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Development and Characterization of RAPD and Microsatellite Markers for Genetic Variation Analysis in the Critically Endangered Yellow Catfish *Horabagrus nigricollaris* (Teleostei: Horabagridae)

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Abstract Random-amplified polymorphic DNA (RAPD) and microsatellite markers were developed and used for the analysis of genetic variability in the critically endangered yellow catfish *Horabagrus nigricollaris*, sampled from the Chalakkudy River, Kerala, India. Eight RAPD and five microsatellite markers were detected to genotype the species. In RAPD, the 73 fragments were 20.55% polymorphic, whereas 4 polymorphic loci (80%) were obtained in microsatellites. In microsatellites, the number of alleles across the 5 loci was 1–5, and the range of heterozygosity was 0.25–0.5. The mean observed number of alleles was 2.4, and the effective number was 1.775 per locus. The average heterozygosity across all investigated samples was 0.29, indicating a significant deficiency of heterozygotes in this species. RAPD and microsatellite methods report a low degree of gene diversity and lack of genetic heterogeneity in the population of *H. nigricollaris,* emphasizing the need for fishery management, conservation, and rehabilitation of this species.

Keywords *Horabagrus nigricollaris* · RAPD · Genetic variation · Microsatellites · Polymorphism

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Introduction

Horabagrus nigricollaris (Pethiyagoda and Kottelat 1994) is a critically endangered (CAMP 1998), endemic, cultivable yellow catfish belonging to the family Horabagridae. It is found only in the Chalakkudy River, originating from the southern part of the biodiversity hotspot of the Western Ghats, Kerala, India. It inhabits hill streams at upper reaches of the river. It is a benthophagic omnivore, and its basic food is terrestrial and aquatic insects (Prasad et al. 2008). *Horabagrus nigricollaris* reaches about 27 cm in length (Menon 1999), and it has a black stripe extending over the neck, forming a black collar, hence the scientific name (Pethiyagoda and Kottelat 1994). Its attractive golden yellow color makes it a valuable species for the aquarium in India and abroad. Over exploitation (especially for trade), destructive fishing methods that lead to habitat alteration, pollution, and related anthropogenic interventions in the natural habitats have considerably reduced the wild population of this species during the last few years (Prasad et al. 2008). The workshop of the Conservation Assessment Management Plan (CAMP) to evaluate the status of freshwater species of India, held at the National Bureau of Fish Genetic Resources in 1997, categorized this species as critically endangered based on criteria of the International Union for Conservation of Nature and Natural Resources (IUCN) (CAMP 1998). So the information on stock structure of *H. nigricollaris* is essential as the species is presently prioritized for ornamental fish culture from Peninsular India (Ponniah and Gopalakrishnan 2000). Proper knowledge of the genetic make-up and variability of critically endangered *H. nigricollaris* stock will help us in the management, conservation, and improvement of stocks and designing captive propagation plans. Lack of definite information on the genetic make-up or stock of fish fauna of a river system is impeding the planning and implementation of proper conservation and management strategies. We have reported the stock structure and genetic variation in another species of the genus (*H. brachysoma*) from different populations using allozymes, RAPD, and microsatellite markers (Muneer et al. 2007, 2008, 2009), but information on the life history traits, stock structure, and basic genetic profile of *H. nigricollaris* is lacking.

Random-amplified polymorphic DNA (RAPD) analysis is a technique based on the polymerase chain reaction (PCR) amplification of discrete regions of genome with short oligonucleotide primers of arbitrary sequence (Welsh and McClelland 1990; Williams et al. 1990). The method is simple, less expensive, and quick to perform, and most important, no prior knowledge of the genetic make-up of the organism is required (Hadrys et al. 1992). RAPD has some disadvantages, like its dominant nature (homozygotes and heterozygotes cannot be distinguished) and low reproducibility (Dinesh et al. 1995). Because the RAPD technique surveys numerous loci in the genome, it is particularly attractive for analysis of genetic diversity and phylogeny (Clark and Lanigan 1993). Stock identification of several species has been carried out using the RAPD technique (Klinbunga et al. 2000; McCormack and Keegan 2000). RAPD has also been used for phylogenetic studies for species and subspecies identification of fish (Bardakci and Skibinski 1994; Borowsky et al. 1995). Few authors have carried out RAPD analyses in catfish (Liu

et al. 1998; Kovacs et al. 2001). Liu et al. (1999a) have reported the usefulness of RAPD markers for gene mapping in channel (*Ictalurus punctatus*) and blue catfish (*Ictalurus furcatus*). Microsatellites are polymorphic DNA sequences containing short tandemly arranged repetitions (Tautz 1989), distributed throughout the genome (Litt and Luty 1989), and found in all prokaryotic and eukaryotic genomes studied until now (Zane et al. 2002). Due to their high variability, these genetic markers have been widely used in genetic mapping (Knapik et al. 1998; Coimbra et al. 2003) and population structure studies (Hatanaka et al. 2006; Primmer et al. 2006). Several investigators have made use of microsatellite markers in various catfishes (Galbusera et al. 1996; Volckaert et al. 1999; Liu et al. 1999b; Usmani et al. 2003; Feng et al. 2009).

In this study, we analyzed the genetic diversity in *H. nigricollaris* collected from the Chalakkudy River, Kerala, using RAPD and microsatellite markers. The microsatellite flanking regions from siluriform species such as *Pangasius hypophthalmus* (Volckaert et al. 1999), *Clarias macrocephalus* (Na-Nakorn et al. 1999), and *Clarias gariepinus* (Galbusera et al. 1996) were used to study the cross-species amplification of microsatellites in *H. nigricollaris.*

Materials and Methods

Sampling

Specimens of *H. nigricollaris* were obtained through commercial catches from the Chalakkudy River at the Vettilappara site (N 10°17′, E 76°32′). Only 21 specimens could be collected for the genetic variability study. Blood samples for DNA extraction were collected from the caudal vein, to avoid contamination, using heparin (Biological E., Hyderabad, India) as anticoagulant from the live fish immediately after capture, and stored in 95% ethanol.

RAPD Marker Analysis

Total DNA was extracted from blood samples following the procedures of Taggart et al. (1992). Thirty decamer primers (20 from the OPA series and 10 from the OPAH series; Operon Technologies, Alameda, Calif.) were used for RAPD analysis. From these, 8 primers were selected by detecting 6–19 sharp, high-intensity, reproducible bands using 10 individuals from each of the populations (Table 1). RAPD-PCR reactions were run in a PTC 200 thermal cycler (MJ Research, Watertown, Mass.) employing the RAPD primers described in Table 1. Amplification was performed in 25 µl reaction volume containing 1× reaction buffer (100 mM Tris, 500 mM KCl, 0.1% gelatin, pH 9.0) with 1.5 mM $MgCl_2$ (Genei, Bangalore, India), 5 pmol primer, 0.2 mM dNTPs, 2 U *Taq* DNA polymerase (Genei, Bangalore, India), and 25 ng template DNA. To check DNA contamination, a negative control was set up omitting the DNA from the reaction mixture. The reaction mixture was preheated at 95°C for 3 min, followed by 40 cycles of 94°C for 3 min, 40°C for 1.30 min, and 72°C for

Table 1 Genetic variation in *Horabagrus nigricollaris* with RAPD markers

Primer code	Sequence (5′-3′)	Number of bands		Number of alleles		Average Heterozygosity
		Total	Polymorphic (%)	Observed	Effective	
OPA-07	GAAACGGGTG	9	2 (22.22)	1.222	1.205	0.107
OPA-09	GGGTAACGCC	9	3 (33.33)	1.333	1.302	0.158
OPA-11	CAATCGCCGT	9	1 (11.11)	1.111	1.109	0.055
OPA-20	GTTGCGATCC	6	1 (16.67)	1.167	1.089	0.058
OPAH-01	TCCGCAACCA	12	2 (16.67)	1.167	1.145	0.078
OPAH-02	CACTTCCGCT	7	2 (28.57)	1.286	1.157	0.093
OPAH-04	CTCCCCAGAC	11	3 (27.27)	1.273	1.217	0.119
OPAH-08	TTCCCGTGCC	10	1 (10.00)	1.100	1.087	0.047
Total	–	73	15 (20.55)	–	–	–
Mean	–	–	–	1.206	1.167	0.090
SD	–	–	–	0.407	0.343	0.182

2 min; and a final extension at 72°C for 10 min. The resulting products were electrophoretically analyzed through 1.5% agarose gels stained with ethidium bromide (5 μg/ml) in TBE buffer (90 mM Tris–borate and 2 mM EDTA, pH 8.0). Gels were documented using an Imagemaster 1D gel documentation system (Amersham Biosciences, USA). The pictures were used for the analysis of the amplified products, and the numbers of bands were counted as seen in the photographs. A binary matrix was produced whereby each DNA fragment for each sample was recorded as present (1, dominant) or absent (0, recessive). Faint or poorly amplified fragments were excluded from the analysis. The DNA marker applied along with RAPD samples helped to determine the molecular sizes of the DNA fractions of the fish samples. The RAPD data were analyzed using Popgene version 1.31 (Yeh et al. 1999).

Microsatellite Marker Analysis

Five primers (microsatellite flanking regions) from other siluriform species (downloaded from the NCBI site) were used to study the cross-species amplification of microsatellites in *H. nigricollaris* (Table 2). Three of these microsatellite primers were from the species *P. hypophthalmus* (Volckaert et al. 1999), and one was from each of two *Clarias* species, *C. macrocephalus* (Na-Nakorn et al. 1999) and *C. gariepinus* (Galbusera et al. 1996). The PCR reactions were run as before, employing the microsatellite primers (Table 2). Amplifications were performed in a 25 μl reaction mixture containing 1× reaction buffer (10 mM Tris, 50 mM KCl, 0.01% gelatin, pH 9.0) with 1.5 mM $MgCl_2$ (Genei, Bangalore, India), 5 pmol each primer, 0.2 mM dNTPs, 2 U *Taq* DNA polymerase (Genei, Bangalore, India), and 25 ng template DNA. To check DNA contamination, a negative control was set up omitting the DNA from the reaction mixture. The reaction mixture was preheated at 94°C for 5 min, followed by 25 cycles of 94°C for 30 s, with annealing temperature

Table 2 Genetic variation at five microsatellite loci in *H. nigricollaris*

Primer	Sequence 5′-3′	Locus	Alleles			Heterozygosity		Inbreeding Coefficient (F_{IS})
			Observed number	Effective number	Frequency	Observed	Expected	
Phy01								
F	CGAACACGCCACAGAGAGTA	*Hn Phy01*	5	3.463	0.450, 0.175	0.350	0.730	0.521
R	CCACACCCACAACACCATAA				0.200, 0.100, 0.075			
Phy05								
F	CCAGCAACCCACATAATTGA	*Hn Phy05*	1	1.000	1.000	0.000	0.000	–
R	CAGCTCAGGGCCAAAAGTAG							
Phy07								
F	AGTCACTTCAGCACCTGCCT	*Hn Phy07*	2	1.600	0.750, 0.250	0.500	0.385	−0.299
R	ATCTCTGTGATGGTGAGCCA							
Cma03								
F	TTCGGATTGTTTCTGTGG	*Hn Cma03*	2	1.406	0.825, 0.175	0.250	0.296	0.155
R	ACACTCTTTACACTGATT							
Cga06								
F	CAGCTCGTGTTTAATTTGGC	*Hn Cga06*	2	1.406	0.825, 0.175	0.350	0.296	−0.182
R	TTGTACGAGAACCGTGCCAGG							
Mean			2.4	1.775	–	0.290	0.341	0.149
SD			–	0.969	–	0.185	0.261	–

depending on the T_m value of the primer (usually 50–60°C), and 72°C for 1 min). The reaction was then subjected to a final extension at 72°C for 2 min. The amplified product was checked in 10% polyacrylamide gel electrophoresis (PAGE). The amplified microsatellite loci were visualized using silver staining (Amersham Pharmacia Kit) of the polyacrylamide gel. The molecular weight of the bands was calculated in reference to the molecular weight marker (*pBR*322 DNA/*Msp*I digest) with the software Image Master ID Elite. In the analysis of microsatellites, the parameters included number of alleles, allelic frequencies, percentage of polymorphic loci, observed and expected heterozygosity, linkage disequilibrium, conformity of allele frequencies to that expected under Hardy–Weinberg equilibrium, and estimates of population differentiation including *F*-statistics. They were estimated using Popgene version 1.31 (Yeh et al. 1999). The expected frequency of null alleles was calculated according to Van Oosterhout et al. (2004, 2006) using Micro-Checker (available from http://www.microchecker.hull.ac.uk).

Results

Genetic Variation in RAPD Markers

A total of 73 different randomly amplified DNA fragments were detected consistently with all eight decamer primers in the population of *H. nigricollaris*. The number of fragments ranged from 6 (OPA-20) to 12 (OPAH-01), and the size range was 150–1800 bp. The average number of bands over all primers was 9.13. All eight amplified primers were polymorphic. Of the 73 RAPD fragments, 58 (79.45%) were shared by all individuals of the species. The remaining 15 fragments were found to be polymorphic (20.55%). The primer OPA-09 (Fig. 1) produced the

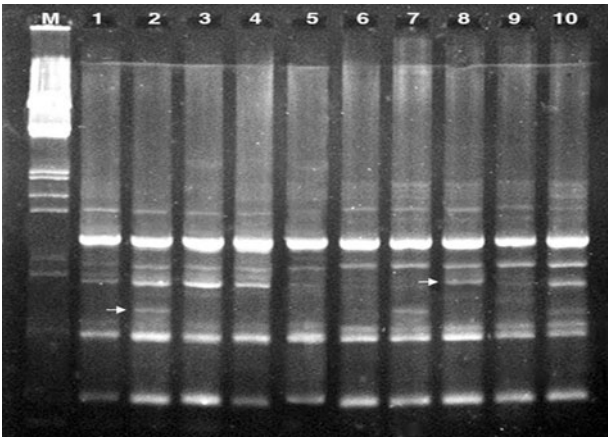

Fig. 1 RAPD pattern of *Horabagrus nigricollaris* with primer OPA-09. *Arrows* Polymorphic RAPD in two samples. *Lane M* molecular weight marker, λDNA with *Eco*R1 and *Hin*dIII double digest

highest percentage of polymorphic bands (33.33%), and OPAH-08 produced only 10% polymorphic bands. For all primers, the observed number of alleles was 1.206 and the effective number was 1.667. Average heterozygosity (*H*; Nei 1978; Khoo et al. 2002), which is also known as average gene diversity index and is analogous to Wright's (1951) F_{ST} statistics (fixation index), for all primers was 0.09 (Table 1).

Genetic Variation in Microsatellite Markers

Cross-species amplification of five microsatellite primers obtained from other catfish species (*P. hypophthalmus*, *C. macrocephalus*, and *C. gariepinus*) revealed five loci, one in each primer: *Hn Phy01*, *Hn Phy05*, *Hn Phy07*, *Hn Cma03*, and *Hn Cga06*. Four were polymorphic (80%). The primer Phy05 did not exhibit any polymorphism. Twelve alleles were obtained at five loci. The total number of alleles per locus ranged from 1 to 5 in a sample size of 21 specimens. The primer Phy01 showed the maximum number of alleles (5), and the primers Phy07, Cma3, and Cga06 had only 2 alleles each. The mean observed number of alleles was 2.4, and the mean effective number was 1.775 (Table 2). The observed heterozygosity (H_{ob}) at all four polymorphic loci in the *H. nigricollaris* population ranged from 0.25 (*Hn Cma03*) to 0.5 (*Hn Phy07*), with a mean of 0.29, whereas the range of expected heterozygosity (H_{ex}) was 0.296 (*Hn Cga06*) to 0.730 (*Hn Phy01*), with a mean of 0.341 (Table 2). The zymogram of locus *Hn Cma03* is shown in Fig. 2. There was no significant association indicative of linkage disequilibrium ($P > 0.05$) between any pair of microsatellite loci; therefore, it was assumed that allelic variation at microsatellite loci could be considered independent. The probability test revealed that the observed allele frequencies in only 50% of the polymorphic loci showed significant deviation from Hardy–Weinberg equilibrium ($P < 0.05$). Positive values of the coefficient of inbreeding (F_{IS}) were shown by two loci (*Hn Phy01* and *Hn Cma03*), and two other polymorphic loci (*Hn Phy07* and *Hn Cga06*) showed a negative F_{IS}. The mean value of F_{IS} was 0.149, and this positive value indicated an extreme deficiency of heterozygotes in the population of *H. nigricollaris.*

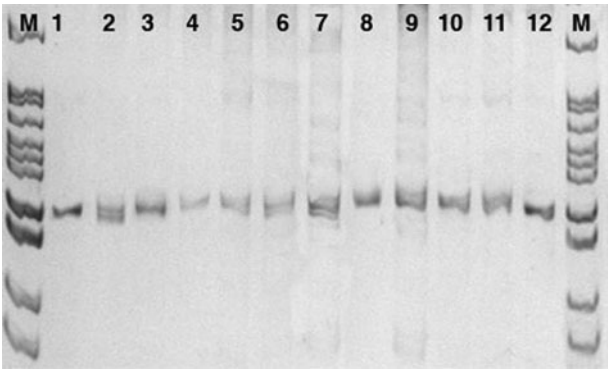

Fig. 2 Microsatellite pattern of locus *Hn Cma03* in *H. nigricollaris*. Two alleles are observed at this locus. *Lane M* molecular weight marker (*pBR*322 with *Msp*I cut)

Frequency of Null Alleles in Microsatellites

The expected frequency of null alleles was calculated using Micro-Checker, and all the genotypes of the loci showing deviation from Hardy–Weinberg equilibrium were tested for null alleles. The estimated null allele frequency was not significant ($P > 0.05$) at all five tested loci using different algorithms, indicating the absence of null alleles and false homozygotes. In addition, there was no instance of nonamplified samples in repeated trials with any of the primer pairs. Therefore, for population genetic analysis, information from all five loci was considered.

Comparison of Genetic Variation in Two Markers

The results derived using two markers (RAPD and microsatellite) in *H. nigricollaris* were compared as follows: The number of loci obtained was 73 in RAPD and 5 in microsatellites. Altogether, 12 alleles were obtained in microsatellites. In RAPD analysis, 15 (20.55%) were polymorphic, whereas in microsatellites 4 loci were polymorphic (80%). For microsatellites, H_{ob} was 0.29 and H_{ex} was 0.341. In RAPD, the average heterozygosity or gene diversity (H) was 0.09, and the coefficient of inbreeding (F_{IS}) for all microsatellite loci was 0.149.

Discussion

Our objective was to analyze genetic variation in the critically endangered yellow catfish *H. nigricollaris* using the most common genetic markers, RAPD (Williams et al. 1990; Welsh and McClelland 1990) and microsatellite (Tautz 1989). As the species is considered to be facing an extremely high risk of extinction in the wild, the analysis of genetic variation and broodstock management is essential. Generally, endangered species show less genetic diversity and heterogeneity within or among populations. This study reveals several significant findings in relation to the genetic diversity of the *H. nigricollaris* population. Low levels of intraspecific variation were observed in this critically endangered catfish. In general, it is predicted that genetic variation within species should positively correlate with population size, and as a consequence, genetic variation in endangered species is expected to be lower than in nonendangered species (Frankham 1996).

Five microsatellite primer pairs developed for three fish species (resource species) belonging to the order Siluriformes (*P. hypophthalmus*, *C. macrocephalus*, and *C. gariepinus*) were evaluated for cross-species amplification of microsatellite loci in *H. nigricollaris*. Successful cross-priming was obtained with five primer pairs, and five loci were revealed. Among these, four loci were polymorphic and ideal for use as markers in stock identification studies. Cross-species amplification of primers of the order Siluriformes in *H. nigricollaris* shows evidence of the remarkable evolutionary conservation of microsatellite flanking regions. Generally, the development of new species-specific microsatellite primers is expensive and time-consuming, but the cross-species amplification of primers is cheap and fast.

There are reports that point to the conservation of flanking sequences of some microsatellite loci among related taxa so that primers developed for one species can be used to amplify homologous loci in related species (Zardoya et al. 1996; Scribner et al. 1996; Yue and Orban 2002; Muneer et al. 2009). We previously reported the successful cross-species amplification of these primers in *H. brachysoma* (Muneer et al. 2009). Certain sequences flanking the tandem repeats could be conserved between the different families of order Siluriformes as reported in other fishes by Scribner et al. (1996) and Zardoya et al. (1996). The remarkable conservation of loci of Siluriformes primers would be helpful to document the evolution of microsatellites contained in these loci and to generate phylogenetic relationships across species of this order, in addition to their application as potential markers in stock identification of *H. nigricollaris.*

In *H. nigricollaris*, microsatellites showed more polymorphism than RAPD. Generally, microsatellites show a high number of alleles. In *H. nigricollaris*, the low level of allele variation was observed in microsatellite loci (Table 2). One reason for the low level of allele variation is probably the small sample size (Galbusera et al. 1996). Ruzzante (1998) suggested a population size of at least 50 individuals for the study of microsatellite loci. As *H. nigricollaris* is very rare, we could collect only 21 specimens for the population analysis. In RAPD, the percentage of polymorphism in *H. nigricollaris* was very low (20.55%, Table 1). Generally, RAPD shows a higher number of polymorphic bands, as seen in the Malaysian river catfish, *Mystus nemurus* (60.48%; Chong et al. 2000) and in the channel catfish *Ictalurus punctatus* and *I. furcatus* (61.05%; Liu et al. 1998).

In microsatellites, the mean heterozygosity values were unusually low ($H_{ob} = 0.290$, $H_{ex} = 0.341$). In *H. brachysoma*, Muneer et al. (2009) reported a mean H_{ob} of 0.472 and mean H_{ex} of 0.649 in three populations. In this study, in *H. nigricollaris*, a significant overall deficiency of heterozygotes was revealed in all polymorphic loci. In *C. macrocephalus*, Na-Nakorn et al. (1999) reported a deficiency of heterozygotes ($H_{ob} = 0.67$, $H_{ex} = 0.76$). Watanabe et al. (2001) and Usmani et al. (2003), however, reported a significant excess of heterozygotes in other bagrid catfishes, *Pseudobagrus ichikawai* ($H_{ob} = 0.54$, $H_{ex} = 0.56$) and *Mystus nemurus* ($H_{ob} = 0.499$, $H_{ex} = 0.482$). The deficiency of heterozygotes in microsatellites in yellow catfish could be due to inbreeding, a situation caused by overexploitation leading to a decline of the species in the wild. Overexploitation leading to drastic decline of the yellow catfish has been recorded in the Chalakkudy River of Kerala since 1997, and the species is now categorized as critically endangered (CAMP 1998). Nonrandom mating and less effective population size could be major reasons for heterozygote deficiency (Van Oosterhout et al. 2004; Donnelly et al. 1999) in the stock of *H. nigricolalris* in this study.

The presence of null alleles could be one of the factors responsible for the observed heterozygote deficiency. Null alleles are alleles that do not amplify during PCR because of mutation events changing the DNA sequence in one of the primer sites (mostly at the 3′ end), so that the primer no longer anneals to the template DNA during PCR (Van Oosterhout et al. 2004, 2006). This results either in no PCR product, if the null allele is homozygous, or in false homozygote individuals, if the locus is heterozygous. This will show apparent significant deviations from Hardy–

Weinberg equilibrium and non-Mendelian inheritance of alleles (Donnelly et al. 1999). The analysis of data using Micro-Checker indicated that occurrence of null alleles in the population is very unlikely for the five primer pairs. Also, there was no instance of nonamplifying samples in repeated trials with any of the primer pairs in *H. nigricollaris*.

The *Horabagrus* were originally placed in the Bagridae family (Talwar and Jhingram 1991), but work carried out by de Pinna (1993) suggested that the genus was more closely related to the Schilbeidae family. Later, he pointed out some features that were distinct from both the Schilbeidae and Bagridae and placed *Horabagrus* in its own family. Recently, Hardman (2005) recommended recognition of this group as the family Horabagridae based on cytochrome b evidence. Sullivan et al. (2006) supported the study by phylogenetic analysis of *rag1* and *rag2* nuclear genes.

Horabagrus nigricollaris is found only in the Chalakkudy River in Kerala, India (Ponniah and Gopalakrishnan 2000). This river is considered one of the richest river systems in the region with regard to freshwater fish diversity (Ajithkumar et al. 1999). Indiscriminate collection for international trade as ornamental species is considered to be one of the major reasons for the massive reduction in the numbers of *H. nigricollaris* (Sivanandan 2004). Extensive habitat alterations due to damming and deforestation, the proliferation of exotic species, and pollution, pesticides, and agrochemicals used in plantations are the other reasons. Tribes and forest-dwelling communities along the basin are also highly dependent on the native species for their livelihoods. Destructive fishing practices are a serious concern in this river (Muneer et al. 2007).

Generating a database on life history traits, ecology, and stock structure is the most important requirement for the conservation and sustainable management of fish fauna. Strong management strategies such as development of captive breeding technology, cryopreservation of germplasm, small-scale aquaculture programs, and bans on destructive fishing practices are other options for the conservation and management of this critically endangered species.

In conclusion, genetic diversity analysis using RAPD and microsatellite markers revealed the low genetic diversity in one population of yellow catfish, *H. nigricollaris*. The potential use of heterologous microsatellite primers was explored, and many of them appeared to be conserved in this bagrid catfish (order Siluriformes). RAPD and microsatellite methods reported a low degree of gene diversity and lack of genetic heterogeneity in the population of *H. nigricollaris*, strongly emphasizing the need for fishery management, conservation, and rehabilitation of this species.

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