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External Morphological Comparison, Taxonomic Revision and Molecular Differentiation of the Four Economically Important Species of Earthworm in Thailand

WIRIYA LOONGYAI[1], PHUWADOL BANGRAK† AND SOMCHAI CHANTSAVANG
Department of Animal Science, Faculty of Agriculture, Kasetsart University, Bangkok 10900 Thailand
†*School of Science, Walailak University, Thasala, Nakhon Si Thammarat 80160 Thailand*
[1]Corresponding author's e-mail: wiriyal@yahoo.com; agrwyl@ku.a.th

ABSTRACT

Four economically important species of earthworm were cultured and the external and internal characters of adult clitellate earthworms were studied. Partial sequences for ribosomal 16S rDNA and subunit one for mitochondrial cytochrome c oxidase (COI) of four earthworm species were obtained. The result of sequence analysis combined with taxonomic characters could distinguish the different species of earthworm. Morphology and nucleotide sequence of two genes for the red worm (*Pheretima peguana*) were distinct from *Eudrilus eugeniae* but were similar to the blue worm (*Perionyx excavatus*) and Lao worm (*P. excavates*) and therefore, it was classified as a new species, *Perionyx* sp. 1. Moreover, *Eudrilus eugeniae* was evidently defined as the same genus and species. Interestingly, the blue worm and Lao worm were morphologically similar to *Perionyx* sp. However, the molecular data of 16S rDNA could not differentiate in taxa of those two species. COI nucleotide sequence analyses showed the presence of divergent lineages between two species, suggesting the blue worm and Lao worm could be described as *Perionyx* sp. 2 and *Perionyx* sp. 3, respectively. © 2011 Friends Science Publishers

Key Word: Cytochrome c oxidase subunit I; *Eudrilus eugeniae; Perionyx* sp.; Ribosomal 16S rDNA

INTRODUCTION

Over 10,000 species of earthworms exist around the world and only 31 described species of earthworms inhabit Thailand (Gates, 1939). The culture of worms on a large scale is in high demand for the production of both protein and biofertilizer. In every region of the world, many species of earthworm are cultured namely *Eisenia fetida, Lumbricus terrestris, Perionyx excavatus* and *Eudrilus eugeniae* in all part of the world. In Thailand, these four economically important species of earthworm are of great importance in the vermicomposting of a wide variety of organic wastes and also a potential source of protein for animal consumption. *E. eugeniae* is widely distributed in warmer parts of the world and cultured as the "African Nightcrawler" (AF). Introduced species are commonly found over a large area of tropical Asia, namely, the blue worm or Indian worm (*Perionyx excavates*), red worm (*Pheretima peguana*) and earthworm from Lao (*P. excavates*) as previously described by Ayamuang (2000). However, the AF earthworm (*E. eugeniae*) and red worm (*P. peguana*) are almost similar in body size and coloration.

The lack of agreement in their ranking as diagnostic characters for taxonomic and genetic purposes, especially the red worm has led to situations in which the same or different species receives names. However, the blue worm (*P. excavatus*) and Lao worm (*P. excavatus*?) are also similar in body size and coloration. Interestingly, the Lao worm has a smaller tail than the blue worm, although they have been identified as same species. Such problems have led to misidentification of cultured earthworms species. The taxonomic technique was used to identify morphological characters of earthworm species. The problem of taxonomic identification is morphological characters, both external and internal, often show high variability between individuals (Pop *et al*., 2003; Reynolds, 2004; Chang *et al*., 2007; Iglesias Briones *et al*., 2009). However, previous reports have shown the evidence that in earthworm systematics, reproductive organs including the clitella, male pores, female pores, testes, ovaries, spermathecae, and prostate glands are believed to be less affected by environmental influences through time and are evolutionarily more conservative (Gates, 1972; Blakemore, 2002).

In recent times, morphological characters still are used to characterize earthworm species. Stephenson (1930) reported that the genital system is much more conservative and resistant to evolutionary change than the somatic system. Thus, molecular techniques are used to improve the

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understanding of disputed taxonomic problems of different earthworm groups that are rather scarce and sporadic (Pop *et al*., 2004; Heethoff *et al*., 2004; Dupont, 2009). Recently, several genes have been chosen for identifying the earthworm species through DNA barcoding such as mitochondrial cytochrome-c oxidase I, COI (Pop *et al.*, 2003; Chang *et al*., 2007; Huang *et al.*, 2007; Pop *et al*., 2007; Iglesias Briones *et al*., 2009; Otomo *et al*., 2009; Pérez Losada *et al.*, 2009; Richard *et al*., 2010), 18S (Pop *et al.*, 2003, 2007), 28S (Pérez Losada *et al.*, 2009) and 16S ribosomal DNA (Pop *et al.*, 2003; Pop *et al*., 2007; Iglesias Briones *et al.*, 2009; Pérez Losada *et al.*, 2009).

Mitochondrial DNA (mtDNA) has been widely used in molecular taxonomy of animals, because it evolves much more rapidly than nuclear DNA and it has been used to identify the differences between closely related species (Brown *et al.*, 1979; Moore, 1995; Mindell *et al.*, 1997; Hebert *et al*., 2003; Otomo *et al*., 2009). Thus, DNA barcodes based on a fragment of the COI and 16S rDNA genes have been demonstrated to work well for species identification. The present study explores the utility of DNA sequences from these genes to identify the four species and taxonomic status of poorly known earthworm species.

MATERIALS AND METHODS

Earthworm culture and preservation: The four economically important species of earthworm, African Nightcrawler, red worm, blue worm and Lao worm were cultured in a plastic box under controlled environmental conditions. Air-dried powdered chicken manure moistened with water and kept for one week for thermal stabilization and microbial initiation of degradation. The culture beds were prepared by transferring stabilized chicken manure as food (in the ratio of 1:50 worm to manure) to round plastic boxes. The moisture content of the food was maintained at 75 to 80% by sprinkling it with tap water whenever required. The food was replaced at monthly intervals by stabilized stock chicken manure, to avoid scarcity of food. The adult clitellate earthworms (six individuals for each population) were killed in 30% ethanol and preserved in 95% ethanol at room temperature. All specimens in this study were identified following the taxonomic key in Gate (1972).

DNA extraction, PCR amplification and DNA sequencing: Total genomic DNA was isolated from one to six individuals per species following the phenol/chloroform method and washing with ethanol. DNA was diluted to the working concentration with TE-buffer. The 16S rDNA fragments were amplified using the primers 16sar (5′-CGCCTGTTTATCAAAAACAT-3′) and 16sbr (5′-CCGGTYTGAACTCAGATCAYGT-3′) (Palumbi *et al.*, 1991). PCR amplification was carried out in a 50 µL total volume, using 1 cycle at 94°C for 1 min, followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 54°C, and extension for 50 s at 72°C, with a final extension at 72°C for 10 min. A fragment of the mitochondrial cytochrome c oxidase subunit I sequences (COI) was amplified using the universal primers HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) and LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) (Folmer *et al.*, 1994) following the same procedures as for 16S rDNA fragments. The PCR products were checked on 1.5% agarose gel electrophoresis and cloned into pGEM-T Easy (Promega, USA). DNA sequencing was performed in both directions by BioDesign Co., Ltd., Thailand.

Sequence analysis: Sequences were aligned using Clustal X, version 1.8 (Thompson *et al.*, 1997), and were improved manually using BIOEDIT 5.0.9 sequence alignment software (Hall, 1999). All alignment illustrations were created with the program GENEDOC, version 2.6.001 (Nicholas & Nicholas, 1997). The nucleotide divergence of 16S rDNA and COI gene from four species was analyzed by PAUP portable version 4.0b10 for Unix (Swofford, 2002).

RESULTS

External and internal characters: The adult clitellate earthworms used in this study are illustrated in Fig. 1. External and internal characters were used to identify different species of earthworm as described by Gates (1972). Different parameters including length, clitellum width, segment number, first dorsal pore position, setae, prostomium, position of female and male pore, penail setae, number and position of spermathecae, position of last heart, position of seminal vesicles and position of prostate glands were used for the identification of earthworm species (Table I). The result showed that *E. eugeniae* was defined as the same genus and species. The morphology of *P. peguana* was similar to *P. macintoshi*, but some differences such as the number of clitellum, position of first dorsal pore, thick septa at position 6-7 and lack of gizzard. Therefore, the red worm (*P. peguana)* was classified as *Perionyx* sp. 1. The species of the blue worm (*P. excavatus*) and earthworm from Lao (*P. excavates*?) were morphologically similar to *Perionyx tenuis*. Many species were compared and it was found that the position of spermathecae was distinct from *P. tenuis*. The two species of the *Perionyx* species complex, the blue worm and Lao worm, were morphologically similar while body length, clitellum width, number of segments and clitellum segments were different. Thus, the blue worm and Lao worm were described as new species identified as *Perionyx* sp. 2 and *Perionyx* sp. 3, respectively.

The 16S ribosomal DNA (16S rDNA): The 16S rDNA sequences were generated from six adult earthworms. The samples used in the alignment analyses and the corresponding GenBank accession numbers of the 16S rDNA sequences (Table II). Nucleotide sites ranging between 482 to 485 bp were sequenced and showed alignments of nucleotide sequences of *E. eugeniae*, *Perionyx* sp. 1, *Perionyx* sp. 2 and *Perionyx* sp. 3 (Fig. 2).

Table I: Morphological comparison of *Eudrilus eugeniae, Perionyx* sp. 1, *P.* sp. 2 and *P.* sp. 3 according to the original descriptions

	Eudrilus eugeniae	Perionyx sp. 1	Perionyx sp. 2	Perionyx sp. 3
Body length (mm)	140-145	122-134	85-120	73-90
Clitellum width (mm)	5.3-5.5	4.0-4.2	1.4-1.7	1.5-1.7
Number of segments	184-188	166-175	149-185	179-181
Clitellum segments	14-18	13-17	13-17	14-16
First dorsal pore position	ND	5-6	ND	ND
Setae	Closely paired	Lumbricine	Lumbricine	Lumbricine
Prostomium	Epilobous	Epilobous	Epilobous	Epilobous
Position of female pore	14	14	13	13
Position of male pore	17-18	18	18	18
Penail setae	-	-	1 pairs	1 pairs
Number of spermathecae	ND	2 pairs	3 pairs	3 pairs
Position of spermathecae	ND	7-8, 8-9	6-7, 7-8, 8-9	6-7, 7-8, 8-9
Position of last heart	12	12	13	13
Position of seminal vesicles	11, 12	11, 12	11, 12	11, 12
Position of prostate glands	17-23	18	18	18

ND: no data

Table II: Genes, Species and GenBank accession numbers for sequenced earthworms

Gene	Species	Nucleotide length (bp)	Accession number
16S rDNA	*Eudrilus eugeniae*	482	HM219175
	Perionyx sp.1	485	HM219176
	Perionyx sp.2	483	HM219177
	Perionyx sp.3	483	HM219178
Cytochrome c oxidase subunit I sequences	*Eudrilus eugeniae*	657	HM219171
	Perionyx sp.1	657	HM219172
	Perionyx sp.2	657	HM219173
	Perionyx sp.3	657	HM219174

Table III: Comparison of nucleotide sequence identity of the 16S rRNA and COI of *Eudrilus eugeniae, Perionyx* sp. 1, *Perionyx* sp. 2 and *Perionyx* sp. 3 to those deposited in GenBank

Species	Accession number	Nucleotide length (bp)	Identity (%)
16S rRNA			
Eudrilus eugeniae			
Diporochaeta sp.	AF406574	463	84
Allolobophora chlorotica	AM774393	479	84
Pontodrilus litoralis	AF406586	468	84
Perionyx sp. 1			
Perionyx excavatus	AF406582	455	95
Diporochaeta sp.	AF406574	463	87
Spenceriella sp.	AF406572	465	87
Perionyx sp. 2			
Perionyx excavatus	AF406582	455	90
Eisenia fetida	DQ257296	455	90
Pontodrilus litoralis	AF406586	468	87
Perionyx sp. 3			
Perionyx excavatus	AF406582	455	90
Eisenia fetida	DQ257296	455	90
Pontodrilus litoralis	AF406586	468	87
Cytochrome c oxidase subunit I			
Eudrilus eugeniae			
Diplocardia komareki	EF156634	601	83
Glossoscolecidae sp.	GU013961	657	82
Amynthas wulinensis	DQ224180	658	82
Perionyx sp. 1			
Megascolecidae sp.	GU013873	657	83
Metaphire nanaoensis	AY960805	1056	83
Metaphire formosae	AY960807	1056	83
Perionyx sp. 2			
Megascolecidae sp.	GU013856	657	83
Metaphire glareosa	AY960803	1056	83
Acanthodrilidae sp.	GU014211	657	83
Perionyx sp. 3			
Megascolecidae sp.	GU013856	657	83
Metaphire glareosa	AY960803	1056	83
Acanthodrilidae sp.	GU014211	657	83

Table IV: Nucleotide structure of 16S rDNA and Cytochrome c oxidase sequences in earthworms

Species	16S rDNA			COI		
	GC%	AT%	GC/AT	GC%	AT%	GC/AT
Eudrilus eugeniae	33.36	66.64	0.50	41.65	58.35	0.71
Perionyx sp. 1	39.18	60.82	0.64	42.60	57.40	0.74
Perionyx sp. 2	37.11	62.89	0.59	40.24	59.76	0.67
Perionyx sp. 3	37.11	62.89	0.59	40.24	59.76	0.67

Table V: The sequence divergence of the 16S rRNA and COI gene between *Eudrilus eugeniae*, *Perionyx* sp. 1, *Perionyx* sp. 2 and *Perionyx* sp. 3

16S rDNA				
	Perionyx sp. 2	*Perionyx* sp. 3	*Perionyx* sp. 1	*E. eugeniae*
Perionyx sp. 3	0.00%			
Perionyx sp. 1	10.59%	10.59%		
E. eugeniae	18.62%	18.62%	17.93%	
Cytochrome c oxidase subunit I				
Perionyx sp. 3	0.16%			
Perionyx sp. 1	18.48%	18.63%		
E. eugeniae	19.88%	19.72%	19.57%	

No variation was observed in the sequences between *Perionyx* sp. 2 and *Perionyx* sp. 3. Interestingly, the nucleotide of *Perionyx* sp. 1 differs from *Perionyx* sp. 2 and *Perionyx* sp. 3 at 32 sites in the nucleotide sequence. Between *E. eugeniae* and *Perionyx* sp., divergent sequences of 16S rDNA were observed (Table V). Sequence divergence between *E. eugeniae* and *Perionyx* sp. 1 was around 18.62%, whereas no sequence divergence between *Perionyx* sp. 2 and *Perionyx* sp. 3 was observed.

The cytochrome c oxidase subunit 1 (COI): Nucleotide sites of 657 bp COI sequences were determined and GenBank accession numbers (Table II). The alignment of nucleotide sequences (Fig. 3) was analyzed. The results showed that the nucleotide sequences of *Perionyx* sp. 2 and *Perionyx* sp. 3 differed at one nucleotide sites (A: *Perionyx* sp. 2 & T: *Perionyx* sp. 3). Therefore, it was found that these worms revealed, which confirmed the variations at the COI region. The result of alignment was also displayed the intra-specific variation (49 sites) among *Perionyx* sp. and the inter-specific variation in nucleotide sequence between *E. eugeniae* and *Perionyx* sp. (Fig. 3). The results of nucleotide divergence of the COI gene (Table V) indicated 19.57% divergence between *E. eugeniae* and *Perionyx* sp. 1. Interestingly, the nucleotide divergence between *Perionyx* sp. 2 and *Perionyx* sp. 3 was 0.16%.

Sequence analysis: The comparison of nucleotide sequence identity of 16S rDNA and COI sequence in the four taxa was shown in Table III. The sequence of *E. eugeniae* exhibited 84% homology with the sequence of *D. komareki* (463 bp compared, GenBank [accession number AF406574]). Similarly, sequence of *Perionyx* sp. 1, *Perionyx* sp. 2 and *Perionyx* sp. 3 showed 95%, 90% and 90% homology to *P. excavatus* (455 bp compared,

Fig. 1: The four economically important species of earthworm: *Eudrilus eugeniae* (A), *Perionyx* sp. 1 (B) *Perionyx* sp. 2 (C) and *Perionyx* sp. 3 (D)

GenBank [accession number AF406582]), respectively. Moreover, the sequence of the COI obtained from the present study, *E. eugeniae* showed 83% homology to *D. komareki* (601 bp compared, GenBank [accession number EF156634]). The sequence of COI determined for isolates of *Perionyx* sp. 1, *Perionyx* sp. 2 and *Perionyx* sp. 3 showed a homology of 83% with the sequence of *Megascolecidae* sp. (657 bp compared, GenBank [accession number GU013856]). The relative nucleotide structure of analyzed genes is presented in Table IV. Nucleotides base composition was similar for all sequences with a strong AT base (%). The percentage of GC content of 16S rDNA ranged from 33.36% to 39.18%, while the GC content of COI gene was stronger than 16S rDNA, the GC content ranged from 40.24 to 42.60%.

DISCUSSION

Recently, four economically important species of earthworm were identified in Thailand, namely, the AF, blue worm, Lao worm and red worm. However, data is lacking for those earthworm species (except *E. eugeniae* & *P. excavatus*) concerning morphological and genetic information that is used to determine the precise species and eliminate continued confusion about their taxonomic. As previously reported (The information was published in Thai journal & Thai website), the AF, blue worm, Lao worm and red worm were identified as *E. eugeniae*, *P. excavatus*, *P. excavatus*? (Ayamuang, 2000) and *P. peguana*, respectively. In the present study, taxonomic identification and molecular techniques were used to identify and improve the understanding of disputed taxonomic problems of the abovementioned four economically important species of earthworm in Thailand. External and internal characters of earthworm were used to identify species in this study and

Fig. 2: The nucleotide sequence alignment of 16S rDNA of *Eudrilus eugeniae* (Eue), *Perionyx* sp. 1 (Psp1), *Perionyx* sp. 2 (Psp2) and *P*erionyx sp. 3 (Psp3)

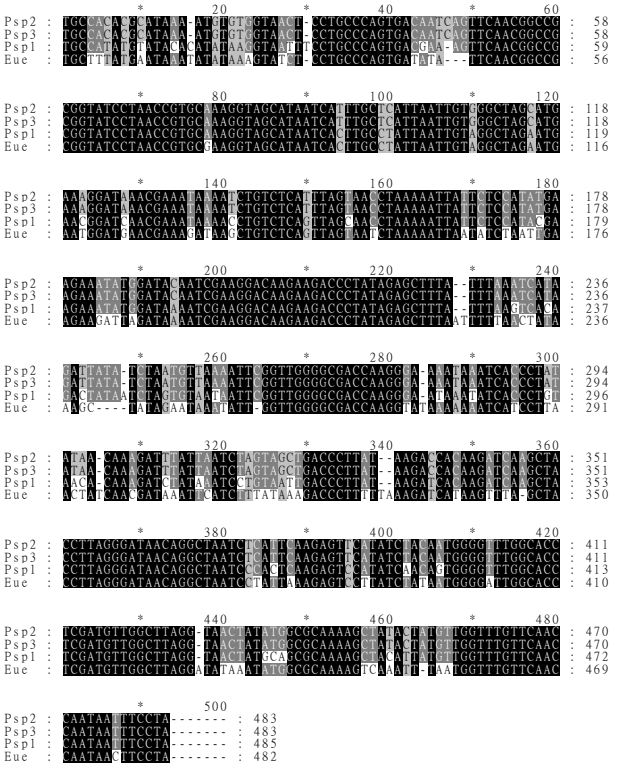
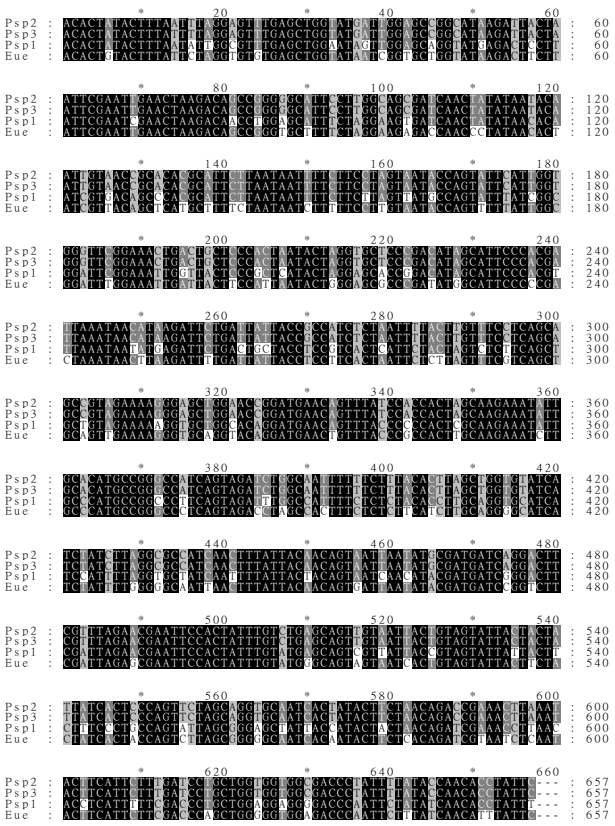


Fig. 3: The nucleotide sequence alignment of cytochrome c oxidase subunit I (COI) of *Eudrilus eugeniae* (Eue), *Perionyx* sp. 1 (Psp1), *Perionyx* sp. 2 (Psp2) and *P*erionyx sp. 3 (Psp3)



the results are shown in Table I. DNA sequences of the mitochondrial COI and 16S rDNA gene were used to evaluate whether the specimens of the four earthworms species that differ in external and internal morphology belong to different genetic lineages. We obtained DNA sequences for two fragments of the mitochondrial COI and 16S rDNA genes for four species.

After comparing larger numbers of specimens, we found that taxonomic identification can lead to correct identification within the AF and red worm species. External and internal characters of these two earthworms were determined and identified the AF as *E. eugeniae* and red worm as a new species, *Perionyx* sp. 1. In addition, the results of sequence analysis of the AF and red worm showed the value of sequence variation was 17.93% and 19.57% for 16S rDNA and COI genes, respectively and the relative nucleotide structure of analyzed genes (Table IV) was different. This abundant evidence indicated that the AF was distinguished as a different genus and species from the red worm. According to the results from the BLAST search (Table III), the sequences of COI and 16S genes for the AF matched *Diporochaeta* sp., because the only a few studies have been published concerning DNA in *E. eugeniae* and no data of COI and 16S rDNA genes have been published. The nucleotide sequence of COI and 16S genes for the red worm (*P. peguana*?) showed homology to *Megascolecidae* sp. (83% identity) and *Perionyx excavatus* (95% identity), respectively but was not matched to the sequence of *Pheretima* sp. present in the NCBI database. Finally, after combining the results of taxonomic characteristics, our study concluded that the AF was defined as the same genus and species as previously described, *E. eugeniae* (Sims & Gerard, 1985) and *P. peguana* was described as a new species, *Perionyx* sp. 1.

On the basis of morphological identification (Table I), the blue worm could be distinguished (*P. excavatus*) from the Lao worm (*P. excavatus*?), and therefore, these earthworms were identified as two new species, *Perionyx* sp. 2 and *Perionyx* sp. 3, respectively. Unfortunately, these species were indistinguishable by molecular techniques for 16S rDNA gene. This indicated that no variation existed in the 16S rDNA sequence over these amplified regions. Although, many report have shown that the 16S rDNA gene could be distinguished among the taxa of same species group (Pop *et al.*, 2003, 2007; Iglesias Briones *et al.*, 2009; Pérez Losada *et al.*, 2009). However, the analyzed region of the mitochondrial 16S rDNA gene (Palumbi *et al.*, 1991) was not suitable to identify the complex species in this

study. Keeping in view the sequence divergence, the mitochondrial COI gene was used as a tool for resolving differences among the many earthworm species as previously described (Pop *et al.*, 2003; Chang *et al.*, 2007; Huang *et al.*, 2007; Pop *et al*., 2007; Iglesias Briones *et al*., 2009; Otomo *et al*., 2009; Pérez Losada *et al.*, 2009; Richard *et al*., 2010). Intra-species sequence polymorphisms of the COI gene between blue worm and Lao worm were the most conserved and presented the identical length with only 0.16% (one base substitution). These results confirmed that the mitochondrial COI gene could be used to distinguish complex species. The sequence identity of nucleotide sequences (COI gene) for the blue worm (83%) compared with the Lao worm (83%) may be related to *Megascolecidae* sp., while the identity of the 16S rDNA gene was related to *P. excavatus* (90%). Additionally, the nucleotide sequences of the 16S rDNA gene for the blue worm (*P. excavatus*?) did not match the data of *P. excavates*, which were reported in GenBank (accession number AF406582).

In conclusion, all these evidences proved that the blue worm and Lao worm are biologically distinct species and have been classified as the new species as *Perionyx* sp. 2 and *Perionyx* sp. 3, respectively.

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REFERENCES

Ayamuang, N.F., 2000. *Potential of Vermiculture in Lao PDR*, www.ist.cmu.ac.riseat/library.php; accessed August 6, 2001

Blakemore, R.J., 2002. *Cosmopolitan Earthworms–an Eco-taxonomic Guide to the Peregrine Species of the World Verm Ecology*, p: 506. PO BOX 414 Kippax, ACT 2615, Australia

Brown, W.M., M.J. George and A.C. Wilson, 1979. Rapid evolution of animal mitochondrial DNA. *PNAS*, 76: 1967–1971

Chang, C.H., Y.H. Lin, I.H. Chen, S.C. Chuang and J.H. Chen, 2007. Taxonomic re-evaluation of the Taiwanese montane earthworm *Amynthas wulinensis* Tsai, Shen and Tsai, 2001 (Oligochaeta: Megascolecidae): polytypic species or species complex? *Organ. Divers. Evol.*, 7: 231–240

Dupont, L., 2009. Perspectives on the application of molecular genetics to earthworm ecology. *Pedobiologia*, 52: 191–205

Folmer, O., M. Black, W. Hoeh, R. Lutz and R. Vrijenhoek, 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol. Mar. Biol. Biotechnol*., 3: 294–297

Gates, G.E., 1939. Thai earthworms. *J. Thailand Res. Soc. Bangkok Nat. Hist. Suppl*., 12: 65–114

Gates, G.E., 1972. *Burmese Earthworm: An Introduction to the Systematics and Biology of Meladrile Oligochaetes with Special Reference to Southeast Asia*, p: 326. The American Philosophical society library of congress catalog, Philadelphia

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Sym. Series*, 41: 95–98

Hebert, P.D.N., A. Cywinska, S.L. Ball and J.R. De Waard, 2003. Biological identifications through DNA barcodes. *Proc. Royal Soc. London B*, 270: 313–321

Heethoff, M., K. Etzold and S. Scheu, 2004. Mitochondrial COII sequences indicate that the parthenogenetic earthworm Octolasion tyrtaeum (Savigny 1826) constitutes of two lineages differing in body size and genotype. *Pedobiologia*, 48: 9–13

Huang, J., Q. Xu, Z.J. Sun, G.L. Tang and Z.Y. Su, 2007. Identifying earthworms through DNA barcodes. *Pedobiologia*, 51: 301–309

Iglesias Briones, M.J., P. Morán and D. Posada, 2009. Are the sexual, somatic and genetic characters enough to solve nomenclatural problems in lumbricid taxonomy? *Soil Biol. Biochem.*, 41: 2257–2271

Mindell, D.P., M.D. Sorenson, C. Huddleston, H.C. Miranda and A. Knight, 1997. Phylogenetic relationships among and within select avian orders based on mitochondrial DNA. *In*: Mindell, D.P. (ed.), *Avian Molecular Evolution and Systematics*, pp: 214–217. Academic Press, New York

Moore, W.S., 1995. Inferring phylogenies from mtDNA variation: mitochondrial gene trees versus nuclear gene trees. *Evolution*, 49: 718–726

Nicholas, K.B. and Jr. H.B. Nicholas, 1997. *GENEDOC: A Tool for Editing and Annotating Multiple Sequence Alignments*. http://www.cris.com/ketchup/genedoc.html, accessed January 24, 2010

Otomo, P.V., B.J. Van Vuuren and S.A. Reinecke, 2009. Usefulness of DNA barcoding in ecotoxicological investigations: resolving taxonomic uncertainties using *Eisenia* Malm 1877 as an example. *Bull. Environ. Contam. Toxicol.*, 82: 261–264

Palumbi, S., A. Martin, S. Romano, W.O. McMillan, L. Stice and G. Grabowski, 1991. *The Simple Fool's Guide to PCR, Version 2.0*. Honolulu, HL 96822: Department of Zoology and Kewalo Marine Laboratory, University of Hawaii

Pérez Losada, M., M. Ricoy, J.C. Marshall and J. Domnguez, 2009. Phylogenetic assessment of the earthworm *Aporrectodea caliginosa* species complex (Oligochaeta: Lumbricidae) based on mitochondrial and nuclear DNA sequences. *Mol. Phyl. Evol*., 52: 293–302

Pop, A.A., M. Wink and V.V. Pop, 2003. Use of 18S, 16S rDNA and cytochrome c oxidase sequences in earthworm taxonomy (Oligochaeta, Lumbricidae). *Pedobiologia*, 47: 428–433

Pop, A.A., G. Cech, M. Wink, C. Csuzdi and V.V. Pop, 2007. Application of 16S, 18S rDNA and COI sequences in the molecular systematics of the earthworm family Lumbricidae (Annelida, Oligochaeta). *European J. Soil Biol*., 43: 43–52

Reynolds, J.W., 2004. The Status of Earthworm Biogeography, Diversity and Taxonomy in North America Revisited with Glimpses into the Future. *In*: Edwards, C.A. (ed.), *Earthworm Ecology*, pp: 63–71. CRC Press, New York

Richard, B., T. Decaéns, R. Rougerie, S.W. James, D. Porco and P.D.N. Hebert, 2010. Re-integrating earthworm juveniles into soil biodiversity studies: species identification through DNA barcoding. *Mol. Ecol. Res*., 10: 606–614

Sims, R.W. and B.M. Gerard, 1985. Earthworms: keys and notes for the identification and study of the species. *In*: Kermack, D.M. and R.S.K. Barnes (eds.), *Synopses of the British Fauna (New Series)*, pp: 41–145. Linnean Society of London, Estuarine and Brackish-water Sciences Association, Great Britain at the Pitman Press, England

Stephenson, J., 1930. *The Oligochaeta*, p: 978. Oxford University, Clarendon Press, England

Swofford, D.L., 2002. *PAUP*. Phylogenetic Analysis using Parsimony (*and other Methods), Version 4.0b10 (Alvitec)*. Sunderland, Massachusetts, Sinauer Associates

Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin and D.G. Higgins, 1997. The Clustal X windows interface; flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res*., 24: 4876–4882

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