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Original Article

Genetic variation between hybrid (*Labeo rohita* ♂, *Cirrhinus mrigala* ♀ and *Labeo rohita*) by RAPD marker

Variação genética entre híbridos (*Labeo rohita* ♂, *Cirrhinus mrigala* ♀ e *Labeo rohita*) a partir do marcador RAPD

M. Butt[a], S. Perveen[b,c,d*], F. Rasool[e], A. Ayub[f], K. Ali[g] A. Sajjad[h], M. F. Nasir[i], S. Kanwal[j], E. Muzammil[k], I. Chaudhary[l] and A. S. Magsi[m]

[a]Chung-Ang University, Department of Life Science, Seoul, Republic of Korea
[b]Institute of Ocean Engineering, Ningbo University, Ningbo, China
[c]Ministry of Education, Key Laboratory of Applied Marine Biotechnology, Ningbo, China
[d]Ningbo University, School of Marine Sciences, State Key Laboratory for Managing Biotic and Chemical Threats to the Quality and Safety of Agro-Products, Ningbo, China
[e]University of Education, Department of Zoology, Faisalabad Campus, Lahore, Pakistan
[f]University of Agriculture Faisalabad, Depalpur, Okara, Pakistan
[g]University of Education, Department of Zoology, Vehari Campus, Lahore, Pakistan
[h]Quaid-I-Azam University, Department of Environmental Sciences, Islamabad, Pakistan
[i]University of Education, Department of Zoology, Division of Science & Technology, Lahore, Punjab, Pakistan
[j]University of Okara, Department of Zoology, Punjab, Pakistan
[k]Shaheed Benazir Bhutto University of Veterinary and Animal Sciences, Department of Poultry Production, Sakrand, Pakistan
[l]University of Veterinary and Animal Sciences, Department of Aquaculture and Fisheries, Lahore, Pakistan
[m]Shaheed Benazir Bhutto University of Veterinary and Animal Sciences, Faculty of Animal Production and Technology, Department of Dairy Technology, Sakrand, Pakistan

Abstract

The present work was to study the genetic variability between the major carps *Labeo rohita* and *Cirrhinus mrigala* and their hybrids of *L. rohita* (male♂) and *C. mrigala* (female♀). Genetic variability was studied by employing RAPD molecular markers. 25 samples of each target species having different sizes with the same age group for the determination of interspecific variation were collected. The morphometric parameters such as body weight, total length, tail length, and lengths of dorsal and anal fins of each individual were recorded and results showed that wet body weight, total length, dorsal fin, anal fin, and tail fin length are positively correlated and then the DNA was extracted using the inorganic salt-based method and conformed by Gel electrophoresis. Twenty-four arbitrary decamer primers were used to get species-specific RAPD analysis Distinct and highly reproducible RAPD profiles with significant genetic variability was detected among species. Only five primers showed amplification. The RAPAD primer OPB-05 produced a total of seven bands out of these 5 monomorphic and 2 polymorphic, so in this case, the percentage polymorphism was 28.57%. The Hybrid show more than a 50% difference from the *Labeo rohita.* This shows that the Hybrid more resembles *C.mrigala*. Phylogenetic analysis demonstrated that hybrid (*L. rohita* ♂ X *Cirrhinus mrigala* ♀) is the closest to *C. mrigala* and the farthest from *L. rohita.* Overall data are presented concerning the applications of RAPD markers for hybrid identification, genetic diversity assessment, and studying taxonomic relationships at a molecular level.

Keywords: genetic variability, hybrids, *Labeo rohita*, RAPD.

Resumo

O presente trabalho teve como objetivo estudar a variabilidade genética entre as carpas maiores *Labeo rohita* e *Cirrhinus mrigala* e seus híbridos de *L. rohita* (machos) e *C. mrigala* (fêmeas). A variabilidade genética foi estudada empregando marcadores moleculares RAPD. 25 amostras de cada espécie-alvo com tamanhos diferentes e com a mesma faixa etária foram coletadas para a determinação da variação interespecífica. Os parâmetros morfométricos como peso corporal, comprimento total, comprimento da cauda e comprimento das nadadeiras dorsal e anal de cada indivíduo foram registrados. O DNA foi extraído através do método à base de sal inorgânico e conformado por eletroforese em gel. 24 primers decâmeros arbitrários foram usados para obter a análise RAPD espécie-específica. Perfis RAPD distintos e altamente reprodutíveis com significativa variabilidade genética foram detectados entre as espécies. Apenas 5 primers apresentaram amplificação. O primer RAPAD OPB-05 produziu um total de 7 bandas,

*e-mail: perveen@nbu.edu.cn
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dessas, 5 monomórficas e 2 polimórficas, portanto, neste caso, o percentual de polimorfismo foi de 28,57%. O *Hybrid* mostrou mais de 50% de diferença do *Labeo rohita*. Isso mostra que o híbrido se parece mais com o *C.mrigala*. A análise filogenética demonstrou que o híbrido (*L. rohita* macho X *Cirrhinus mrigala* fêmea) é o mais próximo de *C. mrigala* e o mais distante de *L. rohita*. Foram apresentados dados relativos à aplicação de marcadores RAPD para identificação de híbridos, avaliação de diversidade genética e estudo de relações taxonômicas ao nível molecular.

Palavras-chave: variabilidade genética, híbridos, *Labeo rohita*, RAPD.

1. Introduction

Aquaculture and fisheries are amongst the most important growing areas (in the food sector) as compared with all the remaining food-related areas (FAO, 2009). Most of the freshwater aquaculture is dominated by three major carps, Rohu *Labeo rohita* Hamilton 1822: Cypriniformes, and Cyprinidae) (FAO, 2019). Among these, (due to its preference by the farmers because of the higher growth rate and increasing demand in the market), rohu is the most important and hence preferred by the consumers (Gjerde et al., 2019). Rohu is an aquaculture spetcies in countries like Pakistan, India, Bangladesh, and Burma. (Rasal and Sundaray, 2020). Thus, this specie holds a prominent position in the Bangladesh and Pakistani aquaculture industry because of its unique taste and fast-growing nature (Alam et al., 2002) and is considered among the top ten aquaculture species globally (Abbasi et al., 2013). It is a dire need time to launch some good managemental practices like, morphological identification, conservation, and assessment of genetic variability (Hussain et al., 2021). Recently rohu has been developed (genetically) to compete with the increasing food demand (Gjerde et al., 2019). However, limited literature is available in Pakistan, as these freshwater species have to face numerous anthropogenic and environmental threats which are reducing the population of these fishes (Gaston et al., 2000). The highest probability of inbreeding is due to using a small number of parents as broodstock for the selection and breeding of related individuals. Unintentionally, the decrease of genetic variability may present in farmed stocks due to aquaculture practices. To minimize the harms, maintain natural levels of genetic diversity, and genetic integrity, and enhance species' genetic diversity, genetic resource conservation is necessary (Rajora and Mosseler, 2001). When environmental conditions have altered for the survival of the species' populations, genetic diversity is essential because it offers the raw material for evolution and adoption. Hybridization is used to transfer desirable characteristics e.g. disease resistance from one group or species to another, combine valuable traits from two species into a single group, e.g., good growth and flesh quality, and produce sterile individuals. In hybridization, they may also involve crosses within a species (also known as line crossing or strain crossing) or crosses between different species, and the mating of genetically differentiated individuals or groups is held. Aquaculturists used this breeding technique to produce aquatic organisms with a general improvement in performance. They produced offspring that performed better than both parental species (hybrid vigor or positive heterosis). The hybrids of Indian major carps are fertile, creating characters in-between parental lines. The most exciting combinations are the strong rohu x Catla and the combined fast growth of Catla with the desirable head shape of rohu. Catla x fringe-lipped peninsula carp hybrid has the desirable head and body shape, improved dressing percentage, and growth rate similar to Catla (Reddy, 2000). At the molecular level, the valuation of genetic variations is constructed upon Information by various sensitive molecular techniques such as Restriction Fragment Length Polymorphism (RFLP), Randomly Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeats (SSR), etc. Random amplified polymorphic DNA (RAPD) is the first introduced technique by Williams et al. (1990).

In providing essential applications in fish, one of the most frequently used molecular methods RAPD technique, is the better for taxonomic and systematic analyses of various organisms (Bartish et al., 2000). The molecular approach by RAPD is very beneficial for providing useful information about species management and genetic conservation of several freshwaters and marine fish including, (*Brycon cephalus*) (Wasko et al., 2004). For individual and pedigree identification, trait improvement in genetics, breeding programs, and pathogenic diagnostics markers that were shown to link to a trait of interest genetically could be used (Holsinger et al., 2002). As the (RAPD) technique no preliminary work such as nucleotide sequencing is required, uses universal sets of primers, and probe isolation is necessary, it has an advantage over other systems of genetic documentation. (Martins et al., 2003). Since the 1990's, polymerase Chain Reaction (PCR) at the molecular level is widely used to assess intra specific genetic variations generated by RAPD markers. RAPD is a technique in which discrete regions of genome amplification on the PCR with short oligonucleotide primers of arbitrary sequence. In this process, several random, short primers (8-12 nucleotides) are used, which proceeds with the PCR using a large template of genomic DNA. A semi-unique profile can be collected from an RAPD reaction by resolving the resulting patterns (Sevindik et al., 2019).

The potential use of RAPD, including aquaculture, population genetics, and genetic mapping, has been broadly documented. For long-term fisheries management, the comprehension of genetic differences of native fish populations is essential Information derived from molecular genetic techniques. It contributes significantly to the sustainable development and preservation of aquatic lives and genetic resources.The results indicated that RAPD could be successfully used for genetic diversity analysis in marine species of prospective value. It is rapid, reliable, and superior to those based on pedigree informatio (Martins et al., 2003).

In Pakistan, the hybrids of Cyprinids are available in the natural waters as a result of in-breeding and on the manufactured hatcheries the hybrids of *L. rohita* (male♂)

and *C. mrigala* (female♀) have been produced with some levels of future viability. The genetic difference studies among the pure and hybrid species on the molecular level are still awaited for the contribution of the fisheries population and molecular geneticists. The present research on genetic variability between *L. rohita* and Hybrid by RAPD technique is a pilot project to proceed further to the phylogenetic studies of the stocks of Cyprinids.

2. Material and Methods

2.1. Experimental species

25-samples of each target species *Labeo rohita*, Hybrid (*L. rohita* ♂ X *Cirrhinus mrigala* ♀) having different sizes with the same age group for the determination of interspecific variation and recording morphometric data were collected from the fish farm, Pattoki, Pakistan. After recording the morphometric parameters and collection of tissue for DNA extraction was stored at -80°C in the Genetics lab of the Department of Fisheries and Aquaculture, UVAS, Ravi Campus, Pattoki, Pakistan.

2.2. Morphometric parameters

Data regarding each individual's morphogenetic parameters viz., body weight, total length, tail length, and lengths of dorsal and anal fins were recorded (Rasool et al., 2013).

2.3. Genomic DNA extraction

Total genomic DNA isolation was carried out from the stored fish samples using the procedure described by (Sevindik et al., 2019) which was modified by NaCl. In this procedure, lysis buffer was used, which carried 50mM tris, which was taken from a stock of 1 M pH: 8 tris buffer, 50mM EDTA taken from a store of 0.5 M pH: 8, 100mM NaCl taken from a stock of 5 M NaCl and 1% SDS. Working lysis buffer was prepared from this lysis buffer by adding 7µL of 200µgmL-1 proteinase K. Stock solution of the proteinase K was prepared by preparing the buffer of 100mM Tris-base, 50mM EDTA, 500mM NaCl, and then Proteinase K was added and dissolved at 200µgmL^{-1}. About 1g of the fish flesh was taken in a 1.5 ml microfuge tube and homogenized in the 550 µL lysis buffer, then 7µL of proteinase K buffer was added to the sample containing Eppendorf microtube. After this, the contents of the tube were incubated in the thermoregulated water bath at 500°C for 12h. After this incubation, 5M NaCl amounting to 600 µL solutions was added and mixed thoroughly and then centrifuged for 10 min at 12000 rpm. A fresh Eppendorf microtube was taken, and the supernatant was transferred into it with the help of a micropipette. Then the DNA was precipitated by adding 700 µL absolute cold ethanol. After mixing the contents of the tube, it was incubated at -20°C for 2 h. The tube was then centrifuged for 10 min at 12000rpm to obtain the pellet of the DNA. All the liquid was discarded, and 300 µL of 70% ethanol was added to remove salts. Then this washing with 70% ethanol was repeated, and the pellet was dried by inverting the tube on dry tissue paper. Air-dried pellet of the DNA was dissolved in 200 µL distilled water.

2.4. DNA confirmation and quantification

For the assessment of the quality of the DNA samples, all the samples were sequestered on 1% agarose gel prepared in 0.5X TAE buffer which was obtained from 50X TAE stock solution prepared by dissolving 121 gm tris base and 28.6 mL glacial acetic acid and 0.5 M EDTA in water and raising its volume to 500 mL. The DNA samples were loaded into the gel after mixing with DNA loading buffer with 0.21% bromophenol blue, 0.21% xylene cyanol FF, 0.2M EDTA, and 50% glycerol. Following RAPD, decamer primers were selected for polymorphic analysis and were synthesized by (micro gen- Humanizing Genomics) (Table 1).

2.5. PCR amplification of the Random Sequences from the fish samples

Polymerase chain reactions were devised with the help of the primers . Each reaction was performed in a 0.2 mL PCR tube and 25 µL reaction mixtures. To prepare this 25 µL reaction mix 2.5 µL 10x PCR buffer, 2 µL 1.6 mM

Table 1. Primer sequences for polymorphic analysis.

Sr. No.	PRIMER NAME	SEQUENCE
1	OPB-01	GTTTCGCTCC
2	OPB-02	TGATCCCTGG
3	OPB-03	CATCCCCCTG
4	OPB-04	GGACTGGAGT
5	OPB-05	TGCGCCCTTC
6	OPB-06	TGCTCTGCCC
7	OPB-07	GGTGACGCAG
8	OPB-08	GTCCACACGG
9	OPB-09	TGGGGGACTC
10	OPB-10	CTGCTGGGAC
11	R1	AGGCCCCTGC
12	R2	ATGCCCCTGT
13	R7	ACCGCCGAAG
14	P5	GAATGCGACG
15	P8	GGGCTAGGGT
16	P9	ACCGGGAACG
17	P10	AGCAGGTGGA
18	P7	CTGAGGAGTG
19	RAN1	GATGACCGCC
20	RAN3	GGCACGTAAC
21	RAN4	GGCATGACCT
22	RAN5	GGGTAACGCC
23	RAN9	GTGCCAAATG
24	RAN11	GTGCCCGTTA

MgCl2, 2µL 10 nM primer, 2 µL 2.5 mM dNTPs, 0.3 µL 5 units/µL Taq polymerase enzyme and 11.2 µL sterilized deionized double distilled water was mixed (Chart 1). Negative control was also run using sterilized water as the template in each reaction. The PCR reaction was carried out in a thermal cycler (BIO-RAD T100). For RAPD primer following conditions were used: One cycle of 5-minute denaturation at 95°C and then 35 cycles of 1 minute at 95°C, 1 min at 37 °C for annealing and 2 min at 72°C for extension, and finally 20 min final extension time was given at 72°C. Then the machine was allowed to hold the reaction contents at 22°C. All the PCR products were analyzed by running them on a 1.8% agarose gel. Agarose gel was prepared in TAE buffer as described in the section (Quantification of DNA). The DNA samples were then loaded on \the gel using the DNA loading buffer. 100 base pair DNA ladder was also loaded in the left and right lanes as a size standard. These gels were visualized in UV light and the image was captured and saved on the computer for further polymorphic analysis by the gel documentation system (UVDI- 254/365-220).

2.6. Statistical analysis

The data for the morphometric parameters were subjected to Pearson correlation analysis by using SAS statistical package version 15.1.1.

3. Results

3.1. Morphometric parameters

The data regarding the morphometric of the species under study viz., *Labeo rohita* and Hybrid and molecular analysis for variability between the populations were subject to statistical analysis (Equations 1 and 2). The outcome/results of the study are as follows. Fish individuals of the same groups are similar regarding morphological characteristics such as body weight, total body length, Dorsal fin length, tail fin length, and anal fin length compared to each other and exhibited distinct variations in body weight in the morphological characteristics.

$$R = Labeo\ rohita \quad (1)$$

Chart 1. PCR recipe.

INGREDIENTS	QUANTITY
Buffer	2.5 µL
Mgcl2	2.0 µL
Primers	2.0 µL
dNTPs	2.0 µL
Taq A polymerase	0.3 µL
DDW	11.2 µL
DNA	5.0µL
Total	25.0µL

$$H = Hybrid\ (Labeo\ rohita♂ \times Cirrhinus\ mrigala♀) \quad (2)$$

A total of 25 samples of the Hybrid (*L. rohita* ♂and *C. mrigala* ♀) with different age groups were collected. The maximum and minimum values of wet body weight, total length, dorsal fin length, tail fin length, and anal fin length of Hybrid were 15.24 g and 24.48 g, 17cm and 13.5cm, 3.5cm and 2cm, 3.5 cm, and 2.5 cm, 2.8 cm, and 1.5 cm respectively. The data for the morphometric parameters were subjected to Pearson correlation analysis using SAS statistical package version 15.1.1. The correlation results showed that wet body weight, total length, dorsal fin length, tail fin length, and anal fin length were positively correlated among species' other morphometric parameters. The P- values were highly significant in wet body weight and the total length, whereas non-significant in tail fin length and dorsal fin length. A total of 25-samples of the *L. rohita* were collected. For the morphometric parameters of wet body weight, total length, dorsal fin length, and tail fin length the higher and lower values were 24.48g and 52.12g, 13.5cm and 12 cm, 2.5 cm and 1.5 cm, 3.5 cm and correlated with total body weight and tail fin length. Whereas negatively correlates with dorsal fin length. The correlation results showed that the dorsal fin length of *L. rohita* was negatively correlated with wet body weight and total body length. Whereas positively correlates with tail fin length. The correlation results showed that the dorsal fin length of *L. rohita* was positively correlated with wet weight total body length and anal fin length. Wet body, total length-weight, dorsal fin length, and tail fin length had non-significant differences among the same morphometric considerations (Tables 2 and 3).

Table 2. Correlation Matrix the Morphometric Parameters of Hybrid.

VARIABLE	WEIGHT	LENGTH	TAIL FIN	DORSAL FIN
	p=<.0001			
TAIL FIN	r=0.59436	r=0.65936		
	p=0.0017	p=0.0003		
DORSAL FIN	r=0.10735	r=0.05562	r=0.35841	
	p=0.6095	p=0.7917	p=0.0785	
ANAL FIN	r=0.54903	r=0.52592	r=0.58877	**R=0.18191**
	P=0.0045	**P=0.0069**	**P=0.0020**	**P=0.3841**

Table 3. Correlation Matrix the Morphometric Parameters of *Labeo rohita*.

VARIABLE	WEIGHT	LENGTH	TAIL FIN
LENGTH	r = 0.20834		
	p = 0.3176		
TAIL FIN	r = 0.36605	r = 0.25521	
	p=0.0719	p=0.2182	
DORSAL FIN	r = -0.31720	r = -0.12435	**R = 0.06819**
	P=0.1223	**P=0.5537**	**P=0.7460**

3.2. Molecular analysis

The results showed that in row 1, samples 1, 2, and 3, and row 2, samples 3, and 4 had a small quantity of DNA as indicated in figure 1. So the samples were repeated. DNA was confirmed from *L. rohita,* and hybrid samples were set as 25 µL reaction in PCR thermal cycler with 10mer micro gen RAPD primers. In 0.2ml PCR tubes, the recipe for the PCR (Chart 1)

The five primers which show the amplification are listed in Chart 2.

The PCR product of the DNA samples was loaded into the gel after mixing with 10X DNA loading buffer with 0.21% bromophenol blue, 0.21% xylene cyanol FF, 0.2 M EDTA, and 50% glycerol. Only the samples of *L. rohita* show amplification, whereas no sample of Hybrid show amplification against any primer. The selected representative picture of the gel electrophoresis after PCR is given in Figure 3.

The primers were species-specific and out of 24 primers, only 5 showed results against samples of *L. rohita*. At the same time, the samples of Hybrid showed no amplification as shown in figure 2. The documentation of the gel of *L. rohita* showed that R7 produced a whole six bands out of these five monomorphic and 1-polymorphic bands in nature, so in this way, the percentage polymorphism remained at 16.67%. The P9 amplified five bands out of these 3 monomorphic and 2 polymorphic bands in nature, so in this way, the percentage polymorphism remained at 40.00%. The P10 produced six bands out of these 5 monomorphic and 1 polymorphic band in nature, so the percentage polymorphism remained at 16.67%. The OPB-06 amplified five numbers of bands out of these four monomorphic and one polymorphic band in nature, so in this way, the % polymorphism remained at 20.00%. The RAPAD primer OPB-05 produced a total of seven bands out of these 5monomorphic and 2 polymorphic, so in this case, the percentage polymorphism was 28.57%. The Hybrid show more than a 50% difference from the *Labeo rohita.* This shows that the Hybrid more resembles *C.mrigala*.

4. Discussion

Nowadays worldwide and in Pakistan, the hybrids of Cyprinids *L. rohita* (male♂) and *C. mrigala* (female♀) have been produced with some levels of future viability. These hybrids show tremendous growth as samples of Hybrid (*L. rohita* ♂ and *C. mrigala* ♀) which were captured from the Pattoki fish hatchery were studied.

Chart 2. Primers with their GC percentage.

PRIMERS	SEQUENCE	GC%
R7	ACCGCCGAAG	70%
P9	ACCGGGAACG	70%
P10	AGCAGGTGGA	60%
OPB-06	TGCTCTGCCC	70%
OPB-05	TGCGCCCTTC	70%

4.1. Morphometric parameters

The data of morphometric parameters were analyzed and analysis of morphometric parameters showed that wet body weight, total length, Dorsal fin, anal fin, and tail fin length are positively correlatedand have a p-value (P<.0001) highly significant. These results are in agreement with the previous studies done by in which the correlation between fish body weight and fork length was highly significant (P<0.001) and positive with all the parameters Rasool et al. (2013) and Naeem et al. (2010). Dorsal fin

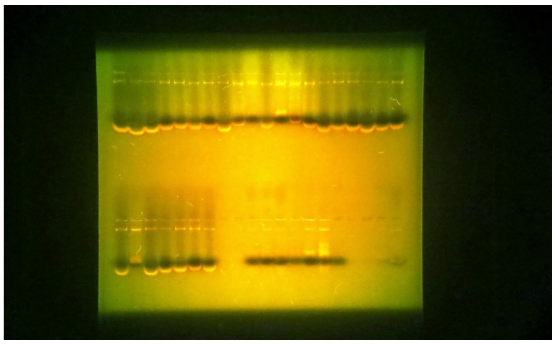

Figure 1. DNA conformation of the sample of Hybrid in Row 1 (R1-R20) and in Row 2 (R1- R7).

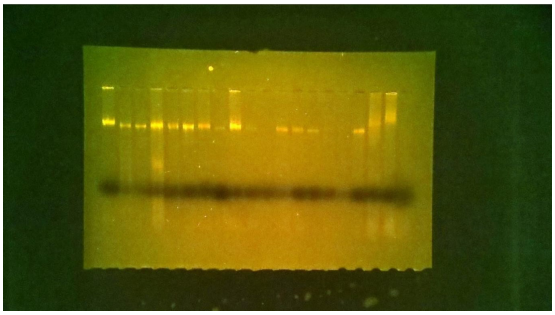

Figure 2. DNA conformation of *Labeo rohita.*

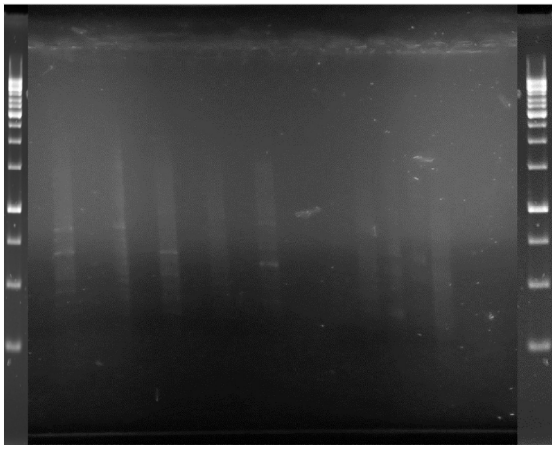

Figure 3. PCR amplification for the samples of *Labeo rohita.*

length and anal fin have non-significant difference and positively correlates with each other and other parameters. These outcomes are conflicting with the study of done by Rasool et al. (2013) anal fin length was significantly different (P<0.05), and all the remaining parameters were highly significantly different (P<0.01) among the sites.. The morphometric parameters of L. rohita were observed and results showed that the wet weight, total length, tail fin length, dorsal fin length, and anal fin were non-significantly different (P>0.05). Wet body weight positively correlates with total length, dorsal fin, anal fin, and tail fin length. Total body length also positively correlates with wet body weight and tail fin length whereas negatively correlates with dorsal fin agreeing with the effects as postulated by Rasool et al. (2013). According to their results the average length of paired pectoral fins, total length, and body weight of *L. rohita* were non-significantly different (P>0.05). Tail fin length positively correlates with wet total length and body weight whereas negatively correlates with a dorsal fin. Dorsal fin positively correlates with tail fin length whereas negatively correlates with wet body weight tail fin length and total length but contradictory in the case of anal fin length which shows a significantly different (P<0.05).

4.2. PCR amplification of the Random Sequences

For molecular analysis of *L. rohita* and Hybrid samples was run in PCR with 24 different primers but only 5 shows amplification against *L. rohita* whereas Hybrid showed no amplification. This result shows that RAPD markers are species-specific aggress with the study of (Mizukami et al., 1996; Zhang and Reddy, 1991).

In the present study, the documentation of the gel of *L. rohita* showed that R7 produced whole six bands out of these 5 monomorphic and 1- polymorphic bands in nature, so in this way, the percentage polymorphism remained at 16.67%. The P9 amplified five bands out of these 3monomorphic and 2 polymorphic bands in nature, so in this way, the percentage polymorphism remained at 40.00%. The P10 produced six bands out of these 5 monomorphic and 1 polymorphic band in nature, so in this way, the percentage polymorphism remained at 16.67%. The OPB-06 amplified five numbers of bands out of these 4 monomorphic and 1 polymorphic band in nature, so the percentage polymorphism remained at 20.00%. The RAPD primer OPB-05 produced a total of seven bands out of these 5monomorphic and 2 polymorphic, so in this case percentage polymorphism was 28.57%. The inquiry of randomly amplified polymorphic DNA (RAPD) of 25 primers; 21 primers produced 230 RAPD bands. The percentage of polymorphic bands in Burullus (24%) and Manzella (29.4%) populations was low compared with Assuit (30.54%), Cairo (33.5%), and Qena (44.84%) populations. The molecular phylogenetic tree constructed by unweighting pair-group analysis shows Manzalla and Burullus populations strongly linked and separate from the Assuit and Cairo populations, with the Qena population as an outgroup. Parallel with the fallouts, the species' polymorphism is lower and higher between the species.

5. Conclusion

In conclusion, worldwide, during aquaculture practices, hybrids show a reasonable growth rate and better resistance towards diseases beneficial for the aquaculture industry, but morphometric studies are at baseline. We used the RAPD method between the Hybrid and pure breeds in this project. Twenty-five primers were used, but only 5 showed amplification on *L. rohita* samples. This amplification indicates that the primers were species-specific. Analysis of bands showed a low level of polymorphism and a high level of similarity. The results suggest that polymorphism within species is common and high between species.

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