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GENETIC CHARACTERIZATION OF BROOD BANK STOCKS OF *CATLA CATLA* (HAMILTON) (CYPRINIDAE:CYPRINIFORMES) COLLECTED FROM THREE DIFFERENT RIVERS OF BANGLADESH

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ABSTRACT

Genetic characterization of the base population of a breeding program is useful in monitoring changes in the genetic structure that might occur over generations. By using microsatellite DNA markers, we have analyzed the genetic population structure of the Jamuna, Padma and Halda river samples of *Catla catla* maintained in a Brood bank of the Department of Fisheries, Bangladesh. We detected a total of 27 alleles at five loci in 90 individuals with an average of 5.4 alleles per locus. The mean allelic richness of the Jamuna, Padma and the Halda population of catla were 4.800, 5.200, and 4.400, respectively. The average observed (*Ho*) and expected (*He*) heterozygosity values for the Jamuna, Padma and the Halda rivers were 0.720, 0.720 and 0.667 and 0.663, 0.678 and 0.643, respectively. The F_{IS} values ranged from -0.341 to 0.121 and found to be nonsignificant while the pairwise F_{ST} values ranged from 0.0142 to 0.0507 and found to be significant. The genetic variations in the Jamuna and the Padma populations are comparable and slightly higher than that of the Halda population. Our study detected an excess of heterozygosity indicating recent genetic bottlenecks in the brood bank founder stocks of *C. catla*.

Key words: Microsatellite, population structure, bottleneck, assignment, family reconstruction.

INTRODUCTION

The three Indian major carp species such as catla (*Catla catla* Hamilton), rohu (*Labeo rohita* (Hamilton), and mrigal (*Cirrhinus cirrhosus* Hamilton) together constitute a traditional and popular aquaculture practice termed as carp polyculture system in Bangladesh. The Bengali common name of *C. catla* is catla. It is the fastest grower among the three major carp species and accounted for 20.00% of the total pond production of the country (Belton and Azad 2012). *C. catla* is an endemic species in Bangladesh, India, Pakistan and Myanmar (Talwar and Jhingran 1991).

Aquaculture in Bangladesh had experienced a massive growth during the last twenty five years and currently 50% of total fish production comes from aquaculture (FRSS 2012) which was only 20% in the 1980's (Dey *et al*. 2008). To meet the increasing demand of fish seed for aquaculture, some 900 carp hatcheries had been established during the last three decades and at present 99% fish spawn are produced in the hatcheries (FRSS, 2012). However, due to improper management practices such as use of smaller size breeders (negative selection), inbreeding and interspecific hybridization, the quality of the hatchery produced seed has been deteriorated (Sarder 2007). With a view to addressing these problems, the Department of Fisheries (DoF), Government of Bangladesh has initiated a Brood Bank for the Indian major carps by stocking spawn (hatchlings) of catla, rohu and mrigal from three major river sources- the Jamuna, the Padma and the Halda. The fishes are being maintained in government-owned fish seed multiplication farms across the country and have already been introduced in the induced breeding programmes of the Government hatcheries.

Preservation of genetic diversity is important for both wild and cultivated species to maintain the evolutionary potential and the fitness of a population (Vandewoestijne *et al*. 2008). The hatchery stocks are smaller and thus prone to inbreeding and genetic drift, two potential features that can destroy the genetic variability over time. The level of success in the hatchery management and also the status of the hatchery brood stock can be assessed by comparing the genetic variability of the hatchery stock with that of a reference natural stock or preferably of the base population (Hansen *et al*. 2000). Therefore, it is recommended that the genetic structure of the base population (e.g. Brood bank collections) be analyzed so that this information can be used to assess the quality of the hatchery stocks in future.

Microsatellites, defined as loci of short DNA (2-6bp) sequences arranged in tandem repeats (simple sequence repeats, SSR) and distributed randomly throughout the genome have been recognized as powerful polymorphic markers for genetic characterization. As versatile genetic markers, microsatellites are applicable in many studies of ecology, evolution and conservation (O'Connell and Wright 1997) and have practical implications in the field of fisheries and aquaculture.

Several sets of microsatellite DNA markers have been isolated from *C. catla* and used for genetic characterization of samples collected from India, Bangladesh and Nepal. (Naish and Skibinski 1998; McConnell *et al*. 2001). Using DNA markers, the population genetic structures of these three river populations of catla had also been studied in the past (Alam and Islam 2005; Hansen *et al*. 2006). However, samples used in all the previous studies were collected randomly and had not been maintained for propagation as a recognized breeding unit. We report here the genetic variation and population structure of the Jamuna, Padma and the Halda river samples of *C. catla* maintained as Brood Bank by the Department of Fisheries. This Brood Bank is planned to serve as the major source to replace the existing brood stocks of the hatcheries in Bangladesh in the future.

MATERIALS AND METHODS

Sample collections and extraction of genomic DNA: The Department of Fisheries initiated a Brood Bank of the Indian major carps by collecting spawn samples from the Padma, Jamuna and the Halda rivers- the natural spawning grounds of carps including catla. The 120 km long Padma river enters from India (Ganges) into north-west Bangladesh. The Jamuna river also enters from India and passes through the middle of the country is 205 km long. The 81 km long Halda river is originated from the northern hilly regions of Chittagong districts. Fin samples were collected from a total of 90 catla of approximately two years old 30 from each of the three river sources of the Brood Bank collections. In order to avoid any incidental mixing among the fishes of the three river sources, the Jamuna, Padma and the Halda fishes were maintained in three separate establishments-Shambhugonj Fish Farm (Mymensingh), the Rajshahi Fish Farm (Rajshahi) and Jangalia Fish Farm (Comilla) respectively. A small amount of tissue from caudal fin was clipped from each fish and preserved in 95% ethanol until used for isolation of genomic DNA. The genomic DNA was extracted from the fin tissue following standard proteinase-K digestion, phenol:chloroform:isoamyl alcohol extraction and alcohol precipitation method as described in Islam and Alam (2004). Quality of the DNA was assessed by electrophoresis on ethidium bromide stained 1% agarose gel and the quantity was measured using a spectrophotometer (Bio Photometer Plus, Eppendorf, Germany).

Microsatellite genotyping: Five pairs of primers, *CcA12, CcG1, CcC3, CcG2* developed by Naish and Skibinski (1998) and *CcC9* developed by McConnel *et al*. (2001) were used for PCR amplification. The PCR reactions contained 50 ng template DNA, 0.25 μM of each primer, 0.25 mM of each of the dNTPs, 1.5 μl 10 × reaction buffer containing 1.5mM $MgCl_2$ and 1 unit of *Taq* DNA (GENEI, India) polymerase in a total volume of 15 µl. PCR amplifications were performed with a temperature profile comprising 3 min initial denaturation at 94 C followed by 35 cycles, each of 30 sec at 94 C, 30 sec at a respective annealing temperature and 1 min at 72 C. Finally, an additional one cycle of 5 min at 72 C was added to allow complete elongation of the amplified products. The PCR products were electrophoresed on 6% denaturing polyacrylamide gel containing 19:1 acrylamide:bis-acrylamide and 6M urea using a Sequi Gen GT sequencing electrophoresis system (BIO-RAD Laboratories, Hercules, CA, USA). The Promega (Madison,WI) protocol DNA fragments silver staining protocol was used for visualizing the bands. The gel plate after staining and drying were scanned, saved in GPEG format and the bands representing particular alleles at the microsatellite loci were scored based on the sizes using the AlphaEase FC, 1D gel image analysis software.

Statistical analysis of microsatellite data: A single matrix of genotype data comprising the five microsatellite loci and three populations was constructed in Microsoft Excel. The software MicroChecker (van Oosterhout *et al*. 2004) was used for examining incidence (if any) of large allele dropout or stutter-bands at the five loci. As measures of genetic variability of the five microsatellite loci, the observed heterozygosity (*Ho*), expected heterozygosity (*He*) (Nei 1987) and the polymorphic information content (PIC) were calculated for each loci in the entire set of samples (n=90) using the software CERVUS version 3.0.3 (Kalinowski *et al*. 2007). The population-wise allelic richness (*Ar*), *Ho* and *He* at each locus were estimated by the software FSTAT 2.9.3.2 (Goudet, 2001). We tested the deviation from Hardy-Weinberg equilibrium at each locus by estimating the exact P-value following the Markov chain method by using the software GENEPOP 4.0 (Rousset 2008) with the following parameters: Dememorization-1000, batches- 100 and iterations per batch- 1,000. The pair-wise F_{ST} values were calculated by means of 10000 permutations of genotypes using the software FSTAT (Goudet 2001). The 95% confidence interval for each pairwise F_{ST} was calculated by bootstrapping on loci with 1000 replications. For multiple comparisons, the nominal significance level (0.05) was subjected to sequential Bonferoni corrections recommended by Rice (1989).

For analyzing population structure, the software STRUCTUE 2.3.2 (Pritchard *et al*. 2000; Falush *et al*. 2003) was used with a burn in length of 50000 and MCMC (Monte-Carlo Markov Chain) iterations of 500000. We conducted four independent runs for each k value, ranging from 1 to 5 and determined the number of cluster according to Evanno *et al*. (2005) with the website based program STRUCTURE HARVESTER (Earl and vanHoldt 2012). We conducted an assignment test according to a Bayesian algorithm by the software GeneClass 2 (Piry *et al*. 2004). We reconstructed full-sib families nested within half-sib families based on the genotype of 90 offspring collected from three different populations for each population separately using the maximum likelihood method implemented in software COLONY 1.2 (Wang 2004). The family reconstruction was performed without information on parental genotypes with the assumption that only the male parent was polygamous. The allelic dropouts and other genotyping errors were accounted for 0.02 and 0.002 respectively. We examined the evidence for genetic bottlenecks in each population using the software BOTTLENECK version 1.2.02 (Cornuet and Luikart 1996) under the infinite allele model (IAM), two-phase model (TPM) and stepwise-mutation model (SMM) with 1000 replications.

RESULTS AND DISCUSSION

Genetic variability in the microsatellite loci: The estimates of variability parameters across the five microsatellite loci are presented in Table 1. A total of 27 alleles with an average of 5.40 alleles per locus were detected in 90 individuals. Examination of genotyping errors using MicroChecker (van Oosterhout *et al*., 2004) revealed no evidence for large allele dropouts or stutter-band scoring at any of the five loci. The locus specific *Ho* ranged from 0.556 (CcC9) to 0.856(CcC3) with a mean of 0.702 and *He* ranged from 0.442 (CcC9) to 0.817 (CcG2) with a mean of 0.675. The polymorphic information content (PIC) ranged from 0.398 (CcC9) to 0.789(CcG2) with a mean of 0.631 (Table 1) which belong to the middle 0.25<PIC<0.5 and highly PIC>0.5 informative marker category (Botstein *et al*. 1980).

Genetic variation within population and conformity to Hardy-Weinberg expectation: All five microsatellite loci analyzed in the samples of three river populations were found to be polymorphic (P_{95}). The allelic richness at five loci in samples of the three populations ranged from 3.000 to 7.000 (Table 2). The mean allelic richness was highest in the Padma (5.20) population and lowest in the Halda population (4.40). One private allele was observed in the Jamuna population ((Locus *CcG2*, frequency=0.083) and two private alleles were detected in the Padma population (Locus *CcA12*- frequencies 0.083 and 0.350). The allelic richness observed in the present study was similar to those reported by Alam and Islam (2005) and Hansen *et al*. 2006) but lesser than those observed by Naish and Skibinski (1998).

The mean *Ho* of the Jamuna and the Padma population was 0.720 which was higher than that of the Halda population (0.667). The mean *He* was found to be the highest in the Padma population followed by the Jamuna and the Halda population respectively (Table 2). The *Ho* and *He* values found in the present study are higher than those reported by Alam and Islam (2005) and Hansen *et al*. (2006). The F_{IS} values were negative at all five loci in the Jamuna and three loci in the Padma and two loci in the Halda population. Deviations from Hardy-Weinberg expectation (HWE) were found to be significant in six out of 15 tests (40%) after sequential Bonferoni adjustments (Rice, 1989) (Table 2). The Jamuna population deviated at three loci (*CcG1*, *CcC3* and CcG2), the Padma population deviated at two loci (*CcA12* and *CcG2)* and the Halda population deviated at one locus (*CcG2*). Alam and Islam (2005) reported significant deviation in 75% cases in river and hatchery stocks of *C. catla*. The observed *Ho* values were higher than the respective *He* values expected from observed number of alleles at 66.67% cases (loci-population combinations) indicating the probability of recent reduction in effective breeding number, a phenomenon popularly termed as genetic bottleneck.

Inter-population genetic variation: The F_{ST} value between the Padma and the Jamuna population pair was the highest (0.0507) and that between the Halda and the Jamuna population was the lowest (0.0142) (Table 3). The F_{ST} values between all population pairs were found to be significant after Bonferoni adjustments (initial k=3). Similar to F_{ST}, Nei's (1972) genetic distance was also highest between the Jamuna and the Padma population (0.142) and lowest between the Jamuna and the Halda population (0.056) (Table 3). The pair-wise F_{ST} values obtained in the present study indicate that the genetic differentiation among the studied populations is low (Balloux and Lugon-Moulin 2002) though the values were found to be significant. Hansen *et al*. (2006) also reported weaker differentiation between the Jamuna, Padma and Halda river populations compared to the hatchery populations of catla.

Population structure: We obtained consistent results across the four independent runs for each k value (k=1-5). The mean estimated log-likelihood value was found to be the highest for k = 3 (Figure 1A). However, delta k, the quantity of the second order rate of change of the likelihood function estimated by the method of Evanno *et al*. (2005), was highest for k=2 (Figure 1B). No admixture was observed up to k= 3; all the individuals were assigned to any of the three populations. The proportions of admixture individuals were 7.77% and 50% when the k value was set at 4 and 5 respectively (data not shown). The number of individuals genotyped and the number of markers used for genotyping play a role in accurate determination of the number of groups. Evanno *et al*. (2005) however, opined that calculating delta k using as low as five microsatellite markers, can allow detection of the real number of groups. For microsatellite-based population genetic studies, Hale *et al*. (2012) proved that a sample size of 25 to 30 individuals per population was enough for accurately estimating the allele frequencies and expected heterozygosity in a population. Our samples in the number of individuals and number of loci comply with both the conditions.

Assignment of individuals into populations and into families: Overall, 63.00% of the individuals were correctly assigned to their original source population and 37.00% were included in other (non-source) population. Out of the 30 Jamuna samples, 21 belonged to the Jamuna, one belonged to the Padma and seven belonged to the Halda; out of the 30 Padma samples, 15 belonged to the Padma and 11 belonged to the Halda and the rest four belonged to the Jamuna population; of the 30 Halda samples, 22 belonged to the Halda, six belonged to the Padma and two belonged to the Jamuna population (Table 4). Several factors determine the efficacy of the assignment methods, including the amount of genetic differentiation among the stocks, the number and sample sizes of populations and the number of loci studied (Manel *et al*. 2002, Piry *et al*. 2004). Manel *et al*., (2002) for example, obtained an almost correct assignment when the F_{ST} values were >0.1. We obtained, on average, a moderate score (63.00%) of assignment of 90 individual catla into their source populations indicating that the populations are not completely isolated from each other. Rather, a good number of individuals were found to be included in non-self populations. Table 5 shows the number of full-sib and half-sib families estimated from the real data of the offspring sampled from the three populations. We estimated half-sib families by assuming the male parent as polygamous. The number of full-sib families ranged from six (HR) to nine (Jamuna and Padma) and the number of half-sib families were six for the Jamuna and three each for the Padma and the Halda river populations. The numbers of full-sib and half-sib families detected in the present study were lower than those reported by Hansen *et al*. (2006) analyzing similar sample sizes.

Bottleneck tests: The tests of mutation-drift equilibrium for detecting genetic bottlenecks revealed signs of population decline in some cases. Sign test could not detect bottlenecks in any of the three populations (P>0.05). The standard difference test (SDT) detected recent bottlenecks in all populations under the IAM and TPM, and none under SMM. The Wilcoxon rank test detected recent bottlenecks in all three populations (P=0.031) under IAM and, in the Padma and Halda populations under TPM and SMM. Mode-shift was observed only in the Halda population (Table 6). The fewer numbers of half-sib and full-sib families in the samples of the three rivers also reflect reduction in effective population size. The production of major carps in the Padma and the Jamuna river systems has declined substantially: 187 MT and 157 MT respectively in the year 2001-2002 (FRSS 2003) while 103 MT and 43 MT respectively in the year 2010-2011 (FRSS 2012). Production of fish spawn in the Halda river also reduced significantly: 2470 kg in 1945 to 234 kg in 2011 with an all time low of only 20 kg in 2004 (Azadi and Arshad-ul-Alam 2012). This information testify the reduction in population sizes of the three major river systems recognized as natural breeding grounds of the Indian major carps also comply with the genetic assessment.

Overall, based on different estimated parameters, we can conclude that the genetic variation in the Halda stock is lower compared to those of the Padma and the Jamuna stocks and there is signs of population decline in all the rivers.

Table 1. Genetic variability parameters at five microsatellite in 90 individuals of *C. catla* collected from three river sources*

Locus	*k*	Allele size range (bp)	*Ho*	*He*	*PIC*
CcA12	7	125-149	0.722	0.783	0.748
CcG1	3	124-132	0.611	0.549	0.470
CcC3	6	130-150	0.856	0.787	0.748
CcC9	3	215-225	0.556	0.442	0.398
CcG2	8	391-433	0.767	0.817	0.7892
Mean	5.40	-	0.702	0.675	0.6309

* k=number of alleles; *Ho*=heterozygosity observed; *He*=heterozygosity expected; PIC=polymorphic information content

Inbreeding and genetic drift are two major consequences that may deteriorate the genetic quality of a captive breeding population maintained in a hatchery or in a brood bank. No inbreeding was detected in the brood bank stocks of catla of the three major river systems. However, at the time of selecting breeders, care should be taken to avoid any change in gene frequencies due to genetic drift. The next generation's breeders should be selected in such a way that the alleles with the minimum frequencies in the base population are not lost because once lost, it may be a loss for ever. For example, the lowest frequency of alleles we detected was 0.017 (data not shown). Since, we are discussing about a brood bank collections, a 99% guarantee should be ensured to save the rare alleles. And if we want to maintain that guarantee for five generations, the *Ne* should be at least 309 (Tave

1993). As the populations apparently have passed through genetic bottlenecks, it is rather recommended to collect new batches of fish from other locations of the respective rivers that may broaden the genetic variability of the base population of the brood bank. This genetic data would serve as baseline information of the brood bank collections and enable efficient monitoring of the impact of natural and/or human interferences on the river populations of *C. catla*.

Table 2. Genetic variation in three river populations of *C. catla*[1]

Microsatellite locus	**Parameters**	**Population**		
		Jamuna	**Padma**	**Halda**
CcA12	*Ar*	5.000	7.000	5.000
	Ho	0.800	0.700	0.667
	He	0.725	0.796	0.667
	F_{IS}	-0.104	0.121	0.044
	HWEP	0.051ns	0.000**	0.835ns
CcG1	*Ar*	3.000	3.000	3.000
	Ho	0.633	0.600	0.600
	He	0.537	0.461	0.603
	F_{IS}	-0.180	-0.300	0.005
	HWEP	0.004**	0.188ns	0.336ns
CcC3	*Ar*	6.000	6.000	5.000
	Ho	0.867	0.867	0.833
	He	0.782	0.806	0.769
	Fis	-0.108	-0.075	-0.084
	HWEP	0.002**	0.912ns	0.241ns
CcC9	*Ar*	3.000	3.000	3.000
	Ho	0.467	0.700	0.500
	He	0.390	0.522	0.410
	F_{IS}	-0.198	-0.341	-0.218
	HWEP	0.806ns	0.049ns	0.594ns
CcG2	*Ar*	7.000	7.000	6.000
	Ho	0.833	0.733	0.733
	He	0.832	0.807	0.768
	Fis	-0.002	0.091	0.045
	HWEP	0.000**	0.000**	0.000**
Mean±SE *Ar* over the loci		4.800±0.79	5.200±0.92	4.400±0.59
Mean±SE *Ho* over loci		0.720±0.07	0.720±0.04	0.667±0.057
Mean±SE *He* over loci		0.653±0.08	0.678±0.07	0.643±0.07
Mean±SE F_{IS} over loci		-0.118±0.03	-0.101±0.09	-0.042±0.05
Polymorphism		100%	100%	100%

[1][allelic richness (*Ar*), Heterozygosity observed (*Ho*), Heterozygosity expected (*He*), Fixation Index (Inbreeding coefficient) (F_{IS}) and probabilities of conformity in Hardy-Weinberg equilibrium (HWEP)]

[a] ns= not significant, *P<0.0167, **P<0.003 (after Bonferoni corrections)

Table 3. Above diagonal: Pair-wise estimates of F_{ST} calculated using FSTAT (Goudet, 2001) and their associated 95% confidence interval calculated based on bootstrapping on loci (1000 replications). Below diagonal: Nei's (1972) genetic distance between the population pairs of *C. catla*.

	Jamuna	**Padma**	**Halda**
Jamuna	-	0.0507(0.0130 – 0.0995)*	0.0142 (0.0017-0.0567)*
Padma	0.142	-	0.0321(0.0011 – 0.0772)*
Halda	0.056	0.098	-

* significant at P<0.05)(P=0.0167)

Table 4. Summary of population assignment outcomes to 'Self' or 'Other' Population[1].

Population		***C. catla***	
Source	**Jamuna**	**Padma**	**Halda**
Jamuna	21	1	7
Padma	4	15	11
Halda	2	6	22
Total correctly assigned:	57		
Percent	63.00%	37.00%	

[1] The underlined figures indicate correct assignment to the respective source population.

Table 5. Number of half-sib (HS) and full-sib (FS) families in the three river stocks of *C. catla* along with the number of individuals assigned to each full-sib families.

Population	**No. of HS families**	**No. of FS families**	**No. of FS families under each HS**	**No. of individuals assigned to each FS families**
Jamuna	5	9	HS-1	FS-1: 5
				FS-2: 2
			HS-2	FS-3: 5
				FS-4: 3
			HS-3	FS-5: 10
				FS-7: 2
				FS-8: 1
			HS-4	FS-6: 1
			HS-5	FS-9: 1
Padma	3	9	HS-1	FS-1: 10
				FS-2: 1
				FS-3: 3
				FS-4: 1
			HS-2	FS-5: 1
				FS-8: 4
				FS-9: 1
			HS-3	FS-6: 4
				FS-7: 5
Halda	3	6	HS-1	FS-1: 10
				FS-2: 3
				FS-3: 2
			HS-2	FS-4: 8
				FS-6: 4
			HS-3	FS-5: 3

Table 6. Results of bottleneck test for *C. catla*. The values indicate probability under the hypotheses of mutation drift equilibrium*.

Population	**Sign Test**			**Standard Different Test (SDT)**			**Wilcoxon Rank Test (two tailed for heterozygosity excess/deficient)**			**Mode shift**
	IAM	**TPM**	**SMM**	**IAM**	**TPM**	**SMM**	**IAM**	**TPM**	**SMM**	
Jamuna	0.058	0.305	0.340	0.011	0.042	0.184	0.031	0.062	0.156	Normal
Padma	0.056	0.064	0.333	0.009	0.036	0.171	0.031	0.031	0.624	Normal
Halda	0.056	0.071	0.321	0.008	0.040	0.170	0.031	0.031	0.156	Shifted mode

* $P<0.05$ indicates evidence of recent bottlenecks. Mode shift normal indicates L-shaped allele frequency distribution, meaning no occurrence of recent bottlenecks

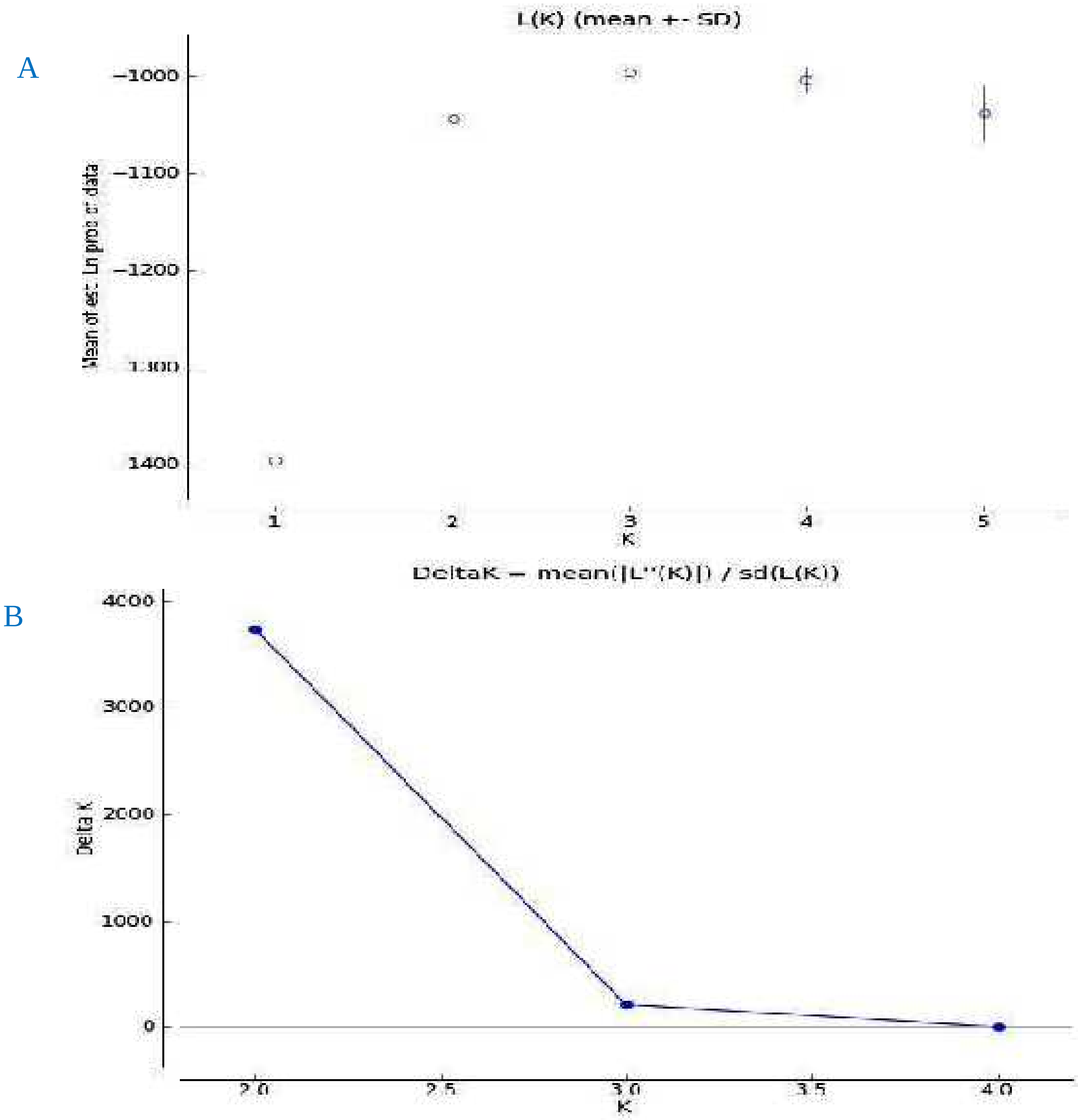

Figure 1. A: Log probability of data L(K) for each k values ranging from 1 to 5 for the admixture and correlated frequencies model (averaged over four independent runs) for the brood bank collections of *C. catla* (k=number of cluster)

B: Delta k values for each of the k inferred clusters of *C. catla* with a maximum value obtained at k=2.

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