RESEARCH PAPER

www.trjfas.org
ISSN 1303-2712
DOI: 10.4194/1303-2712-v18_1_04

Turkish Journal of Fisheries and Aquatic Sciences

Prediction of Potential Hybridization Between Three Major Carps in Ravi River (Punjab, Pakistan) Basin by Using Microsatellite Markers

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Abstract

Three major carps namely; *Labeo rohita, Catla catla, Cirrhinus mrigala* are commercially important aquaculture freshwater fish species of Pakistan. Ascertaining the pure species status of major carps is essential for their appropriate management and conservation. The use of microsatellites DNA markers and Bayesian models based statistical methods have considerably improved the estimation of admixture analyses at individual level. In the present study, a set of twelve heterologus microsatellite markers were employed to assess the extent of potential hybridization in stocks of major carps collected from Ravi River basin. The methods, Structure assignation and Newhybrids status determination statistical analyses were used to determine the status of pure and hybrid individuals. Multidimensional and model-based Bayesian assignment analyses consistently delineated the presence of individuals having mixed-ancestry genome and detected different levels of hybridization between major carps fish species. The clustering analyses showed high efficiency in the detection of F_1 hybrids as well as potential backcross specimens. For the first time, the incidence of natural hybridization between major carp fish species was detected on genetic basis, at frequency rates reaching about 7.77% in Ravi River basin Punjab, Pakistan. The Bayesian model statistical analyses proved to be robust tools in discriminating pure species and hybrid specimens.

Keywords; *Labeo rohita, Catla catla, Cirrhinus mrigala*, Microsatellite markers, Hybridization

Introduction

From last several decades, the role of hybridization in conservation and evolutionary biology has been a central question (Abbott et al., 2013). Natural hybridization plays an important role in the evolution of a species and has been observed in a number of taxa, mainly in fishes (Scribner, Page, & Bartron, 2000) and plants (Whitney, Ahern, Campbell, Albert, & King, 2010). Recently, there is increasing concern on the role of human-mediated hybridization in shaping the existing biodiversity. To determine that the underlying cause of hybridization is natural or anthropogenic is of greater relevance for conservation and management purposes. There is a growing concern that human-induced hybridization is escalating globally, having erratic effects on species integrity (Tallmon, Luikart, & Waples, 2004). Such type of hybridization is in general considered as a dilemma (Rhymer & Simberloff, 1996) while researchers have concentrating on its instant fitness consequences in the first couple of generations (McGinnity et al., 2003; Araki, Cooper, & Blouin, 2007).

Introgressive hybridization also disintegrates the components of genetic diversity that evolve by divergent

www.trjfas.org
ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences DOI: 10.4194/1303-2712-v18_1_04

evolutionary adaptation. To evaluate the risk of biodiversity losses, the consideration of hybridization and the detection of admixed individuals are preliminary steps leading to the risk of outbreeding depression in local populations. Introgressive hybridization in local wild populations simultaneously with loss of ecological structure due to habitat degradation, overexploitation of resources and loss of community structure is recognized as the potential pressure to biodiversity conservation. Hydrological manipulations to natural flow regimes in anthropogenically-impacted river systems have reducing the amount of available habitat for species and probable further limiting the chances to disperse (Frankham, 2010)

The most threatened water regimes in the world are the freshwater ecosystems, being affected by anthropogenic activities such as habitat fragmentation and destruction (Hoagstrom, 2015), introduction of invasive species (Gozlan, Britton, Cowx, & Copp, 2010), fisheries activities (Allan et al., 2005) and aquaculture (Ford, Myers, Palermo, Bailey, & Chen, 2008). Among all animal taxa, freshwater fishes have the highest frequencies of both recent adaptive radiations and hybridization (Scribner et al., 2000). Several instances of hybridization have been renowned in freshwater fishes (Konishi, Hosoya, & Takata, 2003). The ecological impact of hybridization is severing, causing extinction of populations, subspecies, and species by genetic annihilation through introgression (Allendorf, Leary, Spruell, & Wenburg, 2001; Sato et al., 2010).

Hybridization is more prevalent within cyprinids as compared to other fish taxa because species of this group appear to be very closely related with each other as indicated by their karyotypes, isozyme and DNA profile studies. Major carps viz. *Labeo rohita, catla catla* and *cirrhinus mrigala* also belong to this group. A number of inter-specific and inter-generic hybrids from natural ecosystems such as dry bundhs and reservoir have been recorded (Padhi & Mandal, 1997). Moreover, the F_1 hybrids of these species are fertile and can backcross with the parental species thus leading to genetic introgression (Das, Mishra & Srivastava, 1996; Padhi & Mandal, 1997). Due to socio-economic importance of major carps, the wild populations are progressively being replenished with hatchery-reared seed of major carps that is compromising the genetic integrity of the species (Padhi & Mandal, 1997).

Indeed, an important part of any study of hybridization is to confirm the taxonomy of pure-species and hybrids of fish by establishing the reliable methods of identification. Historically, meristic and morphometric measurements were the primary means of identifying naturally occurring hybrids. However, documentation of hybridization based on meristic or morphological criteria often can be misleading when used as the sole source of inference (Neff & Smith, 1979). It is difficult to infer a uniform phenotypical standard on morphological hybrid traits. Considering the amount of information and general reliability in most species, molecular makers are best choice in evaluating whether specific individuals are hybrids. For instance, is it possible by means of molecular markers to trace signs of recent events of introgression, at least a couple of generation backwards (Goodman, Barton, Swanson, Abernethy, & Pemberton, 1999; Verardi, Lucchini, & Randi, 2006).

Carp species; *L. rohita* (Rohu), *C. catla* (Catla) and *C. mrigala* (Mrigal) are commercially important fishes in inland waters of Pakistan. It has been reported that during the last two decades, aquaculturists are facing the problems of high mortality in these species at fingerling stage. Major carps are found in overlapping habitats during the spawning season. The incidences of high level of hybridization between these species have been reported in

www.trjfas.org
ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences DOI: 10.4194/1303-2712-v18_1_04

Bangladesh (Simonsen, Hansen, Mensberg, Sarder, & Alam, 2005). Anecdotal evidences have showed that the conservation value of these fish species in Pakistan is also uncertain and are being genetically deteriorated in many hatcheries and natural reservoirs owing to inadvertent hybridization. The decade-long practice of stocking indigenous carp seed in the rivers and reservoirs to replenish the fisheries resources already made it difficult to determine the negative impact resulting from genetic introgression and inbreeding (Hansen, Bekkevold, Jensen, Mensberg, & Nielsen, 2006). Therefore, better knowledge of ecological and genetic consequences of hybridization is needed to improve the conservation strategies, fisheries and production perspectives that will help in sustainable management of these species in the country. So, the present study was conducted to detect the patterns of potential hybridization and genetic introgression in major carps of River Ravi in the Punjab, Pakistan. Consequently, effective conservation and management of native biota will be enhanced by understanding the extent of potential hybridization and genetic introgression events among these species.

Materials and Methods

Sampling

Thirty individuals each of the three species viz. *L. rohita* (Rohu), *C. catla* (Catla) and *C. mrigala* (Mrigal) were collected from Balloki Head on Ravi River basin. Initially, species identification was based on morphological traits observed on site. Specimens were brought live to the Aquaculture Biotechnology Laboratory, Department of Zoology, Wildlife and Fisheries at University of Agriculture Faisalabad and identified up to species level by using appropriate fish identification key following Mirza and Sharif (1996). A sample of dorsal muscle tissue was taken from each fish and stored at -20 °C for subsequent DNA extraction.

DNA extraction

Traditional proteinase-K digestion and standard phenol/chloroform protocol following the Yue and Orban (2005) was used to extract the total genomic DNA from small amounts (0.2 g) of muscle tissues. The quality of extracted DNA was checked on 0.8% agarose gel in horizontal gel electrophoresis chamber (ME20, Major Science, USA). While the concentration of extracted DNA was checked by using UV-Vis spectrophotometer (NanoDrop 2000c, Thermo Scientific, USA) and final concentration was adjusted by diluting to almost 50 ng/ml.

Genotyping

A set of twelve heterologous microsatellite primers already characterized for *L. rohita* (Patel et al., 2009) *C. catla* (Sahu et al., 2014) *Cyprinus carpio* (Crooijmans, Poel, Groenen, Bierbooms, & Komen, 1997) and *Barbodes gonionotus* (Kamonrat, McConnell, & Cook, 2002) were employed for cross-species amplification. The characteristics of the microsatellite DNA markers used in the present study have been summarized in Table 1. These oligo primers *Lr1, Lr10, Lr31, Lr38, Cc15, Cc19, Cc31, Cc62, Cc70, MFW2, MFW17* and *Bgon22* were purchased from the company Gene-link, USA and used to amplify the target loci from each individual. The PCR reaction was carried out in a 20 µL reaction mixture, which included 0.8 µL of each primer set (10 µM), 0.4 µL of dNTPs (10

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ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences DOI: 10.4194/1303-2712-v18_1_04

mM), 1.5 µL $MgCl_2$ (20 mM), 2.0 µL of 10X PCR buffer (20 mM), 0.4 µL (2 U/µL) Taq polymerase (Sure Bio-Diagonistic and Pharmaceutical), and approximately 50 ng of template DNA using gradient thermal cycler (Multigene Optimax, LabNet, USA).

The PCR amplification of target microsatellite loci was carried out following the PCR reaction conditions as follows: Preheating for five minutes at 94 ℃, 30 cycles consisting of 1 minute denaturation at 94 ℃, 1 minute at a primer-specific annealing temperature, 1 minute at 72 ℃ elongation step, and final extension period was appended for 10 minutes at 72 ℃ . The amplicons were confirmed for the successful amplification through 0.8% agarose gel electrophoresis and subsequently stored at 4 ℃ until analyzed. In case some DNA sample failed to amplify, PCR was repeated for the missing individuals.

Polyacrylamide gel electrophoresis

Five µL of the PCR product of each individual was mixed with 1 µL loading dye (Bromophenol Blue) and loaded on to the polyacrylamide gel along with 3 µL 100 bp DNA ladder (FERMENTAS: MBI #SM0321). The electrophoresis was done in a vertical gel electrophoresis chamber (MV20DSYS, Major Science, USA) at constant voltage (286Volts) for 90 minutes. After electrophoretic resolution of PCR products, removed the plates gently and washed the gel for ethidium bromide staining. The microsatellite alleles were score manually for genotyping and the genotypic data was recorded for statistical analyses.

Data Analyses

Factorial correspondence analysis (FCA) test was implemented to investigate the relationships among individuals based on the extent of allele sharing among major carps and hybrids using the software Genetix version 4.05.2 (Belkhir, Borsa, Goudet, Chikli, & Bonhomme, 2000). Clustering algorithms employing Markov-Chain Monte-Carlo (MCMC) simulations were implemented in the programs Structure version 2.1 (Pritchard, Stephens, & Donnelly, 2000) and Newhybrids version 1.1 (Anderson & Thompson, 2002) to analyze the data from the twelve microsatellite loci scored in all the analyzed fish species.

The admixture analyses made in a Structure 2.1 uses the HWE expectations and linkage disequilibrium to determine whether the suspected hybrids had ancestry from both species, and to investigate undetected mixed-ancestry in individuals of each population. Analyses were performed with 1, 000, 00 Markov-Chain Monte-Carlo step (MCMC) and 100, 00 burn-in steps for K = 1-3 potential species groups. To determine the actual number of species groups method of ΔK statistic (Evanno, Regnaut, & Goudet, 2005) was implemented.

The Newhybrids software was used to probabilistically determine the status of each individual by assign to one of six different categories (P1, P2, F_1, F_2, Bx1, Bx2). Assignment probabilities were predictable with a 50,000 MCMC sweeps and of 10,000 sweeps of burn-in period. Jeffreys (Uninformative) priors were positioned on admixture distributions. Final class assignments were based on the category with the highest probability, usually greater than 90%. The use of probabilities allowed for a transparent estimation of class in light of possible allelic scoring error and the rare presence of fourth- or fifth-generation hybrids.

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ISSN 1303-2712
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Results

The multi-locus genotypic data of each appraisal population was subjected to three consecutive statistical analyses including Factorial Correspondence Analysis (FCA), Structure assignment test and Newhybrids status determination for discriminating the pure, hybrid and genetically introgressed individuals involving the three major carp species and is explained as following.

Multidimensional analysis

The FCA test highlighted the three main clusters, each corresponding to one of the analyzed species depicting the departures among these species as shown in Figure 1. For the assessment of hybridization sign it was observed that some individuals of *L. rohita*, *C. catla* and *C. mrigala* population were fall out of their pure species cluster though were considered morphologically as pure species. The results showed that these individuals were erroneously labeled as pure species and, in fact, they might be hybrids to be tested.

Structure-based admixture analysis

The subsequent statistical test to detect the hybridization was conducted with the program Structure which confirmed the presence of three species clusters: (1) *L. rohita* (2) *C. catla* (3) *C. mrigala* (Figure 2a). The 83 individuals with exception of some individuals powerfully exhibited q-values greater than 0.9 and dispersed to their corresponding pure species. The remaining total of the seven individuals (7.77%) LR-BAH5 (q_{LR}=0.767), LR-BAH12 (q_{LR}=0.640), LR-BAH16 (q_{LR}=0.792), CC-BAH10 (q_{CC}=0.787) and CC-BAH18 (q_{CC}=0.492), CC-BAH26 (q_{CC}=0.660) and CM-BAH19 (q_{CM}=0.7930) showed the membership coefficient values (q) lower than 0.9 which led to the hypothesis that these individuals are of admixed ancestry. Out of these seven putative hybrid individuals, three specimens (LR-BAH5, LR-BAH12, LR-BAH16) in *L. rohita* population and three (CC-BAH10, CC-BAH18, CC-BAH26) in *C. catla* population showed partial assignment to both *L. rohita* and *C. catla* cluster and were considered as LR×CC hybrids. However, in *C. mrigala* stock, only one individual (CM-BAH19) showed partial assignment to both *L. rohita* and *C. mrigala* cluster and was characterized as hybrid of LR×CM category (Figure 2b). The proportion of hybridization was 10% in *L. rohita*, 10% in *C. catla* and 3.33% in *C. mrigala* population. All the hybrid individuals were found to be of LR×CC and LR×CM category.

Newhybrids determination of hybrid categories

In order to categorize the admixed individuals into F_1, F_2 and later hybrid categories the hybrid individuals together with their genitor species were subjected to analysis in the program Newhybrids. All individuals that were labeled as *L. rohita* and *C. catla* showed a posterior probability higher than 0.97 to be composed of one of the two species. Figure 3 shows the Newhybrids statistical analysis that included the *L. rohita*, *C. catla* and their hybrids. Regarding the hybrids between these two species, LR-BAH5, LR-BAH12, LR-BAH21 hybrid individuals were differentiated as backcross of *L. rohita* in *L. rohita* population. In *C. catla* population, a total of three specimens; CC-BAH10 and

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ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences DOI: 10.4194/1303-2712-v18_1_04

CC-BAH26 and CC-BAH18 were characterized as backcross of Catla and F_1 hybrid, respectively. The Newhybrids statistical analysis that included the *L. rohita* and *C. mrigala* is shown in Figure 4. Regarding the hybrid individual (CM-BAH19) between these two species that was differentiated by the program Structure in *C. mrigala* population was categorized as backcross of *C. mrigala*.

Discussion

The current study is the first to detect the extent to which major carps have been hybridizing in wild environment, Ravi River basin in southern Punjab, Pakistan.

From last several decades, hybridization between fish species has become a serious issue in conservation and evolutionary biology. There is a growing concern over the ecological and genetic impacts on the pristine parental populations of a species. In Pakistan, studies on genetic aspects of fish particularly indiscriminate hybridization and genetic introgression are scarce; the utmost required for sustainable management and conservation of fisheries resources. Added to the poor fitness of major carps in growing anthropogenic perturbations, the maintenance of genetic integrity of these species is immensely required in both wild and captive environment. The molecular markers are robust tools to analyze the genetic integrity of populations (Chauhan & Rajiv, 2010). To differentiate the pure and hybrid lineage is a substantial step towards the development of conservation and management strategies for native species (Allendorf et al., 2001; Docker, Dale, & Heath, 2003; Gunnell, Tada, Hawthorne, Keeley, & Ptacek, 2008).

Our results based on twelve microsatellite loci suggested that hybridization between major carps has been occurring in the Ravi River basin. Microsatellites markers have been proven to be very useful tool for identifying major carp hybrids taking into account that the alleles of an individual represent a combination of parental alleles (Chauhan & Rajiv, 2010). Microsatellites have recently been applied in many hybridization studies (Nolte, Gompert, & Buerkle, 2009; Vandeputte & Haffray, 2014). Several researchers have used the microsatellite markers for parentage assignment that is essential for broodstock management (Borrell et al., 2011).

In present study, the microsatellite based genotypic data of major carps of each population was subjected to assignment analyses implemented in programs Genetix, Structure and Newhybrids to investigate undetected mixed-ancestry in individuals of major carps. Bayesian and Maximum likelihood analyses are especially useful in investigating the populations which may not be in equilibrium (Excoffier & Heckel, 2006). These statistical methods do not necessarily require pure representatives of the parental species be available, and do not require that the different groups being analyzed have diagnostic alleles because these are based on the probability of individual genotypes at multiple loci to belong to parental species, taking into account the allele frequencies of parental species. Such methods have been frequently used for hybrid detection and introgression measurement (Lajbner, Linhart, & Kotlik, 2011; Dudu et al., 2011).

Bayesian model-based clustering analyses revealed the presence of three taxa corresponding to the examined species *(L. rohita, C. catla* and *C. mrigala*). The assignment analyses confirmed the mixed nuclear-genome ancestry of one phenotypically identified putative hybrid. Putative mixed ancestry was also detected in three individuals from *L.*

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ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences DOI: 10.4194/1303-2712-v18_1_04

rohita, two from *C. catla* and one specimen from *C. mrigala*, morphologically identified as Rohu, Mrigal and Catla, respectively. Overall, the extent of hybridization was found to be 7.77% in major carp populations of River Ravi. All the hybrids individuals were found to be of LR×CC and LR×CM category. The hybrid individuals detected in *L. rohita* population were differentiated as backcross of *L. rohita*. However, in *C. catla* population, both F1 and backcross individuals were detected while in *C. mrigala*, only backcross individuals were observed.

Due to commercial significance of major carps, the fisheries department of Punjab, Pakistan launches a campaign of releasing fish seed of major carps in natural waters every year under a coordinated programme so that these native species could be conserved on sustainable grounds in the country. About 10 thousand fish seed is released annually at Head Balloki River Ravi under this program. The current introgressive hybridization, however, has not led to a swamping of the gene pool of major carps. Senanan, Kapuscinski, Na-Nakorn and Miller (2004) reported that the release or escape of fertile hybrids in nature results in consequences of introgressive hybridization.

Major carps have spatial reproductive isolating mechanisms. It has been suggested that spatial isolating mechanisms are most frequently circumvented by human activities including water management projects, such as dam and canal construction, water diversion for agriculture activities, dredging, pollution and droughts decreasing stream flows, limiting the spawning habitat (Grady & Cashner, 1988). Carlson, Pflieger, Trial and Haverland (1985) reported that suspected hybrids of Pallid and Shovelnose Sturgeon appear to be more common in areas with reduced habitat diversity, where homogenized habitat may lead to a loss of reproductive isolation. Recent evidence indicates that adaptation to distinct environments has an important or even predominant role in reproductive isolation (Schemske, 2000). Rundle, Nagel, Boughman and Schluter (2000) examined that the three-spine Stickleback fish evolved to benthic and limnetic forms with different sizes and diets. Tests of mating preference revealed that benthics and limnetics from the same or different populations showed reproductive isolation.

Breakdown of reproductive barriers between species leads to the loss of genotypically distinct populations with subsequent mixing of gene pools (Hasselman et al., 2014). Recently, the extinction of several Orestias fish species in the Lake Titicaca has been reported due to their narrow geographic distribution and occurrence in highly exploited habitats (Carson et al., 2014). Recent research has indicated that introgressive hybridization has been so extensive between white suckers and flannel mouth suckers that the genetic integrity of flannel mouth suckers has been eroded to the point where they are at risk of local extinction in Muddy Creek (McDonald, Parchman, Bower, Hubert, & Rahel, 2008)

The occurrence of natural hybridization may be due to anthropogenic activities such as destruction of spawning grounds. The congestion in the spawning ground may result accidently the ova of one species getting fertilized by the sperm of another species. Particularly, in the case of major carps with very compatible genomic structure (Nagpure, Kushwaha, Sriyastava, & Ponniah, 2001), it is more easy and frequent to encounter natural hybrids among major carps when bred together in relatively congregated conditions. Hundred of the carp species breed together in the, bundh-type ponds, a small spawning area, and incidence of natural hybridization is comparatively frequent in these areas. The probable reason for this type of hybridization might be the accidental fertilization of eggs by the sperms of another species due to the overcrowding in the spawning grounds.

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ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences DOI: 10.4194/1303-2712-v18_1_04

The detection of hybrids by employing the statistical methods is secure. In present findings, almost perfect correspondence was seen between the findings of three successive statistical analyses, in majority of the cases based on totally different concepts (Figures 1, 2, 3 and 4). The present preliminary results suggested that introgressive hybridization is occurring between the major carps species in Ravi River basin. As, the observations of farmers and scientists indicated that wild populations of major carps in River Ravi are declining due to habitat loss, pesticide use and over exploitation. Findings of present study suggest that the diagnosis of pure parental and hybrid specimens is a key point in such conservation projects relying on the captive-breeding of wild specimens.

As a precautionary principle, and in order to limit the risks of hybridization, it is recommended that captive-bred populations are screened for purity of major carps on genetic basis. Habitats of major carps are being deteriorated due to increase in anthropogenic pressure and there is a genuine opportunity for hybridization events to increase in numbers and sites. In this context, the development of diagnostic methods to detect hybrids will be an important achievement for the survey of hybridization patterns among species of major carps and related taxa.

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Table 1. Characteristics of twelve microsatellite loci used for cross-species amplification in major carps

Sr. No	[1]-Locus name	[1]-Motif	[1]-Primer sequence (5´……3´)	[1]- Gene-Bank Accession no.	[2]-Fragment size (bp)	[2]-T_a(°C)	[2]-Allele Number
1	[a]*Lr1*	$(TG)_{14}$	F-GACCCTTAACCCTTGACCTT R-TGGGATAATGCAGGGAAAAC	AJ507518	167-171	58	2
2	[a]*Lr10*	$(CA)_{13}$	F-GATCTTCAGCGCCAGCGTG R-GAGGACCTGCCCAGCATG	AJ507523	141-165	60	4
3	[b]*Lr3*	$(CT)_{23}$	F: CACTCTTATCTCGCTGCCCAG R: ACAAGCGGTCTGTGGTGAGTC	AM231180	158-162	60	4
4	[b]*Lr38*	$(GT)_{12}$	F: ATAGCATCACCATCTGTTGGTG R: TCTGCTTCAGTCACTCAGCAC	AM269528	240-250	59	2
5	[c]*Cc15*	$(CA)_6$	F: GGGTTGCTCTCTAAAACTCTGG R: CTCCTTCTGCTCTCTCTGCTCT	KF913010	152-170	57	3
6	[c]*Cc19*	$(TG)_6$	F: CATGTGTATGCTTTGTACTGTGAG R: CAATTCACCACCGATTCTTTTG	KF913011	140-170	54	4
7	[c]*Cc31*	$(GT)_{14}$	F: TGTCTAGGTGTGTTTCTCTGTGG R: GAACATGAGCGGGAAAACTG	KF913013	142-172	59	5
8	[c]*Cc62*	$(GA)_7$	F: TCCAACCATCCATATCAGCTAC R: TGACGACGCTATCTTCTCTCTTT	KF913018	180-210	60	4
9	[c]*Cc70*	$(TG)_6$	F: CGCTCAGGTTACCCAGCATT R: CACACACACACGCAACAGATAC	KF913019	160-186	60	3
10	[d]*MFW2*	$(TG)_6$	F: CACACCGGGCTACTGCAGAG R: GTGCAGTGCAGGCAGTTTGC	---------	170-175	55	4
11	[d]*MFW17*	$(GT)_{14}$	F:CTCAACTACAGAGAAATTTCATC R:GAAATGGTACATGACCTCAAG	---------	216-225	55	5
12	[e]*Bgon22*	$(TCC)_6$	F: TCTTGTTGATCACACGGACG R: GTGACTGTATCAATGAGTCTG	AJ291680	110-113	49	6

[a]- (Das *et al.*, 2005); [b]- (Patel *et al.*, 2009), [c]- (Sahu *et al.*, 2014); [d]- (Crooijmans *et al.*, 1997); [e]- (Kamonrat *et al.*, 2002); F– forward; R – reverse; N- no. of alleles 2) Fragment size, annealing temperature and allele number observed in current study

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ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences DOI: 10.4194/1303-2712-v18_1_04

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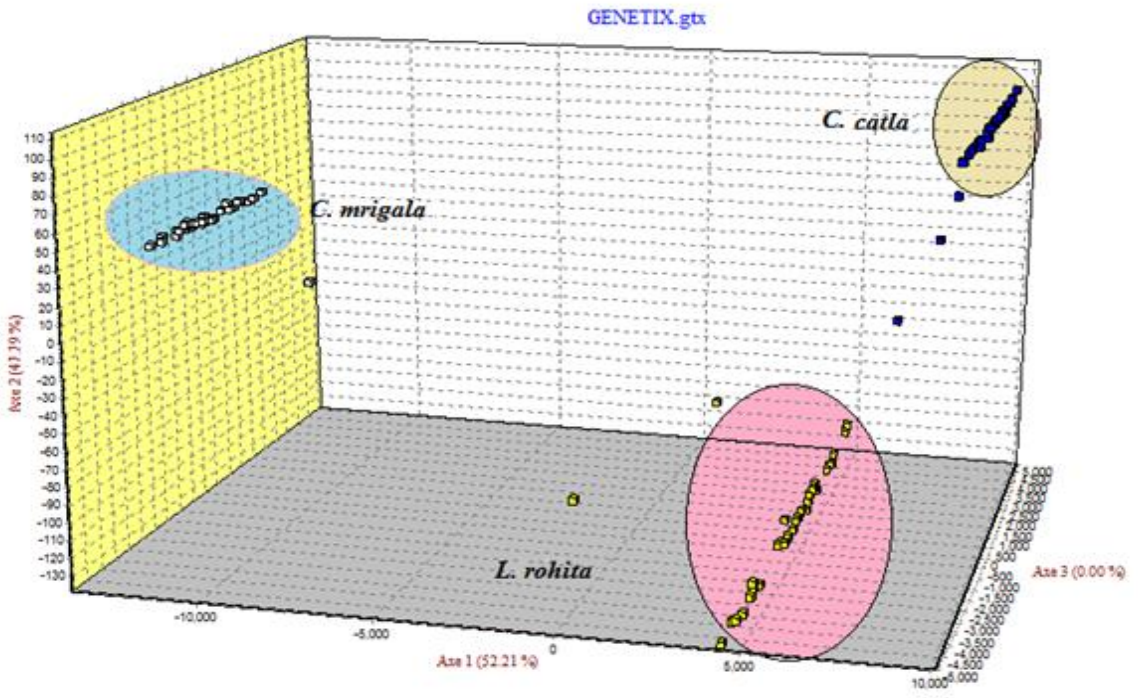

Figure 1. Factorial Correspondence Analysis of all the major carps individuals of River Ravi populations based on microsatellite genotyping

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DOI: 10.4194/1303-2712-v18_1_04

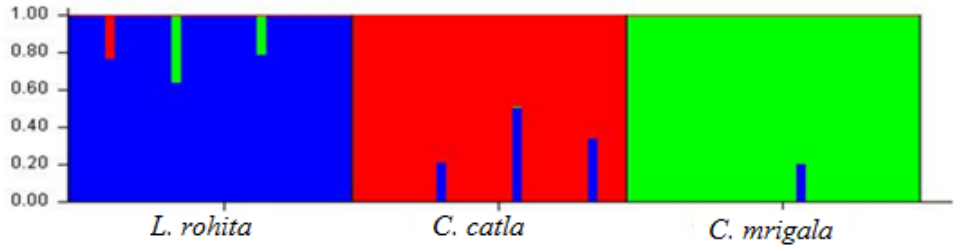

Figure 2a. Histogram showing the clustering on the totality of specimens of River Ravi populations of major carps computed by STRUCTURE with K = 3

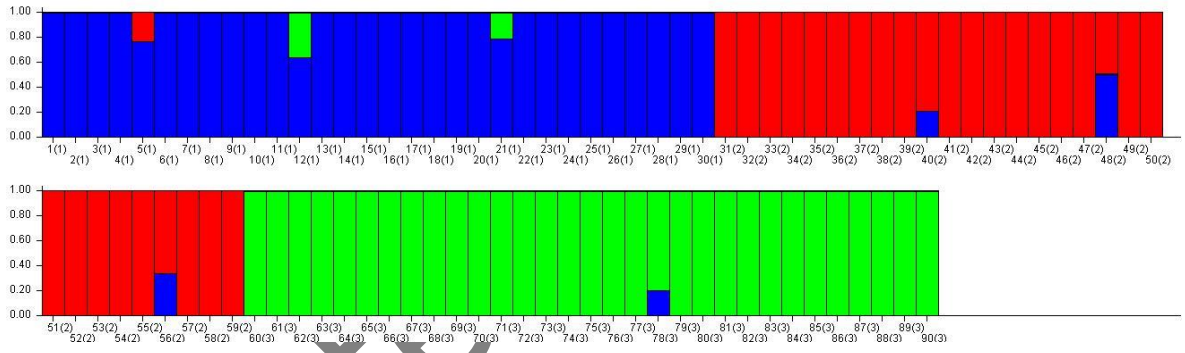

Figure 2b. Proportion of the model-based clusters in the ancestry of each sample of River Ravi populations; in blue: ''*L. rohita*'' cluster; in red: ''*C. catla*'' cluster and in green: *C. mrigala*''. Each individual is represented as a vertical bar. Composite bar, partitioned into two segments whose length is proportional to the estimated membership in the two clusters, represent expected hybrid

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ISSN 1303-2712
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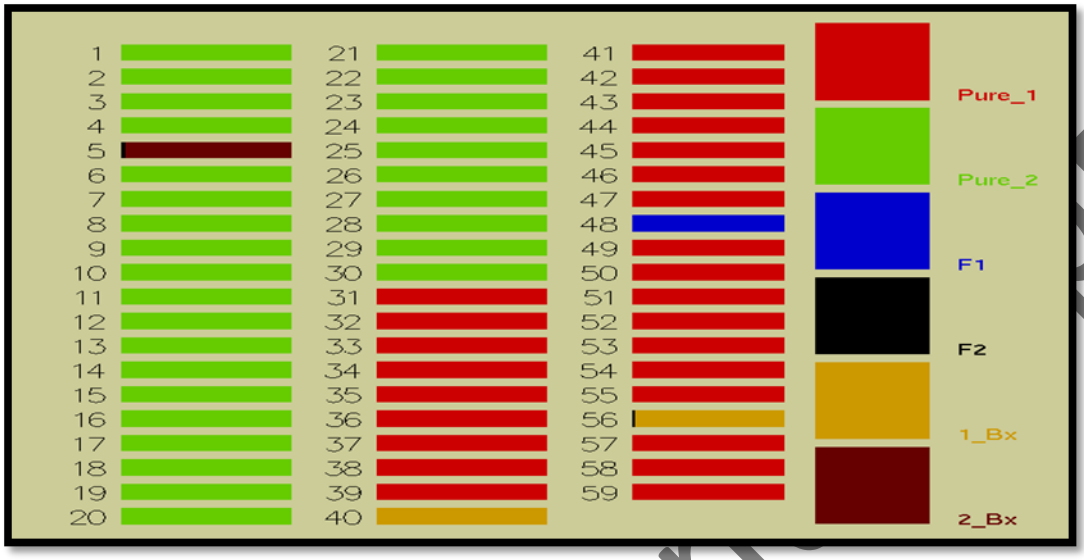

Figure 3. Admixture categorization of the *L. rohita* and *C. catla* specimens of River Ravi populations; green bar: ‘‘Pure *L. rohita*’’; red bar: ‘‘Pure *C. catla*’’; blue bar: F_1 hybrid; yellow bar: backcross of F_1 with pure *C. catla* and brown bar backcross of F_1 with pure *L. rohita*: Each individual is represented as a horizontal bar

Figure 4. Admixture categorization of the *L. rohita* and *C. mrigala* specimens of River Ravi populations; red bar: ‘‘Pure *L. rohita*’’; green bar: ‘‘Pure *C. mrigala*’’; yellow bar: backcross of F_1 with pure *L. rohita* and brown bar backcross of F_1 with pure *C. mrigala*. Each individual is represented as a horizontal bar