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ORIGINAL ARTICLE

Microsatellite Genotyping Corroborated Loss of Genetic Diversity in *Clarias batrachus* as a Result of Lack of Regulatory Reforms in Aquaculture

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Abstract

In India, over the past 50 years, aquaculture practices of species such as those used for *Clarias batrachus* were developed without adequate regulatory oversight. In these situations, it is important to consider the influence that genetic factors can have on such vulnerable aquaculture species. Population genetic structure can be evaluated through the use of neutral molecular markers, and this can aid in predicting the risk of the demise of populations and for framing management strategies to conserve remaining populations. The study presented here reports on the genetic status of *C. batrachus* populations through the analysis of data collected using 22 microsatellite markers from seven natural and one hatchery population. The mean values for observed heterozygosity across loci within populations ranged from 0.242 to 0.485. Measures of genetic differentiation were low overall, with mean values for F_{ST} of 0.270, F_{IS} of 0.113 and F_{IT} of 0.353. An AMOVA analysis revealed that percentages of variation among and within populations were 27.16 and 6.86, respectively, and Bayesian clustering analyses showed a population subdivision consisting of five clusters with admixture of haplotypes from other populations leading to genetic bottleneck. We also examined how hatchery management factors leading to excessive exchanges of fish between river systems through could impact the structure of the *C. batrachus* populations. Overall, this study shows how the systematic use of molecular markers can facilitate the development of management policies for these populations and for the development of a comprehensive set of rules for hatcheries and aquaculture practices, including avoidance of excessive homozygosity by avoiding repeated use of feral broodstock and their interrogation.

Keywords *C. batrachus* · Seed regulation · Hatchery · Markers · Genetic diversity

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Introduction

The regulation of fish seed production and certification of hatcheries is yet again in the discussion stage in several countries that still do have not defined national policies (Bondad-Reantaso 2007). In such situations, the lack of regulation can lead to indiscriminate use of wild germplasm material in hatcheries. When this occurs and species integrity is not maintained in the hatchery breeding stock it may result in interspecies hybridization. This in turn can lead to major shifts in the genetic makeup of natural populations when such hatchery seedlings present in culture facilities escape into adjoining natural water resources. Such uncontrolled population translocations and invasions can have an enormous impact on genetic diversity through disruption of locally adapted genotypes affecting population fitness (Rhymer and Simberloff 1996; Allendorf et al. 2001).

These issues are all highly relevant to populations of the Asian catfish, *Clarias batrachus*, commonly known as Magur (Khedkar et al. 2010, 2014a; Hossain et al. 2006; Islam et al. 2007), a species in high demand in India. *C. batrachus* was once widespread in most of the Indian River systems, but since the late 1980s it has become very scarce in its natural habitats. Only a few small and isolated natural populations of this fish are known to remain, and recent studies have detected the loss of genetic diversity in these populations (Khedkar et al. 2010, 2014b). These problems are further compounded by some of the natural, inherited biological traits that *C. batrachus* is known for including low fecundity and poor larval survival (Hossain et al. 2006). To address these issues and in response to the market demand in India, the Central Institute of Freshwater Aquaculture, Bhubaneshwar took a lead in developing seed production technology for this important aquaculture species (Rao et al. 1994).

However, the aquaculture industry in India is still at an early stage of development and depends heavily on brood fish or seed collections from feral stocks. This, together with the lack of regulatory controls, has led to over exploitation and loss of diversity in this fish. This has also likely contributed to the current status of this species being listed as endangered with the very real possibility of extinction, despite the high level of consumer demand for this fish (Khedkar et al. 2014b).

To better understand the genetic makeup of populations of this fish, we surveyed genetic variability in 22 microsatellite loci in hatchery and natural populations. We determined the distributions of various genotypes to estimate the extent of genetic structuring between populations from different rivers basins and their sub-drainages. We also assessed levels of genetic diversity of wild populations to be able to compare them to hatchery populations. We used the distributions of the various genotypes in conjunction with historical and demographic data to infer the history of colonization and expansion of this species. In addition, the microsatellite variability between sub-drainage populations was used to identify potential barriers to migration which, when considered with the geography of the region, can be used to define management units for informed strategies for control programs and for deciding on appropriate regulatory measures for hatchery seed production.

Material Methods

Ethics Statement

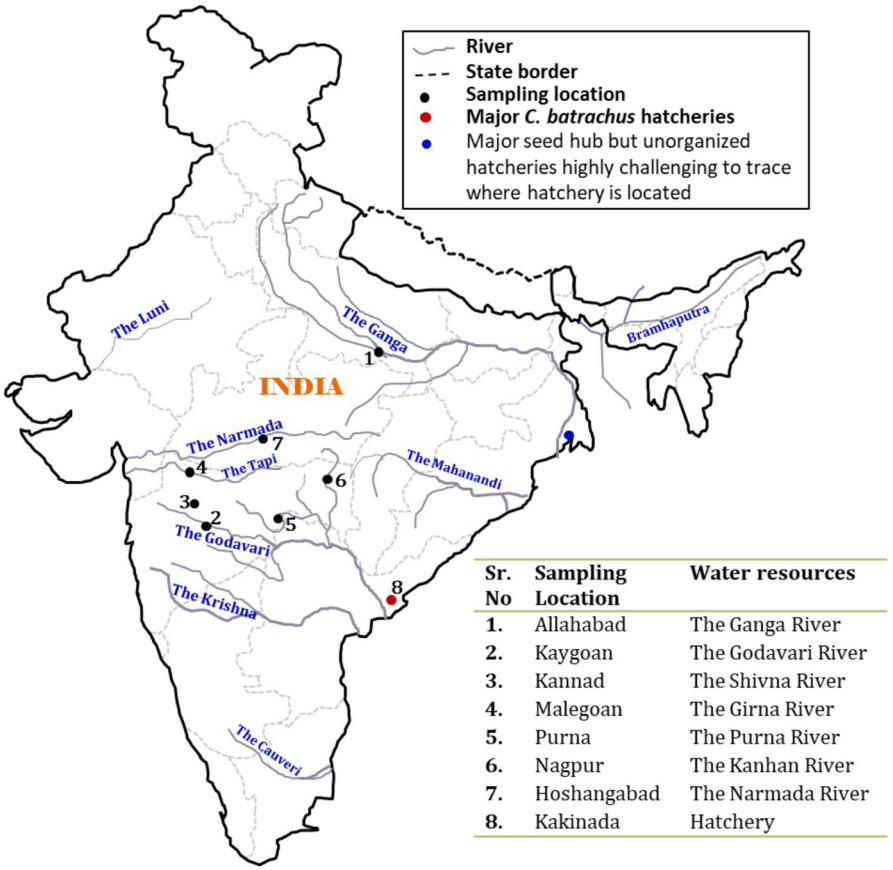
The catfish collected for this study are routinely used as food and no special permissions are required in India.

Sample Collection

Catfish samples were collected during 2010–2013 with the help of local fisherman using various nets and gear. A small fin clip (5 mm^2) was taken from each specimen and placed in absolute ethanol for later analysis. An effort was made to collect at least 20 individuals from each major river catchment. The samples from various riverine resources were collected over a stretch of 2–3 kms using nets and gear with the help of local fisherman. Hatchery samples were also collected from one of the major seed production regions in India. Sample site names, coordinates, sample sizes, etc., are given in Table S1 and a location map is given in Fig. 1. Another major fish seed hub in India is Howrah, Kolkata, where seed for several fish species is traded. But in this environment, determining the origin and number of traders supplying seed was highly challenging. Because efforts to get any official information were unsuccessful for any of these units, therefore, we have not incorporated these samples in our analysis.

PCR and Genotyping

Total genomic DNA was extracted using the wizard genomic DNA purification kit following manufacturer instructions (Promega Wizard kit) and samples were genotyped for 22 di-, tri-, or tetranucleotide microsatellite loci, including Cba02-KUL, Cba06-KUL, Cba08-KUL, Cba09-KUL, Cba01, Cba05, Cba07, Cba20, Cba21, Cba02, Cba06, Cba09, Cba10, Cba11, Cba13, Cba14, Cba17, Cba19, Cba07-KUL, Cba03, Cba04, Cba12 (Volckaert et al. 1999; Yue et al. 2003) (details about the microsatellite markers used is provided in supporting table S2). Forward primers were labeled with fluorescent dyes, viz., FAM, NED, VIC, and PET (Applied Biosystems, Inc. Foster City, California, USA). Each 12.5 µL PCR reaction consisting of 10% trehalose, 1 × PCR buffer 'B', 0.4 mM $MgCl_2$, 0.2 mM dNTP, 0.04 mM forward and reverse primers and 0.028U *Taq* polymerase (Kappa biosystems), 0.8 ng DNA template and Nuclease-free water. Cycling conditions for amplification of loci included an initial denaturation step of 3 min at 94 °C followed by 35 cycles of 30 s. at 94 °C, 40 s., with respective annealing temperature (Table S2) and 30 s at 72 °C, with a final extension at 72 °C for 7 min. For confirmation of amplification PCR products were run on 2% agarose gel (Fig. S1). Primers for Cba07, Cba03, Cba04, and Cba12 were failed in amplification.



Sr. No	Sampling Location	Water resources
1.	Allahabad	The Ganga River
2.	Kaygoan	The Godavari River
3.	Kannad	The Shivna River
4.	Malegoan	The Girna River
5.	Purna	The Purna River
6.	Nagpur	The Kanhan River
7.	Hoshangabad	The Narmada River
8.	Kakinada	Hatchery

Fig. 1 Locations of *C. batrachus* collection from different regions

Fragment Analysis

All PCR products were pooled in the ratio of 1:1:1:1 for FAM: NED: VIC: PET with respective primer sets and sample Ids (Table S2). Pooled PCR fragments were separated on an ABI 3730 sequencer and sized relative to the GeneScan500-LIZ®size standards (Applied Biosystems, Inc., Foster City, California, USA). Alleles were scored using GeneMapper software v4.0 (Applied Biosystems Inc., California, USA).

Statistical Analysis

Allelic Diversity

The range of allele sizes and numbers at each locus were summarized using GenAlEx 6.5 (Peakall and Smouse 2012). Potential deviations of genotype frequencies from Hardy–Weinberg (HW) expectations were analyzed in GENEPOP 4.2.2 (Raymond and Rousset 1995). A large number of sites were tested, and the *P*-values obtained in this analysis were adjusted for multiple tests using the Benjamini–Hochberg (BH) method (Benjamini and Hochberg 1995), to keep type 1 errors to a minimum (Reiner et al. 2003).

Genetic Analysis of Populations

Genetic diversity values for each population and locus were quantified by calculating heterozygosity (H) using GENEPOP 4.2.2. Polymorphisms shown by each locus for each population were assessed in terms of the number of alleles (NA), expected heterozygosity (He) and Polymorphic information content (PIC) as a measure of genetic diversity using GenALEx V6.5 (Peakall and Smouse 2012). Similarly, the data were analyzed to detect deviations from Hardy–Weinberg equilibrium (HWE) in the *C. batrachus* populations as in Raymond and Rousset (1995). Samples were tested for HWE using an exact test as described in Guo and Thompson (1992) using a Markov Chain approach in Genepop version 4.2.2 (Raymond and Rousset 1995) with parameters as follows: dememorization = 100,000; batches = 100 and iterations = 10,000; When the null hypothesis was rejected, the FIS statistic of Wright (1951) estimated following Weir and Cockerham (1984) as an indicator of heterozygote excess or deficiency. Average observed heterozygosity, expected heterozygosity, and inbreeding coefficient values were estimated using Genetix version 4.03 software (Belkhir et al. 1999).

Population Differentiation

Population differentiation and molecular variance values (AMOVA) were examined using ARLEQUIN v.3.1 (Excoffier and Lischer 2010) where pairwise F_{ST} values were used to test the null hypothesis of panmixia. Significance levels were obtained using the exact test as this method is considered robust even when sample sizes are small and when alleles with low frequencies are included (Raymond and Rousset 1995). From the same program, we also used R_{ST} values (Slatkin 1995) to test for genetic differentiation. R_{ST} is a measure analogous to Wright's F_{ST} (Wright 1951) that has been adapted to the high rate of stepwise mutations occurring at microsatellites.

Population Structure

The UPGMA tree was constructed using the POPTREE2 (Takezaki et al. 2010) software package while TreeView v1.6.6 (Page 1996) was used to view the tree.

Patterns of genetic structure were evaluated using STRUCTURE v2.3.4 (Pritchard et al. 2000) which assigns individuals to groups using a Bayesian model-based method. An admixture model with correlated allele frequencies was applied for each STRUCTURE (Evanno et al. 2005) run with 100,000 MCMC repeats preceded by a burn-in of 100,000 steps. The number of clusters was assumed to range from $K=2$ to $K=7$ and then most probable K values for each pairwise comparison were estimated using STRUCTURE HARVESTER 0.6.94 (Earl and von Holdt 2012).

Population Bottleneck

BOTTLENECK V1.2.0217 (Piry et al. 1996) was used to analyse genetic bottleneck effect on populations. We constrained the model by defining 90% of mutations as compliant under a two-phase model (TPM) and 10% as multi-step.

Results

Genetic Diversity

Microsatellite markers were genotyped using eighteen microsatellite loci from a total of 209 individuals of *C. batrachus* from eight populations. Genetic diversity values, including both expected (H_e) and observed heterozygosities (H_o) were obtained for eighteen loci in all population units along with polymorphic information content (PIC) values (Table 1). A total of 151 alleles were analyzed in this study for an average of 8.389 and mean number of individuals typed per locus per population of 3.75.

Observed heterozygosity was 0.434 with expected heterozygosity 0.508 per locus and polymorphic information content value was 0.465. The lowest polymorphism observed in locus Cba02 ($H_o=0.089$, $H_e=0.240$, PIC = 0.226) and highest was in locus Cba20 ($H_o=0.868$, $H_e=0.799$, PIC = 0.769) (Table 1). All populations were shown expected heterozygosity in the range of 0.320–0.505, observed heterozygosity in the range of 0.242–0.485 and number of alleles per population in the range of 2.056–5.167 (Fig. 2). The hatchery population of *C. batrachus* from the Kakinada and the Kanhan River population has shown the positive index of the inbreeding coefficient and all remaining populations were showing negative index (Fig. 2). Inbreeding coefficient (0.5707) recorded from the hatchery population (Kakinada) was highest among all populations studied here.

Hardy–Weinberg Equilibrium

Tests for Hardy–Weinberg Equilibrium (HWE) were performed among all loci in all populations. Our results found significant deviations at 64 loci ($P<0.05$). For 64 loci-population combinations, observed values for heterozygosity (H_o), expected heterozygosity (H_e) inbreeding coefficient (F_{IS}) and the probability of significant deviation from HWE (P) are presented in Table 2. The *C. batrachus* population from

Table 1 Polymorphic parameters of microsatellite loci in *C. batrachus* populations

Locus	*k*	N_a	H_o	H_e	PIC
Cba01	9	3.500	0.332	0.453	0.400
Cba02-KUL	10	4.000	0.625	0.641	0.591
Cba05	10	4.750	0.378	0.577	0.526
Cba06-KUL	10	4.000	0.181	0.462	0.439
Cba07	6	3.500	0.479	0.650	0.585
Cba08-KUL	7	2.750	0.105	0.401	0.354
Cba09-KUL	6	2.250	0.058	0.278	0.266
Cba20	12	7.125	0.868	0.799	0.769
Cba21	7	2.875	0.297	0.337	0.314
Cba02	4	2.125	0.089	0.240	0.226
Cba06	8	3.125	0.696	0.526	0.440
Cba09	9	3.375	0.332	0.293	0.271
Cba10	5	2.875	0.335	0.605	0.555
Cba11	10	4.250	0.308	0.469	0.442
Cba13	10	4.125	0.604	0.648	0.590
Cba14	6	3.250	0.784	0.630	0.555
Cba17	10	4.750	0.433	0.436	0.417
Cba19	12	4.875	0.904	0.692	0.640
Average	8.389	3.750	0.434	0.508	0.465

k number of alleles at each locus, N_a mean number of individuals typed for each locus, H_o mean heterozygosity observed, H_e mean heterozygosity expected (unbiased estimate Nei, 1987), *PIC* polymorphic information content

the Ganga River significantly deviated from HWE at six loci, the Girna at eight, Kakinada and Purna at nine, the Shivna at twelve, the Godawari, and the Narmada at three and Kanhan at ten loci, due either to excesses of homozygotes or heterozygotes (Table 2).

Population Differentiation

Clarias batrachus populations were analyzed for structuring using the AMOVA method. Here, variation among the populations from different localities was 27.16% with a significant inbreeding coefficient of cohesive gametes relative to gametes drawn at random from within a subpopulation ($F_{ST}=0.271$, $P<0.00$). The highest variations were found within individuals (64.63%) with a significant inbreeding coefficient of an individual relative to the total population ($F_{IT}=0.354$, $P=0.00$). Only 6.86% variation was noticed within individuals of each subpopulation, with significant overall genetic divergences among subpopulations ($F_{IS}=0.114$, $P=0.006$) (Table 3).

The overall F_{ST} value was statistically significant ($F_{ST}=0.271$, $P=0.00$) for all population across all loci, reflecting low genetic variation. Pairwise F_{ST} values between all

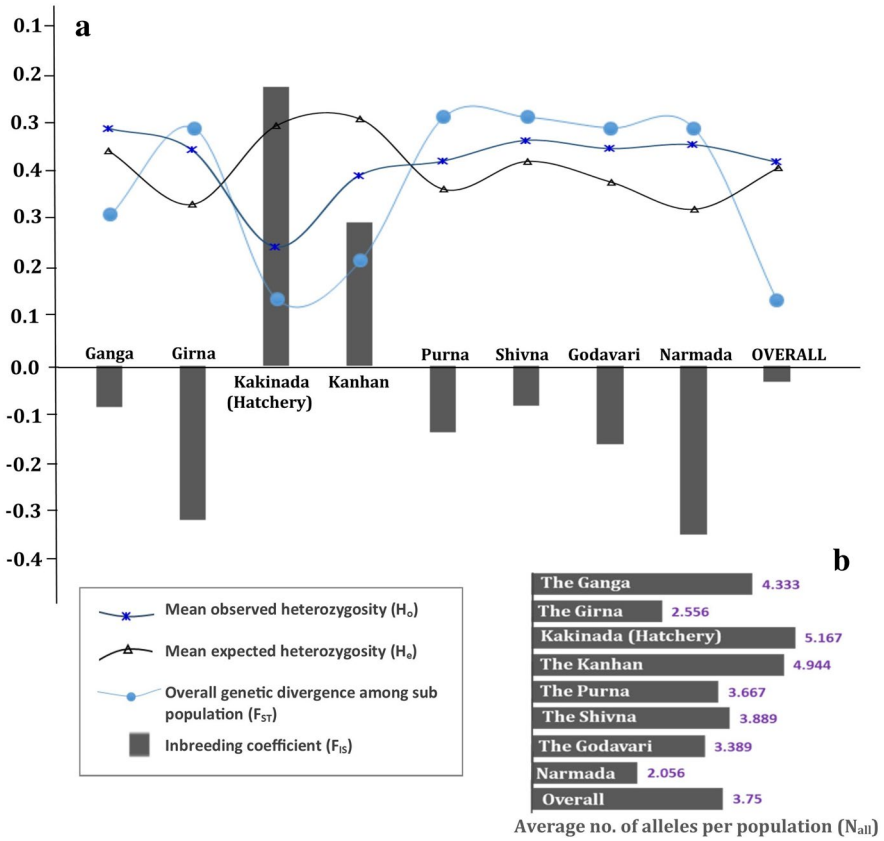


Fig. 2 Genetic diversity *of C. batrachus* population over all loci using 18 polymorphic microsatellite markers

populations are presented in Table 4. The lowest pairwise comparison was found for the populations from the Ganga river and the Shivna river (F_{ST}=0.008) followed by Godavari and Girna (0.015) (Table 4).

In addition, we also used pairwise R_{ST} estimations for comparisons based on allele sizes (Table 5). As compare to F_{ST} value, R_{ST} values for pairwise comparison, the Narmada River and the Ganga, the Girna, the Kanhan, and the Shivna were not differentiated from the other populations.

To consider gene flow among different populations, we conducted further analyses in ARLEQUIN and obtained *M* values (Table 6). Almost 50% of the *M* values were greater than two, suggesting that there is considerable gene flow between these populations.

Table 2 Inbreeding coefficients and deviations from Hardy–Weinberg equilibrium (HWE) observed in *C. batrachus* populations

Population		Cba01	Cba02-KUL	Cba05	Cba06-KUL	Cba07	Cba08-KUL	Cba09-KUL	Cba20	Cba21	Cba02	Cba06	Cba09	Cba10	Cba11	Cba13	Cba14	Cba17	Cba19
Ganga	H_o	0.069	0.500	0.433	0.593	0.217	0.138	0.333	0.862	0.133	0.000	0.900	0.633	0.400	0.517	0.552	0.933	0.567	0.966
	H_e	0.067	0.583	0.533	0.547	0.258	0.161	0.621	0.677	0.214	0.124	0.544	0.452	0.331	0.512	0.602	0.498	0.482	0.713
	F_{IS}	− 0.009	0.161	0.203	− 0.064	0.179	0.161	0.478	− 0.258	0.391	1.000	− 0.645	− 0.38	− 0.192	0.007	0.100	− 0.871	− 0.160	− 0.338
	P	1.000	0.115	0.147	0.132	0.414	0.171	**0.000***	**0.002***	**0.003***	**0.001***	**0.000***	0.070	0.700	0.287	0.220	**0.000***	0.885	**0.00***
Girna	H_o	0.190	0.333	0.048	0.095	0.524	0.190	0.000	0.952	0.000	0.143	1.000	0.476	0.762	0.048	0.810	1.000	0.381	1.000
	H_e	0.354	0.359	0.046	0.172	0.459	0.172	0.000	0.687	0.000	0.414	0.500	0.373	0.489	0.046	0.557	0.500	0.316	0.500
	F_{IS}	0.481	0.097	0.000	0.467	− 0.117	− 0.081	Mono	− 0.365	mono	0.669	− 1.000	− 0.25	− 0.542	0.000	− 0.435	− 1.000	− 0.181	− 1.000
	P	**0.004***	0.273	1.000	0.145	0.665	1.000	Mono	**0.015***	mono	**0.000***	**0.000***	0.632	**0.015***	1.000	**0.028***	**0.000***	1.000	0.000*
Kakinada	H_o	0.240	0.000	0.643	0.000	0.167	0.036	0.000	0.839	0.667	0.000	0.188	0.182	0.097	0.364	0.353	0.300	0.292	0.000
	H_e	0.463	0.500	0.714	0.781	0.764	0.364	0.219	0.835	0.528	0.000	0.375	0.172	0.152	0.810	0.521	0.720	0.266	0.667
	F_{IS}	0.497	1.000	0.118	1.000	0.815	0.905	1.000	0.012	− 0.247	Mono	0.524	− 0.04	0.377	0.583	0.349	0.600	− 0.073	1.000
	P	**0.001***	0.333	0.407	**0.000***	**0.001***	**0.000***	0.067	**0.002***	0.291	Mono	**0.032***	1.000	0.160	**0.000***	**0.016***	**0.000***	1.000	0.067
Kanhan	H_o	0.433	0.571	0.633	0.250	0.192	0.065	0.000	0.692	0.742	0.429	0.200	0.097	0.100	0.467	0.200	0.448	0.474	1.000
	H_e	0.414	0.615	0.592	0.563	0.679	0.152	0.458	0.754	0.679	0.457	0.488	0.093	0.255	0.622	0.449	0.574	0.758	0.500
	F_{IS}	− 0.030	0.107	− 0.054	0.571	0.726	0.586	1.000	0.122	− 0.076	0.080	0.603	− 0.02	0.640	0.266	0.568	0.235	0.398	− 1.000
	P	0.104	0.282	**0.009***	**0.000***	**0.000***	**0.003***	**0.000***	0.436	0.404	**0.027***	**0.000***	1.000	0.158	0.099	**0.001***	**0.000***	**0.002***	1.000
Purna	H_o	0.533	0.808	0.167	0.074	0.793	0.067	0.000	0.966	0.167	0.000	0.800	0.033	0.067	0.267	0.690	0.933	0.500	0.667
	H_e	0.489	0.592	0.321	0.203	0.687	0.065	0.067	0.787	0.153	0.000	0.480	0.033	0.064	0.231	0.631	0.541	0.397	0.762
	F_{IS}	− 0.073	− 0.348	0.494	0.646	− 0.138	− 0.009	1.000	− 0.211	− 0.074	Mono	− 0.657	0.000	− 0.018	− 0.137	− 0.076	− 0.719	− 0.245	0.159
	P	0.498	**0.004***	**0.000***	**0.003***	**0.000***	1.000	**0.018***	**0.001***	1.000	Mono	**0.000***	1.000	1.000	1.000	**0.000***	**0.000***	0.668	0.074
Shivna	H_o	0.333	0.800	0.552	0.071	0.571	0.000	0.000	0.931	0.133	0.033	0.667	0.400	0.367	0.400	0.857	0.900	0.414	0.870
	H_e	0.289	0.721	0.507	0.168	0.610	0.238	0.231	0.748	0.124	0.033	0.444	0.359	0.299	0.442	0.634	0.610	0.401	0.674
	F_{IS}	− 0.135	− 0.090	− 0.072	0.586	0.081	1.000	1.000	− 0.228	− 0.055	0.000	− 0.48	− 0.09	− 0.208	0.112	− 0.336	− 0.462	− 0.014	− 0.270
	P	1.000	**0.005***	**0.001***	**0.002***	**0.000***	**0.000***	**0.000***	**0.038***	1.000	1.000	**0.011***	1.000	0.551	**0.013***	**0.000***	**0.000***	0.072	**0.000***

Table 2 (continued)

Population		Cba01	Cba02-KUL	Cba05	Cba06-KUL	Cba07	Cba08-KUL	Cba09-KUL	Cba20	Cba21	Cba02	Cba06	Cba09	Cba10	Cba11	Cba13	Cba14	Cba17	Cba19
Godawari	H_o	0.542	0.696	0.154	0.042	0.600	0.320	0.042	0.696	0.148	0.000	0.840	0.654	0.385	0.083	0.577	0.852	0.370	1.000
	H_e	0.478	0.515	0.269	0.041	0.449	0.338	0.041	0.693	0.206	0.346	0.500	0.473	0.370	0.157	0.452	0.497	0.318	0.621
	F_{IS}	− 0.112	− 0.331	0.444	0.000	− 0.319	0.075	0.000	0.018	0.300	1.000	− 0.669	− 0.36	− 0.020	0.486	− 0.258	− 0.704	− 0.148	− 0.597
	P	0.683	0.297	**0.009***	1.000	0.184	**0.050***	1.000	0.203	0.069	**0.000***	**0.001***	0.189	0.061	**0.043***	0.399	**0.000***	1.000	**0.000***
Narmada	H_o	0.143	0.714	0.000	0.143	0.429	0.000	0.000	1.000	0.000	0.286	1.000	0.143	1.000	0.000	1.000	0.857	0.429	1.000
	H_e	0.133	0.459	0.000	0.337	0.337	0.000	0.000	0.694	0.000	0.643	0.500	0.133	0.684	0.000	0.500	0.490	0.357	0.500
	F_{IS}	0.000	− 0.500	Mono	0.625	− 0.200	Mono	Mono	− 0.377	mono	0.607	− 1.000	0.000	− 0.400	mono	− 1.000	− 0.714	− 0.125	− 1.000
	P	1.000	0.443	Mono	0.231	1.000	Mono	Mono	0.601	mono	0.087	**0.036***	1.000	0.116	mono	**0.038***	0.158	1.000	**0.037***

*Values are statistically significant at $P < 0.05$ (values are also denoted in boldface letters)

Table 3 AMOVA analysis among groups (8) and populations (20) as sources of variation in *C. batrachus* populations

Source of variation	d. f	Sum of squares	Variance components	Percentage of variation	*F* index	*P* value
Among populations	7	94.124	0.246	27.16	0.271*	0.00
Among individuals within populations	201	148.694	0.076	6.86	0.114**	0.00
Within individuals	209	123	0.589	64.63	0.354***	0.00
Total	417	365.818	0.911	–	–	–

*F_{ST}: Inbreeding coefficient of uniting gametes relative to gametes drawn at random from within a subpopulation

**F_{IT}: Inbreeding coefficient of an individual relative to the total population

***F_{IS}: Overall genetic divergence

Table 4 Pairwise F_{ST} values (below the diagonal) and P values (above the diagonal) in *C. batrachus* populations

	Ganga	Girna	Kakinada	Kanhan	Purna	Shivna	Godawari	Narmada
Ganga	–	0.000	0	0	0	0.225	0.042	0.000
Girna	0.076*	–	0	0	0	0	0.213	0.137
Kakinada	0.209*	0.377*	–	0	0	0	0	0
Kanhan	0.399*	0.479*	0.257*	–	0	0	0	0
Purna	0.098*	0.133*	0.330*	0.513*	–	0.002	0	0.000
Shivna	0.008	0.104*	0.204*	0.383*	0.068*	–	0.001	0.000
Godawari	0.028*	0.01	0.276*	0.392*	0.131*	0.057*	–	0.027
Narmada	0.180*	0.057	0.408*	0.466*	0.291*	0.221*	0.100*	–

*Values are statistically significant ($P < 0.05$)

Table 5 Pairwise R_{ST} values (below the diagonal) and *P* values (above the diagonal) in *C. batrachus* populations

	Ganga	Girna	Kakinada	Kanhan	Purna	Shivna	Godawari	Narmada
Ganga	–	0.259	0.000	0.004	0	0.240	0.892	0.097
Girna	0.005	–	0.000	0.001	0	0.292	0.153	0.155
Kakinada	0.089*	0.151*	–	0.007	0	0.000	0.000	0.020
Kanhan	0.047*	0.072*	0.053*	–	0	0.002	0.007	0.067
Purna	0.094*	0.083*	0.150*	0.084*	–	0.012	0	0.623
Shivna	0.008	0.006	0.117*	0.066*	0.053*	–	0.025	0.267
Godawari	− 0.017	0.013	0.102*	0.048*	0.136*	0.041*	–	0.04
Narmada	0.041	0.029	0.130*	0.064	− 0.019	0.018	0.088*	–

*Values are statistically significant ($P < 0.05$)

Table 6 *M* values by population comparisons for *C. batrachus* populations

	Ganga	Girna	Kakinada	Kanhan	Purna	Shivna	Godawari	Narmada
Ganga	–							
Girna	6.095	–						
Kakinada	1.891	0.826	–					
Kanhan	0.753	0.544	1.448	–				
Purna	4.593	3.252	1.016	0.475	–			
Shivna	62.956	4.301	1.946	0.806	6.872	–		
Godawari	17.382	33.785	1.310	0.775	3.326	8.346	–	
Narmada	2.278	8.306	0.725	0.573	1.220	1.765	4.488	–

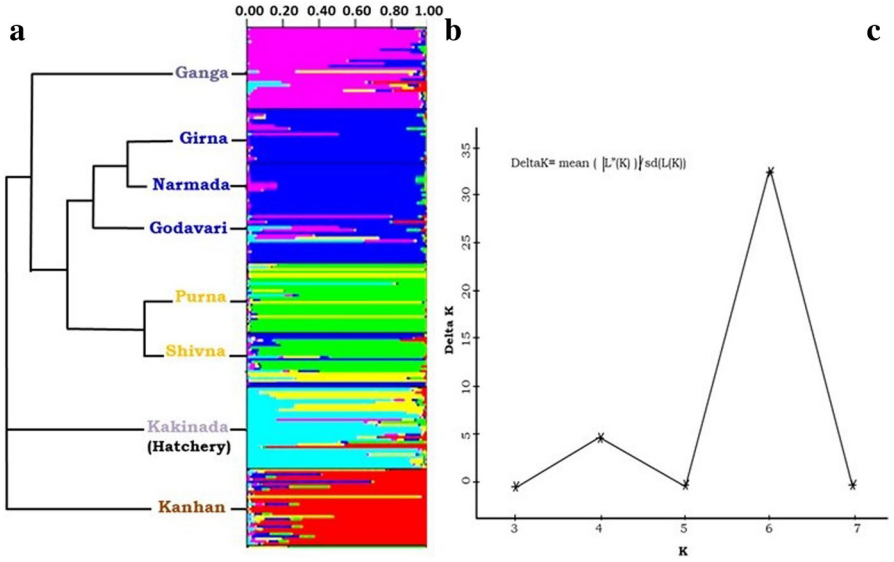


Fig. 3 Population clustering and gene flow of *C. batrachus*. **a** UPGMA based clustering; **b** Bayesian clustering assignment using structure algorithm (colored lines represent genotype of individual of a population. For quick correlation, population names denoted in different colors) and **c** Delta *K* values obtained by the Evanno's method (Evanno et al. 2005) considering $K=2$, where the maximum value of Delta *K* is considered as the highest level of structuring

Population Structure

The UPGMA model-based method presented five group structures (Fig. 3a) with the population from the Ganga, the Kakinada, and the Kanhan forming different clades (Fig. 3a). The Bayesian model-based clustering method showed a *K* value of six indicating that the eight *C. batrachus* populations included in this study can be assigned to six clusters (Fig. 3c). But in actual, only five clusters were recognized (Fig. 3a). The Kanhan, Kakinada, Ganga showed a distinct cluster with some gene

flow from other populations. The Godawari, Girna, and the Narmada populations belong to the same cluster (Fig. 3b). The Purna and Shivna constitute a cluster with low genetic differentiation. Cluster no. 4 is common to three populations, suggesting the possibility that there has been considerable gene flow among these populations studied here.

Test for Bottleneck Effect

To test for population BOTTLENECK, IAM (Infinite Allele Model), TPM (Two-Phase model), and SMM (Stepwise Mutation Model) were applied. Populations exhibiting a significant heterozygosity excess would be presumed to have undergone a genetic bottleneck. Under the TPM and SMM, the results displayed genetic bottleneck effect in most of the populations (Table 7). The sign test and standardized difference test showed a significant heterozygosity excess in all population under the TPM and SMM ($P > 0.05$) except IAM, whereas Wilcoxon test showed nonsignificant heterozygosity excess in all populations under three models tested here. However, this was an indication of true heterozygosity excess, as the IAM is thought to be a less appropriate model for microsatellites than the TPM and SMM (Shriver et al. 1993). These results thus point to the genetic bottleneck in *C. batrachus*.

Discussion

Over time, the decline in the genetic resources of many natural fish populations has become a key issue in fisheries management. Various human activities can lead to these reductions of genetic diversity, and this in turn may result in loss either of populations or entire species. As part of an effort to manage endangered species, microsatellite markers, in combination with advanced statistical methods, have proved to be a useful tool for monitoring levels of genetic diversity in many populations. In India, *C. batrachus* is an important candidate fish species for aquaculture, but efforts to sustain it are still presumed. In addition, Ng and Kottelat (2008) raised questions on our present level of understanding about this species by suggesting that the *C. batrachus* found in India and other south Asian countries actually comprised four subspecies. However, Ng and Kottelat (2008) do not have observed any original material of Indian *C. batrachus* population for comparison and all conclusions were drawn based on Javanese material. Earnestly, the origin of Javanese type specimen observed by Ng and Kottelat (2008) was dubious and it was suspected that the material might have come from India, southern China, or Java. Further confusion arose when a group of scientists at the Indian National Bureau of Fish Genetic Resources in Lucknow recommended that *C. batrachus* be designated as *C. magur*. As these observations cannot be confirmed to date, therefore, we prefer to use the name of catfish under this study as *C. batrachus* as has been prevalent for over two centuries (Hamilton 1822).

In terms of genetic diversity levels described in this study, using microsatellite markers, the total number of alleles found per locus ranged from 2.12 to 4.87. The

Table 7 Results (*P*-values) of genetic bottleneck analysis under three models

Population	Test	Microsatellite mutation models		
		IAM	TPM	SMM
Ganga	Sign test: No. of loci with heterozygosity excess (probability)	9.98 ($p=0.425$)	10.24 ($p=0.003$)*	10.41 ($p=0.000$)*
	Standardized differences test: *T*2 values (probability)	− 0.486 ($p=0.314$)	− 2.767 ($p=0.313$)	− 6.270 ($p=0.00$)*
	Wilcoxon test (probability of heterozygote excess)	0.617	0.998	0.999
Girna	Sign test: No. of loci with heterozygosity excess (probability)	7.76 ($p=0.550$)	8.35 ($p=0.334$)	8.64 ($p=0.140$)
	Standardized differences test: *T*2 values (probability)	1.354 ($p=0.088$)	0.273 ($p=0.393$)	− 0.994 ($p=0.160$)
	Wilcoxon test (probability of heterozygote excess)	0.162	0.391	0.702
Kakinada	Sign test: No. of loci with heterozygosity excess (probability)	9.977 ($p=0.263$)	9.77 ($p=0.263$)	9.86 ($p=0.015$)*
	Standardized differences test: *T*2 values (probability)	− 1.382 ($p=0.084$)	− 4.217 ($p=0.000$)*	− 9.238 ($p=0.00$)*
	Wilcoxon test (probability of heterozygote excess)	0.727	0.905	0.978
Manipur	Sign test: No. of loci with heterozygosity excess (probability)	10.14 ($p=0.216$)	10.52 ($p=0.028$)*	10.57 ($p=0.002$)*
	Standardized differences test: T2 values (probability)	− 0.582 ($p=0.280$)	− 2.373 ($p=0.009$)*	− 5.142 ($p=0.00$)*
	Wilcoxon test (probability of heterozygote excess)	0.739	0.971	0.999
Kanhan	Sign test: No. of loci with heterozygosity excess (probability)	9.63 ($p=0.287$)	9.99 ($p=0.101$)	10.03 ($p=0.00$)*
	Standardized differences test: *T*2 values (probability)	− 1.073 ($p=0.142$)	− 3.721 ($p=0.00$)*	− 8.334 ($p=0.00$)*
	Wilcoxon test (probability of heterozygote excess)	0.785	0.968	0.999
Purna	Sign test: No. of loci with heterozygosity excess (probability)	8.78 ($p=0.555$)	9.19 ($p=0.931$)	9.53 ($p=0.001$)*
	Standardized differences test: *T*2 values (probability)	− 2.05 ($p=0.373$)	− 2.025 ($p=0.022$)*	− 4.549 ($p=0.00$)*
	Wilcoxon test (probability of heterozygote excess)	0.662	0.940	0.994
Shivna	Sign test: No. of loci with heterozygosity excess (probability)	9.40 ($p=0.301$)	9.77 ($p=0.050$)*	10.04 ($p=0.015$)*
	Standardized differences test: *T*2 values (probability)	0.244 ($p=0.404$)	− 1.562 ($p=0.060$)	− 4.315 ($p=0.00$)*
	Wilcoxon test (probability of heterozygote excess)	0.351	0.894	0.991

Table 7 (continued)

Population	Test	Microsatellite mutation models		
		IAM	TPM	SMM
Godavari	Sign test: No. of loci with heterozygosity excess (probability)	9.21 ($p=0.445$)	9.74 ($p=0.143$)	9.93 ($p=0.017$)*
	Standardized differences test: $T2$ values (probability)	− 0.119 ($p=0.453$)	− 2.036 ($p=0.021$)*	− 4.762 ($p=0.00$)*
	Wilcoxon test (probability of heterozygote excess)	0.500	0.849	0.973
Narmada	Sign test: No. of loci with heterozygosity excess (probability)	6.39 ($p=0.040$)*	7.06 ($p=0.085$)	7.45 ($p=0.123$)
	Standardized differences test: $T2$ values (probability)	2.379 ($p=0.009$)*	1.714 ($p=0.044$)*	1.215 ($p=0.112$)
	Wilcoxon test (probability of heterozygote excess)	0.0199*	0.064*	0.270

IAM infinite allele model, *TPM* two-phase model, *SMM* stepwise mutation model

*Statistically significant at $P \leq 0.05$

average genetic diversity values observed in terms of the allele numbers and the expected heterozygosity among eight *C. batrachus* populations was 3.75 and 0.40, respectively. These values were lower than those reported in the meta-analysis of microsatellite polymorphisms in freshwater fish by DeWoody and Avise (2000) who found allele numbers of 9.1 ± 6.1 alleles and values of expected heterozygosity of 0.54 ± 0.25 per population. Clearly, in these categories, the genetic diversity values found in Clarias populations in India are lower, even in comparison to studies of other Clarias populations from Bangladesh, where the average number of alleles was 5.57 and value for expected heterozygosity was 0.63 (Islam et al. 2007). And although in India, Srivastava et al. (2017) did find high genetic diversity among three populations of *C. batrachus* using nine SSR markers, reporting an average of 0.84 alleles and expected heterozygosity of 0.87, only three populations were studied. Also, each group of populations was analyzed independently, and the microsatellite markers used were different from our study.

In populations, loss of genetic diversity may be driven by bottlenecks or natural selection against heterozygotes (Selkoe et al. 2006; Li et al. 2017). In addition, for fish species, ecological factors such as exploitation pressures (Berg and Getz 1989) including water pollution (Dudgeon et al. 2006), destruction or degradation of habitat (Khedkar et al. 2014c) or their combined influences may have great impacts. Over fishing has also been a severe problem during the past decade in India (Khedkar et al. 2010, 2014c) and in China (Kang et al. 2009), and this also known to be a leading cause for the reduction in effective population sizes and yields that in turn can ultimately lead to the loss of genetic diversity and population viability (Hauser et al. 2002; Li et al. 2017). Additionally, in the aquaculture sector in India, the lack of seed regulation policies over seed handling may severely exacerbate the loss of genetic diversity in both culture populations as well as in natural stocks. This can be clearly seen in our analysis where the Kanhan, Kakinada, a hatchery population and the Ganga population showed a distinct cluster. This group appears to be ancient to former populations with significant gene flow suggesting excessive seed exchanges of broods with the hatchery in Kakinada.

In general, loss of genetic diversity in aquaculture stocks (including hatchery stock) in relation to their wild counterparts has also been reported for various fish species in China (eg. *Ctenopharyngodon idella*, Zhang et al. 2006; *Siniperca chuatsi*, Yang et al. 2015) and in India (*Labeo gonius*, Behera et al. 2018). These reductions in genetic diversity may be due to random genetic drift or improper processes used for domestication of stocks that may be occurring in aquaculture populations. Surprisingly, in our study, the number of alleles (N_a) and expected heterozygosity (H_e) for each locus of the wild populations ($N_a = 3.70$, $H_e = 0.43$) was lower than that of the hatchery population (Kakinada; $N_a = 5.16$, $H_e = 0.49$). This may be due to a number of factors such as poor brood stock management as well as loss of diversity though over collection of brood fish from diverse wild resources. Also, the lack of regulation of entry of seed from hatcheries into the same habitat may severely impact the structure of the remaining wild population (Khedkar et al. 2014b). In support of this, Van Der Bank et al. (1992) made similar observations, where possibly due to outcross breeding with genetically diverse individuals, one domesticated stock of *C. garipinus* had relatively high mean heterozygosity values.

The lack of significant genetic differentiation among the Ganga, Purna, Shivna, Godawari, Girna, and Narmada population may suggest either a relatively short domestication history or high levels of gene flow resulting in populations that are almost clonal in structure (Fig. 2) (Reichel et al. 2016). This is further supported by the fact in our study that all eight populations could be organized into five clusters having low F_{ST} values, consistent with the suggestion that they in fact represent a single metapopulation (Fig. 3). On an average we included 20 individuals per populations for genetic analysis. As suggested by Hale et al. (2012), variability in allele frequency (NA) and expected heterozygosity (H_e) among replicates decreased with increasing sample size, but these decreases were minimal above sample sizes of 25–30. Therefore, there appears to be little benefit in sampling more than 25–30 individuals per population for population genetic studies based on microsatellite allele frequencies. In the present study, various analysis has already revealed fairly low genetic variability, denotes that number of individuals used were sufficient to demonstrate loss of genetic diversity. However, for clearer insights for population bottleneck analysis, further sampling would be valuable since Wilcoxon test showed nonsignificant heterozygosity excess.

In our study, in 50% of cases, the values for the absolute number of migrants ($M > 2$) suggests excess gene flow among populations, and this in turn suggests that these populations may originate from the same source. Heterozygote deficiency was also observed at 70 out of 144 loci (Table 2). In wild fish populations, such deficiencies of heterozygotes are generally considered to be the result of several possible factors such as a limited number of founders, random genetic drift, or inbreeding. But here, in part, it may also be correlated to the supplying of seed from a major seed producing region and the use of wild broods in the hatchery.

The STRUCTURE clustering software is used to infer the correct number of subpopulations and for the assignment of individuals appropriately to these subpopulations even when genetic differentiation among groups is low (Latch et al. 2006) and when even a relatively small number of loci are available (Pritchard et al. 2000). In our case, results derived from this program provide strong support for five clustered subdivisions. This seems to be reasonable since over 90% of *C. batrachus* seed used in aquaculturing is supplied from only two regions in India (Kainada, Andhra Pradesh and Howrah, Kolkata) (Khedkar et al. 2010). Also, most of the aquaculture ponds are in the vicinity of reservoirs and the riverine network, and through accidental escapes these individuals are likely have entered most of the water resources in India. Clearly these populations show more genetic homogeneity, and the low average percentage of the ability to make assignment (6.86%) of individuals to the population they were collected from points is consistent with the loss of genetic differences between populations. In addition, the AMOVA indicates that 27.16% of the total genetic variation is between populations while the remaining 64.63% corresponds to differences among individuals. Of course, the extent to which factors such as random genetic drift, regional differences in selective regimes, and different histories of mutation event may or may not contribute to genetic differences in these populations must also be considered in future studies (Qi et al. 2015; Li et al. 2017).

Genetic differences were not correlated with geographic distances among populations (Mantel test) since physical translocation and seed ranching practices do not

align the analysis. This further suggests that management factors such as the limited exchange of brood fish between hatcheries or the transport of seed over large areas could constitute reasons for the existence of the *C. batrachus* population subdivisions. However, the consequences of loss of genetic diversity are becoming more prominent in majority of culture facilities and *C. batrachus* is suffered with poor larval survival, bacterial diseases, and poor growth. In wild populations, reproductive isolation and the consequences of the local use and management practices of the fish will also reduce the effective population size and contribute to the genetic subdivision. From the use of Wright' *F*-statistics, the average value of F_{IS} across all loci was low (0.034, Fig. 2). This suggests that more than inbreeding, processes affecting subdivision could be the cause of the observed genetic differences between populations. Furthermore, the populations analyzed showed deviations from HW equilibrium as revealed by the occurrence of heterozygosity values that were smaller in observed populations than would be expected. This heterozygote deficiency is possibly also a reflection of the subdivided population structure rather than selection against heterozygotes.

Genetic Bottleneck Signature

Bottleneck detection is critical for interpreting the historical demography of populations and is informative for establishing conservation strategies for endangered animals (Cornuet and Luikart 1996; Du et al. 2016). Simulations inferred from three regions of mitrochondrial DNA show that the *C. batrachus* experienced a severe historical demographic decline since past two decades (Khedkar et al. 2014a, b, c). Although the Wilcoxon test detected nonsignificant signature for recent population bottleneck in the *C. batrachus* under all three models via BOTTLENECK in the present study, a recent well-documented decline in the population size of the *C. batrachus* has occurred over the three decades, with steady decline in population size (Khedkar et al. 2014b). Unregulated seed ranching (Mahesh et al. 2020), rampant fishing (Khedkar et al. 2010), introduction of exotics (Khedkar et al. 2014a; Tiknaik et al. 2019) could account for this severe demographic reduction. The bottleneck was not detected using the Wilcoxon test for heterozygous excess probably because the number of loci analyzed was small (Cornuet and Luikart 1996; Piry et al. 1999) or due to an insufficient sample size (Cornuet and Luikart 1996), however, based on the results of TPM and SMM analysis and present population history of *C. batrachus* we accept genetic bottleneck signature.

Overall, the results of our study can be correlated with key technical, economic, and social factors hindering the growth of the aquaculture seed production and management subsectors in India. This key issue may be more germane in refining the seed quality as one of the recommendations by the expert group in Bangaladesh, FAO (2015). For this purpose and based on our study findings, it can be suggested to have a regional brood banking program for *C. batrachus* in India. Nationwide translocation of broods as well as seed supply must be regulated to avoid further inbreeding events leading to further loss of genetic diversity and eliminating genetic

bottleneck in *C. batrachus* populations. Genetic considerations must be taken into account in the hatchery operations and in scheduling breeding programs.

Conclusion

The analysis of population structure done here has shown that in India, *C. batrachus* populations are low in genetic diversity. This has implications for policy regulations and certification of the use of fish seeds for hatcheries, and for management practices such as the extensive exchange of seed/brood fish between different habitats. This is further supported by the levels of gene flow detected and evidence for genetic bottleneck could in turn be a major factor affecting the *C. batrachus* populations. Since, poor growth, poor larval survival. and diseases are the major problems affecting *C. batrachus* culture populations, the potential benefit that each population could contribute to mitigating these effects should be included in conservation decisions. Also, since molecular markers clearly allow for inferring genealogical relationships, the use of markers such as the microsatellites described here should be included in any of the policy criteria used for maintenance of hatchery brood collections. Furthermore, these markers can be used in breeding schemes to improve desirable traits and to avoid the deterioration of culture populations. Finally, the systematic use of these markers can also be included in a program designed for the comprehensive management of endangered natural populations.

Compliance with Ethical Standards

Conflict of interest The funding from Department of Biotechnology, Government of India, New Delhi (vide File No. 102/IFD/SAN/4469/2011-2012 Dated Feb 17, 2012) and DR. Babasaheb Ambedkar Marathwada University Aurangabad, India (Ref. No. STAT/IV/RC/Dept./2019-20/295-96 dated 13.06.3019) to G. D. Khedkar are greatly acknowledged. We declare that we have no financial or personal relationships relating to the work submitted here that could influence or bias this work.

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