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Comparative study of genetic biodiversity in carp fish (*Cyprinous carpio*)

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Abstract
In the present study the genetic variations in the two countries population viz. India and Iraq of the Common carp, *Cyprinus carpio* were analyzed by Random Amplified Polymorphic DNA (RAPD) markers and the protein polymorphism. According to RAPD analysis the percentage of gene similarity (s) and the gene diversity (h) estimates were 93.75% and 68.75% for the India respectively and the percentage of gene similarity (s) and the gene diversity (h) estimates were 83% and 50% for Iraq respectively indicating the existence of a relatively high level of genetic variation in the India and Iraqi river population. According to protein analysis, the genetic similarity (s) and the gene diversity (h) estimates were 100 % and 67% for the Indian population respectively. The genetic similarity indices and genetic distance values indicated that Indian-Iraq populations of some common carp were genetically closer and some were genetically diverse. The minimum genetic distance between the species indicated that population was a reproductive isolated population. The data suggest that the RAPD technique being able to screen more easily a larger part of the nuclear genome than allozymes. The common carp i.e. *Cyprinus carpio* present a classified case of a species in a highly active state of differentiation and evolution and genetic variation between Indian and Iraq population.

Keywords: *Cyprinus carpio*, Random Amplified Polymorphic DNA (RAPD)

Introduction
Carp (cyprinids) contribute over 20 million metric tons to fish production worldwide and account for approximately 40% of total global aquaculture production and 70% of total freshwater aquaculture production. They have emerged as the most economically important teleost family. In comparison to other major aquaculture species, such as salmon and shrimp, carp are recognized as an ecofriendly fish because most are omnivorous filter-feeders and thus consume much less fish meal and fish oil (FAO, 2006).

The Indian fish fauna is divided into two classes, viz., Chondrichthyes and Osteichthyes. The Chondrichthyes are represented by 131 species under 67 genera, 28 families and 10 Orders in the Indian region. Common carp (*Cyprinus carpio* L.) belongs to Cyprinidae, the largest family among freshwater teleosts (Nelson, 1994) [13], for which the world's annual total catch in 1999 was estimated above 15.6 million metric tons, compared to the 2.3 million tons of salmonids (FAO, 2001) [9]. The carp is a triploid gynogenetic species, which provides a unique model system for understanding evolutionary genetics and for elucidating the regulatory mechanisms underlying diverse reproduction modes in vertebrates. Several features, such as the existence of males (Fan and Shen, 1990) [7], and two reproduction modes (Zhou *et al*., 2000b), place this particular silver crucian carp on an intermediate evolutionary step between species with uni and bisexual reproduction systems. Common carp (*Cyprinus carpio*) varieties (e.g. races, landraces, strains, breeds and stocks) ''developed through a combination of forces including geographic isolation, adaptation accumulation of mutations and natural as well as human selection pressures'' (Hulata, 1995) [10]. Classical taxonomic analysis divides the currently existing common carp forms into three categories: European (*Cyprinus carpio carpio*), FarEastern (*C. carpio haematopterus*) and South East Asian (*C. carpio viridiviolaceus*). It is however, essential to know the genetic structure of natural populations to be used as a founder or replacement stock so that any change in gene and genotype frequencies that may happen during the operation period can be assessed. Genetic variation is useful for stock improvement breeding programs, management for suitable yield and conservation of diverse gene pool (Tassanakajon *et al*., 1997).

Genetic markers come in a variety of formats in modern molecular biology, although initial marker systems were based on protein polymorphism and morphological characteristics. There is a wide array of DNA based molecular marker types (Davis and Hetzel, 2000) [6]. Isozyme electrophoresis, restriction fragment length polymorphism (RFLP) and microsatellites have been so far used to analyze genetic similarity and diversity in genetics and breeding research of fish/invertebrates. Also, molecular markers from random amplified polymorphic DNA (RAPD) have recently been used to evaluate genetic diversity and/or similarity in several organisms (Yoon and Park, 2002) [18, 19].

Despite the commercial importance of the species, genetic data on common carp stocks are relatively scarce. Analysis of protein poly-morphisms was performed on some populations However, reports have only been published recently on common carp genotypes using RAPDs (Dong and Zhou, 1998) and microsatellite markers (Crooijmans *et al*., 1997; Aliah *et al*., 1999; Tanck *et al*., 2000; 2001;) [5, 3, 16]. The genetic analysis of two famous common carp varieties from Hungary and their comparison with over 100 individuals collected from various other sources has been reported by

Bartfai *et al*. (2003) [4].

Material and Methods

Sample collection, DNA extraction, Primer selection, amplification and electrophoresis

The 12 samples of common carp i.e. *Cyprinus carpio* fish were collected from Mukundwadi Fish Market, Aurangabad and the Central Naka Fish Market, Aurangabad, Maharashtra, India. The samples were preserved at 20^0C in the deep freezer.

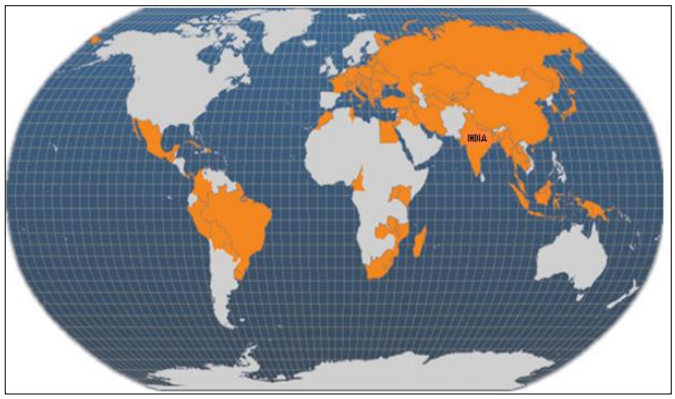


Fig 1: Main producer countries of Cyprinus carpio (FAO Fishery Statistics, 2006)

The DNA from the fish was isolated by using the phenol: chloroform: Isoamyl alcohol extraction and ethanol precipitation method (Alam *et al*., 1996) [1]. The tissue were then homogenized by using a micro homogenizer in extraction buffer (100 mM Tris.HCl, 10 mM EDTA, 250 mM NaCl and 1% SDS, pH=8). After homogenization 25 µl of 20 mg/ml Proteinase-K was added to the homogenate and incubated at 37°C overnight. The following morning the lysates were extracted once with equal volume of Phenol: Chloroform: Isoamyl Alcohol (25:24:1) and once with equal volume of Chloroform: Isoamyl Alcohol (24:1). DNA was precipitated using 0.6 volume of Isopropanol and then resuspended in TE buffer (10 mM Tris.HCl, 1 mM EDTA, pH=8). The concentrations of DNA samples were determined by a UV-spectrophotometer.

One kit (designated a), containing 20 decamer primers of random sequence, was obtained from Sigma Aldrich Private Limited, New Dehli, India. Initially, the genomic DNA of one individual from each sample was used as template for amplification of RAPD markers with each of the 20 decamer primers. Ten primers having high intensity of bands and no smearing were further used for amplification of DNA of two individuals from each population. A final subset of four primers (Table 1) exhibiting the highest quality banding patterns and sufficient variability for population analysis were taken for analysis of all the samples.

Experiments were run to optimize DNA, dNTPs and Taq DNA polymerase concentrations and to determine the optimum annealing temperature. The ten RAPD primers were taken for RAPD analysis. The PCR reaction were performed on each DNA sample in a 25µl mixtures containing 2µl of 10 X assay buffer with 25mM $MgCl_2$, 1µl 10mM dNTP mixture, 1µl of 10mM primer, 1µl of template DNA and 0.5µl of 3U/l Taq Polymerase. All these ingredients dissolve in the suitable amount of molecular biology grade water. The DNA amplification was perfomed in an oil free thermal cycler (Master Cycler Gradient, Eppendorf). The reaction mix was preheated at 94^0C for 3 mins followed by 40 cycles of 1 min denaturation at 94^0C, 1 min annealing at 36^0C and 2 min extension at 72^0C. After the last cycle, a final step of 7 min at 72^0C was added to allow complete extension of all amplified fragments.

The amplified products were separated by electrophoresis on 2% agarose gel (Himedia, Mumbai, India) containing ethidium bromide in 1XTAE buffer at 120 V for 1½ h. One molecular weight marker DNA, High Throughput DNA 200 bp ladder (Himedia, Mumbai, India) was electrophoresed alongside the RAPD reactions. DNA bands were then observed on UV-transilluminator and photographed by a Gel Cam Polaroid camera in the Gel Documentation System.

Protein Extraction and SDS-PAGE

The proteins from the tissue were isolated from the tissue of fish. The tissue were homogenize in the Phosphate saline buffer (50mM NaH_2PO_4, 50mM Na_2HPO_4, 150mM NaCl and 1mM EDTA) containing protease inhibitor such as EDTA. The homogenize tissue solution was centrifuged at high speed and supernatant used as a protein source. These proteins were mixed with 10 X Laemilli Buffer and heated for the 10 min at 98^0 C. The 40 µl sample was loaded on the 15% polyacrylamide gel. The electrophoresis was carried out at 100 V for an hour to run the sample. The gel was stained in the 0.2% (w/v) Co-omassie Brilliant Blue R-250 dye for overnight. After that the stained gel was destained in the destainer until the gel become transparent and the protein band appeared clear.

RAPD data analysis

Fragments were scored as 1 if present and 0 if absent. The scores were then pooled for constructing a single data matrix. This was used for comparing the frequencies of all polymorphic RAPD markers and estimating gene diversity (h), gene similarity (S), genetic distance (D) and constructing a UPGMA (unweighted pair group method of arithmetic means) dendrogram among populations with simulated samples using NTSYS (version 2.02) computer program. Bandsharing based similarity indices between the populations samples were calculated for all possible comparisons by using the method of Lynch (1991).

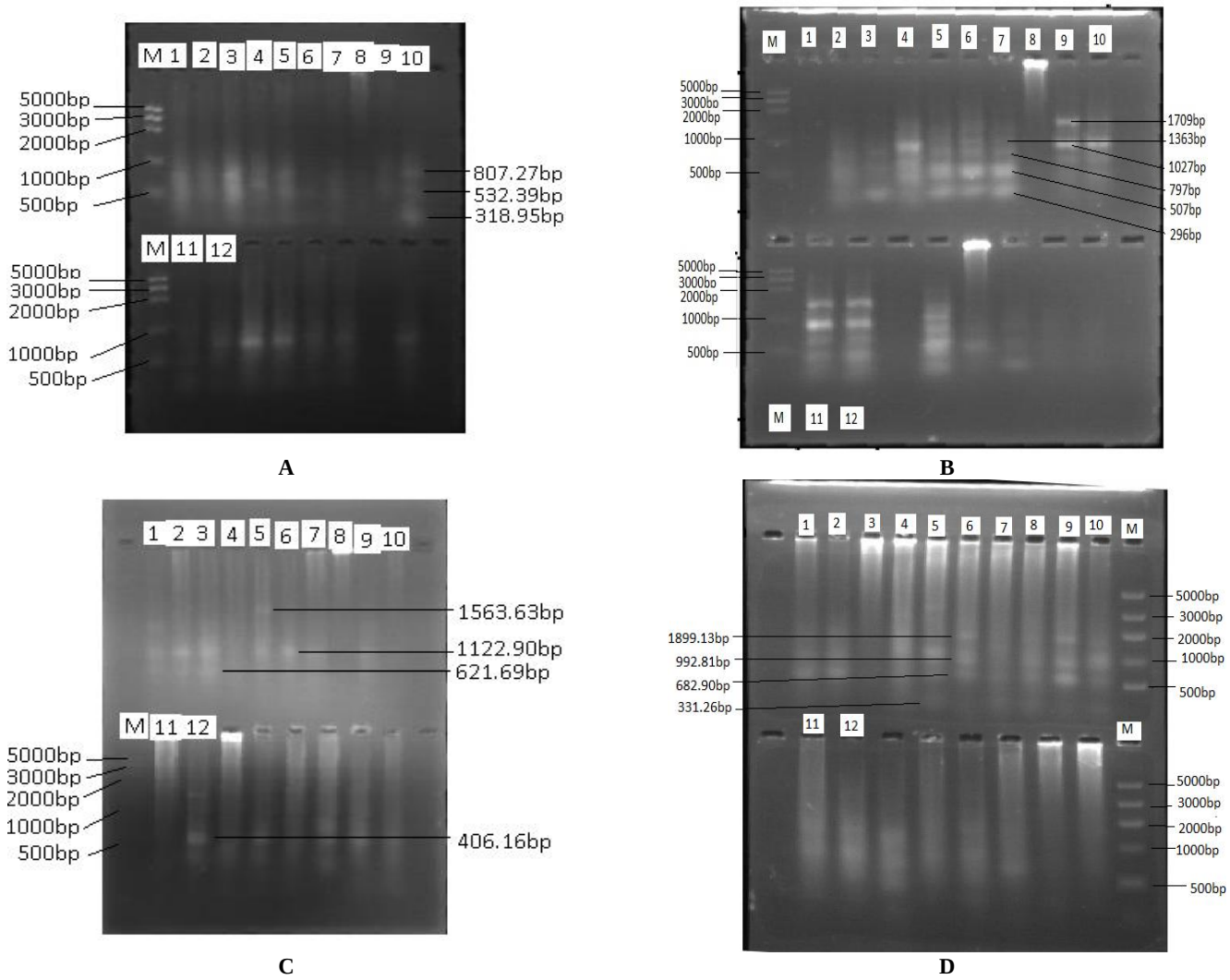


Fig 2: RAPD profiles for the *C. carpio* for the OPA-1 (A), OPA-4 (B), OPA-7 (C), and OPA-8 (D) primers from the Sigma Aldrich Kit along with the 500bp DNA ladder (M) (Himedia).

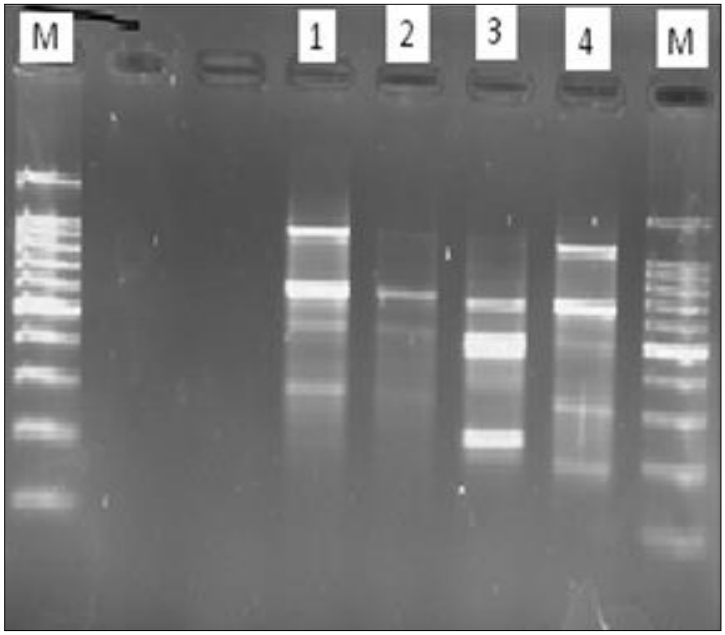
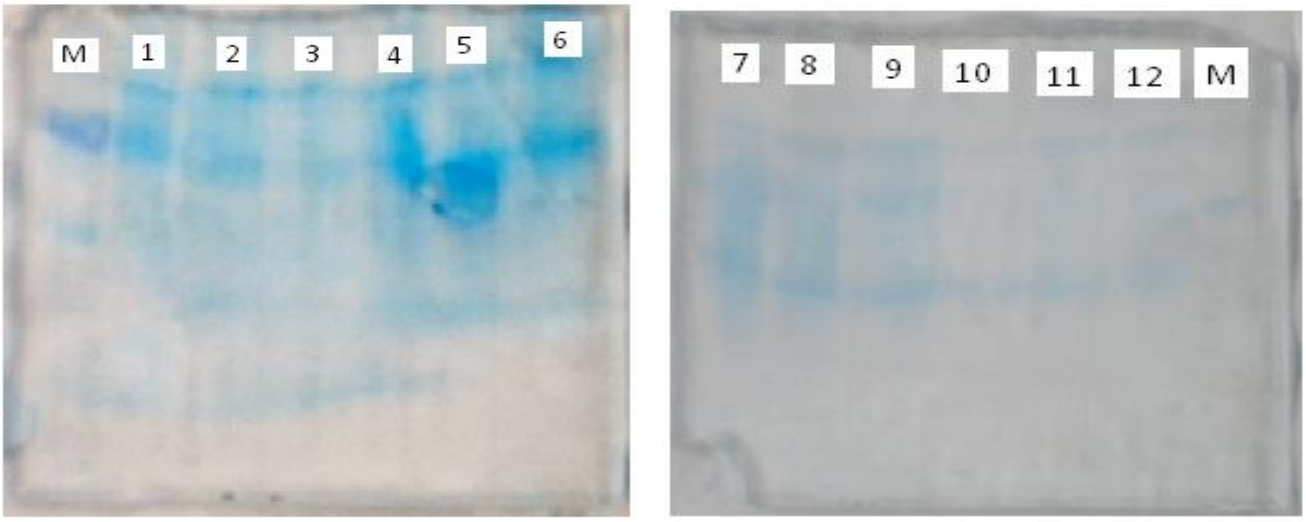


Fig 3: RAPD profiles of *Cyprinus carpio* for the RAPD primer along with the high throughput DNA ladder (200 bp) from Iraq.



A B

Fig 4: Protein profile obtained after the SDS polyacrylamide gel electrophoresis of cyprinus fish 1-6 (A) and 7-12 (B).

Results and Discussion

Table 1: Primers used to generate RAPD profiles from *C. carpio* DNA

Primer	Sequence (5´-3´)	Total scorable bands	Size range (bp)
OPA - 1	5'- CAGGCCCTTC - 3'	6	296-1709
OPA - 4	5'- AATCGGGCTG- 3'	3	318-807
OPA - 7	5'- GAAACGGGTG- 3'	4	406-1563
OPA - 8	5'- GTGACGTAGG- 3'	3	331-1899

DNA profiles generated by RAPD primers

All the four primers produced different RAPD patterns and the number of fragments amplifies per primer varied. Among the four primers OPA-1 gave DNA profiles with more numerous bands than the other three primers (Table - 1). The total numbers of the polymorphic fragments obtained from these four primers were 16 (Table 1). The RAPD profile obtained in this analysis are shown in the figure 2.

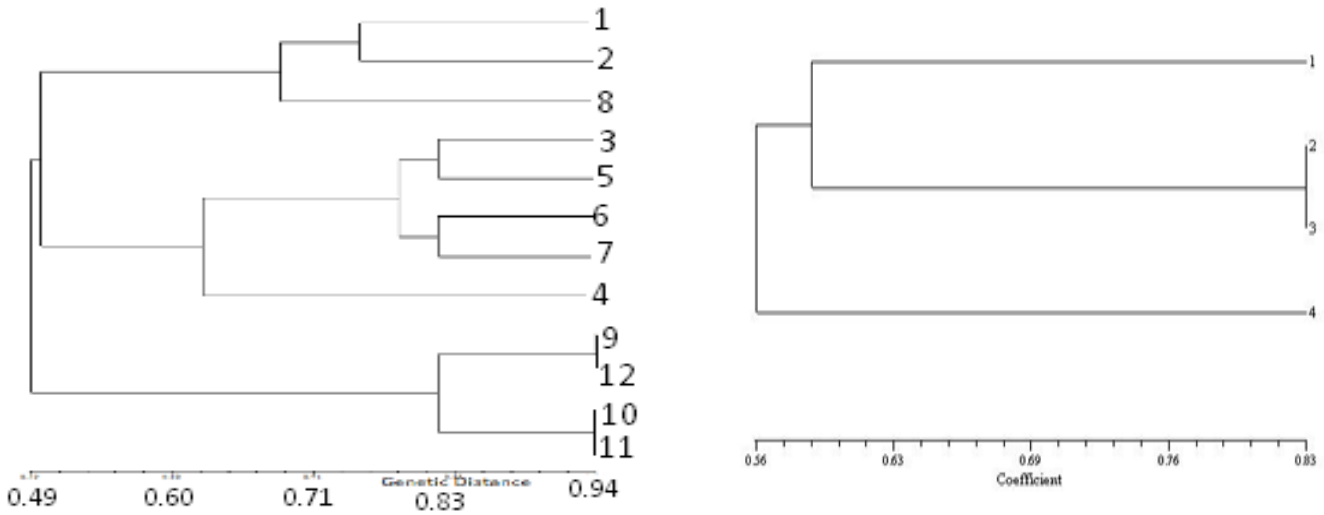


Fig 5: UPGMA dendrogram based on Nei's (1978) unbiased genetic distance, summarizing the data on differentiation between *C. carpio* populations (A) from India and (B) from Iraq, according to RAPD analysis.

The dendrogram (Figure 4 (A) along with the similarity indices table shows that there is the significant similarity and diversity found between the 12 cyprinus fish samples. The fish sample 9 and 12, 10 and 11 showed the highest gene similarity (S) amongst each other i.e. 93.75% gene similarity (s) these samples shows the lowest gene diversity (h) i.e. 6.25% with each other. The fish sample 7 and 10, 8 and 12 shows the lowest gene similarity (s) with each other i.e. 31.25% so means that the fish sample 7 and 10, 8 and 12 are the highest genetically diverse (h) with each other i.e. 68.75% gene

diversity (h) found between these samples.

Comparison of the genetic diversity between Indian *Cyprinus carp* fish and the Iraqi *Cyprinus carp* fish

The polymorphism was studied among the Indian *Cyprinus carp* fish and the Iraqi *Cyprinus carp* fish on the basis of dendrogram along with the similarity indices values. (Figure 6) For this study the dendrogram was plotted by using the NTSYS computer program (version 2.02).

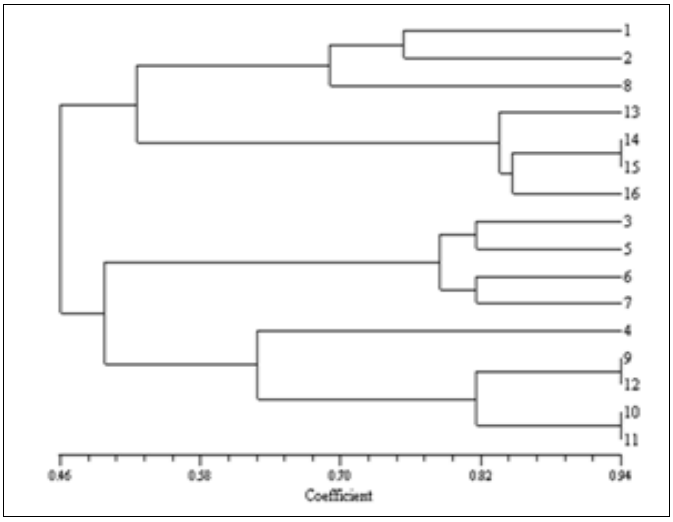


Fig 6: UPGMA dendrogram based on unbiased genetic distance, summarizing the data on differentiation between *Cyprinus carpio* populations from india as well as Iraq, according to RAPD analysis

The values obtained from dendrogram (Figure 6) along with similarity indices, the comparison can be made between Indian cyprinus carp fish with the Iraqi cyprinus carp fish. The sample 12 from India and 14 from Iraq were found to be most similar with each other. The similarity between these two samples was found to be 87.50%. Other than these fishes, the sample 4 from India and 16 from Iraq and the sample no 8 from India and 14 from Iraq were found to be 75% similar with each other which showing the highest similarity in the India as well as Iraq cyprinus fish.

But the genetic diversity among the Indian as well as Iraq fishes was also found to be very high in case of some fishes. The fish sample 15 from the Iraq was found to be most genetic diverse or dissimilar with the fish sample 12 and 9 from India. The genetic diversity between these samples was found to be 87.50 % and 81.25%.

Protein Analysis

The observation obtained after the analysis of the dendrogram of proteins (Figure 7) and the similarity indices values was found to be different than that of RAPD analysis. The dendrogram obtained (Figure 7) in case of protein polymorphism was divided into the three parts. The sample 10, 11 and 12 were found to be 100 % similar with each other in the 1st part. In 2nd part of the dendrogram the sample 2, 4, 5, 6, 7, 8, and 9 was found to be completely similar with each other and in the 3rd part sample 1 and 3 showed the 100% similarity with each other. The sample 1 and 3 was found to be most diverse with fish sample 10, 11 and 12. The dissimilarity or genetic diversity (h) between these samples was found to be 67%.

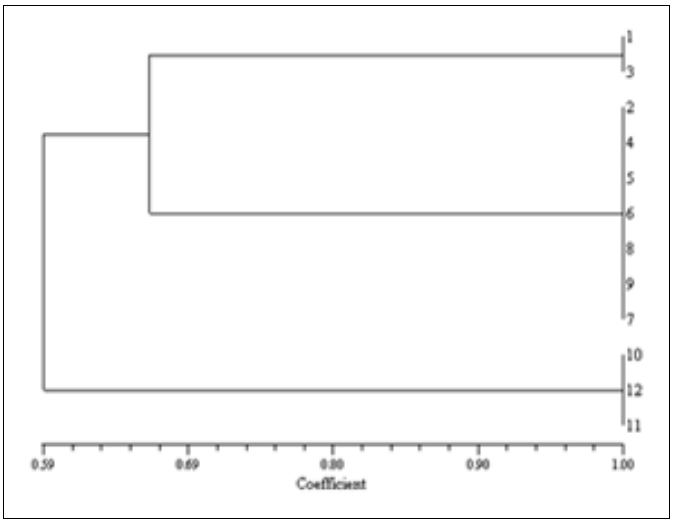


Fig 7: UPGMA dendrogram based on unbiased genetic distance, summarizing the data on differentiation between Cyprinus carpio populations, according to protein analysis.

In case of RAPD analysis the sample, the sample 1 showed the dissimilarity with sample 3 i.e. about 62.5% dissimilar and but in the case of protein polymorphism sample 1 was totally similar with sample 3. The sample 4, 5, 6 and 7 showed the highest similarity between each other in case of both DNA as well as protein polymorphism. The sample 9, 10, 11 and 12 showed highest similarity with each other in case of DNA polymorphism but in protein polymorphism sample 10, 11 and 12 were totally similar with each other while sample 9 found to 66 % similar with sample 10, 11 and 12.

Discussion

Report on genetic structure of different *cyprinus carpio* and populations in Aurangabad region are very scarce. It is also unknown how much the different dams in these area populations are genetically differentiated from each other. The RAPD method uncovered polymorphic loci from four primers selected for population analysis. The result is consistent with the fact that the RAPD technique, being able to screen more easily a larger part of the nuclear genome than allozymes, may assess higher levels of genetic variation. Analysis of the proportions of gene diversity, gene similarity indices for within samples and finally the gene diversity estimates indicate that a relatively high level of genetic variation exists in the population. The finding is consistent with the fact that the dams in the Aurangabad area is a geographically isolated spawning ground of the major carps from where naturally spawned eggs of carps are collected.. The higher similarity and lower level of frequency of polymorphic loci and gene diversity estimates for the population could be an indication of comparatively closer relationship among individuals. The higher between population similarities S = 93.75 %. Therefore, there is a great possibility of mixing between the individuals of the two dams constructed on same river. Nei's (1978) genetic distance was also used to evaluate the genetic variability and relatedness among cyprinus populations. The

results are consistent with the band-sharing based similarity indices. The greatest genetic distance exists between populations that are the most geographically distant. These results support the hypothesis that geographical distance is an important factor influencing the genetic relatedness of populations (Wright, 1943) [17]. The value of coefficient of gene differentiation (0.12) reflects some degree of genetic differentiation among three studied populations indicating the usefulness of RAPD markers in discriminating different populations of cyprinus. The calculation of a genetic distance between two populations gives a relative estimate of the time that has passed since the populations existed as single cohesive units. Small estimations of distance may indicate population substructure (i.e., subpopulations in which there is random mating but there is a reduced amount of gene flow). However, small estimation of distance may also be present because the populations are completely isolated but have only been separated for a short period of time. (Pandey *et al*., 2002) [15] The minimum genetic distance between the species indicated that population was a reproductive isolated population.

Conclusion

This study presents step towards the investigation of genetic variability of cyprinus in the dams among Aurangabad region by a DNA marker and proteins. The fishes from various dams are genetically more diversified with each other. The present study may serve as a reference point for future examinations of genetic variations within the populations of fishes which are commercially important but also play a significant role in food chain in lentic as well as lotic habitats. Thus the common carp i.e. *Cyprinus carpio* present a classified case of a species in a highly active state of differentiation and evolution. Our study further showed the comparison of genetic variation between Indian and Iraq population.

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