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Genetic diversity of loach *Misgurnus anguillicaudatus* (Cantor, 1842) 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 *Misgurnus anguillicaudatus* (Cantor, 1842), collected from six localities (A Luoi, Phong Dien, Phu Vang, Phu Loc, Quang Dien, and Huong Thuy) along Thua Thien Hue in Vietnam. The six primers used in RAPD analysis amplified 91 loci, 88 (96.70%) of which were polymorphic. The percentages of polymorphic loci observed in the six populations were: 61.54% (A Luoi), 58.24% (Phong Dien), 57.14% (Phu Vang), 39.56% (Phu Loc), 63.74% (Quang Dien), and 47.25% (Huong Thuy). Data for observed and effective number of alleles, Nei's (1973) genetic diversity, and Shannon's information index, for all the six populations were 1.55, 1.30, 0.18 and 0.27, respectively. The value for total genotype diversity among populations was 0.2239 while within populations diversity was found to be 0.1769. The mean coefficient of gene differentiation value and the estimate of gene flow across the populations were found as 0.2100 and 1.8812, respectively. The Nei (1978) measures of genetic distance and identity between pairs of loach populations indicate thatthe population originated from Phong Dien and Quang Dien has the highest genetic

identity, while the fish originated from Phu Vang and A Luoi showed the greatest genetic distance.

Keywords: genetic diversity, loach, *Misgurnus anguillicaudatus*, RAPD, Vietnam

INTRODUCTION

The loach, *Misgurnus anguillicaudatus* (Teleostei: Cpriniformes: Cobitidae) (**Figure 1**), is a small-sized freshwater, subtropical species native to Southeast Asia. The species was described from Chusan Island, China. The species is considered native to Cambodia, China including Hong Kong, India, Japan and Thailand, Korea, Laos, Myanmar, the Russian Federation, Taiwan, and Vietnam[27]. *M. anguillicaudatus* inhabits in the bottom of the streams, ponds, ditches, estuaries, swamps, rice paddy fields, lakes, giving preference to lentic environments[9, 10, 12, 34].

It is an important commerical food fish, a common live bait fish and also popular as an aquarium fish and introduced in the United States, Germany, Italy, Spain, Palau, Indonesia, Australia, Turkmenistan and Philippines[10], Hawaii[9], Brazil[11], Netherlands and Northern Africa[13]. On the other hand, *M. anguillicaudatus* is one of the most important cultured fish in East Asia, and a promising model animal to study evolutionary biology for polyploidy[36]. It has long been employed as a traditional Chinese medicine for the treatment of many illnesses such as hepatitis, osteomyelitis, carbuncles, inflammations and cancers, as well as for recovery from fatigue[33].

In Vietnam, they are distributed from Northern provinces to Southern Central provinces and Tay Nguyen area[12]. Due to its great food value (tender meat, delicious, nutrient-rich) and high medicinal value, local people in Vietnam prefer to have it for their meals by different ways of cooking: loach soup, fried loach, caramel loach, etc. Study on artificial reproduction of *M. anguillicaudatus* had been carried out[8].

The development of genetic marker technologies have revolutionized the way aquaculture genetics research is conducted. A popular genetic marker in the aquaculture community is Randomly Amplified Polymorphic DNA (RAPD)[16]. RAPD is a simple, easy technique for population genetic studies. RAPD markers have been widely used for analysis of genetic diversity of various aquatic organisms: Malaysian river catfish[7]; silver crucian carp[37]; cupped oysters[15]; fishes *Pimelodus maculatus*, *Prochilodus lineatus*, *Salminus brasiliensis* and *Steindachneridion scripta*[25]; Indian coldwater fishes[28]; tilapia[4, 5]; common tiger prawn[23]; giant freshwater prawn[29]; freshwater mud eel[18]; ornamental gold fish[24];ornamental fish *Badis badis*[20]; fishes *Labeo rohita, Catla catla and Cirrhina mrigala*[21];square head climb perch[32]; loach *M. anguillicaudatus*[1, 6, 19, 35], and other marine and freshwater fishes such as striped red mullet (*Mullus surmuletus* L.), Atlantic coastal striped bass (*Morone saxatilis*), freshwater crayfish (*Austropotamobius pallipes*), Asian arowana (*Scleropages formosus*), etc.[1].

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[2, 16].

The objective of this study is to determine genetic diversity of the loach in Vietnam, for which no data are available at present.

MATERIAL AND METHODS

Sample collection: Ninety-six individual loaches were captured from A Luoi district (n=16), Phong Dien district (n=16), Phu Vang district (n=16), Phu Loc district (n=16), Quang Dien district (n=16), and Huong Thuy district (n=16) in Thua Thien Hue province, Vietnam **(Table 1** and **Figure 2).** 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
A Luoi	16	AL1, AL2, AL3, AL4, AL5, AL6, AL7, AL8, AL9, AL10, AL11, AL12, AL13, AL14, AL15, AL16
Phong Dien	16	PD1, PD2, PD3, PD4, PD5, PD6, PD7, PD8, PD9, PD10, PD11, PD12, PD13, PD14, PD15, PD16
Phu Vang	16	PV1, PV2, PV3, PV4, PV5, PV6, PV7, PV8, PV9, PV10, PV11, PV12, PV13, PV14, PV15, PV16
Phu Loc	16	PL1, PL2, PL3, PL4, PL5, PL6, PL7, PL8, PL9, PL10, PL11, PL12, PL13, PL14, PL15, PL16
Quang Dien	16	QD1, QD2, QD3, QD4, QD5, QD6, QD7, QD8, QD9, QD10, QD11, QD12, QD13, QD14, QD15, QD16
Huong Thuy	16	HT1, HT2, HT3, HT4, HT5, HT6, HT7, HT8, HT9, HT10, HT11, HT12, HT13, HT14, HT15, HT16

Figure 1: Loach *Misgurnus anguillicaudatus* (Cantor, 1842)

Extraction of genomic DNA: Genomic DNA was isolated from fish muscle using modified phenol-chloroform protocol[26]. 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: Six random primers: OPA02, TGCCGAGCTG; OPA04, AATCGGGCTG; OPA09, GGGTAACGCC; OPC05, GATGACCGCC; OPC06, GAACGGACTC; OPC08, TGGACCGGTG **(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.
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[1].
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.

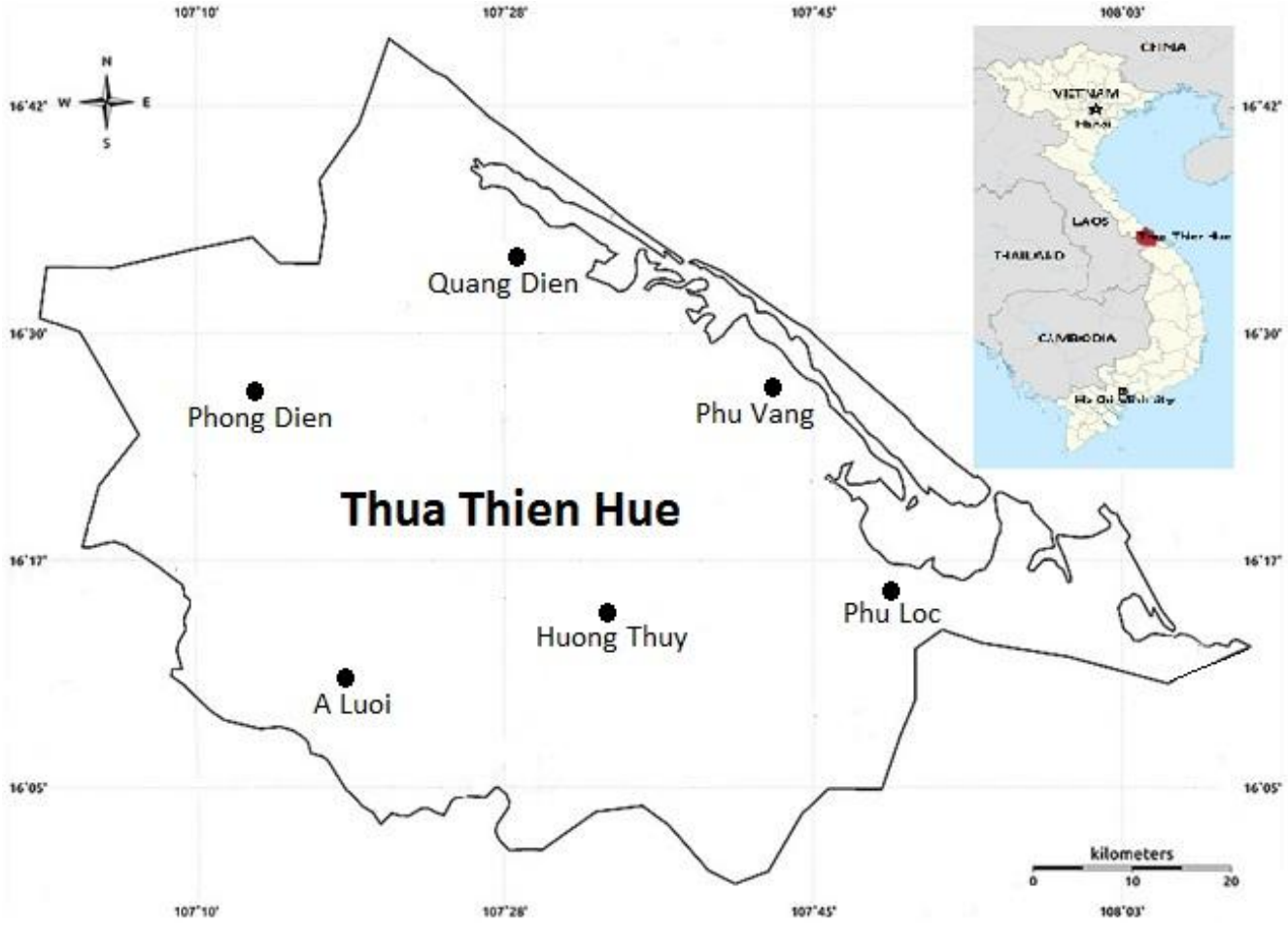

Figure 2: Map of sampling localities for specimens *M. anguillicaudatus* used in this study. Detailed information and voucher codes of locality are shown in Table 1.

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 (1978) genetic distance[22].
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

Table 2: Sequence of RAPD primer, sizes, and number of amplified bands, and percentage of polymorphic bands based on RAPD analysis in six populations of loach *M. anguillicaudatus* in ThuaThien Hue, Vietnam

Primer code	Nucleotide sequence (5'-3')	Size range of amplified bands (bp)	Number of amplified bands	Number of polymorphic bands	Percentage of polymorphic bands
OPA02	TGCCGAGCTG	315-1643	13	11	84.61
OPA04	AATCGGGCTG	278-2533	21	21	100
OPA09	GGGTAACGCC	339-2276	14	14	100
OPC05	GATGACCGCC	225-2249	14	13	92.86
OPC06	GAACGGACTC	322-2674	15	15	100
OPC08	TGGACCGGTG	262-1568	14	14	100
Overall		225-2674	91	88	96.70
Mean			15.17	14.67	96.25

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)[31]. 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 loach populations.

RESULTS AND DISCUSSION

Percentage of polymorphic bands of loach *M. anguillicaudatus*: A series of discrete bands were obtained after amplification of DNA samples of all six populations of loach *M. anguillicaudatus* with six random primers (OPA02, OPA04, OPA09, OPC05, OPC06, and OPC08). The different primers produced different banding patterns. The number of reproducible bands across all investigated samples was 13, 21, 14, 14, 15, and 14 bands for primers OPA02, OPA04, OPA09, OPC05, OPC06, and OPC08, respectively. The largest number of RAPD bands were detected for primer OPA04 (21 bands), while the lowest number was scored for primer OPA02 (13 bands). A total of 91 amplified bands were consistentely generated; out of which, 88 bands were polymorphic, with an average number of bands and average number of polymorphic bands per primer was 15.17 and 14.67, respectively. Size of these amplified bands ranged from 225 bp to 2674 bp. Highest size range was exhibited for OPC06 (322 bp-2674 bp) while it was lowest for OPC08 (262 bp-1568 bp). Percentage of polymorphic bands ranged from 84.61% (OPA02) to a maximum of 100% (OPA04, OPA09, OPC06 and OPC08) with an average of 96.25% polymorphism (**Table 2**). The result shows that high polymorphic levels of these primers in loach *M. anguillicaudatus* in Thua Thien Hue, Vietnam. This was higher to result from study on genetic diversity of loach *M. anguillicaudatus* from the Poyang lake, the biggest fresh water lake in China (59.56%)[35].

Four primers (OPA02, OPC08, OPA04, and OPA09) generated RAPD bands exhibiting fixed frequencies in at least one population. Every primer produced a population-specific marker: OPA02 and OPC08 in Phu Vang population (1447 bp and 400 bp, respectively), OPA04 in Huong Thuy population (1109 bp),

and OPA09 in Phong Dien population (339 bp) **(Table 3).** No population-specific bands were found in Phu Loc, Quang Dien, and A Luoi.

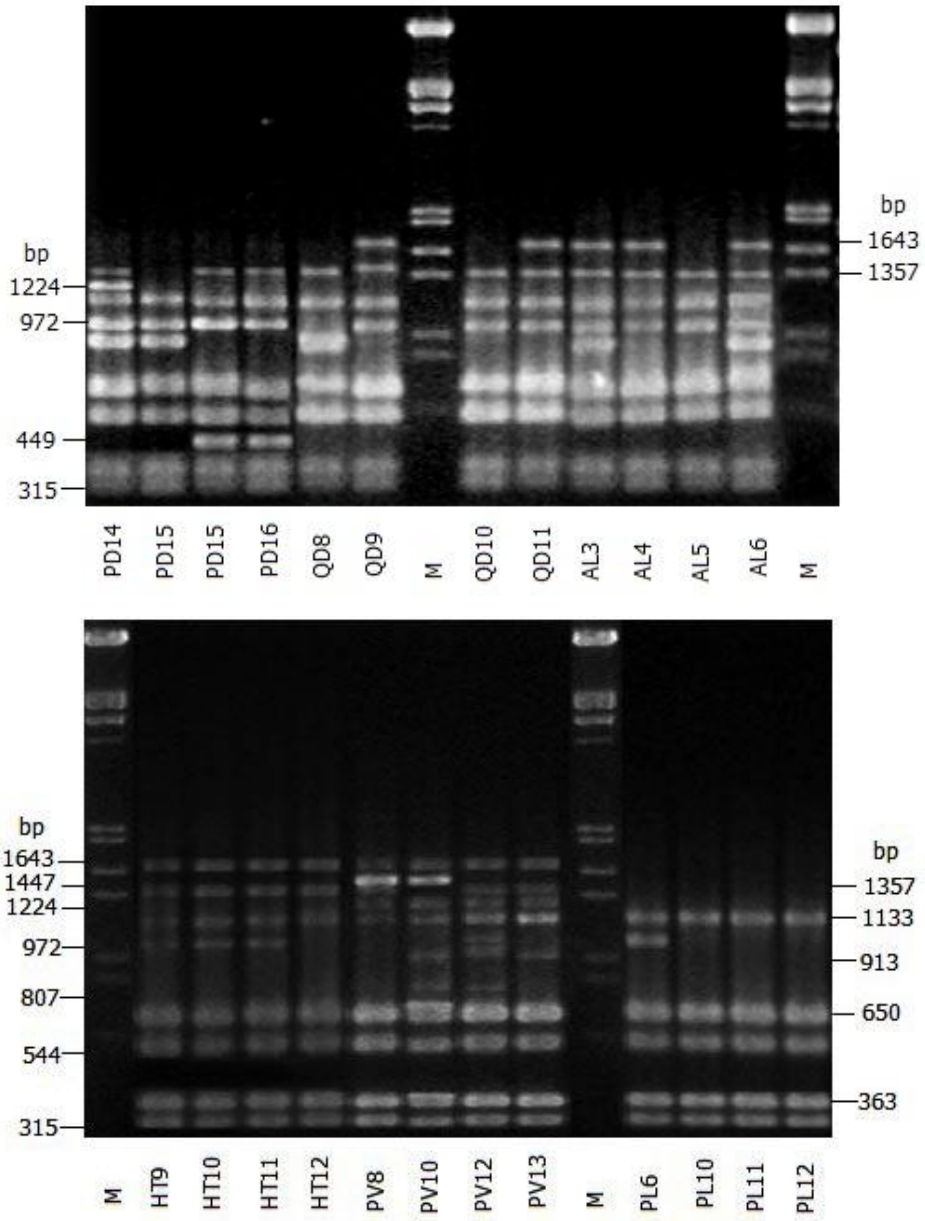

Figure 3: RAPD pattern generated by amplification of *M. anguillicaudatus* DNA with OPA02 primer. M: Molecular weight marker (Phage Lambda DNA *Eco*RI/*Hind*III, Fermentas), AL: A Luoi, HT: Huong Thuy, PD: Phong Dien, PL: Phu Loc, PV: Phu Vang, QD: Quang Dien.

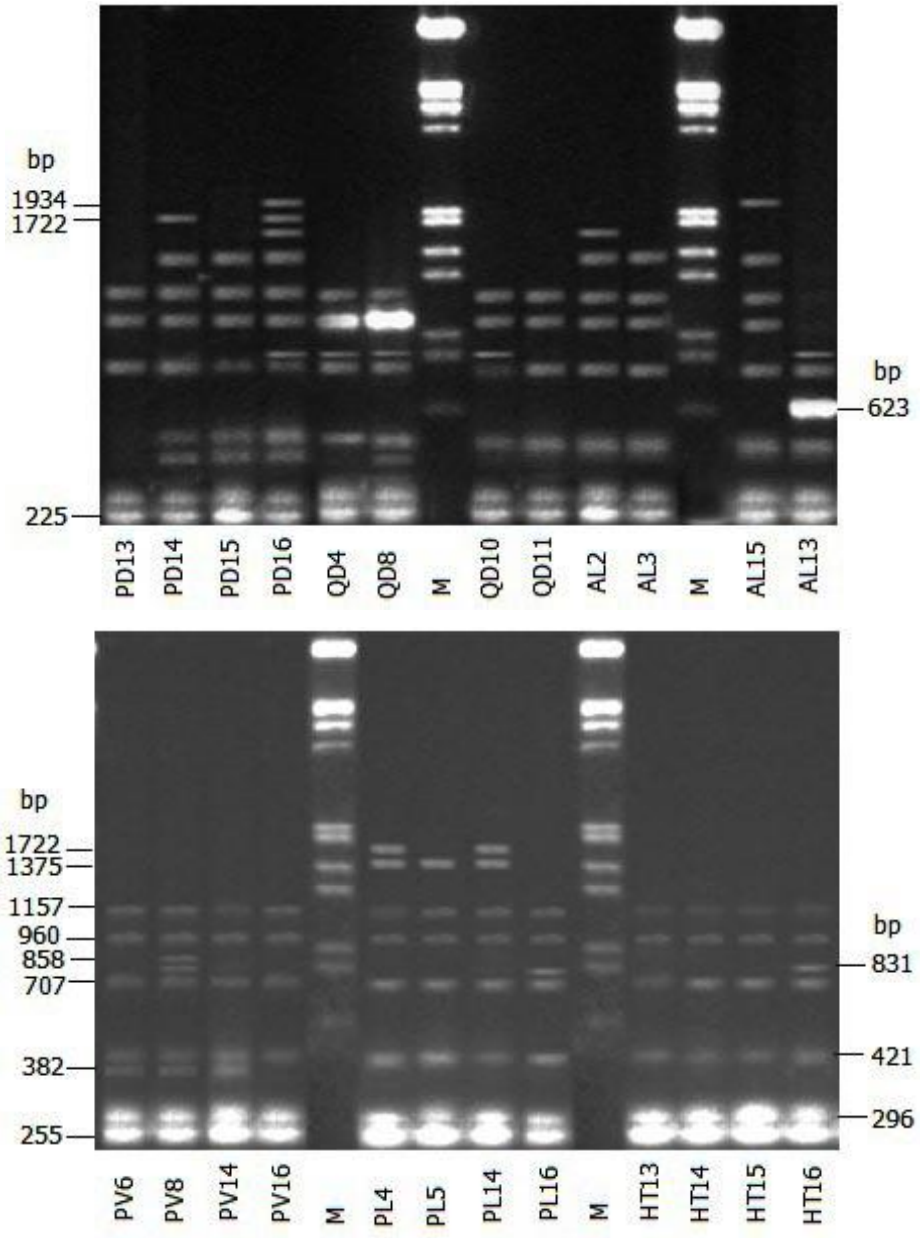

Figure 4: RAPD pattern generated by amplification of *M. anguillicaudatus* DNA with OPC05 primer. M: Molecular weight marker (Phage Lambda DNA *Eco*RI/*Hind*III, Fermentas), AL: A Luoi, HT: Huong Thuy, PD: Phong Dien, PL: Phu Loc, PV: Phu Vang, QD: Quang Dien.

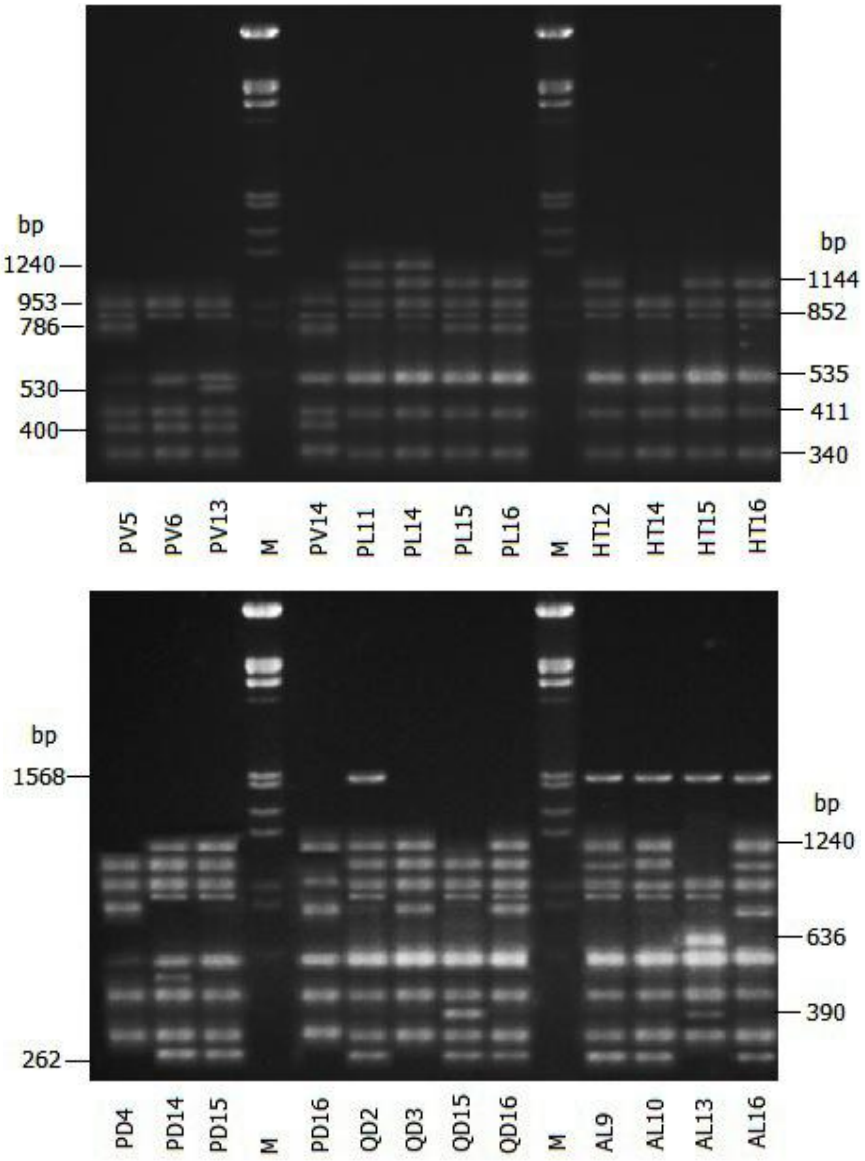

Figure 5: RAPD pattern generated by amplification of *M. anguillicaudatus* DNA with OPC08 primer. M: Molecular weight marker (Phage Lambda DNA *Eco*RI/*Hind*III, Fermentas), AL: A Luoi, HT: Huong Thuy, PD: Phong Dien, PL: Phu Loc, PV: Phu Vang, QD: Quang Dien.

Table 3: Population-specific markers of loach *M. anguillicaudatus* in ThuaThien Hue, Vietnam by RAPD technique

Primer	Population	RAPD marker (bp)
OPA02	Phu Vang	1447
OPC08	Phu Vang	400
OPA04	Huong Thuy	1109
OPA09	Phong Dien	339

Table 4: Number of polymorphic loci and percentage of polymorphic loci in six populations of loach *M. anguillicaudatus* in ThuaThien Hue, Vietnam

Population	Number of polymorphic loci	Percentage of polymorphic loci
Phu Vang	52	57.14
Phu Loc	36	39.56
Huong Thuy	43	47.25
Phong Dien	53	58.24
Quang Dien	58	63.74
A Luoi	56	61.54

The arevage percentage of polymorphic loci of each loach population was ranged from 39.56% to 63.74% **(Table 4).** Quang Dien and A Luoi population exhibited greater levels of polymorphic loci than did Phong Dien, Phu Vang, Huong Thuy and Phu Loc population. This result is lower than that of populations from Ueda city (70.0%), Nasu town (83.3%), Nikko city (69.3%), and Futtsu town (73.6%) in Japan, analysed using RAPD[1]; and Kita village in Japan (78.26%), analysed using microsatellites[3]. Mendel *et al* (2008) studied genetic diversity of *Misgurnus fossilis* populations from the Czech Republic and Slovakia, using microsatellite analysis, and obtained the percentage of polymorphic loci of the population was lower, 31.25% (5 polymorphic loci/16 loci tested)[17]. The average number of alleles per locus in the loach *M. anguillicaudatus* population from Memanbetsu town and that from Kita village in Japan were 2.60 (60 alleles/23 loci) and 2.13 (49 alleles/23 loci), respectively; with average observed heterozygosities (H_O) of around 0.05-0.80, and expected heterozygosities (H_E) of around 0.05-0.85, analysed by microsatellite markers[3]. In the RFLP analysis of loaches (*M. anguillicaudatus*), Khan *et al* (2005) determined genetic variations and relationships among eleven populations (Memanbetsu town, Hashima city, Izumo city, Tomari village, Yuya town, Naruko town, Saito city, Nikko city, Udea city, Fittsu town, and Nasu town). Ten haplotypes were detected using restriction enzymes (*Alu* I, *Hinc* II, *Msp* I, *EcoR* I, *Hinf* I, *Hae* III and *Taq* I). The haplotypic and nucleotide diversities within populations varied from 0 to 0.889 and 0 to 22.222%, respectively. The average nucleotide diversity among eleven populations varied from 0 to 15.255% with a mean of 6.272% and net nucleotide divergence varied from 0 to 15.255% with a mean of 5.312%[14].

Genetic diversity and genetic relationships of loach *M. anguillicaudatus*: Genetic diversity of loach populationswas clearly illustrated in **Table 5** and **Table 6**. 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 six populations were analysed using six RAPD markers and their respective values were found as 1.55, 1.30, 0.18 and 0.27, respectively **(Table 5)**. The value for total genotype diversity among populations (Ht) was 0.2239 and within population diversity (Hs) was found to be 0.1769. The mean coefficient of gene differentiation value (Gst) and the estimate of gene flow across the populations (Nm) was found as 0.2100 and 1.8812, respectively **(Table 6).**

Table 5: Summary of genetic parameters estimate for six populations of loach *M. anguillicaudatus* in Thua Thien Hue, Vietnam using RAPD markers

Population	na	ne	h	I
Phu Vang	1.57	1.34	0.19	0.29
Phu Loc	1.40	1.22	0.13	0.20
Huong Thuy	1.47	1.24	0.14	0.22
Phong Dien	1.58	1.31	0.18	0.27
Quang Dien	1.64	1.36	0.21	0.32
A Luoi	1.62	1.35	0.20	0.30
Mean	1.55	1.30	0.18	0.27

Table 6: Summary analysis of genetic variability across all six populations of loach *M. anguillicaudatus*

Parameter	Ht	Hs	Gst	Nm
Across six populations	0.2239	0.1769	0.2100	1.8812

Using Nei's (1978) genetic distance approach, the values of genetic distance and genetic identity between six loach populations from Phu Vang, Phu Loc, Huong Thuy, Phong Dien, Quang Dien, and A Luoi were calculated and given in **Table 7.** The analysed data indicate that the values of genetic identity between populations were high, ranging from 0.8958 to 0.9557. Genetic identity between Phong Dien and Quang Dien, Huong Thuy and Quang Dien, Huong Thuy and Phu Loc, Huong Thuy and Phu Vang, and A Luoi and Quang Dien were higher than those of Huong Thuy and Phong Dien, Phu Vang and Quang Dien, A Luoi and Huong Thuy, Phu Loc and Phong Dien, A Luoi and Phu Loc, Phu Vang and Phong Dien, A Luoi and Phong Dien, Phu Loc and Phu Vang, and A Luoi and Phu Vang. The populations originate from Phong Dien and Quang Dien had the highest genetic identity (0.9557). These high values of identity might be reflecting the low levels of genetic variability showed by different loach populations analysed here.

Table 7: Nei's (1978) genetic distance (below diagonal) and genetic identity (above diagonal) between six populations of loach *M. anguillicaudatus* in Thua Thien Hue, Vietnam

Population	Phu Vang	Phu Loc	Huong Thuy	Phong Dien	Quang Dien	A Luoi
Phu Vang	-	0.9016	0.9488	0.9186	0.9342	0.8958
Phu Loc	0.1035	-	0.9492	0.9285	0.9444	0.9211
Huong Thuy	0.0525	0.0521	-	0.9398	0.9523	0.9336
Phong Dien	0.0849	0.0742	0.0621	-	0.9557	0.9110
Quang Dien	0.0681	0.0572	0.0488	0.0453	-	0.9425
A Luoi	0.1101	0.0822	0.0688	0.0932	0.0593	-

The values of genetic distance between populations were low, varying from 0.0488 to 0.1101 and the population pairs Phu Vang-A Luoi have the greatest genetic distance (0.1101) (**Table 7**). The greatest genetic distance exists between populations that has the most geographically distant and vice versa. Quang Dien was closely related to Phong Dien whereas Phu Vang and A Luoi were more distant. A Luoi

population and Phu Vang population have been adapted in their respective geographical conditions, which may cause wider genetic variability between them. A Luoi is a mountainous region in the West of Vietnam that borders on Laos and Phu Vang is coastal plain region in the East that borders on South China Sea. These results indicate geographical distance is an important factor influencing the genetic relationship of populations[30].

The dendrogram constructed on the basis of comparative analysis of the total loci obtained with the six RAPD primers across the six loach populations, presented two clusters: five populations from A Luoi, Quang Dien, Phong Dien, Huong Thuy, and Phu Loc in one cluster and the Phu Vang population in the other cluster (**Figure 6**).

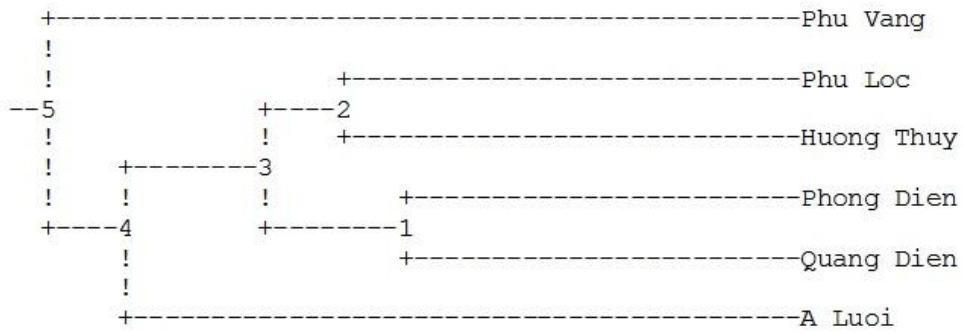

Figure 6: Dendrogram obtained with UPGMA method based on Nei's (1978) distance for six populations of loach *M. anguillicaudatus* in ThuaThien Hue, Vietnam.

CONCLUSION

The observations generated using RAPD markers revealed the high polymorphic levels and the genetic relationships of six populations of loach *M. anguillicaudatus* in Vietnam.

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