. prolobous prostomium), connected testis sacs (vs. separated testis sacs), and the absence of lymph glands (vs. lymph glands starting in 27/28) as well as being more segmented (206–236 vs. 98–119 segments) and slightly larger (91–133 mm vs. 67–101 mm in length; 3.4–6.4 mm vs. 4.0–4.8 mm in diameter).

Several species recorded in Vietnam also have spermathecal pores on dorsal side, such as *M. truongsonensis* (Thai, 1984), *M. amplectens* (Michaelsen, 1934), *M. scitula* (Gates, 1936), and *M. dorsobitheca* (Thai & Huynh, 1992). *Metaphire songbeensis*, new species, is distinguished from *M. truongsonensis* by having 4–8 pairs of genital markings in xvii, xix, and subsequent segments, the first dorsal pore in 9/10 (vs. absence of genital markings in the male region, first dorsal pore in 12/13). The new species is also different from *M. amplectens*, *M. scitula*, and *M. dorsobitheca* in having four pairs of spermathecal pores in 5/6/7/8/9 (vs. three pairs in 6/7/8/9), the presence of genital markings in the male region (vs. absence of genital markings), first dorsal pore in 9/10 (vs. in 12/13). The new species is similar in size to *M. scitula* (91–133 mm vs. 106–120 mm in length; 3.4–6.4 mm vs. 5 mm in diameter), but larger and more segmented than *M. amplectens* and *M. truongsonensis* (91–133 mm vs. 44–52 mm and 54 mm in length; 3.4–6.4 mm vs. 2–3 mm and 2 mm in diameter; 206–236 vs. 90–112 and 76 segments, respectively).

Metaphire setosa, new species

(Figs. 1, 6)

Material examined. Holotype: 1 mature (CTU-EW.179.h01), bushes (11°34′2.2″N, 106°35′46.9″E), 84.9 m, Tan Khai commune, Hon Quan District, Binh Phuoc Province, 26 October 2017, coll. Luong Thi Huynh Tien. Paratypes: 6 matures (CTU-EW.179.p02), same data as holotype.

Diagnosis. Small-sized worm, length 57.0–73.5 mm, average diameter 1.6–3.2 mm. Prostomium not developed. First dorsal pore in 13/14. Three pairs of spermathecal pores in ventral 6/7/8/9. Setae unusual, arranged as two setal rings. Clitellum saddle-shaped, xiv–xvi. Male pores deeply located inside copulatory pouches in the setal ring xix. Five pairs of genital markings in xvi–xviii and xxi–xxii. Holandric. Intestinal caeca simple. Septum 8/9 thick, 9/10 absent.

Description. Body cylindrical, small size, length 57.0–73.5 mm, average diameter 1.6–3.2 mm, weight 0.5–1.6 g, segments 94–121. Body pale. Prostomium not developed. First dorsal pore in 13/14. Setae unusual, arranged as two setal rings, more obvious on ventral side; pre-clitellar setae stouter and denser than post-clitellar ones; setal number 119–135 in viii, 27–42 in xxx; setal distance aa=ab, zz=zy. Clitellum saddle-shaped, xiv–xvi without setae and dorsal pores. Female pore single, on round disc-shaped pad in midventral xiv.

Three pairs of spermathecal pores in dorsal intersegments 6/7/8/9; two bean-shaped pads surrounding each spermathecal pore. No genital markings in the spermathecal region. Male

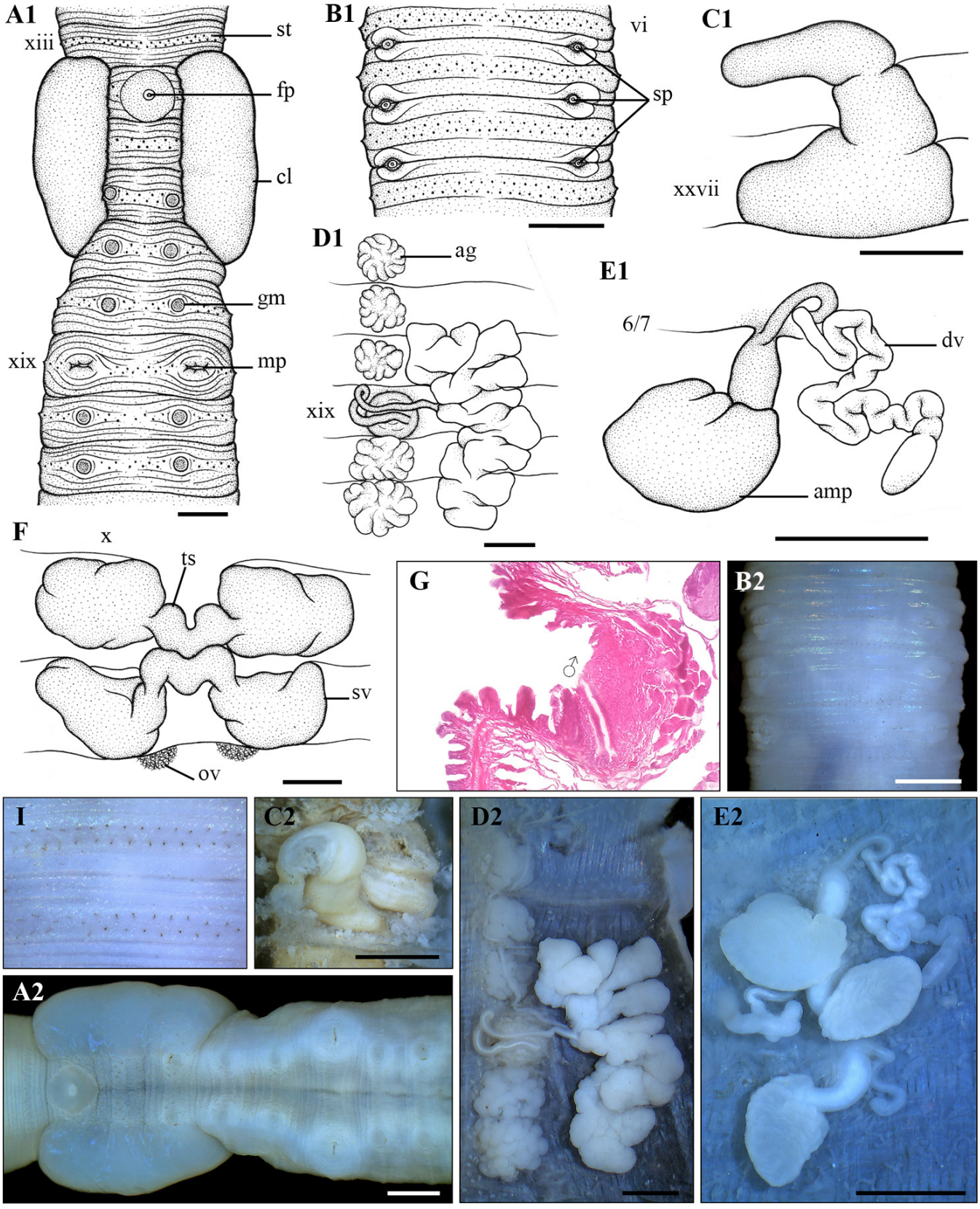

Fig. 6. *Metaphire setosa*, new species, holotype (CTU-EW.179.h01). A1, A2, ventral side of the male region; B1, B2, dorsal side of spermathecal region; C1, C2, intestinal caecum; D1, D2, right prostate gland; E1, E2, left spermathecae; F, testis sacs and seminal vesicles; G, male copulatory pouch, transverse body section; I, ventral side of segments vi–vii. Scale bar = 1 mm.

pores located deeply inside copulatory pouches in the setal ring xix. Ventral distance between two openings of copulatory pouches about 0.35× body circumference. Genital markings paired ventrally in xvi–xviii and xx–xxi, one pair in each segment.

Septa 6/7/8/9 thick, 9/10 absent, 10/11/12/13 thin. Oesophageal gizzard after viii. Intestinal origin at xv; caeca simple, paired in xxvii–xxv. Last hearts in xiii. Pharyngeal micronephridia developed in 5/6/7. Typhlosole simple, lamelliform. Lymph glands absent.

Three pairs of spermathecae in vii–ix. Ampulla large, mushroom-shaped; ducts long, about ½ ampulla length. Diverticula longer than ampulla, folded and directly attached to the base of ampulla duct; seminal chamber long, bullet-shaped. Spermathecal ducts without nephridia. No accessory glands.

Holandric. Testis sacs in x and xi, connected. Seminal vesicles developed in xi and xii. Ovaries in 12/13. Ovisacs invisible. Prostate glands deeply lobuled, paired in xviii–xx; duct long, folded before entering copulatory pouch which is slightly elevated from body wall. Five pairs of accessory glands.

DNA barcode. The amplification of the mitochondrial gene cytochrome oxidase subunit I (COI) failed.

Etymology. The specific epithet "*setosa*" alludes to the unusual setal arrangement on this earthworm. It is used as an adjective.

Remarks. The new species is very unique among known *Metaphire* species, with regard to its saddle-shaped clitellum and setal arrangement. There has been no report on *Metaphire* species with saddle-shaped clitellum. More interestingly, the setal pattern is completely different from all known pheretimoid species in Vietnam. The unusual setal pattern was only seen in the *Amynthas polyperichaeta* (Thai, 1984). The male region was finely coarse with dense setae which was known as the setal zone.

Metaphire houlletoides, new species
(Figs. 1, 7)

Material examined. Holotype: 1 mature (CTU-EW.180.h01), secondary forests (11°28′07.2″N, 107°00′03.7″E), 145.7 m, Tan Hung commune, Dong Phu District, Binh Phuoc Province, 24 October 2017, coll. Luong Thi Huynh Tien. Paratypes: 3 matures (CTU-EW.180.p02), same data as holotype.

Diagnosis. Body cylindrical, small-sized; length 50.2–76.5 mm, average diameter 1.0–2.4 mm. First dorsal pore in 11/12. Prostomium epilobous. Two pairs of spermathecal pores in ventrolateral intersegments 7/8/9. Male pores deeply located inside copulatory pouches in the setal ring xviii. Genital markings absent. Holandric. Intestinal caeca simple.

Description. Body cylindrical, small-sized, length 50.2–76.5 mm, average diameter 1.0–2.4 mm, weight 0.17–0.22 g, segments 68–97. Body white or pale. Prostomium epilobous. First dorsal pore in 11/12. Pre-clitellar setae stouter and sparser than post-clitellar ones; setal number 34–37 in viii, 38–51 in xxx, 3–5 between two openings of copulatory pouches ventrally in xviii; setal distance aa=1.6ab, zz=1.3–2.3zy. Clitellum closed, xiv–xvi with dorsal setae, without dorsal pores. Female single, in midventral xiv.

Two pairs of spermathecal pores in ventrolateral intersegments 7/8/9; ventral distance between two spermathecal pores about 0.35× body circumference. Male pores deeply located inside copulatory pouches. The openings of copulatory pouches oval-shaped in the setal ring xviii; ventral distance between two openings about 0.35× body circumference. Genital markings totally absent.

Septa 5/6/7/8 thick, 8/9/10 absent, 10/11/12/13 thin. Oesophageal gizzard after viii. Intestinal origin at xv; caeca simple, paired in xxvii–xxiii. Last hearts in xiii. Typhlosole simple, lamelliform. Lymph glands from 27/28.

Two pairs of spermathecae in viii and ix. Ampulla large, oval-shaped; duct about ½ ampulla length. Diverticula folded, directly attached to the duct of ampulla. Spermathecal ducts without nephridia. Accessory glands present, small, mushroom-shaped, attached directly to ampulla ducts.

Holandric. Testis sacs developed, but separated in x and xi. Seminal vesicles in xi and xii. Ovaries developed in 13/14, ovisacs invisible. Prostate glands deeply lobuled, paired in xvii–xxi; duct U-shaped, ending at copulatory pouch. Accessory glands present.

DNA barcode. A partial sequence of the mitochondrial gene cytochrome oxidase subunit I (COI) was uploaded to GenBank with the accession number MN514951.

Habitat. The new species was found only in secondary forests on mountain slopes with very thin litter layer.

Etymology. The epithet "*houlletoides*" is used to emphasise its similarity to *Metaphire houlleti* (Perrier, 1872).

Remarks. The new species is particularly similar to *Metaphire houlleti* in the following characters: epilobous prostomium, presence of copulatory pouches, absence of genital markings, but presence of accessory glands in spermathecal region, holandric, simple intestinal caeca. However, the new species differs from *M. houlleti* in having two pairs of spermathecal pores in 7/8/9 (vs. three pairs in 6/7/8/9), smaller size (52–76.5 mm in length, 1–2.4 mm diameter vs. 82–193 mm in length, 3.3–5.4 mm diameter).

Metaphire houlletoides, new species, is assigned to the *Metaphire insulata* group characterised by having two pairs of spermathecal pores in 7/8/9, ducts of accessory glands ending inside copulatory pouches, being holandric, and having simple caeca (Sims & Easton, 1972). This group consists

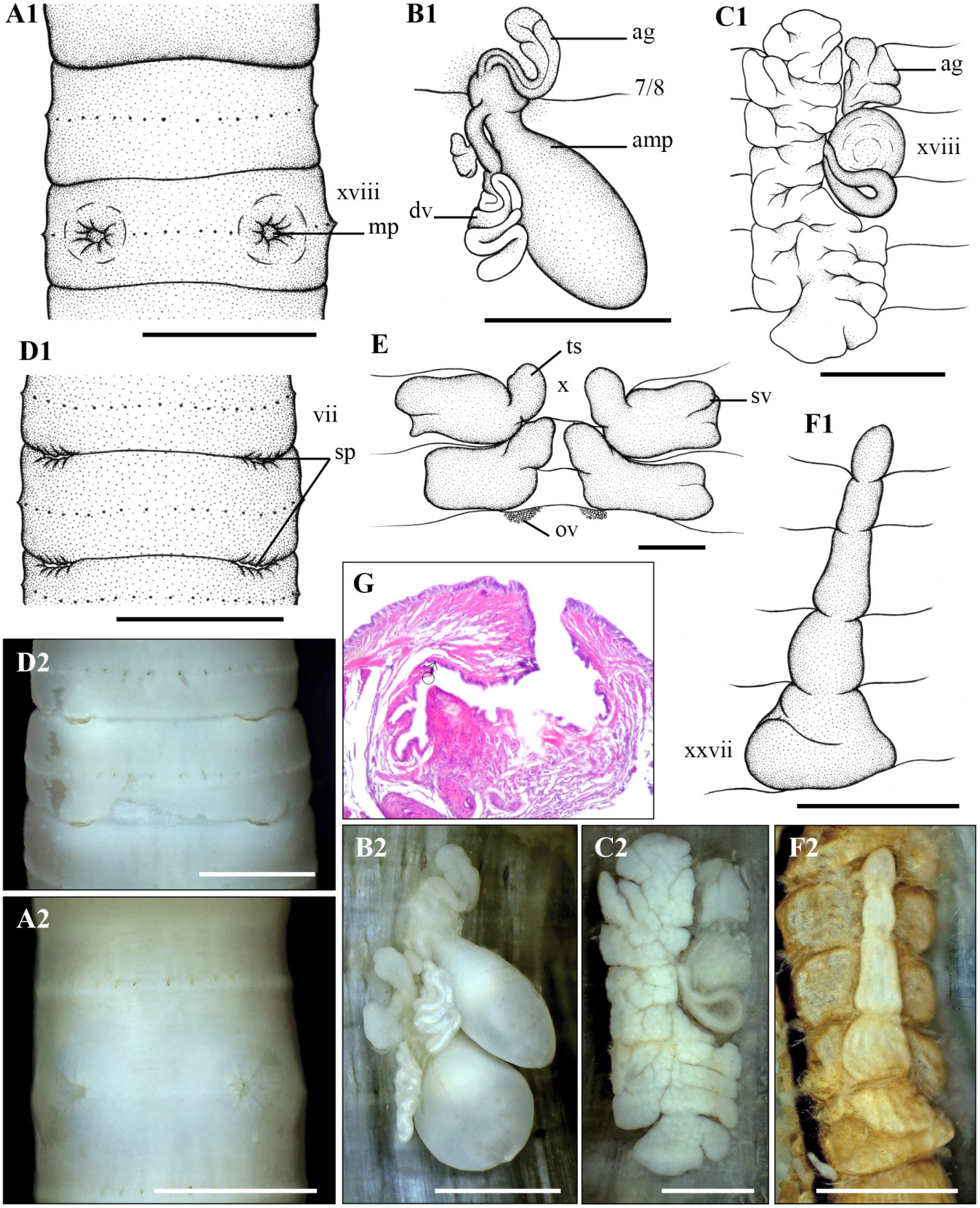

Fig. 7. *Metaphire houlletoides*, new species, holotype. A1, A2, ventral side of the male region; B1, B2, right spermathecae; C1, C2, left prostate gland; D1, D2, ventral side of spermathecal region; E, seminal vesicles; F1, F2, intestinal caecum, right side; G, male copulatory pouch, transverse body section. Scale bar = 1 mm.

Table 2. K2P distance of the COI gene among Metaphire species calculated by MEGA 7.0. *: Sequences were obtained from GenBank.

Species	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
M. bariaensis, new species **(1)**										
M. haui, new species **(2)**	19.4%									
M. songbeensis, new species **(3)**	18%	14.7%								
M. houlletoides, new species **(4)**	19.7%	21.3%	18.3%							
M. houlleti **(5)***	18.5–21%	17.7–19.7%	15.9–19.5%	15.3–18%	0–21.4%					
M. bahli **(6)***	19.9–21.1%	17.7–18.2%	15.2–15.4%	21.1–21.8%	17.9–21%	0.3–1.2%				
M. peguana **(7)***	21.2–21.5%	19–19.2%	20.1–20.3%	22.1–22.4%	21.1–22.6%	16.5–17.2%	0.2%			
M. anomala **(8)***	18.5–19.0%	16.4–17.0%	14.2–14.8%	18.6–18.8%	16.1–20.9%	16.3–17.2%	19–19.4%	0–0.7%		
M. californica **(9)***	19.2%	17.9%	18%	20.4%	18.3–22.4%	21.1–21.3%	21.7–22%	16–16.2%		
M. guillelmi **(10)***	20.8%	22.1%	21.1%	21.1%	18.6–23.3%	20%	19.6–19.9%	19.6–19.9%	21.1%	
Amynthas aspergillus **(11)***	23.2%	20.1%	19.2%	21.9%	19.7–23.8%	20.8%	22.2–22.4%	19.7–20.2%	19.3%	22.1%

of two species, *M. insulata* (Gates, 1930) and *M. leonoris* (Chen, 1946). The new species differs from *M. leonoris* in the position of first dorsal pore (11/12 vs. 12/13), absence of genital markings in spermathecal region (vs. presence of genital markings). It is also distinguished from *M. insulata* by having the first dorsal pore in 11/12 (vs. 12/13) and the absence of genital markings in the spermathecal region (vs. presence of genital markings). *Metaphire houlletoides*, new species, is smaller than *M. insulata* and *M. leonoris* (52–76.5 mm vs. 79 mm and 125 mm in length; 1–2.4 mm vs. 5 mm and 6 mm in diameter, respectively). It is less segmented than *M. leonoris* (68–97 vs. 148 segments), but more segmented than *M. insulata* (68–97 vs. 67 segments).

Genetic distance. The K2P distance among *Metaphire* species is variable and relatively high, ranging from 14.2% to 23.3% (Table 2). The genetic distance between the outgroup (*Amynthas aspergillus*) and *Metaphire* species is also very high, ranging from 19.2% to 23.8%. The mean K2P distance among all examined species is 17.7%. The genetic K2P distance between each new species and other *Metaphire* species varies from 14.7 to 22.4%. It is 18–21.5% (20% in average) for *M. bariaensis*, new species; 14.7–22.1% (18.1% in average) for *M. haui*, new species; 15.3–22.4% (18.8% in average) for *M. houlletoides*, new species; and 14.2–21.1% (17.3% in average) for *M. songbeensis*, new species.

Compared to the COI intergeneric K2P distances calculated by Aspe & James (2018), the distance for *Metaphire* (14.2–22.3%) is somewhat similar to that for *Amynthas* (15.2–22.9%), but slightly lower than that for *Pithemera* (17.1–25.4%) and *Polypheretima* (16.2–23.8%). However, the divergence rate for the genus *Pheretima* (8.9–22.9%) is more widely variable than for the genus *Metaphire* (14.2–22.3%).

Key to the species of *Metaphire* in southeastern Vietnam

1. Male pores in xix 2
– Male pores in xviii 3
2. Spermathecal pores paired lateroventrally in 5/6/7/8/9. Clitellum normal, ring-shaped..... *M. anomala* (Michaelsen, 1907)
– Spermathecal pores paired lateroventrally in 5/6/7/8. Clitellum saddle-shaped (Fig. 6)..... *M. setosa*, new species
3. Genital markings present in the male region 4
– Genital markings absent in the male region..... 12
4. Intestinal caeca manicate ..... *M. pacseana* (Thai & Samphon, 1988)
– Intestinal caeca simple 5
5. Spermathecal pores in 5/6/7/8/9 6
– Spermathecal pores in 6/7/8/9 9
6. Genital markings in intersegment 19/20 and subsequent intersegments. Setal penis present ..... *M. malayanoides* Nguyen & Lam, 2017
– Genital markings present in segments. No setal penis 7
7. Only one pair of genital markings in xviii. Septum 8/9 absent..... *M. grandiverticulata* Nguyen & Lam, 2017
– No genital markings in xviii. Septum 8/9 present 8
8. Spermathecal pores located dorsally. Four to eight pairs of genital markings in xvii, xix, and subsequent segments (Fig. 5)..... *M. songbeensis*, new species

– Spermathecal pores located lateroventrally. Two pairs of genital markings in xvii and xix ..... *M. posthuma* (Vaillant, 1868)
9. Spermathecal pores located dorsally. Genital markings present in xvii and xix (Fig. 2)..... *M. haui*, new species
– Spermathecal pores located not dorsally. Genital markings present in intersegments 17/18 and 18/19.....10
10. Male region strongly concave.....*M. bahli* (Gates, 1945)
– Male region not concave. Genital markings disc-shaped 11
11. The openings of copulatory pouches close to ventromedial line. Septum 10/11 absent ..... *M. saigonensis* (Omodeo, 1957)
– The openings of copulatory pouches not close to ventromedial line. Septum 10/11 present ..... *M. peguana* (Rosa, 1890)
12. Intestinal caeca manicate 13
– Intestinal caeca simple 14
13. Spermathecal pores in 5/6/7/8/9. First dorsal pore in 11/12. Seminal vesicles connected (Fig. 3) ....*M. bariaensis*, new species
– Spermathecal pores in 7/8/9. First dorsal pore in 10/11. Seminal vesicles separated ..... *M. californica* (Kinberg, 1867)
14. Spermathecal pore starting from 6/7 15
– Spermathecal pore starting from 7/8 17
15. Three spermathecal pores in 6/7/8/9..... .....*M. houlleti* (Perrier, 1872)
– Two spermathecal pores in 6/7/8.....16
16. First dorsal pore in 12/13. Accessory glands present, ending at spermathecal ducts ..... *M. guillelmi* (Michaelsen, 1895)
– First dorsal pore in 11/12. Accessory glands absent (Fig. 4).. .....*M. planatoides*, new species
17. Two spermathecal pores in 7/8/9. No setal penis (Fig. 7) ....*M. houlletoides*, new species
– Only one pair of spermathecal pores in 7/8. Setal penis present..... *M. xuanlocensis* Nguyen & Lam, 2017

CONCLUSION

With the discovery and description of six new species (*Metaphire haui*, *M. bariaensis*, *M. planatoides*, *M. songbeensis*, *M. setosa*, and *M. houlletoides*) the number of *Metaphire* species in the southeastern region of Vietnam is increased to 17. The mean K2P distance among *Metaphire* species is 17.7%. The number of *Metaphire* species still does not reflect the true diversity of the genus in Vietnam. More intensive surveys will reveal more new species and more DNA sequences are needed to have better analysis.

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