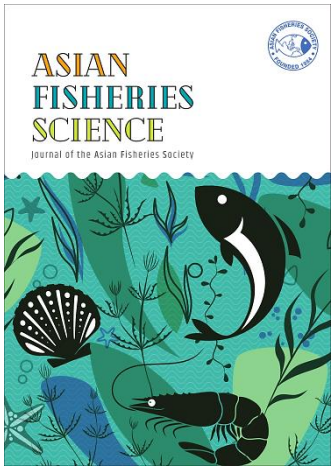

Genetic Diversity and Population Structure of Endangered Catfish *Rita rita* (Hamilton, 1822) Revealed by Heterologous DNA Microsatellite Markers

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Abstract

Genetic information is essential for conservation and future aquaculture development of the endangered catfish *Rita rita* (Hamilton, 1822). Two hundred catfish, *R. rita*, 50 from four rivers, the Old Brahmaputra, Jamuna, Meghna and Kangsa were collected and analysed to evaluate the genetic diversity and population structure using five microsatellite primers (*Cba06-KUL*, *Cba08-KUL*, *Cba09-KUL*, *Phy03-KUL* and *Phy07-KUL*). Four of the five amplified loci were found polymorphic (P_{95}) in all the populations and 46 alleles were recorded with 9 to 14 alleles per locus. Differences were observed in the total number of alleles ranging from 41 to 44, effective number of alleles from 29.96 to 37.46, observed heterozygosity from 0.57 to 0.76, Shannon's information index from 2.09 to 2.30 and polymorphic information content from 0.84 to 0.88 among the four populations. Results exposed the highest levels of genetic diversity in the Meghna population while the lowest in the Kangsa population of *R. rita*. All the populations were significantly deviated ($P < 0.001$) from the Hardy-Weinberg equilibrium for all the loci. Nei's genetic distance between populations ranged 0.007 to 0.017 with low overall genetic difference F_{ST} = 0.011 and high gene flow N_m = 24.333, indicating that *R. rita* populations were not subdivided. This study revealed a high level of gene diversity with deficiency in genetic heterogeneity in all the populations of *R. rita*, emphasising natural management, conservation and rehabilitation measures of this species.

Keywords: Bagridae, cross-species amplification, polymorphism, genetic variation, conservation

Introduction

The catfish *Rita rita* (Hamilton, 1822) belongs to the family Bagridae under the order Siluriformes. The *Rita* genus includes six extant species, *Rita chrysea* Day, 1877, *Rita gogra* Sykes, 1839, *Rita kuturnee* Sykes, 1839, *Rita macracanthus* Ng, 2004, *R. rita*, and *Rita sacerdotum* Anderson, 1879, and one extinct species, *Rita grandiscutata* Lydekker, 1886, are extensively distributed in different countries of the world (Ferraris, 2007), and among them, only *R. rita* is available in Bangladesh (Rahman, 2005; Siddiqui et al., 2007; DoF, 2014). *Rita rita* is also native to Afghanistan, Pakistan, India, Nepal and Myanmar (Froese and Pauly, 2007). It is found in freshwater (Shaji, 1995), estuaries and coastal water bodies (Mirza, 1982; Talwar and Jhingran, 1991; Rahman, 2005; Yashpal et al., 2006; Siddiqui et al., 2007). It is popular not only for its good taste but also from the nutritional and medicinal perspectives. The muscles of the fish contain high protein (17.22–19.55 %), low fat (1.01–2.70 %) and a good amount of minerals (0.89–1.07 %) compared to other teleosts as well as catfishes (Mitra et al., 2017) and rich in vitamin A (Siddiqui et al., 2007). Consequently, the Rita fish fetches high demand and price in the market. Domestication of *R. rita* has not been successfully done and people still depend on the wild sources for seeds. In early studies, it is observed that overfishing, habitat fragmentation and damage, aquatic pollution and other anthropogenic reasons caused severe reduction in the indigenous stock of *R. rita* (Parveen and Faisal, 2002; World Bank, 2005; IUCN, 2015). At present, *R. rita* has been categorised as endangered in Bangladesh (IUCN, 2015), near threatened in India

(CAMP, 1998; Gupta, 2015) and least concerned in the world (IUCN, 2015). Considering the biodiversity status of *R. rita*, it is urgent to conserve and manage the species and for doing so, vital information on relevant population genetics is essential, specifically its genetic diversity and structure. So far, it is the first attempt to analyse genetic diversity and population structure of *R. rita*.

Population decline is the foremost anxiety for conservation genetics due to potentially adverse effect for species. Small and fragmented populations can suffer alteration such as loss of genetic diversity, fixation of harmful alleles, inbreeding, and reduced fitness (Frankham et al., 2009). Such consequences can reduce a given species' to adjust to future ecological changes, escalating the risk of extinction (Frankham, 2005). The levels and patterns of genetic diversity and structure within and between populations and species are evaluated using molecular markers as powerful tools for managing and conserving target species (Allendorf et al., 2010; Chauhan and Rajiv, 2010). Among the molecular markers, microsatellites are popularly used for their highly polymorphic, abundant, short sized nature with repetitive arrays and are conserved between species and families, and hence offer a higher resolution in assessing genetic variation among populations (Cunningham and Meghen, 2001; Waldbieser et al., 2001).

Additionally, microsatellite markers are co-dominant and easy to score by polymerase chain reaction (PCR), and added new dimensions in the field of fisheries and aquaculture as many populations are subjected to bottleneck, inbreeding and genetic drift. As a result, it exhibits low variation that other markers cannot detect (Lui and Cordes, 2004; Chistiakov et al., 2006). However, microsatellites are not always available for the species to be studied, and their isolation could be time-consuming (Lin et al., 2008; Wang et al., 2008). Therefore, researchers frequently rely on cross-species amplification (Chang et al., 2008; Kupper et al., 2008; Lin et al., 2008).

Microsatellite markers have been developed from the genome of several catfish species, including *Clarias gariepinus* Burchell, 1822 (Galbusera et al., 1996), *Clarias batrachus* Linnaeus, 1758 (Yue et al., 2003), *Clarias macrocephalus* Günther, 1864 (Na-Nakorn et al., 1999; 2004), *Mystus nemurus* Valenciennes, 1840 (Usmani et al., 2001), *Ictalurus punctatus* Rafinesque, 1818 (Liu et al., 1999) and used for the study of population genetic structure (Waldbieser and Wolters, 1999). As microsatellite markers have not been isolated from *R. rita*. Whereas the markers developed from other catfish species like *C. gariepinus* (Galbusera et al., 1996), *C. batrachus* and *Pangasius hypophthalmus* Sauvage, 1878 (Volckaert et al., 1999) were used as cross-species amplification to reveal the genetic diversity and compare the genetic variation in four different riverine populations of Rita in Bangladesh. Thus, the present study was aimed to evaluate the genetic diversity and population structure of the endangered catfish *R. rita* from four rivers, the Old Brahmaputra, Jamuna, Meghna and Kangsa.

Materials and Methods

Sample collection and genomic DNA isolation

A total of 200 *R. rita* samples were collected from four different populations (50 from each river), the Old Brahmaputra (Mymensingh), Jamuna (Jamalpur), Meghna (Chandpur) and Kangsa (Netrakona) (Fig. 1) during November 2015 to February 2016. Fish were brought to the Fish Genetics and Biotechnology Laboratory in the Department of Fisheries Biology and Genetics, Bangladesh Agricultural University, Mymensingh. About 30 mg of caudal fin tissue was clipped from each individual. The fin tissues were cut into small pieces with scissors and ground with a mortar and pestle in extraction buffer. The sample was taken into 1.5 mL microcentrifuge tube and digested with proteinase-K (20 mg.mL^{-1}) in TEN buffer (100 mM Tris, 10 mM EDTA and 250 mM NaCl, pH = 8.0 and 1 % SDS) for overnight at 37 °C. After digestion, the genomic DNA was purified by successive extraction with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1, v:v:v) and chloroform: isoamyl alcohol (25:1, v:v), and precipitated using 0.6 volume of isopropanol and ethanol as described by Tonny et al. (2014) with some modifications. After drying, the extracted DNA was resuspended in TE buffer (10 mM Tris and 1 mM EDTA, pH = 8.0). The quality of DNA was tested by electrophoresis on 1 % agarose gel (UltraPure™ Agarose, invitrogen) and quantity was measured by using a spectrophotometer (Eppendorf Bio Spectrotometer, Germany).

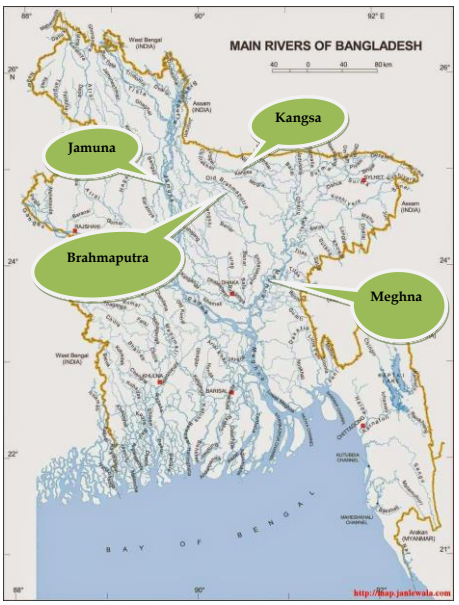

Fig. 1. Sampling locations of *Rita rita* from four populations in Bangladesh to study the genetic diversity and population structure.

Amplification of microsatellite markers by PCR

A total of 12 pairs of microsatellite primers were tested for cross-species amplification in *R. rita*, of which four pairs of primers, *Cba02-KUL*, *Cba06-KUL*, *Cba08-KUL* and *Cba09-KUL* developed from the walking catfish *C. batrachus* (Yue et al., 2003), two pairs of primers, *Cma2* (Na-Nakorn et al., 1999) and *Cma12* (Sukmanomon et al., 2003) originated from *C. macrocephalus*, two pairs of primers, *Cga03* and *Cga06* invented from sharp tooth catfish *C. gariepinus* (Galbusera et al., 1996) and four pairs of primers, *Phy01-KUL*, *Phy03-KUL*, *Phy05-KUL*, *Phy07-KUL* developed from *P. hypophthalmus* (Volckaert et al., 1999). Among them, five microsatellite markers produced clear scorable banding patterns (Table 1). PCR was carried out in a 12 µL reaction volume containing 50 ng of template DNA, 0.25 µM of each primer, 0.25 mM of each dNTPs, 1 unit *Taq* DNA polymerase (TaKaRa, Japan) and 1.2 µL 10× reaction buffer containing 1.5 mM $MgCl_2$. An oil-free thermal cycler (Nexus Mastercycler, Eppendorf, Germany) was used with a temperature profile comprising 3 min initial denaturation at 95 °C, followed by 30 cycles, each of 30 sec at 95 °C, 30 sec at respective annealing temperature (Table 1), and 45 sec at 72 °C for extension. Finally, an additional one cycle of 10 min at 72 °C was added for elongation of the amplified products.

Electrophoretic separation and visualisation of PCR products

About 4.5 µL of amplified PCR products of each microsatellite was mixed with 1.5 µL 6× loading dye (Thermo Fisher Scientific) and run on 2 % standard agarose gel to check the quality of amplification. The PCR products showing good resolution on agarose gel were separated on 10 % polyacrylamide gel containing 29:1 acrylamide: bis-acrylamide with 10 % APS (w.v^{-1}). Electrophoresis was conducted using dual slab electrophoresis chamber in PAGE system (AE-6220, Atto Corporation, Japan) at 120 volts for about 4 h. A molecular weight marker DNA (100 bp DNA ladder, TaKaRa, Japan) was loaded in the gel. After electrophoresis the DNA fragments was visualised using the ethidium bromide (10 mg.mL^{-1}) staining protocol. The gels were viewed in the gel doc (UVIDOC HD5, France) and exposed to UV light. The gel images were saved as jpg files.

Scoring of bands and statistical analysis of microsatellite data

The genotype of each fish was assessed and recorded from the gel stained with ethidium bromide for each microsatellite locus. The marker length and allelic length was estimated from the gel image using the software AlphaEaseFC version 4.0. A single genotypic data matrix was constructed for all the loci. Estimation of allelic variations, values of observed heterozygosity (H_o) and expected heterozygosity (H_e), fixation index, Shannon's information index, deviation from Hardy-Weinberg equilibrium, population homozygosity values (observed and expected), analysis of molecular variance (AMOVA), inbreeding co-efficient (F_{IT}), population differentiation (F_{ST}), gene flow (N_m) and Nei's genetic distance (Nei, 1972) were performed using the software GenAlEx 6.502 and POPGENE version 1.31 (Nei, 1972; Peakall and Smouse, 2012). The polymorphic information content (PIC) was calculated using an online program PolyPICker (https://www.genecalculators.net/pq-chwe-polypicker.html). A dendrogram was drawn based on the genetic distance between the populations by following the unweighted pair group method with arithmetic mean (UPGMA). The Bayesian clustering methodology of population structure was analysed with a burn-in length of 50,000, followed by 500,000 Markov Chain Monte-Carlo (MCMC) iterations using prior information and the admixture model by the STRUCTURE software 2.3.4 (Pritchard et al., 2000; Falush et al., 2003). Ten independent runs were conducted for each k value (where k represents the number of clusters), ranging from 1 to 5. The optimal number of clusters (k value) was determined based on the second-order rate of change of log probabilities (Evanno et al., 2005) using the STRUCTURE HARVESTER software (Earl and vonHoldt, 2012).

Table 1. The details of five microsatellite markers that produced scorable bands.

No.	Locus	Primer sequence (5′→3′)	Tan (°C)	Accession No.	No. of alleles	Range of alleles (bp)
1	*Cba06-KUL*	F:TGGTGGTCTGACCGATGACTCC R:TGTGCAGCAGTTTGTTAAAGTTCC	54	AJ131376	3	221-251
2	*Cba08-KUL*	F:AGTGGTTCTTTATGTGGTAAGTG R:TACAATACTCCGAGGTGGGC	54	AJ131378	7	180-200
3	*Cba09-KUL*	F:CAAGACAACAAGGCTGTGGTGC R:TGCATCTGATGACCCATATTTGC	54	AJ131379	2	208-210
4	*Phy03-KUL*	F: TAGGAGTCAGGAGTCGC R: TGCAACGGTAACAAACC	54	AJ131381	4	166-194
5	*Phy07-KUL*	F: GATCAGTCACTTCAGCACCTGCC R: TCCAAATCTCTGTGATGGTGAGCC	68	AJ131383	5	214-270

Results

Allelic and genotypic variability

A total of 47 alleles were detected from the five analysed microsatellite loci. The size of the alleles for all loci ranged from 127 to 640 bp (400 bp, 490 to 550 bp, 590 to 640 bp, 127 to 175 and 191 to 243 for the loci *Cba06*, *Cba08*, *Cba09*, *Phy03* and *Phy07*, respectively) (Table 2). Only the Meghna population had a private allele (*Cba08* 540 bp). The total number of missed alleles across the five loci was highest in the Kangsa (5) followed by the Old Brahmaputra (3), the Jamuna (2) and the Meghna (2) populations (Table 2).

Table 2. Allele size and frequencies at five microsatellite loci in four populations of *Rita rita*.

Locus name	Allele size (bp)	Old Brahmaputra	Jamuna	Meghna	Kangsa
Cba06	400	1.00	1.00	1.00	1.00
Cba08	490	0.11	0.21	0.06	0.16
	495	0.08	0.15	0.10	0.10
	500	0.28	0.10	0.14	0.24
	505	0.14	0.08	0.13	0.18
	510	0.14	0.13	0.17	0.24
	515	0.16	0.12	0.12	0
	540	0	0	0.10	0
	545	0.05	0.09	0.05	0.04
	550	0.04	0.12	0.13	0.04
Cba09	590	0.14	0.08	0.12	0.14
	595	0.12	0.11	0.10	0.12
	600	0.14	0.15	0.09	0.23
	605	0.20	0.12	0.14	0.21
	610	0.14	0.09	0.14	0.18
	615	0.14	0.16	0.10	0
	620	0	0.10	0.08	0.06
	630	0.04	0.07	0.05	0.03
	635	0.08	0.06	0.08	0.03
	640	0	0.06	0.10	0
Phy03	127	0.09	0	0.05	0
	131	0.03	0.04	0.08	0.01
	135	0.04	0.15	0.12	0.08
	139	0.13	0.13	0.14	0.08
	143	0.12	0.04	0.06	0.13
	147	0.16	0.07	0.10	0.15
	151	0.10	0.21	0.10	0.08
	155	0.06	0.16	0.17	0.06
	159	0.08	0.05	0.08	0.10
	163	0.09	0.04	0.06	0.08
	167	0.04	0.04	0.02	0.15
	171	0.03	0.04	0	0.07
	175	0.03	0.03	0.02	0.01
Phy07	191	0.07	0.05	0.02	0.05
	195	0.06	0.08	0.07	0.02
	199	0.07	0.04	0.08	0.03
	203	0.16	0.10	0.12	0.11
	207	0.09	0.15	0.11	0.09
	211	0.06	0.06	0.13	0.20
	215	0.06	0.08	0.01	0.05
	219	0.04	0.07	0.07	0.04
	223	0.10	0.03	0.08	0.12
	227	0.10	0.13	0.12	0.09
	231	0.10	0.12	0.09	0.12
	235	0.07	0.01	0.07	0.05
	239	0.01	0.04	0	0.02
	243	0.01	0.04	0.03	0.01
No. of missed allele across all loci		3	2	2	5

Four of the five microsatellite loci except *Cba06* were found to be polymorphic (P_{95}). The number of alleles across the loci ranged 9 to 14, while the total number of alleles and the mean number of alleles were 46 and 11.50, respectively. The locus *Phy07* had the highest number of alleles (*N* = 14) in the three populations except for the Meghna, and the *Cba08* had the lowest allele (*N* = 7) in the Kangsa population. The average number of alleles was highest in both the Meghna and the Jamuna populations (11.00) followed by the Old Brahmaputra (10.75) and the Kangsa (10.25) populations (Table 3). While the overall effective number of alleles (*Ne*) across the loci ranged 7.51 to 11.41, the total *Ne* was 38.14 and the mean *Ne* was 9.54. The average *Ne* was highest in the Meghna population (9.37) followed by the Jamuna (8.75), Old Brahmaputra (8.56), and the Kangsa (7.49) populations (Table 3). The average observed (H_o) and expected (H_e)

Table 3. Allelic and genotypic variation at four polymorphic microsatellite loci in the four populations of *Rita rita*.

Microsatellite loci	Parameters	Old Brahmaputra	Jamuna	Meghna	Kangsa	Overall
	N	8	8	9	7	9
	Ne	6.03	7.31	8.14	5.36	7.51
	H_o	0.18	0.42	0.56	0.16	0.33
Cba08	H_e	0.84	0.87	0.89	0.82	0.87
	F_{IS}	0.79	0.52	0.37	0.80	0.62
	I	1.92	2.04	2.14	1.77	2.09
	PIC	0.81	0.85	0.86	0.79	0.85
	N	8	10	10	8	10
	Ne	7.10	8.99	9.35	5.92	8.43
	H_o	0.24	0.38	0.46	0.12	0.30
Cba09	H_e	0.87	0.90	0.90	0.84	0.88
	F_{IS}	0.72	0.58	0.49	0.86	0.66
	I	2.01	2.25	2.27	1.88	2.21
	PIC	0.84	0.88	0.88	0.81	0.87
	N	13	12	12	12	13
	Ne	10.10	7.97	9.42	9.42	10.79
	H_o	1.0	1.0	1.0	1.0	1.0
Phy03	H_e	0.91	0.88	0.90	0.90	0.91
	F_{IS}	- 0.10	- 0.14	- 0.11	- 0.11	- 0.10
	I	2.42	2.26	2.34	2.32	2.45
	PIC	0.89	0.86	0.88	0.88	0.90
	N	14	14	13	14	14
	Ne	10.99	10.71	10.55	9.26	11.41
	H_o	1.0	1.0	1.0	1.0	1.0
Phy07	H_e	0.92	0.92	0.91	0.90	0.91
	F_{IS}	- 0.09	- 0.09	- 0.10	- 0.11	- 0.10
	I	2.49	2.48	2.43	2.39	2.52
	PIC	0.90	0.90	0.90	0.88	0.91
Total *N* over population		43	44	44	41	46
Average *N* over loci		10.75	11.00	11.00	10.25	11.50
Total *Ne* over population		34.22	34.98	37.46	29.96	38.14
Average *Ne* over loci		8.56	8.75	9.37	7.49	9.54
Average H_o over loci		0.61	0.70	0.76	0.57	0.66
Average H_e over loci		0.89	0.89	0.90	0.87	0.89
Average I over loci		2.21	2.26	2.29	2.09	2.32
Average PIC over loci		0.86	0.87	0.88	0.84	0.88
Polymorphism (P_{95})		100	100	100	100	100

(*N* = Number of alleles, *Ne* = Effective number of alleles, H_o = Heterozygosity observed, H_e = Heterozygosity expected, $F_{IS} = 1 - H_o.H_e^{-1}$ = fixation index, I= Shannon's information index and PIC = Polymorphic information content).

heterozygosity values were highest in the Meghna population (0.76 & 0.90) followed by the Jamuna (0.70 & 0.89), the Old Brahmaputra (0.61 & 0.89) and the Kangsa (0.57 & 0.87) populations (Table 3). The fixation index ($F_{IS} = 1 - H_o.H_e^{-1}$) value was found positive at two loci (*Cba08* and *Cba09*) and that was negative at *Phy03* and *Phy07* loci in four different populations. The positive values of F_{IS} pointed out an extreme deficiency of heterozygotes of particular loci (*Cba08* and *Cba09*) in all the populations. Polymorphic information content (PIC) over the loci varied from 0.79 to 0.90 among populations, and their averages were 0.88, 0.87, 0.86 and 0.84 in the Meghna, Jamuna, Old Brahmaputra and Kangsa population, respectively (Table 3).

Deviation from Hardy-Weinberg expectation

Significant deviations ($P < 0.001$) from Hardy-Weinberg Equilibrium (HWE) were detected in all the 16 tests (Table 4). The test for fit to Hardy-Weinberg proportions revealed that all the four studied populations were significantly ($P < 0.001$) deviated at all the polymorphic loci *Cba08*, *Cba09*, *Phy03* and *Phy07* (Table 4).

Inter population genetic structure

Pair-wise homogeneity test

Inter population genetic structure and pair-wise comparisons of different populations of Rita using homogeneity tests revealed that 18 tests out of 24 were found to be significant at different degrees (Table 5). The analysis also showed significant differences between the Old Brahmaputra and the Jamuna population at loci *Cba08*, *Cba09* and *Phy03*; the Old Brahmaputra and the Meghna population at loci *Cba08*, *Cba09* and *Phy03*; the Old Brahmaputra and the Kangsa population at loci *Cba08*, *Cba09* and *Phy03*; the Jamuna and the Meghna population at loci *Cba08* and *Phy07*; the Jamuna and the Kangsa population at all four loci; the Meghna and the Kangsa population at loci *Cba08*, *Cba09* and *Phy03* at varying degrees ($P < 0.05$, $P < 0.01$, $P < 0.001$).

Table 4. Deviation from Hardy-Weinberg genotype frequency expectations at four polymorphic microsatellite loci in four populations of *Rita rita* (χ^2 values, followed by degrees of freedom in parentheses).

Microsatellite loci	Old Brahmaputra	Jamuna	Meghna	Kangsa	Overall
Cba08	285.59***(28)	253.18***(28)	276.77***(36)	219.57***(21)	906.64***(36)
Cba09	277.76***(28)	322.21***(45)	342.14***(45)	273.49***(28)	1225.40***(45)
Phy03	195.75***(78)	145.88***(66)	258.64***(66)	215.02***(66)	695.43***(78)
Phy07	252.26***(91)	227.96***(91)	196.73***(78)	349.88***(91)	652.79***(91)

Statistically significant values are marked with asterisks ***$P < 0.001$.

Table 5. Homogeneity between the samples of *Rita rita* (χ2 values followed by degrees of freedom in parentheses).

Loci	Stocks	Jamuna	Meghna	Kangsa
Cba08	Old Brahmaputra	21.17**(7)	22.02**(8)	20.67**(7)
Cba09	Old Brahmaputra	22.04**(9)	21.26*(9)	25.13**(8)
Phy03	Old Brahmaputra	34.24***(12)	20.56*(12)	22.07*(12)
Phy07	Old Brahmaputra	17.87^{NS}(13)	13.12^{NS}(13)	13.57^{NS}(13)
Cba08	Jamuna		22.91**(8)	32.48***(7)
Cba09	Jamuna		6.81^{NS}(9)	34.42***(9)
Phy03	Jamuna		17.53^{NS}(12)	34.35***(11)
Phy07	Jamuna		22.89*(13)	25.60*(13)
Cba08	Meghna			36.05***(8)
Cba09	Meghna			31.42***(9)
Phy03	Meghna			39.73***(12)
Phy07	Meghna			16.54^{NS}(13)

Statistically significant values are marked with asterisks. NS = Not Significant, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Inbreeding co-efficient (F_{IT}), population differentiation (F_{ST}) and gene flow (N_m)

The inbreeding co-efficient (F_{IT}), population differentiation (F_{ST}) and gene flow (N_m) values between the population pairs have been compared and found that the populations were heterogeneous (Table 6). The highest F_{IT} value (0.329) was observed between the Old Brahmaputra-Kangsa population pair and the lowest value (0.186) was observed between the Jamuna-Meghna population pair (Table 6). The F_{ST} value between the Jamuna and the Kangsa populations was the highest (0.017) and was lowest (0.007) between the Jamuna and the Meghna populations at $P < 0.01$ (Table 6). On the other hand, the gene flow (N_m) was highest between the Jamuna and the Meghna populations (34.748) across all loci and was lowest between the Jamuna and the Kangsa populations (14.618) of Rita (Table 6).

Analysis of molecular variance (AMOVA) showed that only 1 % of the molecular variance appeared among the populations and 99 % appeared within populations (Table 7). While the highest and lowest pair-wise molecular differences (F_{ST}) was observed between Jamuna-Kangsa (0.02075) and Jamuna-Meghna (0.00379) population pairs (Table 8).

Table 6. Multilocus F_{IT}, N_m and F_{ST} values between pairs of four populations of *Rita rita* across all loci.

Populations	Pair-wise F_{IT}	Mean F_{IT}	Pair-wise F_{ST}	Mean F_{ST}	Pair-wise N_m	Mean N_m
Old Brahmaputra-Jamuna	0.267	0.259	0.012	0.011	20.599	24.333
Old Brahmaputra-Meghna	0.237		0.008		30.804	
Old Brahmaputra-Kangsa	0.329		0.010		26.186	
Jamuna- Meghna	0.186		0.007		34.748	
Jamuna- Kangsa	0.283		0.017		14.618	
Meghna-Kangsa	0.253		0.013		19.042	

$P < 0.01$

Table 7. Summary of AMOVA among and within the populations of *Rita rita*.

Source of variation	df	SS	MS	Est. Var.	%
Among Pops	3	23.960	7.987	0.067	1
Within Pops	196	907.740	4.631	4.631	99
Total	199	931.700	12.618	4.698	100

P < 0.01

Table 8. AMOVA showing pairwise differences (F_{ST}) among four populations of *Rita rita*.

	Old Brahmaputra	Jamuna	Meghna	Kangsa
Old Brahmaputra	0.00000			
Jamuna	0.01372	0.00000		
Meghna	0.00787	0.00379	0.00000	
Kangsa	0.00879	0.02075	0.01653	0.00000

P < 0.05

Admixture population structure

Reliable results were generated across the 10 independent runs for each K value (number of clusters) ranging from 1 to 5 using the STRUCTURE program 2.3.4. The mean estimated log-likelihood value reached a plateau at K=2, which was maintained up to K=5 (Fig. 2A). However, the method proposed by Evanno et al. (2005) revealed two clusters among the four Rita samples included in the analysis (Fig. 2B). Among the 200 individuals, 117 (29 from Old Brahmaputra, 35 from Jamuna, 24 from Meghna and 29 from Kangsa) were assigned to one cluster and 83 individuals to another cluster (K=2, Fig. 3). While 110 individuals were admixed at K=2 and the number of admixture individuals increased with the increase in the K value (K= 2-5, Fig. 3).

Genetic difference and identity

Genetic distance ranged from 0.120 to 0.256 and genetic identity ranged from 0.774 to 0.887 (Table 9). The genetic distance was highest between the Jamuna and Kangsa populations (0.256) and lowest between the Jamuna and Meghna populations (0.120) (Table 9). The UPGMA dendrogram based on Nei's (1972) genetic distance resulted in two major clusters. The Jamuna and the Meghna populations formed one cluster while the Old Brahmaputra and Kangsa populations remained in other clusters (Fig. 4).

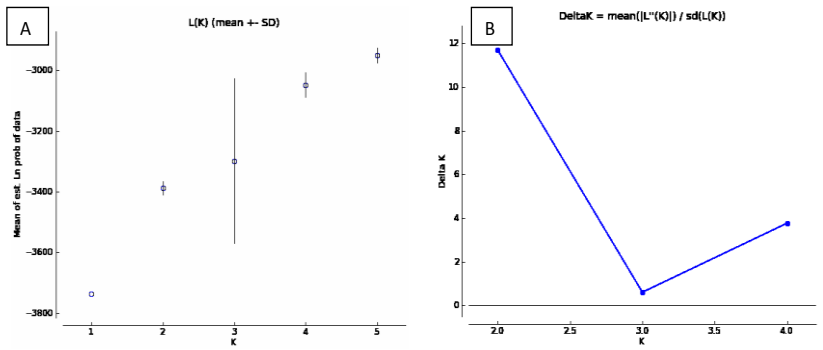

Fig. 2. Determination of the optimal number of clusters (*k*). (A) Mean log probability data L(K) of 10 independent runs for each *k* values ranging from 1 to 5 for the *Rita rita* (*k* represents the number of clusters). Burn-in length=50,000; MCMC iterations=500,000. Vertical bars represent standard deviation. (B) Delta *k* values for each of the *k* inferred clusters, with a maximum value obtained for *k*=2.

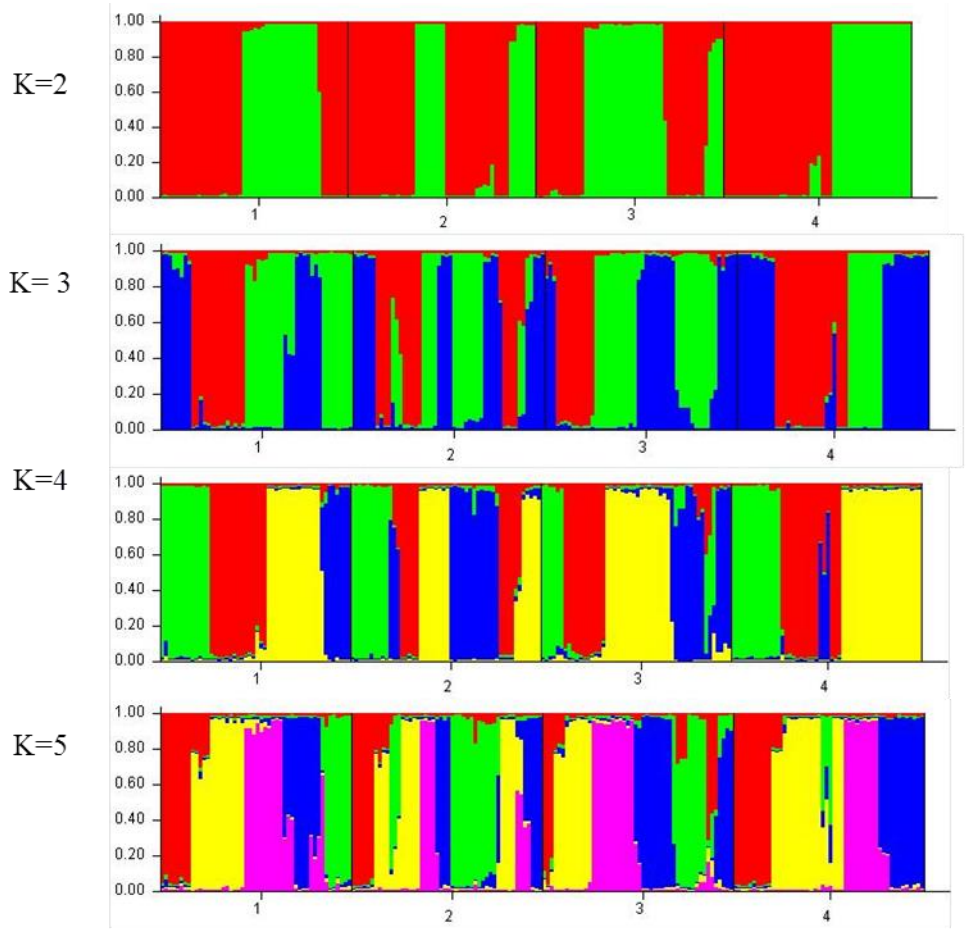

Fig. 3. Population structure across four riverine stocks (1=Old Brahmaputra, 2=Jamuna, 3=Meghna, 4=Kangsa) of *Rita rita* as inferred using Admixture at K=2-5 estimated from the STRUCTURE program. Each vertical line represents one individual, while each colour shows the genetic composition that is assigned into a distinct genetic cluster.

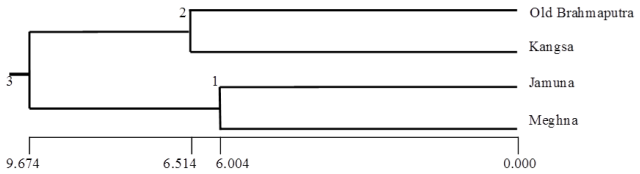

Fig. 4. UPGMA dendrogram based on Nei´s (1972) genetic distance among four stocks of *Rita rita*.

Table 9. Nei´s (1972) original measures of genetic identity (above diagonal) and genetic distance (below diagonal) in four different populations of *Rita rita* (P-value in the parenthesis).

Stocks	Old Brahmaputra	Jamuna	Meghna	Kangsa
Old Brahmaputra	****	0.823	0.878	0.878
Jamuna	0.195 (0.0002)	****	0.887	0.774
Meghna	0.130 (0.0080)	0.120 (0.0087)	****	0.824
Kangsa	0.130 (0.0022)	0.256 (3.90e)	0.193 (5.43e)	****

Discussion

Cross-species amplification

The development of polymorphic molecular markers is an original deed for population genetic studies and similarly cross-species amplification of microsatellite loci is also widely used for every target species (Nasren et al., 2009; Muneer et al., 2011a, b; Ezilrani and Christopher, 2015). Hence, microsatellites isolated from one species usually applied to other closely allied species and also applied in intimately correlated species (Estoup and Angers, 1998; Yue et al., 2003; Islam et al., 2007). Cross-species

amplification of microsatellite loci isolated from *P. hypophthalmus*, *C. macrocephalus* and *C. gariepinus* were effectively used in related catfish species like *Horabagrus nigricollaris* Pethiyagoda & Kottelat, 1994 (Muneer et al., 2011a). Similarly, the 12 microsatellite primer pairs developed from four species of the order Siluriformes viz, *C. batrachus*, *C. macrocephalus*, *C. gariepinus* and *P. hypophthalmus* were tested to cross-species amplification of microsatellite loci in *R. rita* and successful cross-species priming was attained with five primer pairs.

The current study illustrated fruitful cross-priming of microsatellite loci, among the fish species that are distant or unrelated. Several sequences flanking the tandem repeats could be conserved between the various families of the order Siluriformes as indicated in other fishes (Scribner et al., 1996; Zardoya et al., 1996). The cross-amplification results exposed that allelic diversity was higher at all loci except *Cba06* in Rita compared to the original species.

Genetic diversity and variability

Four out of the five loci in *R. rita* were fairly polymorphic, whereas *Cba06* was appeared to be monomorphic in the four populations. A similar result reported previously that seven polymorphic microsatellite markers derived from *C. gariepinus* resulted in three monomorphic loci in *H. longifilis* after cross-amplification (Galbusera et al., 1996), two of the 18 loci were monomorphic in source species *C. batrachus* (Yue et al., 2003), one and two monomorphic loci were recorded from *C. meladerma* and *C. batrachus*, respectively following cross-amplification of *C. macrocephalus* originated eight polymorphic markers (Nazia and Azizah, 2014). In contrast, using heterologous microsatellite markers, all cross-amplified loci were found to be polymorphic in different populations of *C. batrachus* (Islam et al., 2007) and *Heteropneustes fossilis* Bloch, 1794 (Nasren et al., 2009; Sultana et al., 2015) in Bangladesh. Thus, it reveals the differences in the genetic structure among species.

In the present study, at three loci *Cba06* (monomorphic), *Cba08* and *Cba09* (polymorphic) bands produced by cross-species amplification appeared to be much longer than species from the source which is consistent with the findings of *C. fuscus*, *C. gariepinus*, *C. macrocephalus*, *Heterobranchus longifilis* (Valenciennes, 1840), *H. fossilis*, *Phractrocephalus hemioliopterus* (Bloch & Schneider, 1801) and an unclassified catfish for microsatellite loci cross-amplified from *C. batrachus* (Yue et al., 2003). Dissimilar band size was also observed between *C. batrachus* and *C. gariepinus* with the same microsatellite loci (Ezilrani and Christopher, 2015). The present study revealed high levels of allelic diversity from 9 to 14 (average number of alleles per locus 11.50) exist among the four wild stocks. The Meghna and the Jamuna populations contained the highest mean number of alleles (11.00). The Meghna population possessed the highest effective number of alleles (9.37) followed by the Jamuna (8.75), Old Brahmaputra (8.56), and Kangsa (7.49) populations. This might be due to the larger mating population size in Meghna River compared to other populations.

Similar allelic variations were observed in four wild populations of *C. macrocephalus* in Thailand with 8 to 10 alleles at four polymorphic loci (Na-Nakron et al., 1999), 2 to 10 alleles in natural populations of *C. batrachus* (Yue et al., 2003), 6 to 9 alleles in four natural populations of *H. fossilis* (Nasren et al., 2009) and 4 to 13 alleles in two natural and one hatchery stocks of *H. fossilis* (Sultana et al., 2015) in Bangladesh, and 1 to 8 alleles in two critically endangered yellow catfish, *Horabagrus brachysoma* (Günther, 1864) and *H. nigricollaris* in India (Muneer et al., 2011b). The PIC value was higher than 0.5 for all the loci in all the populations (Table 3), indicated that *R. rita* populations have high polymorphism and high genetic diversity. Similarly, a higher PIC value was also observed in *C. gariepinus* using *Heterobranchus bidorsalis* (Geoffroy Saint-Hilaire, 1809) primer in Nigeria (Agbebi et al., 2013), and *C. batrachus* and *C. macrocephalus* primer in India (Ezilrani and Christopher, 2015). The PIC value stands for the number of alleles identified per locus and their frequencies as a higher number of alleles is traced, representing a better genetic population. The Meghna population appeared as a more diversified stock than others. It also possessed a private allele (*Cba08* 540 bp) that indicated its genetic richness. On the other hand, the Kangsa stock contained the lowest total (41) and the average number of alleles (10.25) along with the highest missing alleles (5). The loss of allelic variation has been reported in the natural stock of *C. macrocephalus* in Thailand (Na-Nakron et al., 1999). The lowest genetic diversity observed in the Kangsa population could be postulated that the Rita population in the river is under pressure due to overfishing and worsening of the breeding ecology resulting in less recruitment (Alam and Islam, 2005). As the Kangsa is a small and distantly located from any other rivers sampled, inbreeding due to smaller effective population size could be one of the reasons for a bit lower genetic diversity. The higher allelic diversity observed in the Meghna and Jamuna river populations is generally expected due to their enormous size and vast water area. Therefore, genetically more diversified Rita individuals can be found in the Meghna and Jamuna rivers.

Population bottleneck

The average observed (H_o) and expected (H_e) heterozygosity were found highest in the Meghna stock (0.76 and 0.90) and lowest in the Kangsa stock (0.57 and 0.87), respectively. The average H_o values of all the populations were lower than the corresponding average H_e values for the loci *Cba08* and *Cba09*, while those were higher than the corresponding average H_e

values for the loci *Phy03* and *Phy07*, respectively. These findings are more or less consistent with that of Japanese endangered bagrid catfish *Pseudobagrus ichikawai* Okada & Kubota, 1957 with H_o 0.03 to 0.82 (mean 0.54) and H_e 0.05 to 0.81 (mean 0.56) in the Kawaura river population (Watanabe et al., 2001), Malayasian bagrid catfish *Mystus nemurus* Valenciennes, 1840 (H_o = 0.499, H_e = 0.482) in six river populations (Usmani et al., 2003), and Indian endangered catfish *H. brachysoma* as mean H_o 0.47 and H_e 0.65 in three wild populations (Muneer et al., 2009). On the contrary, a significantly excess H_o were reported in sutchi catfish *P. hypophthalmus* (H_o = 0.734 and H_e = 0.757) in the Mekong River (So et al., 2006), stinging catfish *H. fossilis* (H_o = 0.614 to 0.773 and H_e = 0.774 to 0.839 in four natural populations in Bangladesh (Nasren et al., 2009), bighead catfish *C. macrocephalus* with H_o 0.033 to 0.967 (mean 0.696) and H_e 0.033 to 0.942 (mean 0.789) in the natural population of Malaysia (Nazia and Azizah, 2014).

In *R. rita*, the F_{IS} values were positive in all four populations at the loci *Cba08* and *Cba09* suggesting a significant deficiency of heterozygotes in the populations. The deficiency may be caused by inbreeding or bottleneck effects. The deficiency of heterozygotes (H_o = 0.67 and H_e = 0.76) was also reported in four natural populations of *C. macrocephalus* in Thailand (Na-Nakorn et al., 1999). Conversely, the negative F_{IS} values in Rita at the loci *Phy03* and *Phy07* exposing that the populations had an excess of heterozygosity and individuals could be considered as naturally outbred only at those loci. Negative values of F_{IS} were recorded in almost all populations across all microsatellite loci in *C. batrachus* (Islam et al., 2007). Heterozygote excess in all the populations of Rita was not as common as heterozygote deficiency in the present study. The deficiency of heterozygotes could be due to bottlenecking and/or inbreeding (mean inbreeding coefficient, F_{IT} 0.259 in Table 6), a situation that resulted by over-exploitation leading to a decline of the species in the wild (IUCN, 2015). The high fishing pressure by several types of legal and illegal fishing gears combined with other anthropogenic activities may put the genetic integrity of this species at risk. In addition, non-random mating and less effective population size could be major reasons for heterozygote deficiency (Donnelly et al., 1999; Van Oosterhout et al., 2004) in the riverine stocks of Rita. A similar situation was reported in the bagrid catfish *Chrysichthys auratus* (Geoffroy Saint-Hilaire, 1809) of West Africa (Agne`se, 1991) and in *C. macrocephalus* of Thailand (Na-Nakorn et al., 2004).

Population structure

All four polymorphic microsatellite loci exhibited significant deviations (P < 0.001) from Hardy-Weinberg Equilibrium (HWE). Disappearance from HWE drifted to homozygote excess and possibly due to one or more of the following reasons: 1) missing of alleles or mis-scoring of heterozygote for homozygote. 2) Being thereof a null allele: null allele is an allele that fails to multiply using a microsatellite primer due to mutation happened at a primer site of the loci. It has been highlighted in the genetic studies of *C. macrocephalus* collected from four natural stocks in Thailand (Na-Nakorn et al., 1999). Deviations from HWE are generally ascribed to null alleles, selection, genetic bottleneck, or grouping of gene pools (Walhund effect) (Gibbs et al., 1997). 3) Non-random mating, random change in allele frequency in a population (genetic drift), mutation, migration and natural selection. Nonetheless, a small sample size can be another reason for the failure to detect these alleles. 4) Small sample size: the rationale that makes this hypothesis promising is that all the populations that deviated from HWE in this study increased due to the number of alleles at a locus. As the number of allele at a locus is large, more sample size is required for precise reflection of genotype frequencies (Ruzzante, 1998). It was recommended that the population size should be 50 to 100 individuals or larger for accurate genetic study through microsatellite loci (Ruzzante, 1998). Besides, a severe decline of Rita catfish in natural habitats, the occurrence of inbreeding etc. might result in deficiency of heterozygotes and deviation from HWE (Beaumont and Hoare, 2003). Similar findings were observed in other endangered catfish species such as *H. brachysoma* (Muneer et al., 2009) and *H. nigricularis* (Muneer et al., 2011a) in India owing to over-exploitation resulted in inbreeding.

Significant pair-wise differentiation from homogeneity tests was detected in 18 out of the 24 tests (P < 0.001, P < 0.01 and P < 0.05), indicating all the population pairs were not homogeneous at all studied loci. The study also revealed that all the populations to be genetically differentiated to some extent. Very similar findings were observed in *C. macrocephalus* based on pair-wise exact tests in most of the populations in central Thailand using allozyme and microsatellite markers (Senanan et al., 2004) and *C. batrachus* individuals of the four populations in Bangladesh (Islam et al., 2007). Pair-wise F_{ST} values (0.007 to 0.017) also demonstrated that the population pairs were not homogenous groups, instead, they are differentiated. Excluding the Jamuna and Meghna population pair, the F_{ST} values between all other population pairs widely varied, reflecting a moderate level of genetic variation among individuals of the populations (Table 6). Similar outcomes were recorded in *C. batrachus* (Islam et al., 2007) and *H. fossilis* (Nasren et al., 2009) from a number of individuals of populations in Bangladesh. Comparatively, the highest population differentiation (F_{ST}) and lowest gene flow (N_m) were observed between the Jamuna and the Kangsa population pairs of Rita. Alternatively, the lowest F_{ST} was observed between the Jamuna and Meghna population pair with the highest N_m. The ecological condition, geographical location and physical barrier among the populations

might be the possible reason. Though the Jamuna and Meghna rivers have different origin but the Jamuna River met with Meghna in Chandpur estuary, where both the populations got a chance of mixing with each other. The Kangsa River was geographically separated from the Jamuna River and less possibility of mixing between the two stocks. Substantial population differentiation was also reported in rainbow trout strains collected from broad geographic ranges (Silverstein et al., 2004). In addition, the F_{ST} and N_m values have been influenced by the geographical condition and distance in stream-living brown trout collected from multiple parts of the main stream in Jamtland, Central Sweden (Carlsson et al., 1999). The relatively high level of gene flow (14.618 to 34.748) observed in wild Rita population pairs supported some extent of intermixing among them. The N_m values represented that high levels of gene flow existed among the four populations that might be resulted from some scale of migration during flooding, allowing no incidence of absolute regional genetic isolation of populations. Additionally, the AMOVA summarised in Table 7, genetic variation was mainly attributable to individual level by 99 % within populations, while it was negligible to 1 % among populations; indicated minor genetic variation existed among populations. Moreover, pair-wise molecular differences between populations was also very little (Table 8). Similarly, 96 % and 4 % molecular variation was observed within and among four populations of *Channa lucius* (Cuvier, 1831) in the Mekong Delta, Viet Nam (Sawasawa and Duong, 2020).

Genetic distance and phylogenetic dendrogram

The genetic distance of Rita between the population pairs ranged from 0.120 to 0.256, which indicated that the genetic difference among the studied populations is not prominent. The Jamuna and Kangsa populations maintained the highest genetic distance (0.256), while the lowest genetic distance (0.120) existed between the Jamuna and Meghna populations. These might be caused due to different geographical positions of the stocks. The geographical location seemed to influence the genetic distance in *C. batrachus* (Islam et al., 2007), *H. fossilis* (Nasren et al., 2009; Sultana et al., 2015), where samples were collected from different parts of Bangladesh, and *C. garipienus* in India (Ezilrani and Christopher, 2015). The UPGMA dendrogram based on genetic distance (Nei, 1972) placed the Jamuna and Meghna in one cluster and the Old Brahmaputra and Kangsa in another cluster. The dendrogram showed relatively more distance between the Kangsa and the Jamuna populations. This might be happened due to the geographical isolation between the Kangsa and the Jamuna rivers.

The structuring pattern was also visibly verified in the Bayesian analysis, in which two populations were identified. These populations are distributed along the four tested River basin, where each location represent a mixture of different populations. This result represented the dispersal capabilities of Rita might be due to its reproductive behavior and the geographic proximity of some localities, where many of the sampled sites (like lagoon, marshes, etc.) are interconnected during periods of flooding. Thus, the hypothesis is that the population genetic structure of *R. rita* may be sustained by an occurrence of "reproductive waves" (Jorgensen et al., 2005), performed by genetically segregated groups that breed in the same place at different time periods with some overlap. This idea is supported by Jimenez-Segura et al. (2010).

The limitation of this study was the use of manual sequencing apparatus, and the alleles were separated only by electrophoresis on a denaturing polyacrylamide gel and detected by ethidium bromide staining. While currently, the microsatellite alleles upon PCR amplification usually are separated by sequencing with automatic DNA sequencer/high-throughput technologies in well-structured laboratories of developed countries. However, additional study 1) to develop microsatellite marker for *R. rita* and use for the analysis of genetic variability, 2) involving more numbers of populations and large sample size representing all parts of the country with added microsatellite loci and/or with other types of markers is recommended to mark out a comprehensive genetic structure of this important fish species so that successful conservation can be implemented.

Conclusion

Microsatellite data from four polymorphic primers depicted high levels of genetic diversity and low genetic differentiation of *R. rita* in four wild populations. However, all the populations have deviated from Hardy-Weinberg Expectation emphasising the need for strong fishery management strategies in *in-situ* conservation like habitat protection from pollution, siltation, dam construction, and bans on destructive fishing practices. As well as *ex-situ* conservation, like artificial insemination of the species for aquaculture, can be a viable solution to reduce fishing pressure on Rita for food and developing co-management strategies with the fishing communities. The current study helps to realise the genetic relationship among different populations of Rita inhabiting the River systems and hints in conservation and management of the species. This study is also helpful as reference research for future molecular studies on *R. rita* and other catfishes.

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