TAXONOMY AND SYSTEMATICS OF EARTHWORM GENUS *Pontodrilus* Perrier, 1874 IN THAILAND

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อนุกรมวิธานและซิสเทมาติกส์ของไส้เดือนสกุล *Pontodrilus* Perrier, 1874 ในประเทศไทย

นายธีระพงค์ สีสมุทร์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ธีระพงค์ สีสมุทร์ : อนุกรมวิธานและซิสเทมาติกส์ของไส้เดือนสกุล *Pontodrilus* Perrier, 1874 ในประเทศไทย. (TAXONOMY AND SYSTEMATICS OF EARTHWORM GENUS *Pontodrilus* Perrier, 1874 IN THAILAND) อ.ที่ปรึกษาหลัก : ศ. ดร.สมศักดิ์ ปัญหา

การศึกษาอนุกรมวิธานของไส้เดือนสกุล *Pontodrilus* Perrier, 1874 ได้วิเคราะห์จากตัวอย่างไส้เดือนที่เก็บจากถิ่นอาศัยชายหาดตลอดแนวฝั่งตะวันออกและฝั่งตะวันตกของคาบสมุทรไทย-มลายู รวมถึงถิ่นอาศัยชายหาดจากประเทศญี่ปุ่น มาเลเซีย เมียนมาร์ เวียดนาม สิงคโปร์ และอินโดนีเซีย จากการศึกษาโดยใช้ลักษณะทางสัณฐานวิทยาพบว่าไส้เดือนมีลักษณะทางสัณฐานวิทยา 2 แบบ คือ ตัวอย่างส่วนใหญ่เป็นไส้เดือนชายหาดที่รู้จักกันโดยทั่วไปชนิด *P. litoralis* สำหรับอีกชนิดหนึ่งมีลักษณะของไดเวอร์ติคิวลัมในส่วนของถุงเก็บสเปิร์มแตกต่างจากชนิดแรก จากการวิเคราะห์ทางสัณฐานวิทยาร่วมกับการศึกษาทางชีววิทยาโมเลกุลจึงสามารถแยกไส้เดือนชายหาดชนิดดังกล่าวออกเป็นชนิดใหม่และบรรยายในชื่อ *P. longissimus* การวิเคราะห์มอร์โฟเมตริกจากการวัดรูปร่างของไส้เดือนชายหาดชนิด *P. litoralis* ทั้งหมด 14 กลุ่มประชากรที่เก็บได้จากเอเชียตะวันออกเฉียงใต้และญี่ปุ่น พบว่าความยาวของลำตัวและเส้นผ่านศูนย์กลางมีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบระหว่างกลุ่มประชากร อย่างไรก็ตามความแตกต่างดังกล่าวไม่ได้สอดคล้องกับผลการวิเคราะห์ทางความสัมพันธ์เชิงวิวัฒนาการเชิงโมเลกุลด้วยยีน COI นอกจากนี้การศึกษาทางชีววิทยาโมเลกุลของไส้เดือนชายหาดชนิด *P. longissimus* จากจำนวนทั้งสิ้น 136 ตัวอย่าง โดยใช้เครื่องหมายดีเอ็นเอตำแหน่งยีน COI ขนาด 658 คู่เบสพบว่าแฮโพลไทป์ที่มีความถี่สูงสุดพบได้จาก ประชากร 4 กลุ่มจากฝั่งทะเลอันดามัน และพบว่ากลุ่มประชากรฝั่งทะเลอันดามันมีความแปรผันทางพันธุกรรมมากกว่ากลุ่มประชากรจากฝั่งอ่าวไทย จากการวิเคราะห์แบบเบย์เซียน หลักความเป็นไปได้สูงสุดและเครือข่ายแฮโพลไทป์ได้จำแนกประชากรไส้เดือน *P. longissimus* ออกเป็นสองกลุ่มอย่างชัดเจน ประชากรกลุ่มหนึ่งจำกัดอยู่เพียงชายฝั่งอ่าวไทยและอีกกลุ่มจำกัดอยู่ทางชายฝั่งทะเลอันดามัน ความแตกต่างและลักษณะการกระจายตัวที่พบน่าจะจำกัดด้วยรูปแบบของกระแสน้ำรอบ ๆ คาบสมุทรมลายู

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KEYWORD: Pontodrilus, new species, phylogeny, morphometric, COI, genetic variation

Teerapong Seesamut : TAXONOMY AND SYSTEMATICS OF EARTHWORM GENUS *Pontodrilus* Perrier, 1874 IN THAILAND. Advisor: Prof. SOMSAK PANHA, D.Sc.

A taxonomic study of the littoral earthworm genus *Pontodrilus* Perrier, 1874 was critically conducted based on material collected from marine sandy coasts along the east and west sides of the Thai-Malay Peninsula (Thailand and Malaysia) and selected localities in Japan. Comparative material from other parts of Southeast Asia, including Myanmar, Vietnam, Malaysia, Singapore and Indonesia, were also collected. Morphological examination identified almost all of the specimens as *Pontodrilus litoralis*, but as two morphotypes with some character differences in the diverticulum between them. A combined morphological and molecular phylogenetic analyses supported one of these morphotypes as a new species, *P. longissimus*. Statistical inference on morphometric data revealed significantly different size variations in the body length and diameter among the 14 populations of *P. litoralis* from Southeast Asia and Japan. One-way analysis of variance revealed significant difference in the body length and diameter among specimens from the different geographical sites. However, this distinction was not congruent with the phylogenetic relationship based on mitochondrial cytochrome c oxidase subunit 1 (mtCOI) gene sequence analysis. In addition, molecular analysis of 136 samples of *P. longissimus* using the 658 bp DNA fragment of COI revealed that the most frequent haplotype was present in four populations from the Andaman Sea, with a greater degree of genetic variation than in the Gulf of Thailand (GOT) clade. Bayesian inference, maximum likelihood and haplotype network analyses clearly showed that there were two geographically isolated populations of the *P. longissimus* lineages, one restricted to the GOT and the other to the Andaman Sea. The divergence and distribution of the worms are probably restricted by tidal circulation patterns around the peninsula and vicinities.

Field of Study: Biological Sciences Student's Signature ....

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Chapter 1

Introduction

Earthworms are cited as the most important soil engineers (González et al., 2003) due to their ability to move through the soil and build organo-mineral structures with specific chemical, physical and microbiological properties. Earthworms have been described as one of the main groups of soil engineers and keystone species (Blondel and Aronson, 1995) in temperate and tropical ecosystems because of their ingestion of soil, mixing of mineral particles and humus, and the production of worm castings, which are rich in nutrients and microorganisms (Lavelle et al., 1992). Terrestrial earthworms belong to the order Oligochaeta, which contains more than 700 known genera with the largest family, Megascolecidae, containing in excess of 2000 recognized species.

Among the Megascolecidae, the genus *Pontodrilus* Perrier, 1874 shows various unique characteristics, and in particular its occurrence mostly in marine sandy coastal areas, at the ecotone between terrestrial and marine habitats. *Pontodrilus litoralis* (Grube, 1855) and *P. primoris* Blakemore 2000 occur in marine littoral habitats, such as sandy beaches, estuaries, areas under seaweed debris, damp mud under stones, and areas with wet sand mixed with mud (Blakemore, 2007; Easton, 1984). The euryhalinity of *Pontodrilus* is a specific character that has been reported with great attention by researchers (Jansson, 1962, Sanders et al., 1965). There are some hypotheses trying to explain those characters, for example that the worms acquire a certain degree of euryhalinity as an insurance against fluctuating environmental conditions (Delamare, 1960). However, there are still no appropriate answers on how worms survive in such euryhaline habitats.

Pontodrilus is characterized by the distinct tubular prostate duct structure connected to male pores on segment 18, and holoic nephridia that are absent in anterior segments (Michaelsen, 1900, Stephenson, 1930, Gates, 1972, Easton, 1984). Based on these morphological characteristics, since 1855 only five nominal species have been recorded from terrestrial and aquatic habitats worldwide: (i) the cosmopolitan coastal species *Pontodrilus litoralis* (Grube, 1855); (ii) *P. lacustris* (Benham, 1903), a freshwater species from Lake Wakatipu, New Zealand; (iii) *P. agnese* Stephenson, 1915, a terrestrial species from Horton Plains near Adam's Peak and Elk Plains, Sri Lanka; (iv) *P. sinensis* Chen and Xu, 1977 from Yuanshan, China, and (v) *P. primoris* Blakemore, 2000 from a beach in northeastern Tasmania, Australia.

In Thailand, oligochaete fauna exhibit a high species diversity, especially in terrestrial earthworms that belong to the genera *Amynthas* Kinberg, 1867 and *Metaphire* Sims & Easton, 1972, within the Megascolecidae family (Gates, 1972, Somniyam and Suwanwaree, 2009, Bantaowong et al., 2014, Bantaowong et al., 2015, Bantaowong et al., 2016). In addition, fifteen species of semi-aquatic freshwater earthworm species in the genus *Glyphidrilus* Horst, 1889 have been reported (Chanabun et al., 2012a, Chanabun et al., 2012b, Chanabun et al., 2013, Chanabun et al., 2017). However, littoral earthworms in Thailand have not received much attention so far. To date the only report on them was by Panha et al. (2007), who reported the first occurrence of the littoral earthworm *P. litoralis* in Thailand from Khanom, Nakhon Si Thammarat. This discovery supported that *P. litoralis* is widely distributed along coastal areas of tropical regions, as previously suggested (Gates, 1972, Blakemore, 2002). However, the negative impacts from aquaculture activities on the coastal ecosystem are now leading to the loss of major habitats for some important species (Chua and Paw, 1987). For this reason, these earthworms in Thailand need to be urgently investigated.

Accordingly, this study aimed to provide the first in-depth study of *Pontodrilus* in Thailand, which may be useful for further species conservation and understanding of their role in the ecosystem. *Pontodrilus* were surveyed and collected from along the coastal areas of Thailand, both the Gulf of Thailand (GOT) and the Andaman Sea. Moreover, comparative material from Japan, Myanmar, Vietnam, Malaysia, Singapore and Indonesia were collected. Traditional morphology-based taxonomy was used for the initial stage of the study. Then molecular analysis was conducted to explain the genetic diversity, support (cross-validate) species identification and determine the population structure using nucleotide sequence variations in the mitochondrial cytochrome c oxidase subunit 1 (mtCOI) gene fragment. The habitat characteristics of each sampling site were observed and recorded to determine the likely real ranges of habitat characteristics, especially for some physical factors, such as salinity.

The literature review of the dissertation as follows:

Earthworms are oligochaetes (Annelida: Clitellata), which are mostly terrestrial, except for a few aquatic taxa, such as earthworms in the family Almidae and *Eiseniella tetraedra* in freshwater habitats, and *Pontodrilus litoralis* and *P. primoris* in marine littoral habitats (Blakemore, 2007). The earthworm genus *Pontodrilus* Perrier, 1874 belongs to the family Megascolecidae, in which the main family characteristic is a male pore combined with prostatic pore on segment 18 (Blakemore, 2002). Littoral earthworms occur in sandy habitats with a high content of organic matter and salt, and have been recorded from sandy beaches, salty mud margins of estuaries, brackish lakes, mangrove swamps and under seaweed (Gates, 1972, Blakemore, 2002, Gobi et al., 2004).

Morphological characteristics have been the most commonly used feature in the identification of *Pontodrilus.* Taxonomic studies on *P. litoralis* have been reported from several parts of India as follows. Aiyer (1929) reported littoral earthworms from Kovalam, Gobi et al. (2004) reported species from Tuticorin backwaters, Satheeshkumar et al. (2011) published the first record of a littoral earthworm in Pondicherry mangroves, along the southeast coast of India, and Narayanan et al. (2014) reported the second record from the Kerala state, southern India. These reports all used morphological characters, such as body length, number of segments and the location of the clitellum and nephridia, for species identification.

Gates (1972) studied Burmese earthworm taxonomy using morphological characters and identified *Pontodrilus bermudensis* (a synonym of *P. litoralis*), which was found under logs on sandy soil away from the seashore in Myanmar. The important characters used by Gates in species identification were the digestive, vascular and excretory systems. Moreover, Shen et al. (2005) conducted earthworm surveys in various parts of Taiwan, and they identified two megascolecid earthworms as *P. litoralis* (Grube, 1855) and *Metaphire houlleti* (Perrier, 1872), and a lumbricid earthworm *Eiseniella tetraedra* (Savigny, 1826). Forty-nine specimens of *P. litoralis* were studied using external and internal characters, such as the clitellum, setae, male pores, spermathecal pores, prostate glands and nephridia.

In Japan, Iizuka (1898) discovered a littoral earthworm from Matsushima Bay, Miyagi Prefecture and described it as *P. matsushimensis*, but this species was later synonymized with the cosmopolitan earthworm *P. litoralis* (Easton, 1984). *Pontodrilus matsushimensis* was subsequently reported by Yamaguchi (1953) from Miyakojima in Miyagi Prefecture. Subsequently, the distribution of this species in Japan was further studied (Ohno, 2003), and *P. litoralis* has since been recorded in more than 20 localities in Honshu, Shikoku, Kyushu and Ryukyu (Oba et al., 2011, Oba et al., 2015). In addition, the occurrence of *P. litoralis* was also recorded on the beaches of Awaji Island, located between Honshu and Shikoku (Hara et al., 2016).

In common with other animal taxa, in addition to morphological identification, which has been used for hundreds of years, molecular techniques have increasingly been used more recently to confirm systematic identifications at both the species and intraspecies levels in oligochaetes and applied to define higher taxa classification within earthworms (Jamieson, 1988, James and Davidson, 2012). Molecular markers, typically mitochondrial and nuclear gene sequences, have been used to analyze evolutionary relationships, distinguish species and reveal cryptic species (Hebert et al., 2003, Erséus, 2005, James et al., 2010). Within clitellates, molecular phylogenetic analyses have been widely used to solve taxonomic problems and propose phylogenetic and biogeographic hypotheses since the early 1990s, with a total of 19 publications on earthworm phylogeny or systematics between 1996 and 2002 (Chang and James, 2011). The first molecular phylogenetic study that included earthworms was a study focusing on leeches and their relatives (Siddall et al., 2001). Thereafter, Jamieson et al. (2002) reported the first phylogenetic study of earthworms to confirm the hypothesis of the clade Crassiclitellata and supported the monophyly of the family Megascolecidae using 12S and 16S mitochondrial rRNA and 28S nuclear rRNA data. In addition, DNA barcoding of the standardized 658 bp region of the mtCOI gene is a powerful tool for identifying earthworm species (Chang and James, 2011, Jeratthitikul et al., 2017).

DNA barcode analysis can sustain decisions in alpha taxonomy, including synonymies or resurrections of species names, and descriptions of new taxa (James et al., 2010, Decaëns et al., 2013). Furthermore, it can reveal the diversity of cryptic species (Novo et al., 2010, Richard et al., 2010). In addition, the levels of genetic diversity can be inferred in several organisms and used to compare its extent within and among populations (genetic structure) as well as between species, and tested to see if the observed pattern was congruent with geography (phylogeography), could recognize isolated or small populations, and get rough estimates of gene flow between populations and demographic or range expansions (Excoffier, 2004). However, molecular methods have not really been used to confirm species delimitation among *Pontodrilus*.

Although Thailand is located in the heart of a biodiversity hotspot, littoral earthworms have been poorly surveyed in this region, and many problems regarding their taxonomy still await to be resolved, such as the synonyms, verifying misidentifications and comparison to type specimens. The combined use of morphological characters and molecular phylogenetic analyses are necessary to more reliably clarify the classical taxonomy and reveal the evolutionary relationships among these littoral earthworm species. Moreover, the geographical layout of the Thai peninsula allows the opportunity to investigate gene flow, population connectivity and phylogeography of the littoral earthworm populations between the GOT and Andaman Sea coastal areas.

Objectives

i) To explore the taxonomy of earthworm genus *Pontodrilus* in Thailand

ii) To construct a molecular phylogeny of *Pontodrilus* in Thailand

The structure of the dissertation is arranged as follows:

Chapter 2 Morphological and molecular evidence reveal a new species of the earthworm genus *Pontodrilus* Perrier, 1874 (Clitellata, Megascolecidae) from Thailand and Peninsular Malaysia

Published in Zootaxa 4496 (1): 218–237 (2018).

This chapter re-describes the previously known species *Pontodrilus litoralis* and describes a new species *P. longissimus* Seesamut and Panha, 2018. Specimens were compared morphologically and by molecular phylogenetics, using COI sequences.

Chapter 3 Size variation and geographical distribution of the luminous earthworm *Pontodrilus litoralis* (Grube, 1855) (Clitellata, Megascolecidae) in Southeast Asia and Japan

Published in Zookeys 862: 23–43 (2019).

This chapter investigates the pattern between the size and genetic (COI) variations in *P. litoralis* samples from 14 populations across Southeast Asia and Japan, and reports new data on the distribution and habitat types of *P. litoralis*.

Chapter 4 Mitochondrial genetic population structure and variation of the littoral earthworm *Pontodrilus longissimus* Seesamut and Panha, 2018 along the coast of Thailand

Published in European Journal of Soil Biology 93 (2019).

This chapter reveals the phylogeography, genetic diversity and population structure of *P. longissimus* using nucleotide sequence variation of the mtCOI gene fragment. It then includes a discussion on the tidal circulation patterns around the Thai-Malay peninsula as a potential geographical barrier between the Gulf of Thailand and Andaman sea.

Chapter 2

Morphological and molecular evidence reveal a new species of the earthworm genus *Pontodrilus* Perrier, 1874 (Clitellata, Megascolecidae) from Thailand and Peninsular Malaysia

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Abstract

A new species of the megascolecid earthworm genus *Pontodrilus* Perrier, 1874, *Pontodrilus longissimus* **sp. n.**, is described from seashores of Thailand and Peninsular Malaysia. The new species differs from congeners, especially the cosmopolitan *P. litoralis* (Grube, 1855) in the size of the body, number of segments and the shape of the spermathecae. *P. litoralis* is redescribed, based on specimens collected from the same region and the same type of habitat. DNA fragments of the mitochondrial cytochrome oxidase subunit I of both species were sequenced. Morphological as well as DNA sequence based comparisons confirm that *P. longissimus* **sp. n.** is a lineage distinct from *P. litoralis* and in fact a new species. The illustrated descriptions are accompanied by a key to species of *Pontodrilus*.

Key words: littoral earthworm, systematics, taxonomy, DNA barcode

Introduction

In Thailand, the oligochaete fauna exhibits a high species diversity, especially in terrestrial earthworms that belong to *Amynthas* Kinberg, 1867 and *Metaphire* Sims & Easton, 1972, genera within the Megascolecidae family (Gates, 1972, Somniyam and Suwanwaree, 2009, Bantaowong et al., 2014, Bantaowong et al., 2015, Bantaowong et al., 2016). In addition, fifteen species of semi-aquatic freshwater earthworm species in the genus *Glyphidrilus* Horst, 1889 have been reported (Chanabun et al., 2012a, Chanabun et al., 2012b, Chanabun et al., 2013, Chanabun et al., 2017). However, marine littoral earthworms have received no attention in Thailand until Panha et al. (2007) reported the first occurrence of the littoral earthworm *Pontodrilus litoralis* (Grube, 1855) in Thailand from Khanom, Nakhon Si Thammarat. This discovery supported the notion that *P. litoralis* is widely distributed in tropical coastal ecosystems (Gates, 1972, Blakemore, 2002). There are numerous taxonomic studies and records of *P. litoralis* from Asia, namely from the Indian subcontinent (Aiyer, 1929, Gobi et al., 2004, Satheeshkumar et al., 2011, Narayanan et al., 2014), Myanmar (Gates, 1972), China including Taiwan (Shen et al., 2005) and Vietnam (Nguyen et al., 2016). *Pontodrilus litoralis* occurs in sandy habitats with a high content of organic matter and salt, and has been recorded from sandy beaches, salty mud margins of estuaries, brackish lakes, mangrove swamps and under seaweed (Gates, 1972, Blakemore, 2002, Gobi et al., 2004).

The genus *Pontodrilus* Perrier, 1874 is an earthworm taxon that primarily inhabits marine littoral ecosystems. This genus was diagnosed by the absence of holoic nephridia from the anterior segments and the distinct tubular prostatic duct structure that opens to the male pore on XVIII (Gates, 1972, Easton, 1984). At present, *Pontodrilus* contains five species worldwide, which are from both terrestrial and aquatic habitats. They are *P. lacustris* (Benham, 1903) from Lake Wakatipu of New Zealand; the terrestrial species *P. agnesae* Stephenson, 1915 (Stephenson 1915b) from Horton Plains near Adam's Peak and Elk Plains, Sri Lanka; *P. sinensis* Chen & Zhifang, 1977 from Yunnan in China, which is either terrestrial or lacustrine; the littoral species *P. primoris* Blakemore , 2000 from a beach of Tasmania, Australia, and the cosmopolitan species *P. litoralis* (see Blakemore (2007)). *Pontodrilus lacustris* is distinguished from *P. litoralis* by four pairs of spermathecal and penial setae, *P. agnesae* by the presence of penial setae; *P. sinensis* by the presence of four pairs of seminal vesicles in IX–XII (Easton, 1984); and *P. primoris* by the short spermathecal diverticula relative to ampullae (Blakemore, 2007).

DNA barcoding of the standardized region of 658 bp of the mitochondrial cytochrome *c* oxidase I (COI) gene is a powerful tool for identifying species of earthworms (Chang and James, 2011, Jeratthitikul et al., 2017). DNA barcode analysis can sustain decisions in alpha taxonomy, including synonymies or resurrections of species names, and descriptions of new taxa (James et al., 2010, Decaëns et al., 2013); it can furthermore reveal the diversity of cryptic species (Novo et al., 2010, Richard et al., 2010). In this study, we re-describe the previously known cosmopolitan littoral earthworm species *P. litoralis* and we describe a new species of the genus *Pontodrilus*. Both species were collected from the same habitat, seashores of Thailand and Peninsular Malaysia. Specimens were compared morphologically and at the DNA level, using COI sequences. The illustrated descriptions and an accompanying key to species of *Pontodrilus* serve as an identification tool for taxonomists.

Materials and methods

Specimen collection and morphological examination.

Earthworms were collected throughout the coastal areas of Thailand, both the Gulf of Thailand (east coast) and the Andaman Sea (west coast), and also some parts of Peninsular Malaysia (Fig. 2.1) from January 2015 to February 2016. The specimens were carefully dug up along the sandy beaches at the level of tide using hand sorting. Adults, juveniles and cocoons were collected. The GPS coordinates of the sampling locality were recorded and the habitat type photographed. The worms were cleaned and then killed in 30% (v/v) ethanol, photographed, and fixed in 95% (v/v) ethanol for morphological and molecular studies. The descriptions of each species were made during observation under an OLYMPUS SZX16 stereomicroscope. Drawings were made of the external characters and internal organs. The specimens have been deposited in the Zoological Museum of Chulalongkorn University (CUMZ), The Natural History Museum, London, UK (NHMUK) and the Biozentrum Grindel und Zoologisches Museum, University of Hamburg, Germany (ZMH).

DNA extraction, PCR and DNA sequencing.

Details of the specimens selected for molecular analysis are provided in Table 2.1. The genomic DNA was extracted from the integument tissue of the posterior part of the earthworms using a Geneaid^{TM} DNA extraction kit. DNA was eluted in elution buffer and kept at -20 °C until use. For the phylogenetic analysis, a 658 bp region of the COI mitochondrial gene was used following its PCR amplification and sequencing. Each PCR amplification mixture consisted of 0.6–1 μl of DNA template, 2.5 μl (5 μM) each of the LCO1490 (forward) and HCO2198 (reverse) universal primers (Folmer et al., 1994), 25 μl of Ultra-Pure *Taq* PCR Master Mix with emerald dye and 19–19.4 μl of double distilled H_2O. The PCR reaction mixture was heated to 94 °C for 2 min, followed by 35 cycles of 94 °C for 1 min, 48 °C for 1 min and 72 °C for 2 min, and then followed by a final extension at 72 °C for 5 min. The amplified PCR products were checked on 1% (w/v) agarose gel electrophoresis in 0.5x TBE buffer and detected with SYBR safe DNA gel staining under UV transillumination. PCR products were purified using QIAquick purification kit (QIAGEN Inc.). Purified PCR products were sent for commercial sequencing using an Applied Biosystems Automatic Sequencer (ABI 3730XL) at Macrogen, Inc. (Korea).

Molecular analyses.

The COI sequences were aligned with MEGA version 6 (Tamura *et al.* 2013) and adjusted manually. The sequences were checked with NCBI database using the BLASTn algorithm to avoid contamination (http://ww.ncbi.nlm.nih.gov). The phylogenetic analyses were performed using maximum likelihood (ML) and Bayesian inference (BI). The ML trees were inferred with RAxML-HPC2 on XSEDE v.8.2.4 (Stamatakis, 2014) through the CIPRES Science Gateway (Miller et al., 2010), using 1000 bootstrap replicates to assess branch support. The BI tree was constructed by MrBayes v 3.2.2 (Ronquist et al., 2012) in the CIPRES Science Gateway. The analysis was run for 2 million generations (default heating parameter), and sampled every 100 generations. Then 50% of the sampled trees were discarded as a burn-in and support for nodes was defined as posterior probabilities. Moreover, a distance-based analysis was conducted in MEGA6 using the Neighbor-Joining (NJ) algorithm with distances corrected with the Kimura-2 parameter model and a bootstrap re-analysis of 1000 pseudoreplicates. In this study, *Metaphire peguana* (CUMZ 3297) and *Metaphire bahli* (CUMZ 3298) were used as outgroups (Prasankok et al., 2013).

Anatomical abbreviations.

The following abbreviations used in the figures are as appeared in Bantaowong et al. (2016) and Chanabun et al. (2017): **mp**, male pores; **fp**, female pores; **gm**, genital markings; **he**, hearts; **np**, nephridia; **sp**, spermathecal pores; **sc**, spermathecae; **sv**, seminal vesicles; **pg**, prostate glands.

Results

Systematics

Family Megascolecidae Rosa, 1891

Genus *Pontodrilus* Perrier, 1874

Type species. *Lumbricus litoralis* Grube, 1855

Diagnosis. Dorsal pores absent. Tubular prostatic duct structure to male pore on segment XVIII. Female pores in XIV. Setae lumbricine. Nephridia absent in the anterior segments. Gizzard absent; calciferous glands absent; typhlosole absent. Penial setae present or absent. Spermathecae 2–4 pairs.

Distribution. One cosmopolitan species, *P. litoralis*, occurs over a very wide range of sub-temperate and tropical coastal areas all over the world. Another littoral species, *P. primoris* in Tasmania; two species, *P. agnesae*, and *P. sinensis*, found inland in Sri Lanka and China, respectively, and the lacustrine species *P. lacustris* in Lake Wakatipu, New Zealand.

Pontodrilus litoralis (Grube, 1855)

(Figures 2.2, 2.3, 2.7B; Tables 2.2, 2.3)

Lumbricus litoralis Grube, 1855: 127–129, pl. 5, figs 5–10.

Pontoscolex arenicola Schmarda , 1861: 11–12, pl. 18, fig. 157.

Pontodrilus marionis Perrier, 1874: 1582–1586. Perrier 1881: 176–179, pls 13–17, pl. 18; figs 40–43.

Pontodrilus bermudensis Beddard, 1891: 96. Michaelsen 1894: 183–184. Beddard 1895: 469–471. Michaelsen 1910: 84–89. Michaelsen 1913: 417. Michaelsen 1921: 12. Stephenson 1923: 180–182. Gates 1926: 150. Stephenson 1931: 51.

Gates 1942: 90. Gates 1943: 99–100. Gates 1954: 240–241. Gates 1972: 47–48. Jamieson & Wampler 1979: 665–666. Sathianarayanan & Khan 2006: 139–144.

Cryptodrilus insularis Rosa, 1891: 387–288, pl. 14, fig. 11.

Pontodrilus arenae Michaelsen, 1892: 222–223, pl. 13, fig. 9. Michaelsen 1900: 181. Michaelsen 1903: 87.

Pontodrilus hesperidium Beddard, 1894: 37–40. Beddard 1895: 471. Michaelsen 1900: 182. Michaelsen 1903: 87.

Pontodrilus litoralis (Grube). Beddard 1895: 469. Michaelsen 1900: 180. Cognetti 1901: 16. Michaelsen 1910: 89–91. Easton 1984: 114–116. Jamieson 2001: 1002–1007, figs 34.1–34.2. Gobi et al. 2004: 1712, fig. 1. James et al. 2005: 1022–1023. Shen et al. 2005: 12–13, fig. 1. Blakemore 2007: S3–S8, figs 1–3. Csuzdi & Pavlicek 2009: 12. Satheeshkumar et al. 2011: 406–409, fig. 1. Blakemore et al. 2012: 302–303, fig. 3. Narayanan et al. 2014: 473–476, table 1. Oba et al. 2015: 1–10, figs 1–2. Hara et al. 2016: 5–7, fig. 1. Nguyen et al. 2016: 10–11.

Pontodrilus insularis (Rosa). Beddard 1895: 471–472. Michaelsen 1897: 173–174. Michaelsen 1900: 181–182. Michaelsen 1903: 87.

Pontodrilus michaelseni Eisen, 1895: 73–84, pl. 33, figs 24–28; pls 34–39; pl. 40, figs 74–76. Michaelsen 1900: 182. Michaelsen 1903: 87.

Pontodrilus ephippiger Rosa, 1898: 281–283, pl. 9, figs 4–5. Michaelsen 1900: 180. Michaelsen 1903: 87. Michaelsen 1907a: 187. Michaelsen 1907b: 43. Cognetti 1908: 81–82. Stephenson 1914: 256–259.

Pontodrilus matsushimensis Iizuka, 1898: 21–26, pl. 2, figs 1–6. Beddard 1899: 192–193. Michaelsen 1900: 179–180. Easton 1981: 45–46.

Pontodrilus ephippiger laysanianus Michaelsen, 1899a: 217–220. Michaelsen 1899b: 28–29, fig. 2. Michaelsen 1900: 181.

Pontodrilus matsushimensis chathamianus Michaelsen, 1899a: 220–221. Michaelsen 1900: 180. Michaelsen 1910: 91.

Pontodrilus michaelseni hortensis Eisen, 1900: 241–243.

Pontodrilus chathamensis (sic! pro *chatamianus)* Michaelsen. Benham 1901: 136–140. Lee 1952: 25.

Pontodrilus laccadivensis Beddard, 1903: 374–375.

Pontodrilus crosslandi Beddard, 1906: 561, fig. 78.

Pontodrilus albanyensis Michaelsen, 1907a: 185–187, pl. 2, fig. 26. Jackson 1931: 93.

Pontodrilus bermudensis ephippiger (Rosa). Stephenson 1915a: 145. Stephenson 1915b: 61. Stephenson 1916: 311. Stephenson 1917: 375. Stephenson 1920: 202. Jackson 1931: 95–96, pl. 14, fig. 6.

Pontodrilus albanyensis var. *cygni* Jackson, 1931: 94–95, pl. 14, figs 2, 3, 4, 9.

Plutellus (*Pontodrilus*) *matsushimensis indica* Michaelsen, 1935: 106–107.

Pontodrilus gracilis Gates, 1943: 100–102. Gates 1954: 241.

Type locality. Villa Franca on the French Riviera (Grube 1855).

Material examined.

Thailand. 112 adults and 51 juveniles, CUMZ 3462, Ao Talkoo, Laem Ngob, Trat (12° 12' 14.4" N, 102° 16' 47.1" E) on 7 August 2015. 21 adults and 56 juveniles, CUMZ 3463, Hat Laem Sing, Laem Sing, Chanthaburi (12° 28' 48.5" N, 102° 03' 46.7" E) on 7 August 2015. 8 adults and 14 juveniles, CUMZ 3464, Hat Chao Lao, Thamai, Chanthaburi (12° 33' 52.8" N, 101° 54' 25.1" E) on 8 August 2015. 38 adults and 48 juveniles, CUMZ 3465, Hat Mae Rumphueng, Ban Phe, Rayong (12° 36' 12.6" N, 101° 23' 52.2" E) on 8 August 2015. 38 adults and 8 juveniles, CUMZ 3466, Hat Nam Rin, Ban Chang, Rayong (12° 40' 31.1" N, 101° 05' 03.4" E) on 9 August 2015. 26 adults and 49 juveniles, CUMZ 3467, Ao Noi, Mueang, Prachuap Khiri Khan (11° 49' 10.7" N, 99° 47' 58.1" E) on 29 August 2015. 53 adults and 34 juveniles, CUMZ 3468, Maejo University at Chumphon, Lamae, Chumphon (9° 46' 48.8" N, 99° 08' 27.2" E) on 29 August 2015. 1 adult and 7 juveniles, CUMZ 3469, Hat Pak Meng, Sikao, Trang (7° 30' 14.3" N, 99° 19' 6.8" E) on 30 August 2015. 89 adults and 30 juveniles, CUMZ 3470, Ao Noon, La Ngu, Satun (6° 50' 16.0 "N, 99° 45' 37.2" E) on 30 August 2015. 131 juveniles, CUMZ 3471, Khrua Suan Son Restaurant, Pak Phanang, Nakhon Si Thammarat (8° 10' 51.8" N, 100° 17' 30.4" E) on 1 September 2015. 1 adult and 18 juveniles, CUMZ 3472, Ban Koh Kaew Naruemit, Pak Phanang, Nakhon Si Thammarat (8° 14' 28.1" N, 100° 16' 41.2" E) on 3 September 2015. 1 adult and 29 juveniles, CUMZ 3473, Laem Talumphuk, Pak Phanang, Nakhon Si Thammarat (8° 19' 19.7" N, 100° 15' 19.9" E) on 3

September 2015. 7 adults and 60 juveniles, CUMZ 3474, Pak Mae Nam Tepa, Sichon, Nakhon Si Thammarat (8° 55' 51.2" N, 99° 54' 47.5" E) on 3 September 2015. 45 juveniles, CUMZ 3475, Klong Bang Siap, Patiew, Chumphon (10° 39' 29.9" N, 99° 18' 39.1" E) on 4 September 2015. 16 adults and 23 juveniles, CUMZ 3476, Hat Nang Kam, Don Sak, Surat Thani (9° 18' 53.7" N, 99° 45' 37.9" E) on 2 December 2015. 170 adults and 32 juveniles, CUMZ 3477, Hat Lamai, Koh Samui, Surat Thani (9° 28' 16.3" N, 100° 03' 10.3" E) on 3 December 2015. 42 adults and 16 juveniles, CUMZ 3478, Koh Samui Hospital, Koh Samui, Surat Thani (9° 31' 17.3" N, 99° 56' 08.5" E) on 3 December 2015. 24 adults and 9 juveniles, CUMZ 3479, Hat Sai Ngoen, Klong Yai, Trat (12° 02' 43.5" N, 102° 45' 01.8" E) on 15 December 2015. 16 adults and 36 juveniles, CUMZ 3480, Hat Ban Chuen, Klong Yai, Trat (11° 52' 56.9" N, 102° 47' 58.7" E) on 15 December 2015. 41 adults and 1 juvenile, CUMZ 3481, Wat Huang Som, Klong Yai, Trat (11° 50' 47.4" N, 102° 49' 31.9" E) on 15 December 2015. 86 adults and 29 juveniles, CUMZ 3482, Lung Chalerm Bungalow Koh Chang, Koh Chang, Trat (12° 05' 52.4" N, 102° 21' 27.9" E) on 16 December 2015. 81 adults and 39 juveniles, CUMZ 3483, Laem Sing, Laem Sing, Chanthaburi (12° 30' 59.0" N, 102° 01' 37.7" E) on 17 December 2015. 25 adults and 13 juveniles, CUMZ 3484, Laem Mae Nok Kaew, Na Yai Arm, Chanthaburi (12° 40' 46.7" N, 101° 48' 41.2" E) on 17 December 2015. 38 adults and 5 juveniles, CUMZ 3485, Wat Tanon Kaprao, Klaeng, Rayong (12° 41' 06.1" N, 101° 40' 27.2" E) on 17 December 2015. 6 adults and 6 juveniles, CUMZ 3486, Laem Mae Pim, Klaeng, Rayong (12° 38' 37.6" N, 101° 38' 05.1" E) on 17 December 2015. 23 adults and 4 juveniles, CUMZ 3487, Ao Dong Tai, Sattahip, Chonburi (12° 38' 46.6" N, 100° 55' 47.4" E) on 18 December 2015. 80 adults and 19 juveniles, CUMZ 3488, Hat Jomtien, Pattaya, Chonburi (12° 50' 25.1" N, 100° 54' 18.3" E) on 18 December 2015. 42 adults and 6 juveniles, CUMZ 3489, Hat Na Klua, Pattaya, Chonburi (12° 58' 08.0" N, 100° 54' 04.4" E) on 18 December 2015. 53 adults and 5 juveniles, CUMZ 3490, Hat Bang Saen, Mueang, Chonburi (13° 15' 10.9" N, 100° 55' 45.4" E) on 18 December 2015. 28 adults and 21 juveniles, CUMZ 3491, Bo Nok, Kui Buri, Prachuap Khiri Khan (11° 59' 47.8" N, 99° 51' 50.2" E) on 9 January 2016. 22 adults and 7 juveniles, CUMZ 3492, Hat Sang Arun, Tub Sakae, Prachuap Khiri Khan (11° 34' 10.9" N, 99° 39' 48.7" E) on 9 January 2016. 34 adults and 28 juveniles, CUMZ 3493, Ban Ma Phaw Resort, Bang

Saphan Noi, Prachuap Khiri Khan (11° 08' 08.1" N, 99° 29' 11.9" E) on 9 January 2016. 10 adults and 5 juveniles, CUMZ 3494, Hat Koey, Kaper, Ranong (9° 37' 26.7" N, 98° 28' 08.6" E) on 10 January 2016. 16 adults, CUMZ 3495, Hat Manee Naka, Kaper, Ranong (9° 28' 40.4" N, 98° 26' 02.8" E) on 10 January 2016. 2 adults, CUMZ 3496, Wat Tet Tum Nava, Thai Muang, Phangnga (8° 21' 21.9" N, 98° 15' 29.9" E) on 10 January 2016. 36 adults and 4 juveniles, CUMZ 3623, Ao Thalen, Mueang, Krabi (8°09'58.0"N 98°44'53.0"E) on 11 January 2016. 8 juveniles, CUMZ 3624, Hat Yao, Nuea Klong, Krabi (7° 58' 49.4" N, 98° 56' 46.6" E) on 11 January 2016. 10 juveniles, CUMZ 3625, Hat Samran, Hat Samran, Trang (7° 14' 02.9" N, 99° 32' 19.4" E) on 12 January 2016. 52 adults and 30 juveniles, CUMZ 3626, Hat Bo Chet Look, La Ngu, Satun (6° 53' 32.6" N, 99° 41' 12.5" E) on 12 January 2016. 20 adults and 10 juveniles, CUMZ 3627, Hat Kao Seng, Mueang, Songkhla (7° 10' 58.9" N, 100° 36' 59.5" E) on 12 January 2016. 17 adults and 3 juveniles, CUMZ 3628, Hat Tumbon Kao Roob Chang, Mueang, Songkhla (7° 09' 06.5" N, 100° 38' 25.0" E) on 12 January 2016. 1 adult and 61 juveniles, CUMZ 3629, Hat Na Tub, Jana, Songkhla (7° 04' 26.3" N, 100° 41' 34.6" E) on 12 January 2016. 19 adults and 51 juveniles, CUMZ 3630, Hat Sai Kaew, Singha Nakhon, Songkhla (7° 15' 13.1" N, 100° 32' 53.1" E) on 13 January 2016. 2 adults and 1 juvenile, CUMZ 3631, Hat Ma Ha Rat, Satingpra, Songkhla (7° 28' 10.1" N, 100° 26' 50.1" E) on 13 January 2016. 40 adults and 12 juveniles, CUMZ 3632, Wat Hua Rawa, Ranod, Songkhla (7° 43' 30.3" N, 100° 22' 55.4" E) on 13 January 2016. 1 adult, CUMZ 3633, Klong Loe, Tha Sala, Nakhon Si Thammarat (8° 45' 02.0" N, 99° 56' 13.4" E) on 14 January 2016. 119 adults and 81 juveniles, CUMZ 3634, Klong Klai, Tha Sala, Nakhon Si Thammarat (8° 46' 36.1" N, 99° 56' 06.4" E) on 14 January 2016. 67 adults and 11 juveniles, CUMZ 3635, Laem Sai, Chaiya, Surat Thani (9° 25' 26.2" N, 99° 17' 05.5" E) on 14 January 2016. 49 adults and 26 juveniles, CUMZ 3636, Klong Pak Nam Thamueng, Tha Chana, Surat Thani (9° 33' 15.9" N, 99° 12' 39.2" E) on 14 January 2016. 63 adults and 22 juveniles, CUMZ 3637, Hat Sam Roi Yot, Sam Roi Yot, Prachuap Khiri Khan (12° 16' 50.6" N, 99° 58' 23.8" E) on 15 January 2016. 50 adults and 93 juveniles, CUMZ 3638, Hat Cha-am, Cha-am, Phetchaburi (12° 49' 36.2" N, 99° 59' 40.3" E) on 15 January 2016.

Malaysia. 5 adults and 54 juveniles, CUMZ 3639, Tanjung Rhu, Sepang, Selangor (2° 38' 07.2" N, 101° 37' 26.8" E) on 21 January 2016. 154 adults and 62 juveniles, CUMZ 3640, Jalan Klebang, Besar Dataran1, Melaka (2° 13' 13.7" N, 102° 11' 09.3" E) on 22 January 2016. 71 adults and 85 juveniles, CUMZ 3641, Tanjung Kling, Pantai Kundur, Melaka (2° 14' 54.9" N, 102° 08' 15.3" E) on 22 January 2016. 64 adults and 148 juveniles, CUMZ 3642, Kampung Pasir Putih, Pasir Gudang, Johor (1° 26' 16.8" N, 103° 55' 32.9" E) on 23 January 2016. 149 adults and 65 juveniles, CUMZ 3643, Highway Senai Desaru, Sungai Johor, Johor (1° 32' 02.6" N, 104° 01' 47.8" E) on 23 January 2016. 102 adults and 57 juveniles, CUMZ 3644, Tanjung Balau, Desaru, Johor (1° 36' 55.8" N, 104° 15' 26.1" E) on 23 January 2016. 59 adults and 22 juveniles, CUMZ 3645, Sungai Jemaluang, Mersing, Johor (2° 23' 03.5" N, 103° 52' 40.8" E) on 23 January 2016. 95 adults and 96 juveniles, CUMZ 3646, Kampung Air Puteri, Mersing, Johor (2° 24' 56.0" N, 103° 51' 32.5" E) on 24 January 2016. 33 adults and 42 juveniles, CUMZ 3647, Kampung Janglau, Pantai Kuala Rompin, Pahang (2° 43' 17.2" N, 103° 34' 24.9" E) on 24 January 2016. 20 juveniles, CUMZ 3648, Kampung Pandon, Pekan, Pahang (3° 05' 40.8" N, 103° 26' 26.2" E) on 24 January 2016. 83 adults and 10 juveniles, CUMZ 3649, Kampung Tanjung Lumpur, Kuantan, Pahang (3° 48' 25.0" N, 103° 20' 29.4" E) on 25 January 2016. 38 adults and 46 juveniles, CUMZ 3650, Pantai Chendor, Cukai, Terengganu (4° 10' 36.7" N, 103° 25' 15.6" E) on 25 January 2016. 16 adults and 7 juveniles, CUMZ 3651, Teluk Bidara, Sungai Dungun, Dungun, Terengganu (4° 47' 03.9" N, 103° 25' 39.0" E) on 25 January 2016. 79 adults and 22 juveniles, CUMZ 3652, Kampung Batin Seberang Takir, Kuala Terengganu, Terengganu (5° 20' 42.7" N, 103° 07' 40.7" E) on 26 January 2016. 52 adults and 4 juveniles, CUMZ 3653, Sungai Besut Kampung Nail, Besut, Terengganu (5° 49' 25.5" N, 102° 33' 24.7" E) on 26 January 2016. 82 adults and 26 juveniles, CUMZ 3654, Pantai Melawi, Sungai Ger Bachok, Kelantan (5° 59' 35.7" N, 102° 25' 54.2" E) on 26 January 2016. 49 adults and 27 juveniles, CUMZ 3655, Tanjung Dawai, Merbok, Kedah (5° 40' 47.4" N, 100° 22' 12.8" E) on 28 January 2016. 107 adults and 46 juveniles, CUMZ 3656, Tanjung Bungah, Pulau Pinang (5° 28' 06.7" N, 100° 16' 41.0" E) on 28 January 2016.

Description of new material. Length 28–136 mm, diameter 1–5 mm, body cylindrical, 76–128 segments. Body colour red to pink, pale brown around clitellum (newly collected specimens after placement in 30% (v/v) ethanol for narcotization). Prostomium epilobous. Clitellum saddle in XIII–XVII or XVIII, setae present. Setae lumbricine, ab absent on XVIII, aa > bc, ab < cd, aa > cd, dd > aa. Female pores paired in setal line b on XIV. Male pores minute and superficial on XVIII, close to sites of b setae, penial setae absent (or not found). Spermathecal pores minute, intersegmental, two pairs in 7/8 and 8/9, ventrolateral, in line with setae b. Dorsal pores totally absent. Genital markings large, medio-ventral, transversely oval across 19/20 (some in 20/21), center depressed.

Septa 5/6–12/13 thickened. Gizzard and calciferous glands absent. Intestine beginning in XVI or XVII. Intestinal typhlosole absent. Esophageal hearts seen in VI–XIII. Nephridia absent in the anterior segments and begin in XIII or XV with a pair of coiled tubules, smaller after clitellum. Spermathecae two pairs in VIII and IX, spermathecal diverticula slender, narrower at the junction with ampulla. Tubular prostate single pair, muscular duct curved and narrowed at each end, coil in XVIII. Ovaries present in XIII. Testes funnels in X and XI, seminal vesicles paired and large in XI–XII. Accessory glands absent.

Distribution. Worldwide, sub-tropical and tropical coastal areas. Easton (1984) and Blakemore (2002) provide distribution records; Figure 2.1 shows the locations of our finds of the species in Thailand and Peninsular Malaysia.

Habitat. Sandy beaches at the intertidal zone, mangrove swamps, coastal salt marsh of estuaries and brackish water. Found in the top soil to 20 cm depth where the salinity ranges from 1–33 ppt.

Remarks. *Pontodrilus litoralis* shows some variations in body dimensions, patterns of the genital markings, and shape of the spermathecal diverticula, especially if the descriptions of the many junior synonyms and redescriptions of the nominal species are taken into account. Characters from synonyms of *P. litoralis* in the Old World territory, including Australia, are presented in Table 2.2. Variations in body dimensions were reported by Jamieson (2001), Satheeshkumar et al. (2011), Shen et al. (2011) and Narayanan et al. (2014); the values are as follows: Jamieson (2001): length 32–120 mm, width 2–4mm, segments 78–120; Satheeshkumar et al. (2011) and Narayanan et al. (2014): length 50–130 mm, clitellum width 2–4mm, segments 81–115; Shen et al. (2011): length 50–130 mm, diameter 1–2 mm, segment number 81–115. Considering genital markings, Jamieson (2001) notes the following variations, based on specimens from Peel Island, and including data of Jackson (1931), Gates (1972) and Easton (1984): genital markings in several furrows of 11/12–20/21. Specimens described by Jamieson (2001) from Nornalup, Western Australia, have markings at 17/18, at the anterior midventral margin of XVIII, at 18/19, or midventral at 18/19 and 19/20. Satheeshkumar et al. (2011); Narayanan et al. (2014) observed large genital markings medio-ventral at 19/20 in specimens from India; specimens of *P. litoralis* from coasts of the Penghu Island and the southwestern Taiwan have one large genital marking, transversely oval across 19/20, center depressed (Shen et al. 2011). Considering the diverticulum, Jamieson (2001) described it as digitiform to club-shaped, whereas in specimens from India and Taiwan it is slender. The specimens of *P. litoralis* investigated in this study are within the variation range as reported in the literature: body length 28–136 mm, diameter 1–5 mm, segments 76–128; genital markings large, medio-ventral, transversely oval across 19/20 (some in 20/21). We include drawings (Fig. 2.2) and colour plates (Fig. 2.3) of selected specimens.

Pontodrilus longissimus Seesamut & Panha, sp. n.

(Figures 2.4, 2.5, 2.7A; Table 2.3)

Type specimens. Holotype: CUMZ 3670 (Fig. 2.4), Hat Pak Meng, Sikao, Trang, Thailand (7° 30' 14.3" N, 99° 19' 06.8" E, 7 m above mean sea level), coll. T. Seesamut, C. Sutcharit, R. Srisonchai & A. Pholyotha, 30 Aug 2015. Paratypes: CUMZ 3671, 64 adults and 37 juveniles; NHMUK, 2 adults; ZMH, 2 adults; same collection data as holotype.

Other materials examined.

Thailand. 23 adults and 57 juveniles, CUMZ 3657, Hat Chao Lao, Thamai, Chanthaburi (12° 33' 52.8" N, 101° 54' 25.1" E) on 8 August 2015. 4 juveniles, CUMZ 3658, Ban Koh Kaew Naruemit, Pak Phanang, Nakhon Si Thammarat (8° 14' 28.1" N, 100° 16' 41.2" E) on 3 September 2015. 17 juveniles, CUMZ 3659, Klong Bang Siap, Patiew, Chumphon (10° 39' 29.9" N, 99° 18' 39.1" E) on 3 September 2015. 33 adults and 67 juveniles, CUMZ 3660, Hat Mai Khao, Talang, Phuket (8° 05' 47.9" N, 98° 17' 55.5" E) on 5 December 2015. 30 adults and 40 juveniles, CUMZ 3661, Nang Thong Bay Resort, Takua Pa, Phangnga (8° 38' 43.4" N, 98° 14' 50.1" E) on 5 December 2015. 3 adults and 12 juveniles, CUMZ 3662, Hat Bang Sak, Takua Pa, Phangnga (8° 47' 03.4" N, 98° 15' 46.1" E) on 5 December 2015. 5 adults and 11 juveniles, CUMZ 3663, Hat Sai Ngoen, Klong Yai, Trat (12° 02' 43.5" N, 102° 45' 01.8" E) on 15 December 2015. 6 adults and 33 juveniles, CUMZ 3664, Hat Koey, Kaper, Ranong (9° 37' 26.7" N, 98° 28' 08.6" E) on 10 January 2016. 74 juveniles, CUMZ 3665, Hat Yao, Nuea Klong, Krabi (7° 58' 49.4" N, 98° 56' 46.6" E) on 11 January 2016. 20 juveniles, CUMZ 3666, Hat Samran, Hat Samran, Trang (7° 14' 02.9" N, 99° 32' 19.4" E) on 12 January 2016. 29 adults and 45 juveniles, CUMZ 3667, Hat Bo Chet Look, La Ngu, Satun (6° 53' 32.6" N, 99° 41' 12.5" E) on 12 January 2016.

Malaysia. 10 juveniles, CUMZ 3668, Pantai Chendor, Cukai, Terengganu (4° 10' 36.7" N, 103° 25' 15.6" E) on 25 January 2016. 1 juvenile, CUMZ 3669, Pantai Melawi, Sungai Ger Bachok, Kelantan (5° 59' 35.7" N, 102° 25' 54.2" E) on 26 January 2016.

Description of holotype. Length 161 mm, diameter 3.47 mm at segment X, 3.10 at segment XX, 3.90 mm at clitellum, body cylindrical with 183 segments. The body colour ranges from red to pink and pale brown around clitellum in newly collected specimens after placement in 30% (v/v) ethanol for narcotization. Prostomium epilobous. Clitellum saddle in XIII–XVII, setae present. Setae lumbricine, ab absent on XVIII, aa:ab:bc:cd:dd = 1.8:0.3:1.6:1.1:5.0 in XX. Female pores paired, medio-ventral in XIV. Male pores paired in XVIII, distance between male pores 2 mm, penial setae absent (or not found). Spermathecal pores intersegmental two pairs in 7/8 and 8/9, ventrolateral, in line with setae b, distance between spermathecal pores 2 mm. Dorsal pores absent. Genital markings present at intersegmental boundary in 17/18 and 18/19.

Septa 4/5–12/13 thickened. Gizzard and calciferous glands absent or not developed. Intestine enlarged from XV. Intestinal typhlosole absent. Esophageal hearts eight pairs in VI–XIII. No nephridia distinguishable in first fourteen segments, beginning in XV with a pair of coiled tubules. Two pairs of spermathecae in VIII and IX. Ampulla as large ovoid sac, without diverticulum. Two pairs of seminal vesicles in XI and XII. No mature (iridescent) sperm observed in spermathecae or seminal vesicles. Ovary in XIII. Tubular prostate single pair, muscular duct in XVIII. Accessory glands absent.

Variation. Holotype measures 161 mm body length with 183 segments. Body length of paratypes and non-types (adult specimens) range in size from 125–165 mm, with 160–191 segments. Clitellum in XIII, XIV–XVII. Intestine origin in XV–XVII. Nephridia from XIII or XV.

Distribution. The new species is known from the type locality and along the coastal areas of Thailand, both the Gulf of Thailand and the Andaman Sea, and also some parts of Peninsular Malaysia (Fig. 2.1).

Habitat. Found in the coastal salt marsh of estuaries at about 10–30 cm depth, in mud with a high content of organic matter and a salinity ranges from 1–33 ppt.

Etymology. The specific epithet "*longissimus*" in Latin means "the longest". This name refers to the length of this species compared to the other species in this genus.

Diagnosis. Length 125–165 mm, 160–191 segments. Setae lumbricine, penial setae absent. Prostomium epilobous. Dorsal pores totally absent. Clitellum saddle shape, in XIII, XIV–XVII. Male pores paired in XVIII; female pores paired in XIV. Genital markings present in 17/18 and 18/19. Spermathecae as large ovoid sacs in VIII and IX, without diverticulum. Two pairs of seminal vesicles in XI and XII. Ovary in XIII. Esophageal hearts eight pairs in VI–XIII. Tubular prostates in XVIII.

Remarks. The new species, *P. longissimus* **sp. n.**, differs from the cosmopolitan littoral species *P. litoralis*, based on the specimens from Thailand and Peninsular Malaysia, by differences in segment number (160–191 and 76–128 for *P. longissimus* **sp. n.** and *P. litoralis*, respectively), and body length (125–165 mm and 28–136 mm for *P. longissimus* **sp. n.** and *P. litoralis*, respectively). Additionally, they are easily distinguished by the spermathecal diverticulum, absent in the new species but present in all other currently accepted species of the genus. *Pontodrilus litoralis* has a long and slender diverticulum, *P. lacustris* has a small globular diverticulum, *P. agnesae* has a spindle or club-shaped diverticulum, *P. primoris* has a short diverticulum relative to the ampulla. Among the many descriptions of *P. litoralis* and its synonyms, only *P. insularis* (Rosa, 1891) from Aru Islands, Indonesia (“Insel Aru”) is without spermathecal diverticula. Michaelsen (1897) identified tentatively two specimens from Sri Lanka (“Ceylon”) as *P. insularis* but conjectured that the absence of diverticula may be due to the fact that his and Rosa's specimens were subadult without clitellum, and he suggested synonymy with *P. bermudensis* Beddard, 1891, considering their overall similarity. Beddard (1895) also noted that the specimens were immature. However, Rosa (1898) maintained the possibility that the absence of diverticula is a taxonomic character and not caused by the immature state of the specimens, since “even in a series of sections no traces could be found of an organ which in the adult reaches so great a development” (id.: 283). Nevertheless, Michaelsen (1910) established the synonymy with *P. bermudensis*. Later, Easton (1984) synonymized *P. bermudensis* with *P. litoralis*. Even though Michaelsen's decision was not questioned in the subsequent taxonomic literature, the possibility remains that Rosa's species is distinct from *P. litoralis* (see Rosa 1898) and that it is characterized by the absence of diverticula. *Pontodrilus insularis* as originally described is much smaller than *P. longissimus* **sp. n.** (length 50 mm, diameter 3 mm, ca. 100 segments) and the first nephridia are found in XIII. *Pontodrilus longissimus* **sp.**

n. is easily distinguished from other *Pontodrilus* species by the morphometric characteristics shown in Table 2.3. Cocoons, presumably of this earthworm, were collected from the littoral zone of the Andaman Sea at Hat Bo Chet Look, La Ngu, Satun. The cocoons are lemon-shaped, yellowish green in colour (Fig. 2.5C).

Molecular analysis

The aligned COI sequence data matrix contained 22 specimens (excluding outgroups) with a length of 658 base pairs. Of these, 153 nucleotides were parsimony informative and 175 were variable sites. The majority consensus tree of the Bayesian analysis of the combined dataset is shown in Fig. 2.6, but the topology was congruent with the ML analysis. Phylogenetic trees based on the NJ, ML and BI methods were divided into two well-separated clades, which agree with both species of *Pontodrilus* by 100% bootstrap values and 1.0 posterior probability supports, while the NJ tree (not shown) shows different topology within each species from ML/BI trees. The mean intraspecific variations for COI in the recognized species *P. litoralis* was 7.34% and *P. longissimus* **sp. n.** was 2.15%. Mean interspecific distance between both species was 17.77%. Comparison of *P. longissimus* sequences with published sequences of *P. litoralis* showed a similar divergence value (data not shown here). Interspecific distance between *P. longissimus* **sp. n.** and the outgroups was 18.16%.

Discussion

The cosmopolitan littoral species *Pontodrilus litoralis* is widely distributed on shorelines in the tropics and warmer parts of continents and islands in all the world's oceans; further species of the genus are lacustrine in New Zealand (*P. lacustris*), terrestrial in Sri Lanka (*P. agnesae*), terrestrial or lacustrine in China (*P. sinensis*), and littoral in Tasmania, Australia (*P. primoris*) (Easton, 1984, Blakemore, 2002, 2007). The new littoral species described here was found in eleven locations in Thailand and two locations in Peninsular Malaysia. With respect to other *Pontodrilus* species, *P. longissimus* **sp. n.** is the longest (Fig. 2.7), and has the highest number of segments.

Apart from the body dimensions, this new species can obviously be distinguished from the others by absence of the diverticulum.

The littoral earthworms from Thailand and Peninsular Malaysia presented in this paper, *P. longissimus* **sp. n.** and *P. litoralis*, occurred mostly on marine sandy coastal areas, which is the ecotone between terrestrial and marine habitats, in coastal salt marshes with a high organic matter and salinity ranges from 1–33 ppt. *P. longissimus* **sp. n.** was mainly found in habitats with substrates on the surface layer containing muddy sand, while *P. litoralis* occurred at habitats containing sandy mud. At Hat Pak Meng, Sikao, Trang, Thailand (type locality), *P. longissimus* **sp. n.** was found in the estuary at a salinity of 28 ppt and at 10–20 cm depth in muddy sand. Curiously, we did not detect casts on the soil surface, yet there were some grass-like plants and some small shrubs (*Ipomoea pes-caprae* (L.) R.Br. and *Sesuvium portulacastrum* (L.) L.) growing on the soil surface. In addition, both species were found under roots of trees, leaf litter and areas that had organic matter near the shore of the sea. At some localities (Fig. 2.1), *P. longissimus* **sp. n.** was found co-existing with the cosmopolitan littoral species *P. litoralis.* Those areas might contain a high-enough load of organic matter as a food source for both species of earthworms to survive without competition. Even though we could find these earthworms in the same areas, *P. litoralis* was found at the beach surface (under seaweed and debris), while *P. longissimus* **sp. n.** was found deeper than *P. litoralis*; at a depth of more than 10 cm.

The molecular analysis revealed a high interspecific genetic distance between *P. litoralis* and *P. longissimus* **sp. n.**, which is comparable to other earthworm DNA barcoding studies. Huang et al. (2007) indicated that the COI sequence divergence between species in earthworms in their study was greater than 15% in all cases. Furthermore, Chang and James (2011) concluded that the consensus among phylogenetic studies of earthworms showed that any two specimens with a Kimura 2-parameter distance of COI higher than 15% can be unambiguously assigned to two different species. According to Szederjesi et al. (2017), by comparing the molecular and morphological data, sequence divergence of 17% or higher can be treated as interspecific, but only when morphological differences have been detected. Thus, the DNA sequences of the mitochondrial COI region confirm the taxonomic status of *P. longissimus* **sp. n.** as a species different from *P. litoralis*. Overall, the combined evidence from both morphological and molecular results support *P. longissimus* **sp. n.** as a new species.

Key to species of earthworm genus *Pontodrilus* Perrier, 1874

1 Four pairs of spermathecae *P. lacustris* (Benham, 1903)

- Two pairs of spermathecae 2

2 Four pairs of seminal vesicles *P. sinensis* Chen & Zhifang, 1977

- Two pairs of seminal vesicles 3

3 Seminal vesicles at IX and XII *P. agnesae* Stephenson, 1915

- Seminal vesicles at XI and XII 4

4 Spermathecal diverticula absent *P. longissimus* **sp. n.**

- Spermathecal diverticula present 5

5 Spermathecal diverticula longer than 1/2 ampulla length *P. litoralis* (Grube, 1855)

- Spermathecal diverticula shorter than 1/2 ampulla length *P. primoris* Blakemore, 2000

Acknowledgements

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Table 2.1 Specimens used in the molecular analysis with sampling localities and GenBank accession numbers, including outgroups. The abbreviations used in the data analysis are shown (Abbr.)

Abbr.	Species	Collection locality	GenBank ID (COI)
E1	*P. litoralis*	Lung Chalerm Bungalow Koh Chang, Koh Chang, Trat, Thailand	MF488722
E2	*P. longissimus* **sp. n.**	Hat Sai Ngoen, Klong Yai, Trat, Thailand	MF488723
E3	*P. longissimus* **sp. n.**	Hat Chao Lao, Thamai, Chanthaburi, Thailand	MF488724
E4	*P. litoralis*	Hat Jomtien, Pattaya, Chonburi, Thailand	MF488725
E5	*P. litoralis*	Ao Talkoo, Laem Ngob, Trat, Thailand	MF488726
S1	*P. litoralis*	Hat Cha-am, Cha-am, Phetchaburi, Thailand	MF488727
S2	*P. longissimus* **sp. n.**	Klong Bang Siap, Patiew, Chumphon, Thailand	MF488728
S3	*P. litoralis*	Maejo University at Chumphon, Lamae, Chumphon, Thailand	MF488729
S5	*P. litoralis*	Hat Kao Seng, Mueang, Songkhla, Thailand	MF488730
S7	*P. litoralis*	Hat Bo Chet Look, La Ngu, Satun, Thailand	MF488731
S8	*P. longissimus* **sp. n.**	Hat Pak Meng, Sikao, Trang, Thailand **(Holotype)**	MF488732
S9	*P. longissimus* **sp. n.**	Hat Mai Khao, Talang, Phuket, Thailand	MF488733
S10	*P. longissimus* **sp. n.**	Hat Koey, Kaper, Ranong, Thailand	MF488734
S11	*P. litoralis*	Hat Koey, Kaper, Ranong, Thailand	MF488735
M1	*P. litoralis*	Tanjung Bungah, Pulau Pinang, Malaysia	MF488736
M2	*P. litoralis*	Tanjung Dawai, Merbok, Kedah, Malaysia	MF488737
M3	*P. litoralis*	Jalan Klebang, Besar Dataran1, Melaka, Malaysia	MF488738
M4	*P. litoralis*	Kampung Pasir Putih, Pasir Gudang, Johor, Malaysia	MF488739
M5	*P. longissimus* **sp. n.**	Pantai Chendor, Cukai, Terengganu, Malaysia	MF488740
M6	*P. litoralis*	Pantai Chendor, Cukai, Terengganu, Malaysia	MF488741
M7	*P. longissimus* **sp. n.**	Pantai Melawi, Sungai Ger Bachok, Kelantan, Malaysia	MF488742
M8	*P. litoralis*	Pantai Melawi, Sungai Ger Bachok, Kelantan, Malaysia	MF488743
	Metaphire peguana	Outgroup specimen	KC404843.1
	Metaphire bahli	Outgroup specimen	KC404844.1

Table 2.2 Comparison of *Pontodrilus litoralis* from Thailand and Peninsular Malaysia with nominal species of *Pontodrilus* described from that area and now in synonymy with *P. litoralis*.

Character	*P. litoralis* (Grube, 1855)*	*Cryptodrilus insularis* Rosa, 1891	*P. ephippiger* Rosa, 1898	*P. matsushimensis* Iizuka,1898	*P. laccadivensis* Beddard, 1903	*P. albanyensis* Michaelsen, 1907a
Length (mm)	28–136	50	43–47	90–110	90–110	50
Segment	76–128	100	85–100	100–105	?	86
Clitellum	XIII–XVII, XVIII	?	XIII–XVII	XII–XVII	XIII–XVII	?
Spermathecal pore	7/8–8/9	7/8–8/9	7/8–8/9	7/8–8/9	7/8–8/9	7/8–8/9
Genital marking	19/20 or 20/21	?	XIX–XX	XIX–XX	XII/XIII, XIV; XI/XII, XII/XIII	17/18, 18/19
First nephridia	XIII	XIII	?	XIII	XIII	?
Last heart	XIII	?	XIII	XIII	XIII	?
Intestinal origin	XVI or XVII	?	XVIII	XIV	?	?
Spermathecae	VIII–IX	VIII–IX	VIII–IX	VIII–IX	?**	?**
Diverticulum	slender	absent	narrow tubular	tubular	tubular	tubular
Type locality	Villa Franca on the French Riviera, France	Aru Island, Indonesia	Christmas Island, Australia	Matsushima Bay, Rikuzen, Japan	Laccadive and Maldive Islands	Princess Royal Harbor, Australia

Table 2.3 Comparison of the morphological characteristics of *P. longissimus* **sp. n.** with other species of the genus.

Character	*P. litoralis** (Grube, 1855)	*P. lacustris* (Benham, 1903)	*P. agnesae* Stephenson, 1915	*P. sinensis* Chen & Zhifang, 1977	*P. primoris* Blakemore, 2000	*P. longissimus* **sp. n.***
Length (mm)	28–136	35–40	65	30.5–41	60–120 (120)	125–165
Segments	76–128	85–90	116	64–94	100–150 (118)	160–191
Clitellum	XIII–XVII, XVIII	½XIII–XIV	½XIII–½XVII	XIII–XVIII	absent	XIII, XIV–XVII
Penial setae	absent	present	present	?	absent	absent
Spermathecal pore	7/8–8/9	VI–IX	7/8–8/9	7/8–8/9	7/8–8/9	7/8–8/9
Genital marking	19/20 or 20/21	?	absent	?	XIV–XVIII, 18/19–19/20	17/18–18/19
First nephridia	XIII	XV	XII or XIII	XIII	XIV	XIII
Seminal vesicle	XI and XII	IX and XI	IX and XII	IX–XII	XI and XII	XI and XII
Intestinal origin	XVI or XVII	XIII	?	XIV or XV	XV	XV or XVI or XVII
Spermathecae	VIII–IX	VI–IX	?	?	VIII–IX	VIII–IX
Diverticulum	slender	globular	club-shaped	?	short	absent
Type locality	Villa Franca, French Riviera, France	Lake Wakatipu, South Island, New Zealand	Sri Lanka	Kunming, Yunnan, China	Tasmania, Australia	Hat Pak Meng, Sikao, Trang, Thailand

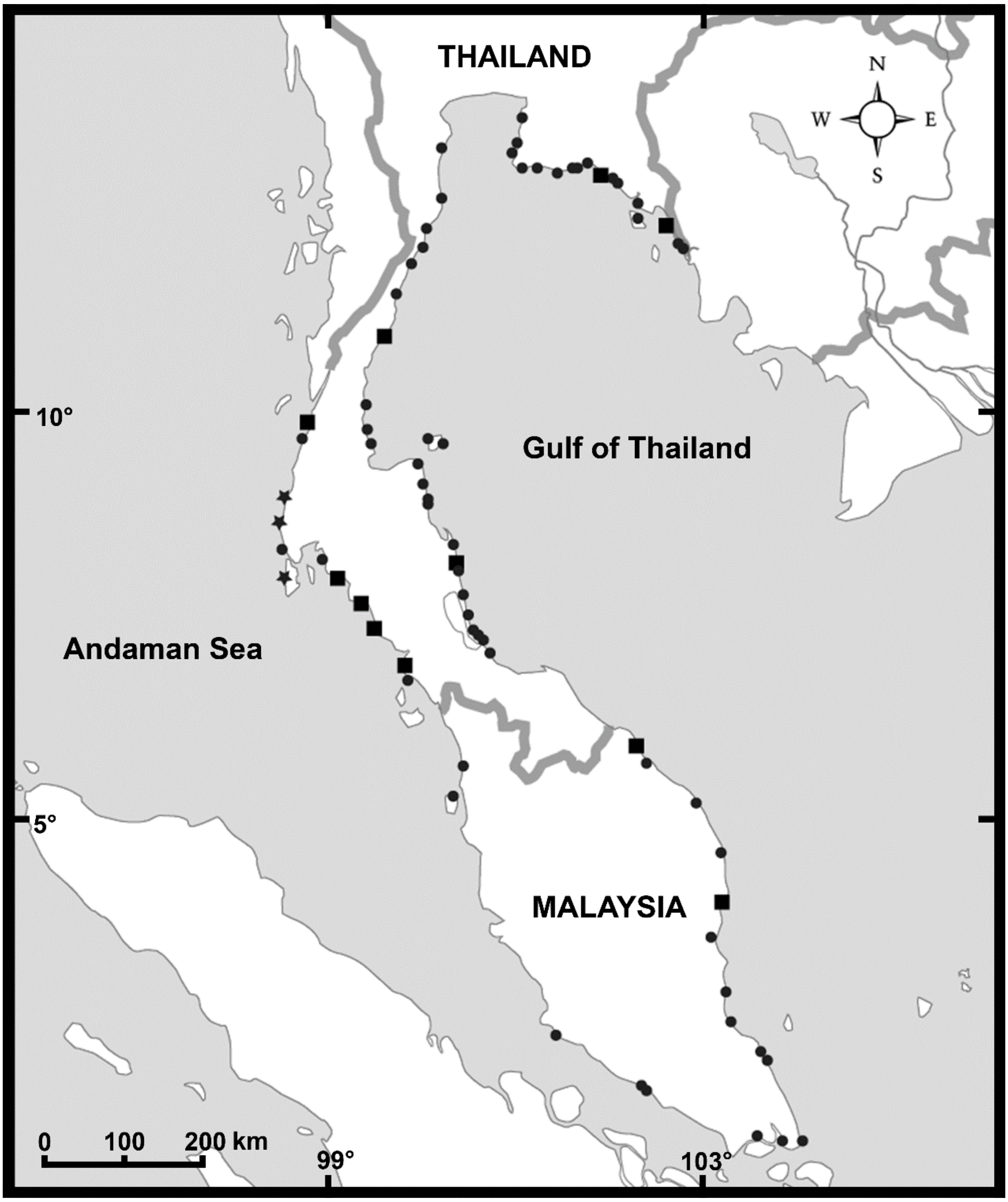

Figure 2.1 Map showing the localities of sampling sites for *Pontodrilus.* Circle and star symbols represent the localities of *Pontodrilus litoralis* and *Pontodrilus longissimus* sp. n., respectively, while square symbols are the localities where both *Pontodrilus* species were found

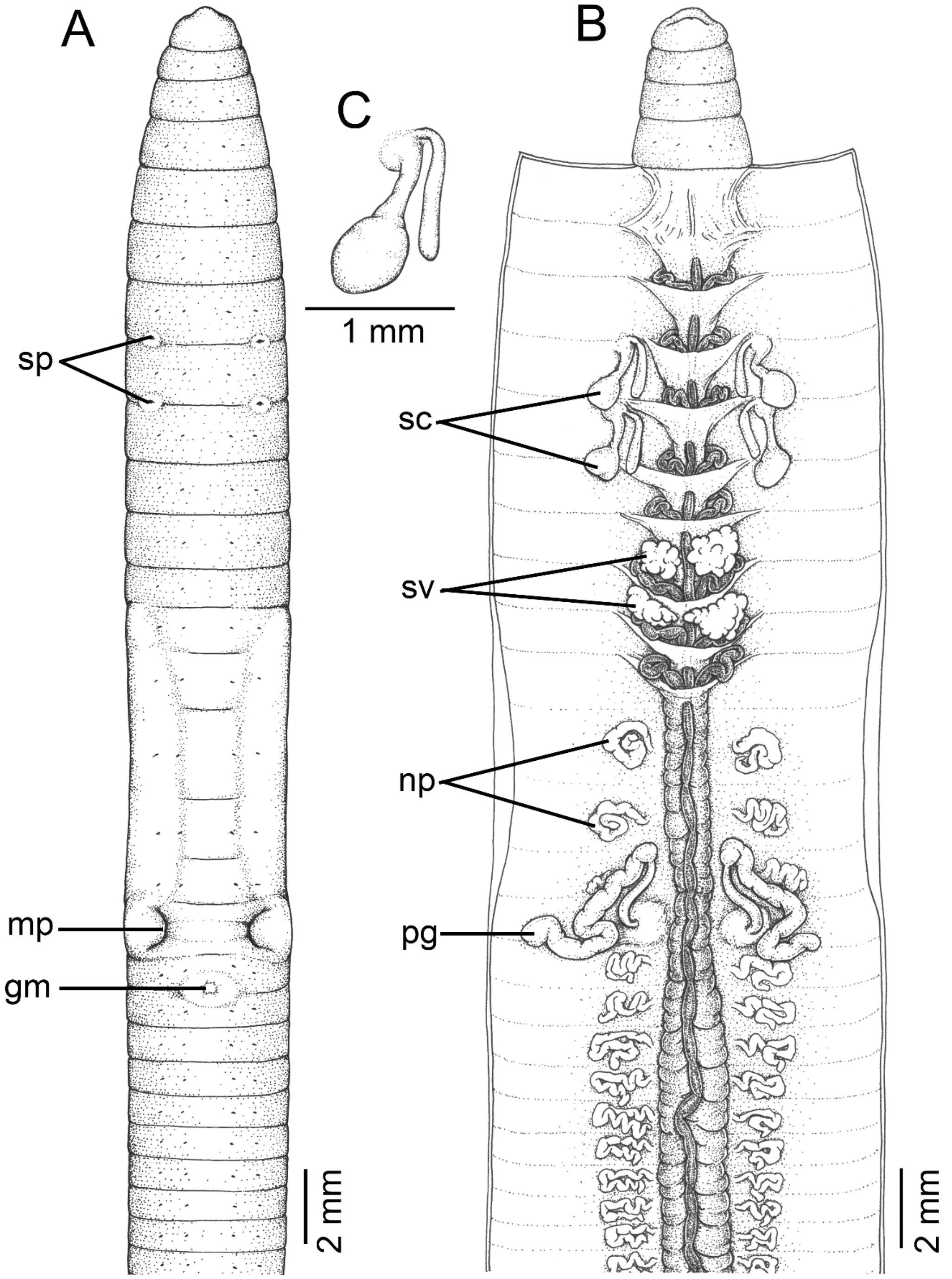

Figure 2.2 External and internal morphology of *Pontodrilus litoralis* (CUMZ 3462) at Ao Talkoo, Laem Ngob, Trat, **A.** External ventral view; **B.** Internal dorsal view; **C.** Spermatheca.

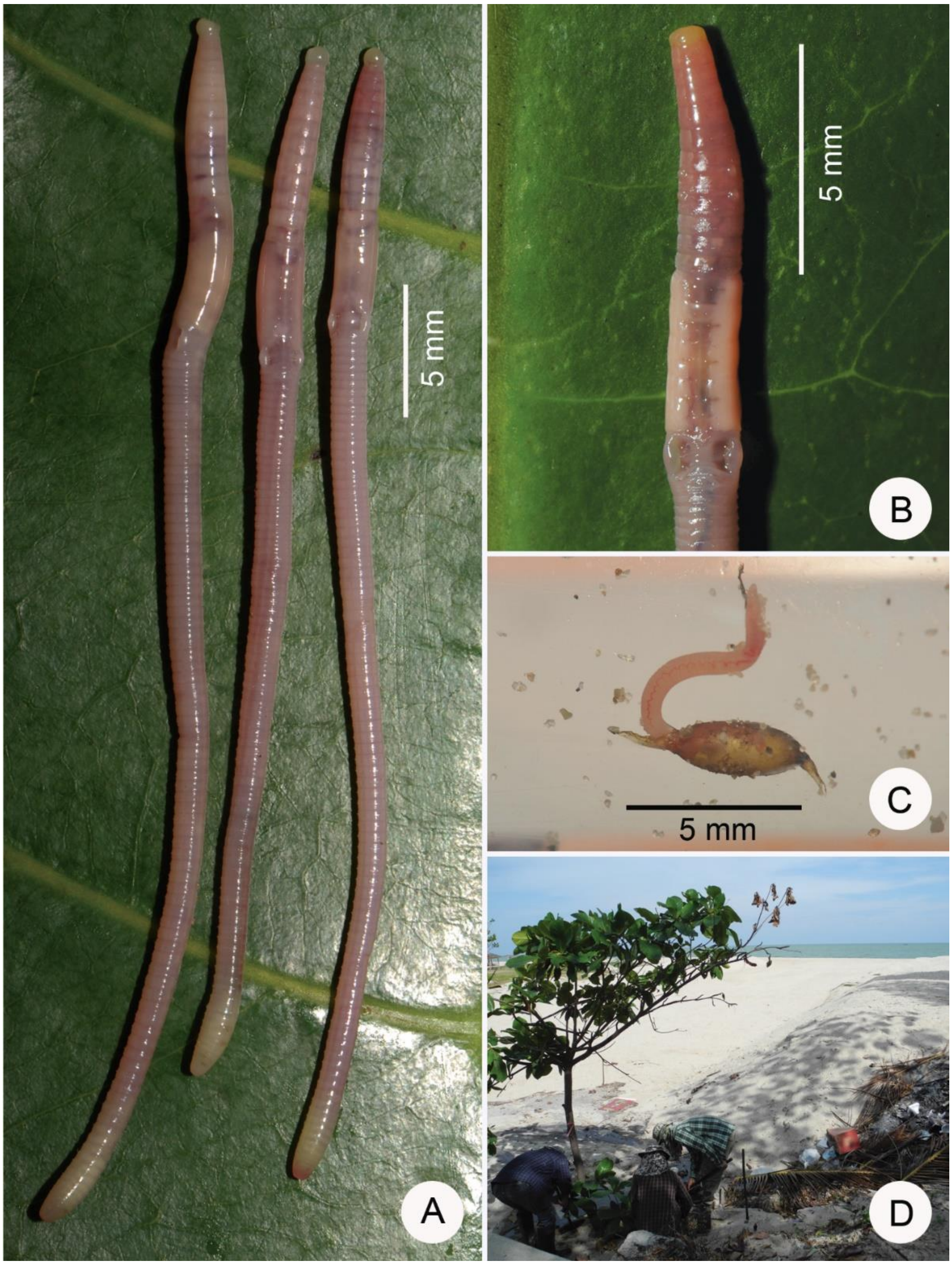

Figure 2.3 Photographs showing: **A.** *Pontodrilus litoralis* (CUMZ 3462) just after the first step preservation in 30% (v/v) ethanol; **B.** Ventral view of anterior portion; **C.** Juvenile hatching from a cocoon; **D.** The microhabitat of *Pontodrilus litoralis* at Hat Cha-am, Cha-am, Phetchaburi, Thailand.

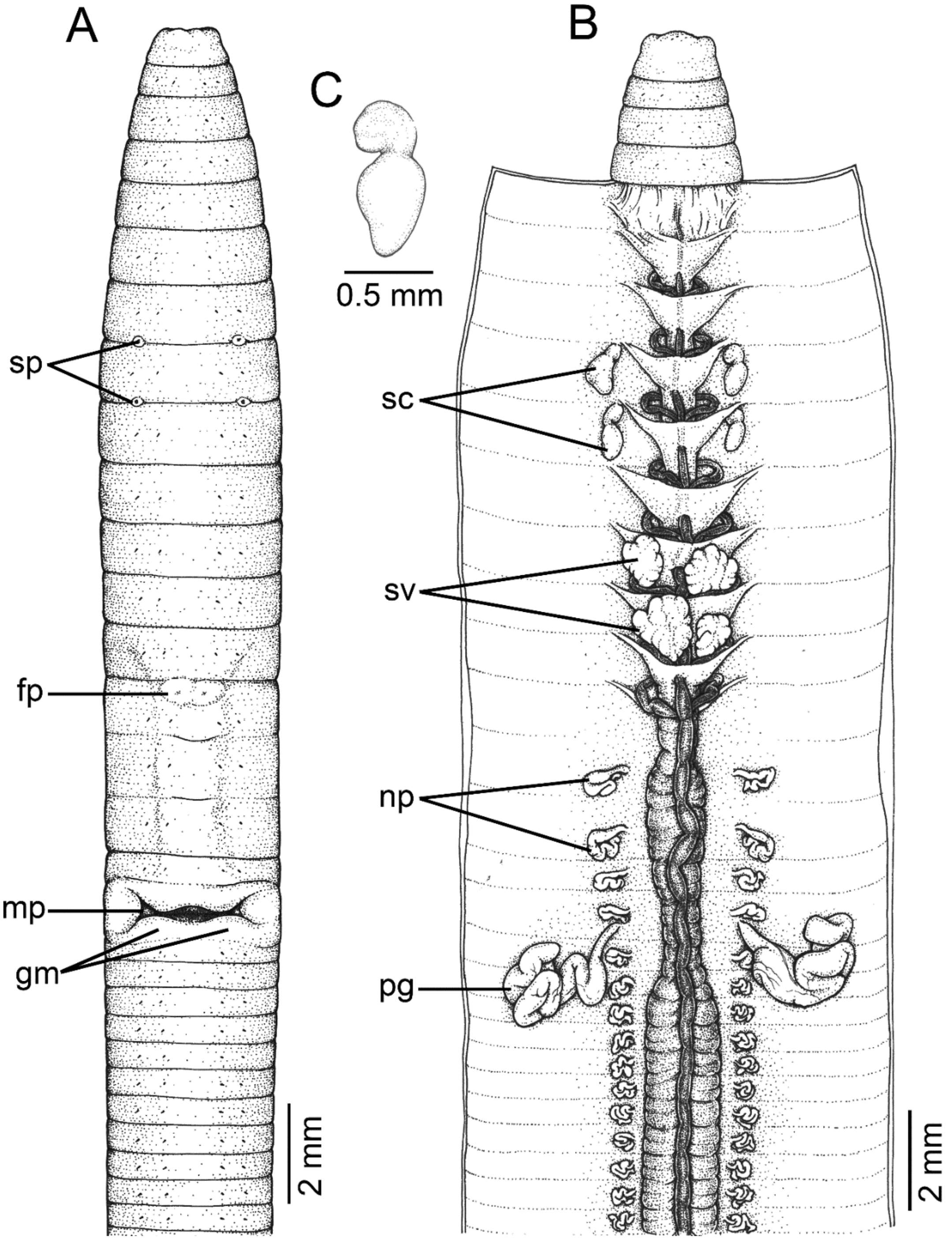

Figure 2.4 External and internal morphology of the holotype (CUMZ 3670) of *Pontodrilus longissimus* **sp. n.** **A.** External ventral view; **B.** Internal dorsal view; **C.** Spermatheca.

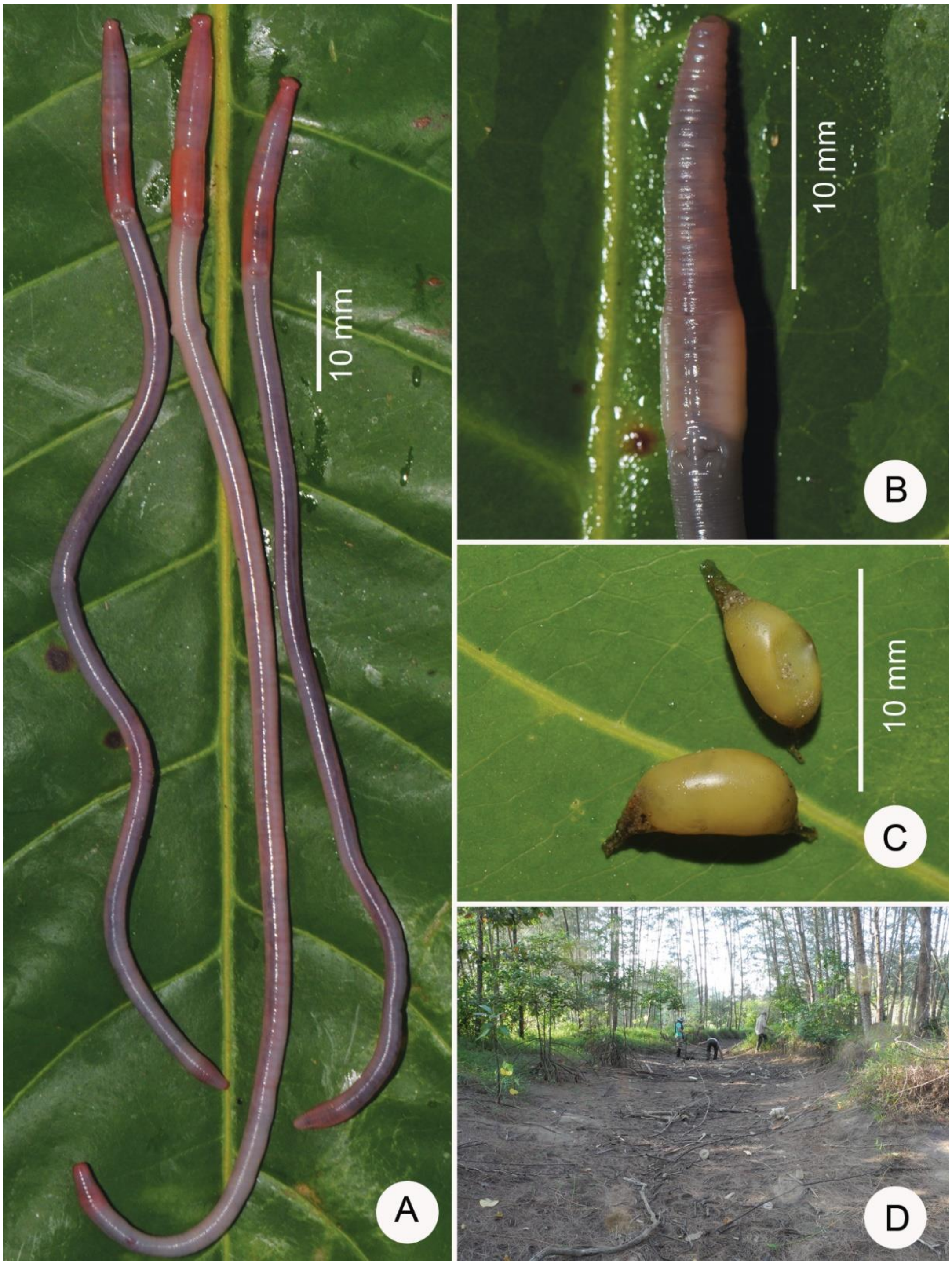

Figure 2.5 Photographs showing: **A.** *Pontodrilus longissimus* **sp. n.** (CUMZ 3670, CUMZ 3671) just after the first step preservation in 30% (v/v) ethanol; **B.** Ventral view of anterior portion; **C.** Cocoons; **D.** The type locality of *Pontodrilus longissimus* **sp. n.** at Hat Pak Meng, Sikao, Trang, Thailand.

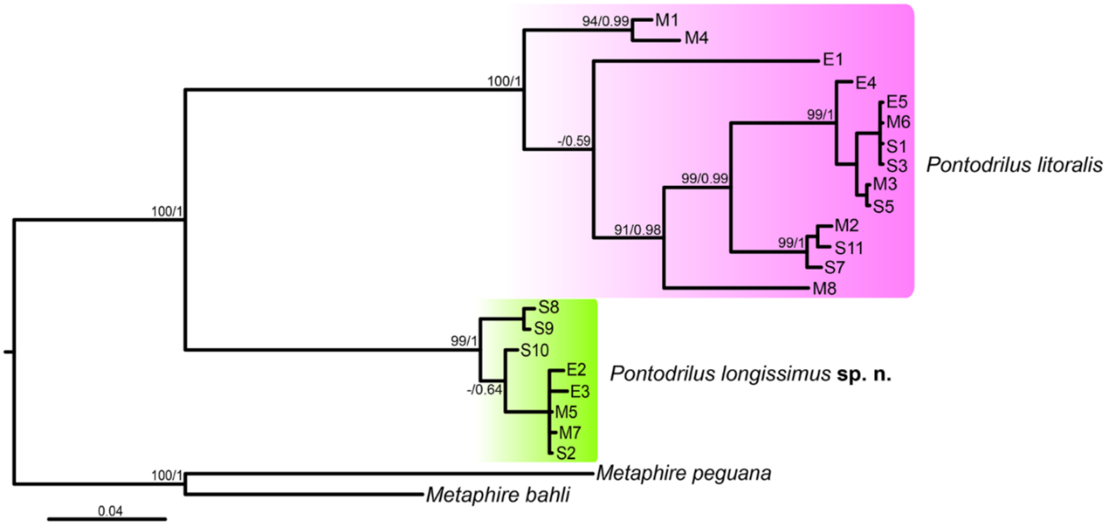

Figure 2.6 Bayesian inference tree based on the partial COI sequence (658 bp) of two *Pontodrilus* species and outgroups. Bootstrap values of >50% and posterior probability values of >0.5 are shown on the tree as ML/BI values. Specimen names correspond to those in Table 1. Scale bar represents the number of nucleotide substitutions per site.

Figure 2.7 Specimens of *Pontodrilus* at Hat Koey, Kaper, Ranong, Thailand, showing the comparative size and length of **A**. *Pontodrilus longissimus* sp. n. and **B**. *Pontodrilus litoralis*.

Chapter 3

Size variation and geographical distribution of the luminous earthworm *Pontodrilus litoralis* (Grube, 1855) (Clitellata, Megascolecidae) in Southeast Asia and Japan

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Abstract

The luminous earthworm *Pontodrilus litoralis* (Grube, 1855) occurs in a very wide range of sub-tropical and tropical coastal areas. Morphometrics on size variation (number of segments, body length and diameter) and genetic analysis using the mitochondrial cytochrome oxidase subunit 1 (COI) gene sequence were conducted on 14 populations of *P. litoralis* from Southeast Asia and Japan. Statistical inference on morphometric data revealed significantly different size variations in the body length and diameter among these 14 populations of *P. litoralis*. However, discordance between the morphometric and mitochondrial COI gene-based phylogenetic analyses was evident, where the size variations in *P. litoralis* showed a different pattern from the COI genetic differences. The update on the current distribution of *P. litoralis* is reported and revealed different aspects of the littoral habitat characteristics between Southeast Asia and Japan.

Keywords: *Pontodrilus litoralis*, morphometrics, COI, distribution, habitat

Introduction

Earthworms are considered as both ecosystem engineers (Jones et al., 1994) and keystone species (Blondel and Aronson, 1995), and they function as decomposers, consumers, and food resources of animals (Lavelle et al., 1992). Earthworms are terrestrial oligochaetes (Annelida: Clitellata), except for a few semi-aquatic taxa, such as earthworms in the family Almidae and *Eiseniella tetraedra* (Savigny, 1826) in freshwater habitats, and *Pontodrilus litoralis* (Grube, 1855), *P. primoris* Blakemore, 2000 and *P. longissimus* Seesamut and Panha, 2018 in marine littoral habitats (Blakemore, 2007, Seesamut et al., 2018).

Pontodrilus litoralis has a wide distribution in the tropical and sub-tropical coastal habitats of the Atlantic, Indian and Pacific Oceans. In Thailand, the first record of the littoral earthworm *P. litoralis* was from Khanom District, Nakhon Si Thammarat Province (Panha et al., 2007). Recently, Seesamut et al. (2018) re-examined the littoral earthworms in Thailand and described a new species *P. longissimus* based on distinct morphological characteristics and molecular genetic distances from *P. litoralis*. In Japan, the littoral earthworm was first discovered from Matsushima Bay, Miyagi Prefecture and described as *P. matsushimensis* by Iizuka (1898), but later this species was synonymized with the cosmopolitan earthworm *P. litoralis* (Easton, 1984). Yamaguchi (1953) reported *P. matsushimensis* from Miyakojima in the Miyagi Prefecture, Misaki and Akashi in the Hyogo Prefecture, Ranshima (Hokkaido) and Fukuoka (Kyushu island). Subsequently, the distribution of this species in Japan was been further studied (Ohno, 2003), with *P. litoralis* being recorded in more than 20 localities in Honshu, Shikoku, Kyushu and Ryukyu (Oba et al., 2011, Oba et al., 2015). In addition, the occurrence of *P. litoralis* was also recorded on the beaches of Awaji Island, located between Honshu and Shikoku, (Hara et al., 2016). Together, these reports indicate that the littoral earthworm *P. litoralis* is a cosmopolitan species and occurs in a very wide range of sub-temperate and tropical coastal areas (Gates, 1972, Easton, 1984, Seesamut et al., 2018).

The study of body size can be helpful in identifying earthworm species, as morphometric characters have been represented as one of the keys for confirming their systematic positions (Chang et al., 2007, James et al., 2010). Morphometric analyses, which use mathematical definitions of size and shape, could be used as an addition to other evolutionary analyses, and the results of which could be interpreted in relation to developmental biology and genetics (Klingenberg, 2002). Size variation has been studied in many earthworm species, in order to investigate their morphological variation and apply the results towards the identification of the earthworm species. Oboh et al. (2007) reported that populations of the terrestrial earthworms *Eudrilus eugeniae* from Lagos, Nigeria were separated into three distinct groups based on the statistical analysis of their morphometric parameters in terms of their body weight, length of clitellum, diameter of posterior and anterior ends, total body length, body size diameter, and total number of segments. In addition, the examination of body size and segment number can be used to separate the terrestrial earthworms *Lumbricus terrestris* and *L. herculeus* into two distinct nominal species, which was also supported by DNA sequence analysis (James et al., 2010). The body size and coloration were also used to separate the *Amynthas wulinensis* species complex into three species (*A. lini*, *A. meishanensis*, and *A. wulinensis*) that were otherwise similar in morphological characters, and this was supported by DNA sequence analysis of the mitochondrial cytochrome oxidase subunit 1 (COI) gene (Chang et al., 2007).

Many distribution records have reported size variation within the cosmopolitan littoral earthworm *P. litoralis* (Gates, 1972, Easton, 1984, Seesamut et al., 2018). However, none of the studies have yet revealed whether the size variation indicated different species or only morphological variation within the same species. In addition, it is believed that there is only one single cosmopolitan species *P. litoralis*, which led us to test this hypothesis based on their size variation coupled with a genetic analysis. The objective of this study, therefore, was to investigate the pattern between the size variations (number of segments, body length and diameter) and genetic (mitochondrial COI) variations in the littoral earthworm *P. litoralis* from 14 populations across Southeast Asia (Thailand, Myanmar, Vietnam, Malaysia and Indonesia) and Japan. Moreover, we report new data on the distribution and habitat types used by this species.

Materials and methods

Field collection, preservation and identification

From August 2011 to September 2018, samples of *P. litoralis* were collected throughout the coastal areas of both the east and west sides of the Thai-Malay Peninsula (Thailand and Malaysia) and Japan (Honshu, Kyushu, and Ryukyu islands). Moreover, samples from Myanmar, Vietnam, Malaysia, Singapore, and Indonesia, were collected (Figures 3.1 and 3.2). Both adult and juvenile stages of the worms were collected by digging suitable habitats, including sandy beaches at both low and high tide levels, estuaries, areas under seaweed debris, damp mud under stones, and areas with wet sand mixed with mud. The living specimens were washed with water, soaked in 30% (v/v) ethanol, photographed and then killed in 30% (v/v) ethanol. Earthworm specimens were then fixed in 95% (v/v) ethanol for morphological and molecular analyses.

Coordinates of each locality were recorded using a GPS receiver, and salinity values were recorded using an ATAGO refractometer. For species identification, the specimens were carefully identified using the taxonomic literature of , Easton (1984) and Seesamut et al. (2018). Small adults (specimen length < 50 mm) and juvenile stages of earthworms were observed under an OLYMPUS SZX16 stereomicroscope. Juveniles were identified by the position of male pores (segment XVIII) showing the inner wall of a longitudinal depression and the internal characters, such as prostate grands on XVIII and absent of nephridia on anterior segments.

Morphometric analysis

Fourteen populations of *P. litoralis* were selected based on being from different geographic regions (Table 3.1). At least nine adult worms from each population were then selected and this resulted in a total of 212 specimens used in the morphometric analysis. Only sexually mature earthworms, as determined by the presence of clitellum, were measured and used to plot the frequency of the length distribution. Total body length, body size diameter, and total number of segments were measured and counted following Ng et al. (2017). Analysis of variance (ANOVA) and principal component analysis (PCA) were performed to assess the significant variation among the three morphometric characters. The mean length and diameter were calculated separately both within each locality and a country scale, and those mean differences were analyzed by one-way ANOVA. The clustering analysis (CA) of the sampling sites was performed to construct a dendrogram depicting the morphological relationship based on the three morphometric measurements, CA were tested based on complete linkage and Euclidean distances. All statistical analyses were performed using the MINITAB software v. 18.1 (Minitab, Inc.).

Molecular analysis

Three specimens were chosen from each of the same 14 populations as in the morphometric analysis resulting in the total of 42 samples used for the molecular analysis (Table 3.2). The total genomic DNA of each worm was extracted from a posterior body part using a Lysis Buffer for PCR (Takara) DNA extraction kit. The mitochondrial COI gene fragment was amplified using the Tks GflexTM DNA Polymerase (Takara) and the universal primers (Folmer et al., 1994). Each PCR reaction was comprised of 1 μL of Tks Gflex DNA polymerase (1.25 unit/μL), 25 μL of 2x Gflex PCR buffer (Mg^{2+}, dNTP plus), 1 μL each of 10 μM LCO1490 (forward) and HCO2198 (reverse) universal primer, 19.5 μL of sterilized distilled water and 2.5 μL of crude lysate (ca. 500 ng/μL DNA) with Lysis buffer. Thermal cycling was performed at 94 °C for 2 min, followed by 35 cycles of 94 °C for 1 min, 48 °C for 1 min and 72 °C for 2 min and then a final 72 °C for 5 min. The concentration and quality of the amplicons were determined visually after coresolution through a 1% (w/v) agarose gel against a DNA standard marker in 1x TAE buffer and detected under UV transillumination.

For sequencing, the PCR products were directly sent to Macrogen Inc. (Japan) without purification. All COI sequences were aligned using the ClustalW algorithm in MEGA7 v. 7.0.18 (Thompson et al., 1994, Kumar et al., 2016) and manually checked by eye. The sequences were aligned, checked and compared with other sequences available in the GenBank databases at the National Center for Biotechnology Information (NCBI), obtained using the BLASTn similarity search tool (http://www.ncbi.nlm.nih.gov). Corrected genetic distances were calculated using the Kimura two-parameter (K2P) model (Kimura, 1980) as implemented in MEGA7. Phylogenetic reconstruction was performed using the maximum likelihood (ML) analysis in RAxML v. 8.1.20 (Stamatakis, 2014), and 1,000 bootstraps were used to estimate the node reliability as bootstrap support values. Bootstrap values lower than 75% for each node were considered as insignificant (Okanishi et al., 2018).

Pontodrilus longissimus was used as the outgroup (Accession number MK642683 and MK642684).

Results

Size variation of *P. litoralis*

The measurement of 212 individuals of *P. litoralis* earthworms from all 14 sampling sites revealed a length range between 31.1–125.4 mm (Figure 3.3). The length between 60.1–69.9 mm occurred at the highest frequency (n = 72), followed by that between 50.0–59.6 mm (n = 44) and 70.5–79.6 mm (n = 38). Two specimens from JP2 were recorded as having a length > 120 mm. The relationship between the total number of segments and the body length of *P. litoralis* (Figure 3.4), had a low correlation between them ($R^2 = 0.0922$). The longest length of *P. litoralis* was 125.4 mm, found in Japan (JP2), while the shortest was 31.1 mm, found in Vietnam (VT2). The mean ± S.D. and median length of *P. litoralis* were 62.6 ± 14.2 mm and 63.1 mm, respectively. The highest number of segments in *P. litoralis* was 119, found in Thailand (TA1) and Myanmar (MY1), while the lowest was 81, found in Indonesia (IN1). The mean ± S.D. and median of the total number of segments of mature *P. litoralis* were 101.7 ± 8.9 and 102, respectively. In addition, the largest body diameter of *P. litoralis* was 4.08 mm, found in Japan (JP2), while the smallest was 1.21 mm, found in Vietnam (VT2). The mean ± S.D. and median *P. litoralis* diameter were 2.12 ± 0.52 mm and 2.02 mm, respectively.

The ANOVA analysis revealed a significant difference ($p < 0.05$) in the mean length and diameter of *P. litoralis* among the 14 locations (Figure 3.5). The JP2 population from Japan showed the highest mean body length (93.0 ± 22.4 mm) and diameter (3.39 ± 0.6 mm), while the VT2 population from Vietnam showed the lowest mean body length (39.5 ± 5.4 mm) and diameter (1.55 ± 0.18 mm). Moreover, the highest mean body length (73.6 ± 16.4 mm) and diameter (2.86 ± 0.47 mm) were found in all the Japanese populations (JP1, JP2, and JP3), while the lowest mean body length (52.4 ± 14.2 mm) and diameter (1.66 ± 0.25 mm) were found in all the Vietnamese populations (VT1, VT2, and VT3).

Cluster analysis of the 14 populations based on the three morphometric data revealed two clusters, one of which contained 13 populations and was further divided into two subclusters, and the other contained only the JP2 population from Japan (Figure 3.6). The PCA showing the first principal component (PC1) explained more than 60.6% of the variation in the dataset and had a variance (eigenvalue) of 1.8174. The second and third PCs (PC2 and PC3) had a variance (eigenvalue) of 0.8882 and 0.2944, respectively, which accounted for 29.6% and 9.8% of the data variability, respectively. The PC1 revealed that all the loadings were positive, whereas the PC2 showed both positive (number of segment) and negative loadings (body length and diameter). The loadings from the PC2 were less similar among themselves compared to the PC1. The PC1 had a large positive association with the body length and diameter as determined by loadings > 0.5, so this PC1 primarily measured the size of the earthworms (Table 3.3). The scatter diagram of PC1 versus PC2 (Figure 3.7) indicated that the size variation within populations of JP2 and VT2 were distinct from other populations.

Genetic analysis

The COI DNA sequences (658 bp) from 42 individuals, three specimens from each of the 14 populations used in the morphometric analysis, were analyzed. The analysis yielded 158 variable (polymorphic) sites and 139 parsimony informative sites. No insertions, deletions, or stop codons were observed in any of the sequences. The K2P genetic distances among the 14 geographical locations within *P. litoralis* ranged from 0.3–12.8 % (Table 3.4). The highest divergence was estimated between TA1 and JP1; MA2 and JP1 (12.8%), while the lowest was estimated between TA1 and MA2 (0.3%). The genetic distance within group ranged from 0–9%. The highest was estimated in TA2 (9%), whereas the lowest was estimated in VT1 and VT3 (0.0%). The ML tree (Figure 3.8) did not show any pattern congruent with the variation in the sizes of *P. litoralis* (Fig. 3.6). For instance, the analyses on the size variation between the shortest population (VT2) and the longest population (JP2) samples clearly showed a significant difference in their body length (39.5 mm and 93.0 mm for VT2 and JP2, respectively), and body diameter (1.55 mm and 3.39 mm for VT2 and JP2, respectively) ($p < 0.05$), while the cluster analysis confirmed that the two clusters were separated, one contained 13 populations (included VT2) and the other contained only JP2. However, the genetic distance analysis showed a low genetic distance between VT2 and JP2 population (5.6%; Table 3.4) and the COI ML tree suggested a sister relationship between 4 samples from VT2 population (VT2, VT2_B) and JP2 population (JP2, JP2_A) (Figure 3.8). The nucleotide sequences reported of *P. litoralis* in this study are deposited at GenBank under accession as showing in Table 3.2.

Distributions and habitats of *P. litoralis*

In Southeast Asia, *P. litoralis* was found scattered over the coastal areas in Thailand, Myanmar, Vietnam, Malaysia, Singapore, and Indonesia (Figures 3.1 and 3.2). The northernmost sampling site was at Nghệ An Province, Vietnam (18° 45' 46.1" N, 105° 45' 23.54" E), whereas the southernmost site was in Bantan, Indonesia (6° 00' 51.3" S, 106° 40' 38.4" E). In this study, we reported the first record of *P. litoralis* in Singapore despite only juveniles being collected from the beach in West Coast Park (1° 17' 45.0" N, 103° 45' 43.1" E). Among the localities in the sub-tropical areas, *P.*

litoralis specimens were collected from various beaches in Japan, and the northernmost site was Matsushima Kaihin Koen in the Miyagi prefecture, where the synonym of *P. litoralis* (*P. matsushimensis*) was originally described from. In total, 29 localities were recorded in the distribution range of *P. litoralis* within Japan, including in the Honshu, Kyushu, and Ryukyu islands.

Based on field collections within Thailand and some parts of Southeast Asia, *P. litoralis* was found to occupy several types of habitats (Table 3.5; Figure 3.9), such as estuaries, brackish, damp mud under stones, under the trash or leaf litter on sandy beaches, mangrove swamps of the intertidal zone, sanitary sewer links, and fresh waterways linked between the mainland and the sea. However, collections of *P. litoralis* in the Japanese coastal areas showed that *P. litoralis* was abundant and mostly found in sandy beaches facing the ocean and lives in the sand mixed with seaweed debris (Figure 3.10). Records of the salinity values during the field collections showed an average salinity between 12–22 ‰ (Table 3.5).

Discussion

This present study is the first attempt to integrate morphometric variations and molecular marker analyses together in the cosmopolitan littoral earthworm *P. litoralis*. The specimens investigated in this study were within the variation range previously reported by Jamieson (2001) (body length 32–120 mm, diameter 2–4 mm, and number of segments 78–120) and Seesamut et al. (2018) (body length 28–136 mm, diameter 1–5 mm, and number of segments 76–128).

According to the results of the one-way ANOVA, there was a significant difference in the body length and diameter among specimens from the different geographical sites. In addition, the PCA results supported that length and diameter had a higher influence than the number of segments in the 14 studied populations of *P. litoralis*. However, the phylogenetic tree did not show any congruent pattern with the size variation of the specimens analyzed in this study. For instance, in both the PCA and cluster analysis the longest (JP2) and the shortest samples (VT2) formed separate groups with statistical differences in their size, whereas a low genetic distance between the two samples from each respective population was detected, revealing that the size variation of *P. litoralis* was independent of the genetic (COI gene) differences.

Differences in the body length, diameter, and number of segments have also reported in other earthworms. The terrestrial earthworm *Metaphire peguana* (Rosa, 1890) from Penang and neighboring states of Malaysia revealed significant differences in their morphometric variations that were not matched by their genetic difference but rather were affected by the type of habitat (Ng et al., 2017). However, Heethoff et al. (2004) reported a strong correlation between the size of *Octolasion tyrtaeum* (Savigny, 1826) earthworms from Germany and Canada and their mitochondrial cytochrome *c* oxidase II (COII) sequences, showing that small and large individuals were genetically distinct.

This study is a comprehensive report on the occurrence, distribution and habitat characteristics of the luminous littoral earthworm *P. litoralis* in the coastal areas of Thailand, Japan (Honshu, Kyushu, and Ryukyu islands), and some parts of Southeast Asia (Myanmar, Vietnam, Malaysia, Singapore, and Indonesia) based on field collections. This survey supported the assumption that *P. litoralis* is widely distributed in sub-tropical and tropical coastal ecosystems (Gates, 1972, Jamieson and Wampler, 1979, Oba et al., 2015, Seesamut et al., 2018), and aligns with the worldwide distribution records (Easton, 1984, Blakemore, 2002).

In general, the distribution of earthworms is mostly affected by environmental factors, such as the temperature, organic matter content, and soil moisture (Johnston et al., 2014). This survey of *P. litoralis* habitats in Thailand and Southeast Asia revealed that the earthworms live in various habitat types with a relatively wide range of salinity and diverse sources of water. The earthworms were mostly found in the ecotone between the terrestrial and marine habitats, such as the mangrove swamps of the intertidal zone, sanitary sewer links to the sandy beach, estuaries, salty mud under stones near the shore, and under the trash or leaf litter on the sand beach. This indicated that *P. litoralis* mostly prefers to inhabit the ecotone between terrestrial and marine habitats. The earthworms were found to occupy the soil column that ranged from the top soil down to 30 cm deep, and on humid substrates in contact with tidal seawater, the level of which is an important factor governing the distribution of intertidal species (Penas and Gonzalez, 1983). In this survey, the habitats of *P. litoralis* in Japan, where the worms were collected, were mostly in sand mixed with seaweed debris on the sandy beaches facing the ocean, whereas we did not collect any littoral earthworms from this type of microhabitat in Southeast Asian shores.

In Japan, beach-cast seaweeds have been reported as important habitats and food for a diverse community of marine and terrestrial organisms, such as amphipods, isopods, and copepods (Okuda, 2008). The habitats of *P. litoralis* in Japan are similar to those reported in Western Australia coastal areas, where the earthworms were recorded in high density within the wrack material, seaweed, and debris deposited on arid beaches, which provided a rich food resource and resulted in a high abundance of earthworms (Blakemore, 2007, Coupland and McDonald, 2008). Carlo et al. (2012) reported the preference of *P. litoralis* to inhabit sites with an accumulation of macrodetritic matter that provided abundant organic matter contents and shade that helped to keep the soil surface cool during daytime. Moreover, the salinity of the *P. litoralis* habitats recorded in this study indicated that *P. litoralis* can survive a wide range of salinity between 1–33 ‰ (Seesamut et al., 2018), the upper bound of which is near the salinity of seawater in general (35 ‰; Schmidt et al. 2018). Taken together, we suggest that the habitat preference of *P. litoralis* is primarily determined by the abundance of organic matter contents but not the salinity.

In conclusion, although morphometric examinations of size variation could make reliable distinctions among different populations of *P. litoralis*, this distinction was not congruent with the phylogenetic relationship based on COI gene sequence analysis, reflecting that the size variation of *P. litoralis* did not correlate with their genetic (COI) differences. Thus, we propose that the food resource is the key factor underlying size variation in *P. litoralis*. Future analyses on the type of habitats, sand texture and components of the food resources are necessary. Moreover, studies on salinity tolerance are needed to confirm the habitat preference of this littoral earthworm species.

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Table 3.1 Sampling localities, GPS coordinates and number of specimens of *P. litoralis* used in the morphometric analysis.

Locality		Latitude, Longitude	Number of adult samples
Thailand (TA)	1. Petchaburi (TA1)	12°49'36.2"N, 99°59'40.3"E	16
	2. Trat (TA2)	12°05'52.4"N, 102°21'27.9"E	20
	3. Chonburi (TA3)	12°50'25.1"N, 100°54'18.3"E	15
	4. Songkhla (TA4)	7°43'30.3"N, 100°22'55.4"E	18
Malaysia (MA)	5. Pulau Pinang (MA1)	5°28'06.7"N, 100°16'41.0"E	16
	6. Pahang (MA2)	3°48'25.0"N, 103°20'29.4"E	18
Myanmar (MY)	7. Dawei (MY1)	14°07'43.5"N, 98°05'50.1"E	10
Indonesia (IN)	8. Banten (IN1)	6°00'51.3"S, 106°40'38.4"E	13
Vietnam (VT)	9. Bến Tre (VT1)	9°48'11.0"N, 106°37'42.2"E	15
	10. Huế (VT2)	16°13'38.9"N, 108°04'58.4"E	16
	11. Nghệ An (VT3)	18°46'06.1"N, 105°45'31.0"E	16
Japan (JP)	12. Aichi (JP1)	34°48'00.2"N, 136°51'30.3"E	18
	13. Hiroshima (JP2)	34°17'45.0"N, 132°19'08.0"E	9
	14. Okinawa (JP3)	26°28'20.0"N, 127°49'54.1"E	12
Total			212

Table 3.2 Details of *P. litoralis* samples using DNA sequencing, and accession numbers of the COI sequences.

Locality	abbreviation	GenBank accession number
1. Petchaburi, Thailand(TA1)	TA1	MK642691
	TA1_A	MK714106
	TA1_B	MK714107
2. Trat, Thailand (TA2)	TA2	MK642690
	TA2_A	MK714108
	TA2_B	MK714109
3. Chonburi, Thailand (TA3)	TA3	MK642689
	TA3_A	MK714110
	TA3_B	MK714111
4. Songkhla, Thailand (TA4)	TA4	MK642688
	TA4_A	MK714112
	TA4_B	MK714113
5. Pulau Pinang, Malaysia (MA1)	MA1	MK642694
	MA1_A	MK714100
	MA1_B	MK714101
6. Pahang, Malaysia (MA2)	MA2	MK642693
	MA2_A	MK714102
	MA2_B	MK714103
7. Dawei, Myanmar (MY1)	MY1	MK642692
	MY1_A	MK714104
	MY1_B	MK714105
8. Banten, Indonesia (IN1)	IN1	MK642698
	IN1_A	MK714092
	IN1_B	MK714093
9. Bến Tre, Vietnam (VT1)	VT1	MK642687
	VT1_A	MK714114
	VT1_B	MK714115
10. Huế, Vietnam (VT2)	VT2	MK642686
	VT2_A	MK714116

	VT2_B	MK714117
11. Nghệ An, Vietnam (VT3)	VT3 VT3_A VT3_B	MK642685 MK714118 MK714119
12. Aichi, Japan (JP1)	JP1 JP1_A JP1_B	MK642697 MK714094 MK714095
13. Hiroshima, Japan (JP2)	JP2 JP2_A JP2_B	MK642696 MK714096 MK714097
14. Okinawa, Japan (JP3)	JP3 JP3_A JP3_B	MK642695 MK714098 MK714099

Table 3.3 PCA percentage of the explained variance and weights of morphometric ratios for the 14 populations of *P. litoralis*.

Variable	**PC1**	**PC2**	**PC3**
Length	0.675	-0.143	0.724
Diameter	0.638	-0.380	-0.670
Segment number	0.371	0.914	-0.165
Eigenvalue	1.8174	0.8882	0.2944
%Total variance	60.6	29.6	9.8

Table 3.4 Between groups mean genetic distances corrected with the Kimura-2 parameter model among the 14 population of *P. litoralis*. The bold values represent the genetic distance within group. Sampling site codes are given in Table 3.1.

	IN1	JP1	JP2	JP3	MA1	MA2	MY1	TA1	TA2	TA3	TA4	VT1	VT2	VT3
IN1	**0.002**													
JP1	0.102	**0.001**												
JP2	0.087	0.073	**0.072**											
JP3	0.079	0.108	0.043	**0.011**										
MA1	0.089	0.101	0.103	0.102	**0.002**									
MA2	0.064	0.128	0.100	0.083	0.112	**0.001**								
MY1	0.048	0.109	0.088	0.076	0.109	0.073	**0.002**							
TA1	0.067	0.128	0.099	0.082	0.111	0.003	0.074	**0.005**						
TA2	0.100	0.111	0.112	0.110	0.108	0.119	0.101	0.118	**0.090**					
TA3	0.064	0.125	0.093	0.075	0.106	0.018	0.068	0.017	0.113	**0.001**				
TA4	0.071	0.109	0.074	0.055	0.107	0.065	0.078	0.067	0.114	0.067	**0.067**			
VT1	0.067	0.127	0.098	0.081	0.109	0.005	0.074	0.004	0.116	0.013	0.066	**0.000**		
VT2	0.073	0.113	0.056	0.035	0.103	0.067	0.071	0.066	0.109	0.061	0.060	0.063	**0.050**	
VT3	0.067	0.119	0.088	0.072	0.102	0.030	0.060	0.030	0.105	0.024	0.066	0.026	0.052	**0.000**

Table 3.5 Salinity records (mean ‰ ± SD) and habitat characteristics of the sampling sites of *P. litoralis* in this study.

Locality	Collection time	Salinity (‰)	Habitat
Thailand	January 2015 – March 2018	19.29 ± 12.14	Estuaries, brackish, damp mud under stones, under the trash or leaf litter on the sandy beach, mangrove swamps of the intertidal zone, sanitary sewer link to the sandy beach
Myanmar	April 2016	18 ± 12.82	Estuaries, under the trash on the sandy beach
Malaysia	January 2016	15.94 ± 9.85	Estuaries, damp mud under stones and the beach, under the trash or leaf litter on the sandy beach
Vietnam	May 2018 – July 2018	19.38 ± 10.57	Estuaries, under the trash or leaf litter on the sandy beach
Indonesia	August 2017	12	Sanitary sewer link to the sandy beach
Singapore	December 2017	22	Under the root of the tree near the shore
Japan	August 2011 – September 2018	17.5 ± 9.85	Sand beach facing to the ocean (sand mixed with seaweed debris), estuaries

Figure 3.1 Location and distribution of *P. litoralis* habitats (sampling sites) in Thailand, Malaysia, Myanmar, Singapore, Indonesia and Vietnam (based on our field collections).

Figure 3.2 Location and distribution of *P. litoralis* habitats (sampling sites) in Japan (based on our field collections).

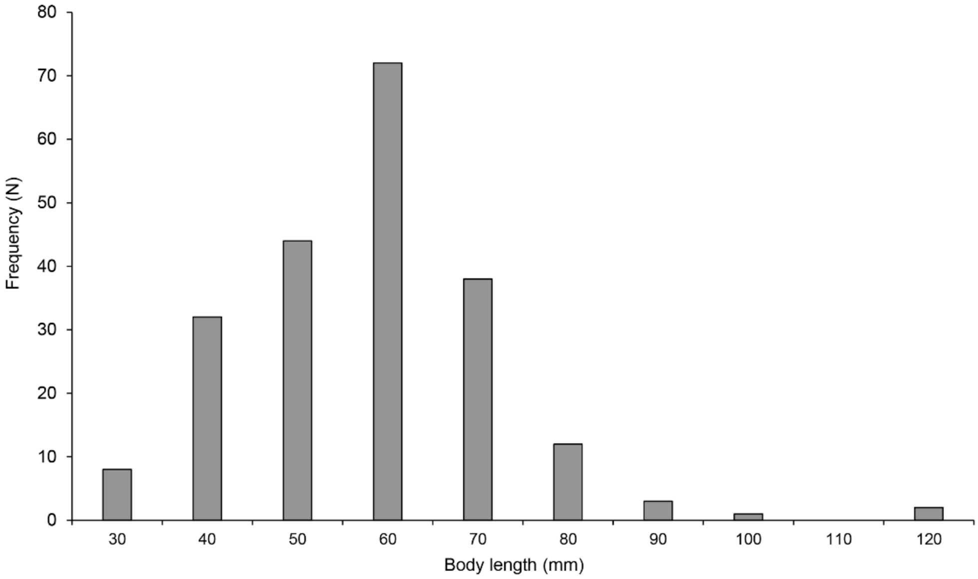

Figure 3.3 Histogram showing the length frequency distribution of the 212 *P. litoralis* samples from all 14 sampling sites.

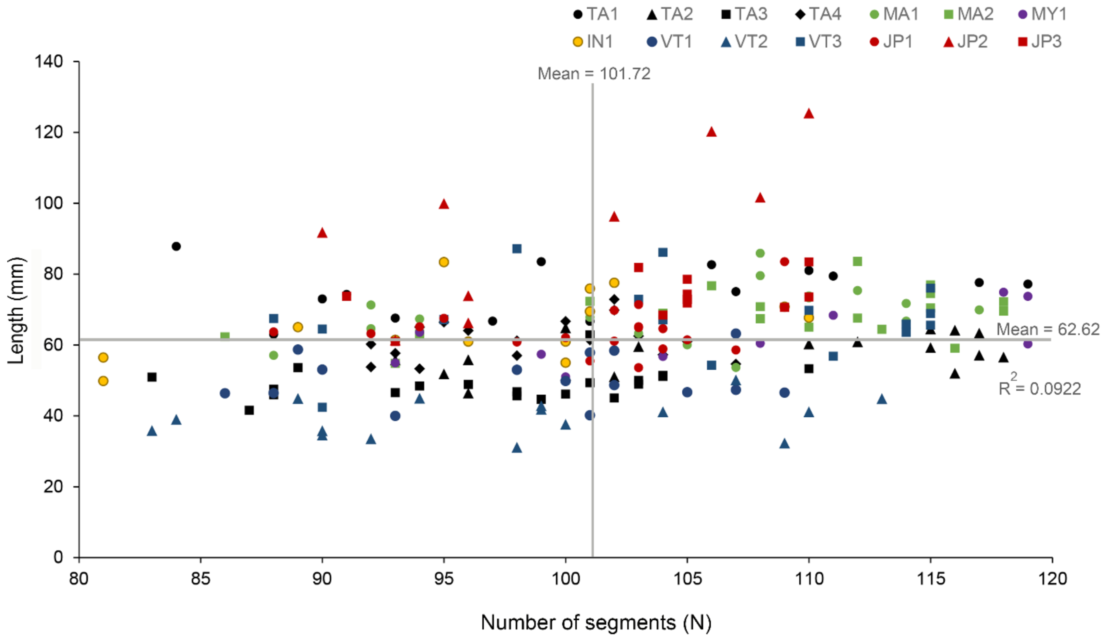

Figure 3.4 Scatter plot between the length and number of segments of *P. litoralis* (212 samples, 14 locations).

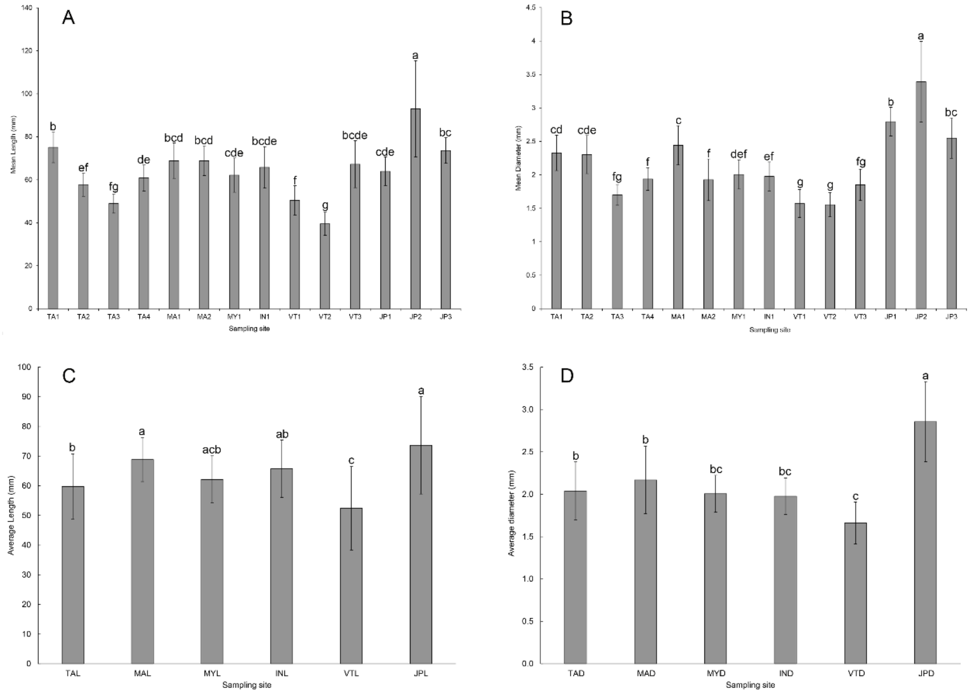

Figure 3.5 Mean (A, C) length and (B, D) diameter of *P. litoralis* samples within each (A, B) locality and (C, D) country sampled in this study. Sampling site codes are given in Table 1. Different letters above the bar indicate a significant difference ($P < 0.05$; one-way ANOVA).

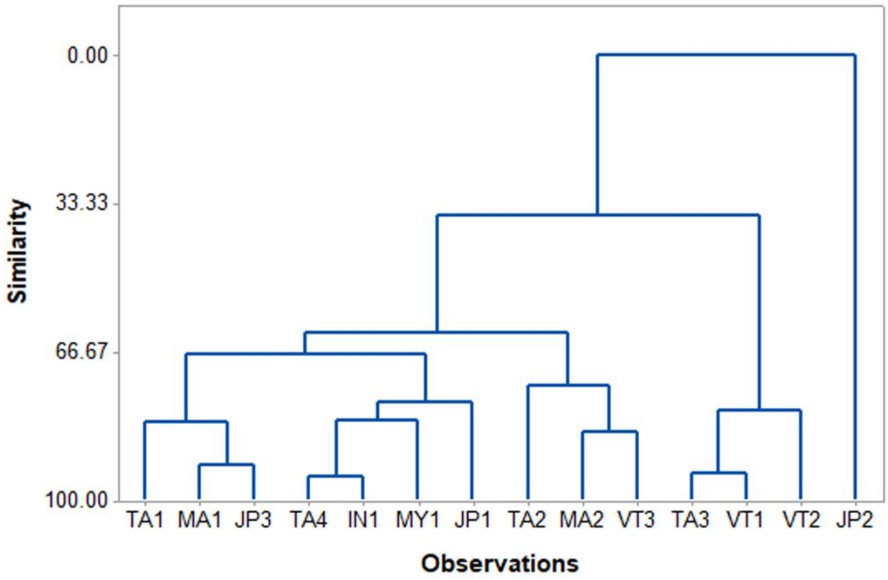

Figure 3.6 Cluster analysis based on the Euclidean distances among the 14 populations of *P. litoralis*. Sampling sites codes are given in Table 3.1.

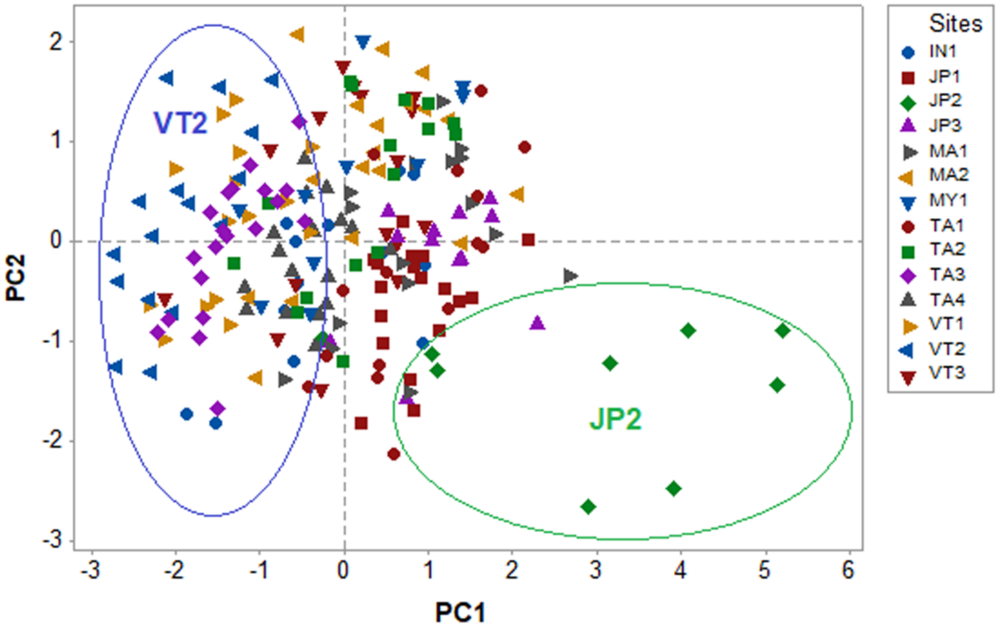

Figure 3.7 PCA plot between PC1 and PC2 using the three morphometric variables (number of segments, body length, and diameter). Sampling sites codes are given in Table 3.1.

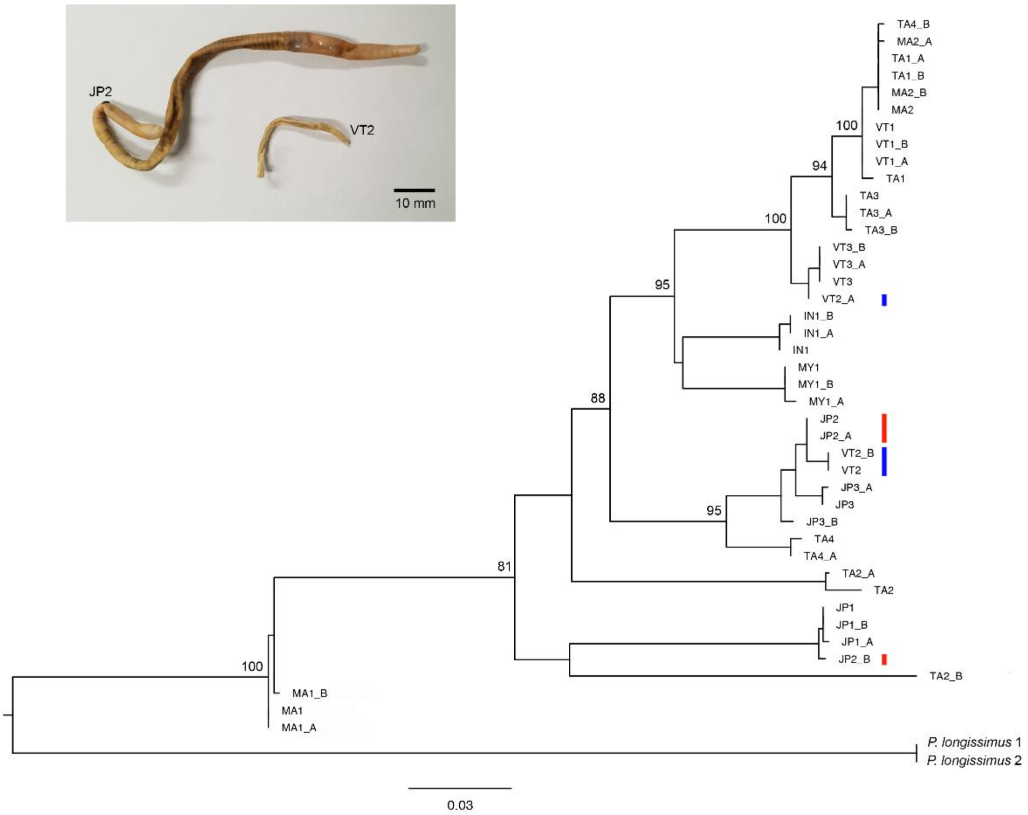

Figure 3.8 ML phylogenetic tree of *P. litoralis* based on the mitochondrial COI gene (658 bp) with *Pontodrilus longissimus* as the outgroup. Only bootstrap values > 70% are indicated at each node. Scale bar represents the number of nucleotide substitutions per site. The sample names correspond to those in Table 5. Photograph on the top left shows comparative size of the shortest and the longest samples in this molecular study. The longest population (JP2) is shown in red and the shortest population (VT2) is shown in blue.

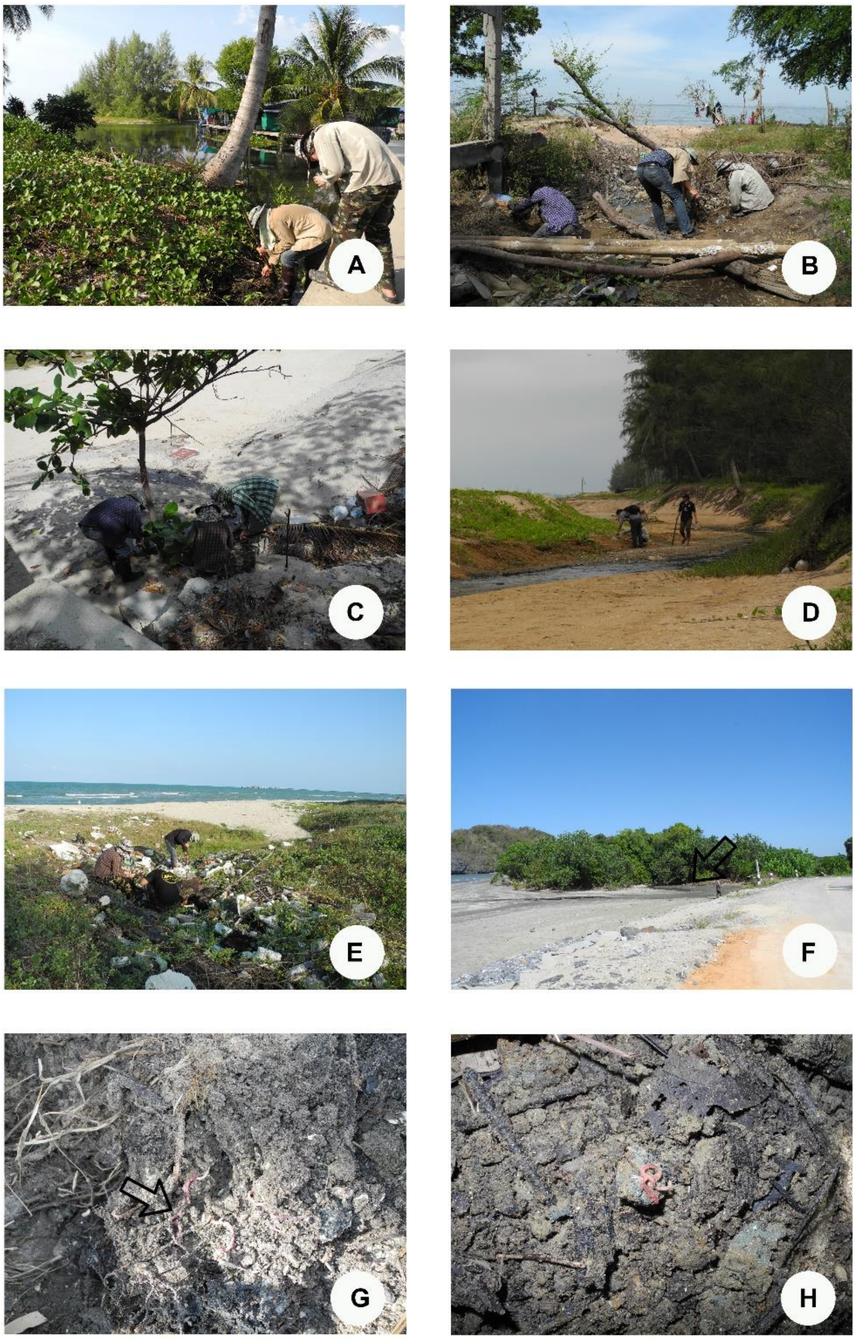

Figure 3.9 Photographs showing the habitats of *P. litoralis* in Thailand: **A.** Trat Province; **B.** Chonburi Province; **C.** Petchaburi Province; **D.** Chumphon Province; **E.** Songkhla Province; **F.** Satun Province; **G.** Petchaburi Province; and **H.** Satun Province

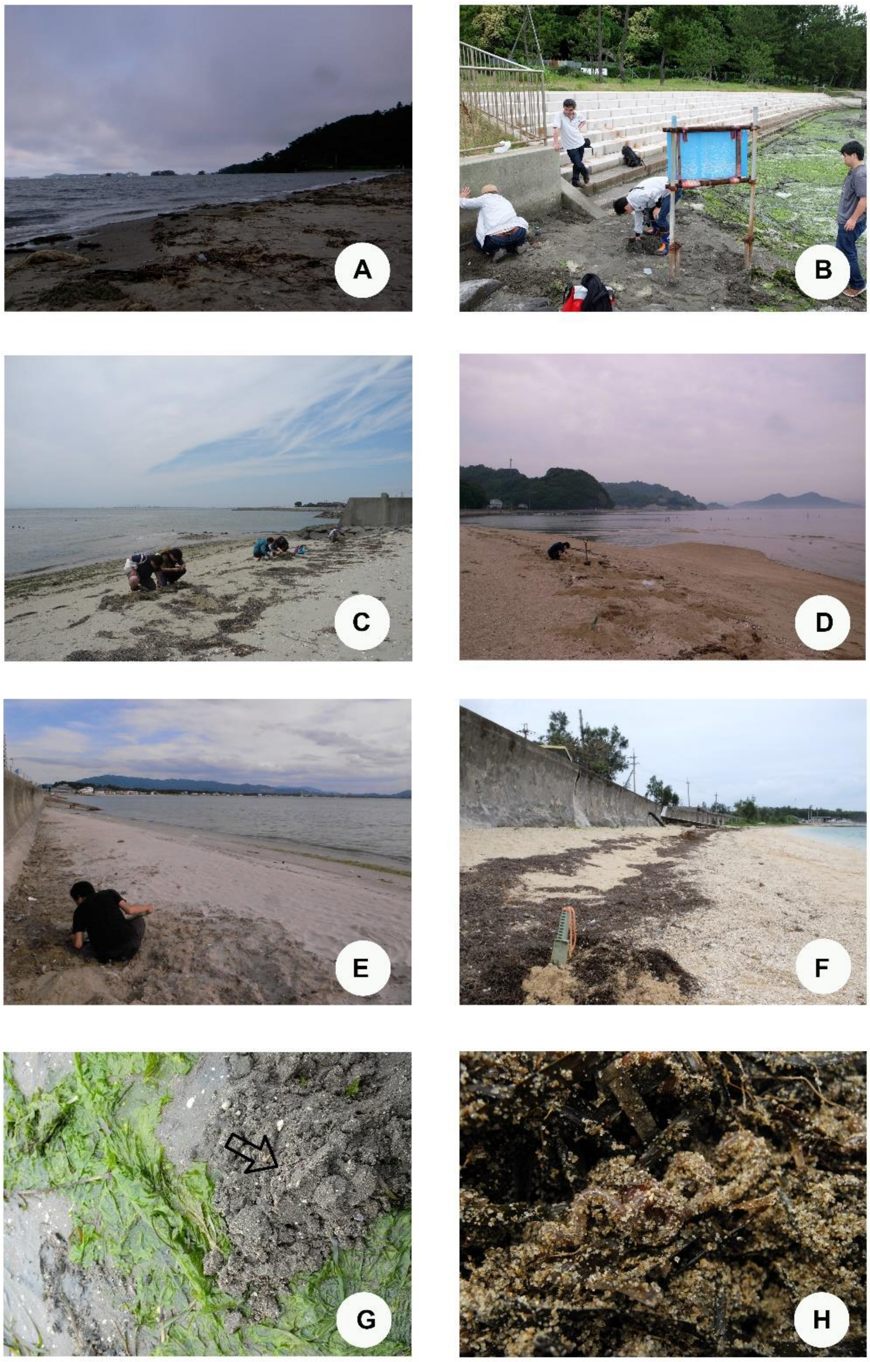

Figure 3.10 Photographs showing the habitats of *P. litoralis* in Japan: **A.** Miyagi Prefecture; **B.** Kanagawa Prefecture; **C.** Aichi Prefecture; **D.** Hiroshima Prefecture; **E.** Fukuoka Prefecture; **F.** Okinawa Prefecture; **G.** Kanagawa Prefecture; and **H.** Aichi Prefecture

Chapter 4

Mitochondrial genetic population structure and variation of the littoral earthworm *Pontodrilus longissimus* Seesamut and Panha, 2018 along the coast of Thailand

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Abstract

Nucleotide sequences of a 658 bp DNA fragment of the cytochrome *c* oxidase subunit 1 were used to investigate the population structure, phylogeographical pattern and genetic variation of *Pontodrilus longissimus* Seesamut and Panha, 2018 sampled from 16 localities along the coastline of Thailand. Bayesian inference, maximum likelihood analyses and the application of a haplotype network showed clearly that there were two geographically isolated populations of *P. longissimus*, one restricted to the Gulf of Thailand (GOT) and the other to the Andaman Sea, in which the latter clade revealed a higher level of genetic variation. The analyses of molecular variance also supported the genetic differentiation between the GOT and Andaman Sea groups. The genetic divergence of this littoral earthworm was probably attributed to restricted gene flow by the Malay Peninsular acting as a significant geographical barrier, while different tidal circulation patterns around the peninsula may restrict the population connectivity between the two sides of the peninsula.

Keywords: *Pontodrilus longissimus*, earthworm, phylogeography, population structure

Materials and methods

Introduction

Earthworms are considered as both ecosystem engineers (Jones et al., 1994) and keystone species (Blondel and Aronson, 1995) due to their contribution to the physical, chemical and biological modifications of soil properties, thus driving key ecosystem services provided by the soil (Blouin et al., 2013). There are many studies on the species richness of earthworms in Thailand, especially of the terrestrial and semi-aquatic earthworm diversity (Gates, 1972, Somniyam and Suwanwaree, 2009, Chanabun et al., 2012a, Chanabun et al., 2012b, Chanabun et al., 2013, Bantaowong et al., 2014, Bantaowong et al., 2015, Bantaowong et al., 2016, Chanabun et al., 2017). In addition, Jeratthitikul et al. (2017) analyzed DNA sequences of a 660 bp fragment of the cytochrome *c* oxidase subunit 1 (COI) gene for DNA barcoding to delimit the species of Thai terrestrial earthworm species in the genera *Amynthas* and *Metaphire*. However, there is still only one study of the littoral earthworm genus *Pontodrilus* in Thailand (Seesamut et al., 2018). As previously reported, *P. longissimus* Seesamut & Panha, 2018 is the longest littoral earthworm and its distinction from its widespread and cosmopolitan congener *P. litoralis* (Grube, 1855) was supported by both morphological and molecular analysis. *Pontodrilus longissimus* occurs in marine littoral sandy habitats and can tolerate broad salinity ranges from marine sandy coastal areas to the ecotone between marine and terrestrial-freshwater aquatic habitats (e.g. the coastal salt marsh of estuaries). Its distribution range covers both sides of the Gulf of Thailand (GOT) and the Andaman Sea coastal areas in Thailand and some parts of Peninsular Malaysia.

The Thai coastline extends along the GOT and the Andaman Sea in 23 provinces, and has a rich and diverse coastal ecosystem. The GOT is situated in the southwestern part of the South China Sea, boarded by Cambodia, Thailand and Vietnam, while the other coastal side of Thailand faces the Andaman Sea which is connected to the Indian ocean. The two different coastal areas have been reported to be without current connectivity (Wichachucherd et al., 2014), which probably influences the gene flow and population connectivity along the Thai-Malay Peninsula.

Molecular genetic data have been used to investigate the evolutionary history of species and populations through phylogenetic, phylogeographic and population-level approaches (Avise, 2009). Mitochondrial DNA (mtDNA) was first used as a molecular marker in the 1970s, and since then has had a profound impact on studies in population genetics and evolution (Avise et al., 1979, Brown et al., 1979, Avise, 2009). Many researchers have used mtDNA data to study the different levels of genetic diversity in several organisms, compare its extent within and among populations as well as between species and test if the observed pattern was congruent with their geography. Moreover, isolated or small populations could be recognized, and the extent of gene flow between populations and demographic or range expansions could be estimated (Excoffier, 2004).

Likewise, mtDNA has been shown to be a valuable tool in the evaluation of the biogeographic events of earthworms. Chang et al. (2008) investigated the systematics and phylogeography of the *Metaphire formosae* species group using three mitochondrial regions; COI, 16S ribosomal (r)RNA and NADH dehydrogenase subunit 1. Shekhovtsov et al. (2015) studied the phylogeography of the earthworm *Eisenia nordenskioldi nordenskioldi* in Northeastern Eurasia using a COI region, while the mtDNA of five genes was used to investigate the molecular phylogeny and paleogeography of the genera *Metaphire* and *Amynthas* in Hainan Island, China (Zhao et al., 2015). Moreover, two mitochondrial gene fragments (COI and 16S rRNA) were used to examine the relationships among populations of Asian megascolecid earthworms in the genus *Amynthas* in the northeast United States (Schult et al., 2016).

Therefore, this present study aimed to analyse the phylogeography of *P. longissimus* inhabiting both coastal sides of Thailand and to determine the genetic diversity and population structure using nucleotide sequence variation of the mtCOI gene fragment. In addition, this study may infer the gene flow, population connectivity and phylogeography of the littoral invertebrate populations between the GOT and Andaman Sea coastal areas.

Sample collection and acquisition of DNA sequence data

During January 2015 to January 2018, 136 samples of *P. longissimus* were collected from 16 localities in Thailand along the coasts of the GOT and the Andaman Sea (Table 4.1, Fig. 4.1). External and internal morphological characters were used to confirm the identification to the species level following Seesamut et al. (2018). A small piece of muscle tissue behind the clitellum region of each specimen was fixed in 95% (v/v) ethanol solution and stored at room temperature until DNA extraction.

Genomic DNA was extracted using a DNeasy Tissue Kit (Qiagen Inc., Valencia, CA, USA). Polymerase chain reaction (PCR) was performed to amplify a 658 bp region of the mtCOI gene fragment. Each PCR amplification mixture (50 µL total) consisted of 0.6–1 μL of DNA template, 2.5 μL (5 μM) each of the LCO1490 (forward) and HCO2198 (reverse) universal primers (Folmer et al., 1994), 25 μL of Ultra-Pure *Taq* PCR Master Mix with emerald dye and 19–19.4 μL of double distilled H_2O. The thermal cycling was heated to 94 °C for 2 min, followed by 35 cycles of 94 °C for 1 min, 48 °C for 1 min and 72 °C for 2 min, and then followed by a final extension at 72 °C for 5 min. The amplified PCR products were assessed by 1% (w/v) agarose gel electrophoresis in 0.5x TBE buffer and detected with SYBR safe DNA gel staining and ultraviolet light transillumination. The PCR products were purified using a QIAquick purification kit (QIAGEN Inc.) and sent for commercial sequencing at Macrogen, Inc. (Korea).

DNA sequence analyses

All COI sequences were aligned using the ClustalW algorithm in MEGA7 v. 7.0.18 (Thompson et al., 1994, Kumar et al., 2016) and manually checked by eyes. The sequences were checked with the NCBI database using the BLASTn algorithm to avoid contamination (http://ww.ncbi.nlm.nih.gov). Genetic distances within/between populations based on the Kimura 2-parameter model (Kimura, 1980) were calculated using MEGA7 in order to compare with other earthworm genetic studies. The relationship among the haplotypes (haplotype network) was obtained and visualized using the median joining approach (Bandelt et al., 1999) performed in PopART v. 1.7 (Leigh and Bryant, 2015). The Tamura 3-parameter model was selected as the best-fit model of nucleotide substitution for the mtCOI gene by the “Find best fit models” option of MEGA7.

Phylogenetic analyses were performed using Bayesian inference (BI) in MrBayes v. 3.2.2 (Ronquist et al., 2012) to obtain the Bayesian posterior probabilities (PPs) and by maximum likelihood (ML) analysis using RAxML v. 8.1.20 (Stamatakis, 2014), where 1,000 bootstraps were used to estimate the node reliability to obtain bootstrap support values. For the BI, the Markov-Chain Monte-Carlo (MCMC) process was run with four chains for 10,000,000 generations, with trees being sampled every 100 generations. Then, 50% of the sampled trees were discarded as a burn-in and support for nodes was defined as PPs. Bayesian PP values lower than 0.95 and bootstrap values lower than 75% for each node were considered as not significant (Okanishi et al., 2018). *Pontodrilus litoralis* was used as the outgroup in the phylogenetic tree (accession no. MK319540 and MK319541). Number of haplotypes, number of segregating sites, haplotype diversity and nucleotide diversity were calculated using DnaSP6 v. 6.11.01 (Rozas et al., 2017).

The genetic differentiation of each pair of populations was evaluated using the F_{ST} pairwise fixation index (Weir and Cockerham, 1984), with a significance test of *F*-statistics using 10,000 random permutations and performed using ALEQUIN v.3.5 (Excoffier and Lischer, 2010). Analysis of molecular variance (AMOVA) was used to analyse the genetic differentiation between the GOT and the Andaman Sea groups, which was run in ALEQUIN v.3.5 and the associated *F*-statistic analogs, including Φ_{CT}, Φ_{SC} and Φ_{ST}, were estimated at the different hierarchical levels. The significance of each Φ-statistic was tested by 10,000 permutations ($p < 0.05$) and the *P* values were adjusted according to the sequential Bonferroni method (Rice, 1989).

Results

The COI gene fragment from all 136 samples were amplified and sequenced. A total of 46 variable sites were detected, seven of which were singleton variable sites, whereas 39 were parsimony informative sites. No indel was observed in the examined sequences. The overall base composition of the COI gene fragment was T (29.8%), C (23.0%), A (30.5%) and G (16.7%). The A + T content (60.3%) was higher than C + G content (39.7%), showing AT bias. In addition, 21 haplotypes were defined from both the Andaman Sea and the GOT populations. The number of haplotypes, the number of segregating sites, haplotype diversity value (*h*) and nucleotide diversity value (π) of all 16 populations are reported in Table 4.2. The haplotype diversity from the GOT and the Andaman Sea populations were 0.835 ± 0.024 and 0.79 ± 0.039, respectively, while the nucleotide diversity was 0.0034 ± 0.0021 and 0.0089 ± 0.0048, respectively. The genetic diversity values for each of the 16 populations ranged from 0–0.6 for haplotype diversity and from 0–0.0129 for nucleotide diversity.

The highest haplotype and nucleotide diversity were found in the A5 population (Hat Yao, Nuea Klong, Krabi, Thailand) from the Andaman Sea coastal area. The nucleotide sequences reported of *Pontodrilus longissimus* in this study were deposited at GenBank under accession numbers MK319542–MK319677.

The COI haplotype network showed two main groups of haplotypes (Fig. 4.2), in which one group represented populations from the Andaman Sea coastal area and the other group was populations from the GOT. The composition of haplotypes within either the Andaman Sea or the GOT coastal area populations is given in Table 4.3. Two haplotypes were shared by two populations (Hap4: G4 and G5; Hap5: A2 and A3), while the most frequent haplotype (Hap8) was present in four populations (A5, A6, A7 and A8). The other populations each had their own private haplotype (Table 3). The analyses of genetic differentiation between populations of *P. longissimus* from the Andaman Sea and the GOT were conducted using the mtCOI gene sequence data. The results of every pairwise F_{ST} between any geographic-based populations are shown in Table 4.4. The K2P genetic distances within the GOT group and the Andaman Sea group ranged from 0–0.8% and 0–1.6%, respectively. Moreover, the genetic distances between the GOT and the Andaman Sea groups ranged from 2.7–4.1% (Table 4.4). The AMOVA analysis based on haplotype frequencies showed that the highest percentage of genetic variation (79.7%) was attributed to the comparison between the GOT and the Andaman Sea groups and its corresponding *F*-statistic (Φ_{CT} = 0.79715) was significant (p = 0.00) (Table 4.5). The phylogenetic trees generated from both the BI and ML analyses showed the same topology, and so only the BI tree is shown in Fig. 4.2. There were three highly supported clades retrieved from the phylogenetic analyses; one from the GOT (G) and two from the Andaman Sea (AS1 and AS2). However, combining the two Andaman sea clades into one clade was not supported (Fig. 4.2). Clade AS1 consisted of most of the individuals from the Andaman Sea, whereas clade AS2 consisted of four samples from Hat Koey (Kaper, Ranong) and four samples from Hat Yao (Nuea Klong, Krabi).

Discussion

The present study represents the first to describe the genetic diversity and population structure of the littoral earthworm *P. longissimus* among 16 populations from Thai coastal areas (eight populations each from the GOT and the Andaman Sea). The most frequent haplotype (Hap8), which was present in four populations from the Andaman Sea, might be the ancestral haplotype of the *P. longissimus* populations in this region. The Andaman Sea clade revealed more genetic variation than the GOT clade, exemplified by the greater K2P genetic distance (0–1.6%) and nucleotide diversity (π: 0.0089) than those calculated from the GOT clade (0–0.8%; and 0.0034, respectively). The COI intraspecific K2P genetic distance of *P. longissimus* populations collected from littoral habitats along the GOT and the Andaman Sea coasts was 2.1%, which is lower than the average intraspecific variation in the cosmopolitan littoral earthworm species *P. litoralis* (7.34%) (Seesamut et al., 2018). In addition, AMOVA revealed that the lowest partition of genetic variation was contributed to the variation within populations (6.63%).

That very few haplotypes were shared among populations of *P. longissimus* was possibly due to the low dispersal ability of the earthworms. The dispersal rates among the earthworm species correlatewith their different ecological niches or mode of reproduction (James, 2004). Moreover, most populations exhibited an extremely low intra-population COI variation and are comprised of only one haplotype per population. This might be due to inbreeding within relatives and/or self-fertilization. Torres-Leguizamon et al. (2014) reported the patterns of genetic population structure in *Aporrectodea icterica* using microsatellite and COI markers, and indicated that the sequenced fragment of the COI gene of this species showed a low genetic variation compared to other earthworm species. Two major explanations suggested for the low level of polymorphism in *A. icterica* were the occurrence of recent population bottlenecks and/or recurrent inbreeding due to reproduction between relatives. In addition, parthenogenesis could be possibly another cause, as there are some parthenogenetic species reported in the Megascolecidae (Díaz Cosín et al., 2011, Minamiya et al., 2011), the family to which *Pontodrilus* belongs. In contrast, a low genetic distance has also been reported in Thai terrestrial earthworms, where Prasankok et al. (2013) reported that *Metaphire peguana* had a high degree of gene flow (allozyme and mtCOI) between populations across the different geographic regions. Thus, the geographic barriers did not appear to exert any significant restriction to gene flow in *M. peguana*, but rather may reflect anthropogenic movement of *M. peguana* throughout Thailand.

Although it is recommended to sample a minimum of 20 to 30 individuals per population, as in other earthworm population genetic research (Peles et al., 2003), a recent study reported that sample sizes above eight individuals are sufficient for accurate estimation of genetic diversity and as few as two individuals are needed in order to obtain good estimates of population differentiation (Nazareno et al., 2017). Thus, in this study, the 3–10 individuals sampled per population was regarded as sufficient, and reflect the challenge of obtaining the samples from the very specific habitat that this earthworm inhabits. In addition, Goodall-Copestake et al. (2012) reported that a sample size of more than five individuals per population is sufficient to generate accurate haplotype diversity (*h*) and nucleotide diversity ($\boldsymbol{\pi}$) for the mitochondrial gene COI comparisons.

The mtCOI data showed evidence of genetic divergence of *P. longissimus* between the coastal areas of the Andaman Sea and the GOT. There was a clear separation between the GOT and the Andaman Sea populations which did not share any of the same haplotypes. Rather nine haplotypes were found in the GOT and not in the Andaman sea populations, while the other 12 haplotypes were found in the Andaman Sea but not in the GOT populations. The AMOVA revealed that the largest partition of genetic variation was contributed by the differentiation between these two groups. These results suggested that populations of *P. longissimus* from the Andaman Sea and the GOT have evolved separately as at least two major evolutionary lineages. In comparison to the cosmopolitan littoral earthworm *P. litoralis*, Blakemore (2007) reported that the dispersal mechanisms of this species might involve the cocoons, which are euryhaline (like other life stages) and dispersed by ocean currents or transported in beach sand ballast. Moreover, the worms could also be dispersed via rafting on floating debris. Although cocoons and all life stages of littoral earthworms have high dispersal capabilities that can promote gene flow between populations, geographic barriers and distance can still limit gene flow.

The coastal areas of the Andaman Sea and the GOT, located on the two sides of the Thai-Malay peninsula, have been reported to have different topographic and oceanographic variations (Nakthon, 1992). These factors may cause the restricted gene flow due to the position of the Malay Peninsula as a geographical barrier and lead to the genetic divergence found in *P. longissimus*. The only connection between the GOT and the Andaman Sea is the narrow Strait of Malacca. Wichachucherd et al. (2014) reported that the gene flow between the two populations of *Padina boryana* occupying either side of the Thai-Malay Peninsula were greatly obstructed due to the restricted seawater flow from the Andaman Sea into the GOT, while the reverse seawater flow was not reported. The circulation patterns of oceanic currents may have acted as effective barriers to gene flow between the GOT and the Andaman Sea populations of *P. longissimus*. Both the position of the Malay Peninsula, as the geographical barrier, and the different oceanic currents between the two coastal sides were reported to cause genetic divergence between populations of other marine organisms in the Andaman Sea and the GOT, such as the Oceanic Paddle Crab (*Varuna litterata*), using mitochondrial gene analysis (Suppapan et al., 2017), horseshoe crabs, using mitochondrial and nuclear genes analysis (Obst et al., 2012), the surf clam (*Paphia undulata*), using inter simple sequence repeat markers (Donrung et al., 2011), and the orange spotted grouper (*Epinephelus coioides*), tropical abalone (*Haliotis asinina*) and the black tiger shrimp (*Penaeus monodon*), using microsatellite markers (Supungul et al., 2000, Tang et al., 2004, Antoro et al., 2016).

Moreover, in the GOT, wind is the most significant driving force and a counter-clockwise circulation develops during the northeast monsoon, where the effect of clockwise and counter-clockwise circulations reduce the connectivity to the open ocean (Buranapratheprat, 2008). Consequently, these current patterns might restrict the dispersal ability of the organisms from the GOT into the Andaman Sea. In this study, the lower K2P genetic distances of the *P. longissimus* specimens examined within the GOT group may associate with the higher gene flow due to the effects of clockwise and counter-clockwise circulations in the GOT.

Conclusion

Sequence analysis of a mtCOI gene fragment was used to analyze the genetic diversity and population structure of *P. longissimus* from 16 populations in the coastal areas of Thailand, and revealed two divergent clades and genetic differentiation between the GOT and the Andaman Sea groups. Therefore, we propose the Malay Peninsula as the significant geographical barrier and that additionally the different tidal circulation patterns may restrict the population connectivity between the two sides of the peninsula.

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Table 4.1 Sampling localities of *P. longissimus* and their GPS coordinates.

Coastal area	Locality	Abb.	Latitude (N)	Longitude (E)
GOT	Hat Chao Lao, Thamai, Chanthaburi, Thailand	G1	12°33'52.8"	101°54'25.1"
GOT	Wat Tanon Kaprao, Klaeng, Rayong, Thailand	G2	12°41'06.1"	101°40'27.2"
GOT	Hat Sai Ngoen, Klong Yai, Trat, Thailand	G3	12°02'43.5"	102°45'01.8"
GOT	Klong Bang Siap, Patiew, Chumphon, Thailand	G4	10°39'29.9"	99°18'39.1"
GOT	Hat Sai Ree, Sawi, Chumphon, Thailand	G5	10°10'59.7"	99°10'56.8"
GOT	Ban Koh Kaew Naruemit, Pak Phanang, Nakhon Si Thammarat, Thailand	G6	8°14'28.1"	100°16'41.2"
GOT	Hat Khan Thuli, Tha Chana, Surat Thani, Thailand	G7	9°40'47.5"	99°09'41.9"
GOT	Hat Na Tub, Chana, Songkhla, Thailand	G8	7°01'18.8	100°43'54.6"
Andaman Sea	Hat Koey, Kaper, Ranong, Thailand	A1	9°37'26.7"	98°28'08.6"
Andaman Sea	Nang Thong Bay Resort, Takua Pa, Phangnga, Thailand	A2	8°38'43.4"	98°14'50.1"
Andaman Sea	Hat Bang Sak, Takua Pa, Phangnga, Thailand	A3	8°47'03.4"	98°15'46.1"
Andaman Sea	Hat Mai Khao, Talang, Phuket, Thailand	A4	8°05'47.9"	98°17'55.5"
Andaman Sea	Hat Yao, Nuea Klong, Krabi, Thailand	A5	7°58'49.4"	98°56'46.6"
Andaman Sea	Hat Samran, Hat Samran, Trang, Thailand	A6	7°14'02.9"	99°32'19.4"
Andaman Sea	Hat Pak Meng, Sikao, Trang, Thailand	A7	7°30'14.3"	99°19'06.8"
Andaman Sea	Hat Bo Chet Look, La Ngu, Satun, Thailand	A8	6°53'32.6"	99°41'12.5"

Table 4.2 Genetic haplotype and nucleotide diversity of *P. longissimus* populations along the Thai coastal areas based on the mtCOI region.

Population	Sample size	Number of haplotypes	Number of segregating sites	Haplotype diversity (*h*) (mean ± SD)	Nucleotide diversity (π) (mean ± SD)
G1	9	2	3	0.222 ± 0.166	0.001 ± 0.0009
G2	3	1	0	0	0
G3	10	1	0	0	0
G4	10	1	0	0	0
G5	10	2	1	0.2 ± 0.154	0.0003 ± 0.0004
G6	3	1	0	0	0
G7	8	1	0	0	0
G8	10	1	0	0	0
A1	10	2	12	0.533 ± 0.095	0.0097 ± 0.0056
A2	10	2	3	0.556 ± 0.075	0.0025 ± 0.0018
A3	6	1	0	0	0
A4	10	2	6	0.356 ± 0.159	0.0032 ± 0.0022
A5	10	3	16	0.6 ± 0.131	0.0129 ± 0.0073
A6	8	3	2	0.464 ± 0.2	0.0008 ± 0.0008
A7	9	2	1	0.222 ± 0.166	0.0003 ± 0.0004
A8	10	1	0	0	0
Total: GOT	63	9	13	0.835 ± 0.024	0.0034 ± 0.0021
Total: Andaman Sea	73	12	25	0.79 ± 0.039	0.0089 ± 0.0048

Table 4.3 Frequency distribution of COI haplotypes in *P. longissimus* populations from 16 localities along the Thai coastal area.

Haplotype	Gulf of Thailand (G)								Andaman sea (A)							
	G1	G2	G3	G4	G5	G6	G7	G8	A1	A2	A3	A4	A5	A6	A7	A8
Hap1	-	-	-	-	-	-	-	-	4	-	-	-	-	-	-	-
Hap2	-	-	-	-	-	-	-	-	-	-	-	8	-	-	-	-
Hap3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-
Hap4	-	-	-	10	9	-	-	-	-	-	-	-	-	-	-	-
Hap5	-	-	-	-	-	-	-	-	-	5	6	-	-	-	-	-
Hap6	-	-	-	-	-	-	-	-	6	-	-	-	-	-	-	-
Hap7	-	-	-	-	-	-	-	-	-	5	-	-	-	-	-	-
Hap8	-	-	-	-	-	-	-	-	-	-	-	-	6	6	8	10
Hap9	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-
Hap10	-	-	-	-	-	-	8	-	-	-	-	-	-	-	-	-
Hap11	-	-	-	-	-	-	-	10	-	-	-	-	-	-	-	-
Hap12	-	-	-	-	-	-	-	-	-	-	-	-	3	-	-	-
Hap13	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hap14	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-
Hap15	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-
Hap16	-	-	10	-	-	-	-	-	-	-	-	-	-	-	-	-
Hap17	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-
Hap18	8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hap19	-	-	-	-	-	3	-	-	-	-	-	-	-	-	-	-
Hap20	1		-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hap21	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-
Total	9	3	10	10	10	3	8	10	10	10	6	10	10	8	9	10

Table 4.4 Pairwise F_{ST} estimates (below diagonal) and genetic distances corrected with the Kimura-2 parameter model (above diagonal) among the populations of *P. longissimus*. Sampling abbreviations are listed in Table 4.1.

	G1	G2	G3	G4	G5	G6	G7	G8	A1	A2	A3	A4	A5	A6	A7	A8
G1		0.002	0.007	0.002	0.002	0.005	0.004	0.005	0.027	0.033	0.033	0.037	0.031	0.037	0.037	0.037
G2	0.632*		0.008	0.003	0.003	0.006	0.005	0.006	0.028	0.034	0.035	0.038	0.032	0.038	0.038	0.038
G3	0.927*	1.000*		0.005	0.005	0.008	0.006	0.008	0.030	0.035	0.036	0.037	0.033	0.040	0.039	0.039
G4	0.761*	1.000*	1.000*		0.000	0.003	0.002	0.003	0.027	0.034	0.035	0.035	0.031	0.038	0.038	0.038
G5	0.704*	0.923*	0.967*	0.000		0.003	0.002	0.003	0.027	0.034	0.035	0.036	0.031	0.038	0.038	0.038
G6	0.845*	1.000	1.000*	1.000*	0.923*		0.005	0.003	0.030	0.037	0.038	0.039	0.034	0.041	0.041	0.041
G7	0.848*	1.000*	1.000*	1.000*	0.898*	1.000*		0.005	0.028	0.035	0.036	0.037	0.032	0.040	0.039	0.039
G8	0.905*	1.000*	1.000*	1.000*	0.952*	1.000*	1.000*		0.030	0.037	0.038	0.039	0.034	0.041	0.041	0.041
A1	0.788*	0.727*	0.831*	0.814*	0.809*	0.743*	0.805*	0.833*		0.011	0.012	0.013	0.013	0.014	0.014	0.014
A2	0.942*	0.937*	0.962*	0.961*	0.956*	0.942*	0.958*	0.964*	0.415*		0.002	0.010	0.014	0.010	0.010	0.010
A3	0.980*	1.000*	1.000*	1.000*	0.994*	1.000*	1.000*	1.000*	0.526*	0.316		0.013	0.016	0.013	0.012	0.012
A4	0.938*	0.929*	0.954*	0.953*	0.948*	0.930*	0.949*	0.956*	0.489*	0.716*	0.837*		0.012	0.006	0.005	0.005
A5	0.757*	0.683*	0.801*	0.783*	0.778*	0.700*	0.772*	0.803*	0.112	0.449*	0.514*	0.318*		0.010	0.010	0.010
A6	0.975*	0.984*	0.991*	0.990*	0.986*	0.985*	0.990*	0.991*	0.591*	0.830*	0.964*	0.619*	0.282*		0.001	0.000
A7	0.981*	0.992*	0.995*	0.995*	0.991*	0.993*	0.995*	0.995*	0.614*	0.851*	0.983*	0.652*	0.305	0.005		0.000
A8	0.986*	1.000*	1.000*	1.000*	0.995*	1.000*	1.000*	1.000*	0.636*	0.871*	1.000*	0.686*	0.328	0.029	0.012	

Table 4.5 AMOVA of mtCOI haplotypes of *P. longissimus* populations between the GOT and the Andaman Sea groups.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*p*-value
Among groups	1	625.750	9.03258	79.72	Φ_{CT} = 0.79715* (p = 0.00)
Among populations within groups	14	192.632	1.54677	13.65	Φ_{SC} = 0.67295* (p = 0.00)
Within populations	120	90.206	0.75171	6.63	Φ_{ST} = 0.93366* (p = 0.00)
Total	135	908.588	11.33106		

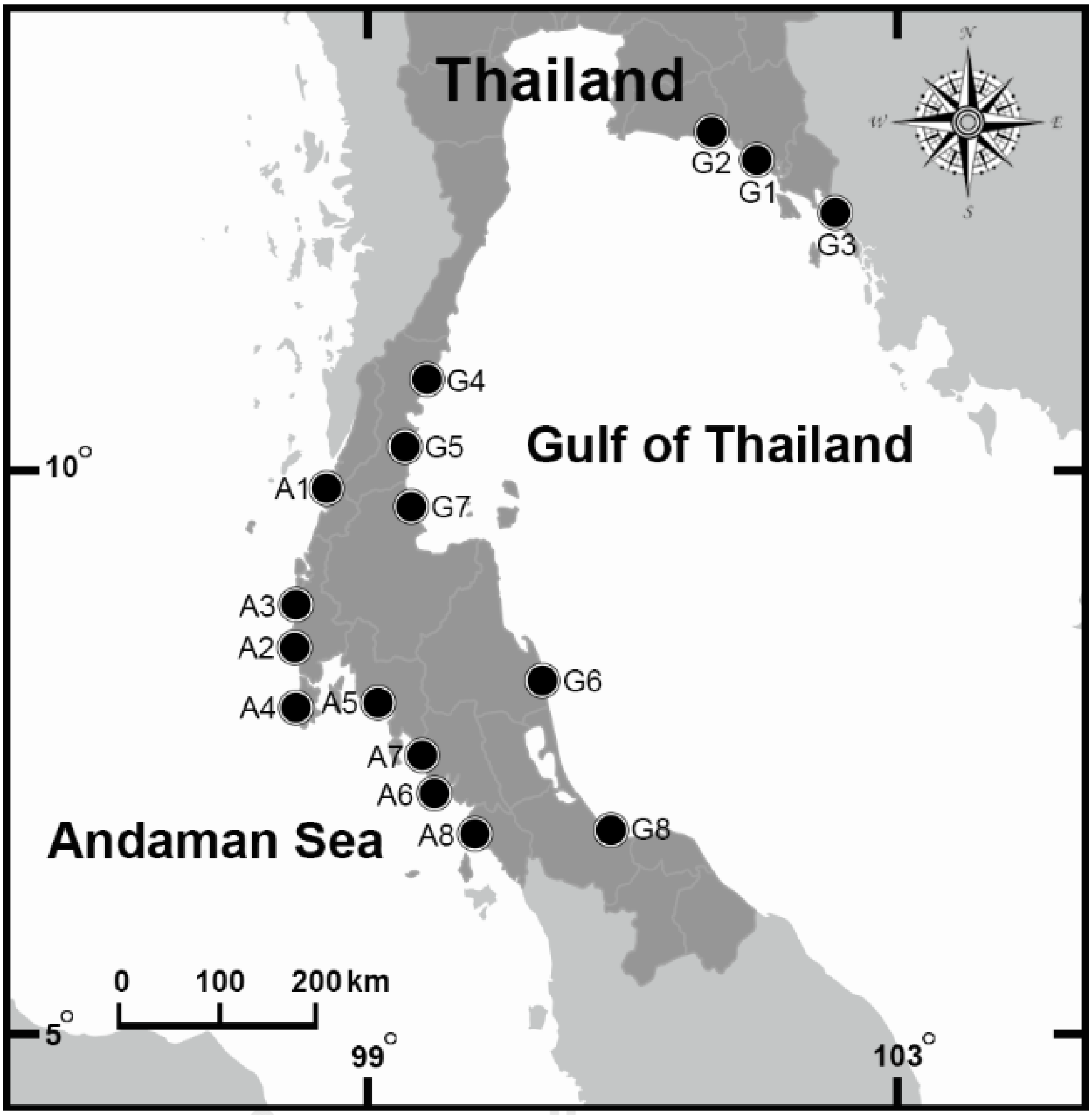

Figure 4.1 Collection sites of *P. longissimus* along the Thai coastal areas.

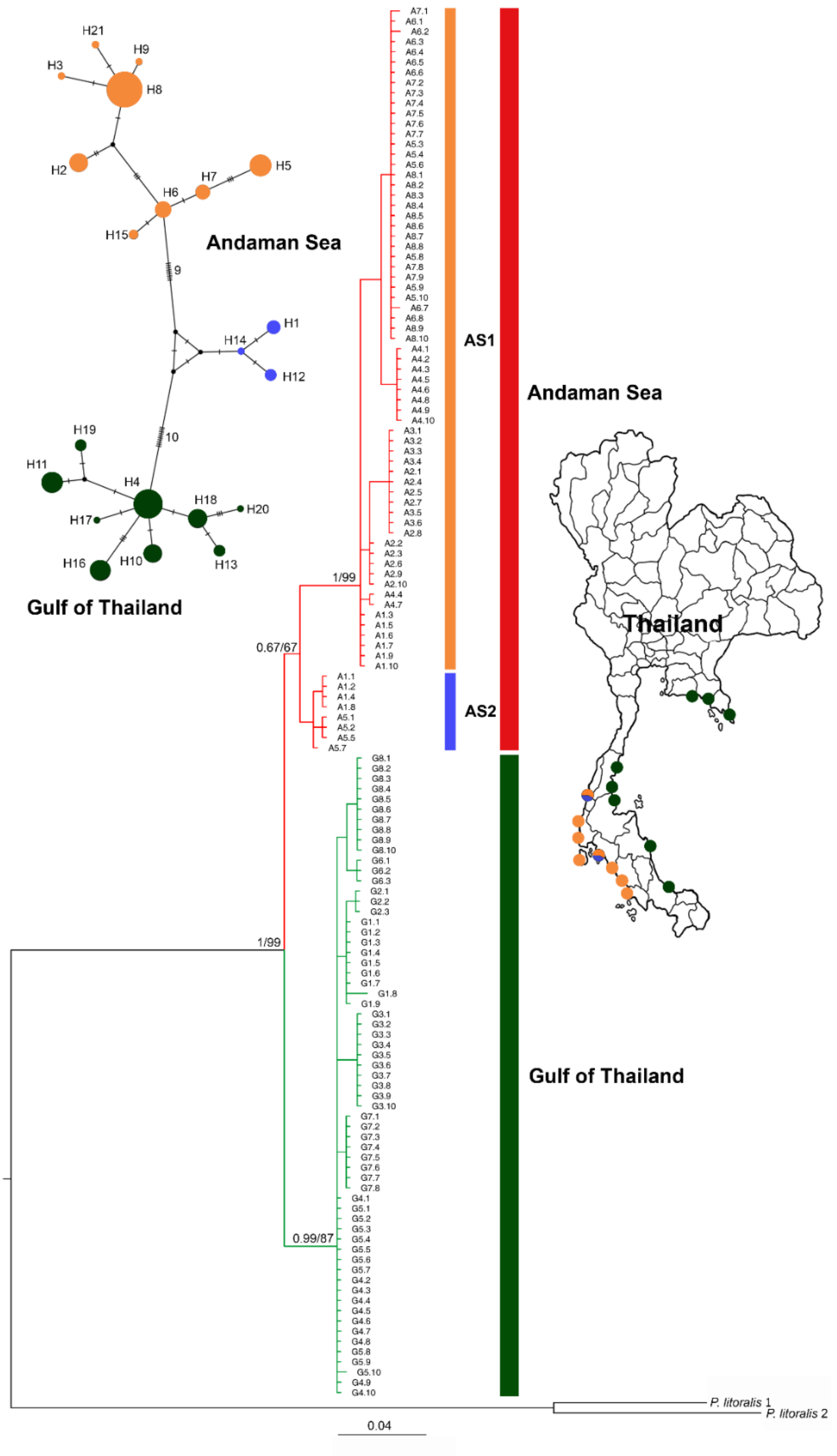

Figure 4.2 Haplotype network and phylogenetic tree resulting from the analysis of the COI dataset. Median-joining networks of COI haplotypes from 16 populations of *Pontodrilus longissimus*; each circle represents a haplotype, its size is proportional to its total frequency. Hatch marks indicate one base pair change. Bayesian inference

tree of COI sequences of *Pontodrilus longissimus*. Supporting values on each node are shown as Bayesian posterior probability / ML bootstrap value. *P. litoralis* was used as outgroup. Scale bar represents the number of nucleotide substitutions per site. On the right side, geographical distribution of populations within each *P. longissimus* clade is shown in the inset map. The Gulf of Thailand clade (G) is shown in green and the Andaman Sea clade (A) is shown in red (AS1 and AS2 are shown in orange and blue, respectively).

Chapter 5

Discussion and Conclusion

Taxonomy and systematics of the earthworm genus *Pontodrilus* Perrier, 1874 was previously poorly known in Thailand. Only Panha et al. (2007) reported the occurrence of the littoral earthworm *Pontodrilus litoralis* (Grube, 1855) in Thailand from Khanom, Nakhon Si Thammarat. In this study, comprehensive surveys were conducted along the east and west coastal areas of the Thai-Malay Peninsula, and selected localities in Japan. Comparative materials from other parts of Southeast Asia, including Myanmar, Vietnam, Malaysia, Singapore and Indonesia, were also collected. The littoral earthworm genus *Pontodrilus* occurs in several types of habitats, such as estuaries, brackish, damp mud under stones, under the trash or leaf litter on sandy beaches, mangrove swamps of the intertidal zone, sanitary sewer links and fresh waterways linked between the mainland and the sea (Aiyer, 1929, Gates, 1972, Gobi et al., 2004, Shen et al., 2005, Panha et al., 2007, Satheeshkumar et al., 2011, Narayanan et al., 2014, Nguyen et al., 2016).

In order to confirm the validity of species, morphological and molecular analyses were combined in this study. A comprehensive report was produced based on the occurrence, distribution and habitat characteristics of the cosmopolitan *P. litoralis* collected from the coastal areas of Thailand, Japan (Honshu, Kyushu and Ryukyu islands) and some parts of Southeast Asia (Myanmar, Vietnam, Malaysia, Singapore and Indonesia). This survey supported the assumption that *P. litoralis* is a cosmopolitan earthworm species, widely distributed in sub-tropical and tropical coastal ecosystems (Gates, 1972, Jamieson and Wampler, 1979, Oba et al., 2015) and aligns with the worldwide distribution records (Easton, 1984, Blakemore, 2002).

Morphological examination of the collected littoral earthworms, based on distinct morphological characteristics, resulted in two nominal species of *Pontodrilus*; *P. litoralis* and the new species *P. longissimus* Seesamut and Panha, 2018 that was then formally described (Seesamut et al., 2018; chapter 2). The morphology of these *P. litoralis* specimens conformed to several previous taxonomic studies (Gates, 1972, Easton, 1984). The new species (*P. longissimus*) was described from Hat Pak Meng, Sikao, Trang province in Thailand, and differed from *P. litoralis*, based on the specimens from Thailand and Peninsular Malaysia, in the segment number and body length. Additionally, *P. longissimus* is easily distinguished by the absence of a spermathecal diverticulum, which is present in all other currently accepted species of the genus. The molecular analysis, based upon 658 bp fragment of mtCOI, revealed a high interspecific genetic distance between *P. litoralis* and *P. longissimus*, which is comparable to the differences between other earthworm species noted in DNA barcoding studies (Chang and James, 2011, Szederjesi et al., 2017).

The occurrence of the littoral earthworm genus *Pontodrilus* in Southeast Asia and Japan was recorded. *Pontodrilus litoralis* was found scattered over the coastal areas in Thailand, Myanmar, Vietnam, Malaysia, Singapore and Indonesia, with the southernmost site in Bantan, Indonesia and the northernmost site at Nghệ An Province, Vietnam. This included the first reported occurrence of *P. litoralis* in Singapore. Among the localities in the sub-tropical areas, *P. litoralis* specimens were surveyed and collected from various beaches in Japan, including Honshu, Kyushu and Ryukyu, where the northernmost site was Matsushima Kaihin Koen in the Miyagi prefecture, where the synonym of *P. litoralis* (*P. matsushimensis*) was originally described from. On the other hand, *P. longissimus* was only recorded in Thailand, Malaysia and Vietnam, and was mainly found in habitats with substrates on the surface layer that contained muddy sand. Based on the habitat preference of this species, it is hypothesized that the worms prefer the ecotone habitats between marine and terrestrial-freshwater aquatic, such as the coastal salt marsh of estuaries.

Based on field collections within Thailand and some parts of Southeast Asia, *P. litoralis* was found to occupy several types of habitats, including under trash or leaf litter on sandy beaches, mangrove swamps in the intertidal zone, estuaries, under stones in brackish, damp mud, near sanitary sewer links and fresh waterways linked between the mainland and the sea. However, field collections of *P. litoralis* in the Japanese coastline showed that it was mostly found in sandy beaches facing the ocean where it lives in the sand mixed with seaweed debris. These habitats of *P. litoralis* in Japan were similar to those reported in Western Australia coastal areas, where a high abundance of earthworms inhabited wreck materials, seaweed and debris deposited on arid beaches, which provide a rich food resource (Blakemore, 2007, Coupland and McDonald, 2008). The salinity of habitats where *P. litoralis* was collected from in the field surveys varied from 1–33‰, the upper bound of which is near the salinity of seawater in general (35‰; Schmidt et al., 2018), which indicated that *P. litoralis* can survive a wide range of salinity. Rather the occurrence records from this study suggested that the habitat preference of *P. litoralis* is primarily determined by the abundance of organic matter contents and not the salinity.

Various studies of the morphometric characters of earthworms have suggested that the body size is one of the key characters for confirming earthworm systematic positions and can be helpful in identifying earthworm species via their morphological variation and taxonomy (Chang et al., 2007, Oboh et al., 2007, James et al., 2010). In this study, morphometric analysis of the size variation (body length, body size diameter and number of segments) of 212 samples of *P. litoralis* from 14 sampling sites (representing different geographic regions) revealed that there was a significant difference in the body length and diameter among specimens from different geographical sites. However, this distinction was not congruent with the phylogenetic relationship based on mtCOI gene sequence analysis, reflecting that the size variations in *P. litoralis* do not correlate with their genetic differences. Ng et al. (2017) found significant differences in the morphometric variations of the terrestrial earthworm *Metaphire peguana* (Rosa, 1890) that were not matched by their genetic difference, but rather were affected by the type of habitat. For this reason, future studies on the habitat types, sand texture and components of the food resources of *P. litoralis* are necessary.

Mitochondrial DNA has become a valuable tool in phylogenetic constructions to examine the relationships among populations and the biogeographic events of earthworms (Chang et al., 2008, Shekhovtsov et al., 2015, Zhao et al., 2015, Schult et al., 2016). In this study, the molecular analysis of 136 samples of *Pontodrilus longissimus* from 14 localities using the 658 bp DNA fragment of mtCOI was performed in order to analyze the phylogeography of *P. longissimus* inhabiting both coastal sides of Thailand and to investigate the genetic diversity and population structure.

The Thai populations from the Andaman Sea coastal area at Hat Yao, Nuea Klong and Krabi had the highest haplotype and nucleotide diversity and might be the ancestral haplotype of the *P. longissimus* populations in this region. The Andaman Sea clade revealed a higher genetic variation than the GOT clade. The phylogenetic trees generated from both BI and ML analyses, along with the application of haplotype network analysis, clearly showed that there are two geographically isolated populations; one from the Andaman Sea coastal area and the other from the GOT. These results suggested that populations of *P. longissimus* from the Andaman Sea and the GOT are evolving separately as at least two major evolutionary lineages, since the GOT and the Andaman Sea populations did not share any haplotypes.

In comparison to the cosmopolitan *P. litoralis*, the dispersal mechanism of *P. longissimus* might involve cocoons, which are euryhaline (like other life stages), and dispersed by ocean currents or transported in beach sand ballast. Moreover, the worms could also be dispersed via rafting on floating debris (Blakemore, 2007). Although the cocoons, and indeed all life stages of littoral earthworms, have high dispersal capabilities that can promote gene flow between populations, distance and geographic barriers can still limit gene flow. The genetic divergence between populations of marine organisms in the Andaman Sea and the GOT have been proposed to be caused by the circulation patterns of oceanic currents that may act as effective barriers to gene flow. Thus, along with the position of the Malay Peninsula acting as a geographical barrier, the different oceanic currents between the two coastal sides (Supungul et al., 2000, Tang et al., 2004, Donrung et al., 2011, Obst et al., 2012, Antoro et al., 2016, Suppapan et al., 2017) act as an effective gene barrier between the Andaman Sea and GOT populations.

Moreover, since very few haplotypes were shared among populations of *P. longissimus* along the Andaman Sea or the GOT, this may reflect a low dispersal ability of the earthworms. James (2004) explained that the dispersal rates among earthworm species correlate with their different ecological niches or mode of reproduction. In addition, inbreeding within relatives and/or self-fertilization might occur in this earthworm species, since most populations exhibited an extremely low intra-population COI variation and were comprised of only one haplotype per population.

In conclusion, this study revealed the species diversity and systematics of littoral earthworms in the genus *Pontodrilus* in Thailand and some adjacent countries. An in-depth study on *Pontodrilus* in Thailand could potentially be used to improve the fundamental knowledge of *Pontodrilus* species and is important for their conservation and the sustainable management of coastal areas. The discovery of a new species, *P. longissimus*, and distinct phylogenetic differences among populations indicated that such differences may be common among fauna on both sides of the peninsula, and more efforts are necessary to ensure conservation of their genetic diversity, and to protect coastal habitats and avoid human impacts, like building the Kra canal. Lastly, an intensive study on the phylogenetic relationships involving additional genetic markers (nuclear DNA) or even increasing the samples to a worldwide scale will be useful to fulfill the fundamental knowledge of littoral earthworm species

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