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Genetic Diversity and Population Structure of *Monopterus* spp. of Assam and Manipur states of Northeast India using Microsatellite Markers

Papari Devi, MA Laskar, Chittaranjan Baruah and DK Sharma

Abstract
Eleven microsatellite markers were used to sort out genetic diversity and population differentiation of *Monopterus species* complex of Northeast India. Samples of morphologically identified *M. albus* (50 nos.) and *M. cuchia* (180 nos.) were collected from Manipur and Assam, were allocated into four populations based on the geographical proximity of the water bodies. Polymorphism Information Content (PIC) across the eleven microsatellite loci ranged from 0.588 to 0.852. The mean single locus estimate of Wright's inbreeding indices F_{IT} and F_{IS} were 0.132 and 0.177 and mean single locus estimate of Wright's fixation index F_{ST} was 0.052. The average numbers of observed alleles (NA) and effective alleles (NAe) across 11 microsatellite loci were 9.73 and 6.33 respectively, suggestive of moderate heterozygosity. The mean expected heterozygosity within and among individuals of *M. albus* was 0.573 and 0.709 respectively while these values for *M. cuchia* ranged between 0.695 to 0.748 and 0.692 to 0.769, approves moderate genetic diversity in *M. albus* and *M. cuchia*. Population structure analysis placed *M. albus* and *M. cuchia* in two distinct clusters.

Keywords: Mirosatellite markers; Monopterus albus; Monopterus cuchia, genetic diversity; genetic admixture

1. Introduction
The freshwater air-breathing mud eel, *Monopterus cuchia* (Hamilton, 1822) [1] and swamp eel, *Monopterus albus* (Zuiew, 1793) [2], often exhibit differences within and among population from different parts of their range [3]. They belong to the synbranchid genus *Monopterus* [4, 5] and mostly considered as species complex and demands taxonomic revision [6]. Both *M. cuchia* and *M. albus* bear cylindrical body with compressed tail tapering to a point. Only minor difference exists, that is *M. cuchia* bears smooth, tiny cycloid and indistinct scales embedded in the skin but *M. albus* is scale less**.** Therefore, *Monopterus albus* and *Monopterus cuchia* are regarded as species complex and require taxonomic revision [2].
Both *M. cuchia* and *M. albus* are economically important freshwater fishes, recorded from India, Nepal, Bangladesh, Myanmar and Pakistan [7, 8, 9]. Both are assumed to be Least Concern status of the IUCN Red List [2]. With ecological importance and high nutritional components this fish can play a unique role for the development of socio-economic status of fishermen as well as with short of culture practice [10]). However, these valued fishery resources have declined in recent years due to overfishing and environmental pollution [11, 12]. In the effort to determine how to conserve and sustainably exploit these resources, population genetic studies are needed. Although some research has investigated population differentiation in *M. albus* population using RAPD [13] and isozymes [14]), yet, little is known about the genetic diversity of *M. albus* and *M. cuchia* in northeast India. According to IUCN [6], taxonomic investigation is needed to clarify confusion between *M. cuchia* and *M. albus* within India, which could impact upon the species.
DNA-based markers have gained popularity in recent years in the assessment of genetic relationship among species. Of these markers, SSR (Simple Sequence Repeat) markers are used in population-level studies vary often [15, 16]. SSRs are scored as co-dominant markers and inherited in Mendelian fashion [17]. The SSR regions scattered evenly throughout the genome [18] and yielding a large number of polymorphic bands, which are interpreted as band present or band absent [19].

The absence of band signifies as primer divergence or loss of a locus through the deletion of the SSR site or chromosomal rearrangement [20].

Techniques of mtDNA sequencing have been widely employed for aquaculture and fisheries-related genetic studies because this molecule has several special features (such as absence of introns, maternal inheritance, single-copy orthologous genes, lack of recombination events, and high mutation rate) that make it effective for detecting recent population isolation [21] and for establishing genealogical relationships among populations within species [22, 23].

Since both the species are not so far genetically distinguished as well established species and morphologically regarded as species complex [2,6], in the present study, an attempt has been made to study the *Monopterus* population of Assam and Manipur states of Northeast India together, to study population genetic relationships of *Monopterus cuchia* and *Monopterus albus*. The present study will be of use in understanding genetic variation between morphologically identified individuals of *M. cuchia* and *M. albus,* to clarify taxonomic uncertainties [2]), however, could be used in the fine-scale population structure analysis of *Monopterus* population.

Materials and methods

1. Sample collection

Field work was carried out during January, 2015 to June, 2017 in different parts of Assam and Manipur in Northeast India in certain suitable habitats like paddy-field and swamps in order to collect the *M. cuchia* and *M. albus* samples (Table 1; Figure 1). A total of 230 *Monopterus* individuals were sampled from 3 water bodies of Manipur and 18 water bodies of Assam with varying geographical co-ordinates. *Monopterus* individuals were morphologically identified, based on twenty three (23) reliably measurable morphometric characters [24]. The individuals were geographically allocated into four populations based on the proximity of the water bodies i.e. each *Monopterus* population was sampled at gap of about 100-400 km away from any other population (Table 2).

2. DNA extraction

From each captured specimen, approximately 1 cm of tail tissue was removed with forceps and was placed it in a sterile 1.5 ml microtube containing 95% ethanol and was stored at –20 °C until processing. The eels were released at their points of capture. The samples were placed in an ice chest during transport to the laboratory. Genomic DNA was isolated from the tissue using the Chloroform-Octanol method [25, 26].

3. Microsatellite Genotyping

The sequences of 11 *Monopterus albus*–specific microsatellite primers were obtained from Li *et al*. [27], Lei *et al*. [28]. Polymerase Chain Reactions (PCRs) were carried out in 25 µL of reaction volume containing 20 ng of genomic DNA, 1.25 U *Taq polymerase*, 200 µM dNTP, 0.2 µM primer, and 1X amplification buffer (containing 2 mM $MgCl_2$). The condition for amplification was an initial denaturation temperature 94 ˚C for 5 min, followed by 36 cycles of 94 ˚C for 30 sec, then by 30 sec at appropriate annealing temperature (Table 1) followed by 72˚C for 45 sec and then by a final extension at 72 ˚C for 10 min. Amplicons and DNA size marker were resolved on 7.5% nondenaturing polyacrylamide gels containing acrylamide and N, N'-methylenebisacrylamide in 19:1 ratio. Electrophoresis was done at 8 V/cm for 120 min. DNA bands were stained using ethidium bromide 10 mg/ml solution for 30 min and visualized with Ultraviolet Gel Document System [29, 30]. The sizes of the microsatellite bands were measured by using the software UVItec ver 12.8.

4. Data analysis

Polymorphism Information Content (PIC) of the microsatellite markers were calculated according to Bolstein *et al*. [42]. Exact tests implemented in the software Genepop 4.2.2 [31] were used to analyse the agreement of genotype frequencies with the expected Hardy-Weinberg equilibrium, and also to test the existence of linkage disequilibrium. Estimates of gene diversity parameters, H_e (expected heterozygosity or gene diversity corrected for sample size) [32] and NA_e (effective number of alleles, Neilsen *et al*. [33] were computed using SPAGeDi [34]. Genepop 4.2.2 was used to compute the single locus estimates of Wright's F-statistic parameters F_{IT}, F_{IS} and F_{ST} [35, 36]. The ANOVA approach of Weir and Cockerham [36] was implemented to compute the single locus estimates of $F_{IT,}$ F_{IS} and F_{ST}.

The programs Structure 2.3.4 [37] and Structure Harvester [38] were used to determine the most probable number of genetic clusters (K) to which the *Monopterus* spp. of the present study were subdivided. CLUMPP 1.1.2 [39, 40] was used to determine the most appropriate proportional genetic memberships (Q) of these individuals in each cluster. The population Q matrices and individual Q matrices (twenty of each) associated with the K at which the modal value of ΔK was located were selected for performing permutations by CLUMPP. The means of permuted population and individual Q matrices were then input into the cluster visualization program *distruct* [41] to obtain a bar plot.

Results and Discussion

1. Descriptive Statistics

Reproducible results were obtained from the nine primer pairs that represented nine microsatellite loci characterized by Li *et al*. [27] in *M. albus* and two primers pairs that represented two microsatellite loci characterized by Lei *et al*. [28] also in *M. albus*. The names given by Li *et al*. [27] and Lei *et al*. [28] to the respective microsatellite loci were retained in the present study (Table 1). A total of 107 alleles were recorded across the 11 microsatellite loci of *Monopterus* spp. of the present study. The lowest number of alleles (6) were amplified from the locus Mal05 and the highest number (15) were amplified from locus Mal11 (Table 1). The PIC values ranged between 0.588 (Mal05) to 0.852 (Mal02).

According to Botstein *et al*. [42] genetic markers are informative if they have PIC values higher than 0.5. Exact test for Hardy-Weinberg equilibrium revealed significant deviations in each of the 11 loci ($P<0.05$). Exact test revealed absence of linkage disequilibrium among all pairs of microsatellite loci.

The single locus estimates of Wright's inbreeding coefficients, F_{IT}, F_{IS} and F_{ST} are presented in Table 1. Inbreeding coefficients are defined in terms of probability of identity in state of different pairs of genes; in other words the probability that two genes are of identical allelic type [43]. Microsatellites loci may be characterized in terms of allele size [43, 44] represented probabilities of identity of state at different genic hierarchical structure by Q; Q_1 among genes within individuals, Q_2 among genes in different individuals within populations and Q_3 among genes in different

individuals among populations. F_{IS} was defined by those authors as equivalent to $(Q_1 - Q_2)/(1- Q_2)$, F_{IT} as equivalent to $(Q_1 – Q_3)/(1- Q_3)$, F_{ST} as equivalent to $(Q_2 – Q_3)/(1- Q_3)$. '1' in the above definitions is probability of identity of a gene with itself (Rousset, 2002). In present work the term gene(s) is replaced by microsatellite allele(s).

Single locus estimates of F_{IT}, ranged between -0.325 (Mal05) and 0.474 (Mal043); the across loci estimate was 0.132. These results indicate that the *Monopterus* populations of the present study do not suffer from high inbreeding. Single locus estimates of F_{IS} ranged from -0.051 (Mal10) to 0.272 (Mal043), the across locus estimate was 0.177. The negative values of F_{IS} (Table 1) underline the fact that heterozygosity was in 'excess' or did not conform to the Hardy-Weinberg proportions. Values of $F_{ST,}$ which is the index of genetic differentiation [36], ranged from 0.052 (Mal10) to 0.277 (Mal043). The value of F_{ST} estimated across loci (0.052) indicated the existence of a moderate degree t of differentiation among the *Monopterus* spp. However, the F_{ST} estimate for one locus, Mal043 was 0.277; this value, according to Wright's guidelines [35] is an indicator of high differentiation or fixation of alleles. The F_{IT} and F_{IS} values, 0.474 and 0.272 also pointed towards fixation of alleles at the locus Mal043. The Jackknifed estimators (mean ±SE) of all three parameters strongly supported the F-statistics estimates for each of the eleven loci (Table 1).

The genetic diversity indices based on the effective number of alleles (NAe) in a given population [45] showed equivalent values for observed allele number (NA), which indicates frequencies are distributed equally or nearly so; whereas, lower NAe values underline skewed distribution of allele frequencies, since more lopsided distribution is the result of more differences between NA and NAe, which is suggestive of lower heterozygosity [33]. It could well be inferred from the values of NA and NAe (Table 1) that moderate heterozygosity exists in all the four eel population could be supported by the mean expected heterozygosity (Table 2); Because the mean expected heterozygosity (H_e) within individuals ranged from 0.573 (*M. albus* population-1, Manipur) to 0.748 (*M. cuchia* of population-4, Assam) while the mean expected heterozygosity among individuals within population ranged between 0.692 (*M. cuchia* population-2, Assam) to 0.770 (*M. cuchia* of population-4, Assam). The expected H_e, within the range of 0.58 to 0.7 was established in establishing higher genetic variation in wild common carp [27] could justify the findings in *Monopterus* species. Genetic markers are informative if they have PIC values higher than 0.5[42]. Exact test for Hardy-Weinberg equilibrium revealed significant deviations in each of the 11 loci ($P<0.05$). Exact test revealed absence of linkage disequilibrium among all pairs of microsatellite loci.

Li *et al*. [5] showed that there were too 11 loci of great significance of disequilibrium in wild common carp population. It was shown that there were 9.44% loci in significant disequilibrium ($P<0.05$), which in fact support the present findings. Pan and Yang [46] recorded H_0 and H_e at 0.710 and 0.674 respectively in the fish population of *Polydactylus sexfilis.*

2. Population Structure

Structure Harvester identified the modal value of ΔK at K = 2; however, a second peak was present at K = 5 (Figure 2). The height of the model values of ΔK indicates the strength of population subdivision signal [40]; hence a Mann-Whitney U test was performed using the Pr (X/K) values associated with K=2 and with K=5. The results revealed a significance difference between the two sets of posterior probability figures. Thus, K=2 for which the higher ΔK value had been obtained was chosen as the true cluster to which *Monopterus spp*. of the present study were subdivided.

Mean of permuted population Q matrices across 20 replicates that were generated by CLUMPP for two clusters are presented in Table 3. The proportional membership for K= 2 is represented graphically in Figure 3. For K = 2 the *M. albus* of Manipur (population-1) had an overwhelming representation in cluster 1 (Q value = 0.992) in which the membership of *M. cuchia* was much less (Q values ranged from 0.008 to 0.274). The *M. cuchia* of population-2 had higher representation in cluster 2 (Q value = 0.993) as compared to those from population-3 and population-4 (Q values = 0.761 and 0.726 respectively). The bar plot in Figure 3, depicts that genome of *M. cuchia* of population-3 and population-4 has been admixed with *M. albus* genome of Manipur but the reverse was not true; there has been very little admixture of *M. albus* genome with *M. cuchia* genome.

The sampling sites of the study area in Assam and Manipur are connected by the Barak river system and there is a possibility of admixture among the *Monopterus* population of paddy fields, wetland and tributaries of Brahmaputra river system and Barak river system during flood. The two river systems also combine in Bangladesh to form the Meghna Basin [47].The admixture could be probably due to frequent migration and connection of wetlands during flood leading to hybridization as migration in *Monopterus spp.* is towards upstream tributaries for spawning [48]. Hybrid speciation may be a component of species complexes, when a reproductively isolated species arises from hybridization [49]. As an air-breathing fish, there are possibilities for migration of *M. cuchia* from rivers to paddy fields and wetlands or from wetlands to rivers for breeding purpose. The genetic admixture in *M. cuchia* might be due to interbreeding or hybridization among isolated populations of wetland, historic ponds and rivers.

Genetic variation is a key tool for assessing biological potential of an organism. A population with high level of genetic variation may be better capable of dealing with changes in its surroundings such as fluctuations in water temperature, epidemics, etc. But yet, the occurrence of highly polymorphic microsatellite and the untranslated region of EST is a potential useful source of gene-associated polymorphism represents the genetic signature of divergent region. Since *M. cuchia* and *M. albus* are spatially separated population that inhibits in differ different environment and different ecological niche. However, Wielgoss *et al.* [50] were able to develop certain testimony after characterization of 12 dinuclotide microsattelite markers for understanding conservation genetics of typical eels, to identify interspecies admixture.

Significant decrease in the observed heterozygosity and number of alleles might be an attribution of genetic bottleneck, however, not evident in the present investigation. The high level of variability in the SSR loci was tested in Atlantic cod [33], which could be supportive of the present data set. Microsatellite DNA variability within and among populations of resident rainbow trout (*Oncorhyncus mykiss*) and sea run Steelhead (*O. mykiss*) from British Columbia was investigated. The allelic diversity was observed to be moderate to high for the two probes used (61% for Ssa1, a

multilocus probe; 80% for T34) with the confirmation of significant heterogeneity in between the individuals of this species [51].

Li *et al.* [27] developed 30 microsatellite loci showed moderate to high level of polymorphism in a test population of *M. albus* in China, with the H_O ranging from 0.3125 to 0.9688 (mean 0.7140), which has extended well supported genetic variation with mean allele of 7.9/locus in *M. albus*, which infact could suggest fine-scale population structure and reproductive ecology of the species.

There have been some studies on genetic variation and population differentiation in *M. albus* and related species in China using random amplified polymorphic DNA (RAPD)[13], isozyme [14] and microsatellite primers from common carp (*Cyprinus carpio*) which is a distantly related species [52]. Alam *et al*. [53] used RAPD- fingerprinting assay to examine genetic variability in the freshwater mud eel, *Monopterus cuchia* in Bangladesh with the aid of the development of 30 polymorphic RAPD loci. The RAPD markers showed a high degree of intra-specific polymorphism in terms of the proportion of polymorphic (76.92%) loci, probably with the drawn of heterozygosity inferences. However, no such effort has been reported from Northeast India and the present study on SSRs revealed that high degree of intra-specific polymorphism exists within *M. cuchia*, which could well supports the RAPD analysis [53].

The present findings thus could extend fine-scale population structure and reproductive ecology of two *Monopterus* species (*M. cuchia* and *M. albus*) in four *Monopterus* populations in Northeast India. However, the exact test for Hardy-Weinberg equilibrium (HWE) demonstrated that majority of this microsatellite were within the limit of HWE, yet Mal01 present significant departure from HWE ($P < 0.00385$).

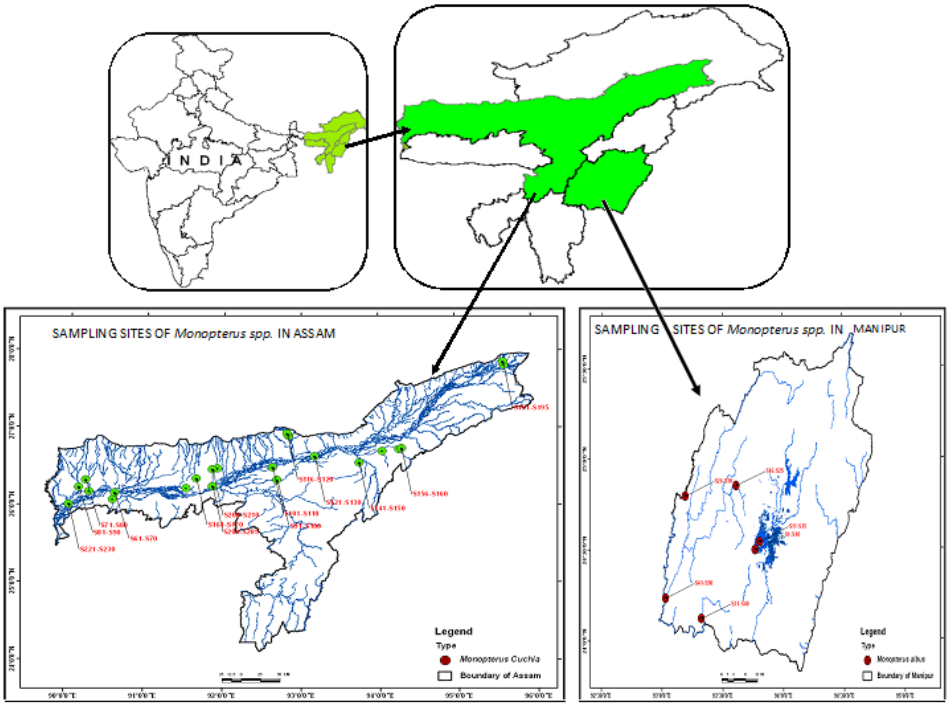

Fig 1: Map of study area showing the sampling sites.

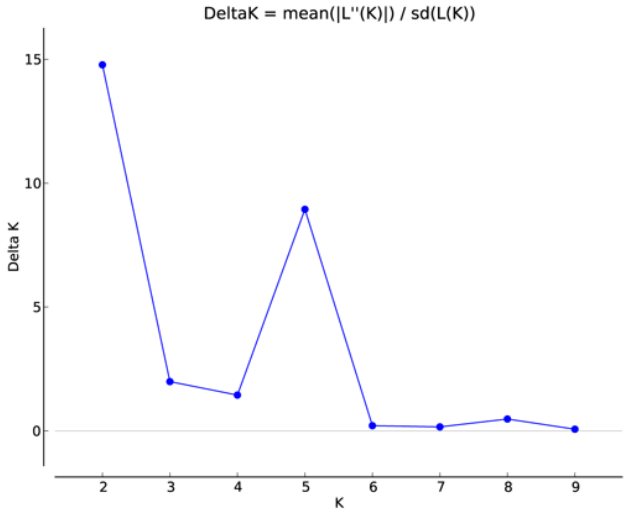

Fig 2: Plot of Delta K vs. K produced by Structure Harvester (Earl and vonHoldt, 2012)

Table 1: Alleles amplified from microsatellite loci

Sl. No.	SSR Locus	GenBank Accession no.	Repeat Motif	PCR Primers	Tm (°C)	NA	NAe	Allele Size Range (bp)	PIC	F-statistics Estimators		
										FIT	FIS	FST
1	Mal01	DQ987569	(TG)2CA(TG)4(TG)11	F: ATATTTGCAAGCGAGCCTGT R: CTCTTCAATGCAGGCACAAA	57	8.00	6.28	640 - 728	0.764	0.164	0.044	0.125
2	Mal02	DQ987574	(AC)23	F: TTGAAGAGCTCCGAGAGCAG R: TTTGGTGCTGGAAAACTGGT	55	11.00	7.55	626 - 740	0.852	0.236	0.114	0.137
3	Mal04	DQ987576	(CA)21	F: CCACTGAGATTCACCCTCGT R: GCTCTGTCTGTGTCGTCTGG	64	9.00	4.06	556 - 602	0.732	0.199	0.066	0.143
4	Mal05	DQ987579	(GT)10	F:TATTTCTCCAAGGGCTGGTG R:CACATGCACACAAACAGCA	48	6.00	2.67	218 - 248	0.588	-0.325	-0.410	0.060
5	Mal07	DQ987581	(CA)4(TA)6(CA)13	F:CCCATACAGACATTTGCCACT R: CCAGACAACCTCTTTCCACA	55	11.00	7.42	678-828	0.85	0.336	0.226	0.142
6	Mal09	DQ987583	(TC)21(CA)20	F: GCCCAGATGATACAAGTC R: TGGGAGAAAGGAAGCACAAG	55	10.00	6.98	664-884	0.84	0.223	0.092	0.145
7	Mal10	DQ987584	(TC)10(AGG)6	F: GTGGGCCTTTATCTGCATGTG R: TGCTTGTCTGTCCCTTACGA	62	7.00	4.88	665 - 710	0.713	0.004	-0.051	0.052
8	Mal11	DQ987590	(TG)11	F: GTGGGCCTTTATCTGCATGT R: CTGTCCCTTACGCACCTCTC	65	15.00	11.37	572 - 704	0.836	0.072	-0.058	0.123
9	Mal13	DQ987593	(TG)19	F: CAGCAAAAGTAAAGCCGACTA R: TGTCGCATTTCTGCAAGTTT	65	9.00	6.87	562 - 700	0.82	0.255	0.130	0.143
10	Mal008	DQ987582	(AC)13	F:AGCTCACTATTCCCGTCTGTCTGA R:CCTGCCCTTTCTCACATTACAAAC	55	13.00	5.83	424 - 718	0.808	0.155	0.082	0.079
11	Mal043	JN712692	(TG)20	F: CAGCCGCAGAGTTAAACATCACCA R: CCTAAACCCCAAAGGTCCCAGAAA	55	8.00	5.75	674-762	0.801	0.474	0.272	0.277
						Across loci F-statistics				0.132	0.177	0.052
				Jackknifed estimators of F-statistics (Mean ±SE)						0.133±0.018	0.178±0.054	0.052±0.047

NA: Observed Alleles; NAe: Effective Alleles; PIC: Polymorphism information content (Botstein *et al*. 1980); *All loci departed significantly from Hardy-Weinberg Equlibrium;
#Primer Reference: Li *et al.* (2007): Sl No.1- 9; Lei *et al.* (2012): Sl No.10- 11.

Table 2: Summary of Heterozygosity Statistics of *Monopterus spp*.

Population Name	Population Code	Sampling Site(s)	Coordinates	NS	N	Mean Expected Heterozygosity	
						Within Individuals	Among Individuals within Populations
Manipur	1	Loktak Lake	24.3°N, 93.5°E	15	50	0.573	0.709
		Ijei River, Longmai, Tamenglong	24.5°N, 93.4°E	15			
		Tuivel River, Manipur	24.08°N, 93.2°E	20			
Assam	2	Wetlands, Hallawgaon, Sadiya, Tinsukia	27.49°N, 95.38°E	10	45	0.695	0.692
		Rice field, Mariani, Jorhat	27.52°N, 95.37°E	5			
		Bhogdoi River, Jorhat	26.77°N, 94.22°E	10			
		Kakodonga River, Golaghat	26.43°N, 94.3 °E	10			
		Dhansiri River, Golaghat	26.35°N, 93.35°E	10			
	3	Kolong River, Nagaon	26.36°N, 92.69°E	20	40	0.7424	0.7423
		Jia Bhorali River, Sonitpur	26.69°N, 92.87°E	10			
		Bishwanath Ghat, Sonitpur	26.66°N, 93.17°E	10			
	4	Kulshi river, Kamrup	26.03°N, 91.26° E	10	95	0.7483	0.7695
		Chandubi beel, Kamrup	25.51°N, 91.21° E	5			
		Puthimari river, Kamrup	26.14°N, 91.08°E	10			
		Rice field, Hajo, Kamrup	26.22°N, 91.4°E	10			
		Rice field, Nalbari	25.89°N, 90.01°E	10			
		Wetland, Manikpur, Bangaigaon	26.45°N, 90.80°E	10			
		Rice field, Bilaishipara, Dhubri	26.11°N, 90.16°E	10			
		Rice field, Dhubri	26.01°N, 89.6°E	10			
		Urpad Beel, Goalpara	26.06°N, 90.35°E	10			
		Rice field, Dudhnoi, Goalpara	25.98°N, 90.79°E	10			

Table 3: CLUMPP-generated mean of permuted Q matrices across 20 replicates for two genetic clusters.

Sampling area	Population code	*Cluster (K)	
		1	2
Manipur	Population-1	0.992	0.0085
Assam	Population-2	0.008	0.9925
	Population-3	0.239	0.7613
	Population-4	0.274	0.7263
	*Vide text for details.		

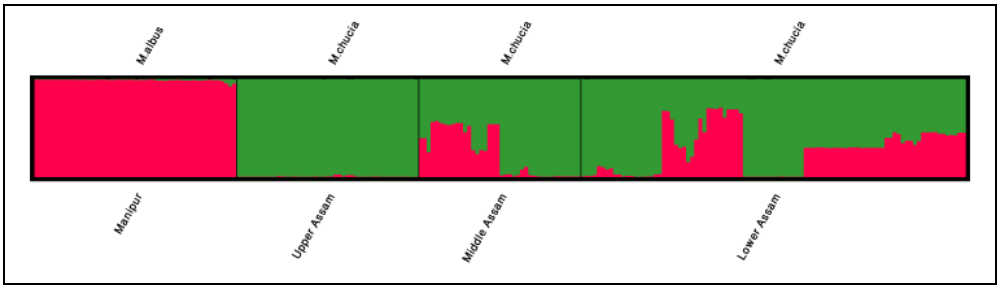

Fig 3: Graphical representation of proportional memberships (Q) of Monopterus spp. of Manipur and Assam states in two genetic clusters represented by red and green colours.

Conclusion

In this study, the genetic variability of the four *Monopterus* population of Northeast India was analyzed for the first time by using SSR markers. Moderate levels of genetic diversity and differentiation were observed. *M. albus* and *M. cuchia* were placed in separate genetic clusters, but admixture was observed. The study has laid the foundation for conservation and management of *M. albus* and *M. cuchia* in Northeast India.

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