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Intra-specific and inter-generic phylogenetic relationships in endangered catfish (*Clupisoma garua* and *Eutropiichthys vacha*) of family schilbeidae

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Abstract
The present study was designed to evaluate the patterns of intra and inter-specific genetic variations and phylogenetic relationship using RAPD-PCR technique in two endangered catfish, *Clupisoma garua* and *Eutropiichthys vacha* collected from different rivers characterized by different environmental conditions. The number of RAPD bands found to be less in *C. garua* as compared to *E. vacha*. RAPD markers showed a high percentage of polymorphic bands among and between all analyzed individuals of fish than monomorphic. The phylogenetic tree generated on the basis of the distance matrix, images of RAPD gel and ladder indicated intra-specific variations among the various samples in *E. vacha* and *C. garua* of schilbeidae in the present study. Interestingly the degree of conservation in *E. vacha* and *C. garua* was found to be less between the samples of same species of the same geographical region or same river but the strong relationship was noted between the samples of different rivers.

Keywords: *Clupisoma garua*, *Eutropiichthys vacha*, RAPD-PCR

1. Introduction
The catfish, *Eutropiichthys vacha* and *Clupisoma garua* are commercially important food fish which have gained popularity among consumers due to their high nutritional value and good taste [1]. The fish are known to occur in rivers, streams, canals, reservoirs, lakes and swamplands of both freshwater and brackish-water bodies in subtropical regions of Asia and throughout the Indian subcontinent including Bangladesh, India, Pakistan, Nepal, Myanmar and Thailand [2]. The fish, *E. vacha* is commonly known as Bacha in Bangladesh [3], Batchwa or vacha in India [4], Cherki in Nepal [5], and Challi in Pakistan [6]. The fish, *E. vacha* and *C. garua* are heavily exploited as a food fish because of high demand in the markets which exceed the supply. The fish sold in the markets come from natural stocks. The overexploitation and overfishing are considered to be major causes of the decline of the abundance of catfish, *E. vacha* and *C. garua* resulted into threatened status in India [7, 8]. The natural environment is damaged and populations of several species are either overexploited or depleted, so the situation demands the necessity of useful genetic information for the management under the selective breeding program for the conservation of threatened species [9, 10]. Biological diversity is the variability among living organisms from all sources, including terrestrial, marine and other aquatic ecosystems and the ecological complexes. This includes diversity within species, between species and of ecosystems [11]. Various organisms including the plankton play a significant role in the dynamics of an ecosystem [12]. RAPD (Random amplified polymorphic DNA) and microsatellite markers are among the molecular markers used to analyze genetic diversity of fish. Both of these markers may be analyzed by PCR (Polymerase chain reaction). RAPD technique is the one of the most frequently used molecular methods for taxonomic and systematic analyses of various organisms and has provided important applications in catfish [13]. Several workers used RAPD as a molecular marker to evaluate the genetic variations in freshwater and marine fish, and notable among them [14-18]. The study of genetic diversity is essential for the long-term survival of the species by the assessment of intra-specific variations because it provides the raw material for adoption and evolution, especially when environmental conditions have changed [19].

The ability of the RAPD technique to revealed the intra-specific variations which can be used in screening for the degree of inbreeding in animal species to prevent an increase in the frequency of deleterious recessive alleles in the populations [20-23]. Information derived from molecular techniques will contribute significantly to the preservation of aquatic genetic resources and their sustainable development [24]. The results indicated that RAPD could be effectively used for genetic diversity analysis in wild species of prospective value as it is reliable, rapid and superior to those based on pedigree information [25]. This is the first study which is based on intra-specific and inter-genic phylogenetic relationships in endangered catfish namely *Clupisoma garua* and *Eutropiichthys vacha* of family Schilbeidae.

2. Materials and Methods

A total of 10 samples of each species of *Clupisoma garua* and *Eutropiichthys vacha* of family Schilbeidae were randomly collected during one year from August, 2014 to June, 2015 from rive Ganga at Allahabad (Latitude-25^0 25'17.31"N, Longitude- $81^0$50'50.25"E, Altitude 285ft) and Gomti at Lucknow (Latitude- 26^0 51'36.83" N, Longitude- $80^0$55'46.28" E, Altitude- 375ft) with the help of local fisherman through netting process. Five samples were collected from each river. All the samples were living in the natural body of water but fish were dead when obtained.

The genomic DNA was isolated from the whole blood using the method as per [26] and analyzed on 1% agarose gel. PCR reaction mixture contained 10× Taq buffer 5µl, 2µl dNTPs mix, RAPD primer 2 µl, 0.5µl Taq DNA polymerase (3U/□µl), and 1µl genomic DNA in a final reaction volume of 50 µl with 39.5 µl water. The PCR cycling were 94 °C for 5 min, 40 cycles of 94 °C for 1 min, 45°C for 1min, 72 °C for 2 min and finally 72 °C for 2min. Genomic DNA was taken for PCR amplification using fluorescent labeled RAPD-PCR with pooling of 3 primers. Primer1:5'CCCHGCAMCTGMTCGCACHC3'; Primer2: 5'AGGHCTCGATAHCMGVY3'; Primer3: 5'MTGTAMGCTCCTGGGGATTCHC3'. The fluorescent labeled PCR products were run on 2% agarose gel. The distance matrix table and family tree were constructed using UPGMA (Unweighted Pair Group Method with Arithmetic Mean). Genetic similarity indices (SI) were calculated from Dice formula in RAP Distance Package Software (Ver. 1.04) [27-29]. Genetic similarity indices values were used to construct the unweighted pair-group method with averages (UPGMA) dendograms, using PHYLIP (Ver. 3.69), to determine the genetic distance between intra-specific and inter-genic populations taken for analysis.

3. Results and Discussion

The present study utilized RAPD marker technique to investigate intra-specific and inter-generic phylogenetic relationships in endangered catfish species i.e. *C. garua* and *E. vacha* of family Schilbeidae. RAPD markers showed a high percentage of polymorphic bands between all analyzed individuals of *C. garua* and *E. vacha* in the present study. In *C. garua*, 9 bands out of 260 allelic bands were found to be unique and other bands were polymorphic, similarly in *E. vacha* 10 bands were unique and other were polymorphic. The RAPD fingerprinting used for discriminating the samples collected from different rivers and between the species indicated the occurrence of phylogenetic relationships within and between the species in *E. vacha* and *C. garua* of family schilbeidae. Less number of bands was noted in *C. garua* as compared to *E. vacha* in the present study. The details of RAPD fingerprinting for the 10 samples of both the species collected from river Ganga are given in Figs.1&2 and band lengths shown in Fig.3. The reproducible bands generated in DNA fingerprinting in the present study were considered for genetic variations within and between the species of schilbeidae. The number and sizes of the bands generated were dependent on the nucleotide sequences of the primers used in RAPD. The 16 DNA fragments in the size range between 35-500 nucleotides (35, 50, 75, 100, 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490 and 500 nucleotides) were single stranded. Interestingly, the degree of conservation in *E. vacha* and *C. garua* was not found to be more between the samples of same geographical region or same river. E1, E3, E2, E9 originated from a different branch and E5, E7 and E4, E8 and E6, E10 were found to be closely related in the phylogeny tree constructed. A marked variation was observed between the samples of *E. vacha* collected from the same geographical region. On the other hand CLG10 and CLG 13, CLG 12 and CLG 8 were more closely related as compared to CLG7, CLG 9, CLG 11, CLG 14, CLG 15, CLG 16.The details of genetic distance of each species are given in Fig. 4 and Fig. 5. In the species of *E. vacha* the highest genetic distance was recorded in E1 (0.08) and lowest in E6 (0.11) and E10 (0.12). Similarly in the samples of *C. garua* the highest genetic distance was recorded in CLG15 (0.09) and lowest in two samples CLG 10 (0.22) and CLG13 (0.24). The phylo-genetic tree generated on the basis of distance matrix (Figs.6, 7), images of RAPD gel and ladder indicated intra-specific variations among the various samples in *E. vacha* and *C. garua* and between the genuses of schilbeidae in the present study.

Genetic distance between populations was determined using indices as given by Nei and Li [28], which showed a significant correlation between genetic identity and geographical distance. The present finding is in consistent with the conclusion of Ikeda *et al*. [30] who reported that the population structure of freshwater organisms was dependent on the distribution of river systems. The presence of genetic variability within species (among populations and also between individuals in the same population) is essential for the survival of species and considered to be a successful response to environmental changes [31]. The study also showed similarity with the findings of Marimuthu *et al*. [32]. The results are considered to be useful to know the genetic structure of both the species. Generally, a genetic diversity study is required for populations that considered to be more adaptive with the environmental changes and can be measured using an array of molecular methods [33]. The present study showed that RAPD primers were informative in detecting the species-specific DNA markers and degree of conservation between the samples.

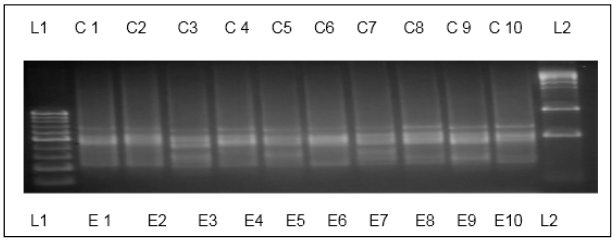

Fig 1: RAPD gel image of 10 samples of *C. garua* from Ganga River (C1 - C5) and Gomti River (C6 - C10). L1: 100 bp ladder, L2: 500 bp ladder.

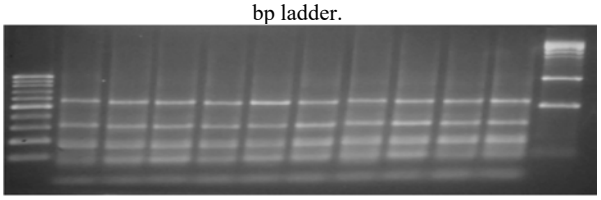

Fig 2: RAPD gel image of 10 samples of *E. vacha* from Ganga River (E1 - E5) and Gomti River (E6 - E10). L1: 100 bp ladder, L2: 500 bp ladder.

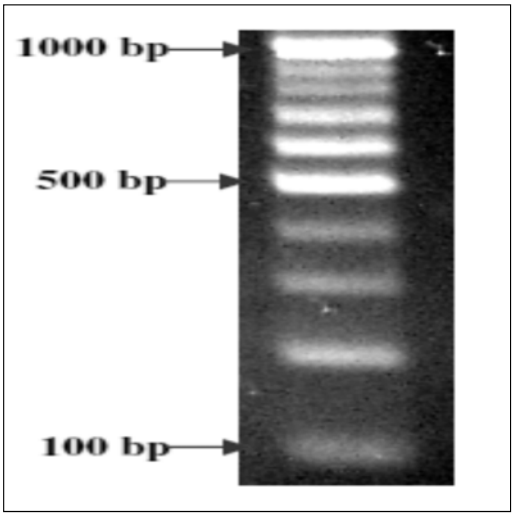

Fig 3: Ladder contains 10 DNA fragments of size 100 bp, 200 bp, 300 bp, 400 bp, 500 bp, 600 bp. 1 ladder= 100 bp.

Table 1: Distance matrix table prepared by UPGMA tree construction method on the basis of RAPD binary output data for 10 samples of *C. garua*. Ganga River (CLG7 – CLG11) and Gomti River (CLG12 - CLG16) 700 bp, 800 bp, 900 bp and 1kb

Nei and Li / Dice **Tree-construction method** **UPGMA * neighbor-joining**										
	CLG 7	**CLG 8**	**CLG 9**	**CLG 10**	**CLG 11**	**CLG 12**	**CLG 13**	**CLG 14**	**CLG 15**	**CLG 16**
CLG7		0.60	0.63	0.22	0.35	0.63	0.24	0.35	0.09	0.30
CLG8	0.60		0.50	0.61	0.63	0.31	0.61	0.60	0.58	0.62
CLG9	0.63	0.50		0.61	0.64	0.57	0.59	0.62	0.55	0.67
CLG10	0.22	0.61	0.61		0.43	0.63	0.30	0.32	0.57	0.40
CLG11	0.35	0.63	0.64	0.43		0.66	0.39	0.42	0.61	0.39
CLG12	0.63	0.31	0.57	0.63	0.66		0.63	0.65	0.64	0.65
CLG13	0.24	0.61	0.59	0.30	0.39	0.63		0.33	0.56	0.38
CLG14	0.35	0.60	0.62	0.32	0.42	0.65	0.33		0.58	0.35
CLG15	0.09	0.58	0.55	0.57	0.61	0.64	0.56	0.58		0.61
CLG16	0.30	0.62	0.67	0.40	0.39	0.65	0.38	0.35	0.61	

Table 5: Distance matrix table prepared by UPGMA tree construction method on the basis of RAPD binary output data for 10 samples of *E. vacha*. Ganga River (E1 - E5) and Gomti River (E6 - E10)

Nei and Li / Dice **Tree-construction method** **UPGMA * neighbor-joining**										
	E1	**E2**	**E3**	**E4**	**E5**	**E6**	**E7**	**E8**	**E9**	**E10**
E1		0.65	0.72	0.09	0.08	0.17	0.25	0.36	0.21	0.12
E2	0.65		0.63	0.68	0.09	0.80	0.09	0.34	0.20	0.26
E3	0.72	0.63		0.69	0.06	0.78	0.06	0.38	0.26	0.36
E4	0.09	0.68	0.69		0.09	0.31	0.07	0.35	0.27	0.31
E5	0.08	0.09	0.06	0.09		0.18	0.26	0.31	0.24	0.34
E6	0.17	0.80	0.78	0.31	0.18		0.27	0.36	0.25	0.34
E7	0.25	0.09	0.06	0.07	0.26	0.27		0.11	0.07	0.05
E8	0.36	0.34	0.38	0.35	0.31	0.36	0.11		0.72	0.35
E9	0.21	0.20	0.26	0.27	0.24	0.25	0.07	0.72		0.32
E10	0.11	0.26	0.36	0.31	0.34	0.34	0.05	0.35	0.32	

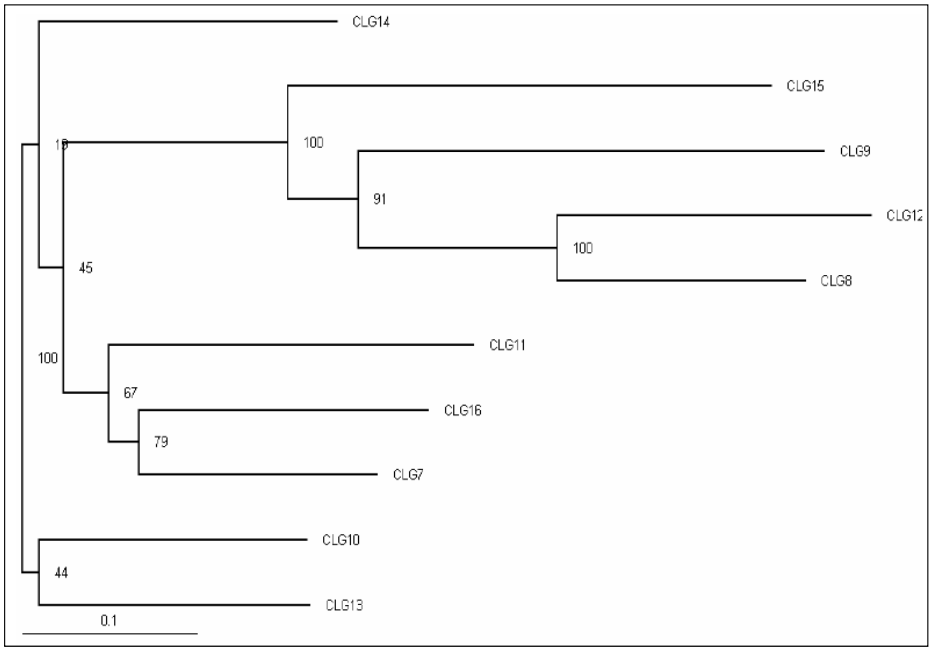

Fig 6: Phylogenetic tree based on total 10 samples of *C. garua* using UPGMA method. Ganga River: CLG7 – CLG11; Gomti River: CLG12 - CLG16.

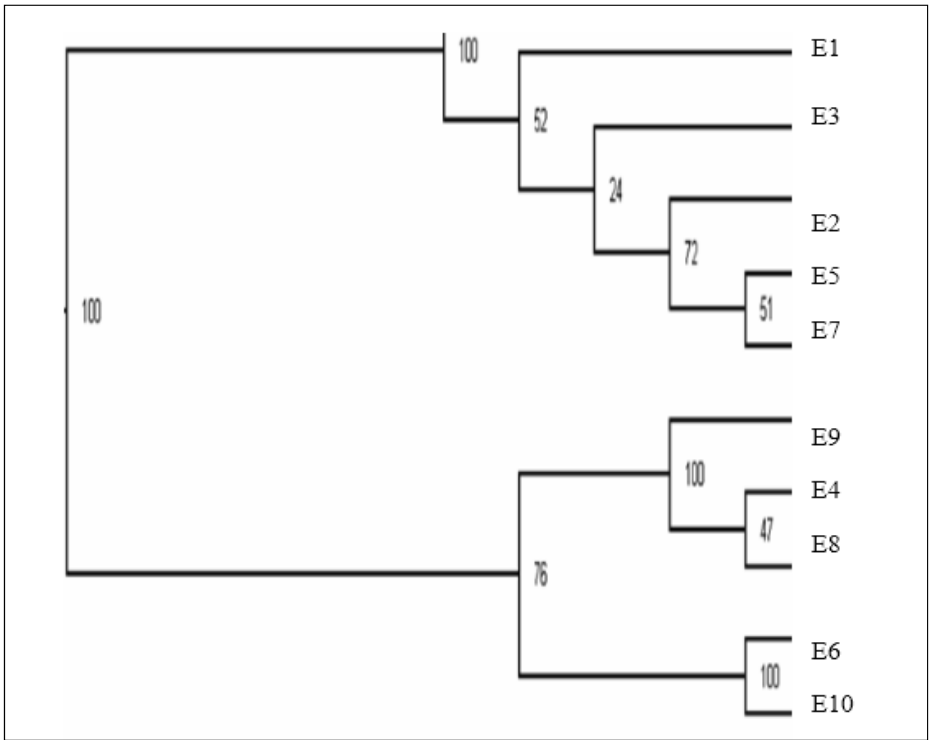

Fig 7: Phylogenetic tree based on total 10 samples of *E. vacha* using UPGMA method. Ganga River: E1 - E5; Gomti River: E6 - E10.

4. Conclusion

The individuals of the same river may not be genetically closely related to each other. The present findings are in contrast to those who considered the individuals of the same environment or geographical region are closely related to each other.

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