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A New Earthworm Species of the Genus *Metaphire* Sims & Easton, 1972 (Oligochaeta: Megascolecidae) from Southern Vietnam

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ABSTRACT. – A new species, *Metaphire acampanulata* sp. nov., was described from Southern Vietnam. The species belonging to *Metaphire houlleti* species-group, can be characterized by having three pairs of spermathecal pores in 6/7/8/9, genital markings invisible, first dorsal pore in 11/12 or 12/13, spermatheca mushroom-shaped, ampulla duct L-shaped, and penial setae present. The K2P genetic distance based on a 600 bp fragment of the mitochondrial gene, COI, between the new species and its closely related one, *M. houlleti*, was 18.8%, and the intraspecific distance between its populations was from 0.3 to 9.8%.

KEYWORDS: *Metaphire houlleti*, *Metaphire* cf. *campanulata*, morphological variations, genetic variations, new species, taxonomy, Southern Vietnam

INTRODUCTION

Perrier (1872) described a new species, *Perichaeta houlleti*, from Calcutta (India) with some typical characters including three pairs of spermathecal pores in 6/7/8/9, absence of genital markings in the male region, esophageal gizzard bulb-shaped without esophageal pouch, and stalked accessory gland attached to the base of ampulla ducts. More different types of this species were described in subsequent years, for example Gates (1926) proposed three subspecies, namely *Pheretima houlleti typica* (typical form, small, first dorsal pore in 9/10), *Pheretima houlleti tourtuosa* (penial setae present), and *Pheretima houlleti rugosa* (lack of one or more spermathecae). One year later, Gates (1927) assigned *Pheretima houlleti tourtuosa* to *Pheretima campanulata*, which was described by Rosa (1890). He also described a new subspecies, *Pheretima campanulata penetralis*, which has penial setae trifid (vs spine of *typica*). Then, Gates (1932) named another subspecies *Pheretima campanulata meridiana*, which was close to *penetralis* but without penial setae. Furthermore, Gates (1932) transferred *Pheretima houlleti rugosa* to *Pheretima campanulata rugosa.*

Sims and Easton (1972) created *Metaphire houlleti*-species group characterized by three thecal segments in 6/7/8/9, male pore on xviii, bithecate, and post-cllitelar genital markings absent. The group now contains 40 species and is widespread all over the world (Sims and Easton, 1972; Thai et al., 1992; Thai and Le, 1993; Blakemore, 2002; Bantaowong et al., 2016; Ng et al., 2018). He considered as two different species *Metaphire campanulata* including three subspecies *M. campanulata campanulata*, *M. campanulata meridiana*, and *M. campanulata penetralis*, and *Metaphire houlleti* also with three subspecies *M. houlleti houlleti*, *M. houlleti rugosa*, and *M. houlleti tortuosa* in their revision. However, Gates (1972) considered *M. campanulata* (Rosa, 1890) as a junior synonym of *M. houlleti* (Perrier, 1872), and his opinion was followed by Blakemore (2002, 2006, 2012), Shen and Yeo (2005), Chang et al. (2009), Bantaowong et al. (2011), Nguyen et al. (2016), and Tiwari et al. (2020).

In Vietnam, Thai (1983) firstly identified Vietnamese specimens as *Metaphire campanulata* (Rosa, 1890) based on H morph (penial setae present) in Gates (1972). Later, other Vietnamese researchers also followed Thai (1983) for distinguishing *M. campanulata* (Rosa, 1890) and *M. houlleti* (Perrier, 1872) (Tran, 1985; Do, 1994; Nguyen, 1994; Le, 1995; Pham, 1995; Huynh, 1996, 2005; Thai, 2000; Thai et al., 2007; Nguyen and Tran, 2008; Nguyen and Nguyen, 2010; Nguyen, 2014; Nguyen et al., 2010, 2017, 2019).

Recently, Nguyen et al. (2020) reported an earthworm form, which was similar to *M. campanulata* sensu Gates (1972) and *M. houlleti* (Perrier, 1872), but there were still differences. The form was recognized as *M.* cf. *campanulata*. After that, the intensive studies on earthworms have shown that *M.* cf. *campanulata* is widely found in Southern Vietnam and all samples known as *M. campanulata* collected in the region corresponding to the new form (Nguyen et al., 2021a, 2022; Lam et al., 2021). Unfortunately, the form has not been still exactly identified and properly named.

Therefore, this work is devoted to re-examined specimens of *M.* cf. *campanulata* and describe a new species, *M. acampanulata* sp. nov.

MATERIALS AND METHODS

A total of 75 clitellate individuals of *M.* cf. *campanulata* and 163 mature specimens of *M. houlleti* were collected from Southern Vietnam (Table 1). Earthworms were collected and treated following Nguyen et al. (2022). All specimens were housed in the Laboratory of Zoology, Department of Biology, School of Education, Can Tho University.

The earthworms' morphological characters were externally examined under a Motic microscope (model: DM143) and dissected from the dorsal side for internal observations of which, the data of eight followings measurable traits were collected: length (L), diameter (D), number of segments (S), number of setae between male pores (MS), the setae number ratio of the post-clitellar region to the pre-clitellar region (st), the proportion of the male pores distance to the body circumference (MpD), the proportion of spermathecal pores distance to the body circumference (SpD), and the proportion of twice the typhlosole height to the intestinal circumference (T). All data were compared using one-way ANOVA (parametric), Kruskal-Wallis test, and Mann-Whitney test (nonparametric). Principal Component Analysis was run to identify any morphological differences correlated to collecting sites. All statistical analyses were peformed on MINITAB software version 16.

Total DNA was extracted from muscle tissues of earthworm specimens using the commercial DNeasy Blood & Tissue Kits (Qiagen, Valencia, CA, USA). The primer set LCOI490 and HCO2198 was used to amplify a 680 fragment of the COI (Folmer et al., 1994). The amplification conditions follow Nguyen et al. (2022). Successfully amplified samples were purified and sequenced at the FirstBase Company (Malaysia). Genetic distances were calculated using the K2P (Kimura 2 parameter) model performed in MEGA 7.0 (Kumar et al. 2016). The phylogenetic diagram was constructed using the Maximum Likelihood (ML) method performed in IQTREE ver. 1.6.12 (Nguyen et al., 2015).

RESULTS

SYSTEMATICS

Family Megascolecidae Rosa, 1891

Genus *Metaphire* Sims & Easton, 1972

TABLE 1. Mean±SD and Kruskal-Wallis test of physico-chemical parameters among study sites in Lao PDR.

No.	Label codes	Number of individuals	COI sequence	GPS Coordinates		Location	Time
				Latitude (N)	Longitude (E)		
***Metaphire acampanulata* sp. nov.**							
1.	EW.018.02	6	0	11°19'55.4	107°09'28.3	Vinh Cuu Distr., Dong Nai Prov. (DN)	17/10/2012
2.	EW.018.06	7	0	10°44'24.2	106°58'55.8	Long Thanh Distr., Dong Nai Prov. (DN)	21/10/2014
3.	EW.018.23	7	1	10°43'38.0	106°49'38.0	Nhon Trach Distr., Dong Nai Prov. (DN)	24/10/2016
4.	EW.018.09	7	1	10°40'00.0	107°14'54.0	Chau Duc Distr., Ba Ria Vung Tau Prov. (BRVT)	25/10/2016
5.	EW.018.10	2	1	10°39'42.7	107°09'23.0	Chau Duc Distr., Ba Ria Vung Tau Prov. (BRVT)	25/10/2016
6.	EW.018.13	23	0	11°50'27.3	107°16'42.4	Bu Dang Distr., Binh Phuoc Prov. (BP)	24/10/2017
7.	EW.018.24	7	0	11°03'39.6	106°29'31.6	Cu Chi Distr., Ho Chi Minh City (HCM)	25/09/2019
8.	EW.018.05	10	0	10°30'31.9	105°30'03.1	Gieng Isl., An Giang Prov. (AG)	09/2009
9.	EW.018.41	7	0	10°05'03.7	105°44'55.1	Son Isl., Can Tho City (CT)	09/10/2020
***Metaphire houlleti* (Perrier, 1872)**							
10.	EW.006.02	20	0	09°43'28.0	105°42'41.1	Phung Hiep Distr., Hau Giang Prov. (HG)	09/10/2009
11.	EW.006.03	30	0	10°47'34.9	107°31'32.5	Xuan Loc Distr., Dong Nai Prov. (DN)	10/2012
12.	EW.006.08	30	0	09°58'33.0	104°51'19.3	Hon Tre Isl., Kien Giang Prov. (KG)	16/10/2014
13.	EW.006.29	10	0	10°06'11.1	104°00'51.6	Phu Quoc Isl., Kien Giang Prov. (KG)	05/11/2016
14.	EW.006.11	20	1	10°39'06.0	107°14'27.0	Chau Duc Distr., Ba Ria Vung Tau Prov. (BRVT)	25/10/2016
15.	EW.006.12	10	1	10°39'45.1	107°15'56.9	Chau Duc Distr., Ba Ria Vung Tau Prov. (BRVT)	25/10/2016
16.	EW.006.21	30	2	11°37'28.5	106°39'06.5	Hon Quan Distr., Binh Phuoc Prov. (BP)	26/10/2017
17.	EW.006.48	13	0	11°22'17.3	106°15'17.4	Duong Minh Chau Distr., Tay Ninh Prov. (TN)	25/09/2019

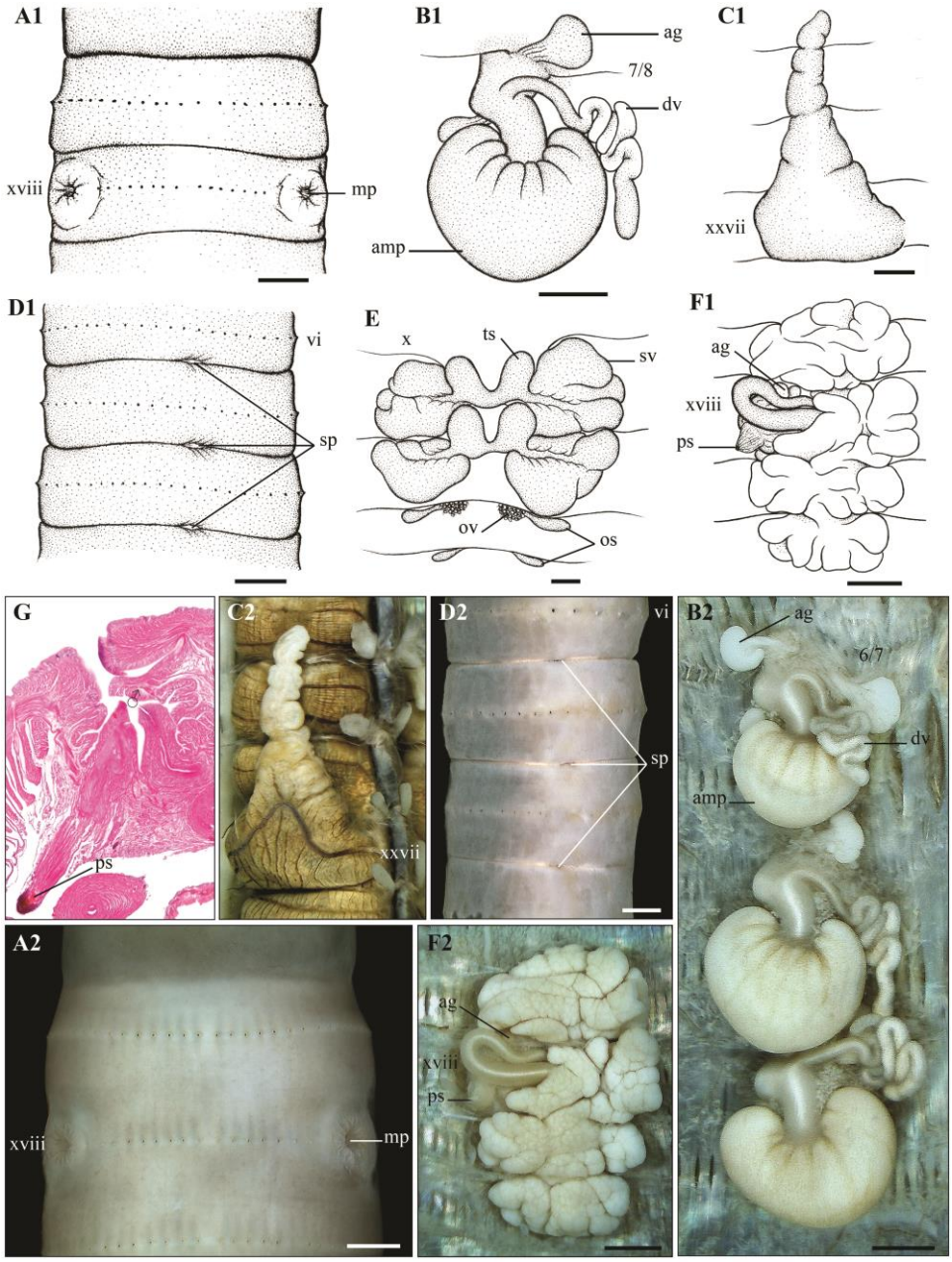

FIGURE 1. *Metaphire acampanulata* sp. nov., **holotype** (**CTU-EW.018.h01**). **A1** and **A2.** Ventral view of male region. **B1** and **B2.** Left spermathecae. **C1** and **C2.** Intestinal caecum. **D1** and **D2.** Lateral view of spermathecal region. **E.** Dorsal view of male organs. **F1** and **F2.** Right prostate gland. **G.** Male pore region transverse body section (**CTU-EW.018.p02**). Abbreviations: **amp**, ampulla; **ag**, accessory gland; **dv**, diverticulum; **mp**, male pore; **os**, ovisacs; **ov**, ovary; **ps**, penial seta; **sp**, spermathecal pore; **sv**, seminal vesicle; **ts**, testis sac. Scale bar = 1mm.

***Metaphire acampanulata* sp. nov.**
(Fig. 1)

Pheretima sp.8 – Nguyen 2013: 72, 2014: 111.

Pheretima campanulata – Nguyen and Tran 2008: 184; Nguyen and Nguyen 2010: 123; Nguyen and Huynh 2011: 1018; Nguyen et al. 2011: 1025, 2012: 146.

Metaphire campanulata – Nguyen et al. 2017: 98; Nguyen et al. 2019: 120.

Metaphire cf. *campanulata* – Nguyen N.Q. et al. 2020: 15; Lam et al. 2021: 17699; Nguyen et al. 2021a: 105, 2022: 105.

Metaphire houlleti (part) – Nguyen T.T. et al. 2016: 58, 2020: 8.

Etymology: An epithet '*acampanulata'* is formed by the prefix 'a' and '*campanulata'* to emphasize the wrong name '*campanulata'* recorded in Vietnam.

Vietnamese name: giun khác campanulata

Materials examined: *Holotype*: 1 clitellate (CTU-EW.018.h01), banana garden (10°40'00.0N; 107°14'54.0E), 135 m amsl, Bau Chinh commune, Chau Duc District, Ba Ria – Vung Tau Province, 25/10/2016, coll. Nguyen Quoc Nam.

Paratypes: 8 clitellates (CTU-EW.018.p02) and 1 clitellate (CTU-EW.018.DNA.p02) same data as for holotype.

Other materials: 6 clitellates (CTU-EW.018.02), rubber trees plantation (11°19'55.4N; 107°09'28.3E), 69 m amsl, Phu Ly commune, Vinh Cuu District, **Dong Nai Province**, 17/10/2012, coll. Duong Chi Trong; 7 clitellates (CTU-EW.018.06), shrubs and bushes (10°44'24.2N; 106°58'55.8E), 58 m amsl, Long Phuoc commune, Long Thanh District, **Dong Nai Province**, 21/10/2014, coll. Le Van Nhan; 7 clitellates (CTU-EW.018.23) and 1 clitellate (CTU-EW.DNA.018.23), shrubs and bushes (10°43'38.0N; 106°49'38.0E), 35 m amsl, Dai Phuoc commune, Nhon Trach District, **Dong Nai Province**, 24/10/2016, coll. Nguyen Quoc Nam; 23 clitellates (CTU-EW.018.13) and 5 clitellates (CTU-EW.DNA.018.13), coffee trees plantation (11°50'27.3N; 107°16'42.4), 340 m amsl, Doan Ket commune, Bu Dang District, **Binh Phuoc Province**, 24/10/2017, coll. Nguyen Quoc Nam; 2 clitellates (CTU-EW.018.10) and 1 clitellate (CTU-EW.DNA.018.10), cashew trees plantation (10°39'42.7N; 107°09'23.0E), 107 m amsl, Lang Lon commune, Chau Duc District, **Ba Ria – Vung Tau Province**, 25/10/2016, coll. Nguyen Quoc Nam; 7 clitellates (CTU-EW.018.24) and 2 clitellates (CTU-EW.DNA.018.24), rubber trees plantation (11°03'39.6N; 106°29'31.6E), 3 m amsl, Nhuan Duc commune, Cu Chi District, **Ho Chi Minh City**, 25/09/2019, coll. Nguyen Quoc Nam; 7 clitellates (CTU-EW.018.05), mango garden (10°30'31.9N; 105°30'03.1E), 4 m amsl, Gieng islet, Cho Moi District, **An Giang Province**; 09/2009, coll. Ho Minh Thuan; 2 clitellates (CTU-EW.DNA.018.31), *Bfigaccaurea racemosa* garden (10°30'11.2N; 104°59'08.6E), 520 m amsl, Cam Mountain, Tinh Bien District, **An Giang Province**, 07/10/2019, coll. Nguyen Thanh Tung; 7 clitellates (CTU-EW.018.41) and 2 clitellates (CTU-EW.DNA.018.41), roadside bamboo bush (10°05'03.7N; 105°44'55.12E), 2 m amsl, Son islet, Binh Thuy District, **Can Tho City**, 09/10/2020, coll. Lam Hai Dang.

Diagnosis: Medium size, length 77–198 mm, diameter 4.03–6.91 mm, number of segments 56–144. Prostomium epilobic. First dorsal pore in 11/12 or 12/13. Three pairs of spermathecal pores in 6/7/8/9, ventrolaterally or laterally. Male pore in xviii, copulatory pouches present; ventral distance between openings of the pouches about 0.21–0.37 circumference. Genital markings inconspicuous. Intestinal caeca simple. Septa 8/9/10 absent. Spermathecal ampulla mushroom shaped with longitudinal grooves on the surface, duct L-shaped. More than two stalk accessory glands attach anteriorly and posteriorly to the base of the ampulla duct. Penial setae present. Holandric. Testis sacs connected. Ovisacs paired in 12/13/14.

Description:

External characters. Cylindrical body, medium size, length 77–198 mm, diameter 4.03–6.91 mm, segments 56–144 (holotype: length 172 mm, diameter 5.27 mm, segments 92). Dorsum greyish brown, ventrum paler, gradient from dorsum to ventrum; pre-clitellar segments darker than the post; clitellum light grey; iridescent, no pigmentation. Transverse grooves present, one in both anterior and posterior of the setal ring; no longitudinal grooves. Prostomium epilobic (1/2 or 2/3). First dorsal pore in 11/12 or 12/13. Pre-clitellate setae stouter and sparser than post-clitellar ones; setae more crowded ventrally; setae number: 32–57/viii, 38–70/xxx, 8–25 between male pores ventrally; setae distance: aa > ab, zz > zy; 1–3 penial setae pinned to the ventral side of the roof of each of the copulatory pouches. Clitellum annular (xiv-xvi), without seta and dorsal pore. Female pore single at mid-ventral xiv.

Three pairs of spermathecal pores located ventrolaterally or laterally in intersegments 6/7/8/9; ventral distance between spermathecal pores about 0.36–0.50 body circumference. Male pores inside copulatory pouches in xviii, and the openings slit or round shaped; ventral distance between these two openings about 0.21–0.37 body circumference. Genital markings are inconspicuous in both spermathecal pore and male pore regions.

Internal characters. Septum 5/6/7/8/9 and 10/11/12/13 thick, 8/9/10 absent. Esophageal gizzard between 7/8 and 10/11. Micronephridia meroic, well developed in 4/5/6/7. Last heart in xiii. Calciferous gland directly opens into esophageal cavities in xi-xiii. Intestine swelling at xv; caeca simple, originating from xxvii and anteriorly extending to xxiv, rarely longer to xxii or shorter to xxv. Lymph glands lobuled, present from 16/17 onward. Typhlosole lamelliform, T=0.04-0.30. Longitudinal muscle layer fasciculate.

Three pairs of spermathecae in vii-ix. Ampulla mushroom-shaped with longitudinal grooves on the surface; ampulla ducts short, L-shaped, without nephridia, about 1/3-1/4 of ampulla length, enlarged to the base; diverticulum curved basally and attached to the middle of ampulla duct, seminal chamber sinusoidal shaped. Two to four stalked accessory glands, attached anterior and posterior to the base of each ampulla duct.

Holandric. Testis sacs in x and xi, subesophageal, connected ventrally. Seminal vesicles paired in xi and xii. Ovaries paired, attached posterior face of 12/13. Ovisacs paired in 12/13 or 12/13/14. Prostate glands racemose, deeply lobuled, one pair within xvii-xx; prostatic duct hairclip shaped. Three to four accessory

glands pour in anterior and posterior wall of each copulatory pouch in segment xviii.

Distribution: Binh Duong (Tan Uyen, Bau Bang, Dau Tieng, Phu Giao) (Nguyen et al., 2015), Ho Chi Minh City (Cu Chi), Binh Phuoc (Dong Xoai, Dong Phu, Bu Dang, Phuoc Long, Bu Gia Map NP., Phu Rieng, Bu Dop, Loc Ninh, Hon Quan), Tay Ninh (Trang Bang, Duong Minh Chau, Tan Chau, Ba Den Mt.), Dong Nai (Vinh Cuu, Xuan Loc, Tan Phu, Dinh Quan, Long Khanh, Trang Bom, Thong Nhat, Nhon Trach, Long Thanh, Cam My), Ba Ria – Vung Tau (Chau Duc, Xuyen Moc, Dat Do) (Lam et al., 2021). Kien Giang (Phu Quoc Isl., Hon Dat Mt., Chua Hang Mt., Ba Trai Mt., Da Dung Mt., Thach Dong Mt., Ta Bang Mt. and To Chau Mt.), An Giang (Sam Mt., Phu Cuong Mt., Ba Doi Mt., Cam Mt., Co To Mt. and Cho Moi), Hau Giang (Phung Hiep), Vinh Long (Long Ho, Vung Liem), Tien Giang (Cai Be, Cai Lay, Chau Thanh), Dong Thap (Tan Long Islet, Cao Lanh, Lap Vo), Can Tho (commonly found), Ben Tre (Binh Dai), Tra Vinh (Duyen Hai), Soc Trang (Tran De, Long Phu), Bac Lieu (Hoa Binh) (Nguyen, 2014; Nguyen et al., 2021a, 2022).

Habitats: The new species was found from all terrains of mountainous to the coastal areas. They live under leaf litter or deep into the soil up to 10 cm.

Morphological variations: The body length of *Metaphire acampanulata* sp. nov. was normally distributed while other traits were not. Morphological comparisons (One-way ANOVA and Kruskal-Wallis test) of 76 individuals of *Metaphire acampanulata* sp. nov. from six populations showed that worms in Ba Ria – Vung Tau has the significantly the longest length (155.10 ± 32.30 mm) while the shortest were from An Giang (114.40 ± 9.48 mm) (One-way ANOVA, P = 0.004); individuals from Binh Phuoc has significantly the widest male pores distance (0.32 ± 0.02 body circumference) while the individuals with the closest male pores were from Dong Nai (0.28 ± 0.03 body circumference); samples from An Giang have significantly most number of setae between male pores (18.40 ± 2.84 setae), the widest distance between spermathecal pores (0.49 ± 0.03 body circumference), and the largest T proportion (0.22 ± 0.04) compared to the least number of setae between male pores were from Ho Chi Minh (13.29 ± 3.55 setae), the nearest distance between spermathecal pores were from Can Tho (0.43 ± 0.01 body circumference), and the smallest T proportion were from Binh Phuoc (0.13 ± 0.04) (Kruskal-Wallis test, P value from 0.000 to 0.004). There was no significant difference between populations in terms of the diameter (Kruskal-Wallis test, P = 0.875), the number of segments (Kruskal-Wallis test, P = 0.334), and setae ratio (Kruskal-Wallis test, P = 0.308) (Table 2).

The PCA analysis showed that the first component (PC1) has a variance (eigenvalue) of 2.1189, and accounts for 26.5% of the total variance. The first two and three components represent 46.9% and 60.8%. The morphometric data of populations not grouped in biplot supported no significant morphological differences of earthworms among locations (Fig. 2). The PC1 was shown by the following formula:

PC1 = 0.408 MS + 0.393 SpD + 0.334 T + 0.151 D + 0.061 st - 0.180 MpD – 0.478 L – 0.530 S

In addition, *Metaphire acampanulata* sp. nov. also has variation in the position of first dorsal pore when most specimens in 11/12 but specimens from An Giang 12/13. The spermathecae are sometimes poorly developed in vii (47 specimens), viii (5 specimens), or vii and viii (4 specimens) (Fig. 3).

TABLE 2. Mean±SD, Oneway ANOVA, and Kruskal-Wallis test of morphologies among *Metaphire acampanulata* sp. nov. in Southern Vietnam.

No.	Morphological traits	Dong Nai (20)	Binh Phuoc (23)	Ba Ria-Vung Tau (9)	Ho Chi Minh (7)	Can Tho (7)	An Giang (10)
1.	Length (mm)	132.25 ± 24.22^{ab}	146.17 ± 17.60^{a}	155.10 ± 32.30^{a}	131.0 ± 31.60^{ab}	116.40 ± 28.90^{b}	114.40 ± 9.48^{b}
2.	Diameter (mm)	5.00 ± 0.50	5.16 ± 0.68	5.18 ± 0.45	5.30 ± 0.90	5.06 ± 0.36	5.07 ± 0.59
3.	Number of segments	96.30 ± 20.10	104.91 ± 15.52	99.78 ± 21.05	97.57 ± 19.84	98.86 ± 19.63	89.80 ± 19.17
4.	Male setae	13.50 ± 2.98^{b}	14.13 ± 3.38^{b}	14.78 ± 2.22^{ab}	13.29 ± 3.55^{b}	13.71 ± 1.60^{b}	18.40 ± 2.84^{a}
5.	**st** ratio	1.37 ± 0.18	1.34 ± 0.12	1.45 ± 0.20	1.36 ± 0.21	1.31 ± 0.10	1.27 ± 0.15
6.	Spermathecal pores distance	0.44 ± 0.05^{cd}	0.44 ± 0.03^{bcd}	0.45 ± 0.04^{abcd}	0.49 ± 0.02^{abc}	0.43 ± 0.01^{d}	0.49 ± 0.03^{a}
7.	Male pores distance	0.28 ± 0.03^{c}	0.32 ± 0.02^{a}	0.31 ± 0.02^{ab}	0.30 ± 0.02^{abc}	0.31 ± 0.04^{ab}	0.28 ± 0.02^{bc}
8.	**T** ratio	0.20 ± 0.04^{a}	0.13 ± 0.04^{c}	0.14 ± 0.02^{bc}	0.20 ± 0.02^{a}	0.19 ± 0.03^{ab}	0.22 ± 0.04^{a}

(*): Numbers in parenthesis are the number of individuals surveyed

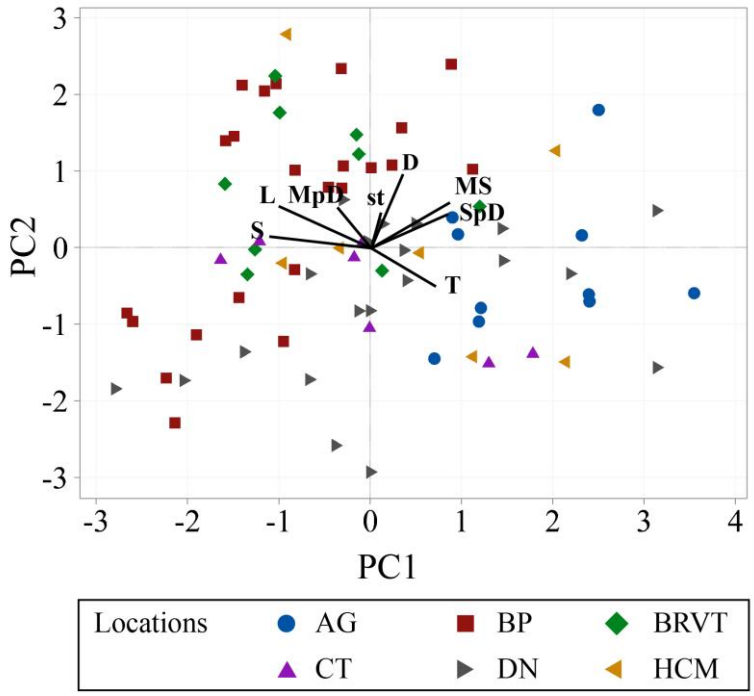

FIGURE 2. PCA analysis of the correlation between morphometric data of the sampling site of *Metaphire acampanulata* sp. nov. Traits codes as given in material and method. Abbreviations: **L**, length; **D**, diameter; **S**, number of segments; **MS**, number of setae between male pores; **MpD**, ventral distance of male pores/body circumference; **SpD**, ventral distance of spermathecal pores/body circumference; **st,** setae ratio between post-clitellar and pre-clitellar segments; **T**, ratio of twice the typhlosole height to intestinal circumference.

FIGURE 3. Variation in the spermatheca of *Metaphire acampanulata* sp. nov. **A.** Fully developed. **B.** Poorly develop in vii. **C.** Poorly developed in viii. **D.** Poorly developed in vii and viii. Scale bar = 1mm.

DISCUSSION

The new species belongs to the *Metaphire houlleti* species-group (Sims and Easton, 1972) that now includes 40 species, of which, nine were found in Vietnam are *M. amplectens* (Michaelsen, 1934), *M. catbaensis* (Thai & Le, 1993), *M. dawydovi* (Michaelsen, 1934), *M. dorsobitheca* (Thai & Huynh, 1992), *M. honbaensis* (Gates, 1941), *M. houlleti* (Perrier, 1872), *M. scitula* (Gates, 1936) (Nguyen et al., 2016), and *Metaphire acampanulata* sp. nov. *Metaphire houlelti*-species group containing species that hard to be distinguished from each other, however, there were still differences between *M. acampanulata* sp. nov. and other members of the group. It is clearly seen that *M. acampanulata* sp. nov. charaterized by very unique traits such as ampulla mushroom-shaped, spermatheca duct L-shaped, and penial seta present

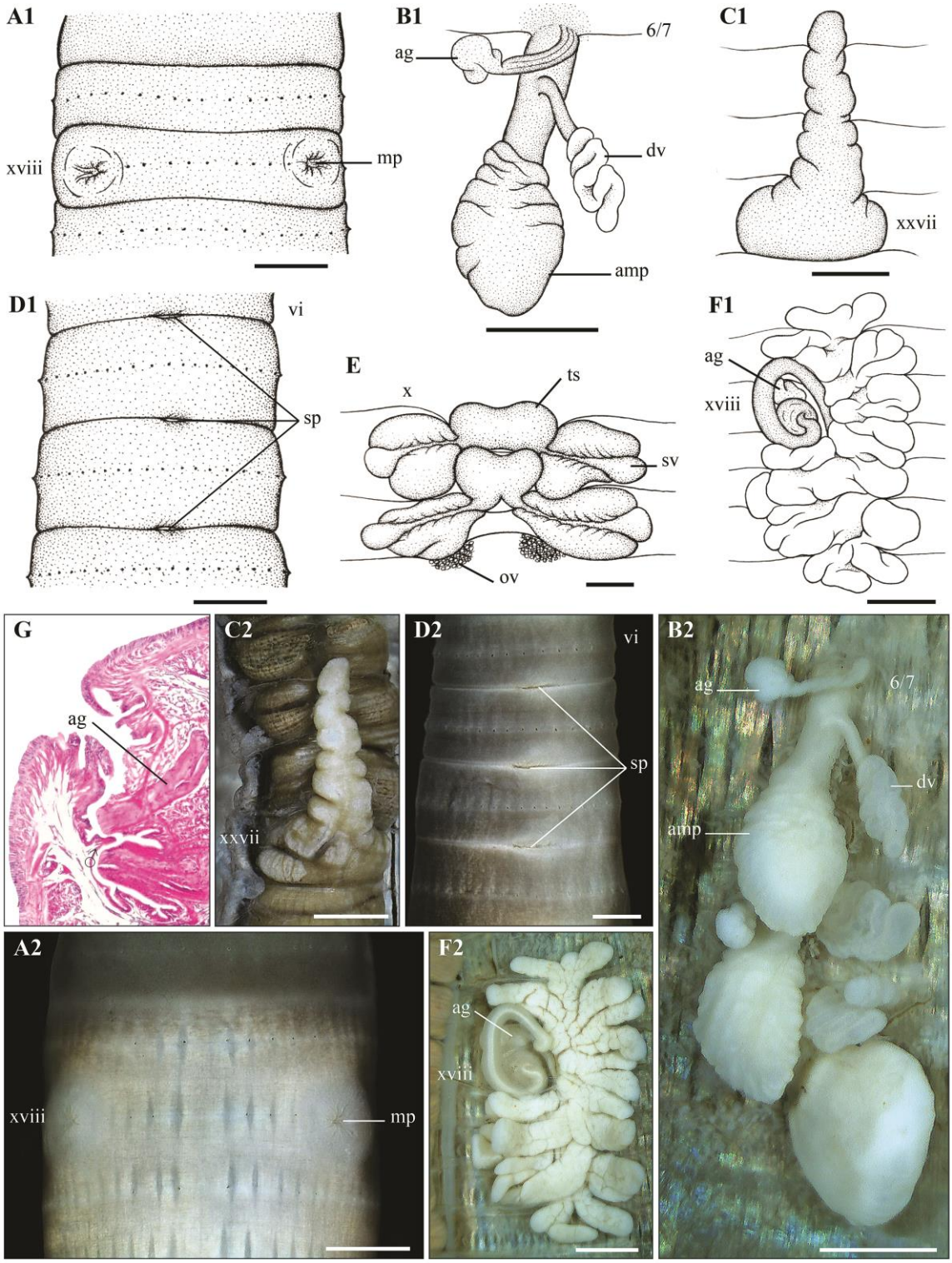

FIGURE 4. *Metaphire houlleti* (Perrier, 1872), **CTU-EW.006.11. A1** and **A2.** Ventral view of male region. **B1** and **B2.** Left spermathecae. **C1** and **C2.** Intestinal caecum. **D1** and **D2.** Lateral view of spermathecal region. **E.** Dorsal view of male organs. **F1** and **F2.** Right prostate gland. **G.** Male pore region transverse body section. Abbreviations: **amp**, ampulla; **ag**, accessory gland; **dv**, diverticulum; **mp**, male pore; **os**, ovisacs; **sp**, spermathecal pore; **sv**, seminal vesicle; **ts**, testis sac. Scale bar = 1mm.

while other *houlleti's* species having ampulla not in mushroom-shaped, spermatheca duct I-shaped (except *M. ukedemi* (Michaelsen, 1892)), and penial seta absent (except *M. houlleti* H morph).

Morphologically, *M. acampanulata* sp. nov. is closed similar to *M. houlleti* (Fig. 4) by having spermathecal pores paired in 6/7/8/9, prostomium epilobic, genital markings invisible outside,

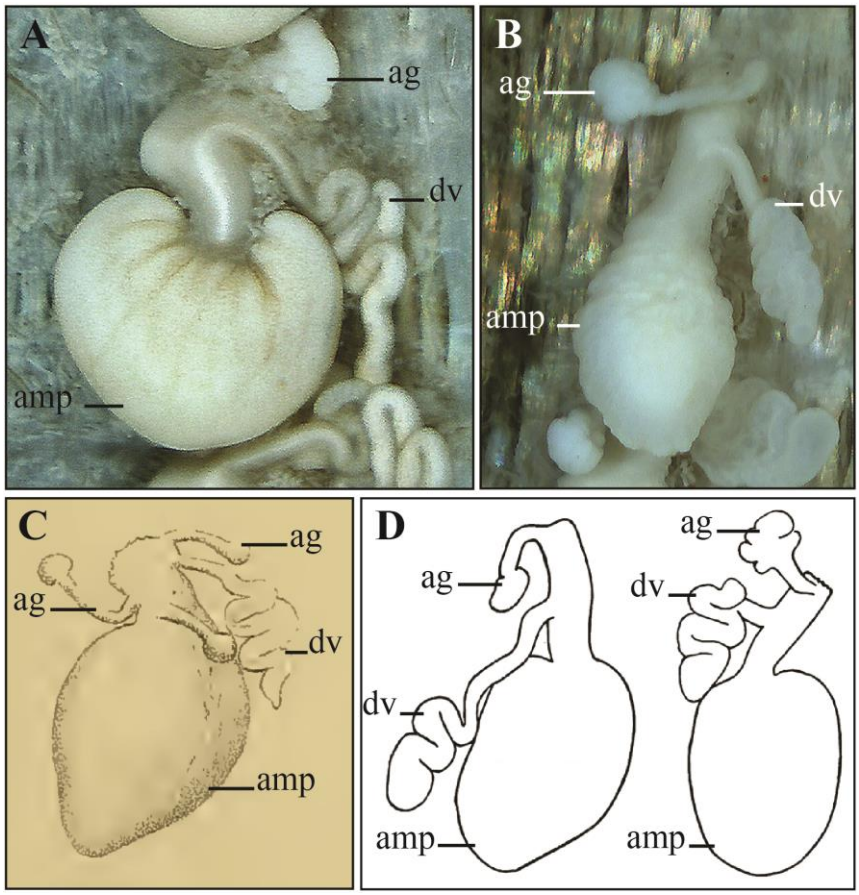

FIGURE 5. Spermathecae of *M. acampanulata* sp. nov. (**A**). *Pheretima houlleti* (**B**) from Southern Vietnam. *Perichaeta campanulata* Rosa, 1890 (**C**). and *Pheretima campanulata penetralis* Gates, 1931 (**D**). Abbreviations: **amp**, ampulla; **ag**, accessory gland; **dv**, diverticulum.

diverticulum attached to the middle of the spermathecal duct, accessory glands stalked in both spermathecal and male pores regions, septa 8/9/10 absent, testis sacs connected ventrally, intestine swelling at xv, intestinal caeca simple and typhlosole lamelliform. However, *M. acampanulata* differs from *M. houlleti* by the location of first dorsal pore, the distance of spermathecal pores, the presence of penial setae, the shape of the spermathecae (Fig. 5), the position of diverticulum on ampulla duct, the number of accessory glands of each spermatheca, the presence of ovisacs, the shape of prostatic duct, and the differences on diameter. The differences between two species are detailed in Table 3.

Morphometric comparison of 239 individuals by Mann-Whittney test showed that *M. acampanulata* have significant differences from *M. houlleti* in six of eight examined traits such as longer length (135.25 ± 26.23mm vs. 78.71 ± 15.75 mm, P = 0.000), greater diameter (5.11 ± 0.59 mm vs. 3.68 ± 1.06 mm, P = 0.000), more segments (98.82 ± 18.73 vs. 92.72 ± 14.35 segments, P = 0.002), more seta between male pores (14.49 ± 3.29 setae vs 5.22 ± 2.94 setae, P = 0.000), larger st ratio (1.35 ± 0.16 vs. 1.29 ± 0.13, P = 0.003), and smaller spermathecal distance (0.45 ± 0.04 vs. 0.5, P = 0.000). However, there were no significant differences in the distance of male pores (0.30 ± 0.03 vs. 0.30 ± 0.02 body circumference, P = 0.521) and the T proportion (0.17 ± 0.05 vs. 0.18 ± 0.04 intestinal circumference, P = 0.842). The PCA analysis showed that the first component (PC1) has a variance (eigenvalue) of 3.1190, and accounts for 39.0% of the total variance. PC1 well divided the morphometric data of *M. acampanulata* and *M. houlleti* and represented the main differences between two species are diameter, length, male setae, number of segments, and distance of spermathecal pores (Fig. 6). The coefficients listed under PC1 show the scores of each variables tested:

PC1 = 0.516 D + 0.512 L + 0.454 MS + 0.221 S + 0.162 st + 0.078 MpD – 0.082 T – 0.422 SpD

Genetical analysis

Genetically, genetic variations were quite large between two taxa. *Metaphire acampanulata* sp. nov. had intraspecific K2P distance varied from 0.3% (EW.018.09 and EW.018.10) to 9.8% (EW.018.09 and EW.018.23), but the maximum distance is less than

TABLE 3. Morphological comparisons between *M. acampanulata* sp. nov. and *M. houlleti* recorded in Southern Vietnam.

No.	Morphological traits	*M. acampanulata* (Figure 1) (76)	*M. houlleti* (Figure 4) (163)
1.	Length (mm)	77-198	48-115
2.	Diameter ca. (mm)	4.03-6.91	2.42-3.89
3.	Number of segments	56-144	53-123
4.	Seta between male pores	8-25	2-15
5.	First dorsal pore	11/12 (63C) or 12/13 (15C)	9/10 (76C) or 10/11 (87C)
6.	Penial seta	3-6	Absent
7.	Distance between spermathecal pores	0.36-0.50X body circumference	0.50X body circumference
8.	Spermathecal ampulla	Mushroom shaped with longitudinal wrinkles	Ovoid with latitudinal wrinkles
9.	Ampulla duct	L shaped, about 1/3 ampulla length, enlarged basally	Straight, about 1/2 ampulla length, cylindrical
10.	Position of diverticulum on ampulla duct	Attached onto ampulla duct at the middle	Attached onto ampulla duct near the base
11.	Prostatic duct	Hairclip shaped	C shaped
12.	Ovisac	1-2 pairs, 12/13 (66), 12/13/14 (11)	absent
13.	Spermathecal accessory glands	1-4, anterior and posterior of ampulla ducts	Only one, anterior of ampulla ducts

(*): Numbers in parenthesis are the number of individuals surveyed

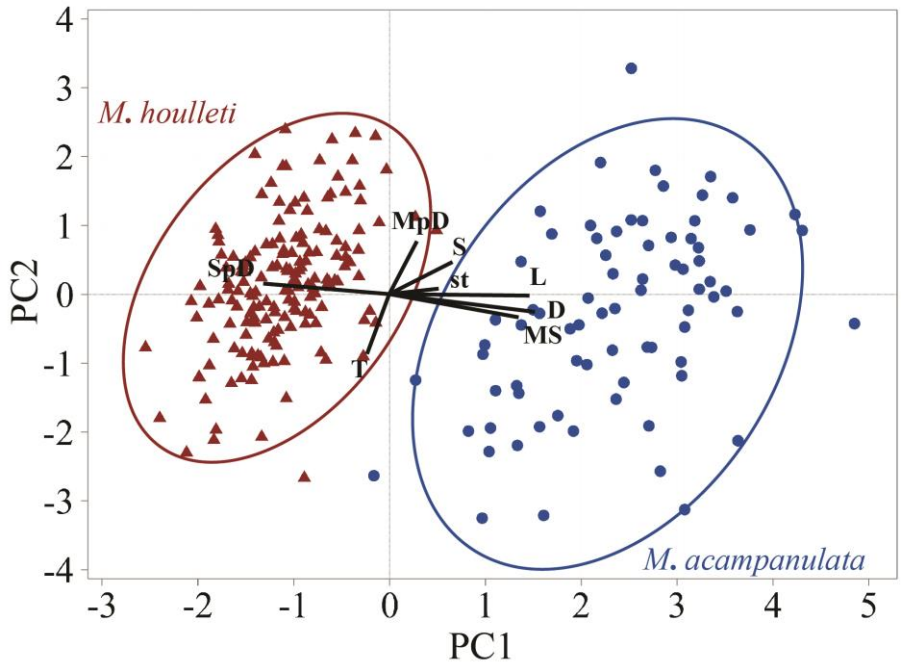

FIGURE 6. PCA analysis of the correlation between morphometric data of *M. acampanulata* sp. nov. and *M. houlleti*. Traits codes as given in the material and method part. Abbreviations: **L**, length; **D**, diameter; **S**, number of segments; **MS**, number of setae between male pores; **MpD**, ventral distance of male pores/body circumference; **SpD**, ventral distance of spermathecal pores/body circumference; **st,** setae ratio between post-clitellar and pre-clitellar segments; **T**, ratio of twice the typhlosole height to intestinal circumference.

species divergence 13% reported by Nguyen et al. (2021b). Besides, *M. houlleti* has no variation recorded. However, two taxa had a crucial difference with the K2P distance of 18.8%, higher than species divergence of 13% in Nguyen et al. (2021b) or 13.8% mentioned in Jeratthitikul et al. (2017). Moreover, the phylogenetic tree showed that the two taxa are separated from each other. *Metaphire acampanulata* is a sister clade of *M. houlleti* with the strong support of a bootstrap value of 84%. Two subclades of *M. acampanulata* are well supported by a bootstrap value of 98% (Fig. 7).

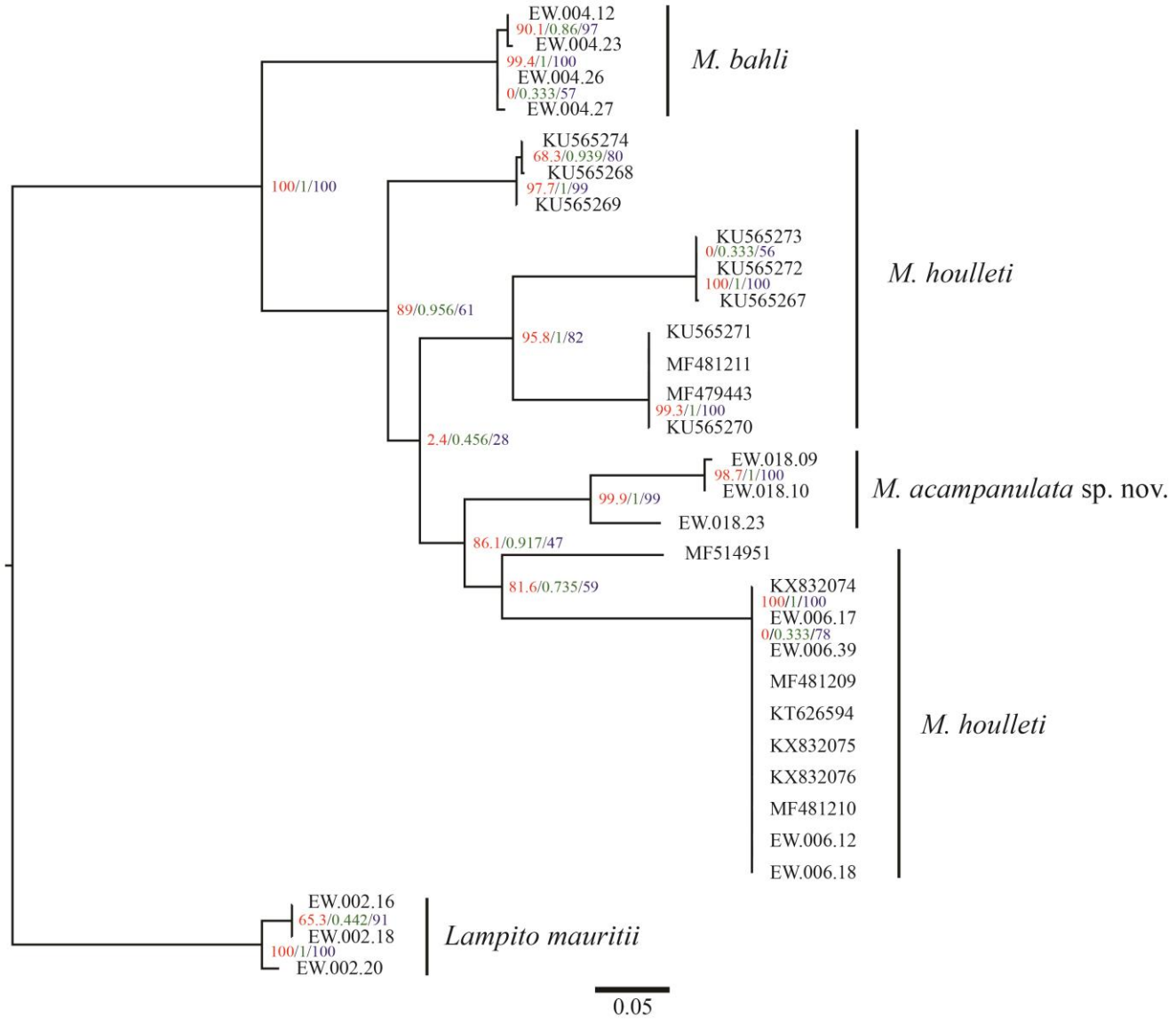

FIGURE 7. The phylogenetic diagram of COI fragments of *M. acampanulata* sp. nov. and *M. houlleti* using using the Maximum Likelihood (ML) and Bayesian Inference (BI) analysis. The values at node show the bootstrap and BI.

The differences between *M. acampanulata*, *Metaphire campanulata* Rosa, 1890, *Metaphire houlleti* sensu Gates (1972) H morph, and other species of the *houlleti* species-group

Metaphire acampanulata is closely similar to *Metaphire campanulata* Rosa, 1890 (described by Rosa (1890) and Gates (1927)) and *Metaphire houlleti* sensu Gates (1972) for H morph (*Metaphire campanulata penetralis* and *Metaphire campanulata typica*) by having three pairs of spermathecal pores in 6/7/8/9, first dorsal pore usually in 11/12, diverticulum attached onto ampulla duct, numerous accessory glands per each of spermatheca, penial setae present, testis sacs connected ventrally, intestine swelling at xv, and caeca simple. However, *Metaphire acampanulata* and above taxa are distinguished from each other by several important morphologies such as the presence of genital markings in spermathecal region, the shape of ampulla and its ducts (Fig. 5), the position of accessory glands on ampulla ducts, and the shape of prostatic ducts. The differences between taxa are shown in Table 4.

Metaphire acampanulata sp. nov. also differs from other species with three pairs of spermathecal pores in 6/7/8/9, male pores, and without male genital markings that belonged to *Metaphire houlleti* species-group followed Sims and Easton (1972) by followings specific traits: penial setae present (vs absent, except *M. dorsobitheca* sensu Nguyen T.T. et al. 2020), accessory glands absent in the spermathecal region (vs present, except *M. hijauensis* Ng & Panha, 2018, *M.*

TABLE 4. Morphological comparisons between *Metaphire acampanulata* sp. nov. from Southern Vietnam with *Metaphire campanulata* Rosa, 1890, and *Metaphire houlleti* sensu Gates (1972) H morph.

No.	Morphological traits	*M. acampanulata*	*Metaphire campanulata*		*Metaphire campanulata penetralis**	*Metaphire campanulata typica**
			Rosa (1890)	**Gates** (1927)	**Gates** (1931)	**Gates** (1932)
1.	Length (mm)	77-198	160	130-180	107-144	≤ 200
2.	Diameter (mm)	4.03-6.91	6	4-6	4-6	4-7
3.	Number of segments	56-144	107	107-136	113-129	107-136
4.	Spermathecal genital markings	Absent	?	?	Present	Present
5.	Spermatheca	Mushroom shaped	Sacs shaped[1]	?	Ovoid[2]	?
6.	Ampulla duct	L shaped, 1/3 ampulla length	Straight, 1/4 ampulla length[1]	?	Straight, 1/3 ampulla length[2]	?
7.	Position of accessory gland	Only at base of ampulla duct	At base and onto ampulla duct	At base and onto ampulla duct	At base and onto ampulla duct	At base and onto ampulla duct
8.	Prostatic duct	Hairclip shaped	?	Coiled	?	C or U shaped

[1, 2] Determined by figures in the original descriptions; * H morph in Gates (1972)

kelantanensis (Michaelsen, 1900), and *M. quelparta* (Kobayashi, 1937)), ampulla duct L-shape (vs straight), diverticulum attached to the middle of ampulla duct (vs at the base, except *M. mendosa* (Gates, 1931)).

The differences mentioned above shows that *M. acampanulata* separated from *M. campanulata* (Rosa, 1890) and *M. houlleti* (Perrier, 1872) sensu Gates (1972) both by morphologies and genetics. Therefore, this study named the new species *Metaphire acampanulata* sp. nov. replacing *M.* cf. *campanulata* recorded in Southern Vietnam previously.

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