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Research article

Population diversification in geographically varied population of *Lonchura punctulata* in India based on 12SrRNA and morphometry

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ABSTRACT

Morphological characters of birds imitate their adaption occurred due to evolution and pressure of environmental for their ecological requirements. These characters are also significant towards their phylogenetic relationships in a genus and family. *Lonchura* genus is represented by colorful passerine birds widely distributed in Asia, Africa and Australia. One of their member Spotted munia is widely distributed in India in same type of habitat with similar kind of morphological appearances. However the populations show great variability in their physiology according to their altitudinal distributions. In the present study investigations were made to find the genetic and morphometric variation in the geographically varied population of spotted munia in India. Wild birds were caught, samples and data were collected for genetic and morphometric comparison. Data were analysed statistically and comparisons were made at population levels. In morphometric comparison we observed that the lengths northern population (101.8 mm) is high as compared to southern population (96.5 mm). We significantly observed the differences in other parameters too. In genetic comparisons separate population clusters were seen according to geographical locations. We observed morphometric variation according to their altitude. Higher altitude populations are big in size in compare to lower altitude population. It might be due to environmental pressure on their flight, food, prey and breeding. Similar differences were seen in genetic parameters too. Northern populations were seemed to be more genetically varied in compare to southern populations, indicate the ongoing process of speciation.

Keywords: 12S r RNA, Altitude, Haplotype, Morphometric, Phylogeography, Spotted Munia

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INTRODUCTION

The morphological characters of a species are a reflection of an adaptation of that species to their local environments encountered, and to the definite form of life [1]. It has been well predicted that birds have an optimal body size which maximizes their energy intake for particular resources [2, 3]. However, there are some other factors are also exists which can decide the body size of a species for e.g. Prey densities, food availability, nest site, nest size and intraspecific competition, breeding etc [4- 7]. Because such different factors, selective pressures may act in different dir-

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ections to altered the morphology of a species to more economize for local environments and can form different genetic population groups within a single land mass. The study of morphological variation along the altitudinal gradient and comparison with genetics can be useful in understand the role of geographical pressure.

In earth geology pleistocene era characterized by worldwide climatic fluctuations that resulted in the development of large ice sheets and changes in the landscape and ecology of high-altitude continental areas of northern hemisphere [8, 9]. Pleistocene glaciations had extensive impact on phylogeography pattern within and among closely related species of many vertebrates. The phylogeographic studies in Europe and America suggested species dispersal expanded from southern location to survive in refugia and then expanded to north, during interglacial periods when climate recovered to or exceeded current mean global temperatures [10, 11,12] .This glacial activity had a profound impact on species distribution by causing range contractions during period of glacial advance and allowing range expansions during periods of climatic amelioration[13]. However climatic amelioration in Asia during interglacial periods did not seem to resemble that of Europe primarily during continued uplift of Himalaya in late Tertiary. In Western Himalaya (Indian side) glacial advance was not as extensive as in Eastern Himalaya (Tibet, China side) due to monsoon in Indian subcontinent [14]. Flora and fauna of Western Himalaya were located at higher northern latitude than others. Despite the harsh (upper range) and mild climate (mid & lower range), species distribution were affected by climatic fluctuations during Pleistocene. Studies carried out in East Asia address the issues of Pleistocene effect on species distribution but remain limited both in terms of number of model species studies and geological timescale.

The Spotted munia, *Lonchura punctulata* generally known as spice finch is a widespread species found in Indian subcontinent, Indonesia, Taiwan China, and the Philippines. In India it is distributed from 10^0 N to 30^0 N. The spotted munia are found in open habitat including gardens and agricultural fields where they forage in groups for grass seeds. Plumage color variation and size identified 11 subspecies in their distribution range, among those two subspecies *L punctulata punctulata* and *L punctulata subundulata* are found in India [15]. These

birds have been widely subjected to physiological and behavior studies by Chandola-Saklani group [16, 17, 18, 19, 20, 21]. Wide distribution 10^0N to 30^0N and 73^0E to 94^0E, variations in breeding cycle, varied ecophysiological response render this bird as an ideal model for phylogeographic studies.

In the present work, we used Mt DNA 12S rRNA gene for to assess genetic diversity within and among the population and compare it with morphological data.

MATERIALS and METHODS

Morphology

The Spotted munia were caught during the pre monsoon season (Feb-Apr) in two geographical location which are different in altitude. Birds were caught from high altitude area (225 sea level range), Bijnor region (Northern population) and low altitude region (6.7 sea level), Chennai region (Southern population). Both the location has paddy fields surrounded by settlement with very few types of vegetation.

A total of 83 adult Spotted munias were measured for this study; 35 of which were from high altitude, and 48 were from low altitude. Five measurements were taken from each individuals and measured solely by Devinder Kumar in order to minimize biases resulted from observers' error. The measurements were made to the nearest mm with a rule. The method used in the study followed those mentioned in King *et al*, [22]. This objective & methodology of the study was duly approved by the research committee of host institution & wild life Dept of Uttarakhand.

The wing length was measured from the wrist bend to the tip of the longest primary, while the wing was flattened and straightened sideways. The tail length was measure from the tip of the longest feathers to the back, while it was bent at right-angles to the back, and the ruler was placed on the base of the tail which was gently pulled. The culmen length was measured from the tip of the skull, to the nearest 0.1 mm with a dial caliper; from the notch on the back of the inter-tarsal joint to the lower edge of the last complete scale before toe diverge. The body weight was measured to the nearest of 0.1 gt using 50 gr Pesola spring balance.

Genetic Samples

As a source of DNA, blood, tissue or feather samples were collected from living birds and those accidentally killed. Birds were caught with standard techniques using mist nets with the permission of forest department (vide permission Forest & Wildlife Department, Govt of Uttarakhand/ LaCONES, CCMB). Blood samples were taken from the brachial vein of living birds with the help of a conventional syringe, and the animals were released immediately afterwards. All samples were stored in EDTA buffer or 70% ethanol and kept at –20°C until further processing. A total 420 samples were collected from different geographical locations covering Sub Himalayan regions. The molecular wet lab work has been carried out in CCMB, Hyderabad as a part of DST project work (SR/SO/AS-80/2007).

DNA Isolation

Isolation of total genomic DNA followed standard protocols [23], Small aliquots of sample material were digested for several hours at 48°C in lysis buffer in the presence of 20% SDS and 1 mg of proteinase K. Cell fragments and proteins were precipitated with saturated NaCl solution and subsequent centrifugation or by standard phenol/chloroform extraction. DNA was precipitated from the supernatant by adding 1/10th volume of sodium acetate (pH 5.2) and equal volume of ice-cold isopropanol. The extracted DNA was washed twice with 70% ethanol, dried and re-dissolved in TE buffer. DNA stock solutions were kept at 4°C until further analysis. In cases of very limited sample material and low DNA yield, the protein pellet was redigested by addition of guanidine isothiocyanate buffer to extract DNA that was trapped in the pellet. In this case, digestion was followed by extraction twice with phenol/chloroform, then once with chloroform/isoamyl alcohol and subsequent precipitation and washing of the DNA as explained above. To determine the approximate concentration and quality of

the extracted DNA, 2 µl of each DNA solution were loaded onto a 0.8% agarose gel containing ethidium bromide. DNA concentration was estimated by comparison of fluorescence intensities to samples of known DNA content.

Amplification of target gene

Sorenson [24] published primers were used for the amplification of 12S rRNA gene. PCR amplification was standardized using different PCR conditions and PCR reaction components in order to get best results with minimal primer dimmer and nonspecific amplification. PCR was performed in 15µl volume containing 1X PCR buffer, 25nM MgCl2, 2.5nM dNTPs, 1X BSA, 2.5pM of Forward and reverse primers, 0.5 units of Taq polymerase. 50-100 ng of the template DNA. PCR reactions were performed in a mastercycler ep gradient S thermal cycler (Eppendrof, Hamburg, Germany) with following temperature profiles according to primer used: 95.0^{0}C 10 min with 30 cycles of 94.0^{0}C 45 sec, 50-60^{0}C 45 sec, 72.0^{0}C 1 min, final extension at 72.0^{0}C 10 min and 4.00C ∞. PCR products were electrophoresed in 2.5% agarose gel by loading molecular weight marker in one well. The gel was visualized under UV light in the Gel documentation system (Biorad). Amplified PCR product size was observed by matching PCR band of samples with molecular ladder.

Sequencing

Sequencing was performed on ABI 3700 DNA analyzer (Applied Biosystem, USA) by Big Dye Terminator kit following the manufacturer's instructions. The reactions were run in a Biometra thermoblock under the following temperature profiles. 30 cycles of 96°C 1 min, 55°C 5 sec, 60°C 4 min, 04°C ∞.

Genetic Data Analysis

The obtained sequences were aligned manually in autoassembler software. To check for sequence errors, all sequences from closely related populations were carefully compared and all variable sites extracted with the program package MEGA ver. 5 [25] were checked individually.

Data were calculated for the number of segregating sites, haplotype diversity and nucleotide diversity for each population and all populations combined using DnaSP 5.0 [26]. For hierarchical partitioning of genetic variation among population and regions, AMOVA test, Neutrality test: Tajima's D and Fu's Fs, Mismatch distribution, and F test, was performed in Arlequin 3.5 [27].

For population structure, haplotype network was constructed in Network 4.6. The Median-Joining algorithm was implemented. Bayesian algorithm was also implemented to detect the lineages.The resultant matrix was then converted into a dendogram using the Neighbour- Joining algorithm [28] provided with MEGA 5 [25]. For hierarchical distribution of genetic variation was characterized using analysis of molecular variance (AMOVA) in Arlequin 3.5. This method apportions genetic variation within and among groups, estimating F-statistics [29]. Four level AMOVA was conducted using Fst estimator under infinite site model. Gametic phase of haplotypes within individuals was inferred using ELB algorithm [30] and permutation tests (1000 permutations) to examine whether levels of differentiation were significantly greater than zero. These tests included permutation of inferred haplotypes among groups (Fct), among populations within groups (Fsc), among individuals within population(Fis) and within individuals(Fit). Principal coordinate analysis (PCA) was also performed to obtain the genetic relationship. Pairwise PhiPT matrix was used to perform PCA, implemented in GenAlex 6.41.

RESULTS

Morphology

Total 83 birds were caught by mist nest from sampling site and after measurements were released safely. The total lengths of the birds were higher in northern population (101.8mm) as compred to souther population (96.5mm). Accordingly the birds of north were also heavier (14.23gm vs 13.4 gm). There is a significant difference in tarsus length. It was 14.23 mm in north population and 10.5 mm in south population. Wing length was 53.14mm in north population and 48.06mm in south population (**Table 1**).

Table 1. Measurements of characters in the Spotted munia

Characters	Northern Population (Mean ±SD)	Southern Population (Mean ±SD)	t	P
Total length	101.8±0.24	96.5±0.21	16.78	0.0001
Culmen	14.71±0.2	13.4±0.14	5.67	0.0001
Body weight	14.23±0.14	11.7±0.07	14.55	0.0001
Tarsus length	14.06±0.19	10.5±0.07	18.96	0.0001
Wing length	53.14±0.14	48.06±0.14	24.75	0.0001
Tail length	41.71±0.11	38.25±0.11	21.61	0.0001

Mitochondrial sequence data

Partial sequences of 12S (461 nucleotides) was obtained from *Lonchura punctulata*. All sequences appear to be genuine mitochondrial DNA, rather than nuclear copies. The sequences contain no insertions or deletions, and they exhibit nucleotide frequencies and codon site variation typical of mitochondrial DNA [31].

Genetic polymorphism

The overall nucleotide base frequencies in entire *Lonchura punctulata* populations were as follows: A = 33.0%, C =30.9%, G = 15.3%, and T = 20.9%. Of the total 461 total nucleotide sites, 09 are variable (1.9%), of which 06 (1.3%) are potentially parsimony informative. Total number of polymorphic (segregating S) sites 9, total mutation (Eta) 10. Total number of haplotypes, h 29, haplotype (gene) diversity, Hd 0.950 ± 0.009 were found in all populations

Uncorrected proportional distances (p-distances). Distances from Bijnor region to all sites average 0.025% (range:0.003% to 0.005%), from Chennai to all sites average 0.032% (range: 0.004% to 0.008%), from Bihar to all sites average 0.031% (range: 0.004% to 0.008%), from Mirzapur to all sites average 0.032% (range: 0.004% to 0.008%), and from Nepal to all sites average 0.036 (range: 0.005% to 0.009%). The average distance from *L. punctulata* to outgroup *L. Malacca* is 0.008 (range: 0.005% to 0.011%) and to *L. malabarica* is 0.005% (range: 0.003% to 0.007%). The highest number of polymorphic site and

number of mutations was found in Nepal (S 7, Eta 8) followed by Chennai (S 7, Eta 7) and Bihar (S 6,Eta 6) respectively. Overall nucleotide diversity was 0.00521, highest in Nepal (0.00621±0.00039) followed by Bihar (0.00512±0.00037) and Chennai population (0.0044 ± 0.00071). The average number of nucleotide differences and theta were also found maximum in Nepal (k 2.862, Theta 0.0047). The average number of nucleotide diversity between populations was high in Bijnor-Nepal and Mirzapur-Nepal (0.00581). The average number of nucleotide substitution per site between population (Dxy) was high in Mirzapur-Nepal (0.00585) followed by Chennai-Nepal (0.00562). the number of net nucleotide substitution per site petween population (Da) ws high in Bijnor-Nepal (0.00076) and low in Nepal-Bihar (-0.00004) (Table 1.6), Total 29 haplotypes were found. Maximum number of haplotypes was found in Nepal (19) population followed by Bihar (16) and Chennai (11). Haplotype diversity found maximum in Nepal (0.984±0.017) followed by Bihar (0.938±0.023) and Mirzapur (0.936±0.051)

Phylogeography and population structure

The haplotype network exhibits a major clustering between Bihar and Nepal populations. Haplotype 2 emerged as a common haplotype in all populations. Chennai population shared haplotype with northern population whereas haplotype 5 and 6 were distinct haplotype in this population. Nepal population also shared haplotype with others where as maximum number of haplotype were found in this population. Bijnor, Mirzapur and Bihar populations do not show any distinct haplotypes (**Fig 1**).

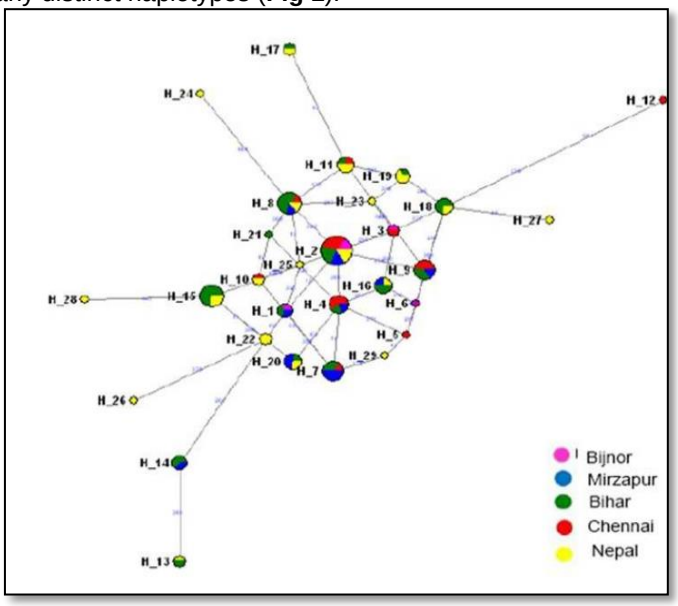

Fig 1. Median-joining network of Lonchura punctulata of india based on MtDNA 12S rRNA gene sequence variations. Number on lines joining haplotypes are polymorphic sties. The size of haplotypes circles are correspond to number of samples.

The Bayesian tree also exhibits a similar topology, where two major lineages were found. Both lineages contain the sequences from all samp-ling populations. These clustering indicate the admixture of populations (**Fig 2**).

A three level AMOVA, partitioned 5.28 % variation among groups, -0.18% among population within groups and 94.9 % variation within populations. The overall Fst was low 0.05098, Fsc, which describe the variation among groups within regions was 0.0598. Finally Fct describing the variation of groups among regions was 0.05275 (**Table 2**). The pairwise Fst values between groups were high between Mirzapur and Nepal (0.08473), whereas its low between Bijnor-Nepal (-0.00193) and Nepal-Bihar (-0.00577) (**Fig 3**).

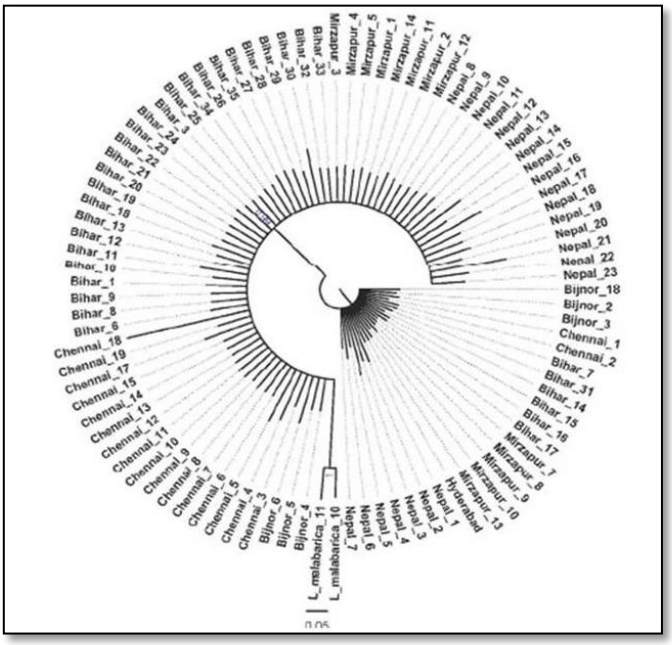

Fig 2. Phylogenetic relationship among the different populations of Lonchura punctulata of india based on MtDNA 12S rRNA gene.

Table 2. AMOVA design and results inferred from mitochondrial 12S rRNA gene.

Source of variation	d.f	Sum of squares	Variation components	Percentage of variation
Among groups	2	5.460	0.05469 va	5.28
Among populations within groups	2	2.256	-0.00217 vb	-0.18
Within populations	85	96.917	1.16373 vc	94.90
Fixation Indices Fsc: -0.00187 Fst: 0.05098 Fct: 0.05275				

The average pairwise difference between population (PiXY) was high in Nepal and Mirzapur (2.69565), followed by Chennai-Bihar (2.6256). The average number of pairwise difference within population (PiX) range from 1.0-2.86166), high in Nepal (2.86166) followed by Bihar (2.35887) and Chennai (2.02614). p value was non significant in all cases (**Fig** 4). The expected heterozygosity was high in Nepal (0.31796) followed by Bihar (0.26210) and Chennai

(0.22513). Locus wise heterozygosity high in locus 7 in Nepal (0.56126), locus 6 (0.53846), locus 1 (0.51282) in Mirzapur (**Fig** 5).

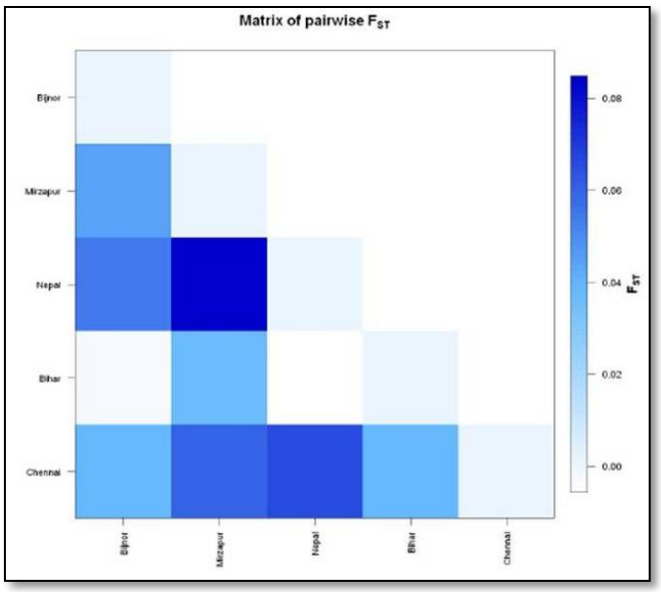

Fig 3. Pair wise Fst between the population of Lonchura punctulata of india based on MtDNA 12S rRNA gene.

Mismatch distribution pattern shows unimodel pattern, where raggedness index and tau high in Bijnor (r 0.5278, Tau 2.401) and low in Nepal (r 0.0671, 2.026, **Fig 6**), indicating recent demographic expansion in populations. Neutrality test were performed using Tajima's D and Fu's Fs. In whole data Tajima's D was 0.5716, high in Nepal (1.60862) and Bihar (1.63848).In Bijnor and Chennai population Tajima's D was -0.7099 and -0.01501 respectively (p value is not significant). The overall Fu's Fs was -1.39176 (p>0.01) was high in Bijnor (-0.08873), followed by Mirzapur (-5.20922) and Chennai (-6.646009).

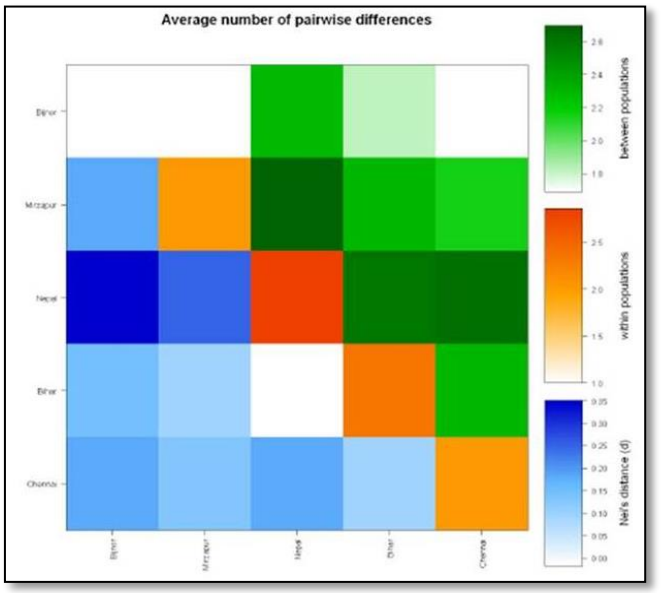

Fig 4. Average number of Pairwise differences within and between the population of Lonchura punctulata of india based on MtDNA 12S rRNA gene.

DISCUSSION

The morphology of Spotted munia differed significantly between northern and southern populations. These differences seemed to be related to the way they face the contrasting seasonality of their breeding time. The northern birds (high altitude) seems to be little longer than compared to lower southern population (lower altitude). The wing and tail in the birds from high altitude were longer than those from low altitude, and the body weight of those in high altitude was heavier than that of low altitude (**Table 1**).

It has been suggested that interspecific competition cause functional width decrease, and determines the partitioning of resources. Further, it can lead to the amusement of diet of species as found in other passerines. Along with other selective pressures, interspecific competition may have determined the morphology of the Spotted munias, at least in the study areas. Selective pressures may vary geographically, and this may result in geographical differences in the morphology of a species. This was well observed in the Passerines whose morphology varies geographically. In this house sparrow, *Passer domestics*, this size of most parts of its body as well as its body weight vary geographically [32, 33]. Our results also indicate the geographical variation in the morphological characters of birds from both altitudes were different in that those of high altitude have longer wing and tail, and heavier body weight than those from low altitudes. It has been found that such morphological characters as wing, tail and tarsus related to foraging behavior. There are some other factors such as species density, competition and resources availability which may govern the morphological variation in this species.

The DNA sequence of 12S revealed northern populations are genetically more diverse compared to that of southern. The northern populations were found to be tightly clusterd. There is a well defined genetic structure but admixture can also be seen. The northern and southern populations sharing show one point radiations of this species. This species has restricted distributed in Indian and South-East Asia. Our Mt DNA sequences data indicated that the most common ancestor of this species was present in

Indian plate in down south and from there it might have migrated towards eastern region of India. From east a northward migration in the Himalayan foothills indicated. Demographic history also suggested the recent expansion in Northern populations. The 16S rRNA marker suggests the recent bottleneck in Sub Himayan population (bijnor) and in southern population (Chennai). It also suggests the Sub Himalayan population (Nepal) is may be ancestor population for Bhabar valley (Bijnor). The high polymorphism, haplotype diversity and neutrality test indicate population expansion in north after bottleneck. The neutrality test also indicates the population's size expansion in recent past in South. The phylogram suggested the clustering of populations with presence of individuals of other populations, indicating high rate of variation in individuals, this leads to low variation among populations within groups supported by AMOVA. The haplotypic MJ network tree also suggest a strong association of southern and northern population with new emerges in northern side. It indicates the impact of glaciations in forming the population

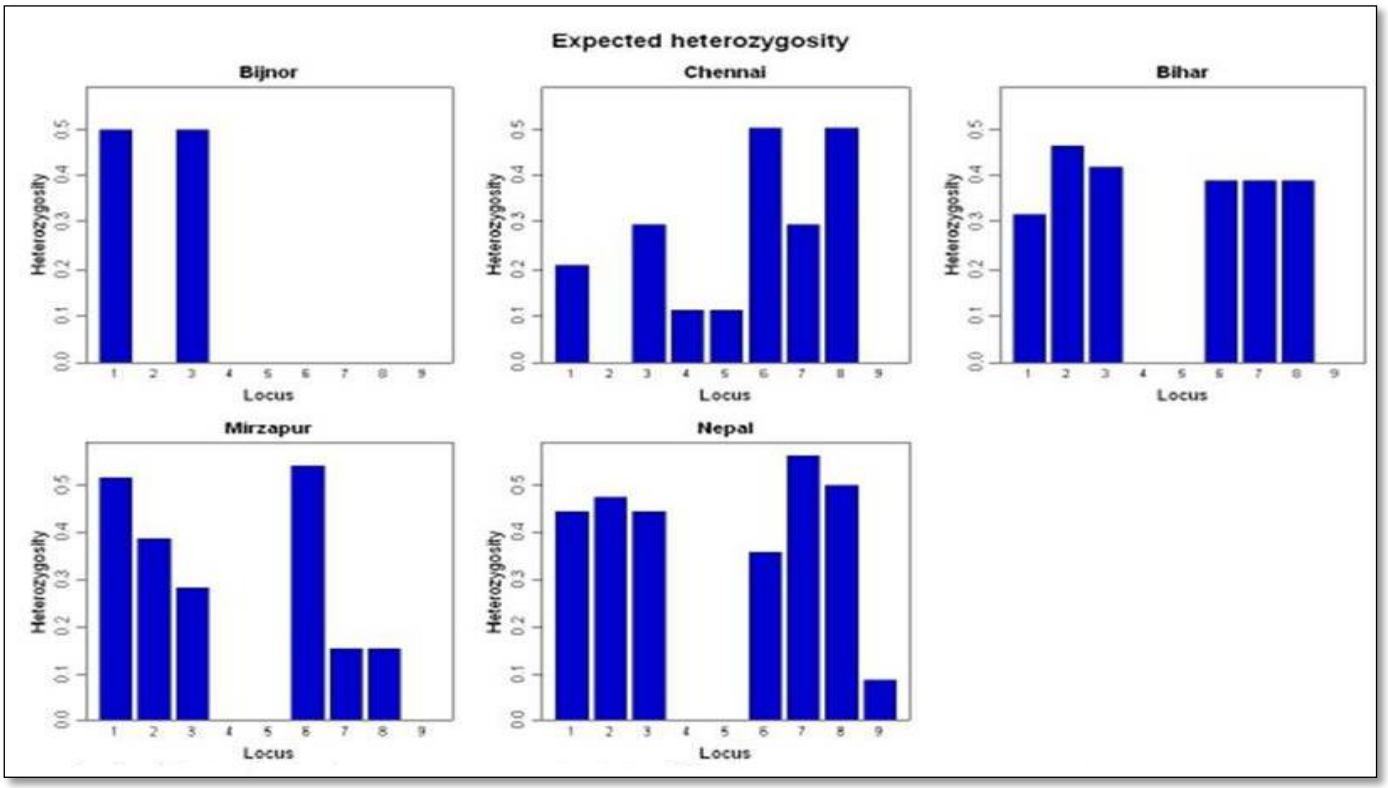

Fig 5. Expected heterozygosity in different populations of Lonchura punctulata of india based on MtDNA 12S rRNA gene.

group of punctulata in India. In comparison of molecular and morphological both population structures can be seen clearer. Therefore based on its previously physiological and behavioral studies with the current results we can hypothesized that in Indian subcontinent this species is still in under the process of speciation. However in depth genetic studies with some more genetic markers are needed for draw actual conclusions.

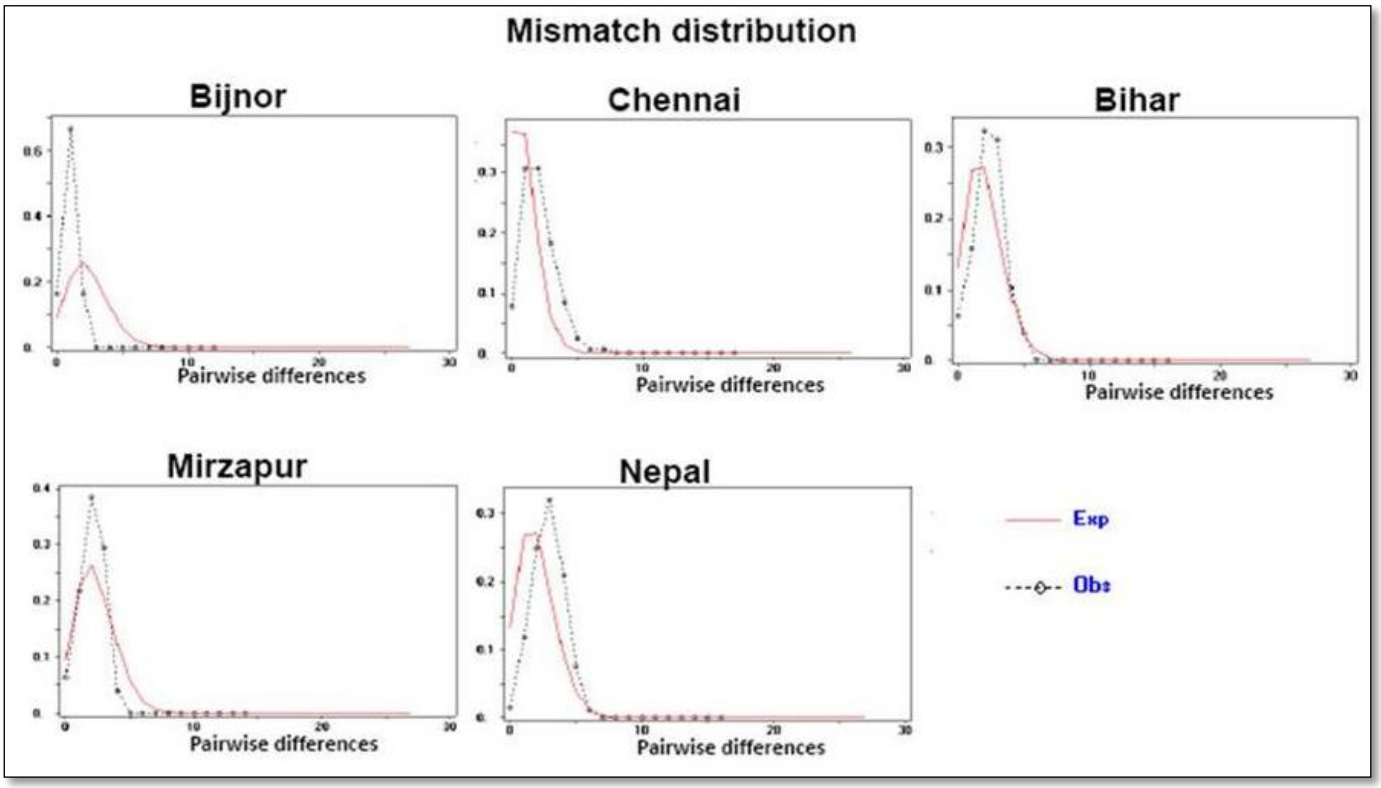

Fig 6. Pairwise mismatch distribution in different populations of Lonchura punctulata of india based on MtDNA 12S rRNA gene.

Conflict of interest

The authors declare that they have no conflict of interests.

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