Genetic Relationship of Two Pheretimoid Earthworm Species in Vietnam Using RAPD-PCR

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Abstract: In this study, Random Amplified Polymorphic DNA (RAPD) markers were applied to analyse the genetic relationship of samples of the *Amynthas rodericensis* (Grube, 1879) and *Amynthas modigliani* (Rosa, 1889), collected from five localities in Vietnam (Quang Ninh belongs to Quang Binh province; Phong Dien, Hue, Huong Tra, and Huong Thuy belong to Thua Thien Hue province). The eight primers used in RAPD analysis amplified 102 bands, 97 (95.10%) of which were polymorphic. The percentages of polymorphic bands observed in the two populations were 86.59% (*A. rodericensis*) and 87.95% (*A. modigliani*). The higher genetic diversity was found within the *A. rodericensis* population (0.2805), and lower genetic diversity was found for the *A. modigliani* (0.2667). The Shannon index ranged from 0.3972 (*A. modigliani*) to 0.4100 (*A. rodericensis*). The analysed data indicate that the values of genetic identity and genetic distance between populations were 0.6636 and 0.4101, respectively. Phylogenetic analysis by RAPD showed two distinct but related clusters between the two earthworm populations. The similarity index value within the individuals of *A. rodericensis* obtained was 0.288-0.943 while in *A. modigliani* individual's ranges from 0.255-0.627. The results showed that RAPD markers revealed congruent interspecific relationship and intraspecific relationship as well as variations among two pheretimoid earthworm populations *A. rodericensis* and *A. modigliani* in Vietnam.
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Key words: *Amynthas modigliani, Amynthas rodericensis*, earthworm, RAPD, genetic relationship, Vietnam.

1. Introduction

Earthworms, the members of the order Opisthophora, class Oligochaeta, phylum Annelida, are distributed worldwide in different habitats where soil water and temperature are favourable. They are very important and beneficial components of soil fauna since they dominate the invertebrate biomass in the soil (Thai, 1983). Earthworms play an significant role in enhancing soil fertility (Aina, 1984; Sharma et al., 2009; Nugroho, 2010; Ansari and Ismail, 2012; Grdisa et al., 2013; Jalal et al., 2014; Lemtiri et al., 2014). They help in organic matter decomposition (Kaplan et al., 1980; Nozaki et al., 2008; Kale and Karmegam, 2009; Nugroho, 2010; Sharma et al., 2009; Ansari and Ismail, 2012; Lemtiri et al., 2014), in vermicomposting (Kale and Karmegam, 2009; Sharma et al., 2009; Ansari and Ismail, 2012; Giraddi et al., 2014; Usman et al., 2016) and in bioremediation (Blouin et al., 2013; Sharma et al., 2009; Sharma, 2017; Usman et al., 2016). Earthworms affect soil structure and functioning (Sharma et al., 2017; Usman et al., 2016; Blouin et al., 2013) and have been utilised as useful bioindicators of soil quality (Biruntha et al., 2015; Kale and Karmegam, 2009; Lima and Brussaard, 2010; Frund et al., 2011; Ansari and Ismail, 2012). Earthworms have also been used for medicinal purposes (Grdisa, 2013; Li, 2010; Sharma et al., 2009). On the other hand, they as a source protein for human (Sharma et al., 2009; Grdisa et al., 2013; Li et al., 2010); pig (Sharma et al., 2009), chickens (Sharma et al., 2009; Tagoba, 1980), quails (Istiqomah et al., 2017), ducks (Li et al., 2010), geese, fishes (Beg et al., 2016; Sharma et al., 2009; Sogbesan et al., 2007; Vassilli and Saurat, 1996), etc. In addition, they are familiar to the fisherman as the best baits (Li et al., 2010).

The two pheretimoid earthworm species, *Amynthas rodericensis* (synonym: *Pheretima rodericensis*) and *Amynthas modigliani* (synonym: *Pheretima modigliani*), belong to the family Megascolecidae, class Clitellata, phylum Annelida. These species seem to be widely distributed in Central Vietnam (*viz.* Quang Binh, Quang Tri, Thua Thien Hue, etc.) (Tung et al., 2016).

The genetic diversity and relationship in any species along with earthworms are assessed by PCR-RAPD techniques that are extremely helpful and potent (Yadav and Mullah, 2017). In recent years RAPD markers have been widely used for analysis of genetic diversity in various of earthworm species such as *Aporrectodea* spp. (Dyer et al., 1998); *Eisenia fetida* (Sharma et al., 2011); *Eudrilus eugeniae* (Biradar et al., 2013; Sharma et al., 2011; Meenatchi et al., 2009); *Pontoscolex corethrurus*; *Drawida* spp.; *Chaetocotoides* sp., *Dichogaster affinis, Lennogaster* sp.; and *Megascolex conkanensis* (Biradar et al., 2013); *Perionyx ceylanensis, P. excavates* (Biruntha et

al., 2013); *Lumbricus terrestris*, *Arion lusitanicus* and *Microtus arvalis* (Kautenburger, 2006a, 2006b); etc.

Our previous study indicated genetic diversity of earthworm *A. rodericensis* in Vietnam by RAPD analysis. Using the RAPD markers revealed the high polymorphic levels and the genetic relationships of four populations of earthworm *A. rodericensis* in Vietnam (Nguyen et al., 2018).

The aim of this study is to analyse genetic relationship of two pheretimoid earthworm populations, *A. rodericensis* and *A. modigliani* in Central Vietnam, for which no data are available at present.

2. Materials and Methods

Sample collection

Earthworms were collected from Quang Ninh district belonging to Quang Binh province (n=5); Phong Dien district (n=2), Huong Tra district (n=1), Huong Thuy district (n=1), and Hue city (n=2) belonging to Thua Thien Hue province, Vietnam (Table 1 and Figure 1). Samples were collected by digging and manual soil sorting. The earthworms were anaesthetized by soaking in 70% ethanol, and then washed to remove soil. Specimens were preserved in the laboratory at the Department of Genetics, Faculty of Biology, College of Education, Hue Universiy, Vietnam and stored in 98% alcohol, -20°C for DNA extraction.

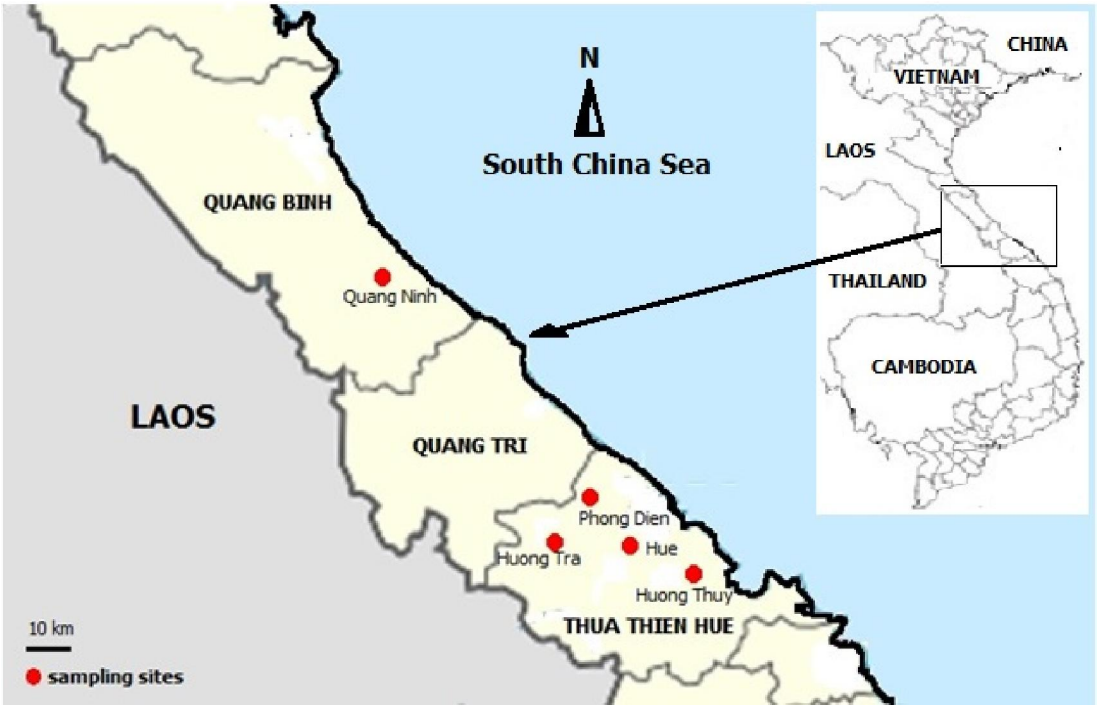

Figure 1. Map of sampling sites.

Genomic DNA extraction

Genomic DNA from earthworm muscle tissue was extracted by using modified phenol-chloroform protocol (Sambrook et al., 1989). The quantity and quality of extracted DNA were determined by measuring its absorbance value at 260 nm and estimating the ratio of absorbance values at 260 nm and 280 nm, respectively. Purified DNA was stored at -20°C till futher analysis.

Table 1. Sampling sites, voucher code and sample size of earthworm populations

Population	Sample size (n)	Voucher code	Sampling sites
A. rodericensis	2	PD2, PD6	Phong Dien, Thua Thien Hue
	1	HTr	Huong Tra, Thua Thien Hue
	2	H1, H4	Hue, Thua Thien Hue
	1	HT1	Huong Thuy, Thua Thien Hue
A. modigliani	5	QN5, QN6, QN8, QN10, QN13	Quang Ninh, Quang Binh

PCR-RAPD reaction

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. Amplifications were 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.

The amplification reactions were performed in a thermocycler (MJ Research, USA) programmed at 94°C for 4 minutes; 92°C for 1 minute, 35°C for 1 minute, 72°C for 2 minutes for 43 cycles and finally 72°C for 10 minutes.

The PCR products were analysed by electrophoresis on 1.5% agarose gels, visualized by staining with ethidium bromide and photographed by a Gel Documentation.

Data analysis

The RAPD patterns of individuals were compared within and among earthworm populations. RAPD bands were scored as 1 if present or 0 if absent. The sizes of the RAPD bands were estimated by using the Quantity One software (ver. 4.1, Bio-rad, USA). The genetic identity and genetic distance between earthworm populations was expressed using Nei's (1972) genetic distance (Nei, 1972).

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) (Yeh et al., 1999). RAPD data were analysed using the NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System) software.

Dendrograms were generated by the UPGMA (Unweighted Pair-Group Method with Arithmeticmean) clustering method to estimate the relationships between two earthworm populations.

3. Results and Discussion

Percentage of polymorphic bands

A series of discrete bands were obtained after amplification of DNA samples of all two populations of pheretimoid earthworm *A. rodericensis* and *A. modigliani* 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 16, 15, 14, 13, 12, 11, 9, and 12 bands for primers OPA03, OPA04, OPB01, OPB18, OPD11, OPF04, OPG17 and OPN06, respectively. The largest number of RAPD bands were detected for primer OPA03 (16 bands), while the lowest number was scored for primer OPF04 (9 bands). A total of 102 amplified bands were consistentely generated, of which 97 (95.1%) bands were polymorphic, with an average number of bands and average number of polymorphic bands per primer was 12.75 and 12.13, respectively. The size of these amplified bands ranged from 200 bp to 1900 bp. The highest size range was exhibited for OPB01 (200 bp-1900 bp) while it was lowest for OPG17 (200 bp-1050 bp). Percentage of polymorphic bands ranged from 83.33% (OPD11) to a maximum of 100% (OPA04, OPB01, OPB18, OPF04 and OPN06) with an average of 94.97% polymorphism (Table 2). The result shows that high polymorphic levels of these primers in two pheretimoid earthworms, *A. rodericensis* and *A. modigliani* in Vietnam.

Table 2. Sequence of RAPD primers, sizes, and number of amplified bands based on RAPD analysis in populations of earthworm *A. rodericensis* and *A. modigliani* in Vietnam.

Primer code	Nucleotide sequence (5'-3')	Number of amplified bands	Number of polymorphic bands	Percentage of polymorphic bands (%)	Size range of amplified bands (bp)
OPA03	AGTCAGCCAC	16	14	87.50	270-1600
OPA04	AATCGGGCTG	15	15	100	200-1900
OPB01	GTTTCGCTCC	14	14	100	240-1400
OPB18	CCACAGCAGT	13	13	100	300-1600
OPD11	GGTGATCAGG	12	10	83.33	300-1600
OPF04	ACGACCGACA	11	11	100	250-1600
OPG17	AGCGCCATTG	9	8	88.89	200-1050
OPN06	GAGACGCACA	12	12	100	300-1350
Overall		102	97	95.10	200-1900
Mean		12.8	12.1	94.97	

This result is comparable to the results of studies carried out by other authors. Sharma (2011) also employed ten random primers in two earthworm species (*Eisenia fetida* and *Eudrilus eugeniae*) from ten different localities representing different soil types in India and observed a total of 94 bands were produced. Of those, 90 (95.74%) bands were found to be polymorphic and the range of amplified bands varied from 5 to 14 and the size of band produced varied from 240 to 1900 bp. Kautenburger (2006a) used three random primers in *Lumbricus terrestris* from five different locations in Western Germany and found the total numbers of amplified bands was 61; of those, 49 (80.3%) which were polymorphic and the range of amplified bands varied from 15 to 36 with a size ranging from 300 bp to 1950 bp. In other research, Kautenburger (2006b) also used RAPD markers to measure the genetic variability of the two selected model earthworm species (*Arion lusitanicus* and *L. terrestris*) in two agricultural landscapes in Western Germany (one site in Northern Saarland and the other in Western Rhineland-Palatinate). Three random primers were employed in 4 earthworm populations of *L. terrestris* and observed a total of 60 bands were produced; of those, 55 (91.7%) which were polymorphic and the range of amplified bands varied from 17 to 25 and the size of band produced varied from 290 to 1610 bp. Similarity, four random primers were used in ten populations of *A. lusitanicus* and observed a total of reproducible 94 bands; of those, 92 (97.9%) were polymorphic and and the range of amplified bands varied from 19 to 26 and the size of band produced varied from 380 to 1940 bp. In our study, the percentage of polymorphic bands was 95.1%. This was higher to results from study on earthworms *L. terrestris* in Germany, 80.3% (Kautenburger, 2006a) and 91.7% (Kautenburger, 2006b) but little lower than those of earthworms *E. fetida* and *E. eugeniae* from India (95.74%) (Sharma *et al.*, 2011) and earthworms *A. lusitanicus* in Germany (97.9%) (Kautenburger, 2006b).

The arevage percentage of polymorphic loci of *A. rodericensis* and *A. modigliani* were 86.59% and 87.95%, respectively (Table 3). The sample gels were obtained from primer OPA03, OPB01, OPG17 and OPN06 which are presented in Figure 2, 3, 4, and 5, respectively.

Table 3. Number of polymorphic loci and percentage of polymorphic loci in two pheretiomoid earthworm populations in Vietnam.

Populations	Number of amplified bands	Number of polymorphic loci	Percentage of polymorphic loci (%)
A. rodericensis	82	71	86.59
A. modigliani	83	72	87.95

Two primers (OPG17 and OPN06) generated RAPD bands exhibiting fixed frequencies in at least one population. Every primer produced a population-specific marker: OPG17 in *A. modigliani* (200 bp), and OPN06 in *A. rodericensis* population (750 bp) (Table 4). Data for observed number of alleles (na), effective number of alleles (ne), Nei's (1973) genetic diversity (h), Shannon's information index (I), for the two populations were analysed using the RAPD markers and their respective values were found as 1.6961, 1.4946, 0.2805 and 0.4100 in *A. rodericensis*; and 1.7059, 1.4475, 0.2667 and 0.3972 in *A. modigliani* (Table 5). The higher genetic diversity was found within the *A. rodericensis* population (0.2805), and lower genetic diversity was found for the *A. modigliani* (0.2667). This means that *A. rodericensis* population have a higher proportion of heterozygous genotypes than the *A. modigliani* population, which was in accordance with the result of Shannon's information index (I) (Table 5). The Shannon's information index ranged from 0.3972 (*A. modigliani*) to 0.4100 (*A. rodericensis*).

Table 4. Size of the diagnostic bands for each pheretimoid earthworm population (bp).

Primer	***A. rodericensis***	***A. modigliani***
OPG17	-	200
OPN06	750	-

Table 5. Summary of genetic parameters estimate for two pheretimoid populations of earthworm in Vietnam using RAPD markers.

Population	na	ne	h	I
A. rodericensis	1.6961	1.4946	0.2805	0.4100
A. modigliani	1.7059	1.4475	0.2667	0.3972

In the present study, the value for total genotype diversity among populations (Ht) was 0.3958 while within population diversity (Hs) was found to be 0.2736. The mean coefficient of gene differentiation (Gst) value and the estimate of gene flow across the populations (Nm) was found as 0.3087 and 1.1195, respectively.

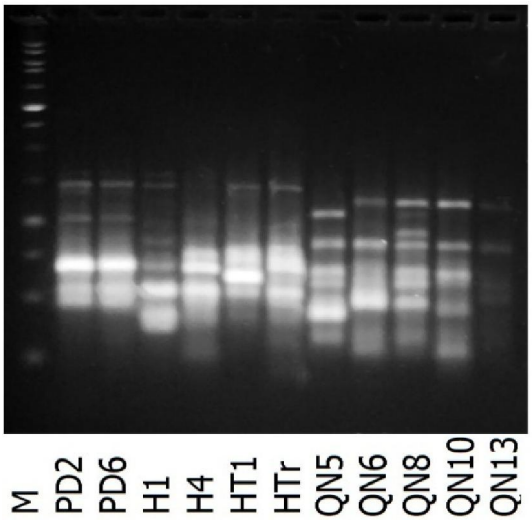

Figure 2. RAPD pattern generated by amplification of *A. rodericensis* DNA and *A. modigliani* DNA with OPA03 primer. M: Molecular weight marker (Phage Lambda DNA *Eco*RI/*Hind*III, Fermentas), PD: Phong Dien, H: Hue, HT: Huong Thuy, HTr: Huong Tra, QN: Quang Ninh.

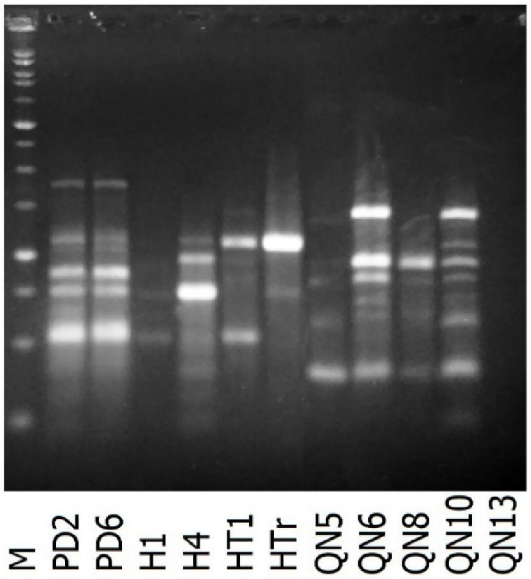

Figure 3. RAPD pattern generated by amplification of *A. rodericensis* DNA and *A. modigliani* DNA with OPB01 primer. M: Molecular weight marker (Phage Lambda DNA *Eco*RI/*Hind*III, Fermentas), PD: Phong Dien, H: Hue, HT: Huong Thuy, HTr: Huong Tra, QN: Quang Ninh.

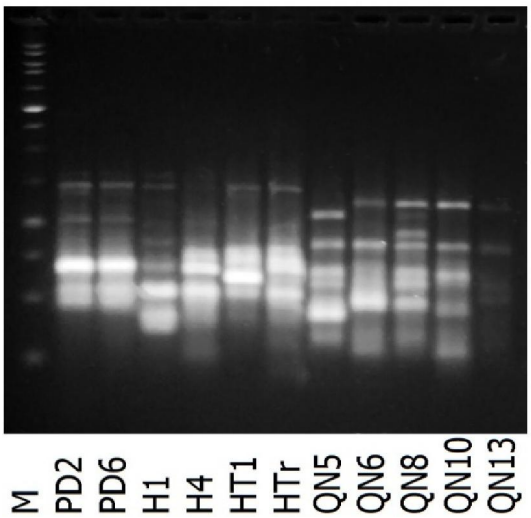

Figure 4. RAPD pattern generated by amplification of *A. rodericensis* DNA and *A. modigliani* DNA with OPG17 primer. M: Molecular weight marker (Phage Lambda DNA *Eco*RI/*Hind*III, Fermentas), PD: Phong Dien, H: Hue, HT: Huong Thuy, HTr: Huong Tra, QN: Quang Ninh.

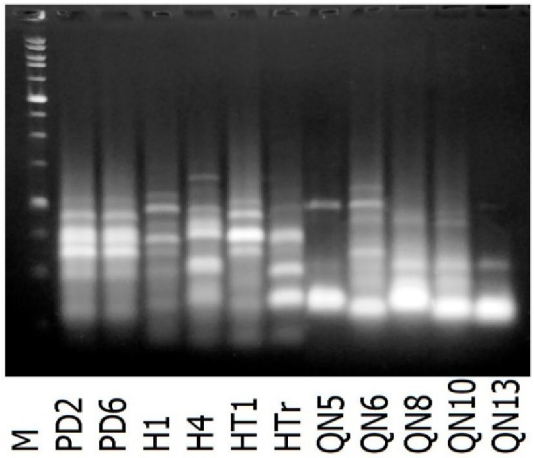

Figure 5. RAPD pattern generated by amplification of *A. rodericensis* DNA and *A. modigliani* DNA with OPN06 primer. M: Molecular weight marker (Phage Lambda DNA *Eco*RI/*Hind*III, Fermentas), PD: Phong Dien, H: Hue, HT: Huong Thuy, HTr: Huong Tra, QN: Quang Ninh.

Genetic distances and genetic relationships

Using Nei's (1972) genetic distance approach, the values of genetic distance and genetic identity between two earthworm populations from Thua Thien Hue and Quang Binh were calculated and given in Table 6. The analysed data indicate that the value of genetic identity between two populations was 0.6636. The value of genetic distance between two populations was 0.4101.

The dendrogram constructed on the basis of comparative analysis of the total loci obtained with the RAPD primers across the two pheretimoid earthworm populations, presented one cluster (Figure 6).

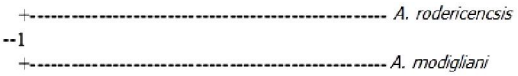

Figure 6. Dendrogram obtained with UPGMA method based on Nei's (1972) genetic distance for two populations of *A. rodericensis* and *A. modigliani* in Vietnam.

Table 6. Nei's (1972) genetic distance (below diagonal) and genetic identity (above diagonal) between two populations of pheretimoid earthworm in Vietnam.

Population	***A. rodericensis***	***A. modigliani***
A.rodericensis	****	0.6636
A. modigliani	0.4101	****

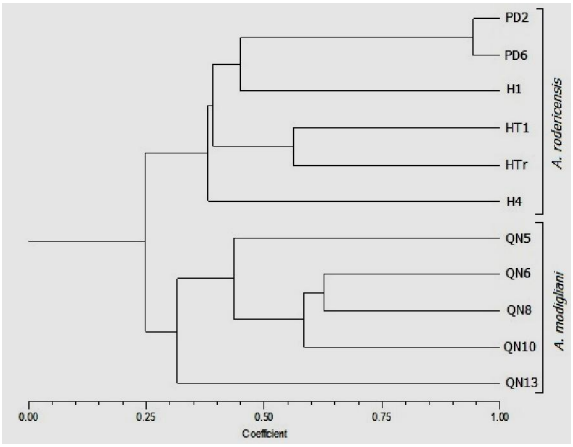

Figure 7. Dendrogram obtained with UPGMA method based on Nei's (1972) genetic distance for two populations of *A. rodericensis* and *A. modigliani* in Vietnam.

The similarity index between all possible pair wise comparisons of individuals from all primers was calculated and phylogenetic relationship between individuals of *A. rodericensis* and *A. modigliani* samples was constructed using cluster analysis. The results showed clustering of *A. rodericensis* (PD2, PD6, H1, HT1, H4 and HTr) and *A. modigliani* (QN5, QN6, QN8, QN10 and QN13) clearly separated from each with two separate clusters and within that each individual were separated by separate cluster. Thus, all the individuals of each species formed monophyletic species clusters (Figure 7 and Figure 8).

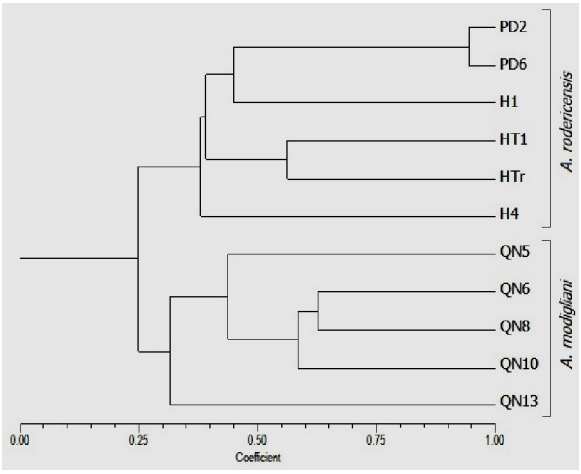

Figure 8. Dendogram obtained by the Jaccard similarity coefficient and method of UPGMA for two earthworm populations of *A. rodericensis* and *A. modigliani* in Vietnam.

Phylogenetic analysis by RAPD showed two different clusters between two populations, *A.rodericensis* and *A. modigliani*. Again Jaccard similarity coefficient values gave different clusters for each of the individual samples used for the study. The similarity coefficient between all possible pair wise comparisons of individuals were calculated. The similarity coefficient value within the individuals of *A. rodericensis* obtained was 0.288-0.943 while in *A. modigliani* individual's ranges from 0.255-0.627. This indicates that *A.rodericensis* individuals have wide range of similarity coefficient value. The similarity coefficient value between *A.rodericensis* and *A. modigliani* ranges from 0.136-0.333 (Table 7). So, the similarity coefficient value within the individuals of *A. rodericensis* and *A. modigliani* were significantly higher than those among populations. These results exhibited great variation of the similarity coefficient value within and among populations by RAPD markers. The similarity coefficient values greatly varied found in our study agree with some published works such as 0.51-0.79 within individuals of *E. fetida*, 0.32-0.57 within individuals of *E. eugeniae*, and 0.19-0.46 among *E. fetida* and *E. eugeniae* populations (Sharma et al., 2011); 0.40-0.90 among *E. eugeniae* populations (Meenatchi et al., 2009).

In contrast, the similarity coefficient values within and among *L. terrestris* populations were similar at widely separate locations (Staden, Burbach, Dillingen, Trier, Solling) in Western Germany (Kautenburger, 2006a); the similarity coefficient values within and among *L. terrestris* populations and the similarity coefficient values within and among *A. lusitanicus* populations were similar at two locations

(Wahlen and Herl/Trier) in Western Germany (Kautenburger, 2006b). Similarity, four different earthworm species in Australia were examined with RAPD-PCR and found that the species *Aporrectodea trapezoides* was genetically very similar, due to parthenogenetic reproduction (Dyer et al., 1998).

Table 7. Matrix obtained by the Jaccard similarity coefficient for *A. rodericensis* and *A. modigliani* by using NTSYS-pc.

	PD2	PD6	H1	H4	HT1	HTr	QN5	QN6	QN8	QN10	QN13
PD2	1.000										
PD6	0.943	1.000									
H1	0.443	0.459	1.000								
H4	0.391	0.406	0.323	1.000							
HT1	0.452	0.468	0.288	0.355	1.000						
HTr	0.406	0.400	0.333	0.424	0.560	1.000					
QN5	0.299	0.275	0.327	0.227	0.230	0.271	1.000				
QN6	0.279	0.291	0.333	0.316	0.241	0.241	0.418	1.000			
QN8	0.278	0.259	0.243	0.267	0.254	0.236	0.509	0.627	1.000		
QN10	0.250	0.262	0.247	0.269	0.274	0.292	0.385	0.569	0.600	1.000	
QN13	0.138	0.136	0.154	0.172	0.170	0.192	0.255	0.323	0.352	0.328	1.000

Conclusion

The results of this study indicate that RAPD markers revealed congruent interspecific relationship and intraspecific relationship as well as variations among two pheretimoid earthworm populations *A.rodericensis* and *A. modigliani* in Vietnam.

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