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Research article

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Notes on the earthworm species, *Metaphire anomala* (Michaelsen, 1907) (Clitellata, Megascolecidae) in Southern Vietnam, with descriptions of two new species

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Abstract. Integrative taxonomy was employed to exploit the differences between the known *Metaphire anomala* (Michaelsen, 1907) and other specimens collected in Vietnam. The results brought to light two new species, namely *Metaphire iranomala* sp. nov. and *Metaphire decemtheca* sp. nov. The former is easily recognised by having male pores on xix and four pairs of spermathecal pores on 5/6/7/8/9 while the latter is distinguished by having five pairs of spermathecal pores on 4/5/6/7/8/9. The K2P distances of the fragment of the cytochrome c oxidase subunit I (COI) gene are 13.1% between *M. iranomala* sp. nov. and *M. anomala* (Michaelsen, 1907) and 18% between *M. decemtheca* sp. nov. and *Metaphire grandiverticulata* Nguyen & Lam, 2017. The intraspecific divergences are 1.5%–10.6% for *M. iranomala* sp. nov. and 2.1%–11.4% for *M. decemtheca* sp. nov.

Keywords. *Metaphire*, taxonomy, biodiversity, COI genetic divergence, Vietnam.

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Introduction

Michaelsen (1907) described a new species, namely *Pheretima anomala*, from only eight specimens collected from the Botanical Gardens of Sibpur, Calcutta (India). The species is very specific without spermathecae and male pores on segment xx. Later, Gates (1925) commented on the species based on specimens collected from Rangoon (Myanmar). His specimens were slightly different from the original description (length 116–162 mm vs 80–90 mm, papillae on xviii–xxii vs on xvii–xxiii). Gates (1925) also described a new species, *P. insolita*. The species contained six types (A, B, C, D, E, F) distinguishable in length, diameter, number of segments, location and numbers of papillae in the male region. *Pheretima insolita* was very similar to *P. anomala* in terms of traces of male pores on xx, but differs in having spermathecal pores on 5/6/7/8 and variations of papillae in the male region. Stephenson (1929) discussed all types of *P. insolita* and *P. anomala*. He commented that the original specimens (*P. anomala*) possibly acted as functional males while the functional females would be *P. insolita*. Stephenson (1929), therefore, suggested to unite all the forms of *P. anomala* and *P. insolita* as one species, *P. anomala*. Michaelsen (1907)'s specimens would constitute the forma typical.

Metaphire anomala (Michaelsen, 1907) is widely distributed in India, Myanmar, Thailand, Laos, Yunnan (China) and Northern Vietnam (Michaelsen 1907; Gates 1925, 1972; Stephenson 1929; Nguyen *et al.* 2016; Yuan *et al.* 2019). However, *M. anomala* is highly variable due to parthenogenetic degeneration, and often specimens without spermathecae or without male pores are dominant (Gates 1925, 1972; Stephenson 1929). If present, the spermathecal pores are in 5/6/7/8 and/or male pores are in xx.

The species *Metaphire anomala* has previously been reported in Southern Vietnam as 'so-called' *Pheretima anomala* Michaelsen, 1907. It is widely distributed in Southern Vietnam: Ba Ria-Vung Tau (Con Lon Isl.), Dong Nai (Cat Tien), Kien Giang (Lai Son Isl., Phu Quoc Isl.), An Giang (Nhon Mts, Phu Cuong Mts, Ba Doi Mts, Cam Mts, To Mts), Dong Nai (Vinh Cuu, Xuan Loc, Dinh Quan, Trang Bom, Cam My, Long Thanh, Cat Tien NP), Tay Ninh (Ba Den Mts) (Thai *et al.* 2004; Nguyen 2014; Nguyen *et al.* 2016, 2017a, 2017b, 2019, 2020).

During our re-examination of previously and newly collected specimens housed at Can Tho University, we found that the specimens collected from Southern Vietnam were very different from their original description (Michaelsen 1907), Gates (1925, 1972) and recent re-description (Bantaogwon *et al.* 2011). An integrative approach combining morphological and molecular data was used to confirm the taxonomic status of the Vietnamese specimens. Two new species were discovered and named in this study.

Material and methods

Earthworms were manually searched and previously collected in Vietnam. After collection, specimens were cleaned with tap water, killed in formalin 2%, temporarilly fixed in formalin 4% for 12 hours, then transferred to new formalin 4% for long-term preservation for morphological study. Specimens for molecular study were preserved in ethanol 90%. Specimens including holotypes and paratypes were deposited in Laboratory of Zoology, Department of Biology, CTU. Some were shared with the Department of Soil Ecology, Institute of Ecology and Biological Resources (IEBR), Hanoi, Vietnam.

Morphological examination

Material was examined under a Motic Digital microscope (model: DM143-FBGG-C) and dissected from the dorsal side for internal observation. Transverse body sections were processed using the classical method of haematoxylin and eosin. Selected segments were cleaned and dehydrated using graded ethanol concentrations. Segments were treated with paraffin, then cut using a microtome Sakura Accu SRM 200CW. The cut sections were stained using haematoxylin and eosin Y (Feldman & Wolfe 2014), then transferred onto glass slides.

Colour images were taken using a camera attached directly to the microscope. Line drawings and colour images were improved and grouped into plates using Photoshop CS6.

DNA extraction, PCR reaction and phylogenetic analysis

Total genomic DNA was extracted from the body walls of segments 30–35 using the DNAeasy Blood & Tissue Kit (QiagenTM). A 680bp fragment of the cytochrome c oxidase subunit I (COI) mitochondrial gene was amplified using the polymerase chain reaction (PCR) method with the universal primer set (HCO-2190/COI-E and LCO-1498/LCO-1498m) (Folmer *et al.* 1994). The PCR conditions for amplification were as follows: 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 using electrophoresis in 1% Agarose-TBE 1X. Successfully amplified PCR products were purified and sequenced at FishBase, Inc. (Malaysia) on an Applied Biosystems automatic sequencer (ABI3130 XL). Each successful sequence was manually checked using BioEdit ver. 7.1 (Hall 1999) and confirmed using BLASTN 2.6.0+ searches (Zhang *et al.* 2000). All confirmed sequences were aligned with MUSCLE (Edgar 2004). All nucleotide sequences have been deposited in GenBank (NCBI).

The dataset contained COI sequences from two new species and five other *Metaphire* sequences (*M. anomala*, *M. bahli* [Gates, 1945], *M. grandiverticulata* Nguyen & Lam, 2017, *M. megascolidioides* [Goto & Hatai, 1899] and *M. peguana* [Rosa, 1890]) (Table 1). *Polypheretima elongate* (Perrier, 1872) was selected as an outgroup, as *Polypheretima* is a sister genus of *Metaphire* (see Sims & Easton 1972). The genetic distance was calculated using the Kimura 2 parameter (K2P) model performed in MEGA ver. 7.0 with 1000 bootstrap replicates (Kumar *et al.* 2016).

ModelFinder performed in IQ-TREE ver. 1.6.12 was applied to find the best-fit maximum likelihood site substitution model (Kalyaanamoorthy *et al.* 2017). The substitution model, TIM2+F+I+G4, was selected using a likelihood ratio test with –lnL = 3509.852 and BIC score = 7513.414. A maximum likelihood bootstrap analysis was conducted using IQ-TREE ver. 2.1.1 with 1000 pseudoreplicates (Minh *et al.* 2020). A Bayesian inference (BI) tree was inferred using MrBayes ver. 3.2 (Ronquist *et al.* 2012) with 10 million generations, heating parameter of 0.06, burnin of 10% and sampling every 1000 generations.

Abbreviations

ag = accessory gland
amp = ampulla
B = Bayesian Inference
CTU = Can Tho University
dv = diverticulum
IEBR = Institute of Ecology and Biological Resources
gm = genital marking
ML = Maximum Likelihood
mp = male pore
ov = ovary
os = ovary sac
sp = spermathecal pore
sv = seminal vesicle
ts = testis sac

Table 1. Specimen vouchers and accession numbers deposited in GenBank

No.	Species	Locality	Specimen voucher	Accession number
1	*Metaphire bahli* (Gates, 1945)	Ba Ria-Vung Tau Prov.	CTU-EW.004.12	MW076178
2	*Metaphire bahli* (Gates, 1945)	Binh Phuoc Prov.	CTU-EW.004.23	MW076179
3	*Metaphire bahli* (Gates, 1945)	Binh Duong Prov.	CTU-EW.004.26	MW076180
4	*Metaphire bahli* (Gates, 1945)	Ho Chi Minh city	CTU-EW.004.41	MW076181
5	*Metaphire bahli* (Gates, 1945)	Tay Ninh Prov.	CTU-EW.004.42	MW076182
6	*Metaphire bahli* (Gates, 1945)	Dong Nai Prov.	CTU-EW.004.43	MW076183
7	*Metaphire bahli* (Gates, 1945)	Ba Ria-Vung Tau Prov.	CTU-EW.004.44	MW076184
8	*Metaphire peguana* (Rosa, 1890)	Ba Ria-Vung Tau Prov.	CTU-EW.009.06	MW076185
9	*Metaphire peguana* (Rosa, 1890)	Binh Duong Prov.	CTU-EW.009.07	MW076186
10	*Metaphire peguana* (Rosa, 1890)	Binh Phuoc Prov.	CTU-EW.009.10	MW076187
11	*Metaphire peguana* (Rosa, 1890)	Ho Chi Minh city	CTU-EW.009.13	MW076188
12	*Metaphire peguana* (Rosa, 1890)	Dong Nai Prov.	CTU-EW.009.15	MW076189
13	*Metaphire peguana* (Rosa, 1890)	Ba Ria-Vung Tau Prov.	CTU-EW.009.16	MW076190
14	*Metaphire iranomala* sp. nov.	Tay Ninh Prov.	CTU-EW.020.16	MW076191
15	*Metaphire iranomala* sp. nov.	Chau Duc Distr., Ba Ria -Vung Tau Prov.	CTU-EW.020.19	MW076193
16	*Metaphire iranomala* sp. nov.	Con Son isl., Ba Ria -Vung Tau Prov.	CTU-EW.020.20	MW076194
17	*Metaphire iranomala* sp. nov.	Con Son isl., Ba Ria -Vung Tau Prov.	CTU-EW.020.20b	MW076195
18	*Metaphire iranomala* sp. nov.	Vinh Cuu Distr., Dong Nai Prov.	CTU-EW.020.18	MW076192
19	*Metaphire iranomala* sp. nov.	Kien Giang Prov.	CTU-EW.020.23	MW076196
20	*Metaphire grandiverticulata* Nguyen & Lam, 2017	Ho Chi Minh city	CTU-EW.089.4a	MW076200
21	*Metaphire grandiverticulata* Nguyen & Lam, 2017	Ho Chi Minh city	CTU-EW.089.4a1	MW076199
22	*Metaphire decemtheca* sp. nov.	Con Son isl., Ba Ria -Vung Tau Prov.	CTU-EW.183.p02	MW076203
23	*Metaphire decemtheca* sp. nov.	Con Son isl., Ba Ria -Vung Tau Prov.	CTU-EW.183.03	MW076202
24	*Metaphire decemtheca* sp. nov.	Con Son isl., Ba Ria -Vung Tau Prov.	CTU-EW.1183.06	MW076201
25	*Polypheretima elongata* (Perrier, 1872)	Ho Chi Minh city	CTU-EW.026.02	MW076197
26	*Polypheretima elongata* (Perrier, 1872)	Ho Chi Minh city	CTU-EW.026.02b	MW076198
27	*Metaphire bahli* (Gates, 1945)	Prasankok *et al.* (2013)		KC404844
28	*Metaphire bahli* (Gates, 1945)	Blakemore (2016a)		KT626598
29	*Metaphire peguana* (Rosa, 1890)	Prasankok *et al.* (2013)		KC404831
30	*Metaphire peguana* (Rosa, 1890)	Jeratthitikul *et al.* (2017)		KU565288
31	*Metaphire anomala* (Michaelsen, 1907)	Bantaowong *et al.* (2011)		KC565251
32	*Metaphire anomala* (Michaelsen, 1907)	Bantaowong *et al.* (2011)		KC565252
33	*Metaphire anomala* (Michaelsen, 1907)	Bantaowong *et al.* (2011)		KC565253
34	*Metaphire anomala* (Michaelsen, 1907)	Bantaowong *et al.* (2011)		KC565254
35	*Metaphire megascolidioides* (Goto & Hatai, 1899)	Blakemore (2016b)	Sequence is presented in the paper	
36	*Metaphire megascolidioides* (Goto & Hatai, 1899)	Minamiya *et al.* (2009)		AB482107

Results

Descriptions of new species

Class Clitellata Michaelsen, 1919
Subclass Oligochaeta Grube, 1850
Order Opisthopora Michaelsen, 1929
Suborder Crassiclitellata Jamieson, 1988
Family Megascolecidae Rosa, 1891
Genus *Metaphire* Sims & Easton, 1972

Metaphire iranomala sp. nov.

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Figs 1–3, Tables 2–3

Pheretima anomala (non *Pheretima anomala* Michaelsen, 1907) – Thai *et al.* 2004: 758. – Nguyen 2014: 108. – Nguyen *et al.* 2017a: 893, fig. 7; 2017b: 98, fig. 3; Nguyen *et al.* 2019: 120, fig. 3.
Metaphire anomala – Nguyen *et al.* 2016: 50.

Diagnosis

Medium-sized worm, length 157–228 mm, diameter 6.5–8.0 mm, segments 85–145. Prostomium epilobous. Clitellum annular, within xiv–xvi. First dorsal pore in 12/13. Four pairs of spermathecal pores on ventrolateral intersegments 5/6/7/8/9. Male pores on the setal ring of segment xix; copulatory pouches present. Genital markings absent. Holandric. Testis sacs connected. Intestinal caeca simple. Septa 8/9/10 absent.

Etymology

The epithet '*iranomala*' is formed by the prefix '*ir*' and '*anomala*' to emphasise the wrong name '*anomala*' recorded in Vietnam.

Material examined

Holotype

VIETNAM • mature spec.; Ba Ria-Vung Tau Province, Con Dao National Park; 8°42′12″N; 106°35′41″ E; 120 m a.s.l.; 19 Oct. 2019; Nguyen Thanh Tung and Nguyen Thi Bao Ngoc leg.; natural forest; CTU-EW.020.h01.

Paratypes

VIETNAM • 9 matures; same collection data as for holotype; CTU-EW.020.p02.

Other material

VIETNAM – **Ba Ria-Vung Tau Province** • 16 matures; same collection data as for holotype; CTU-EW.020.03 • 10 matures; Con Son Island; 8°41′59″ N, 106°36′54″ E; 10 m a.s.l.; 18 Oct. 2019; Nguyen Thanh Tung and Nguyen Thi Bao Ngoc leg.; residential gardens; CTU-EW.020.04 • 33 matures; Con Son Island; 8°39′53″ N, 106°34′00″ E; 20 m a.s.l.; 19 Oct. 2019; Nguyen Thanh Tung and Nguyen Thi Bao Ngoc leg.; natural forest; CTU-EW.020.05. – **Kien Giang Province** • 1 mature; Da Dung mountain; 10°25′07″ N, 104°28′46″ E; Nov. 2010; Nguyen Thanh Tung leg.; natural forest; CTU-EW.DNA.020.23 • 49 matures; Lai Son Island; 09°48′01″ N, 104°39′18″ E; Nov. 2013; Trinh Thi Kim Binh leg.; natural forest; CTU-EW.020.11. – **An Giang Province** • 57 matures; Cam mountain; 10°30′36″ N, 105°00′09″ E; Nov. 2010; Nguyen Thanh Tung leg.; natural forest; CTU-EW.020.12 • 2 matures; Cam mountain; 10°30′36″ N, 105°00′09″ E; Nov. 2010; Nguyen Thanh Tung leg.; natural forest; IEBR-EW.020.22. – **Dong Nai Province** • 2 matures; Vinh Cuu Nature Reserve; 11°06′46″ N, 107°03′12″ E; Oct. 2019; Nguyen Quoc Nam leg.; natural forest; IEBR-EW.020.18a • 3 matures; Vinh Cuu Nature Reserve; 11°06′46″ N, 107°03′12″ E; Oct. 2019; Nguyen Quoc Nam leg.; natural forest; CTU-EW.DNA.020.18b • 7 matures; Cat Tien National Park; 11°26′05″ N, 107°25′45″ E; Oct. 2013; Le Van Nhan leg.; natural forest; CTU-EW.020.07. – **Tay Ninh Province** • 1 mature; Duong minh Chau District; 11°22′59″ N, 106°12′00″ E; Sep. 2019; Nguyen Quoc Nam leg.; home garden; IEBR-EW.020.16 • 6 matures; Ba Den mountain; 11°23′26″ N, 106°09′19″ E; Sep. 2019; Nguyen Quoc Nam leg.; mango gardens; CTU-EW.020.17.

Description

Body cylindrical, large-medium size, length 157–228 mm, diameter 6.5–8.0 mm, segments 85–145, weight 3.0–8.4 gr. Dorsum slightly dark grey, ventrum paler. Prostomium epilobous. First dorsal pore in 12/13. Perichaetine, setae at pre-clitellar segments stouter and sparser than that at post-clitellar segments; setal numbers: 55–62 in viii, 65–71 in xxx, 10–16 between male pores on xix. Setal distance: aa > ab, zz > zy. Clitellum annular, within xiv–xvi, darkish brown, without dorsal pores and setae. Female pore single, mid-ventral in xiv.

Four pairs of spermathecal pores on ventrolateral intersegments 5/6/7/8/9. Ventral distance between spermathecal pores ca 0.27–0.3 body circumference. Male pores on copulatory pouches in xix; ventral distance between male pores ca 0.2–0.3 body circumference. Genital markings absent in both spermathecal and male pores region.

Septa 5/6/7/8 thickened, 8/9/10 absent, 10/11/12 thin. Oesophageal gizzard between 7/8 and 10/11. Intestine origin at xv; caeca simple, short within xxvii-xxiv. Last hearts in xiii. Pharyngeal micronephridia developed in 5/6/7. Lymph glands absent. Typhlosole simple, lamelliform.

Spermathecae paired in vi–ix. Spermathecal ampulla large, mango-shaped; duct about a quarter of ampulla length. Diverticula attached to the base of ampulla ducts; distal part strongly coiled, swollen into coiled sinusoidal seminal chambers. Spermathecal ducts without nephridia. Accessory glands absent in the spermathecal region.

Holandric. Testis sacs in x and xi, connected ventrally. Seminal vesicles well developed in xi and xii. Ovaries on septum 12/13 posteriorly. Prostate glands, deeply lobuled, paired in xvii–xxi; prostatic ducts C-shaped. Accessory glands massed, covered the copulatory pouches.

DNA barcode

COI barcode data (partial) is for the paratypes uploaded to GenBank under the accession numbers MW076191, MW076192, MW076193, MW076194, MW076195, and MW076196. The new species shares the identity of 88.13% and 88.33% with *Metaphire anomala* (KU262251, KU565252, KU565253, KU565254).

Habitat

The species was found in leaf-litters or in the top-soil layer, especially in moist places (near streams) or in rocky holes with organic matter. *Metaphire iranomala* sp. nov. has a soft body, violet light skin when alive. Its moving behavior is similar to a caterpillar locomotion. The species was commonly located in hilly/mountainous areas, but occasionally found in deltas.

Variations

Metaphire iranomala sp. nov. has two slightly different morphological types. The first type is more likely to be distributed in islands or in coastal provinces in Vietnam; the other type is found in mainland provinces.

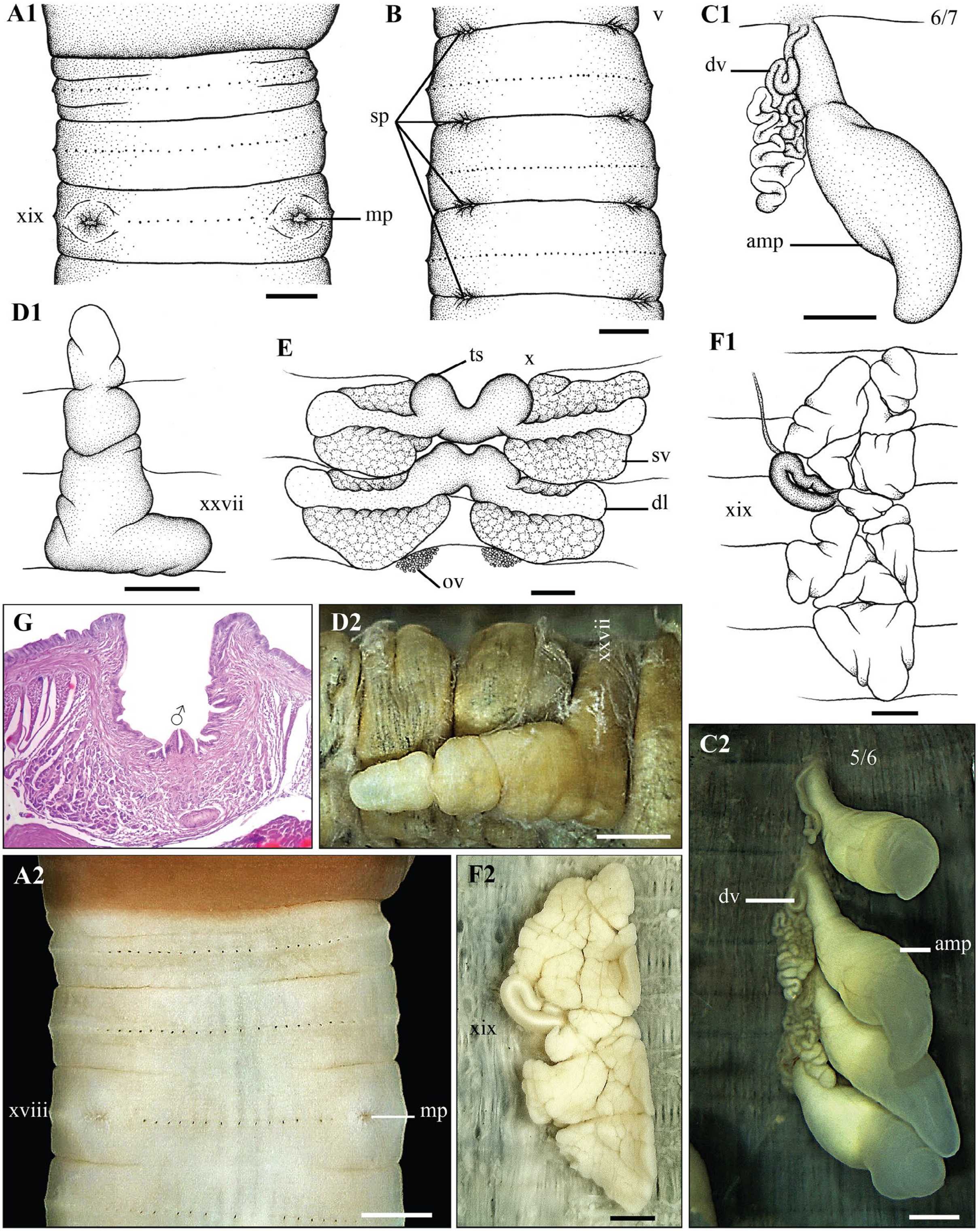

Fig. 1. *Metaphire iranomala* sp. nov., holotype (CTU-EW.020.h01). **A1, A2**. Ventral view of male pores region. **B**. Lateral view of spermathecal pores region. **C1, C2**. Right spermathecae. **D1, D2**. Intestinal caecum. **E**. Testis sacs and seminal vesicles. **F1, F2**. Right prostatic gland. **G**. Copulatory pouch transverse body section. Scale bars = 1 mm.

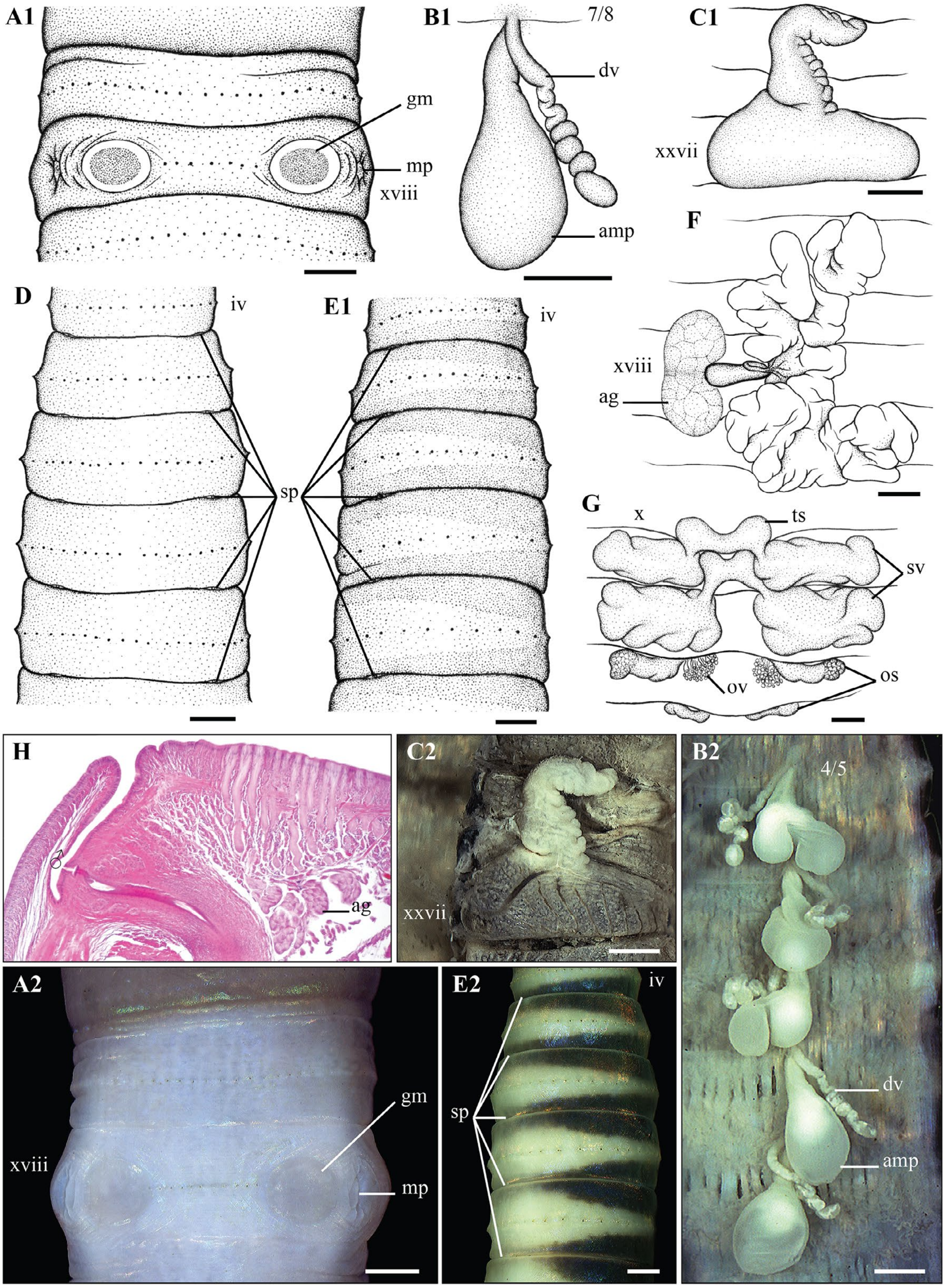

Fig. 2. *Metaphire decemtheca* sp. nov., holotype (CTU-EW.183.h01). **A1, A2**. Ventral view of male pore region. **B1, B2**. Right spermathecae. **C1, C2**. Intestinal caecum. **D**. Ventral view of spermathecal pore region. **E1, E2**. Lateral view of spermathecal pores region. **F1, F2**. Left prostatic gland. **G**. Testis sacs and seminal vesicles. **H**. Male pore region transverse body section. Scale bars = 1 mm.

Table 2. Comparison between *M. iranomala* sp. nov. and *M. anomala* (Michaelsen, 1907) from Thailand, Myanmar and India (its original description).

Characteristics	**Vietnam**[1]	**Thailand**[2]	**Myanmar**[3]	**Original description (India)**[4]
Length	157–228	134–154	116–200	80–90
Diameter	6.5–8.0	4.6–6.7	3.0–7.0	5.0–5.5
Segment	85–145	116–126	119–130	130
Spermathecal pores	four pairs in 5/6/7/8/9	3 pairs in 5/6/7/8	absent or 5/6/7/8	absent
Pre-clitellar setae	46–53 (viii)	84–96	90–96 (viii)	70 (v)
Post-clitellar setae	62–71 (xxx)	58–67	61–70 (xx)	74 (xxv)
Male pores	xix	xx	xx	xx
Ventral distance between male pores	0.2–0.3	0.33	–	0.17
Genital markings	absent	3–6 pairs in xvii–xix, xxi–xxiii or absent	present in xvii–xix and xxi–xxiv	4 pairs in xviii–xix, xxi–xxii
Septum 8/9	absent	absent	present	present
Testis sacs	connected	separated	separated	separated

[1] Freshly collected specimens; [2] Bantaowong *et al.* (2011); [3] Gates (1925, 1972) and Stephenson (1929); [4] Michaelsen (1907).

Table 3. Comparison between *M. iranomala* sp. nov., *M. megascolidioides* (Cognetti, 1908) and *M. isselii* (Goto & Hatai, 1899) sharing location of male pores not in segment xix.

Characteristics	***M. iranomala***	***M. megascolidioides***[1]	***M. isselii***[2]
Length	157–228	240	155–180
Diameter	6.5–8.0	15	10
Segment	85–145	118	94–115
Spermathecal pore	lateroventral, 5/6/7/8/9	lateroventral, 4/5/6/7/8/9	ventral, 6/7/8/9
Setal number between two male pores	10–16	9	0
Ventral distance between two male pores	0.2–0.3	?	< 0.25
Genital markings in male region	absent	3 pairs in xvii, xviii and xx	2 pairs in 18/19 and 19/20
Septum 8/9	absent	absent	thin
Intestinal caeca	simple	manicate	simple
Intestinal origin	xv	xv	xvi

[1] Goto & Hatai (1899) and Blakemore (2016b); [2] Cognetti (1908).

There are not many differences between the two types except the ventral distance between the male pores (0.2–0.22 vs 0.25–0.3). However, the COI genetic distance also distinguishes two types (see below).

Remarks

The new species has been previously identified as *Metaphire anomala*. It is widely distributed in Southern Vietnam (Thai *et al.* 2004; Nguyen 2014; Nguyen *et al.* 2017a, 2017b, 2019, 2020). However, this species is very different from both the original description (Michaelsen 1907), and re-description of *M. anomala* from Myanmar (Gates 1925, 1972), Thailand (Bantaowong *et al.* 2011) in the position of male pores, number and position of spermathecal pores, genital markings in male and spermathecal regions and body size. These differences are summarised in Table 2.

A few *Metaphire* species have been known to exhibit male pores not in segment xviii. Only *M. anomala* has male pores on xx, whereas two other species, *M. isselii* Cognetti, 1908 and *M. megascolidioides* Goto & Hatai, 1899, have male pores on xix. The new species is similar to these two species by having male pores on xix, a first dorsal pore in 12/13 and the absence of genital markings in the spermathecal region. However, the new earthworm species is clearly distinguished by body size, the number and position of spermathecal pores, the morphology of its male region, the status of septum 8/9, and they type of intestinal caeca. The differences are summarised in Table 3.

Metaphire decemtheca sp. nov.

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Figs 2–3

Diagnosis

Medium-sized worm. Prostomium epilobous. First dorsal pore in 12/13. Clitellum annular, within xiv–xvi. Five pairs of spermathecal pores on ventrolateral intersegments 4/5/6/7/8/9. Male pores on the setal ring of xviii; copulatory pouches present. A pair of genital markings in xviii. Holandric. Testis sacs connected. Intestinal caeca simple. Septum 8/9 absent or very thin, 9/10 absent.

Etymology

The epithet ‘*decemtheca*’ emphasises the number of spermathecae of the new species.

Material examined

Holotype

VIETNAM • mature; Ba Ria-Vung Tau Province, Con Son Island; 8°42′17″ N, 106°35′28″ E; 50 m a.s.l.; 19 Oct. 2019; Nguyen Thanh Tung and Nguyen Thi Bao Ngoc leg.; natural forest; CTU-EW.183.h01.

Paratypes

VIETNAM • 9 matures; same collection data as for holotype; CTU-EW.183.p02.

Other material

VIETNAM – **Ba Ria-Vung Tau Province** • 38 matures; same collection data as for holotype; CTU-EW.183.03 • 23 matures; Con Son Island; 8°43′50″ N, 106°37′31″ E; 10 m a.s.l.; 18 Oct. 2019; Nguyen Thanh Tung and Nguyen Thi Bao Ngoc leg.; natural forest; CTU-EW.183.04 • 55 matures; Con Son Island; 8°43′59″ N, 106°37′21″ E; 10 m a.s.l.; 18 Oct. 2019; Nguyen Thanh Tung and Nguyen Thi Bao Ngoc leg.; residential gardens; CTU-EW.183.05.

Description

Body cylindrical, medium-sized worms. Length 64–185 mm, diameter ca 3.0–6.0 mm, segments 79–124, weight 1.1–3.9 gr. Dorsum slightly dark grey with reddish browns stripes or absent, ventrum paler. Prostomium epilobous. First dorsal pore in 12/13. Perichaetine; setae at pre-clitellar segments stouter and sparser than that at post-clitella segments; setal numbers: 38–51 in viii, 46–57 in xxx, 8–13 between two male pores on xviii. Setal distance: aa > ab, zz = zy. Clitellum annular (3/4xiv–3/4xvi), slightly brown, without dorsal pores and setae. Female pore single, mid-ventral in xiv.

Five pairs of spermathecal pores on ventrolateral intersegments 4/5/6/7/8/9. Ventral distance between spermathecal pores ca 0.38–0.45 body circumference. Genital markings absent in spermathecal region. Male pores on line with setal ring in xviii; copulatory pouches present; ventral distance between male pores is 0.38 body circumference. One pair of flat ellipsoidal genital markings in xviii located next to male pores ventrally.

Septa 5/6/7/8 thick, 8/9 absent or membrane (if present), 9/10 absent, 10/11/12/13 thin. Oesophageal gizzard between 7/8 and 10/11. Intestine origin at xv; caeca simple, somewhat rudimentary lobulated dorsally, within xxvii–xxv. Last hearts in xiii. Pharyngeal micronephridia developed in 5/6/7. Lymph glands sac-like, present from 27/28. Typhlosole simple, lamelliform.

Spermathecal paired in v–ix. Spermathecal ampulla oval-shaped, ducts stout, short, about one-third of ampulla length. Diverticula shorter than ampulla, strongly and constrainedly waved, attached to the base of ampulla

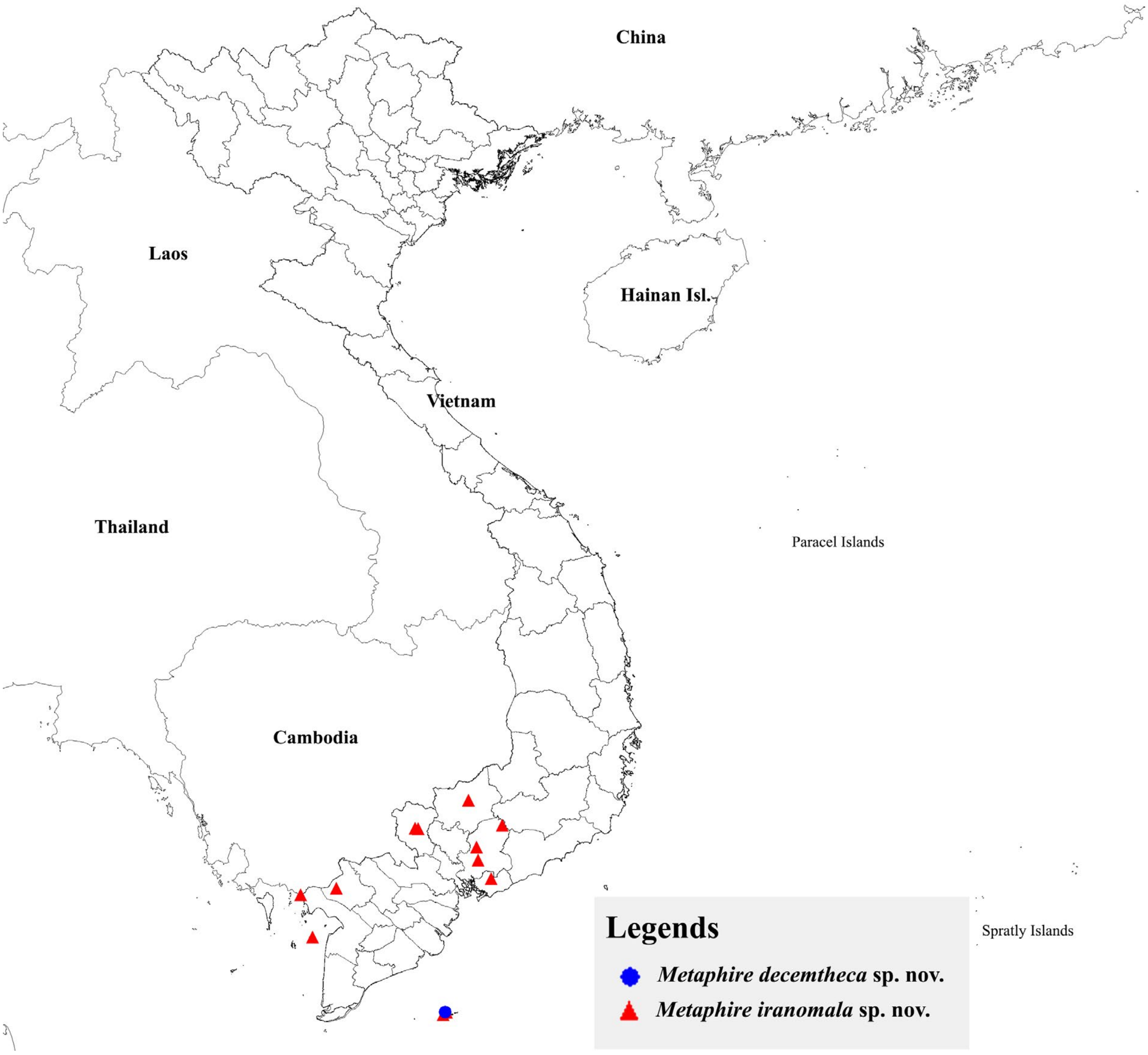

Fig. 3. The distribution of the two new species in Southern Vietnam.

ducts; seminal chamber small oval-shaped. Spermathecal ducts without nephridia. Accessory glands absent in the spermathecal region.

Holandric. Testis sacs in x and xi, connected ventrally. Seminal vesicles developed in xi and xii. Ovaries on septum 12/13 posteriorly. Ovisacs developed on 12/13, poorly developed on 13/14. Prostate glands deeply lobuled within xvi-xx; duct S-shaped, enlarged basally. Accessory glands sessile massed corresponding to external genital markings.

DNA barcode

COI barcode data (partial) is for the paratypes uploaded to GenBank under the accession numbers MW076201, MW076202 and MW076203. The new species shares the identity of 85.8% and 86.1% with *Amynthas* sp. (KT252973, KT205464).

Habitat

The species was found in the leaf litter of soil layer at a depth of 0–10 cm, near streams, and scattered on Con Son Island.

Variations

There are two morphological types found on Con Son Island. The first type was found in natural forests, with the presence of reddish-brown stripes, a bigger size (l = 133–170 mm, d = 5.0–6.5 mm), the first dorsal pore in 13/14 and ventral distance between spermathecal pores ca 0.38–0.4 body circumference. The second type found in residential gardens and forest edges exhibited no stripes, a smaller size (l = 64–122 mm, d = 3.0–3.4 mm), the first dorsal pore in 12/13 and ventral distance between spermathecal pores ca 0.43–0.45 body circumference. The COI genetic distance also showed the variations of 2.2–11.3%.

Remarks

A few *Metaphire* species have been described with five pairs of spermathecal pores, including *M. megascolidioides* and *M. fordi* Michaelsen, 1934. The new species is very similar to *M. megascolidioides* with its first dorsal pore in 12/13 and an absence of genital markings in the spermathecal region. However, *M. decemtheca* sp. nov. is distinguished by having male pores on xviii, a pair of genital markings in xviii, separate intestinal caeca and a small size (l = 64–185 mm, d = 3.0–6.0 mm) whereas *M. megascolidioides* has male pores on xix, three pairs of genital markings in xvii, xviii and xx, manicate intestinal caeca and a larger size (l = 240 mm, d = 15 mm).

The new species is also distinguished from *M. fordi* by having a larger size (l = 64–185 mm, d = 3.0–6.0 mm vs l = 50–64 mm, d = 1–1.5 mm) and the first dorsal pore in 12/13 (vs 11/12). Additionally, *M. decemtheca* sp. nov. has no genital markings in the spermathecal region, but does have one pair of large, round genital markings located next to the openings of copulatory pouches in xviii. By contrast, *M. fordi* has small circular papillae paired in both spermathecal and male regions.

In terms of the morphology of the male region, the new species is very similar to *M. grandiverticulata* Nguyen & Lam, 2017 by having a pair of large, round genital markings located next to the openings of its copulatory pouches and an absence of genital markings in the spermathecal region. However, *M. decemtheca* sp. nov. has five pairs of spermathecal pores on 4/5/6/7/8/9, a larger size (l = 64–185 mm, d = 3.0–6.0 mm), connected testis sacs, oval-shaped spermathecal ampulla, stout ducts, strongly and constrainedly waved diverticula and a small oval-shaped seminal chamber. Meanwhile, *M. grandiverticulata* has four pairs of spermathecal pores on 5/6/7/8/9, a smaller size (l = 69–92 mm, d = 2.3–2.7 mm), separated testis sacs, a small heart-shaped spermathecal ampulla, extremely short muscular ducts, stout and long unwaved diverticula and a bullet-shaped seminal chamber.

Table 4. The K2P intra- and interspecific genetic distances between *Metaphire* species using the 609 bp COI fragment. The intraspecific distances are in bold.

M. bahli	***M. peguana***	***M. iranomala***	***M. grandiverticulata***	***M. decemtheca***	***M. anomala***	***M. megascolidioides***	***Po. elongata***
0.008 ± 0.002							
0.166 ± 0.017	**0.028 ± 0.005**						
0.183 ± 0.017	0.185 ± 0.017	**0.062 ± 0.007**					
0.209 ± 0.019	0.235 ± 0.022	0.206 ± 0.019	**0.023 ± 0.005**				
0.179 ± 0.017	0.199 ± 0.018	0.175 ± 0.016	0.183 ± 0.017	**0.081 ± 0.011**			
0.177 ± 0.018	0.194 ± 0.019	0.130 ± 0.014	0.202 ± 0.020	0.178 ± 0.017	**0.003 ± 0.002**		
0.224 ± 0.021	0.191 ± 0.018	0.173 ± 0.017	0.201 ± 0.020	0.177 ± 0.018	0.172 ± 0.018	**0.003 ± 0.002**	
0.175 ± 0.018	0.180 ± 0.018	0.181 ± 0.017	0.194 ± 0.020	0.166 ± 0.016	0.177 ± 0.018	0.187 ± 0.018	**0.002 ± 0.002**

Molecular analysis

Statement of DNA dataset

The COI dataset comprises a 609 bp fragment from 36 sequences of 8 earthworm species including an outgroup species, *Polypheretima elongata* (Table 1). The nucleotide frequencies of A, T, G and C were 29.7%, 28.7%, 18.6% and 23.0%, respectively. The GC content was 41.6%. The combined dataset contained 210 (34.5%) parsimony informative and 216 (35.5%) variable sites.

Genetic distance

The average K2P distance is 16.0% between earthworm morphospecies. The K2P distance between species varies from 13.0% (*Metaphire iranomala* sp. nov. and *M. anomala*) to 23.5% (*M. peguana* and *M. grandiverticulata*). The intraspecific distance varies from 0.2% (*Polypheretima elongata*, *M. megascolidioides*) to 8.0% (*M. decemtheca* sp. nov.). The new species, *M. decemtheca* sp. nov., has the K2P divergence of 17.7% with *M. megascolidioides* and of 18.3% with *M. grandiverticulata* (Table 4).

The species *Metaphire iranomala* sp. nov. has genetic variations of 0.2% to 9.4%. The maximum distance between two types is 9.4%, but it is considered to be less than the average distance of 16%. Therefore, it is suggested that two morphological types still reflect one species. Similarly, the species *M. decemtheca* sp. nov. also has genetic divergences of 2.2% to 11.3% corresponding to morphological variations.

The K2P species divergences were reported, but were different for earthworm groups, for example, 13-15% for *Allolobophora* Eisen, 1874 (King *et al.* 2008), 13.2% for *Eisenia fetida*/*andrei* complex (Römbke *et al.* 2016), more than 14% in *Octolasion lacteum* Örley, 1885, *Lumbricus rubellus* Hoffmeister, 1843 (Klarica *et al.* 2012), more than 18.7% in pheretimoid species (Chang *et al.* 2008), 19.8% in the genus *Lumbricus* Linnaeus, 1758 (James *et al.* 2010), 8.9–22.9% in the genus *Pheretima* Kinberg, 1867, 14.2–21.9% in the genus *Amynthas* Kinberg, 1867, 7.5–18.0% in the genus *Pithemera* Sim & Easton, 1972 and 11.7–21.0% in the genus *Polypheretima* Michaelsen, 1934 (Aspe & James 2018). Recently, Jeratthitikul *et al.* (2017) also calculated the average species distance of 20% for *Amynthas* and *Metaphire* species in Thailand. Our calculation of 16% only applies to the *Metaphire* species.

Phylogenetic relationship

A phylogenetic tree was reconstructed for a 609 bp dataset using the Likelihood ML and Inference BI analysis (Fig. 4). Two new species are clearly separated from their congeners. *Metaphire iranomala* sp. nov.

is a sister clade of *M. anomala* with strong supports of bootstrap and BI values (90% and 1.00 bpp, respectively). Two subclades of *M. iranomala* sp. nov. are also well supported with bootstrap and BI values (89% and 0.92 bpp, respectively).

Metaphire decemtheca sp. nov. is related to *M. grandiverticulata* and *M. megascolidioides* with moderate bootstrap support and strong BI values of 75% and 0.95 bpp. Within the clade of *M. decemtheca* sp. nov., two morphological variations also represent two linages: CTU-EW.183.06 and CTU-EW.183.p02 + CTU-EW.183.03. The relationship is well supported with bootstrap of 96% and BI value of 1.00 bpp.

Discussion

Nguyen *et al.* (2016) provided the comprehensive checklist of 212 earthworm species in Vietnam. Earthworms have been well surveyed in most areas in Vietnam, but not in highly mountainous or remote areas. For example, earthworms on islands still have been poorly recognised, except some data in Nam Du (Michaelsen 1934), Con Son (Thai *et al.* 2004), Lai Son, Hon Tre, Nam Du and Phu Quoc (Nguyen *et al.* 2017a, 2020). Several species were also recently described from Lai Son Island and Phu Quoc Island (Nguyen *et al.* 2017a, 2020). It is thus suggested that more intensive surveys in islands of Vietnam would bring more new taxa to discovery.

Nguyen *et al.* (2016) also suggested some species should be revised or rechecked to confirm their taxonomic status because those species, which were recorded in Vietnam, differentiated from original

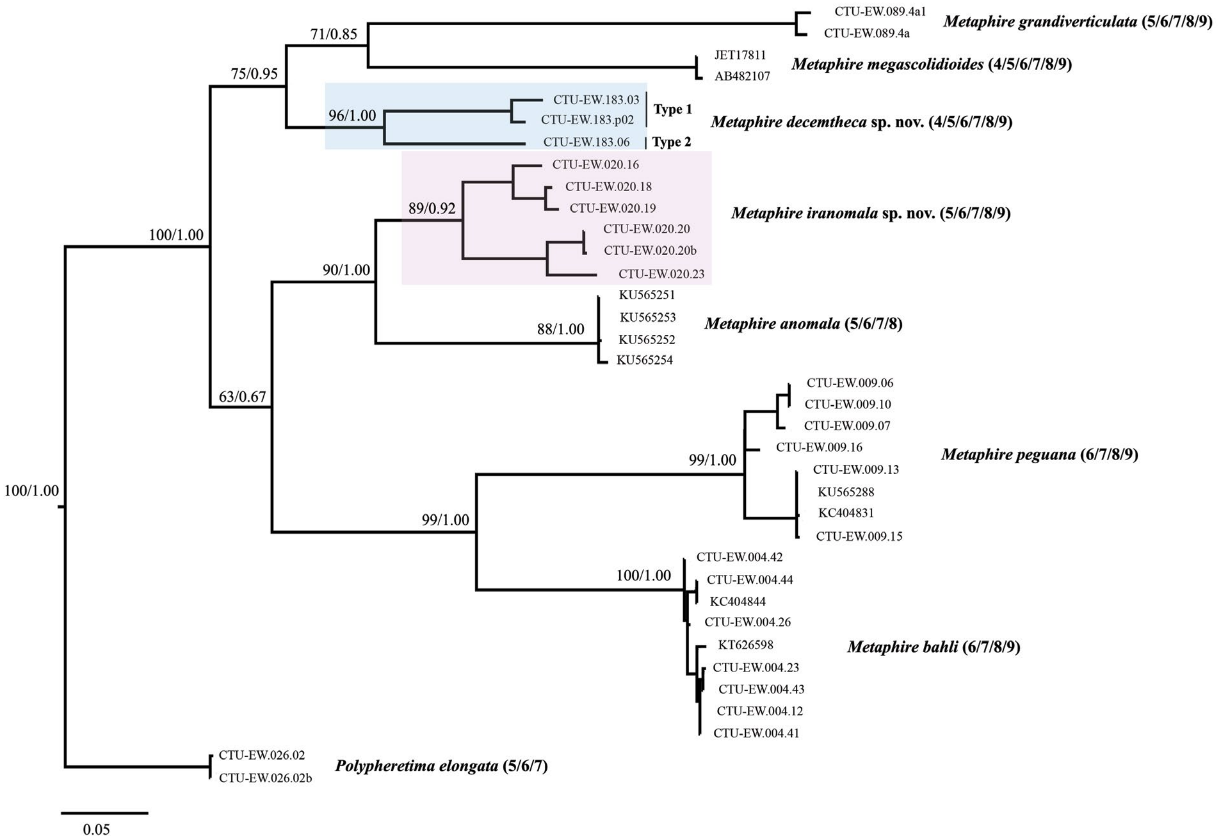

Fig. 4. The phylogenetic diagram inferred from the 594 bp dataset using the Maximum Likelihood (ML) and Bayesian Inference (BI) analysis. The values at node show the bootstrap and BI. The information in parentheses are locations and number of spermathecal pores.

descriptions, e.g., *Metaphire multitheca* (Chen, 1938), *M. anomala* (Michaelsen, 1907). These records of *M. multitheca* and *M. anomala* in Vietnam have been corrected and found to be new species, *M. erroneous* Nguyen & Nguyen, 2015 and *M. iranomala* sp. nov.

The high intraspecific divergences in *Metaphire iranomala* sp. nov. and *M. decemtheca* sp. nov. may suggest more Vietnamese species containing these cryptic forms, such as *M. houlleti* (Perrier, 1872) (as discussed in Nguyen *et al.* 2018). The cryptic speciation events have also been reported in many earthworm species using either mitochondrial data (King *et al.* 2008; James *et al.* 2010; Novo *et al.* 2010) or nuclear data (Rougerie *et al.* 2009). The COI barcode was also applied to discriminate earthworm species and to suggest cryptic forms (Jeratthitikul *et al.* 2017).

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