Sequence–related amplified polymorphism (SRAP) molecular marker: DNA fingerprinting studying on economic and agricultural earthworms in North of Thailand

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ABSTRACT

The terrestrial earthworms in agricultural areas were collected in Chiang Mai and Lamphun province from May to the middle of June. They could be divided into two major families (family Megascolecidae and family Glossoscolecidae) by morphology. Moreover, the economic species which are *Perionyx excavatus* (blue strain), *Eudrilus euginiae* (African night crawler strain), *Eisenia fetida* (tiger strain) and *Perionyx* sp. 1 (Ketare Sankampang strain), were provided by commercial farms. Consequently, the sequence–related amplified polymorphism (SRAP) fingerprinting showed that the banding DNA in each earthworm species differ in totals species except blue strain and Ketare Sankampang strain. Hence, the genetic relationship of these earthworms using SRAP marker from 13 couples of primers showed 321 polymorphic banding scores. Interestingly, all earthworms could also be divided into 7 groups by UPGMA algorithm: 1) *Metaphire peguana* species in group 1, 2) *M. posthuma* species in group 2, 3) *Amynthas alexandri* species in group 3, 4) *Perionyx excavatus* (blue strain) and Ketare Sankampang strain in group 4, 5) *P. correthrurus* species in group 5, 6) the tiger strain in group 6 and 7) the African night crawler strain in group 7. In addition, the value of genetic similarity in group 4 ranged from 0.912 to 0.997 with high bootstrap score value (100%). Hence, we expected that they are the same species on *Perionyx* sp. However, this is the first report to study the discovery of the earthworm genetic relationship by SRAP marker. Therefore, the SRAP molecular technique showed the potential as a molecular tool. It could identify and categorize the terrestrial earthworm including families, genera, species and strain taxa which will be developed to use and apply in agricultural purposes in Thailand.

Keywords: SRAP marker, DNA fingerprinting, agriculture, earthworm, UPGMA, bootstrap

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INTRODUCTION

The earthworms have been raised increasingly throughout the world. They have also been selected to study in terms of the utilities in agriculture (Fragoso *et al*., 1999). In Thailand, both local and exported earthworms such as African night crawler strain, an economic species in *Eudrilus euginiae* from Central Africa (Sivasankari *et al*., 2013), and tiger strain, an economic species in *Eisenia fetida* from Europe (Song *et al*., 2009), are being raised increasingly. Furthermore, the

earthworms from blue strain, an economic species in *Perionyx excavatus*, can also be found throughout Asia and this species can also be used effectively in agriculture in Thailand (Loongyai *et al*., 2011). However, it was found that there was a problem regarding adapting themselves to the environment in Thailand. Moreover, they have to be raised only on the farm.

Maejo University has collected many species of local earthworms from agricultural areas to study, especially "Ketare" which was collected from farmers in Sankampang district, called Ketare Sankampang (*Perionyx* sp. 1). This species can transform organic waste into fertilizer and can grow very rapidly. The Ketare strain is similar to *Metaphire peguana*, a species found in agricultural areas in the northern part of Thailand, causing confusion between these 2 species. Moreover, Ketare Sankampang has characteristics which are similar to blue strain, but these two strains cannot be distinguished by their morphology because this approach can be applied when the earthworms are fully mature. Hence, a molecular marker has been widely used to solve this problem regarding genetic diversification and relationship of earthworms, such as random amplified polymorphic DNA (RAPD) molecular marker (William *et al*., 1990) or amplified fragment length polymorphism (AFLP) molecular marker (Vos *et al*., 1995). However, it was found that RAPD molecular marker had irregular outcomes in repeated experiments while AFLP one could contribute to high accuracy, yet with complications. Accordingly, Li and Quiros (2001) have developed sequence–related amplified polymorphism (SRAP) molecular marker, which can classify the genetic relationship among animals in the population effectively.

The purpose of this research was to study the potential of SRAP molecular marker to provide DNA fingerprinting and studying the genetic relationship of earthworms (4 economic strains, i.e., African night crawler, tiger, blue and Ketare Sankampang) in agricultural areas in Chiang Mai and Lamphun province. The results of this research will enable us to group the earthworms in agricultural areas and commercial species more precisely. Additionally, this can solve the problem regarding a misunderstanding in earthworm classification between the raised one and the natural one, which can be useful in future development.

MATERIALS AND METHODS

Earthworm Sampling Sites

A total of 73 earthworm samples from agricultural areas, i.e., paddy field, vegetable bed, dairy farm and fruit plantation, in Chiang Mai and Lamphun province were collected from the beginning of May to the middle of June 2014 by hand sorting. The 29 samples from 4 types of economic earthworms (Figure 1) were also collected which composed of 10 samples of African night crawler, 3 samples of Ketare Sankampang from Earthworm Research and Development Center, Meajo University, Chiang Mai, Thailand, 8 samples of blue strain and 8 samples of tiger strain from Siam Worm farm in Chanthaburi province (Table 1). All earthworms were gently soaked in 30% (v/v) of ethanol solution at room temperature before preserving them in 95% (v/v) of ethanol solution at room temperature. The earthworms from agricultural areas were all fully mature and were categorized according to Gate's key (Gate, 1972) together with Sim and Easton' key (Sims and Easton, 1972) before extracting DNA.

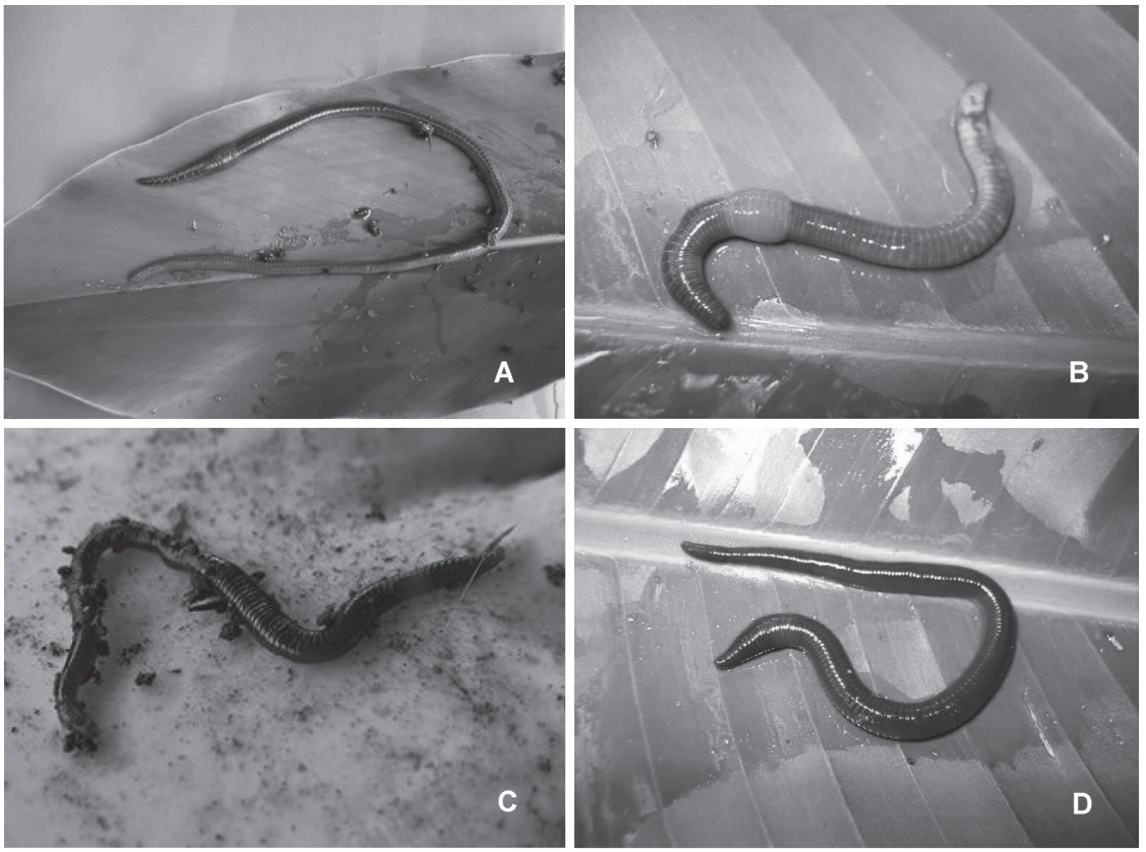

Figure 1 External morphology of *Eudrilus euginiae* (African night crawler strain) (A), *Eisenia fetida* (tiger strain) (B), *Perionyx excavatus* (blue strain) (C) and *Perionyx* sp. 1 (Ketare Sankampang strain) (D)

Table 1 Earthworm samples used in this study

Earthworms	Number (samples)	Location
Metaphire peguana	10	Agricultural areas
Metaphire posthuma	2	Agricultural areas
Amynthas alexandri	10	Agricultural areas
Pontoscolex corethrurus	1	Agricultural areas
African night crawler	10	Siam Worm farm
Ketare Sankampang	3	Cow manure from Sankampang
Blue strain	8	Siam Worm farm
Tiger strain	8	Siam Worm farm

DNA Extraction

The earthworm samples, 23 agricultural and 29 economic earthworms, were preserved in 95% ethanol before DNA extraction. Genomic DNA was extracted from the clitellum area by using GF–1 tissue DNA extraction kit version 3.1 (Vivantis, Malaysia). DNA product was electrophoresed under 0.8% (w/v) agarose gel at 220 voltage for 35 min. Concentration and purity of DNA were measured by spectronanometer at the absorbance of 260/280 nm ratio. Then the DNA samples were diluted to 5.0 ng and ready to use for downstream experiments.

Sequence-Related Amplified Polymorphism Fingerprinting

Thirteen primer pairs were selected from our preliminary screening by using a hundred primers (data not shown). The selected primers were considered to be suitable for DNA fingerprinting, i.e., M1E4, M2E2, M2E4, M2E7, M3E4, M4E3, M4E7, M5E7, M6E3, M7E7, M8E8, M10E1 and M10E6. The sequences of SRAP primer are presented in Table 2. A total of 15 µL of PCR reaction consisted of 1.5 µM reverse primer (1 µL), 1.5 µM forward primer (1 µL), 100 µM dNTP (1.5 µL), 1X buffer (1.5 µL), molecular grade water (6.9 µL), 0.5 unit of *Tag* DNA polymerase (0.1 µL) and 15 ng of DNA template (3.0 µL). The PCR was set up in 5 cycles of denaturation 5 min at 94°C, annealing 1 min at 35°C and extension 1 min at 72°C followed by 35 cycles of denaturation 5 min at 94°C, annealing 1 min at 52°C and extension 10 min at 72°C. Then, 5 µL of PCR product was mixed with 1 µL of red–safe color detector (Ulrapower, Japan) and separated by electrical in electrophoresis system with 1.8% (w/v) agarose gel at 220 voltage for 70 min. Finally, bands of PCR products were detected by an LED light source and photographed by a digital camera.

Table 2 SRAP primer sequences

Forward primer	Sequence	Reverse primer	Sequence
M–1	TGAGTCCAAACCGGAAA	E–1	GACTGCGTACGAATTAAC
M–2	TGAGTCCAAACCGGAAG	E–2	GACTGCGTACGAATTAAT
M–3	TGAGTCCAAACCGGAAC	E–3	GACTGCGTACGAATTGAC
M–4	TGAGTCCAAACCGGAAT	E–4	GACTGCGTACGAATTGCA
M–5	TGAGTCCAAACCGGAGC	E–5	GACTGCGTACGAATTCAA
M–6	TGAGTCCAAACCGGACA	E–6	GACTGCGTACGAATTCAG
M–7	TGAGTCCAAACCGGACC	E–7	GACTGCGTACGAATTCAC
M–8	TGAGTCCAAACCGGATA	E–8	GACTGCGTACGAATTCTG
M–9	TGAGTCCAAACCGGTAG	E–9	GACTGCGTACGAATTTGA
M–10	TGAGTCCAAACCGGTCA	E–10	GACTGCGTACGAATTTGC

Genetic Relationship of the Agricultural and Economic Terrestrial Earthworms

DNA fingerprints of the agricultural and the economic earthworms were parallelly analyzed based on an occurrence of the band on gel electrophoresis. Similar or different DNA band position was scored as 1 or 0, respectively. Similarity coefficiency was then determined by using PHYLIP software (model 3.695) (Felsenstein, 1995). The earthworm samples were clustered by an Unweighted Pair-Group Method, Arithmetic average (UPGMA) (Mega 7). To obtain a phylogenetic tree, all dataset was calculated using 1,000 bootstrap replicates by Seqboot (PHYLIP 3.695). Then the tree was constructed and visualized by using TreeView software (model 1.6.6).

RESULTS AND DISCUSSION

In Thailand, *Metaphire peguana* from natural sources has various common names, one is also known as "Ketare", which is generally called by farmers in the north of Thailand. The Ketare derived from the organic waste around the cow manure areas from Sankampang district, Chiang Mai province, Thailand is called "Ketare Sankampang". They have been studied in many agricultural purposes under the Earthworm Research and Development Center of Meajo University for a long time. Interestingly, sometimes they are called "Ketare" as the short name which is similar to Ketare from natural sources (*M. peguana*). But Ketare from the natural source (e.g., agricultural areas) and the earthworms from Sankampang district display clearly difference in external morphologies when identified them by naked eyesight and under the stereomicroscope, especially in term of body size, body color, male pore position, female pore position and type of clitellum (Table 3). For example, the body color, Ketare from natural sources appears the body color ranging from grey to dark brown but Ketare from Sankampang district from the cow manure in dairy farm areas appears the body color ranging from red to dark red.

Table 3 Important morphological examination between Ketare from Sankampang strain and Ketare from natural sources

Morphological examination	Earthworms		
	Ketare from Sankampang (*Perionyx* sp.)	**Ketare from Fang (*Perionyx* sp.)**	**Ketare from natural sources (*Metaphire peguana*)**
Body length	110–138 mm	98–102 mm	133–141 mm
Clitellum position	13–18	13–17	14–16
Male pore	18	18	18
Female pore	14	14	14
Spermathecal pore	–	–	6/7/8/9
Genital marking	–	–	17/18, 18/19
Prostate gland	Racemose	Racemose	Racemose
Skin color	Red	Dark red	Grey
Location	Cow manure	High latitude	Agricultural areas

The appearance as red on body skin of the earthworms from cow manure in Sankampang district led local peoples and farmers in the north of Thailand to call them "red worm" but this special name (red worm) has been conducive to confusing in an identified strain of red worm of ethnic group people (ethnic group people involved Haw, Shan (Tai Yai), Black LaHu and Pa Long) in Fang district, Chiang Mai province, Thailand who have been provided this earthworm to co–cultured with various economic botanical plants to their product improvements under promoting from the Royal Agricultural Station Angkhang, where is the first research station of Royal Project Foundation in Fang district.

Consequently, the red worms both from Sankampang district and Fang district showed the same in external morphology as *Perionyx* sp. (Table 3) which similar to the commercial name of *Perionyx excavatus* (blue strain) from the Siam worm farm. Previously, the Thai researcher reported that many commercial earthworms in Thailand (African night crawler strain, blue strain and Loa strain) could be

identified by the 16s ribosomal DNA (16s rDNA) and cytochrome c oxidase subunit I sequence (COI) gene. These genes could also separate the *Perionyx excavatus* (blue strain and Loa strain) from the African night crawler and concluded these red worms as the "*Perionyx* sp. 1" (Loongyai *et al.*, 2011). As above mentioned, our thought is that these red worms both from the Royal Agricultural Station Angkhang and cow manure from Sankampang district may be originated from the *Perionyx* sp. 1 (red worms) on the ground areas.

However, the problematic of common names of *Metaphire peguana* (Ketare) and *Perionyx* sp. 1 (Ketare Sankampang or red worm) are confusing to identify for the only farmers and ethnic groups in Thailand. Nevertheless, in this experiment, the *E. eugeniae* (African night crawler) showed the difference in external morphology from *E. foetida* (tiger strain) and others (*Perionyx* sp. 1, *M. peguana*, *M. anomala*, *M. hollecti*, *Amynthas alaxandri* and *Pontoscolex corethrurus*).

Genetic Relationship of the Agricultural and Economic Terrestrial Earthworms

The 13 combination primers (M1E4, M2E2, M2E4, M2E7, M3E4, M4E3, M4E7, M5E7, M6E3, M7E7, M8E8, M10E1 and M10E6) were selected for fingerprinting. These combination primers generated 19, 30, 14, 31, 28, 18, 20, 24, 35, 29, 19, 24 and 30 bands, respectively (Table 4). It showed 321 polymorphic banding DNA resulting in a matrix of Nei's genetic distances which was used to construct the UPGMA dendrogram (Figure 2).

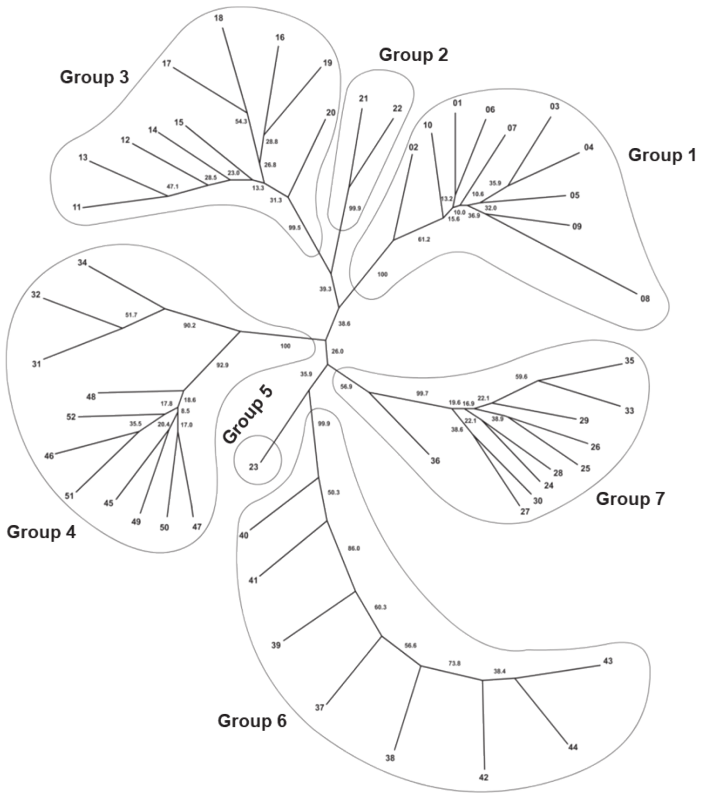

Figure 2 The phylogenetic tree showing the genetic relationship of terrestrial earthworms. Group 1: *M. peguana*, group 2: *A. alexandri*, group 3: *M. posthuma*, group 4: *P. excavatus* (blue strain) and *Perionyx* sp. 1 (Ketare Sankampang strain), group 5: *P. corethrurus*, group 6: *E. fetida* (tiger strain), group 7: *E. euginiae* (African night crawler strain)

According to the dendrogram, all terrestrial earthworms were categorized into 7 groups. All of *M. peguana* (Ketare) (1–10) were grouped in cluster 1 and having a similarity index ranging between 0.932 and 0.997 with 100% bootstrap value. All samples of *M. posthuma* (21–22) were grouped in cluster 2 which the similarity index ranged from 0.937 to 0.948 with 99.9% bootstrap value. In cluster 3, *A. alexandri* (11–20) were categorized together and the similarity index ranged from 0.991 to 0.996 with 99.8% bootstrap value. For cluster 4, 2 earthworm species were categorized together which were *P. excavatus* (blue strain) (45–52) and *Perionyx* sp.1 (Ketare Sankampang strain) (31, 32, 34), similarity index ranged from 0.912 to 0.997 with 100% bootstrap. This result demonstrated that these species were in *Perionyx* species group. The *P. corethrurus* (23) was grouped in cluster 5. All *E. fetida* (tiger strain) (37–44) were categorized in cluster 6 relating with similarity index ranging between 0.912 and 0.997 with 99.9% bootstrap value. For cluster 7, *E. euginiae* (African night crawler) (24–30, 33, 35, 36) were grouped and the similarity index ranged from 0.923 to 0.998 with 56.9% bootstrap value.

Table 4 Banding number of DNA in each primer pairs

Primer pairs	Number of banding DNA
M5E7	24
M1E4	19
M8E8	19
M2E2	30
M6E3	35
M10E6	30
M4E7	20
M2E4	14
M10E1	24
M4E3	18
M7E7	29
M2E7	31
M3E4	28
Total bands	321
Average of banding DNA	24.69

As the result, 13 SRAP fingerprints indicated the different banding patterns among all earthworm species, except for *P. excavatus* and *Perionyx* sp. 1 which identically exhibited in DNA banding patterns as shown in Figure 3. There were 3 monomorphic banding patterns from M6E3 combination primer that indicated the similarity of these two earthworm species. We hypothesized that *P. excavatus* and *Perionyx* sp. 1 were the same species because of the following 2 reasons. First, in 1939, Gates observed and reported 31 earthworm species in Thailand (Gates, 1939). Secondly, both of *P. excavatus* and *Perionyx* sp. 1 have displayed identical external appearance. These reasons made us think that *Perionyx* sp. 1 is possibly the same species with *P. excavatus,* but they were found in different areas and time.

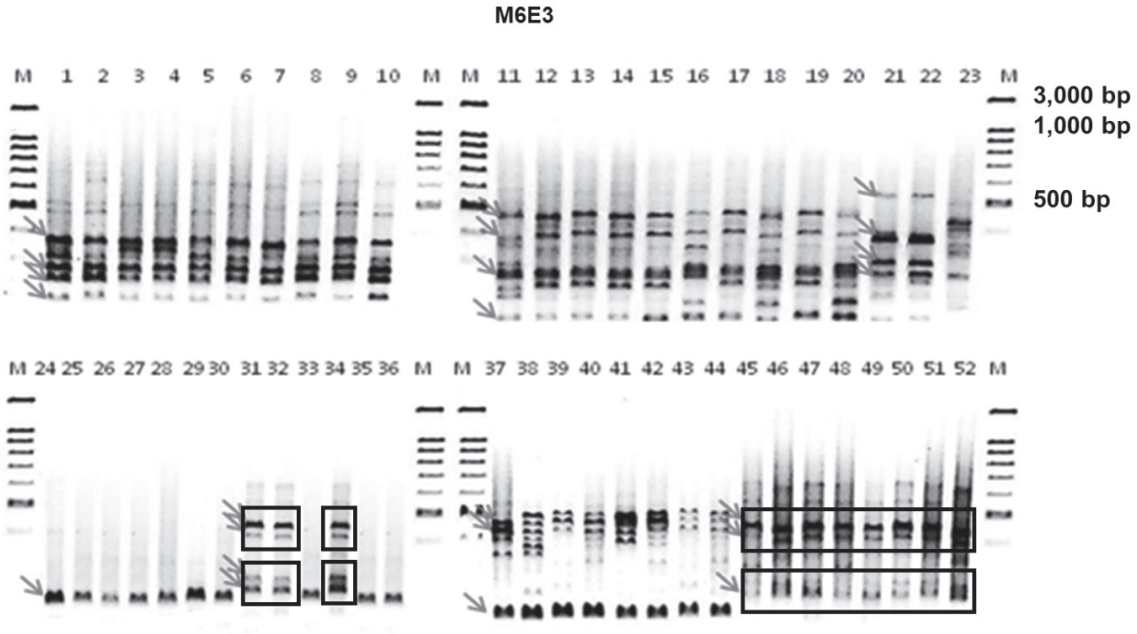

Figure 3 DNA fingerprinting of M6E3 combination primers. Lane 1–10: *M. peguana*, lane 11–20: *A. alexandri*, lane 21–22: *M. posthuma*, lane 23: *P. corethrurus*, lane 24–30, 33, 35, 36: African night crawler strain, lane 31, 32, 34: Ketare Sankampang strain, lane 37–44: tiger strain, lane 45–52: blue strain. The arrow shows monomorphic DNA banding pattern of each terrestrial earthworm. Bands in square block show similar DNA bands of *Perionyx* sp. 1 (Ketare Sankampang strain) and *P. excavatus* (blue strain)

According to the UPGMA dendrogram, terrestrial earthworms were categorized into 7 clusters which were reflected in the values of Nei's genetic distance ranging from 0.874 to 0.999. Interestingly, the data from either morphology or DNA fingerprint not noticeably indicated a non–significant matter between *Perionyx excavatus* and *Perionyx* sp. 1. There was no significance, supported by the value of Nei's genetic distance ranging from 0.912 to 0.997 with 100% bootstrap values. On the other hand, *M. peguana* exhibited a clear difference in DNA fingerprint from the *Perionyx* sp. 1. Moreover, the UPGMA dendrogram supported that Ketare from natural sources (*M. peguana*) was categorized in a different group from Ketare Sankampang. Therefore, these researches could be concluded that Ketare Sankampang strain (*Perionyx* sp. 1) and blue strain (*P. excavatus)* might be classified in the same genus or *Perionyx* sp. and Ketare from natural sources (*M. peguana)* was not classified in the *Perionyx* sp.

CONCLUSIONS

The results of this study provided data of terrestrial earthworms from agricultural areas and farms. The data of earthworm in agricultural areas found that *M. peguana* was found the most in this survey. For genetic relationship of all earthworms, SRAP molecular marker from 13 combination primers appeared 321 polymorphic banding DNA, were detected which can be clustered into 7 groups by UPGMA relating with a morphology similarity index of 0.874–0.999. Importantly, Ketare Sankampang

strain (*Perionyx* sp. 1) and blue strain (*Perionyx excavatus*) were categorized in the same cluster (group 4). However, Ketare Sankampang strain (*Perionyx* sp. 1) and Ketare from natural sources (*M. peguana*) were not categorized in the same cluster. An SRAP molecular marker is a good tool for identifying terrestrial earthworms of this experiment which would be applied in various farming and agricultural purposes.

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REFERENCES

Felsenstein, J. 1995. PHYLIP (Phylogeny Interference Package). Department of Genetic, University of Washington, Washington, USA.

Fragoso, C., P. Lavelle, E. Blanchart, B.K. Senapati, J.J. Jimenez, M. de los Angeles Martinez, T. Decaëns and J. Tondoh. 1999. Earthworm communities of tropical agroecosystems: origin, structure and influence of management practices, pp. 27–55. *In*: P. Levelle, L. Brussaard and P. Hendrix, (Eds.), Earthworm Management in Tropical Agroecosystems. CABI Publishing, Wallingford, UK.

Gate, G.E. 1939. Thailand earthworms. J. Thai. Res. Soc. 12: 65−114.

Gate, G.E. 1972. Burmese earthworm, an introduction to the systematics and biology of megadrile oligochaetes with special reference to Southeast Asia. Trans. Am. Philos. Soc. 62: 1–326.

Li, G. and C.F. Quiros. 2001. Sequence–related amplified polymorphism (SRAP) a new marker system based on a simple PCR reaction: its application to mapping and gene tagging in *Brassica*. Theor. Appl. Genet. 103: 455–461.

Loongyai, W., B. Phuwadol and C. Sonchai. 2011. External morphological comparison, taxonomic revision and molecular differentiation of the four economically important species of earthworm in Thailand. Int. J. Agric. Biol. 13(4): 553–558.

Sims, R.W. and E.G. Easton. 1972. A numerical revision of the earthworm genus *Pheretima auct*. (Megascolecidae: Oligochaeta) with the recognition of new genera and an appendix on the earthworms collected by the Royal Society North Borneo Expedition. Biol. J. Linn. Soc. Lond. 4: 169–268.

Sivasankari, B., S. Indumathi and M. Anandharaj. 2013. A study on life cycle of earthworm *Eudrilus eugeniae*. Int. J. Res. Pharm. Life Sci. 1(2): 64–67.

Song, Y., L.S. Zhu, J. Wang, J.H. Wang, W. Liu and H. Xie. 2009. DNA damage and effects on antioxidative enzymes in earthworm (*Eisenia foetida*) induced by atrazine. Soil Biol. Biochem. 41(5): 905–909.

Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper and M. Zabeau. 1995. AFLP a new technique for DNA fingerprintings. Nucleic Acids Res. 23(21): 4407–4414.

William, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski and S.V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18(22): 6531–6535.