ACADEMIA JOURNAL OF BIOLOGY 2024, 46(4): 103–119
DOI: 10.15625/2615-9023/20476

MORPHOLOGICAL AND GENETIC VARIATIONS OF TWO EARTHWORM SPECIES *Metaphire bahli* (Gates, 1945) AND *Metaphire peguana* (Rosa, 1890) (Oligochaeta: Megascolecidae) IN VIETNAM

Phan Thanh Quoc[1,2], Nguyen Duc Anh[3,4], Truong Xuan Lam[3,4],
Thai Thi Ngoc Dung[2], Lam Hai Dang[5,*]

[1]Institute of Food and Biotechnology, Can Tho University, Can Tho, Vietnam
[2]Hau Giang Community College, Hau Giang, Vietnam
[3]Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Ha Noi, Vietnam
[4]Graduate University of Science and Technology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Ha Noi, Vietnam
[5]School of Education, Can Tho University, Can Tho, Vietnam

Received 31 March 2024; accepted 2 December 2024

ABSTRACT

The paper presents the analysis of morphological and genetic variations of two species, *Metaphire bahli* (Gates, 1945) and *Metaphire peguana* (Rosa, 1900), from Vietnam. As a result, *M. bahli* has two morphological forms based on the shape of the male region and state of genital markings while *M. peguana* has two subspecies, *M. peguana peguana*, and *M. peguana laisonensis*. *M. peguana peguana* had genital markings disc-shaped in 17/18/19 and a diverticulum attached to the base of the ampulla duct while *M. peguana laisonensis* had genital papillae slit-like shaped in xvii and xix, and diverticulum end at the middle of ampulla duct.

Keywords: Morphological variation, genetic divergence, earthworms, *Metaphire bahli*, *Metaphire peguana*, Vietnam.

Citation: Phan Thanh Quoc, Nguyen Duc Anh, Truong Xuan Lam, Thai Thi Ngoc Dung, Lam Hai Dang, 2024. Morphological and genetic variations of two earthworm species *Metaphire bahli* (Gates, 1945) and *Metaphire peguana* (Rosa, 1890) (Oligochaeta: Megascolecidae) in Vietnam. *Academia Journal of Biology*, 46(4): 103–119. https://doi.org/10.15625/2615-9023/20476

[*]*Corresponding author email*: lamhaidangct@gmail.com

INTRODUCTION

Metaphire peguana (= *Perichaeta peguana*) was described by Rosa (1890) for samples from Myanmar (as Burma). The species was originally characterized by two pairs of genital papillae in intersegmental 17/18/19, three pairs of spermathecal pores in 6/7/8/9, and bithecate. To date, *M. peguana* is a cosmopolitan species, and found in many regions in the world, such as French Guiana (Csuzdi & Pavlíček, 2010), Bangladesh (Sarkar & Bandyopadhyay, 2012), India (Stephenson, 1923), Myanmar (Gates, 1925, 1972), Cambodia, Laos (Thai & Do, 1989), Indonesia, Japan (Ishizuka, 1999), Singapore (Shen & Yeo, 2005), Thailand (Blakemore et al., 2006, 2013), Malaysia (Ng et al., 2017), and Australia (Easton, 1982).

At present, a total of 252 earthworm species have been recorded in Vietnam (Nguyen et al., 2016, 2017, 2020, 2021a, 2021b, 2022a, 2022b, 2022c, 2022d, 2023; Lam et al., 2024), and of these species, *M. peguana* (Rosa, 1890) and *Metaphire bahli* (Gates, 1945) were two of the most common *Metaphire* species, especially in the South. These species have been found commonly in the mainland and islands of Southern Vietnam (Nguyen et al., 2016, 2017, 2020, 2021a, 2021b, 2022d, 2023; Lam et al., 2021).

Previous studies indicated that the morphology of *M. peguana* was stable with two pairs of genital markings in 17/18 and 18/19, and three pairs of spermathecal pores in 6/7/8/9 (Gates, 1925, 1972; Prasankok et al., 2013; Blakemore, 2016; Nguyen et al., 2017). The molecular data also reported that the species has low variations with a COI genetic distance of 0.03 (samples from Vietnam) or 0.048 (samples from Thailand and Malaysia) (Prasankok et al., 2013; Nguyen et al., 2023). Recently, based on specimens collected from Lai Son island (Kien Giang province), Nguyen et al. (2017) named a new subspecies *M. peguana laisonensis*. That new morphological type was distinguished from *M. peguana peguana* by having two pairs of genital markings in xvii and xix, spermathecal pores widening apart laterally, and diverticulum attached to the middle of ampulla duct (Nguyen et al., 2017).

Gates (1945) found that samples of "*M. peguana*" from Sri Lanka (= Ceylon) have some significant differences from the original description, such as having genital papillae invaginated which opened by slit-like shaped, a smaller number of spermathecal and male setae. Then, he named a new species *M. bahli* for those individuals (Gates, 1945). Gates (1972) implemented that *M. bahli* was characterized by a concaved male region, and this morphological form appeared in all specimens from Myanmar and the Philippines (Gates, 1972). Thai (1983) reported a lot of variations of *M. bahli* from Vietnam including the absence or presence of genital markings in 17/18 or 18/19. Additionally, another form of *M. bahli* was found in Cam Mountain (An Giang province) characterized without having a concaved male region, having genital markings in xvii and xix, and spermathecal pores wider laterally (Nguyen, 2013). Later, samples of the form were also gathered in Lai Son and Hon Tre islands, but these samples were more similar to the original description (Nguyen et al., 2017). To date, *M. bahli* has been found in several places in South and Southeast Asia including India (Narayanan et al., 2019), Sri Lanka (Gates, 1945), Myanmar (Gates, 1972), Cambodia, Laos (Thai & Do, 1991), Philippines (Michaelsen, 1922), and even Australia (Easton, 1982).

This study aims to provide information about morphological variations between populations of *M. bahli* and *M. peguana* in order to compare morphological features and molecular data more thoroughly.

MATERIALS AND METHODS

Materials examined

Earthworm materials were collected from 16 sites during the period of 2013−2020 in Southern Vietnam (Fig. 1). Earthworms were captured directly by hand sorting (Górny & Grüm, 1993), and then cleaned with tap water before killing in formalin 2%. Clean specimens were fixed in formalin 4% for 12 hours, then transferred to new formalin 4%

for long-term preservation. Samples for molecular extraction were directly treated in ethanol 96% after the cleaning step. All earthworm samples were housed at the Laboratory of Zoology, Can Tho University (CTU), Vietnam.

Table 1. Total examined specimens used for morphological analysis of *Metaphire bahli* and *Metaphire peguana* in Vietnam

No.	Label codes	Individuals	GPS Coordinates		Location	Time	Collector
			Latitude (N)	Longitude (E)			
Metaphire bahli (Gates, 1945)							
1	EW.004.02	30	10º50’15.5	107º32’28.5	Xuan Loc distr., Dong Nai prov. (XL)	10/2012	Nguyen V.T.
2	EW.004.05	30	10º35’17.6	104º57'02.0	Nhon Mts., An Giang prov. (NM)	7/11/2010	Nguyen T.T.
3	EW.004.08	30	9º58’11.0	104º50’09.4	Hon Tre Isl., Kien Giang prov. (HT)	16/10/2014	Trinh T.K.B.
4	EW.004.09	30	9º47’57.6	104º37’24.5	Lai Son Isl., Kien Giang prov. (LS)	15/11/2013	Trinh T.K.B.
5	EW.004.11	30	10º30’36.3	105º00’09.0	Cam Mts., An Giang prov. (CM)	9/11/2010	Nguyen T.T.
6	EW.004.13	30	10º42’11.2	107º23’36.3	Chau Duc distr., Ba Ria-Vung Tau prov. (CD)	25/10/2016	Nguyen Q.N.
7	EW.004.34	30	10º41’30.5	106º36’11.0	Binh Chanh distr., Ho Chi Minh City (BC)	24/9/2019	Nguyen Q.N.
8	EW.004.47	30	10º11’06.6	103º58’15.3	Phu Quoc Isl., Kien Giang prov. (PQ)	6/11/2016	Lam H.D.
9	EW.004.49	30	8º43’50.0	106º37’31.0	Con Son Isl., Ba Ria, Vung Tau prov. (CS)	18/10/2019	Nguyen T.T.
10	EW.004.56	15	9º58’01.0	106º21’18.1	Chau Thanh distr., Tra Vinh prov. (CT)	13/10/2020	Lam H.D.
Metaphire peguana laisonensis Nguyen & Nguyen, 2017							
11	EW.087.01	27	9º48’10.5	104º36’51.3	Lai Son Isl., Kien Giang prov. (LS)	15/11/2013	Trinh T.K.B.
Metaphire peguana peguana (Rosa, 1890)							
12	EW.009.04	23	10º41’01.3	107º01’02.1	Long Thanh distr., Dong Nai prov. (LT)	21/10/2014	Le V.N.
13	EW.009.09	15	10º10’48.7	103º58’15.0	Phu Quoc Isl., Kien Giang prov. (PQ)	5/11/2016	Lam H.D.
14	EW.009.12	30	10º43’38.0	106º49’38.0	Nhon Trach distr., Dong Nai prov. (NT)	24/10/2016	Nguyen Q.N.
15	EW.009.13	30	10º23’15.1	106º54’45.2	Can Gio distr., Ho Chi Minh City (CG)	23/9/2019	Nguyen Q.N.
16	EW.009.19	13	10º13’21.6	106º34’44.5	Binh Dai distr., Ben Tre prov. (BD)	13/10/2020	Lam H.D.

A total of 423 mature specimens of two species, *M. peguana* (285 specimens of 10 populations) and *M. bahli* (138 specimens of 6 populations) were examined (Table 1, Fig. 1). The materials were examined under a Motic Digital stereo microscope (Model: DM143-FBGG-C) and dissected from the dorsal side for internal observation. All images were taken using the software MOTIC IMAGES PLUS version 2.0 connected to a camera attached to the stereo microscope. Images were improved and grouped into plates using Adobe Photoshop CS6.

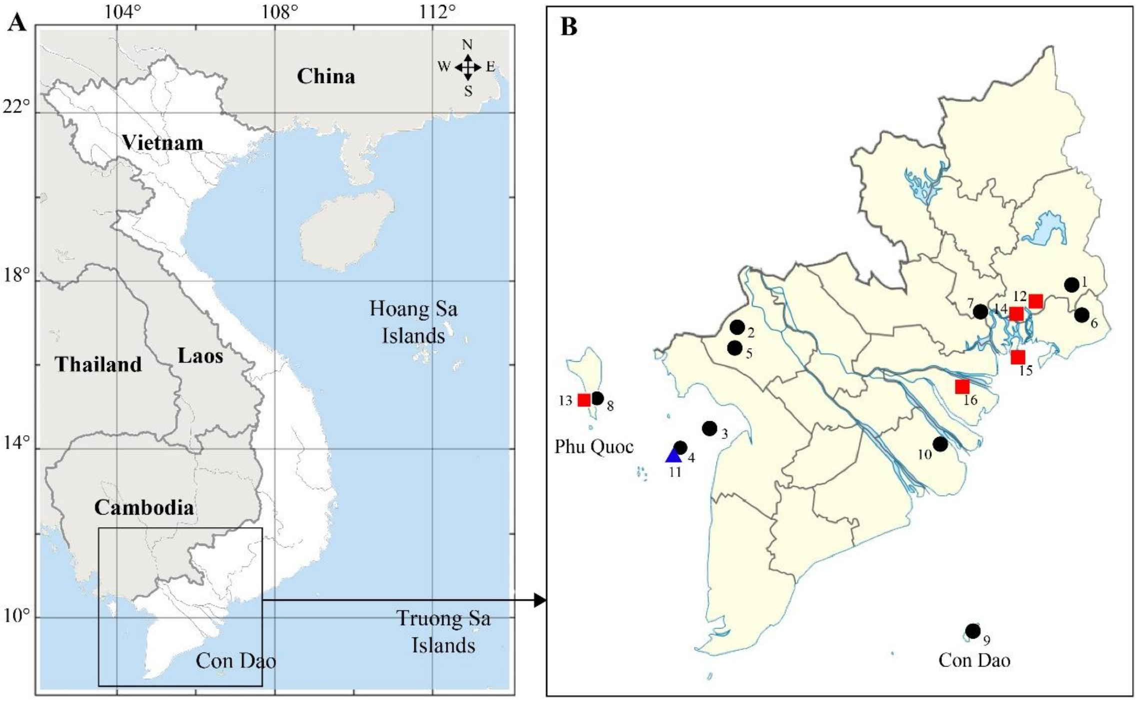

Figure 1. Collecting sites of earthworms in Vietnam. A. Vietnam map, B. South Vietnam map. Circles: *Metaphire bahli*, Rectangles: *Metaphire peguana peguana*, Triangles. *Metaphire peguana laisonensis*

Morphometric analysis

The following characters were examined: body length (Le), body diameter (Di), number of segments (Seg), weight (We), number of setae between two male pores (Set), ventral distance between two male pores (MpD) and ventral distance between two spermathecal pores (SpD). Earthworm diameters and ventral distances were measured through images taken from the software MOTIC IMAGES PLUS version 2.0. Weights were calculated using an electronic scale AND EK-600I with a standard error of 0.01 gr. All data were analyzed using the normality test (Kolmogorov-Smirnov Test) then using the One-way ANOVA analysis with the Tukey Test (normal distribution data) or the Kruskal-Wallis Test and Mann-Whitney Test (non-normal distribution data) performed in the software MINITAB ver. 19.

DNA extraction, amplification, sequencing

Total genomic DNA were extracted from earthworm muscle using a DNAeasy Blood & Tissue Kit (Qiagen TM, USA). Universal primer LCOI490 and HCO2198 were used for amplifying a 680 bp fragment mitochondrial gene COI (Folmer et al. 1994). PCR reactions were performed for 20 μL solution with conditions as follows: an initial denaturation at 95 ^{o}C for 2 min followed by 36 cycles of 95 ^{o}C for 20 sec, 42 ºC for 45 sec, and 72 ºC for 1 min, and a final extension at 72º for 5 min. Successfully amplified products were purified and sequenced at the FirstBase Company (Malaysia). The same primers for the initial PCR were also used as sequencing primers. The dataset combined COI sequences from previous studies and from GenBank (Table 2). The phylogenetic tree of COI fragment gen was constructed by the Bayesian Inference (BI) method using the software MrBayes 3.2.7 (Ronquist et al. 2012) for 10 million generations. *Drawida* sp. (EW.DNA.032.03) was selected as an outgroup.

Table 2. Total COI sequences used for morphological analysis of *Metaphire bahli* and *Metaphire peguana*

No.	Voucher specimen	Accession number	Locality
Metaphire bahli (Gates, 1945)			
1	EW.DNA.004.12	MW076178	Chau Duc distr., Ba Ria-Vung Tau prov.
2	EW.DNA.004.23	MW076179	Hon Quan distr., Binh Phuoc prov.
3	EW.DNA.004.23_a2(1)		Hon Quan distr., Binh Phuoc prov.
4	EW.DNA.004.23_a3(1)		Hon Quan distr., Binh Phuoc prov.
5	EW.DNA.004.26	MW076180 OP324548 OP324549	Tan Uyen distr., Binh Duong prov
6	EW.DNA.004.27	OP324550	Bac Tan Uyen distr., Binh Duong prov.
7	EW.DNA.004.37	OP324551	Trang Bang distr., Tay Ninh prov.
8	EW.DNA.004.41	MW076181	Nha Be distr., Ho Chi Minh City
9	EW.DNA.004.42	MW076182	Tan Chau distr., Tay Ninh prov.
10	EW.DNA.004.43	MW076183	Cam My distr., Dong Nai prov.
11	EW.DNA.004.44	MW076184	Con Son Island distr., Ba Ria, Vung Tau prov.
12	EW.DNA.004.56	OP324552	Chau Thanh distr., Tra Vinh prov.
13	EW.DNA.004.60	ON314427	Hon Tre Isl., Kien Giang prov.
14	EW.DNA.004.61	OP324553	Lai Son Isl., Kien Giang prov.
15	CUMZ 3298	KC404844	Thailand
16	NTM: D1529	KT626598	Thailand
Metaphire peguana peguana (Rosa, 1890)			
17	EW.DNA.009.06	MW076185	Tan Thanh distr., Ba Ria, Vung Tau prov.
18	EW.DNA.009.07	MW076186	Bac Tan Uyen distr., Binh Duong prov.
19	EW.DNA.009.07_b(1)		Bac Tan Uyen distr., Binh Duong prov.
20	EW.DNA.009.10	MW076187	Chon Thanh distr., Binh Phuoc prov.
21	EW.DNA.009.12	OP324554	Nhon Trach distr., Dong Nai prov.
22	EW.DNA.009.13	MW076188	Can Gio distr., Ho Chi Minh City
23	EW.DNA.009.14	OP324555	Cu Chi distr., Ho Chi Minh City
24	EW.DNA.009.15	MW076189	Thong Nhat distr., Dong Nai prov.
25	EW.DNA.009.16	MW076191	Con Son Isl., Ba Ria, Vung Tau prov.
26	EW.DNA.009.18		Tinh Bien distr., An Giang prov.
27	EW.DNA.009.19	ON314432	Binh Dai distr., Ben Tre prov.
Metaphire peguana laisonensis Nguyen, Nguyen, 2017			
28	EW.DNA.004.53(1)		Cam Mts., An Giang prov.

Note: (1) Nguyen et al. (2022d).

Abbreviations: **Le** = length, **Di** = diameter, **Seg** = number of segments, **We** = Weight, **Set** = number of setae between male pores, **MpD** = Ventral distance between male pores/body circumference, **SpD** = Ventral distance between spermathecal pores/body circumference; **amp** = ampulla, **ag** = accessory gland, **dv** = diverticulum, **gm** = genital markings, **mp** = male pore, **sp** = spermathecal pore.

RESULTS

Metaphire bahli (Gates, 1945)

The analysis of 285 matures showed that the values of seven morphological traits vary in length (53–170 mm), diameter (2.43–5.43 mm), number of segments (57–135 segments) weight (0.28–3.03 g), number of male setae (0–13 setae), distance of spermathecal pores (0.15–0.45 body circumference), and distance of male pores (0.11–0.33 body circumference).

Comparing between populations, worms in Lai Son Island have the greatest length (123.17 ± 13.02 mm), heaviest weight (1.77 ± 0.32 mm), and the greatest number of segments (113.40 ± 11.44 segments). The population has the greatest diameter (4.52 ± 0.40 mm) collected in Phu Quoc Island. Specimens from Cam Mountain have the shortest body length (72.53 ± 12.12 mm), the smallest diameter (2.87 ± 0.30 mm), and the lightest weight (0.46 ± 0.15 g) but have the widest distance between spermathecal and male pores, respectively (0.40 ± 0.03 and 0.28 ± 0.04 body circumference, respectively), and the greatest number of male setae (10.70 ± 1.62 setae) (Fig. 2).

Table 3. Comparison between morphological groups of *Metaphire bahli* in Vietnam

No.	Traits	Group 1 (195C)	Group 2 (60C)	Group 3 (30C)
1	Length (mm)	112.60 ± 15.78^{b}	118.50 ± 13.85^{a}	72.53 ± 12.12^{c}
2	Diameter average (mm)	4.14 ± 0.41^{a}	4.21± 0.33^{a}	2.87 ± 0.30^{b}
3	Number of segments	106.35 ± 12.06^{a}	106.32 ± 14,33^{ab}	100.20 ± 9.68^{b}
4	Weight (g)	1.43 ± 0.41^{b}	1.59 ± 0.41^{a}	0.46 ± 0.15^{c}
5	Number of setae between male pores	3.13 ± 1.89^{c}	4.90 ± 2.33^{b}	10.70 ± 1.62^{a}
6	Distance between spermathecal pores/body circumference	0.22 ± 0.03^{c}	0.29 ± 0.04^{b}	0.40 ± 0.03^{a}
7	Distance between male pores/body circumference	0.15 ± 0.02^{c}	0.17 ± 0.02^{b}	0.28 ± 0.04^{a}
8	Genital markings	17/18/19	17/18/19	xvii and xix

Note: (n^{a}): Different letters above numbers indicate a significant difference (P < 0.05; Kruskal-Wallis Test).

Variation between three morphological forms

Kruskal-Wallis Test with a 95% confidential range represented significant differences between three morphological forms in all seven survey traits. Group 3 was significantly different from the others. It had the smallest value in length (72.53 ± 12.12 mm), diameter (2.87 ± 0.30 mm), number of segments (100.20 ± 9,68 segments), and weight (0.46 ± 0.15 g), but had the largest values in male setae (10.70 ± 1.62 setae), male and spermathecal distances (0.40 ± 0,03 and 0.28 ± 0.04 body circumference). In addition, group 3 had genital markings in xvii and xix, while others had that character in 17/18/19 (Fig. 3). Group 2 included the longest (118.50 ± 13.85 mm) and heaviest (1.59 ± 0.41 g) worms, while group 1 had the least value of male setae (3.13 ± 1.89 setae), closest male and spermathecal distances (0.22 ± 0.03 and 0.15 ± 0.02 body circumference) of the worms.

Group 1 and group 2 had no differences in diameter (4.14 ± 0.41 vs. 4.21 ± 0.33 mm) and the number of segments (106.35 ± 12.06 vs. 106.32 ± 14.33 segments), but they were different in other characters with P-value from 0.000 to 0.022.

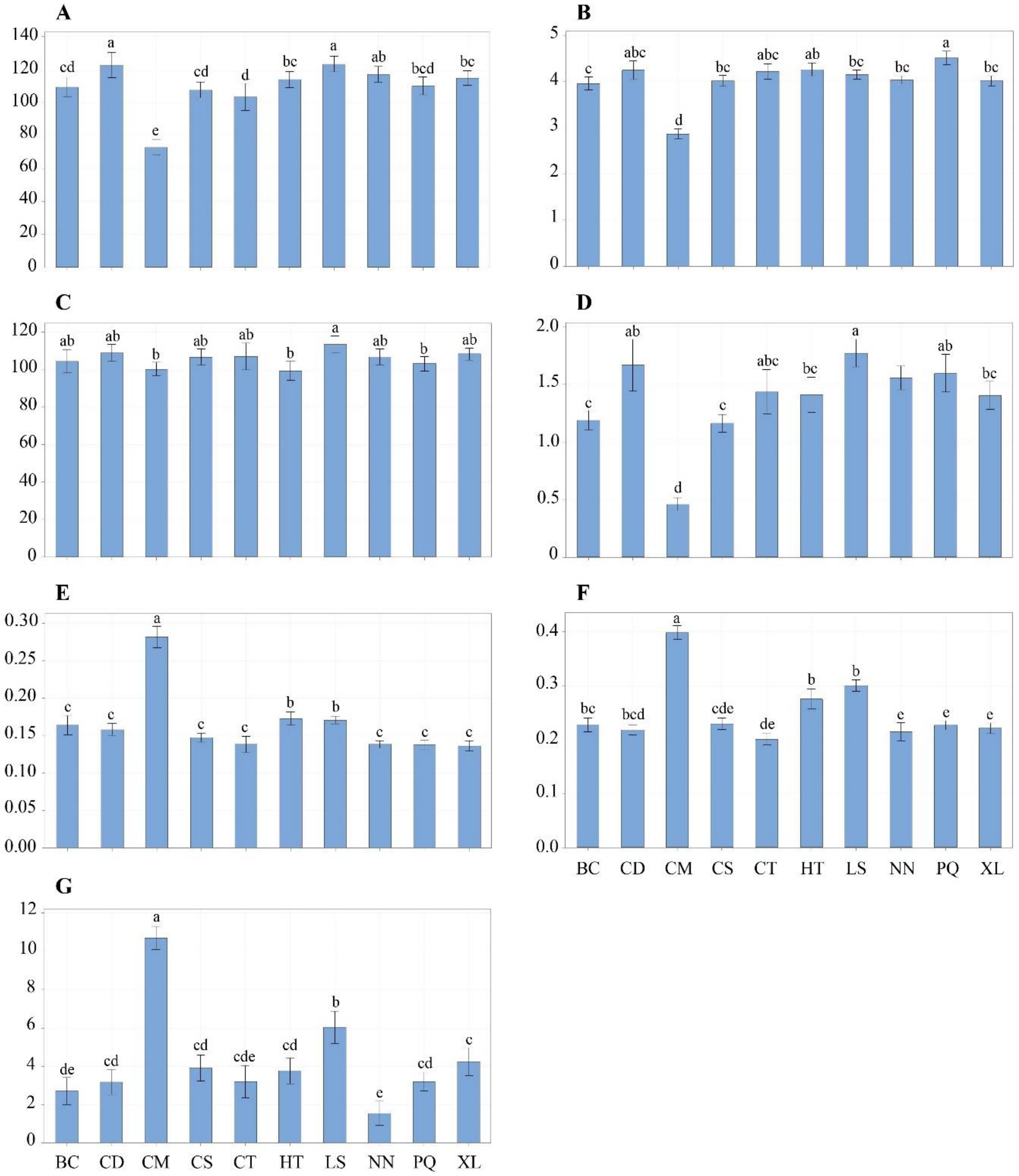

Figure 2. Mean of Length (A), Diameter (B), Number of segments (C), Weight (D), Ventral distance between spermathecal pores/body circumference (E), Ventral distance between male pores/body circumference (F), Number of setae between male pores (G) of *Metaphire bahli*. Different letters above bar chart indicate a significant difference ($P < 0.05$; Kruskal-Wallis Test). Site codes are as given in Table 1

Table 4. Comparison between the location of individuals within the concaved male region group in Vietnam

No.	Traits	Island (60C)	Mainland (135C)	P value
1	Length (mm)	108.58 ± 13.63^{a}	114.39 ± 16.38^{b}	0.017
2	Diameter average (mm)	4.26 ± 0.44^{a}	4.08 ± 0.38^{b}	0.003
3	Number of segments	104.82 ± 11.02^{a}	107.04 ± 12.47^{a}	0.236
4	Weight (g)	1.38 ± 0.41^{a}	1.45 ± 0.41^{a}	0.253
5	Number of setae between male pores	3.55 ± 1.60^{a}	2.94 ± 1.98^{b}	0.038
6	Distance between spermathecal pores/body circumference	$0.23 \pm 0{,}03^{a}$	0.22 ± 0.03^{b}	0.039
7	Distance between male pores/body circumference	0.14 ± 0.02^{a}	0.15 ± 0.03^{a}	0.148

Note: (n^{a}): Different letters above numbers indicate a significant difference ($P < 0.05$; Mann-Whitney Test).

Table 5. Morphological comparison of *Metaphire bahli* found in Hon Tre and Lai Son islands, Vietnam

No.	Traits	Lai Son island (30C)	Hon Tre island (30C)	P value
1	Length (mm)	123.17 ± 13.02^{a}	113.83 ± 13.25^{a}	0.253
2	Diameter average (mm)	4.15 ± 0.26^{a}	4.26 ± 0.38^{a}	0.973
3	Number of segments	113.40 ± 11.44^{a}	99.23 ± 13.53^{b}	0.000
4	Weight (g)	1.77 ± 0.32^{a}	1.41 ± 0.41^{b}	0.003
5	Number of setae between male pores	6.03 ± 2.25^{a}	3.77 ± 1.81^{b}	0.000
6	Distance between spermathecal pores/body circumference	0.30 ± 0.03^{a}	0.28 ± 0.05^{a}	0.087
7	Distance between male pores/body circumference	0.17 ± 0.01^{a}	0.17 ± 0.02^{a}	0.989

Note: (n^{a}): Different letters above bar chart indicate a significant difference ($P < 0.05$; Mann-Whitney Test).

Table 6. Morphological comparison of *Metaphire peguana peguana* found in Phu Quoc island and the mainland of Vietnam

No.	Traits	Phu Quoc island (15C)	Mainland (96C)	P value
1	Length (mm)	111.20 ± 16.37^{a}	113.70 ± 19.68^{a}	0.642
2	Diameter average (mm)	4.79 ± 0.33^{a}	4.56 ± 0.53^{a}	0.103
3	Number of segments	105.40 ± 17.83^{a}	112.57 ± 15.71^{a}	0.109
4	Weight (g)	1.82 ± 0.62^{a}	1.93 ± 0.67^{b}	0.558
5	Number of setae between male pores	8.20 ± 2.88^{a}	9.22 ± 2.09^{b}	0.100
6	Distance between spermathecal pores/body circumference	0.37 ± 0.09^{a}	0.41 ± 0.07^{b}	0.010
7	Distance between male pores/body circumference	0.26 ± 0.02^{a}	0.27 ± 0.03^{a}	0.184

Note: (n^{a}): Different letters above bar chart indicate a significant difference ($P < 0.05$; Kruskal-Wallis Test).

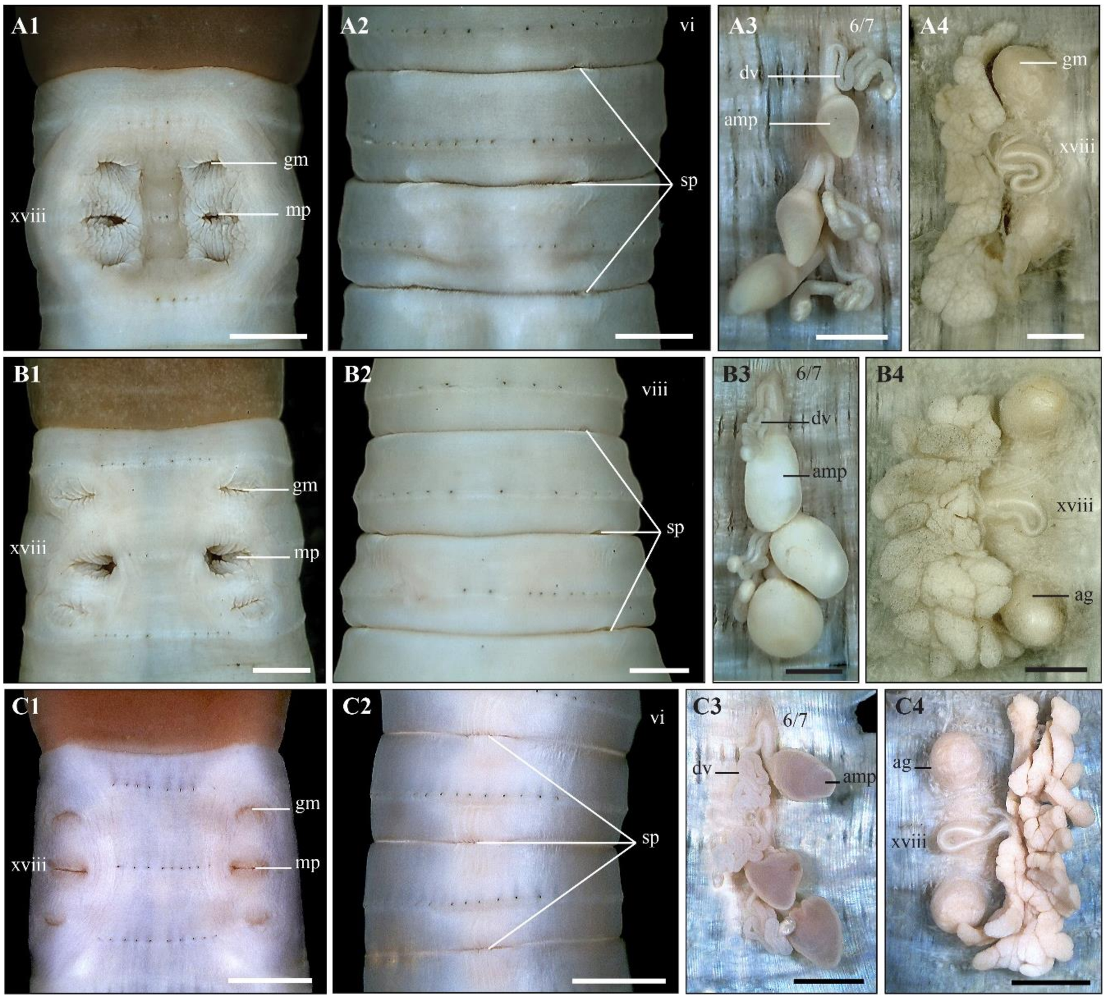

Figure 3. Different morphological group of *Metaphire bahli* (Gates, 1945). **A.** Group 1 (Non-type: **CTU-EW.004.05**); **B.** Group 2 (Non-type: **CTU-EW.004.08**); **C.** Group 3 (Non-type: **CTU-EW.004.11**)

Principle component analysis (PCA) was employed to test the relationship among examined samples. The results showed that group 3 was distinctly separated from group 1 and group 2 (Fig. 4). The first component (PC1) showed a variance (eigenvalue) of 3.9197 and accounts for 56.00% of the total variance. The second component (PC2) well explained that differences between group 1 and 2 had a variance (eigenvalue) of 1.3067, but only explained 18.67% of the total variance (Fig. 4). The first three principal components represented 85.49% respectively of the total variability.

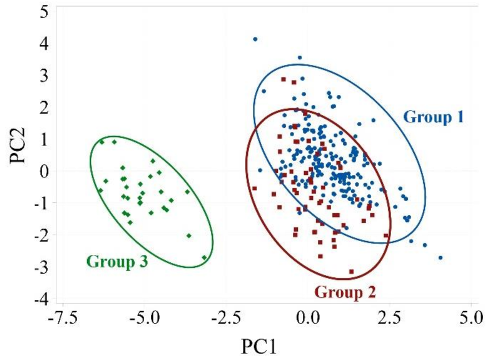

Figure 4. Score plot of morphological traits of *Metaphire bahli* in different groups. Group codes are given as in Table 1

Morphological variation between mainland and island populations

Regarding samples of group 1 (typical form), there were important differences between populations from the mainland and islands. Samples from the mainland were longer (114.39 ± 16.38 vs. 108.58 ± 13.63 mm, $P = 0.017$), smaller (diameter 4.08 ± 0.38 vs. 4.26 ± 0.44 mm, $P = 0.003$), less male setae (2.94 ± 1.98 vs. 3.55 ± 1.60) than samples from islands. The ventral distance between spermathecal pores indicated the difference between the two populations, but these differences were not well enough to separate the two populations (0.22 ± 0.03 vs. 0.23 ± 0.03 body circumference, $P = 0.039$) (Table 4).

The morphological variance between populations of Hon Tre and Lai Son islands

Both populations collected from Hon Tre and Lai Son island morphologically belong to group 2 without a concaved male region. There were no significant differences between the two populations. However, samples in Lai Son have a greater number of segments (113.40 ± 11.44 vs 99.23 ± 13.53 segments, $P = 0.000$), heavier (1.77 ± 0.32 vs. 1.41 ± 0.41 g, $P = 0.003$), more setae between male pores (6.03 ± 2.25 vs. 3.77 ± 1.81 setae, $P = 0.000$) than individuals from Hon Tre (Table 5).

Pairwise genetic distances between populations

The K2P genetic distance of the COI sequences between the sample EW.DNA.004.53 (from Cam Mountain) and other samples were from 0.124 to 0.151. The genetic distance between the sample EW.DNA.004.60 (from Hon Tre Island) and the sample EW.DNA.004.61 (from Lai Son Island) and other samples (except EW.DNA.004.53) were also large (0.087–0.117). Those results also corresponded to the morphological differences between groups. Within the group 1, genetic distances were low (0.000–0.034).

Table 7. Morphological comparison between *Metaphire peguana peguana* and *Metaphire peguana laisonensis* found in Vietnam

No.	Traits	*Metaphire peguana peguana* (111C)	*Metaphire peguana laisonensis* (27C)	P value
1	Length (mm)	113.36 ± 19.22^a	93.26 ± 12.85^b	0.000
2	Diameter average (mm)	4.59 ± 0.51^a	3.77 ± 0.31^a	0.000
3	Number of segments	111.60 ± 16.12^a	100.22 ± 16.70^b	0.001
4	Weight (g)	1.91 ± 0.66^a	0.91 ± 0.20^b	0.000
5	Number of setae between male pores	9.08 ± 2.23^a	8.70 ± 2.54^a	0.444
6	Distance between spermathecal pores/body circumference	0.41 ± 0.07^a	0.49 ± 0.03^b	0.000
7	Distance between male pores/body circumference	0.27 ± 0.03^a	0.25 ± 0.02^b	0.000
8	Genital markings' location	17/18/19	xvii and xix	
9	Genital markings shape	Disc shaped	Slit-like shaped	
10	State of the copulatory pouches	Restricted in body wall	Strong coelomic	
11	Base of diverticulum	Base of ampulla duct	Middle of ampulla duct	

Note: (n^a): Different letters above bar chart indicate a significant difference ($P < 0.05$; Kruskal-Wallis Test).

Metaphire peguana **(Rosa, 1890)**

M. peguana contained two subspecies *M. peguana peguana* (111 samples of 5 populations), and *M. peguana laisonensis* (27 samples from Lai Son Island and 30 samples from Cam Mountain).

M. peguana peguana has a wide range of morphological variations in length (81–157 mm), diameter (3.39–6.27 mm), number of segments (60–157 segments), weight (0.68–3.69 g), number of setae between two male pores (2–13), ventral distance between spermathecal pores (0.33–0.5 body circumference), and the ventral distance between two male pores (0.22–0.38 body circumference).

Comparing between populations, samples in Can Gio (CG) have the greatest length (125.00 ± 12.95 mm), and the number of segments (119.60 ± 13.24 segments) while samples in Binh Dai (BD) have the greatest diameter (4.94 ± 0.52 mm), heaviest weight (2.16 ± 0.49 g), and the greatest number of setae between two male pores (9.92 ± 0.86 setae).

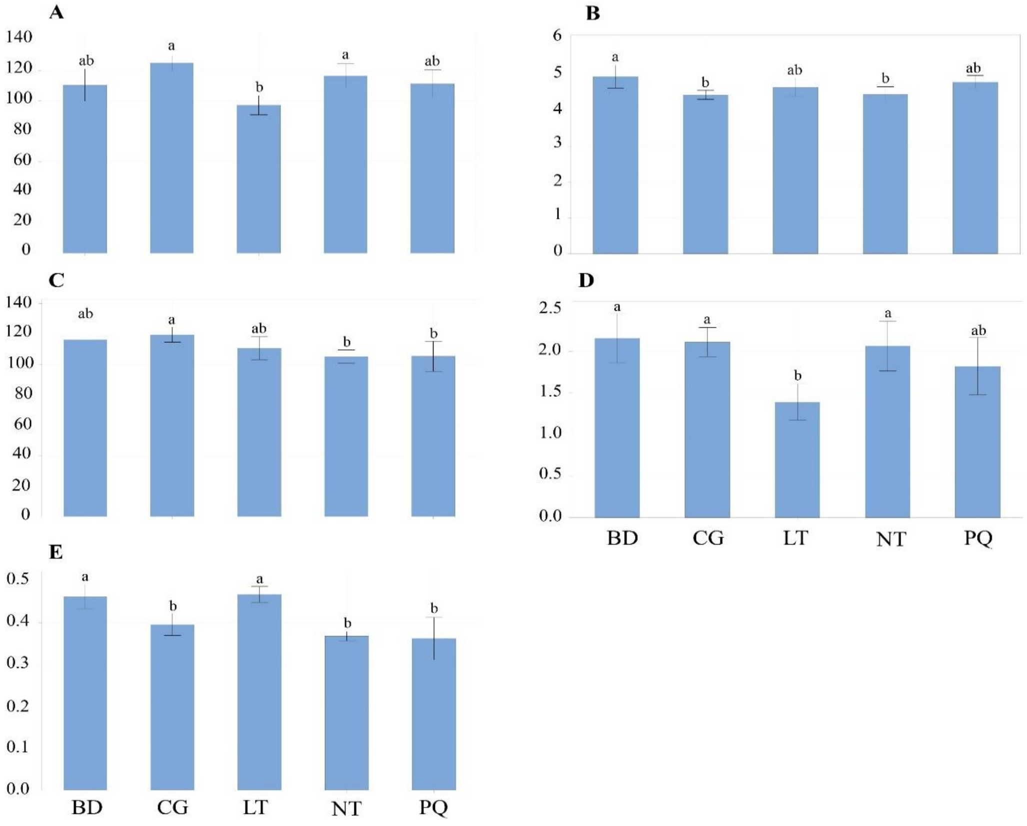

Figure 5. Mean of Length (**A**), Diameter (**B**), Number of segments (C), Weight (**D**), Ventral distance between spermathecal pores/body circumference (**E**) of *Metaphire peguana*. Different letters above bar chart indicate a significant difference ($P < 0.05$; Kruskal-Wallis Test). Site codes are given as in Table 1

Samples from Phu Quoc Island have the closest spermathecal pores (0.36 ± 0.09 body circumference); worms with the shortest length (97.17 ± 14.65 mm) and lightest weight (1.39 ± 0.50 g) are from Long Thanh (LT); samples from Nhon Trach (NT) have the smallest number of segments (105.27 ± 11.66 segments) and male setae (8.12 ± 2.45 setae) while the individuals with smallest diameter (4.43 ± 0.34 mm) is from Can Gio. While the population with the widest distances of spermathecal and male pores (0.47 ± 0.04 and 0.28 ± 0.03 body circumference) is from Long Thanh (LT), those distances are closest in samples from Phu Quoc Island (0.26 ± 0.02 and 0.36 ± 0.09 body circumference) (Fig. 5).

M. peguana laisonensis also varies in length (53–114mm), diameter (2.43–4.46 mm), weight (0.28–1.34 gr), number of segments (62–118 segments), number of setae between two male pores (4–13 setae), ventral distance between spermathecal pores (0.34–0.5 body circumference), and ventral distance between two male pores (0.21–0.33 body circumference).

Morphological variations between mainland and island populations of Metaphire peguana peguana

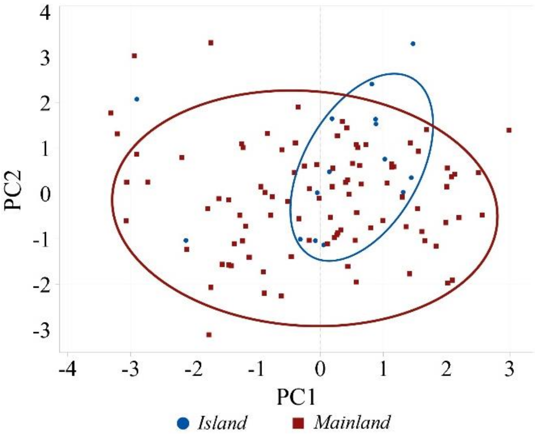

Figure 6. Score plot of morphological traits of *Metaphire peguana peguana* in different locations

Kruskal-Wallis Test of 95% confidence showed that two populations had significant differences in ventral distance between spermathecal pores (0.37 ± 0.09 vs. 0.41 ± 0.07, P = 0.010), but not in other examined characters (P-value from 0.100 to 0.558) (Table 6).

The morphological data of worms on the mainland and island mixed (Fig. 6). PC1 had a variance (eigenvalue) of 2.0783 and explained just 29.69% of the total variability, and the first two and three principal components showed 50.98% and 67.24% of the total variability.

The differences between Metaphire peguana peguana and Metaphire peguana laisonensis

M. peguana laisonensis was described by Nguyen et al. (2017). It differs from *M. peguana peguana* in spermathecal pore distance, location of male genital markings, and the base of the diverticula. Kruskal-Wallis Test with 95% confidence showed that two subspecies were distinguished from each other (except the number of male setae), P-value = 0.000–0.001. *M. peguana peguana* is longer (113.36 ± 19.22 vs. 93.26 ± 12.85 mm), larger diameter (4.59 ± 0.51 vs 3.77 ± 0.31 mm), more segments (111.60 ± 16.12 vs. 100.22 ± 16.70 segments), heavier weight (1.91 ± 0.66 vs. 0.91 ± 0.20 g), the wider distance between male pores (0.27 ± 0.03 vs. 0.25 ± 0.02 body circumference), but having a closer distance between spermathecal pores (0.41 ± 0.07 vs. 0.49 ± 0.03 body circumference) (Table 7). Moreover, *M. peguana peguana* has disc-shaped genital markings, the copulatory chamber restricted within the body wall, and the diverticulum attached to the base of the ampulla duct (Fig. 7) while *M. peguana laisonensis* has ellipsoidal genital markings, the copulatory chamber strongly coelomic, and diverticulum attached to the middle of the ampulla duct (Fig. 8).

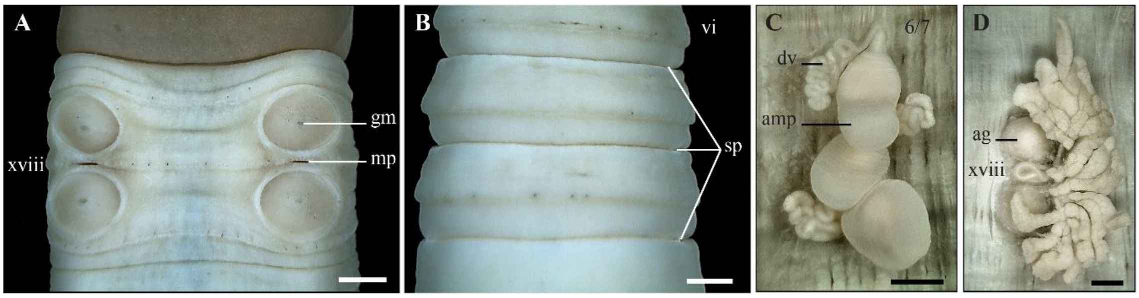

Figure 7. Metaphire peguana peguana (Rosa, 1890) (Non-type: **CTU-EW.009.04**)

Principle component analysis (PCA) indicated that two subspecies were not completely divided into two clusters (Fig. 9). The first principal component had a variance (eigenvalue) of 2.4392 and represented 34.85% total variability and the first two and three principal components showed 53.28% and 76.19% of the total variability.

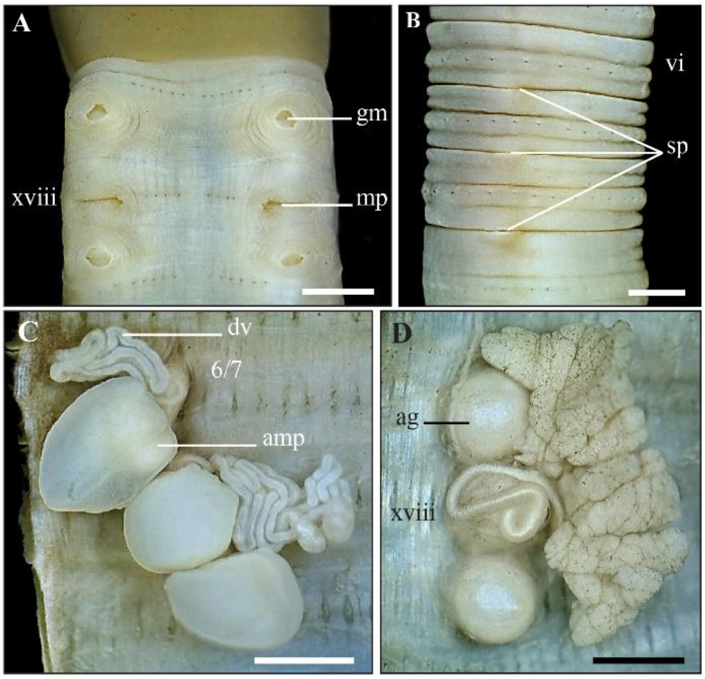

Figure 8. Metaphire peguana laisonensis Nguyen & Nguyen, 2017 (syntype-**CTU-EW.087.01**)

Pairwise genetic distances between populations

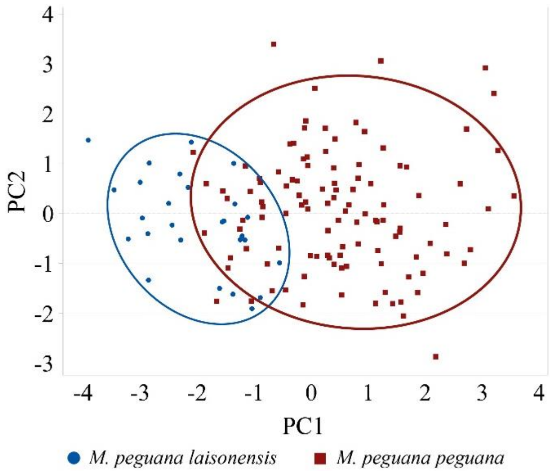

Figure 9. Score plot of the relationship between *Metaphire peguana peguana* and *Metaphire peguana laisonensis*

The K2P genetic distance of the COI sequences of *M. peguana peguana* slightly varies from 0.002 to 0.054.

Among them is the sequence of EW.DNA.009.12 (sample collected from Nhon Trach) is most different from others (the genetic distance from 0.020 to 0.054). The smallest genetic divergence was 0.002 between the specimen from Can Gio (EW.DNA.009.13) and the sample from Thong Nhat (EW.DNA.009.15).

Phylogenetic relationship of *Metaphire bahli* and *Metaphire peguana*

The phylogenetic tree showed that the sample from Lai Son Island (EW.DNA.004.60) was significantly different from other samples in Kimura-2-parameter genetic distance (from 0.124 to 0.151).

The sample from Cam Mountain (EW.DNA.004.53, previously identified as

M. bahli) was also branched within the *M. peguana* clade. The genetic divergence between this sample and other *M. bahli* samples was larger than that of *M. peguana* (0.124 to 0.151 vs. 0.120 to 0.147) (Fig. 10).

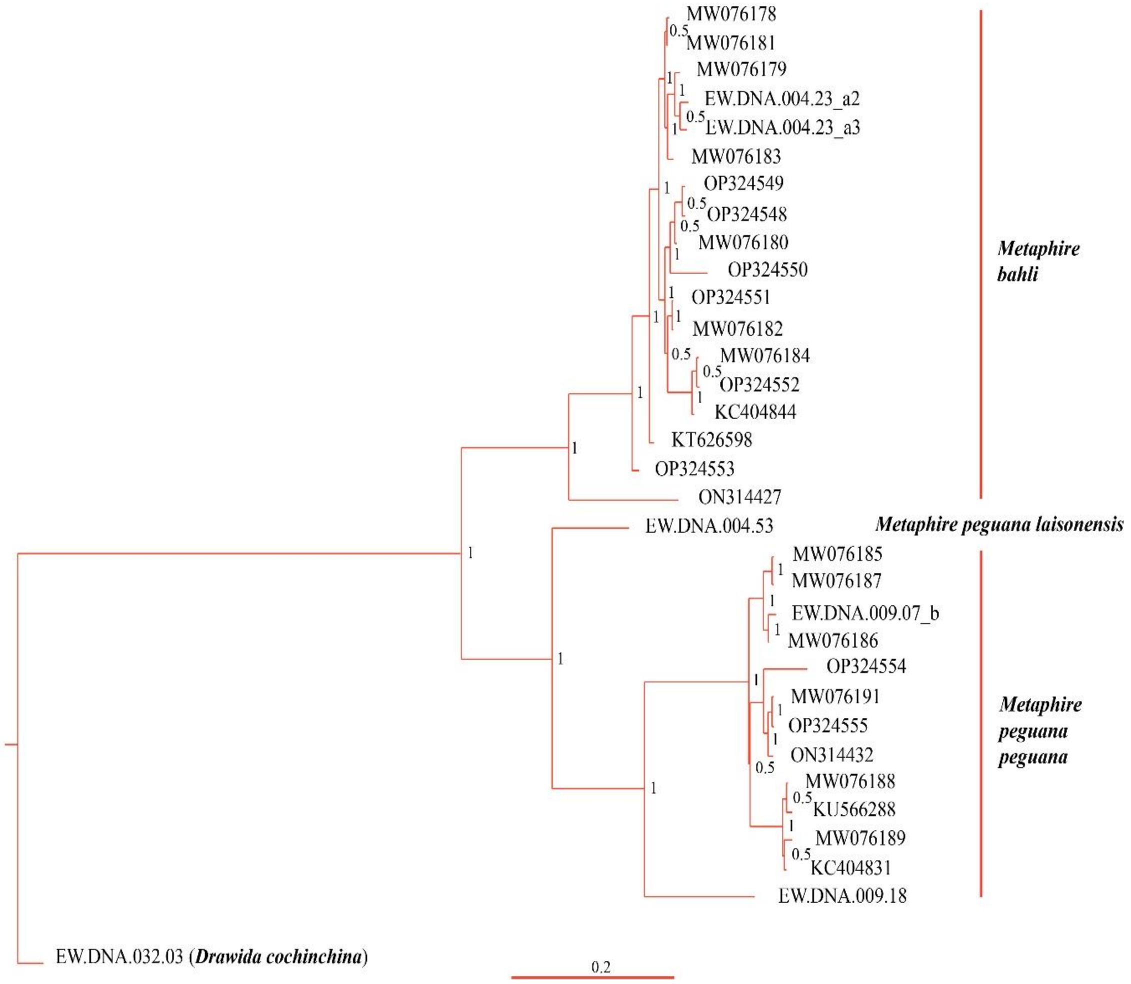

Figure 10. Bayesian Inference tree of *Metaphire bahli* and *Metaphire peguana* based on COI sequences. Values at nodes are bipartition posterior probability (bpp)

DISCUSSION

M. bahli have been commonly found in southern Vietnam, however, the specimens collected in Cam Mountain (Group 3) were misidentified. These samples are easily distinguished from the typical form (Group 1) and samples from Hon Tre and Lai Son islands (Group 2) by the male region's lack of concavity (vs. strongly concaved in group 1) and genital markings in xvii and xix (vs. in 17/18/19 in groups 1 and 2). In addition, these samples also show more male setae (10.70 ± 1.62 vs. 3.13 ± 1.89 and 4.90 ± 2.33 setae), spermathecal more laterally (0.40 ± 0.03 vs. 0.22 ± 0.03 and 0.29 ± 0.04 body circumference), and broader male pores (0.28 ± 0.04 vs. 0.15 ± 0.02 and 0.17 ± 0.02 body circumference). The genetic gaps between samples found in Cam Mountain and other places ranged from 0.124 to 0.151 (compared to species delimitation distance between 0.148 and 0.20 (Chang & James, 2011; Nguyen et al., 2018)). Also, samples in Cam Mountain are closely similar to *M. peguana laisonensis* by the male region shape (not concaved), genital papillae slit-liked, male pores ventrolateral (0.28 ± 0.04 vs. 0.25 ± 0.02 body circumference), spermathecal pores laterally (0.40 ± 0.03 vs. 0.49 ± 0.03 body circumference), and the number of male setae (10.70 ± 1.62 vs. 8.70 ± 2.54 setae). Therefore, samples from Cam Mountain (code EW.004.11) must be *M. peguana laisonensis.*

M. bahli (Group 2) differs from Group 1 in the male region being more flattened rather than concaved. According to their morphological differences, the genetic distance (K2P) between these two morphological variants ranges from 0.087 to 0.117.

M. peguana peguana individuals from the island have a wider distance of spermathecal pores than the mainland samples have (0.41 ± 0.07 vs. 0.37 ± 0.09 body circumference). Between populations, the K2P distance (COI gene) was minimal, ranging from 0.000 to 0.052. Generally, samples of *M. peguana peguana* have narrower spermathecal pores but greater length, diameter, number of segments, weight, and male pores distance. Additionally, *M. peguana peguana* is characterized by having disc-shaped genital markings in 17/18 and 18/19, diverticulum linked to the base of the ampulla duct whereas *M. peguana laisonensis* have slit-liked genital markings in xvii and xix, diverticulum attached to the middle of the duct.

CONCLUSION

The morphology of *M. bahli* varies greatly, including the male region form, the number of setae between two openings of copulatory pouches, the ventral distance between the male pores, and the spermathecal pores. Samples from Lai Son Island and the rest of *M. bahli* individuals can be separated into two subspecies by strong morphological and genetic differences. *M. peguana* species have significant differences between populations. Genital markings of this species can either represent oval disc-shaped on the body surface (*M. peguana peguana*) or invaginate into a horizontal slit-shaped opening (*M. peguana laisonensis*).

Acknowledgements: The authors would like to thank Dr. Nguyen Quoc Nam for his kind help in collecting the samples and completing the paper. This research was supported by a grant from the Vietnam Academy of Science and Technology Code: KHCBSS.02/23-25 for Truong Xuan Lam.

REFERENCES

Blakemore R. J., 2006. A Series of Searchable Texts on Earthworm Biodiversity: Ecology and Systematics from Various Regions of the World. M. T. Ito and N. Kaneko. Yokomaha, Japan, Yokohama National University, CD-ROM.

Blakemore R. J., 2016. Darwin’s earthworms (Annelida, Oligochaeta, Megadrilacea) with review of cosmopolitan *Metaphire peguana*–species group from Philippines. *Opuscula Zoologica*, 47(1): 9–30. https://doi.org/10.18348/opzool.2016.1.9

Chang C.-H. & James S., 2011. A critique of earthworm molecular phylogenetics. *Pedobiologia*, 54: S3–S9. https://doi.org/10.1016/j.pedobi.2011.07.015

Csuzdi C. & Pavlíček T., 2010. Report on the earthworms of the Site du Conservatoire du Littoral “Pripris du Yiyi”, French Guiana. Sepanguy**:** 6.

Easton E. G., 1982. Australian pheretimoid earthworms (Megascolecidae: Oligochaeta): a synopsis with the description of a new genus and five new species. *Australian Journal of Zoology*, 30: 711–735. http://dx.doi.org/10.1071/ZO982071

Folmer O., Black M., Hoeh W., Lutz R., Vrijenhoek R., 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, 3(5): 294–299.

Gates G. E., 1925. Notes on ROSA’S Rangoon earthworms, *Pheretima peguana* and *Eutyphoeus foveatus*. *Annals and Magazine of Natural History*, 9: 561–576.

Gates G. E., 1945. On some earthworms from Ceylon. II. *Spolia Zeylanica*, 24: 69–90.

Gates G. E., 1972. Burmese earthworms: An introduction to the systematics and biology of megadrile oligochaetes with special reference to Southeast Asia. *Transactions of the American Philosophical Society*, 62(7): 1–326. https://doi.org/10.2307/1006214

Górny M. & Grüm L., 1993. Methods in soil zoology. Elsevier, Amsterdam, Neitherland, pp, 459.

Ishizuka, 1999. A review of the genus *Pheretima* s. lat. (Megascolecidae) from Japan. *Edaphologia*, 62: 55–80.

Lam D. H., Nguyen N. Q., Nguyen A. D. & Nguyen T. T., 2021. A checklist of earthworms (Annelida: Oligochaeta) in southeastern Vietnam. *Journal of Threatened Taxa*, 13(2): 17693–17711. https://doi.org/10.11609/jott.6535.13.2.17693-17711

Lam D. H., Tran A.-T. T. & Nguyen A. D., 2024. Two new earthworm species of the genus *Amynthas* Kinberg, 1867 (Annelida, Oligochaeta, Megascolecidae) from the Southeast region, Vietnam. *Zootaxa*, 5406(4): 565–576. https://doi.org/10.11646/zootaxa.5406.4.5

Michaelsen W., 1922. Oligochäten aus dem Rijks-Museum van natuurlijke Historie su Leiden. *Capita Zoologica*, 1(3): 1–72 (in German).

Narayanan S. P., Sathrumithra S., Anuja R. & Christopher G., 2019. First record of the exotic earthworm *Metaphire bahli* (Gates, 1945)(Oligochaeta: Megascolecidae) from India. *Opuscula Zoologica Budapest*, 50(1): 99–103. https://doi.org/10.18348/opzool.2019.1.99

Ng B., Bantaowong U., Chanabun R., Tongkerd P. & Panha S., 2017. Geographic variations in the size and behavior of common earthworms *Metaphire peguana* (Rosa, 1890) in Penang and neighboring states, Malaysia. *European Journal of Soil Biology*, 82: 108–115. https://doi.org/10.1016/j.ejsobi.2017.09.002

Nguyen T. T., 2013. The earthworm fauna of the Cuu Long delta PhD dissertation in Zoology, Hanoi National University of Education.169. (in Vietnamese).

Nguyen T. T., Lam D. H. & Nguyen A. D., 2022a. Two new earthworm species (Annelida, Oligochaeta, Megascolecidae) from the Mekong delta, Vietnam. *Zootaxa*, 5093(4): 483–492. https://doi.org/10.11646/zootaxa.5093.4.6

Nguyen T. T., Lam D.H. & Nguyen A.D., 2023. A revision of the *Metaphire peguana* species-group (Oligochaeta: Megascolecidae) from Vietnam. *Zootaxa*, 5255(1): 113–135.

Nguyen T. T., Lam D. H., Tran B. T. T. & Nguyen A. D., 2022b. Two new *Drawida* (Oligochaeta, Moniligastridae) earthworms from Vietnam. *ZooKeys*, 1099: 41–56. https://doi.org/10.3897/zookeys.1099.72112

Nguyen T. T., Lam D. H., Trinh B. T. K. & Nguyen A. D., 2020. The megascolecid earthworms (Annelida, Oligochaeta, Megascolecidae) in the Phu Quoc island, Vietnam, with descriptions of three new species. *ZooKeys*, 932: 1–25. https://doi.org/10.3897/zookeys.932.50314

Nguyen T. T., Ly V. V., Lam D. H., Nguyen T. V. & Nguyen A. D., 2022c. A New Earthworm Species of the Genus *Metaphire* Sims & Easton, 1972 (Oligochaeta: Megascolecidae) from Southern Vietnam. *Tropical Natural History*, 22: 72–84.

Nguyen T. T., Nguyen A. D., Tran B. T. T. & Blakemore R. J., 2016. A comprehensive checklist of earthworm species and subspecies from Vietnam (Annelida: Clitellata: Oligochaeta: Almidae, Eudrilidae, Glossoscolecidae, Lumbricidae, Megascolecidae, Moniligastridae, Ocnerodrilidae, Octochaetidae). *Zootaxa*, 4140(1): 1–92. https://doi.org/10.11646/zootaxa.4140.1.1

Nguyen T. T., Nguyen N. Q. & Nguyen A. D., 2018. First record of the earthworm genus *Pheretima* Kinberg, 1867 sensu stricto in Vietnam, with description of a new species (Annelida: Clitellata: Megascolecidae). *Zootaxa*, 4496(1): 251–258. https://doi.org/10.11646/zootaxa.4496.1.20

Nguyen T. T., Nguyen N. T. B. & Nguyen A. D., 2021a. Earthworms (Annelida: Oligochaeta) of the Con Dao archipelago. *Academia Journal of Biology*, 43(1): 9–27. https://doi.org/10.15625/2615-9023/15559

Nguyen T. T., Thai B. T., Nguyen A. D. & Lam D. H., 2022d. Earthworms in the Mekong Delta - Vietnam. Can Tho University Publishing House, Can Tho, Vietnam, 233 pp.

Nguyen T. T., Tran T. H. & Nguyen A. D., 2021b. Diversity and distributional characteristics of earthworms in the Cuu Long river Delta, Vietnam. *Journal of Tropical Science and Technology*, 24: 103–114 (in Vietnamese).

Nguyen T. T., Trinh B. T. K., Nguyen L. T. H. & Nguyen A. D., 2017. Earthworms (Annelida: Oligochaeta) from islands of Kien Hai District, Kien Giang Province, Vietnam, with descriptions of two new species and one subspecies. *Journal of Natural History*, 51(15–16): 883–915. https://doi.org/10.1080/00222933.2017.1294213

Prasankok P., Bantaowong U., James S.W. & Panha S., 2013. Low heterogeneity in populations of the terrestrial earthworm, *Metaphire peguana* (Rosa, 1890), in Thailand, as revealed by analysis of mitochondrial DNA COI sequences and nuclear allozymes. *Biochemical Systematics and Ecology*, 51: 8–15. https://doi.org/10.1016/j.bse.2013.07.001

Ronquist F., Teslenko M., van der Mark P., Ayres D.L., Darling A., Höhna S., Larget B., Liu L., Suchard M. A., Huelsenbeck J. P., 2012. MrBayes 3.2: Efficient Bayesian Phylogenetic Inference and Model Choice Across a Large Model Space. *Systematic Biology*, 61(3): 539–542. https://doi.org/10.1093/sysbio/sys029

Rosa D., 1890. Viaggio di Leonardo Fea in Birmania e regioni vicine, XXVI. *Annali del Museo Civico Di Storia Naturale Giacomo Doria*, 10: 107–122 (in Italian).

Sarkar S. & Bandyopadhyay P. K., 2012. Studies on the biodiversity of aseptate gregarine parasite from the oligochaete host of Bangladesh. *Bangladesh Journal of Zoology*, 40(1): 51–58. https://doi.org/10.3329/bjz.v40i1.12894

Shen H. P. & Yeo D. C., 2005. Terrestrial earthworms (Oligochaeta) from Singapore. *The Raffles Bulletin of Zoology*, 53(1): 13–33.

Stephenson J. 1923. Oligochaeta. The fauna of British India. S. A.E. London, Taylor & Francis. XXIV: 518.

Thai B. T., 1983. Earthworms of Vietnam (Systematic, fauna, distribution and zoogeographic) PhD dissertation in Zooglogy, Lomonosov Moscow State University: 292 (in Russian).

Thai B. T. & Do N. V., 1989. Remarks on earthworm fauna in Phnompenh and the neighbouring regions. *Journal of Science of HNUE*, 1(Special issue): 76–78 (in Vietnamese).

Thai B. T. & Samphon K., 1991. Earthworms of some regions skirting the middle part of Mekong river in Laos (From Vientiane to Pacse). *Tap chi Sinh hoc*, 13(4): 1–10 (in Vietnamese).