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Genetic Relationship of Two Agamid Lizard Species in Vietnam by Random Amplified Polymorphic DNA Analysis

Dung Q. Tran[1], Thien V. Tran[2], Quang T. Hoang[3]

[1]Faculty of Biology, College of Education, Hue University, 34 Le Loi Street, Hue City, Vietnam
[2]Nguyen Tri Phương Secondary school, 63 To Huu Street, Hue City, Thua Thien Hue, Vietnam
[3]Institute of Biotechnology, Hue University, Ngoc Anh, Phu Thuong, Phu Vang, Thua Thien Hue, Vietnam
tranquocdung@dhsphue.edu.vn, tranquocdungdhsphue@gmail.com

Abstract: The aim of this study was to apply RAPD technique to analyse the genetic relationship of two agamid lizard species, *Leiolepis guentherpetersi* and *Leiolepis reevesii*, collected from Thuy Phu commune, Huong Thuy town ($16^0$20'N, $107^0$45'E) and Thuan An township, Phu Vang district ($16^0$30'N, $107^0$40'E), belong to Thua Thien Hue province in Vietnam, respectively. The two single primes (428, and CRL5) and one pair-wise combinations of primers (101+268) used in PCR-RAPD analysis. The amplified PCR products were detected on a 1.5% agarose gel and subjected to further analysis to establish the band profiles and genetic relationships using the Quantity One software, the POPGENE software and the NTSYS-pc software. The percentages of polymorphic loci observed in the two populations were 28.26% (*L. guentherpetersi*), and 15.56% (*L. reevesii*). The higher genetic diversity was found within the *L. guentherpetersi* population (0.0756), and lower genetic diversity was found for the *L. reevesii* (0.0566). The Shannon index ranged from 0.0944 (*L. reevesii*) to 0.1207 (*L. guentherpetersi*). The values of genetic identity and genetic distance between populations were 0.8523 and 0.1598, respectively. Phylogenetic analysis by RAPD showed two distinct but related clusters between the two populations. The similarity index value within the individuals of *L. guentherpetersi* obtained was 0.7755-1.0 while in *L. reevesii* individual's ranges from 0.8571-1.0. [Tran QD, Tran VT, Hoang TQ. **Genetic Relationship of Two Agamid Lizard Species in Vietnam by Random Amplified Polymorphic DNA Analysis.** *Life Sci J* 2018;15(10): 36-42]. ISSN: 1097-8135 (Print)/ISSN: 2372613X (Online). http://www.lifesciencesite.com. 4. doi: 10.7537/marslsj151018.04.

Keywords: agamid lizard, genetic relationship, *Leiolepis guentherpetersi*, *Leiolepis reevesii*, RAPD, Vietnam

1. Introduction

The butterfly lizard of the genus *Leiolepis* (Cuvier, 1829), belong to Agamidae family, is widely distributed from Southern China, Vietnam, Laos, Cambodia, Myanmar, and Thailand Southwestward through Peninsular Malaysia (Grismer and Grismer, 2010). In Vietnam, they inhabit from Central to the Southern Vietnam (Hoang and Cao, 2001). The great majority of agamid lizards are oviparous and they were found on the coastal sand and among the bushy vegetation of the coastal slopes and mounds. They play an important role in the balance of an ecosystem. In most ecosystems, they are the vital part of food chains and they play a huge role both as the prey species and the predators in ecosystems (Ngo, 1999). In South Central Vietnam, *Leiolepis* is considered as a delicacy for its nutrional value. Local people, especially in Ninh Thuan province and Binh Thuan province, prefer to have it for their meals by different ways of cooking and soak it in rice wine or grain alcohol for a tonic function. Therefore the populations of this genus might experience a sharp decline in the future (Tran et al., 2011).

Genetic markers have been widely recognized as an ideal supplementary and informative modern tool for studying genetic diversity and genetic relationship of animal species. A popular genetic marker is Randomly Amplified Polymorphic DNA (RAPD).This technique has been developed simultaneously by William et al. (1990) and Welsh et al. (1990). RAPD technique 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 (William et al., 1990; Welsh et al., 1990; Grechko, 2002; Liu and Cordes, 2004; Arif and Khan, 2009; Choi et al., 2018). RAPD markers have been widely used for analysis of genetic diversity and genetic relationship in variety of animal species: fish (Tran et al., 2017), deer (Masseti et al., 1972), cattle and sheep (Kantanen et al., 1995), canine (Olivier, 1999), chicken (Olowofeso et al., 2006), earthworm (Nguyen et al., 2018), trematode (Morozova et al., 2002), etc. and various reptile species such as *Tropiocolotes tripolitanus, T. steudneri, T. nattereri, Tarentola mauritanica and T. annularis* (Sayed, 2012a); *T. tripolitanus*, *T. nattererii*, *Hemidactylus turcicus*, *Cyrtopodion scaber*, *Stenodactylus petrii*, *Ptyodactylus guttatus*, *P. hasselquistii*, *T. mauritanica* and *T. annularis* (Ali, 2012); *Lacerta agilis, L. strigata, L. viridis, L. media* (Grechko *et al*., 2006); 11 species of the genus *Bothrops, B. alternatus, B. neuwiedi, B. cotiara, B. jararacusu, B. insularis, B.*

jararaca, B. erythromelas, B. moojeni, B. leucurus, and B. atrox (Grazziotin and Echeverrigaray, 2005); eight Egyptian snake species, *Psammophis sibilans sibilans, P. sudanensis, P. schokari schokari, P. schokari aegyptiacus, Spalerosophis diadema, Lytorhynchus diadema, Coluber rhodorhachis, C. nummifer* (Sayed, 2012b), *Alligator sinensis* (Wu et al., 2002), turtle *Emydoidea blandingii* (Mockford et al., 1999), *Testudo graeca* (Cemenova et al., 2004), *Phrynops hilarii* and *Trachemys dorbigni* (Guidetti et al., 2015), etc.

In this work, RAPD technique has been used to analyse genetic relationship of two agamid lizard populations *Leiolepis reevesii* and *Leiolepis guentherpetersi* collected from Thua Thien Hue province, Central Vietnam.

2. Materials and Methods

Sample collection

Two agamid lizard populations of *L. guentherpetersi* and *L. reevesii* were captured from Thuy Phu commune, Huong Thuy town ($16^0$20'N, $107^0$45'E) and Thuan An township, Phu Vang district ($16^0$30'N, $107^0$40'E), belong to Thua Thien Hue province, Vietnam (Table 1, Figure 1 and Figure 2). *L. reevesii* is sexual species and *L. guentherpetersi* is asexual species (Darevsky and Kupriyanova, 1993; Grismer and Grismer, 2010). Specimens were stored in the laboratory at the Department of Genetics, Faculty of Biology, College of Education, Hue University, Vietnam and stored in 98% alcohol until further utilized.

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

Population	Number of specimens (n)	Voucher code	Locality (Coordinates)
L. guentherpetersi	16	S1-S16	Thuy Phu commune (16 ^{0}20'N, 107^{0}45'E)
L. reevesii	16	C1-C16	Thuan An township (16 ^{0}30'N, 107^{0}40'E)

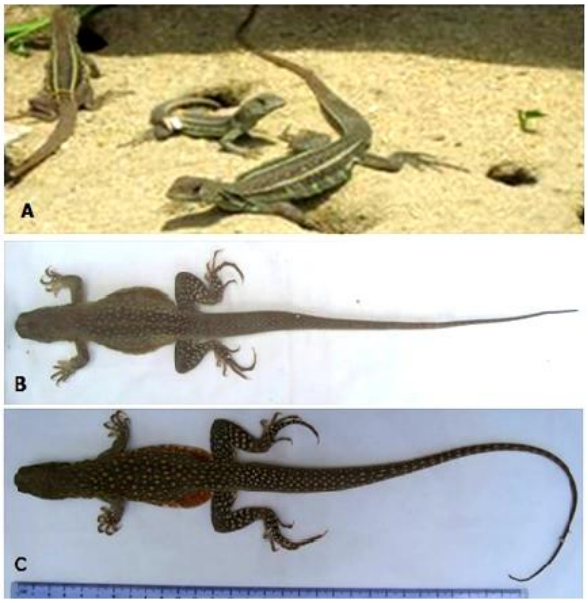

Figure 1. *L. guentherpetersi* (A) and*L.reevesii* (B. female, C. male).

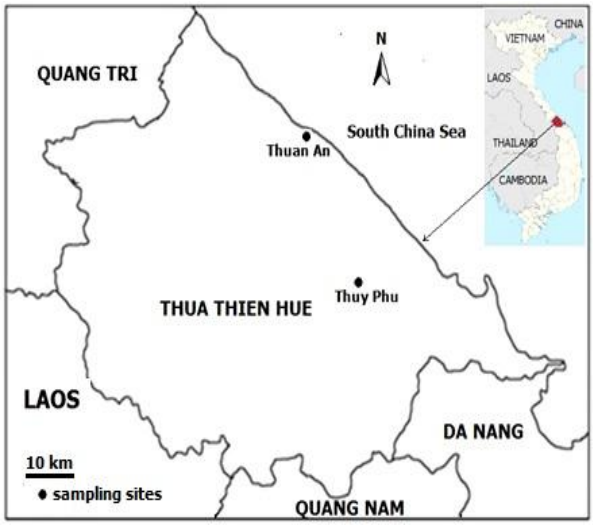

Figure 2. Map of sampling localities for specimens *L. guentherpetersi* and *L. reevesii* used in this study.

Extraction of genomic DNA

Genomic DNA was isolated from tail tissue using 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. DNA was stored at -20°C until analysis.

PCR-RAPD reaction and agarose gel electrophoresis

Twelve single random primers (101, GCGCCTGGAG; 174, AACGGGCAGG; 268, AGGCCGCTTA; 428, GGCTGCGGTA; 457, CGACGCCCTG; 459, GCGTCGAGGG; OPA06, GGTCCCTGAC; OPA09, GGGTAACGCC; OPA14, TCTGTGCTGG; OPA17, GACCGCCTTGT; CRL5, CCAGCGTCCC; and CRL31, CGTGCCCGGC) and three pair-wise combinations of primers (101+268, 101+174, and 268+459) were used to initiate PCR-RAPD amplifications, out of which two single primes (428 and CRL5) and one pair-wise combinations of primers (101+268) (Table 2) that gave better results

were used for final amplification to delineated genetic diversity in two populations. 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.

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 distance and genetic identity between populations was expressed based on 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, 1999). RAPD data were analysed using the NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System) software.

3. Results and Discussion

RAPD diversity

A series of discrete bands were obtained after amplification of DNA samples of two agamid populations, *L. guentherpetersi* and *L. reevesii* with two single random primers (428, CRL5) and one pairwise combinations of primers (101+268). The different primers and one pair-wise combinations of primers produced different banding patterns. The number of reproducible bands across all investigated samples was 14, 22, and 13 bands for primers 428, CRL5 and 101+268, respectively. The largest number of RAPD bands were detected for primer CRL5 (22 bands), while the lowest number was scored for a pair of primers 101+268 (13 bands). A total of 49 amplified bands were consistently generated, out of which, 22 bands were polymorphic, with an average number of bands and average number of polymorphic bands per primer was 16.33 and 7.33, respectively. The size of these amplified bands ranged from 200 bp to 4000 bp. The highest size range was exhibited for 101+268 (300 bp-4000 bp) while it was lowest for 428 (300 bp-2000 bp) (Table 2). This result is comparable to the results of studies carried out by other authors. Sayed (2012b) employs five random primers in eight Egyptian colubrid species (snakes) from different localities of Egypt and find the total numbers of amplified bands as 59 with a size ranging from 250 bp-3000 bp. Similarity, Sayed (2012a) also employs three random primers in five gekkonid species from different locations in Egypt and find the total numbers of amplified bands as eight. Grazziotin et al. (2005) use 20 random primers and observe more numbers of amplified bands (239) in 10 species of the genus *Bothrops*, and two representatives of the genera *Lacchesis* and *Crotalus* with a size ranging from 100 bp-2500 bp.

Table 2. Sequence of RAPD primer, sizes, and number of amplified bands, and percentage of polymorphic bands based on RAPD analysis in two agamid lizard populations

Primer code	Nucleotide sequence (5'-3')	Size range of amplified bands (bp)	Number of amplified bands	Number of polymorphic bands	Percentage of polymorphic bands (%)
428	GGCTGCGGTA	300-2000	14	3	21.43
CRL5	CCAGCGTCCC	200-2700	22	16	72.73
101+268	GCGCCTGGAG AGGCCGCTTA	300-4000	13	3	23.08
Overall		200-4000	49	22	44.90
Mean			16.33	7.33	39.08

The arevage percentage of polymorphic loci of *L. reevesii* and *L. guentherpetersi* were 15.56% and 28.26%, respectively (Table 3). According to polymorphic loci the amount of genetic variation between the two species was *L. reevesii*<*L. guentherpetersi* from low to high. The sample gels were obtained from primer CRL5 and 101+268 primers which are presented in Figure 3 and Figure 4, respectively.

Table 3. Number of polymorphic loci and percentage of polymorphic loci in two agamid lizard populations

Populations	Number of amplified bands	Number of polymorphic loci	Percentage of polymorphic loci (%)
L. guentherpetersi	46	13	28.26
L. reevesii	45	7	15.56

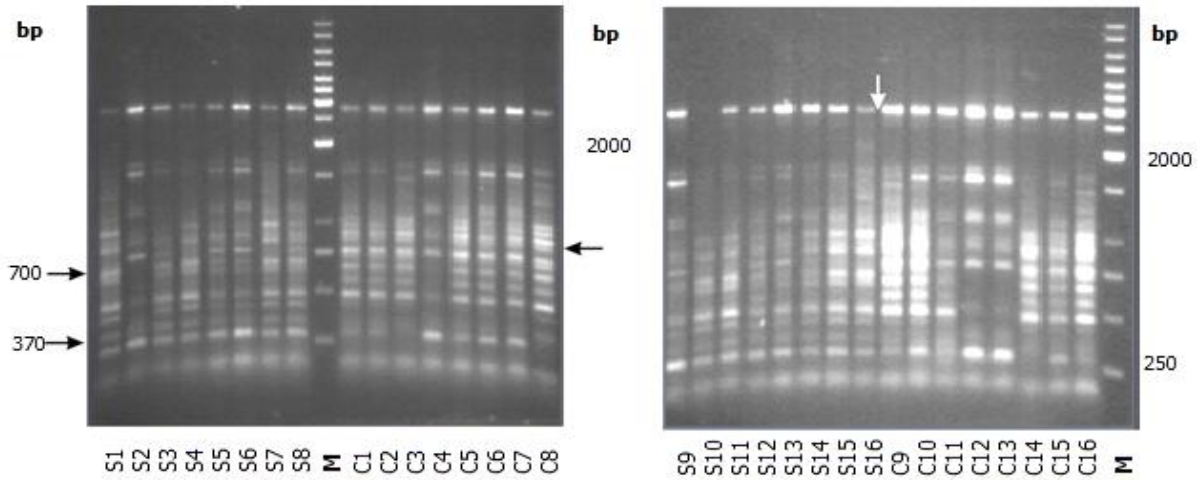

Figure 3. RAPD pattern generated by amplification of *L. guentherpetersi* DNA and *L. reevesii* DNA with CRL5 primer. M: Molecular weight marker (Phage Lambda DNA EcoRI/HindIII, Fermentas), S1-S16: *L. guentherpetersi*, C1-C16: *L. reevesii*.

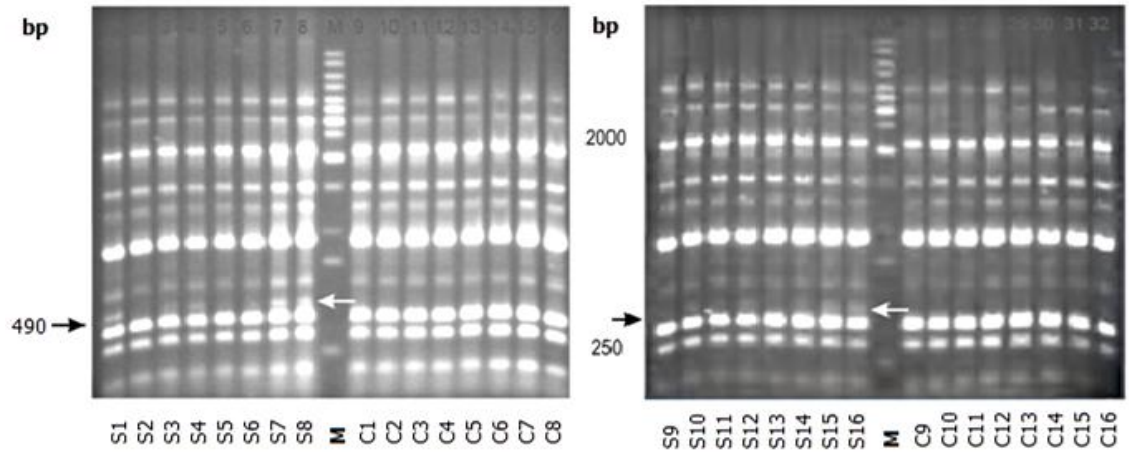

Figure 4. RAPD pattern generated by amplification of *L. guentherpetersi* DNA and *L. reevesii* DNA with 101+268 primers. M: Molecular weight marker (Phage Lambda DNA *Eco*RI/*Hind*III, Fermentas), S1-S16: *L. guentherpetersi*, C1-C16: *L. reevesii.*

Two single primers (428, CRL5) and one pairwise combination of primers (101+268) generated RAPD bands exhibiting fixed frequencies in at least one population. They produced species-specific markers: 428-680 bp in *L. guentherpetersi*, 428-550 bp and 428 in *L. reevesii*, CRL5-380 bp in *L. guentherpetersi*, CRL5-370 bp and CRL5-700 bp in *L. reevesii*, 101+268-490 bp in *L. guentherpetersi*.

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 1.2857, 1.1153, 0.0756 and 0.1207 in *L. guentherpetersi*; and 1.2653, 1.0852, 0.0566, 0.0944 in *L. reevesii* (Table 4). The higher genetic diversity was found within the *L. guentherpetersi* population (0.0756), and lower genetic diversity was found for the *L. reevesii* (0.0566). This meant that *L. guentherpetersi* population had a higher proportion of heterozygous genotypes than the *L. reevesii* population, which was in accordance with the result of Shannon's information index (I) (Table 4). The Shannon's information index ranged from 0.0944 (*L. reevesii*) to 0.1207 (*L. guentherpetersi*). Low genetic diversity of these agamid lizard populations could be due to a number of factors, one of which is overexploitation of local people. Overexploitation of *L. guentherpetersi* and *L. reevesii* as a delicious food and for a tonic function is the main cause of population decline.

Table 4. Summary of genetic parameters estimate for two agamid lizard populations

Population	na	ne	h	I
L. guentherpetersi	1.2857	1.1153	0.0756	0.1207
L. reevesii	1.2653	1.0852	0.0566	0.0944

In the present study, the value for total genotype diversity among populations (Ht) was 0.1351 while within populations (Hs) was found to be 0.0661. The mean coefficient of gene differentiation (Gst) value and the estimate of gene flow across the populations (Nm) was found as 0.5108 and 0.4789, respectively.

Genetic distances and genetic relationships

The values of Nei's (1972) genetic identity and genetic distance between two agamid populations, *L. guentherpetersi* and *L. reevesii* from Thuy Phu commune, Huong Thuy town and Thuan An township, Phu Vang district, Thua Thien Hue province were given in Table 5. The analysed data indicated that the values of genetic identity and genetic distance between populations were 0.8523 and 0.1598, respectively.

Table 5. Nei's (1972) genetic identity (above diagonal) and genetic distance (below diagonal) between two agamid lizard populations

Populations	*L. guentherpetersi*	*L. reevesii*
L. guentherpetersi	-	0.8523
L. reevesii	0.1598	-

The dendrogram constructed on the basis of comparative analysis of the total loci obtained with the RAPD primers across the two agamid lizard populations, presented one cluster (Figure 5).

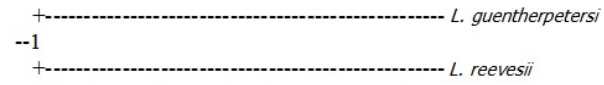

Figure 5. Dendrogram obtained with UPGMA method based on Nei's (1972) genetic distance for two populations of *L. guentherpetersi* and *L. reevesii* in Thua Thien Hue province, Vietnam.

The similarity index between all possible pair wise comparisons of individuals from all primers was calculated and phylogenetic relationship between individuals of *L. guentherpetersi* and *L. reevesii* samples was constructed using cluster analysis. The results showed clustering of *L. guentherpetersi* (S1-S16) and *L. reevesii* (C1-C16) 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 6 and Figure 7).

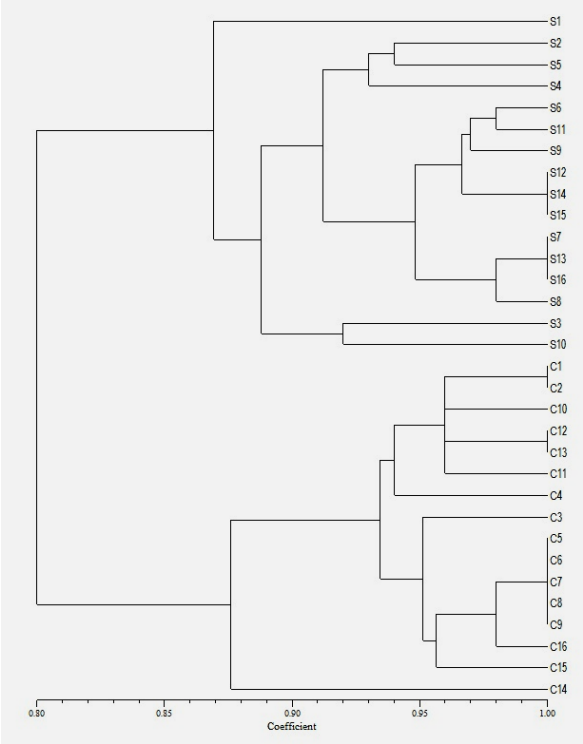

Figure 6. Phylogenetic tree produce by genetic distance between each individual samples of *L. guentherpetersi* (S1-S16) and *L. reevesii* (C1-C16) by NTSYS-pc.

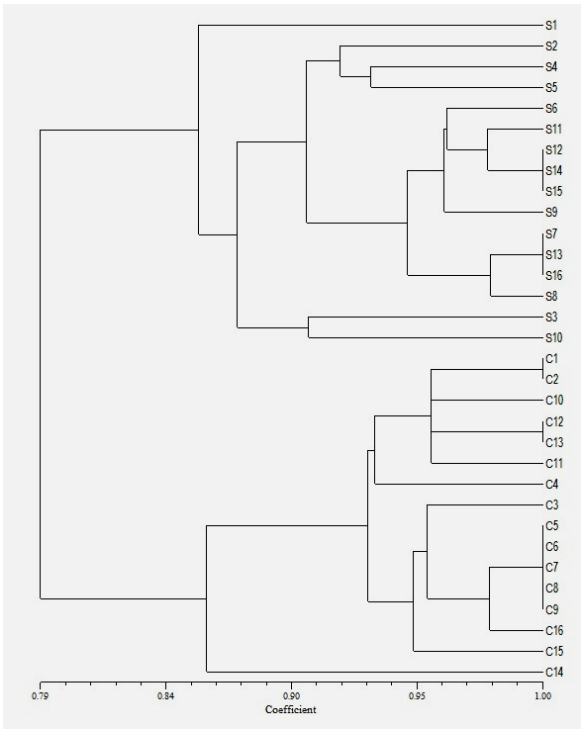

Figure 7. Dendogram obtained by the Jaccard similarity index and method of UPGMA for *L. guentherpetersi* (S1-S16) and *L. reevesii* (C1-C16).

Phylogenetic analysis by RAPD showed two different clusters between two populations (*L. guentherpetersi* and *L. reevesii*). Again Jaccard coefficient values gave different cluster for each of the individual samples used for the study. The similarity index between all possible pair wise comparisons of individuals were calculated. The similarity index value within the individuals of *L. guentherpetersi* obtained was 0.7755-1.0 while in *L. reevesii* individual's ranges from 0.8571-1.0. This indicated that *L. guentherpetersi* individuals had wide range of similarity index value. The similarity index value between *L. guentherpetersi* and *L. reevesii* ranged from 0.6939-0.8776. This result is little lower than that of the two populations *L. guentherpetersi* and *L. reevesii* from the same sampling localities, analyses using mitochondrial *16S rRNA* sequences (91.191.6%) (Tran et al., 2011). Malysheva et al.(2006) use multilocus DNA fingerprinting with microsatellite probes $(CAC)_5$, $(GACA)_4$, $(GGCA)_4$, and $(GATA)_4$ to examine intraspecific variation of *L. reevesii* and *L. guentherpetersi*. The *L. guentherpetersi* were characterized by monophyletyic DNA fingerprint profiles for the loci detected by the $(GACA)_4$, $(GGCA)_4$, and $(CAC)_5$ probes, in terms of intrapopulation similarity index constituting S=0.96. This was different from the individual-specific profiles of *L. reevesii* (S=0.6) at $P<0.001$. The results of DNA fingerprinting analysis of the same *L. guentherpetersi* samples with the $(GATA)_4$ hibridization probe were unexpected variability of *L. guentherpetersi* at the $(GATA)_n$ markers was remarkably higher than that at other DNA markers (S=0.35; $P=3.08\times10^{-11}$), being comparable to the variation of the $(GATA)_n$ DNA markers in *L. reevesii* (P=0.74).

Conclusion

It can be stated that the observations generated using RAPD markers revealed congruent interspecific relatedness and intra-specific relatedness as well as variations among two agamid lizard species *L. guentherpetersi* and *L. reevesii* in Thua Thien Hue province, Vietnam.

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Corresponding Author

Dr. Tran Quoc Dung
Faculty of Biology
College of Education
Hue University, 34 Le Loi, Hue City, Vietnam
Telephone: 84 54 3823183
E-mail: tranquocdung@dhsphue.edu.vn, tranquocdungdhsphue@gmail.com

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