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Genetic diversity of earthworm *Amynthas rodericensis* (Grube, 1879) (Clitellata: Megascolecidae) in Vietnam by randomly amplified polymorphic DNA analysis

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Abstract: In this study, Random Amplified Polymorphic DNA (RAPD) markers were applied to analyse the genetic diversity of samples of the *Amynthas rodericensis* (Grube, 1879), collected from six localities (*viz.*, Hai Lang along Quang Tri province; Phong Dien, Hue, and Huong Thuy along Thua Thien Hue province in Vietnam). The eight primers used in RAPD analysis amplified 71 loci, all of which were polymorphic. The percentages of polymorphic loci observed in the four populations were: 71.83% (Hai Lang), 87.32% (Phong Dien), 73.24% (Hue), and 38.03% (Huong Thuy). Data for observed and effective number of alleles, Nei's (1973) genetic diversity, and Shannon's information index, for all the four populations were 1.6761, 1.4802, 0.2718 and 0.3971, respectively. The value for total genotype diversity among populations was 0.3631 while within populations was found to be 0.2718. The mean coefficient of gene differentiation value and the estimate of gene flow across the populations were found as 0.2515 and 1.4870, respectively. The Nei (1972) measures of genetic distance and identity between pairs of earthworm populations indicate that the population originated from Phong Dien

and Hue has the highest genetic identity, while the earthworms originated from Hai Lang and Huong Thuy show the greatest genetic distance.

Keywords: *Amynthas rodericensis*, earthworm, genetic diversity, RAPD, Vietnam.

INTRODUCTION

Earthworms are very important and beneficial components of soil fauna since they dominate the invertebrate biomass in the soil[1, 2]. Of globally estimated 6500 species of earthworms, only 3500 species are described[3]. Earthworms play an important role in organic matter decomposition[4-10], in vermicomposting[4, 5, 10, 16, 17], in bioremediation[4, 14-16], in improving soil fertility[4, 7, 9-11, 18], in soil structure and functioning[14-16,], and in medicine[10, 17]. Earthworms have been used as bioindicators of soil quality[4, 5, 19-21], and as food for human[11]. On the other hand, earthworms have been used in the feeding of chicken[10, 22], fish[10, 23], duck, goose.

The pheretemoid earthworm, *Amynthas rodericensis* (synonym: *Pheretima rodericensis*), belong to family Megascolecidae, class Clitellata, phylum Annelida. *A. rodericensis*is a cosmopolitan species, known from USA, South America, Pacific Islands, Europe, India, Australia[24]. In Vietnam, they seem to be widely distributed in the highlands and central parts (Quang Binh, Quang Tri, Thua Thien Hue, etc.)[25]

Molecular genetic markers have been widely recognized as an ideal supplementary tool for characterization and analysis of genetic diversity in species. The most commonly used molecular genetic marker for DNA fingerprinting is Randomly Amplified Polymorphic DNA (RAPD). RAPD is a simple, easy technique for population genetic studies[26, 27]. RAPD markers have been widely used for analysis of genetic diversity in variety of species: fish[27]; lizard[28], canine[29], cattle and sheep[30], filarial worm[31], trematode[32], various earthworm species such as *Eisenia fetida*[33]; *Eudrilus eugeniae*[33-35]; *Pontoscolex corethrurus*, *Drawida* spp., *Chaetocotoides* sp., *Dichogaster affinis*, *Lennogaster* sp., and *Megascolex conkanensis*[34]; *Perionyx ceylanensis* and *P. excavates*[19]; *Lumbricus terrestris*, *Arion lusitanicus* and *Microtus arvalis*[36]; *Aporrectodea* spp.[37], etc.

RAPD analysis uses short random primers (usually 8-10 bp in length) that are commercially available and do not require prior knowledge of the DNA sequence of the target organism. Other advantage of RAPD is the ease with which a large number of loci and individuals can be screened[39, 40].

Our previous studies showed distributive characteristics and ofthe earthworm *Amynthas rodericensis* (Grube, 1879) in Hue city[41], and their morphological variations in Thua Thien Hue province[13]. The aim of this study is to determine genetic diversity of *A. rodericensis* (Grube, 1879) in Central Vietnam, for which no data are available at present.

MATERIAL AND METHODS

Sample collection: Twenty-four individual earthworms were captured from Hai Lang district in Quang Tri province (n=8); Phong Dien district (n=8), Huong Thuy district (n=3) and Hue city (n=5) in Thua Thien Hue province, Vietnam **(Table 1** and **Figure 1)**. Specimens were stored in the laboratory at the Department of Genetics, Faculty of Biology, University of Education, Hue Universiy, Vietnam and stored in 98% alcohol, -20°C until further utilised.

Table 1: Specimens for this study with locality and voucher code

Locality	Number of specimens (n)	Voucher code
Hai Lang	8	HL2, HL4, HL5, HL6, HL7, HL8, HL12, HL16
Phong Dien	8	PD2, PD3, PD4, PD5, PD6, PD8, PD9, PD11
Huong Thuy	3	HT1, HT2, HT3
Hue	5	H1, H3, H4, H5, H8

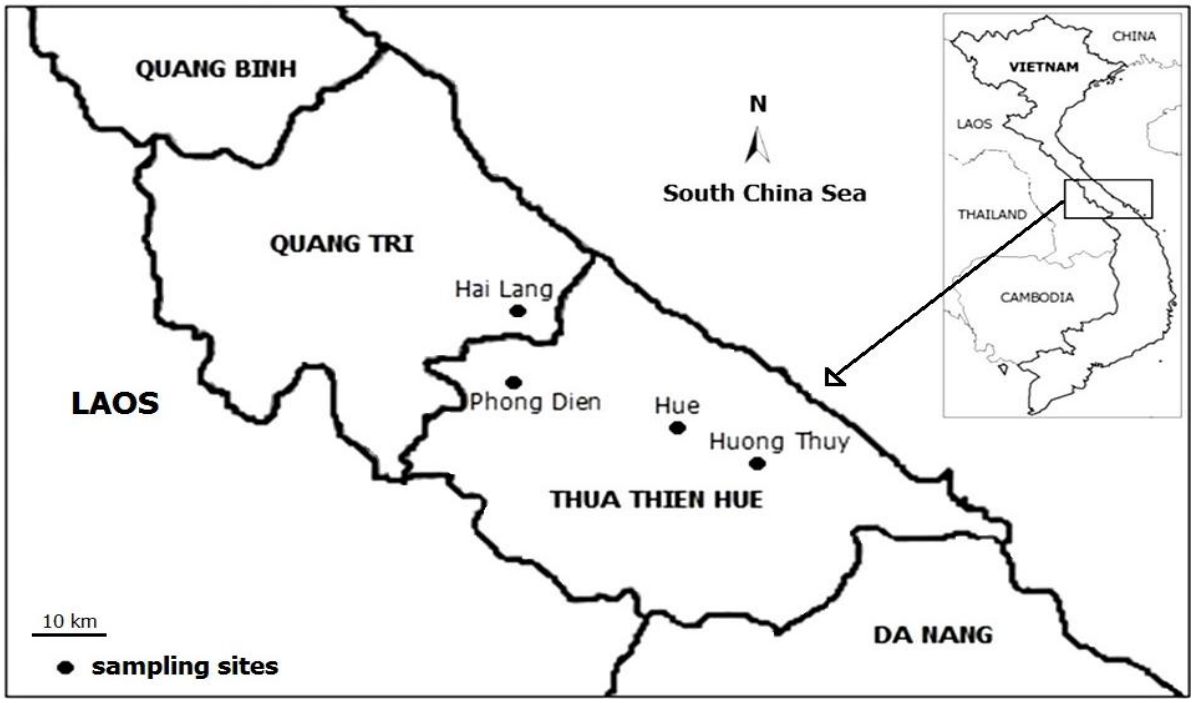

Figure 1: Map of sampling localities for specimen's *A. rodericensis* used in this study. Detailed information and voucher codes of locality are shown in **Table 1.**

Extraction of genomic DNA: Genomic DNA was isolated from muscle using modified phenol-chloroform protocol[42]. The quantity and quality of extracted DNA was determined by measuring its absorbance value at 260 nm and estimating the ratio of absorbance values at 260 nm and 280 nm, respectively. DNA was stored at -20°C until analysis.

PCR-RAPD analysis and agarose gel electrophoresis: Eight random primers: OPA03, AGTCAGCCAC; OPA04, AATCGGGCTG; OPB01, GTTTCGCTCC; OPB18, CCACAGCAGT; OPD11, GGTGATCAGG; OPF04, ACGACCGACA; OPG17, AGCGCCATTG; and OPN06, GAGACGCACA **(Table 2)** were used for PCR-RAPD amplification. PCR-RAPD was carried out in a 20 µl reaction mix volume containing 10 µl GoTaq® Green Master Mix 2× (Promega, USA), 2 µl 10 pmol primers, 2 µl 25 ng of genomic DNA, and 6 µl nuclease-free water.

Table 2: Sequence of RAPD primer, sizes, and number of amplified bands, and percentage of amplified specimens based on RAPD analysis in populations of earthworm *A. rodericensis* (Grube, 1879) in Vietnam

Primer code	Nucleotide sequence (5'-3')	Size range of amplified bands (bp)	Number of amplified bands	Number of poly-morphic bands	Percentage of poly-morphic bands (%)	Number of amplified specimens	Percentage of amplified specimens (%)
OPA03	AGTCAGCCAC	240-1600	12	12	100	22	91.67
OPA04	AATCGGGCTG	200-1900	8	8	100	11	45.83
OPB01	GTTTCGCTCC	240-1800	9	9	100	11	45.83
OPB18	CCACAGCAGT	200-1600	8	8	100	16	66.67
OPD11	GGTGATCAGG	300-1500	9	9	100	18	75.00
OPF04	ACGACCGACA	250-1600	8	8	100	17	70.83
OPG17	AGCGCCATTG	200-1050	9	9	100	20	83.33
OPN06	GAGACGCACA	200-1050	8	8	100	22	91.67
Overall		200-1900	71	71	100	137	71.35
Mean			8.87	8.87	100	17.13	71.35

The amplification reactions were carried out in a thermocycler programmed at 94°C for 4 minutes; 92°C for 1 minute, 35°C for 1 minute, and 72°C for 2 minutes for 43 cycles with a final extension at 72°C for 10 minutes.

The resulting products were electrophoretically analysed through a 1.5% agarose gel, stained with ethidium bromide, obsvered using a UV transilluminator and photographed by a Gel Documentation.

Data analysis: The binary matrix was constructed based on band presence or absence scored by 1 and 0, respectively. The sizes of the RAPD markers were estimated by using the Quantity One software (ver. 4.1, Bio-rad, USA). The genetic identity and genetic distance between populations was expressed using Nei's (1972) genetic distance[43].

Genetic parameters were calculated as observed number of alleles (na), effective number of alleles (ne), the number of polymorphic bands, Nei's (1973) gene diversity (h), Shannon's information Index (I), total genotype diversity in populations (Ht), total genotype diversity within populations (Hs), mean coefficient of gene differentiation (Gst), estimate of gene flow (Nm) for RAPD data using the POPGENE software (ver. 1.31)[44]. RAPD data were analysed using the NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System) package.

A dendrogram was generated based on the Unweighted Pair-Group Method with Arithmeticmean (UPGMA) clustering method to estimate the relationships between earthworm populations.

RESULTS AND DISCUSSION

RAPD diversity: Genetic variation was observed in the PCR-RAPD profile of *A. rodericensis* populations from different geographical locations. A series of discrete bands were obtained after amplification of DNA samples of all four populations of earthworm *A. rodericensis* with eight random primers (OPA03, OPA04, OPB01, OPB18, OPD11, OPF04, OPG17 and OPN06). The different primers produced different banding patterns. The number of reproducible bands across all investigated samples was 12, 8, 9, 8, 9, 8, 9, and 8 bands for primers OPA03, OPA04, OPB01, OPB18, OPD11, OPF04,

OPG17 and OPN06, respectively. The largest number of RAPD bands were detected for primer OPA03 (12 bands), while the lowest number was scored for primers OPA04, OPB18, OPF04, OPN06 (8 bands). A total of 71 amplified bands were consistentely generated, which were polymorphic (100%), and 100% polymorphism was obtained with all of eight used primers. Size of these amplified bands ranged from 200 bp to 1900 bp. Highest size range was exhibited for OPA04 (200 bp-1900 bp) while it was lowest for OPG17 and OPN06 (200 bp-1050 bp). Percentage of amplified specimens was 71.35% **(Table 2)**.

The arevage percentage of polymorphic loci of each earthworm population was ranged from 38.03% to 87.32% **(Table 3)**. Phong Dien, Hue and Hai Lang populations exhibited greater levels of polymorphic loci than did Huong Thuy population. The sample gels were obtained from primer OPA03 and OPA04, OPB01 and OPB18, and OPG17 and OPN06 which are presented in **Figure 2**, **3**, and **4**, respectively. This result shows that high polymorphic levels of these primers in earthworm *A. rodericensis* in Central Vietnam.

Until now, there are no reports in *A. rodericensis* on these aspects in the literature to compare and contrast. However, some reports on genetic diversity of many other earthworm species using RAPD markers have published. Kautenburger (2006) used three random primers in *L. terrestris* from five different locations in Western Germany and found the total numbers of amplified bands was 61 which varied from 15 to 36 with a size ranging from 300 bp to 1950 bp[45]. Sharma *et al.* (2011) employed 10 random primers in earthworms individuals (*Eisenia fetida* and *Eudrilus eugeniae*) from ten different sampling sites representing different soil types in India and observed a total of 94 bands were produced. The range of amplified bands varied from 5 to 14 and the size of band produced varied from 240 bp to 1900 bp[33]. Meenatchi *et al.* (2009) used 20 random primers and observed more numbers of amplified bands (132) in *Eudrilus eugeniae* collected from different geographical locations of South India with numbers of amplified bands varied from 3 to 10[35]. However, Biradar *et al.* (2013) employed only 20 random primers and observed a great of 263 polymorphic bands in earthworms from different locations of Western Ghats of Karnataka, India (*Pontoscolex corethrurus*, *Drawida* spp., *Chaetocotoides* sp., *Dichogaster affinis*, *Lennogaster* sp., *Eudrillus euginae*, and *Megascolex conkanensis*). The numbers of bands were in the range of 5 to 19[34]. The presence of more numbers of amplified bands might be due to the presence of more priming site at the template DNA with the particular series of primers employed in their study.

Table 3: Number of polymorphic loci and percentage of polymorphic loci in four populations of earthworm *A. rodericensis* in Vietnam

Population	Number of polymorphic loci	Percentage of polymorphic loci (%)
Hai Lang	51	71.83
Phong Dien	62	87.32
Hue	52	73.24
Huong Thuy	27	38.03

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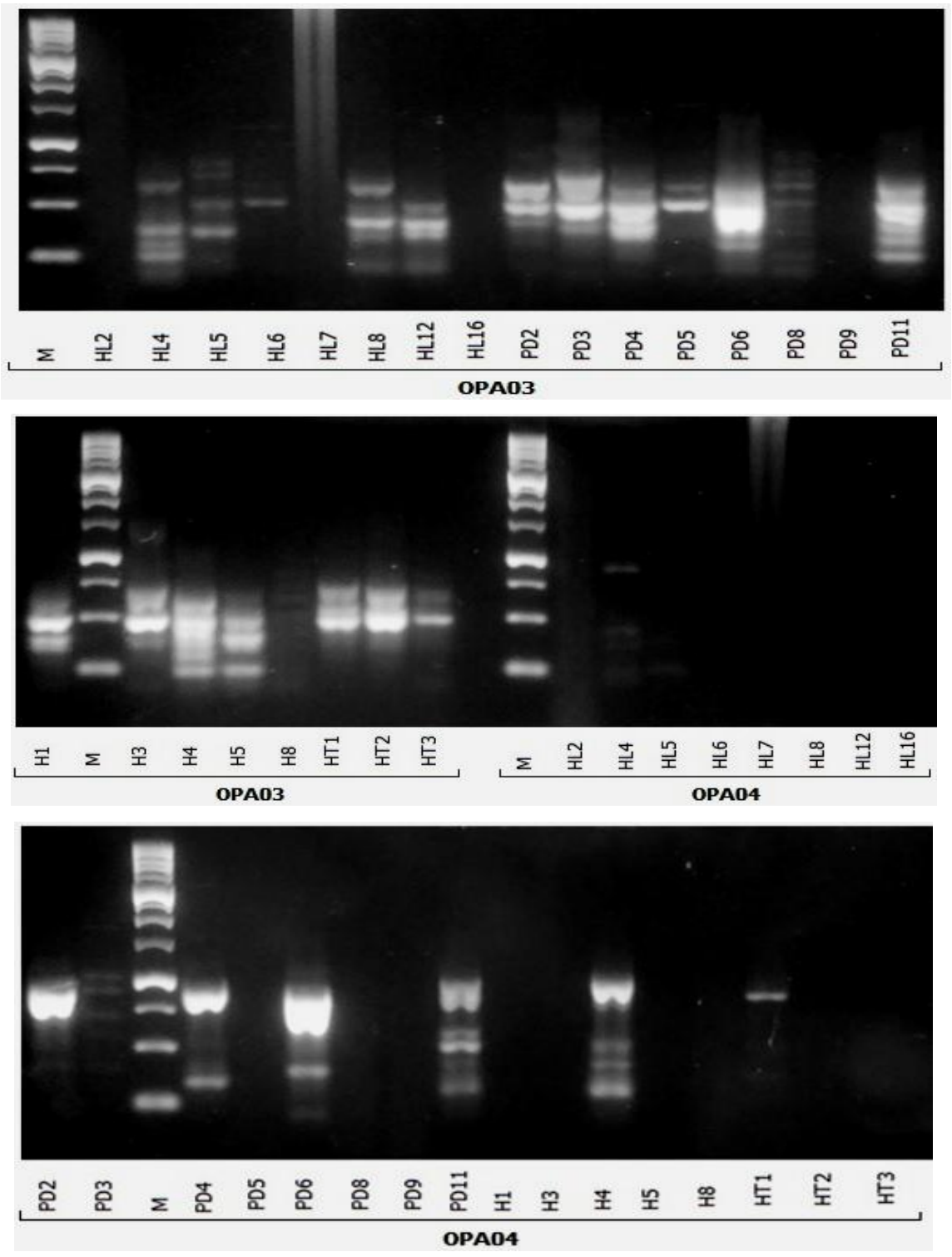

Figure 2: RAPD pattern generated by amplification of *A. rodericensis* DNA with OPA03 and OPA04 primers. M: Molecular weight marker (Phage Lambda DNA *Eco*RI/*Hind*III, Fermentas), HL: Hai Lang, H: Hue, HT: Huong Thuy, PD: Phong Dien

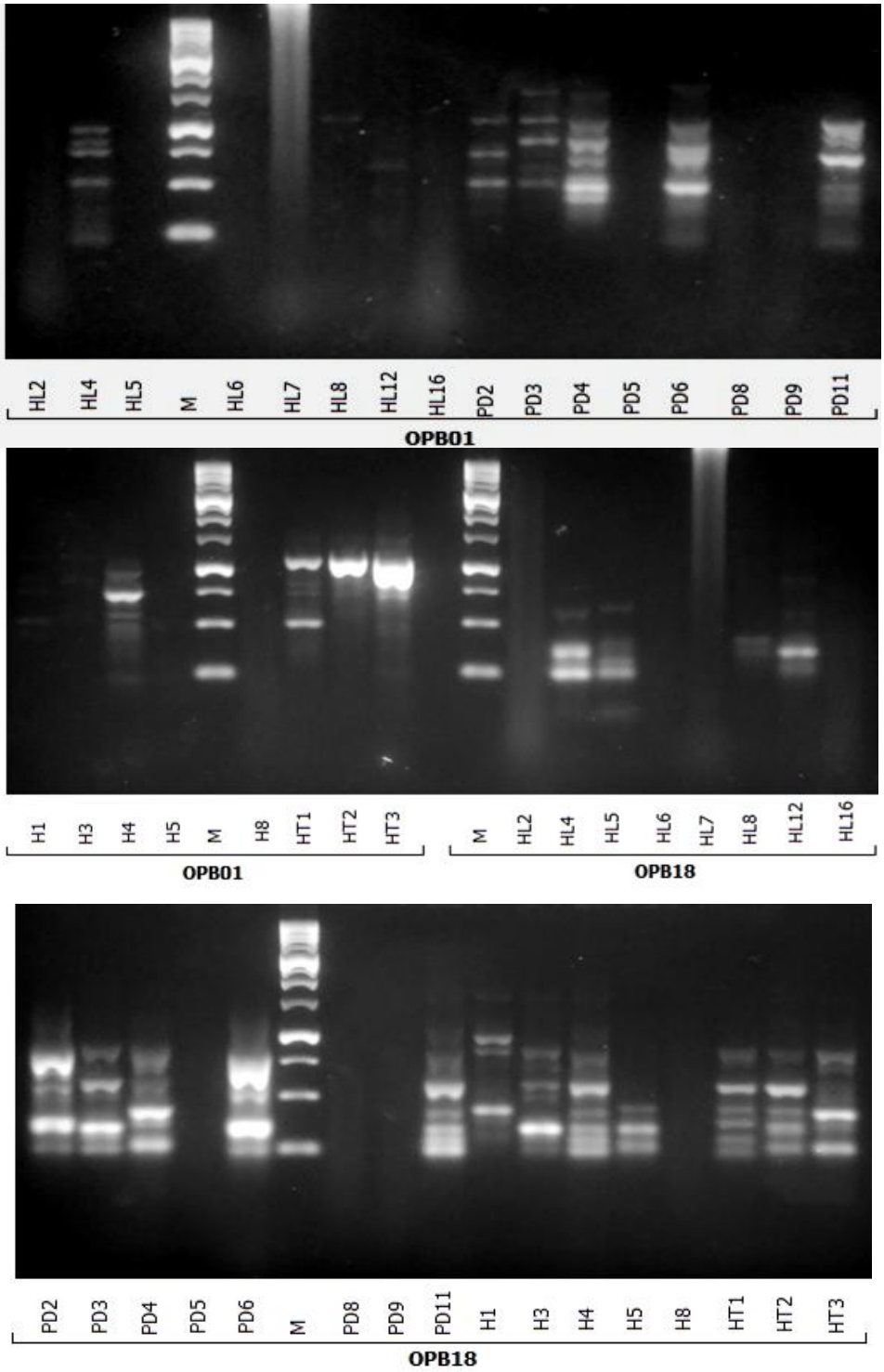

Figure 3: RAPD pattern generated by amplification of *A. rodericensis* DNA with OPB01 and OPB18 primers. M: Molecular weight marker (Phage Lambda DNA *Eco*RI/*Hind*III, Fermentas), HL: Hai Lang, H: Hue, HT: Huong Thuy, PD: Phong Dien.

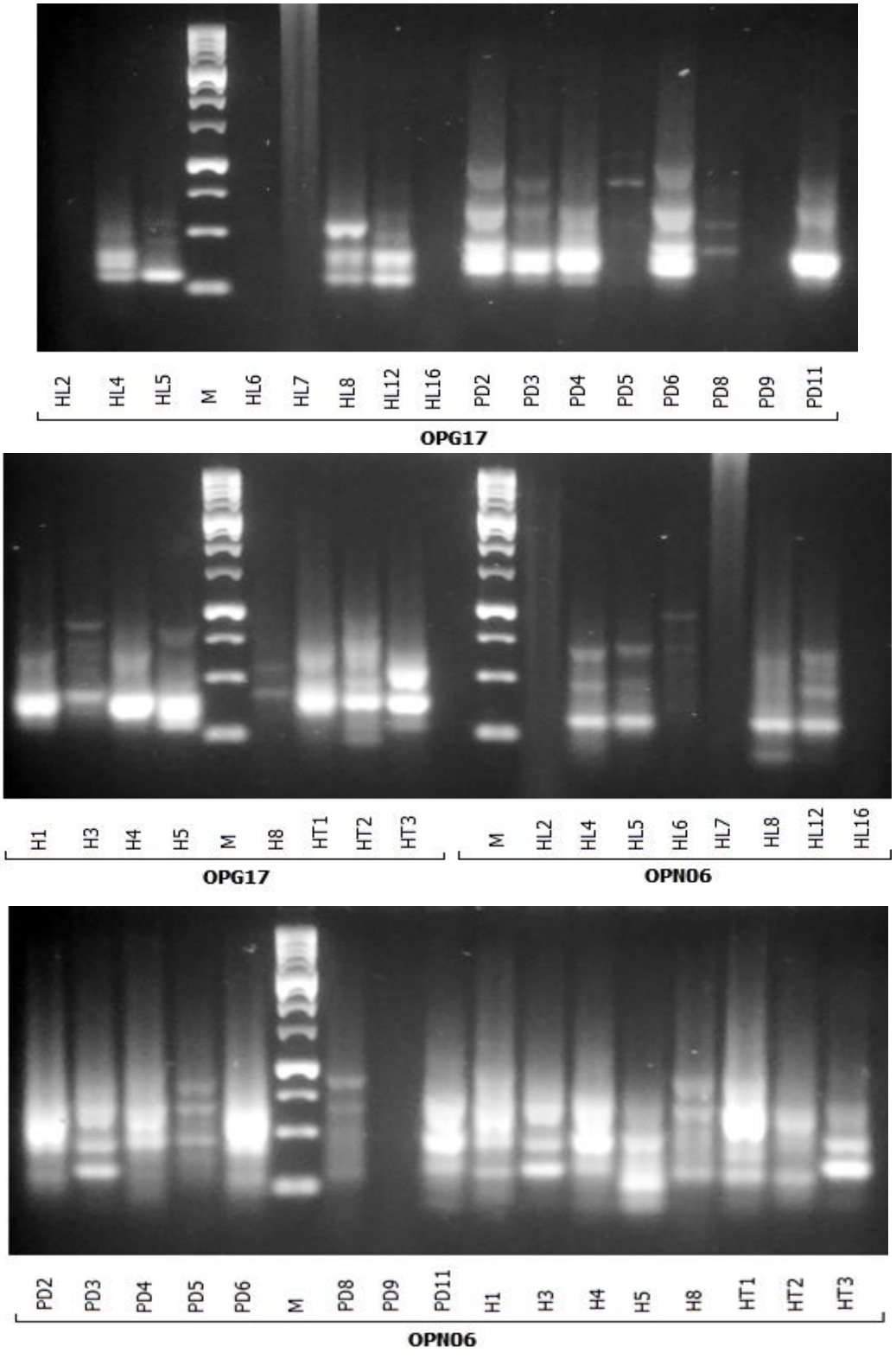

Figure 4: RAPD pattern generated by amplification of *A. rodericensis* DNA with OPG17 and OPN06 primers. M: Molecular weight marker (Phage Lambda DNA *Eco*RI/*Hind*III, Fermentas), HL: Hai Lang, H: Hue, HT: Huong Thuy, PD: Phong Dien.

Genetic diversity of earthworm populations was clearly illustrated in **Table 4** and **Table 5**. Data for observed number of alleles (na), effective number of alleles (ne), Nei's (1973) genetic diversity (h), Shannon's information index (I), for all the four populations were analysed using eight RAPD markers and their respective values were found as 1.6761, 1.4802, 0.2718 and 0.3971, respectively **(Table 4)**. The value for total genotype diversity among populations (Ht) was 0.3631 and within population (Hs) was found to be 0.2718. The mean coefficient of gene differentiation value (Gst) and the estimate of gene flow across the populations (Nm) was found as 0.2515 and 1.4870, respectively **(Table 5)**.

Table 4: Summary of genetic parameters estimate for four populations of earthworm *A. rodericensis* in Vietnam using RAPD markers

Population	na	ne	h	I
Hai Lang	1.7183	1.4257	0.2526	0.3804
Phong Dien	1.8732	1.6678	0.3680	0.5313
Hue	1.7324	1.5231	0.2975	0.4346
Huong Thuy	1.3803	1.3042	0.1690	0.2421
Mean	1.6761	1.4802	0.2718	0.3971

Table 5: Summary analysis of genetic variability across all four populations of earthworm *A. rodericensis*

Parameter	Ht	Hs	Gst	Nm
Across four populations	0.3631	0.2718	0.2515	1.4870

Genetic distances and genetic relationships: Using Nei's (1972) genetic distance approach, the values of genetic distance and genetic identity between four earthworm populations from Hai Lang, Phong Dien, Hue, and Huong Thuy were calculated and given in **Table 6**. The analysed data indicate that the values of genetic identity between populations were high, ranging from 0.7283 to 0.9184. Genetic identity between Phong Dien and Hue, Hai Lang and Hue, Hai Lang and PhongDien, and Phong Dien and Huong Thuy were higher than those of Hai Lang and Huong Thuy, and Hue and Huong Thuy. The populations originate from Hue and Phong Dien had the highest genetic identity (0.9184). These high values of identity might be reflecting the low levels of genetic variability showed by different earthworm populations analysed here.

Table 6: Nei's (1972) genetic distance (below diagonal) and genetic identity (above diagonal) between four populations of earthworm *A.rodericensis* in Vietnam

Population	Hai Lang	Phong Dien	Hue	Huong Thuy
Hai Lang	*****	0.8703	0.9068	0.7283
Phong Dien	0.1389	*****	0.9184	0.8195
Hue	0.0979	0.0851	*****	0.7902
Huong Thuy	0.3170	0.1991	0.2355	*****

The values of genetic distance between populations were low, varying from 0.0851 to 0.3170 and the population pairs Hai Lang-Huong Thuy have the greatest genetic distance (0.3170) **(Table 6)**. The greatest genetic distance exists between populations that has the most geographically distant and vice versa. Phong Dien was closely related to Hue whereas Hai Lang and Huong Thuy were more distant. Hai Lang population and Huong Thuy population have been adapted in their respective geographical conditions, which may cause wider genetic variability between them. These results indicate geographical distance is an important factor influencing the genetic relationship of populations[46].

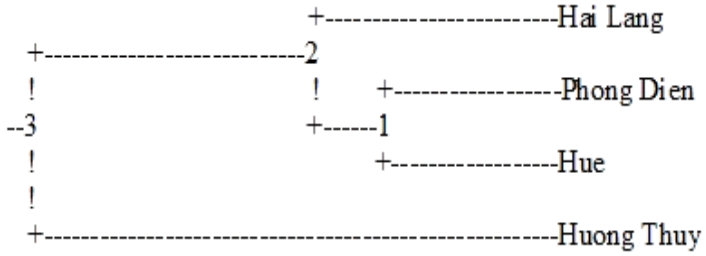

Figure 5: Dendrogram obtained with UPGMA method based on Nei's (1972) genetic distance for four populations of *A. rodericensis* in Vietnam.

The dendrogram constructed on the basis of comparative analysis of the total loci obtained with the eight RAPD primers across the four earthworm populations, presented two clusters: three populations from Hai Lang, Phong Dien, and Hue in one cluster and the Huong Thuy population in the other cluster **(Figure 5)**.

The results of present study indicate that RAPD markers offer a reliable and effective means of assessing genetic variation in earthworm's *A. rodericensis* in Vietnam. On the other hand, studies on genetic diversity using other molecular genetic markers in other earthworms are on record such as *ISSR, URP* in *Eisenia fetida* and *Eudrilus eugeniae*[33]; *COII*, RFLP in *Lumbricus rubellus*[47]; *COI, 5.8S mRNA, ITS1, ITS2* in *Rhinodrilus alatus*[48]; *COI, 16S rRNA*, in *Drawida ghilarovi*[49]; *COI* in *Aporrectodea icterica*[50] and in *Aporrectodea caliginosa, A. trapezoides, Dendrobaena cf. attemsi, Eiseniella tetraedra* and *Octolasion cyaneum*[51]*;18S rRNA* in *Eudrilus eugeniae*[52]; *COI, 16S rRNA, ND1* in *Pheretima formosae*[53], etc. Molecular genetic markers are useful tools which can be utilized for identification, characterization and conservation of earthworm fauna.

CONCLUSION

The observations generated using RAPD markers revealed the high polymorphic levels and the genetic relationships of four populations of earthworm *A.rodericensis* in Vietnam.

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