CLUSTER ANALYSIS IN *CHANNA PUNCTATUS* FISH REVEALED BY MOLECULAR MARKERS IN PUNJAB-PAKISTAN

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ABSTRACT: The studies on genetic diversity of *Channa punctatus* by using molecular markers were carried out amongst the different groups of wild populations to investigate the genetic structure by Randomly Amplified Polymorphic DNA (RAPD) marker and the levels of polymorphism and similarity. The samples were collected from different five locations viz., Chashma Barrage at River Indus near District Mianwali, Qadirabad Barrage at Chenab River, Tehsil Wazirabad, District Gujranwala, Balloki barrage at Ravi River, Tehsil Bhai Pharo, District Kasur, Trimu Barrage at the junction of Chenab and Jhelum Rivers near District Jhang and Taunsa Barrage at Indus River near Tehsil Kot Addu District Muzaffar Garh. RAPD data for Jaccard's coefficient by following the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) for Hierarchical Clustering of the similar groups on the basis of similarity amongst the genotypes. The dendrogram divided the randomly selected individuals of the five populations into four classes/clusters; 17 members in first cluster/class, 4 samples in second cluster/class and 2 samples in the third and fourth class/cluster.

Key words: Variation, Clustering, Wild, Randomly Amplified Polymorphic DNA, *Channa punctatus.*

INTRODUCTION

Fish is an excellent source of high quality protein with high digestible contents and nutritional value. It contains many kinds of vitamins particularly vitamin A and D and minerals like Phosphorus, Magnesium, Selenium, and Iodine (USDA, 2013). During the last few decades, research has revealed that fish meat is also an excellent source of omega 3 fatty acids which are helpful in brain development and anticancerous in nature (Li and Hu, 2009; Kawarazuka, 2010). It is reported that in the year 2007, fish provided 15.7% of the global population's animal protein, more than 1.5 million people with almost 20% of average per capita animal protein intake and 3 billion people with at least 15% of such protein. The high demand of fish led to the development of aquaculture which is continuing to grow with average annual growth rate of 6.6 percent outpacing the human population growth rate (FAO, 2010).

The Family Channidae consists of two genera viz., *Channa* and *Parachanna* (Nelson, 1984). The Asian genus *Channa*, which presently contains 26 valid species, is widely distributed in Iran, southern Asia and the Far East (Musikasinthorn, 2000; Berra, 2001; Musikasinthorn and Taki, 2001; Courtenay and Williams, 2004). The African genus *Parachanna* has three valid species, which are restricted to central West Africa (Bonou and Teugels, 1985; Teugels, 1992). The fish of genus *Channa* (snakehead, murrel) is prominent tropical fresh water fish and is widely used for medicinal and pharmaceutical purposes (Mat Jais *et al.,* 1994; Michelle *et al.,* 2004), in addition to being an important food source in the Asia-Pacific region (Hossain *et al.,* 2008). Members of this carnivorous air-breathing genus are commonly found in a range of water bodies which include rivers, swamps, ponds, canals, drains, reservoirs and rice fields etc. ranging from southern Asia, southern China, Indochina to the Sunda Islands (Mohsin and Ambak,1983; Lee and Ng, 1994; Hossain *et al.,* 2008). In, Pakistan this fish is common in natural water bodies and is normally marketed alive from the catch (Mirza, 1982; Rahman, 1989). The contribution in aquaculture is significant in certain neighboring countries such as Thailand, Taiwan, Philippines, Vietnam, Malaysia, Cambodia and India (Wee and Tacon, 1982; Hossain *et al.,* 2008; Jamaluddine *et al.,* 2011). Amongst the snakeheads, *C. marulius* and *C. punctatus* have been reported by (Hamilton, 1822 and Bloch, 1793) respectively, which are commercially important esteemed table fish and also used for ornamental trade (Ng and Lim, 1990; Courtenay and Williams, 2004).

RAPD technique is one of the most frequently used molecular methods for taxonomic and systematic analysis of various organisms (Bartish *et al.,* 2000), and provides important applications in fish species (Bagley *et al.,* 2001; Barman *et al.,* 2002; Almeida *et al.,* 2003; Sandoval *et al.,* 2007; Bhatti *et al*, 2014). The ease simplicity and low cost involvement in this technique makes it ideal as number of polymorphic markers can be produced easily with no prior knowledge about the genetics of the organism.

Keeping in view the findings of previous studies and prevailing situations the present study was planned to

test the hypothesis that the representatives of the families, Channidae (*Channa punctatus*) are gaining some distinction due to some environmental changes and other unknown factors in natural reservoirs.

MATERIALS AND METHODS

Study Sites: The samples of the target fish species were collected from Chashma Barrage at River Indus near District Mianwali, Qadirabad Barrage at Chenab River, Tehsil Wazirabad, District Gujranwala, Balloki barrage at Ravi River, Tehsil Bhai Pharo, District Kasur, Trimu Barrage at the junction of Chenab and Jhelum Rivers, District Jhang and Taunsa Barrage at Indus River, Tehsil Kot Addu, District Muzaffar Garh (Figure-1).

Sample Collection and Storage: The experimental species from the above mentioned sites were collected with the help of the local fishermen at the site and shifted to experimental laboratory by icing and were stored in the laboratory till analyzed at -80 ^{o}C.

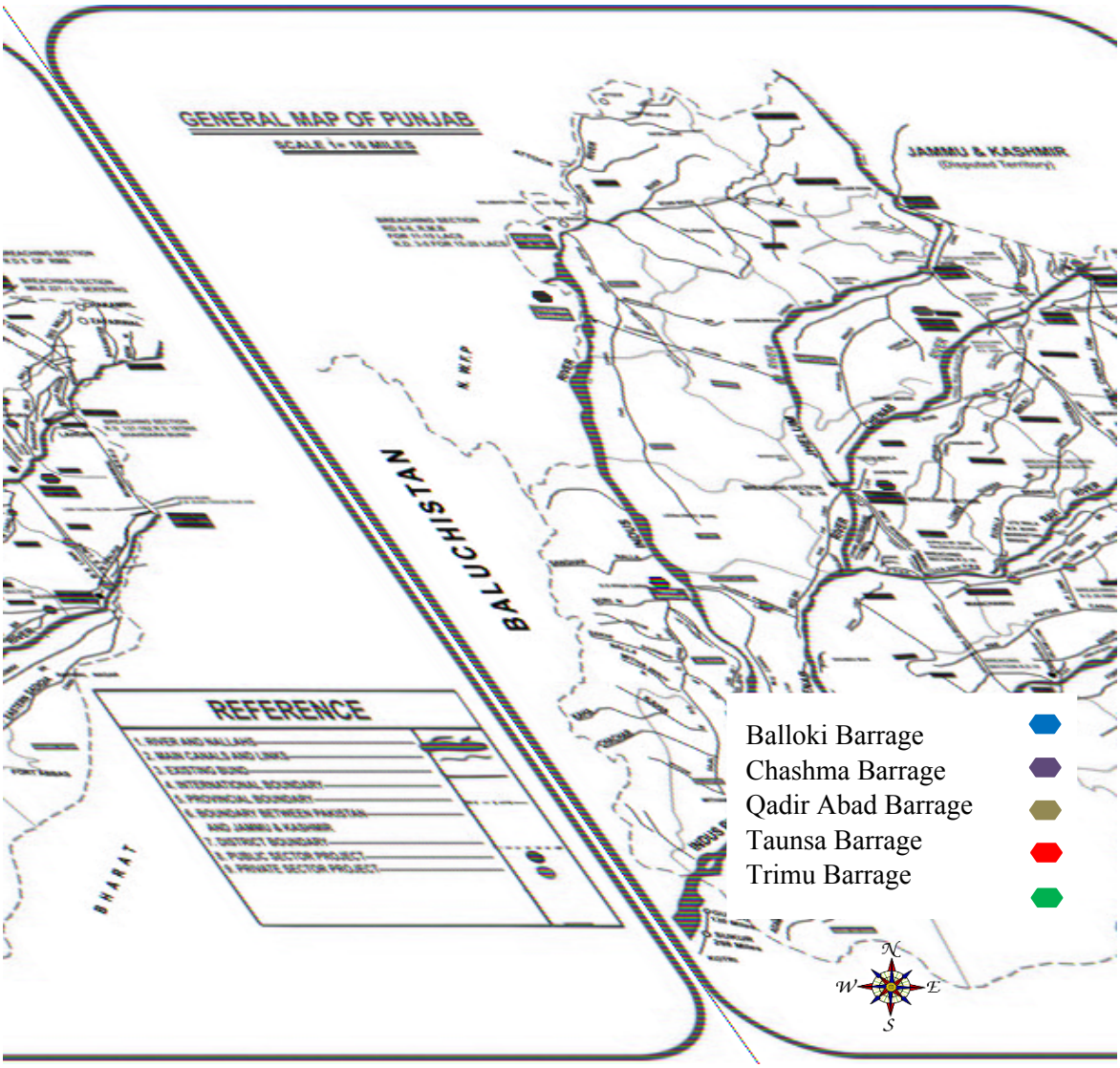

Figure 1: Map showing the sampling sites at different Rivers in Punjab-Pakistan

Location of Research Facilities Used: The collected samples were studied at Molecular Genetics Lab of the Department of Wildlife and Fisheries, Faculty of Sciences and Technology, Govt. College University, Faisalabad.

DNA Extraction: Total genomic DNA isolation was carried out from the stored fish samples (Lopera-barrero *et al.,* 2008). This procedure was based on the protocol (Aljanabi and Martinez, 1997) which was modified by the use of NaCl.

In this procedure, lysis buffer was used which carried 50 mM tris which was taken from a stock of 1 M at pH 8 tris buffer, 50 mM EDTA was taken from a stock of 0.5 M at pH 8, 100 mM NaCl taken from a stock of 5 M NaCl and 1% SDS. From this lysis buffer, working lysis buffer was prepared by adding 7 µl proteinase K from stock solution. Stock solution of the proteinase K was prepared by taking a buffer of 100 mMTris-base, 50 mM EDTA, 500 mM NaCl and then 200 μgL^{-1} Proteinase K was added and dissolved.

About 1 g of fish flesh was taken in a 1.5 ml eppendrof micro tube and homogenized in 550 µL lysis buffer and then 7 µL of proteinase K buffer was added to the sample containing eppendrof micro tube. The contents of the tube were incubated in thermo-regulated water bath at 50 ^oC for 12 h. After this incubation 5M NaCl amounting to 600 µL solution was added and mixed thoroughly and centrifuged for 10 minutes at 12000 rpm.

A fresh eppendrof micro tube was taken and supernatant was transferred into it with the help of micropipette. Then the DNA was precipitated by the addition of 700 µL absolute cold ethanol. After mixing the contents of the tube, it was incubated at -20 ^oC for 2 h. The tube was then centrifuged for 10 minutes at 12000 rpm to obtain the pellet of the DNA. All the liquid was discarded and 300 µl of 70% ethanol was added to remove salt. The washing with 70% ethanol was repeated and the pellet was dried by inverting the tube on a dry tissue paper. Air dried pellet of the DNA was dissolved in 80 µL TE buffer (10 mM tris and 1 mM EDTA). To remove the RNA from these preparations 1µl of 30 μgmL^{-1} of RNAs was added and incubated at 37 ^oC for one hour and then precipitated the DNA with 3.2 M sodium acetate and 2.5 ml volume of absolute alcohol. The pellet was centrifuged, washed with 70% ethanol, dried and dissolved in 50 µL sterilized TE buffer.

Quantification of DNA: Purity of DNA was checked for quantification by using UV spectrophotometer (U-2800, Hitachi) and agarose gel electrophoresis. For this purpose Optical Density (OD) value at 260 nm and 280 nm was taken and calculations were made to determine the concentrations of the DNA samples. For the assessment of the integrity 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 g tris base and 28.6 ml glacial acetic acid and 0.5 molar EDTA in water and raising its volume to 500 ml. The DNA samples were loaded into the gel after mixing with 10X DNA loading buffer with 0.21% bromophenol blue, 0.21% xylene cyanol FF, 0.2 molar EDTA and 50% glycerol.

Primer Selection: Twenty five decamer (Operon) primers designed by Gene Link Ltd., Hawthorne, were used initially in the study to amplify polymorphic DNA randomly. Ten of these with most scorable bands were used for further studies. The sequences of all the used primers are given below

Table 1. Showing the sequences of used primers

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	OPC-11	AAAGCTGCGG
12	OPC-12	TGTCATCCCC
13	OPC-13	AAGCCTCGTC
14	OPC-14	TGCGTGCTTG
15	OPC-15	GACGGATCAG
16	OPC-16	CACACTCCAG
17	OPC-17	TTCCCCCCAG
18	OPC-18	TGAGTGGGTG
19	OPC-19	GTTGCCAGCC
20	OPC-20	ACTTCGCCAC
21	OPD-01	ACCGCGAAGG
22	OPD-02	GGACCCAACC
23	OPD-03	GTCGCCGTCA
24	OPD-04	TCTGGTGAGG
25	OPD-05	TGAGCGGACA

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 0.2 ml PCR tube and 25 µL reaction mixtures. To prepare this 25 µL reaction, 2.5 µL 10x PCR buffer, 2 µL 1.6 mM $MgCl_2$, 2 µL 10 nM primer, 2 µL 2.5 mM dNTPs, 0.3 µL 5 units/µL taq polymerase enzyme and 11.2 µL deionized double distilled water were mixed. In each reaction a negative control was also run using sterilized water as the template.

Profile of the PCR Reaction: PCR reaction was carried out in Personal Autorisieter Master Cycler of the EPPENDORF, Germany. Each reaction profile was of one cycle of 5 minute denaturation at 95 ^oC and then 35 cycles of 1 minute at 95 ^oC, 1 minute at 37 ^oC and 2 minutes at 72 ^oC and finally 20 minutes extension at 72 ^oC. Then the machine was allowed to hold the reaction contents at 22 ^oC for 30 minutes.

Analysis of the PCR products: All the PCR products were analyzed by sequestering them on agarose gel. For this purpose 1.5% agarose gel was prepared in TAE

buffer as described in section 3.8. The DNA samples were then loaded on the gel using the DNA loading buffer. Each gel was run with 100 base pair DNA ladder in the left and right lanes or only on one side. These gels were visualized in UV light and photographs were taken by gel documentation system (Wealtec, Dolphin-DOC).

Statistical analysis: The XLSTAT 2012 version 1.02 (Rasool *et al*. 2012) of the computer software was used to analyze the RAPD data for Jaccard's coefficient by following the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) for Hierarchical Clustering of the similar groups on the basis of similarity amongst the genotypes and the dendrogram generated which is presented in the next section.

RESULTS AND DISCUSSION

The dendrogram (Fig 2 and 3) developed by this method by the presented data of the scorable bands of all the amplified primers divided the randomly selected individuals of the five populations into four classes/clusters. The division of all the randomly selected five population representative *C. punctatus* samples collected from different geographical locations in the three clusters were Blk1, Blk2, Blk3, Blk4, Cha2, Cha3, Cha4, Qbd1, Qbd4, Tsa1, Trm1, Trm3, Trm4, Tsa2, Tsa3 and Tsa4 in first cluster/class, Cha5, Qbd5, Blk5 and Trm2 in second cluster/class, Qbd2, and Trm5 in the third class and Qbd3 and Tsa5 in Fourth class/cluster (Table 5). The results of dendrogram of *Channa punctatus*, it was observed that majority of the samples were in one class and very few of them had got some genetic distinction. These results were in line with the findings of (Cadrin 2000) who concluded that it was often difficult to explain the causes of morphological differences amongest the populations. These differences may be genetic differences, or they may be associated with phenotypic plasticity in response to different environmental factors in each area. The results showed that 66% population was genetically same as making one class. Only a minor level of population was genetically different when 33% samples were divided into three classes. This means that a very minor change was observed in the genetic makeup. So these individuals seem to be distinct from the remaining populations of the same sites and supported the hypothesis that the evolutionary process, environmental condition and other unknown factors were responsible for this condition. These results are also comparable with the results of (Mohindra *et al.,* 2007) who reported genetic variability in three clariid species i.e., *Clarias batrachus, C. gariepinus and C. macrocephalus.* The UPGMA phylogenetic tree also revealed three distinct clusters: *C. batrachus; C. gariepinus* and *C. macrocephalus*.

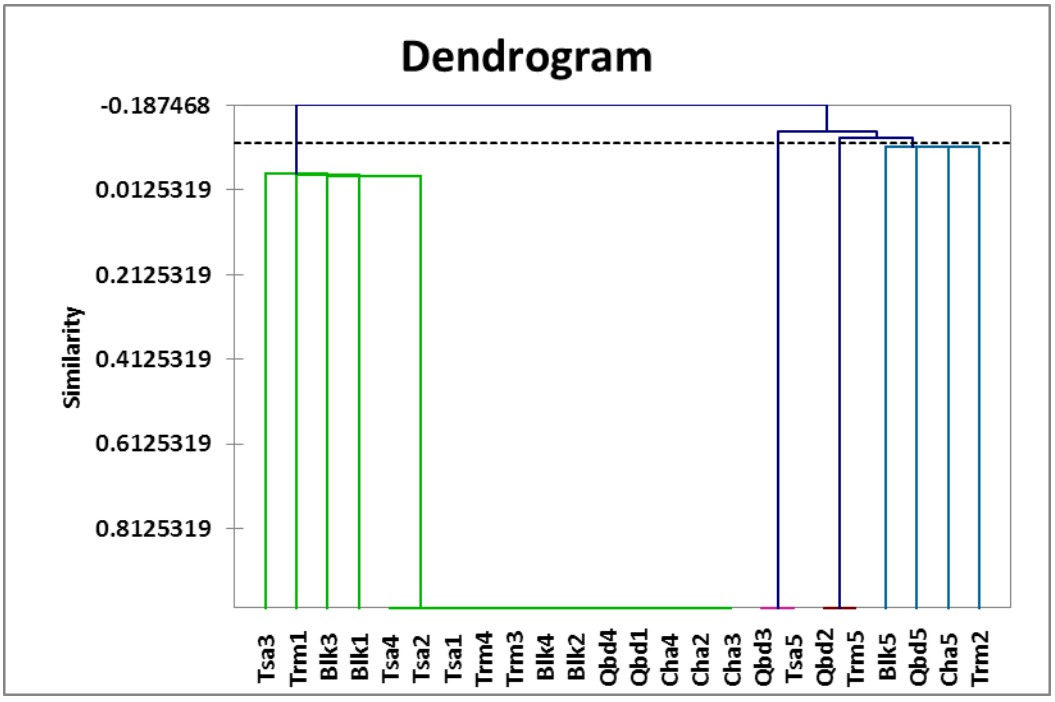

Figure 2: Dendrogram showing classification of *Channa. punctatus.*

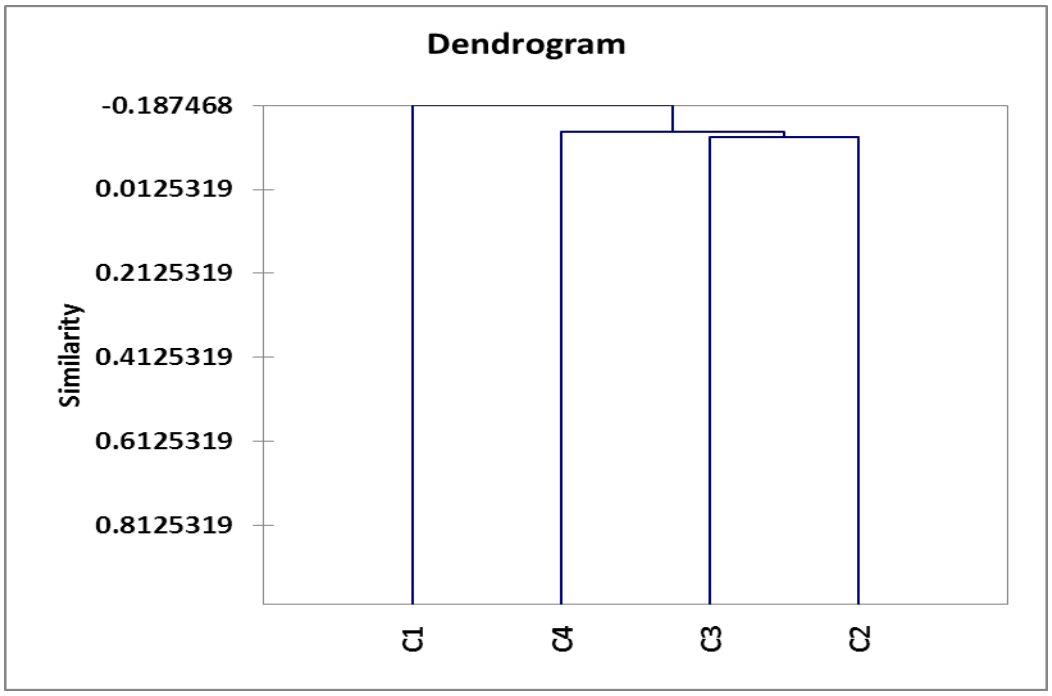

Figure 3: Dendrogram showing classes of *C. punctatus.*

The variance decomposition for the optimum classification values remained as 73.93% for within class variation and 26.07% for the between class differences (Table-2). The distance between the class/clusters centroids remained as 0.718 for class 1 and 2, 1.008 for class 1 and 3, 1.008 for class 1 and 4, 1.225 for class 2 and 3 and 2 and 4 while this distance was 1.414 for class 3 and 4 (Table-3).

Table 2. Showing Variance decomposition for the optimal classification of *C. punctatus.*

	Absolute	Percent
Within-class	0.488	73.93%
Between-classes	0.172	26.07%
Total	0.660	100.00%

Table 3. showing the distances between the class centroids of *C. punctatus*

	1	2	3	4
1	0	0.718	1.008	1.008
2	0.718	0	1.225	1.225
3	1.008	1.225	0	1.414
4	1.008	1.225	1.414	0

In this classification Cha2 from the Samples collected from Chashma Barrage, Cha5 collected from the same location, Qbd2 collected from Qadirabad Barrage and Qbd3 collected from the same location were central objects for class/clusters 1, 2, 3 and 4, respectively. The distances between the central objects of the classes remained as; 1.414 between the central objects of class 1 and 2, 1.000 between the class 1 and 3, 4, respectively, 1.732 between the central objects of class 2 and 3, 4, respectively, while the distance between central objects of class 3 and 4 was 1.414 (Table 4).

Table 4 Showing the Central Objects of Classes of *C. punctatus* and Distances between them

	1 Cha2)	2 Cha5)	3 (Qbd2)	4 (Qbd3)
1 (Cha2)	0	1.414	1.000	1.000
2 (Cha5)	1.414	0	1.732	1.732
3 (Qbd2)	1.000	1.732	0	1.414
4 (Qbd3)	1.000	1.732	1.414	0

The results for the conclusion about 4 classes/clusters with their values for within class variance, minimum distance to centroids, average distance to centroids and maximum distance to centroids are given in Table 5.

The results of this study were completely complementary to the studies of (Rasool *et al*. 2012). In their study on Indian major carp, *C. mirgala* performed Clustering Analysis for Intraspecific Variation amongst the populations of the fish and reported that data of the morphometric parameters divided the populations in to four major clusters or classes. They further reported that variance decomposition for the optimal classification values remained as, 27.28% for within class variations while 72.72% for the between class differences. The

distance between the class/cluster centroids remained as; 50.820 for class one and two, 18.063 for class one and three, 14.564 for class one and four, 68.856 for class two and three, 36.708 for two and four while this distance between class three and four centroids was 32.408.

These results were comparable with Zhu *et al.,* (2009) who concluded that the reason for morphological diversity within the sub populations was a result from the variable environments.

Table 5. Showing the results by class of *C. punctatus*

Class	1	2	3	4
Objects	16	4	2	2
Sum of weights	16	4	2	2
Within-class variance	0.250	2.000	0.000	0.000
Minimum distance to centroid	0.125	1.225	0.000	0.000
Average distance to centroid	0.330	1.225	0.000	0.000
Maximum distance to centroid	0.944	1.225	0.000	0.000
	Cha2	Cha5	Qbd2	Qbd3
	Cha3	Qbd5	Trm5	Tsa5
	Cha4	Blk5		
	Qbd1	Trm2		
	Qbd4			
	Blk1			
	Blk2			
	Blk3			
	Blk4			
	Trm1			
	Trm3			
	Trm4			
	Tsa1			
	Tsa2			
	Tsa3			
	Tsa4			
	Cha1			

The results of the present study showed that most of the individuals of five populations collected from different geographical locations were grouped into same class/cluster, which was the indication that the parental stock of all individuals of the same species were same but minute levels of differentiation were due to some evolutionary process or environmental factors. These results are in line with the studies conducted by (Saini *et al.,* 2010) who examined the genetic variability between the populations of *S. seenghala* in river Satlej and river Beas of Indus river system in India and found following 95% criterion as standard and found that there was 89.06% for Beas population as compared to 95.31% for Satlej population, they found a moderate level of genetic divergence, 0.0486 between both the populations which was the result of substructure of the *S. seenghala* in both the rivers.

These results were also similar with findings of (Danish, *et al.* 2012) on the studies of Molecular characterization of two populations of catfish *Clarias batrachus* L. using RAPD markers and found a similarity within the populations from wild varied from 0.40 to 0.83 with a mean ± SE of 0.57 ± 0.08. The Jaccard's similarity coefficient ranged from 0 to 0.27. At 0.06 similarity coefficient, two major clusters were formed, which indicated that the genotypes belonging to same cluster were genetically similar and those belonging to different clusters were dissimilar. Significant ($P < 0.05$) population differentiation indicated some degree of inter- and intra-population genetic variations in two populations of catfish.

Conclusion: In the present study the difference was very minor between the samples collected from different sampling sites which showed that these fish belong to the same genetic group. Some fishes which have got some distinction may be due to the environmental changes or certain other unknown factors.

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