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ASSESSMENT OF GENETIC DIVERSITY BY RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) AND ATPASE 6/8 GENE IN *Pangasiuspangasius*

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ABSTRACT

The aim of the present study was to use RAPD and ATPase markers to measure and analyze the level of genetic variation within farm cultured populations of introduced Pangas catfish *Pangasius pangasius* to evaluate their potential in ascertaining genetic differentiation. Hence, genetic diversity of farmed *P.pangasius* was carried out from five different sites by using RAPD primer. Primers showed 151 total amplified locus with an average of 37.75 loci. The highest number of bands were observed in *P.pangasius* from population 1, maximum no of bands was observed in OPA-02 (56 bands), while the minimum from OPA-05 (26 bands). Minimum polymorphism was observed in OPA-08 (10.71%), while maximum in OPA-01 (66.67%). The value of heterozygosity of *P. pangasius* ranged from 0.123 to 0.444.A cluster between population 1 and 2, 2nd population 3 and 4, and population 5 with all other as 3rd cluster. Distance found maximum in population 5 that showed heterozygosity. The genetic variations and polymorphism among populations showed the degree of intra and inter-population genetic diversity in *P. pangasius* populations. Study is also done to access the genetic diversity by *ATPase 6/8* gene. ATPase 6/8 showed highest haplotype diversity (0.800) in *the P. pangasius* population of Tariq Khan Faheem Fish Farm, while the lowest (0.700) in Al-Raheem fish Farm. Highest nucleotide diversity (0.5703) in Tariq Khan Faheem Fish Farm, while lowest (0.5026) in Mirza Rizwan Ahmed Fish farm. Higher diversity is important for conservation and management of population.Random amplified polymorphic DNA (RAPD) and ATPase 6/8 markers were used to estimate the genetic diversity and population structure among the samples of farmed *P. pangasius*. Overall, RAPD and ATPase 6/8 proved as valuable tool for reliable and quick stock discrimination and provided information that might be useful regarding management and conservation of *P. pangasius*. The findings will be helpful in developing stock specific management measures for conservation and sustainable utilization of the species.

Key words: Genetic diversity, ATPase 6/8, RAPD, Fish, *P. pangasius*

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INTRODUCTION

Fish are the most diversified vertebrate group (with 33,000 species), occupying nearly every major aquatic habitat type and performing a wide range of ecological activities in ecosystems (Helfman *et al*., 2009). Fish, on the other hand, are more exposed to changed environmental conditions and disturbances as a result of human activities. Overfishing, habitat destruction, pollution, and the introduction of non-native species have all contributed to a decline in marine and freshwater fish biodiversity around the world (Pauly and Zeller, 2016). Methods for detecting and evaluating genetic diversity have evolved in recent years, ranging from morphological trait analysis to biochemical and molecular research. Molecular markers, in particular are essential for the efficient conservation and protection of genetic resources since they allow for the rapid and reliable identification of genetic diversity (Jarvis *et al*., 2000). The technical simplicity, efficiency of the assay, little DNA needs, and low assay cost of RAPD markers make them ideal for detecting genetic diversity. Furthermore, no prior knowledge of the sequence being studied is required. In diversity analysis, RAPD markers have proven to be effective (Surgun *et al*., 2012).Genetic diversity is one parameter that may help estimate which species are more likely to adapt to future situations. In general, genetic diversity refers to any measure that quantifies variation in alternate forms of genes or non-coding locations within a population (Hughes, 2008). Both heterozygosity (the percentage of diploid individuals with two distinct alleles at a single location) and the total number of alleles present in a population might affect a population's ability to adapt and evolve (Frankham *et al*., 2014).Understanding how fish genetic diversity varies across species can help predict which species will adapt to future disturbances while also identifying species that are at risk of extinction (Stockwell *et al*., 2003).

The efficient tool for genetic diversity among population by markers are proved the best due to their unique characteristics, such as pattern of genetic variation (Goios and Alvarez, 2013). Mitochondrial ATPase genes broadly used to analyze the interspecific and intraspecific genetic variation and diversity in fish species (Sarkar *et al*., 2006).

The aim of the present study was to use RAPD and ATPase markers to measure and analyze the level of genetic variation within farmed *Pangasius pangasius* from Pakistan to evaluate their potential in ascertaining genetic differentiation.

MATERIALS AND METHODS

Sample collection and Specie Identification: 50 fish specimens were collected from different fish farms as; Mirza Rizwan Ahmed Fish farm chokiNarool, Tehsil Kabir Wala (Site 1); Tariq Khan Faheem Fish Farm MouzaMumbhal, Kabirwala (Site 2); Nursery unit, Fisheries Department Muzaffargarh (Site 3); Tawakal Fish Hatchery Muzaffargarh (Site 4) and Al Raheem fish Farm Muzaffargarh (Site 5), during September, 2020. Specimens were frozen and transported to the fisheries laboratory, Institute of Zoology, Bahauddin Zakariya University, Multan, Pakistan. Samples of fish muscle tissue were taken, preserved in 95 percent ethanol, and stored at 4°C for later use. Formalin was used to preserve voucher specimens.

DNA Isolation, PCR amplification of RAPD primer and ATPase 6/8: 20 mg of tissue have been cut and collected for DNA extraction. Gently homogenized with a 500 µl of TNES solution (200 mMTris, 100 mM EDTA, 250mMNaCl). Then ten µl of Proteinase K was added and incubated at 56 °C for two hours. Phenol, chloroform, and isoamyl alcohol with a ratio of 25:24:1 were added and centrifuged at 13000 rpm for 10 min. Then its supernatant was collected and transferred to a new MCT. Added the same volume of chloroform: isoamyl alcohol and centrifuged at 13000 rpm for 10 min. The supernatant was collected in another micro-centrifuge tube and added chilled ethanol. Put MCT at 20°C overnight for precipitation. Then centrifuged at 5000 rpm for 10-15 min. DNA pellet was visualized at the tube base. Now added 100µl of 70% ethanol. Centrifuged again for at 5000 rpm 15 min.

Seven random primers,OPA-01, OPA-02, OPA-03, OPA-04, OPA-05, OPA-08 and OPA-09 (Table 1) were used in this study for polymorphism. For PCR reaction, total volume was 25µl (DNA template 1.5µl; PCR Master Mix 12.5µl;Primer 0.3µland 10.8µl nuclease-free water). Conditions for PCR amplification are as follows: Initial denaturation was at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 32°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 5 min. PCR amplified products were run on electrophoresis with 1.5% (w/v) agarose gel and saved by Gel documentation. Different amplified bands were scored accordingly with ladder size. Primer (ATP.2L8331) were used in ATPase 6/8 with denaturation at 94°C for 3min; followed by 33 cycles of denaturation at 94°C for 45sec, annealing at 54°C for 30sec, and elongation at 72°C for 1min respectively in thermal cycler (Eppendorf AG 22331 Hamburg, Germany).

Sequence Alignment and Genetic Diversity Analysis: Genetic diversity was accessed by POPGENE version 1.31 (Yeh *et al*., 1999); FSTAT version 2.9.3.2 (Goudet, 2002). A dendrogram based on the unweighted pair group method with arithmetic average (UPGMA) was used to analyze the genetic distance (Nei and Li, 1979). Haplotype/nucleotide diversity and pairwise distancing were determined using DnaSP 5.1.

RESULTS

In this study amplification of the RAPD primer was performed by using seven different primers OPA-01, OPA-02, OPA-03, OPA-04, OPA-05, OPA-8, and OPA-9. Successful amplification was in OPA-01, 02, 05, and OPA-8, while the remaining primer were unable to amplify because of Mismatch between primer and template region may result in total absence of PCR product. Condition of thermal cycler may also influence the results of PCR products. 1st line of genotype was observed from the individuals of Fish Farm Choki Narool, Kabir Wala (Population 1), 2nd line from Faheem Fish Farm, Mumbhal. Kabirwala (Population 2), 3rd line from Nursery Unit Fisheries Department Muzaffargarh (Population 3), 4th line from Tawakal Fish Hatchery Muzaffargarh (Population 4), and 5th from Al Raheem Fish Farm Muzaffargarh (Population 5). The product of primer amplified was scored manually after run on an agarose gel. Primers showed 151 total amplified locus with an average of 37.75 loci as shown in Table 2; Figure 1.The highest number of bands were observed in *P. pangasius* from population 1, maximum no of bands was observed in OPA-02 (56 bands), while the minimum from OPA-05 (26 bands).

Polymorphism: Recorded percentage of polymorphism were OPA-01 (66.67%)>OPA-05(61.53%)>OPA-05(55.35%)>OPA-08 (10.71%), indicating minimum polymorphism in primer OPA-08 while maximum in primer OPA-01. In OPA-01, highest polymorphism was observed for Population 1 (76.92%), followed by OPA-02 in Population 4 (61.35%), OPA-05 (71.42%) and OPA-08 in Population (16.67%),while lowest polymorphism in Population 3 (40%), OPA-02 in Population 3 (44.44%), OPA-05 in Population 3,5 (50%) and OPA-08 in Population 3,4 (0%). (Table 3). Genetic

variation from the farm population of *P. pangasius* culture from five different populations from five sites 1st from the individual of Fish farm choki Narool, Kabirwala (Population 1), 2nd from Faheem fish farm, Mumbhal. Kabirwala (Population 2), 3rd from Nursery unit Fisheries Department Muzaffargarh (Population 3), 4th from Tawakal Fish Hatchery Muzaffargarh (Population 4), and 5th from Al Raheem fish Farm Muzaffargarh (Population 5) showed variation decline due to the environmental condition/pollution, and human based activities. Allele frequency among each locus in a population of *P. pangasius* from five different localities showed null alleles with a value of 1 in fish from P1, P2, P3 P4, and P5 (Table 4). The heterozygosity value of *P. pangasius* ranged from 0.123 to 0.444 (Table 5), and the phylogenetic showed a tree cluster (Figure 2). 1st cluster between population 1 and 2, 2nd population 3 and 4 and population 5 with all other as 3rd cluster. Distance found maximum in population 5 that showed heterozygosity.

Genetic diversity by *ATPase 6/8* gene:A total of twenty five *P. pangasius* specimens (five from each population group) were barcoded successfully by *ATPase 6/8* gene in the present study. The barcoded sequences were submitted to the GenBank database. BLAST analysis of all *P. pangasius* sequences provided maximum similarity matches with *P. pangasius*. Mitochondrial *ATPase 6/8* gene highest haplotype diversity (Hd) was found 0.800 in *the Pangasius pangasius* population of Tariq Khan Faheem Fish Farm while the lowest haplotype diversity was found 0.700 in the *P. pangasius* population of both Mirza Rizwan Ahmed Fish farm and Al Raheem fish Farm. *ATPase 6/8* gene highest nucleotide diversity (π) 0.5703 was found in *P. pangasius* population of Tariq Khan Faheem Fish Farm while lowest nucleotide diversity was found 0.5026 in *P. pangasius* of Mirza Rizwan Ahmed Fish farm. Haplotype analysis provided a total number of 15 haplotypes for *ATPase 6/8* (304 bp). An average of 280 variable sites were detected for *ATPase 6/8* genes. The average G+C content was observed 0.413 for *the ATPase 6/8* gene (Table 6).

Demographic history and neutrality tests: Tajima's D negative significant values for *ATPase 6/8* gene were shown for all farm populations. *ATPase 6/8* gene, Fu's Fs values were also observed significant for all populations of *P. pangasius*. Tajima's D and Fu's Fs positive significant values were observed among all farm populations. *ATPase 6/8* gene, Tajima's D, and Fu's Fs values are provided in Table 6. *ATPase 6/8* gene, Fu's Fs values were also observed significant for all populations of *P. pangasius*. Tajima's D and Fu's Fs positive significant values were observed among all farm populations. *ATPase* gene intraspecific pairwise genetic distance ranging from 0.00133% to 0.00650% was observed among all populations. The Raggedness index (r) ranged from 0.4700 to 0.5900 for all populations. A summary of pairwise intraspecific genetic distance among the population of *P. pangasius* from five farms is provided in Table 7.

Table 1: Specification of RAPD primer for PCR amplification in *Pangasius pangasius*

Locus	Sequence	G+C (%)	Tm ©
OPA-01	5'-CAGGCCCTTC -3'	60	32
OPA-02	5'-TGCCGAGCTG-3'	60	32
OPA-03	5'-AGTCAGCCAC-3'	60	32
OPA-04	5'- AATCGGGCTG- 3'	60	32
OPA-05	5'-AGGGGTCTTG- 3'	60	32
OPA 8	5'-GTGACGTAGG-3'	60	34
OPA 9	5'-GGGTAACGCC-3'	60	34

Table 2: RAPD amplified Polymorphic and Monomorphic bands of *Pangasius pangasius*

Locus	No. of polymorphic bands	No. of monomorphic bands
OPA-01	30	15
OPA-02	31	25
OPA-05	16	10
OPA-08	3	25
Total	85	75

Table 3: Number of polymorphic bands, amplified fragments, and percentage of polymorphisms by PCR

Primers	Banding Pattern	Populations					Total No. of Bands
		P1	P2	P3	P4	P5	
OPA-01	Poly	10	8	02	03	07	30
	Mono	03	03	03	03	03	15
	% age	76.92%	72.72%	40%	50%	70%	61.92%
OPA-02	Poly	07	07	04	08	05	31
	Mono	05	05	05	05	05	25
	% age	58.33%	58.33%	44.44%	61.53%	50%	54.52%
OPA-05	Poly	03	05	02	04	02	16
	Mono	02	02	02	02	02	10
	% age	60%	71.42%	50%	66.66%	50%	59.62%
OPA-08	Poly	01	01	00	00	01	03
	Mono	05	05	05	05	05	25
	% age	16.67%	16.67%	0%	0%	16.67%	10.002%
Average		42.38%	54.78%	33.6%	44.54%	46.66%	

Table 4: Allele frequencies of five populations of *Pangasius pangasius*

Locus Name	Populations									
	P1		P2		P3		P4		P5	
	Observe Allele	Frequency	Observe Allele	Frequency	Observe Allele	Frequency	Observe Allele	Frequency	Observe Allele	Frequency
OPA-01	50	0.610	52	0.624	52	0.650	54	0.690	50	0.700
	10	0.390	8	0.376	8	0.350	6	0.310	10	0.300
OPA-02	40	0.600	51	0.629	46	0.661	52	0.694	47	0.720
	20	0.400	9	0.371	14	0.339	8	0.306	13	0.280
OPA-03	45	0.620	53	0.620	52	0.655	55	0.670	45	0.670
	15	0.380	7	0.380	8	0.345	5	0.330	15	0.330
OPA-04	51	0.630	55	0.644	53	0.630	56	0.660	55	0.680
	9	0.370	5	0.356	7	0.370	4	0.340	5	0.320
Null allele	1		1		1		1		1	

Table 5: Heterozyosity values among five population of *Pangasius pangasius*

Locus Name	Populations									
	P1		P2		P3		P4		P5	
	Het.	Het. Frq	Het.	Het. Frq	Het.	Het. Frq	Het.	Het. Frq	Het.	Het. Frq
OPA-01	13.31	0.423	12.27	0.422	13.34	0.421	11.73	0.412	11.23	0.425
	13.31	0.423	12.27	0.422	13.34	0.421	11.73	0.412	11.23	0.425
OPA-02	14.22	0.423	12.33	0.434	12.45	0.444	12.36	0.431	14.22	0.423
	14.22	0.423	12.33	0.434	12.45	0.444	12.36	0.431	14.22	0.423
OPA-03	13.10	0.410	11.18	0.411	11.19	0.412	14.14	0.427	13.10	0.420
	13.10	0.410	11.18	0.411	11.19	0.412	14.14	0.427	13.10	0.420
OPA-04	4.44	0.143	13.43	0.432	9.34	0.344	10.92	0.421	4.22	0.123
	4.44	0.143	13.43	0.432	9.34	0.344	10.92	0.421	4.22	0.123

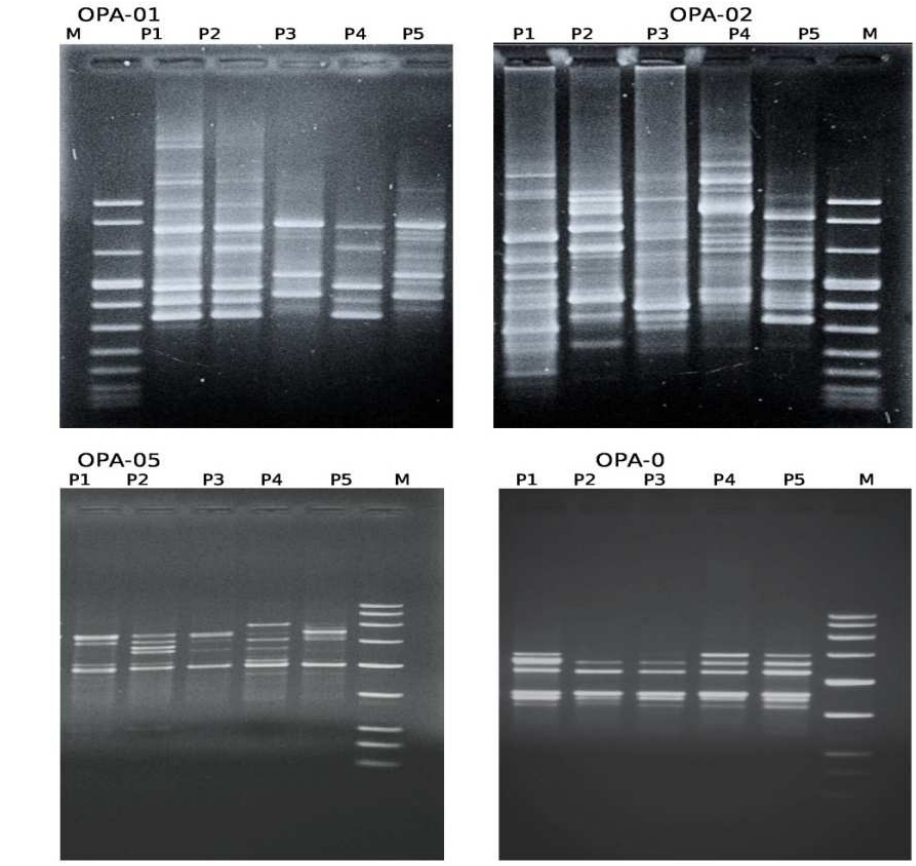
Table 6.Genetic diversity analysis using *ATPase* gene of *Pangasius pangasius* among populations of among five fish farms

Population	N	H	Hd	A variance of Hd ±SD	Pi±SD	S	Eta (s)	G+C content	Tajima's D	Fu's Fs	Raggedness index (r)
Mirza Rizwan Ahmed Fish farm	5	3	0.700	0.04768±0.218	0.5026±0.1564	277	375	0.397	-1.15443	-0.89598	0.4700
Tariq Khan Faheem Fish Farm	5	3	0.800	0.02688±0.164	0.5703±0.1142	274	368	0.401	-0.14012	0.55646	0.5200
Nursery unit Fisheries	5	3	0.730	0.04768±0.219	0.5217±0.1616	280	394	0.400	-1.23299	-0.94277	0.4700
Tawakal Fish Hatchery Muzaffargarh	5	3	0.710	0.04768±0.217	0.5286±0.1626	287	390	0.422	-1.08154	-0.81266	0.4700
Al Raheem fish Farm	5	3	0.700	0.04768±0.218	0.5167±0.1622	285	392	0.445	-1.26120	-0.99627	0.5900

N, Number of the specimen; H, Number of Haplotypes; Hd, Haplotype (gene) diversity: SD, Standard Deviation of Haplotype diversity; Pi, Nucleotide diversity; S, Number of variable sites; Eta(s) Total number of singleton mutations

Table 7. *ATPase 6/8* gene pairwise genetic distance among five fish farms *Pangasius pangasius*

Mirza Rizwan Ahmed Fish farm genetic distance%			Tariq Khan Faheem Fish Farm genetic distance%			Nursery unit Fisheries genetic distance%			Tawakal Fish Hatchery Muzaffargarh genetic distance%			Al Raheem fish Farm genetic distance%		
Min. %	Max. %	Mean	Min. %	Max. %	Mean	Min. %	Max. %	Mean	Min. %	Max. %	Mean	Min. %	Max.%	Mean
0.00139	0.00650	0.00394	0.00148	0.00573	0.00361	0.00136	0.00627	0.00382	0.00133	0.00618	0.00376	0.00138	0.00633	0.00385



M= Lagger (100 bp)
P1= Fish farm choki Narool, Kabir Wala
P2= Faheem fish farm, Mumbhal. Kabirwala
P3= Nursery unit Fisheries Department, Muzaffargarh
P4= Tawakal Fish Hatchery, Muzaffargarh
P5= Al-Raheem fish Farm Muzaffargarh

Figure 1: Random Amplified Polymorphic DNA pattern of *Pangasius pangasius* amplified by different primer from different population samples

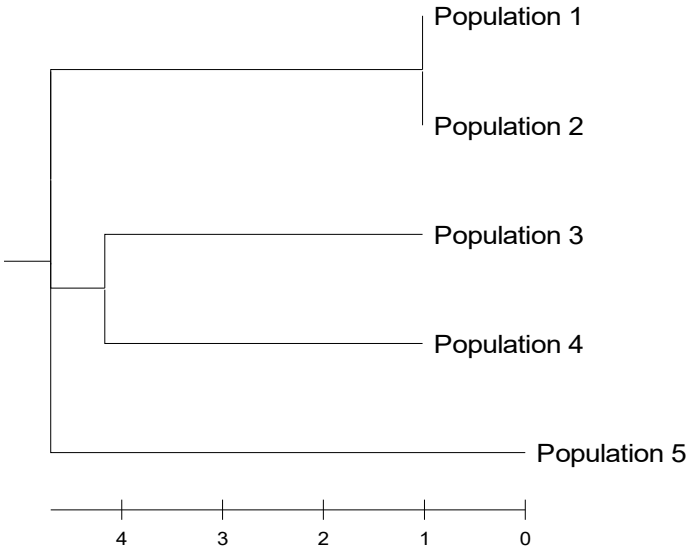


Figure 2: UPGMA dendrogram for genetic distancing among different populations

DISCUSSION

The amplification patterns of the RAPD marker were studied among the different populations of *P. pangasius* in this study. The number and amount of fragments produced are largely determined by the sequence of nucleotides. So as a result the primer and DNA template source results in the uniqueness of the randomized DNA fragments (Welsh *et al*., 1990). *Oreochromis niloticus* specimen's isoenzyme analysis revealed genetic variability within the same region and river (Kohlmann and Kersten, 1999). OPA-08 and OPA-01 found the minimum and maximum polymorphisms. The polymorphism was found higher than the proportion of RAPD analysis by Li and Chu-Wu (2006).

In tilapia, Basavaraju *et al*. (2007) found 57.1 percent polymorphism; Basavaraju *et al.* (2014) studied genetic diversity in *Labeo fimbriatus* using 8 RAPD primers and found polymorphic bands. We found the most genetic variety collected from P2 and the least in P3, indicating that P2 has more heterozygous genotypes in it. Chandra *et al*. (2010) reported comparable findings as well. Ji *et al*. (2014) investigated genetic distance in five *Megalobrama amblycephala* populations and found genetic variance. P3 has the highest heterozygosity in *P. pangasius*. The fish population obtained from four distinct places had a modest amount of genetic variation (Gopalakrishnan *et al*., 2009). These findings are contradicted from Kohlmann and Kersten (1999) findings, which stated that fish stocks fluctuate in variety.

Similar studies by using different RAPD markers in *Prochilodusmarggravii* (Hatanaka and Galletti, 2003) and *Silurisasotus* Korean catfish (Yoon and Kim, 2001) revealed significant difference between populations, which were attributed to varying habitat situations from different sampling sites and, as a result, ecological isolation. In damsels of the Pomacentridae family, such isolations resulted in high rates of inbreeding within populations (Tamang *et al.,* 2012).UPGMA cluster indicates that fish collected from Populations 1 and 2 showed resemblance, similarly in population 3 with 4 that showed distinction from other samples. In a group of three different stocks, Basavaraju *et al.* (2014) discovered two clusters. In the stock of common carp, by Bartfai *et al*. (2003) found no grouping. Due to increased amounts of domestic and industrial pollution, genetic variety. The tilapia's ability to survive habitat degradation caused by anthropogenic activities may be harmed when genetic variety is lost (Milligan *et al*., 1994). *H. nemurus* and *H. macrolepidota* showed similar findings of a closer genetic relationship between freshwater fish species from Borneo and mainland Asia in their biogeography history (Ryan and Esa, 2006). A similar grouping of populations of the migratory fish *B. lundii* was discovered, with 100% band sharing across unisolated populations and 27.3 percent band sharing with the recently separated population (sanches *et al.,* 2012).

Genetic diversity among different populations may used for conservation purposes (Jamsari *et al.,* 2011). Present study data is valuable contribution for accessing genetic diversity among different population of *P. pangasius*. Haplotype diversity was found higher than nucleotide diversity by *ATPase 6/8* gene among all populations, as genetic diversity considered high if value of haplotype is greater than 0.5 (Jamsari *et al.,* 2011). High haplotypic diversity indicates demographic expansion in population size (Sanger *et al*., 1997). In present study haplotype diversity found greater than 0.5 that indicates higher haplotype diversity among different population of *P. pangasius.*

Evolutionary relationships of haplotypes showed diverse clusters which indicated the five genetically diversified genetic stocks in present study (Thompson *et al*., 1994). Neutrality test was used to measure the demographic events which are considered sensitive (Tamura *et al.,* 2011). The Fu's Fs results showed the population expansion with specific patterns of diversity in these farm population.

Genetic variation from the farm population of *P. pangasius* culture from five different populations showed variation decline due to the environmental condition/pollution, and various human based activities. Changes in the environmental conditions and pollution have also degraded biotic potentials of the important freshwater bodies. This has accelerated the rate of loss of freshwater biodiversity (Irshad *et al*.,2008).

In fishes, the degree of genetic diversity is related with its habitat. Population genetic size may effected by habitat destruction and genetic drift which in effectively reduce gene pool and genetic diversity (Excoffier *et al.*, 2010). Populations with low degree of genetic diversity can be at risk of reduction in number of species. This may be due to result of genetic drift in the small population sizes (Böhme, 2004). *ATPase 6/8* gene, Fu's Fs values were also observed significant for all populations of *Pangasius pangasius*. Tajima's D and Fu's Fs positive significant values were observed among all farm populations that indicates a genetic diversity among different farmed population of *P. pangasius.*

Conclusion: RAPD results can be drawn that the genetic diversity existed among the population of *P. pangasius* from different sites through polymorphism. However, sensitive techniques like microsatellite, RAPD,RFLP, mini satellite, and other different sequencing techniques may be used for analyzing genetic analysis. The study suggested that higher concentration reduces growth performance, increases body lesion, mortality, disturbance of various hematological indices, reduction in protein level and increases the concentration of various metals of *P. pangasius*. Besides the traditional way to

identify species, the molecular approach is a precise and effective approach for specie identification. Genetic diversity among various populations of *P.pangasius* was also reported in the present research work by RAPD and *ATPase 6/8* gene analysis. In present study reveals high degree of genetic diversity in farmed different population of *P.pangasius,* as it is important for management and conservation of fish species.

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Author's contributions statement: The authors declare that they have contributed equally to the article.

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