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Genetic diversity and phylogenetic analysis of Indian Rhesus macaque (*Macaca mulatta*) using Mitochondrial D-loop

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Abstract

Indian Rhesus macaques (*Macaca mulatta*) are non-human primates having a wide range of distribution in India. Rhesus macaques are distributed throughout Northern India and Northeast India up to some extent part of South India. In this study, we investigated the genetic diversity and phylogenetic relationship of Indian rhesus macaques in comparison with macaques from other countries using the mitochondrial D-loop region. A total of 61 DNA sequences were generated from five Indian states and compared with 46 sequences from China, Thailand, Japan, and Bangladesh from GenBank. A total of 40 haplotypes were identified in studied samples. The median-joining network showed clustering based on different geographic locations. The Phylogenetic analysis shows two major clades in which the Indian rhesus macaques formed a single clade. There was no geographical cluster found in studied samples. The mismatch and Bayesian skyline plot show recent expansion in the Indian rhesus macaque population. Our results show that the Indian rhesus macaque is undergoing expansion in the population.

Keywords: Macaques; Populations; Expansion; D-loop; Clade

Introduction

Rhesus macaques (*Macaca mulatta*) are widely distributed non-human primates (Fooden, 2000). Its distribution range covers a vast variety of ecologies such as tropical, subtropical, temperate forests, and even rural and urban areas, which could be due to its rich behavioral and genetic features (Zhou *et al*., 2022). The origin and diversification of Indian rhesus macaques began about 2.5 million years ago when the fascicularis species originated and diversified upon reaching the mainland Indo-China region (Fooden, 2006; Stevison and Kohn, 2009). The range and distribution of rhesus macaque are unique. Rhesus macaques have the largest distribution spanning Afghanistan, Bangladesh, Bhutan, India, Nepal, Pakistan, China, Burma, Laos, Thailand, and Vietnam (Brandon Jones *et al*., 2004; Fooden, 2000). In India, the Rhesus macaque occurs in a wide range of habitats from the Himalayas, deserts, tropics, and temperate forests except in southern India where two native species namely the Bonnet macaque (*Macaca radiata*) and lion-tailed macaque (*Macaca Silenus*) (Chaudhuri*et al*., 2006) occur. Rhesus macaques are widely distributed across southern and south-eastern Asia, whereas bonnet macaques are restricted to peninsular India (Kumar *et al*., 2011). Rhesus macaques are common commensals with maximum ecological adaptability (Southwick and Siddiqi, 1988). In India, Rhesus macaques signify historical and cultural aspects of the country. Rhesus macaques have been listed in CITES Appendix II, Schedule I of Part I of the Indian Wildlife (Protection) Act (amended up to 2002). The Indian people view them in terms of food and habitat preferences based on their experiences and religious belief systems. Around 90% considered the macaques to be an incarnation of Lord Hanuman, a Hindu deity. Temple structures also represent Lord Hanuman which is used for worship purposes. Such

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religious beliefs help in the conservation and management of macaques in India (Southwick and Siddiqi, 1988; Pirta*et al*., 1997).

Rhesus macaques are the primary animal model used for research (Smith and McDonough, 2005) and were used extensively for studying human diseases mainly to understand HIV and develop vaccines (Vandeberg and Willams Blangero, 1997). Most of the rhesus macaques were transported from India and China but after 1978 India ceased to supply and China became the main supplier of rhesus macaques to the biomedical research centers in the United States. However, phenotypic differences and significant genetic differences between the Chinese and Indian rhesus macaques have also been noted. Thus, it is necessary to have a detailed understanding of genetic variation within different populations of Rhesus macaques as a part of biomedical research. Regional genetic differences among rhesus macaques may justify the maintenance of separate breeding groups suitable for different animal models. Therefore, it is necessary to characterize rhesus macaques with their geographic distribution (Smith and McDonough, 2005) and understand the distributionand patterns of genetic diversity at the intraspecies level for planning effective conservation management strategies. Although there have been several genetic studies of rhesus macaques in Bangladesh, China, and Nepal which primarily focused on wild populations. There have been no molecular studies on the genetic diversity of rhesus macaques belonging to the Indian subcontinent. Numerous primate habitats have been destroyed due to the increasing human population pressure, industrialization, and urbanization (Feeroz*et al*., 2011), and many rhesus macaque populations have been confined inside human settlements (Hasan *et al*., 2013) causing human monkey conflicts. For this study, non-invasive sampling techniques were performed which provided a new approach in classical taxonomyfor the identification of species and the determination of theirgenetic diversity. This technique includes a collection of naturally shed materials such as hair,faecal pellets, etc., which don'tcause harm or disturb the animals in the wild during sampling (Bhaskar *et al*., 2021).

Phylogeny, evolutionary history, and gene variations in connection to adaptations to local environments will help us understand the biology and evolution of Rhesus macaques. Molecular studies based on mitochondrial DNA genes help in establishing the phylogenetic position and help in understanding divergence and gene flow among the different groups of macaques (Tosi *et al*., 2002; Tosi *et al*., 2003). Mitochondrial DNA is maternally inherited (Giles, 1980) and the control region (CR) evolves at a magnitude more rapidly than nuclear DNA (Brown *et al*., 1979). This region must characterize the intra specific genetic differences in species that are related to geographic distribution (Takahata and Slakin, 1984). The population retrieval of Rhesus macaques in India would help with research and conservation. The data provide in present study will help in genetic resource conservation and management of Indian Rhesus macaques (*Macaca mulatta*). We will discuss the genetic diversity and population differentiation in the collected samples with the overall distribution of mitochondrial D-loop in other country.

Materials and Methods

Sample collection

Faecal materials of Rhesus macaque (*Macaca mulatta*) were collected using a non-invasive technique from five north Indian states namely Uttar Pradesh, Bihar, Assam, Punjab, and Himachal Pradesh during field surveys from the year 2019 to 2022 (Figure1 and Table 1). Sterile forceps were used to place the fecal pellets in sterile containers which contained ethanol or silica gel depending on whether the fecal sample was fresh or dried, respectively, and preserved at room temperature in the laboratory and stored at -80˚C until DNA isolation is done. The Indian rhesus macaque sequences were also compared with China (JF746823-JF746842), Bangladesh (AB275633-AB275639), Thailand (EF208877-EF208892), and Japan (LC585815-LC585818) sequences. The sequences were aligned using the 'CLUSTALW' alignment (Thompson *et al*., 1994).

Figure 1. The dots indicate the various sampling sites of Rhesus macaque in India

Table 1. Samples of Rhesus macaque collected for phylogeographic analysis.

Indian states/ Location ID	Location	Latitude and longitude	No of samples
Uttar Pradesh	Lucknow	26.832 N 80.919 E	20
Himachal Pradesh	Shimla	31.105 N 77.172 E	20
Punjab	Chandigarh	30.759 N 76.771 E	20
Bihar	Kaimur wildlife sanctuary, Kaimur	24.704 N 83.174 E	14
Assam	Guwahati	26.806 N 82.777 E	20

DNA Isolation and PCR amplification of faecal samples

The QiagenQIAmp fast DNA stool kit (QIAGEN, Germany) was used for fecal DNA extraction using the manufacturer's protocol with slight modifications. Samples were processed separately to avoid contamination. The PCR amplification was carried out using DLoop F1 (5′-GCCATAACAATCAAGATCGC-3) and DLoop R1 (5′-TGTTGCGGGTTGGTGTAGAG-3) primers (Zhu and Evans, 2023). PCR amplification was performed using a 3 µl DNA template for a 30 µl PCR reaction using 15 µl master mix, 0.4 µl forward and reverse primer and 12.8 µl nuclease-free water. The PCR wascarried out with an initial denaturation at 94°C for 5 min, 35 cycleseach with denaturation at 94°C for 30 s, annealing at 60°C for 35 s, and extension at72°C for 60 s followed by a final extension at 72 °C for 10 min. After checking the amplification in 1% agarosegel, the successful amplicons were sequencedbidirectionally using BigDyechemistry on an ABI 377 automated sequencer.

Molecular data analysis

The sequences were confirmed throughBLASTn (Basic Local Alignment Search Tool) (https://blast.ncbi.nlm.nih.gov). The obtained sequences were checked andedited manually for ambiguous bases usingBioEdit V7.0 (Hall, 1999) software.

Genetic Variability Analysis

The estimates of genetic polymorphism ie. haplotype numbers (H) and haplotype diversity (Hd) were generated in DNASP V6 (Rozas*et al*., 2017). Pairwise genetic differences were calculated using DNASP V6. Mismatch distribution neutrality tests like the Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997) values were performed in Arlequin v3.5 (Excoffier and Lischer 2010) software for within and between the populations.

Phylogenetic analysis and haplotype network

Phylogenetic analysis was carried out in MEGA v10.2.2 software using the Maximum Likelihood tree method with 1000 bootstrap replicates. We first tested the most suitable model which was found to bethe HYK+G model. The maximum likelihood (ML) was constructed using the best fit substitution model which was selected in MEGAX software accordingly. Branch specific rates and lengths were visualized with Figtree v1.4 (Rambaut, 2009). PopART 1.7software was used in constructing a median-joining (MJ) haplotype network (Leigh and Bryant 2015). We also estimated population genetic differentiation (Fst) between the five different populations of rhesus macaques using Arlequin v3.5 (Excoffier and Lischer 2010).

Population demographic analysis

Mismatch distribution graphs were plotted using the DnaSP Ver. 6.12.03 (Rozas*et al*., 2017) for each geographic sample to infer whether the Indian rhesus macaques had experienced demographic expansion. We evaluated the effective population size over time using the Bayesian skyline plot (BSP) (Ho and Shapiro, 2011) which was implemented in BEAST v2.0 (Bouckaert*et al*., 2014) to understand the historical demographic with the mtDNA. We used the HKY model, empirical base frequencies, and ran the MCMC algorithm for 10,00,000 generations, sampling trees and parameters every 3000 generations. Tracer 1.7.2 (Rambaut*et al*., 2018) was used to visualize the plots to evaluate the convergence of all parameters.

Results

Genetic polymorphism using mitochondrial Dloop

We obtained DNA from 75 fecal samples, out of 94 fecal samples processed and got 61 good quality sequences after the amplification. It showed 64% successful amplification for the mitochondrial D-loop amplification. The final dataset consisted of 595bp of sequences. Generated sequences were submitted in NCBI database (OQ674824- OQ674849, OQ674904- OQ674924, OQ674928- OQ674932). A total of 40 haplotypes (h) were found in the dataset. The total haplotype diversity (Hd) was 0.730. The number of variable sites (S) was 70 and the total number of mutations was (Eta) 72. The nucleotide diversity (Pi) was 0.030 and the average number of nucleotide differences (k) was 16.133. Low haplotype diversity was observed for Assam (0.167) and high haplotype diversity was observed for Bihar (0.833) samples (Table 2).

Table 2. DNA polymorphism data of Indian rhesus macaque samples: sample size (n), haplotype (H), haplotype diversity (h), nucleotide diversity (π).

	n	H	h	π	Tajima D	FuFs
Uttar Pradesh	27	10	0.752	0.002	-1.598 (0.034)	-4.314 (0.003)
Bihar	4	3	0.833	0.019	-0.705 (0.282)	3.504 (0.90)
Assam	12	2	0.167	0.0006	-1.451 (0.067)	0.431 (0.378)
Himachal Pradesh	9	3	0.667	0.001	-0.359 (0.39)	3.040 (0.924)
Punjab	9	5	0.722	0.003	-1.477 (0.076)	-1.185 (0.138)
Total	61	18	0.730	0.003	-2.43** (0.01)	-5.48** (0.02)

Distribution of Haplotypes and phylogenetic relationships

The median joining network was constructed using mitochondrial Dloop sequences. No cluster was found in the study sample. When, we compared our sequences with other countries we found that the Indian rhesus macaque formed a separate cluster (Figure 2). The mtDNA haplotypes of India, China, Bangladesh, Japan, and Thailand formed four distinct haplogroups in the haplotype network shown in Figure 3. Haplogroup1 consisted of haplotypes belonging predominately to China. Haplogroup 2 is consisted of Bangladesh and haplotype 3 is Thailand. Haplogroup4 consisted of haplotypes of India. The phylogenetic tree (ML) of all the haplotypes from these populations showed two major clades. Clade 1 showed the Indian samples whereas Clade 2 showed four subclades consist of Chinese, Bangladesh, Japan, and Thailand samples (Figure 3).

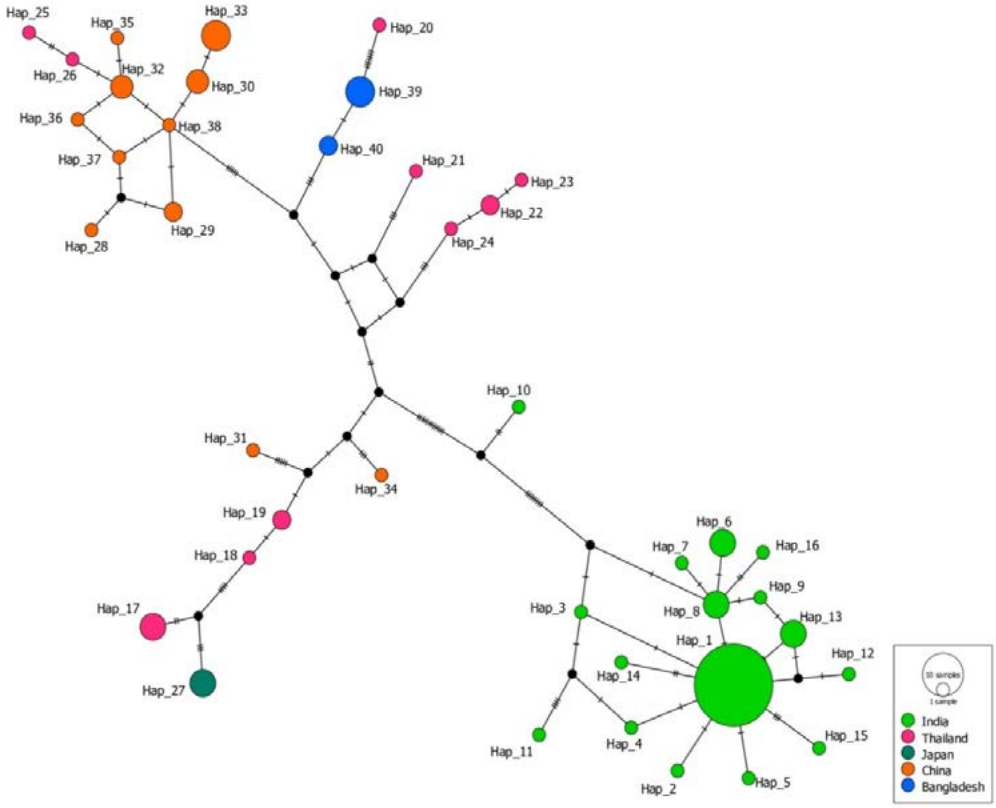

Figure 2. Median-joining network showing separate clusters based on geographic locations of Rhesus macaque.

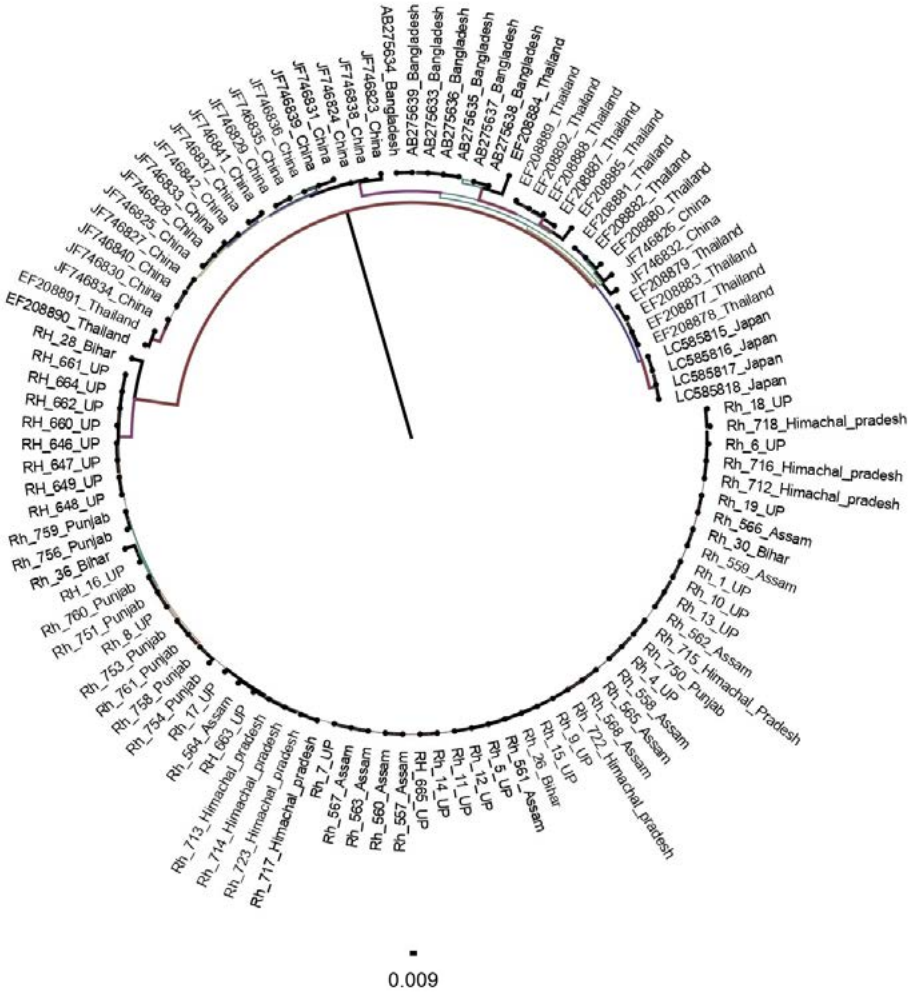

Figure 3. Maximum likelihood (ML) tree of rhesus macaques showing the separate clades between the Indian Rhesus macaque population and the rhesus macaque from other populations.

Genetic distances and population structure

The pairwise genetic distance and FST were calculated in studied samples (Tables 3). Genetic distance and genetic differentiation (Fst) was detected between all the population pair was very low ranged from 0.00 to 0.003 and 0.001 to 0.011 respectively. The pairwise FST and genetic distance were also calculated with other country (Table 4). Low Fst valoue was observed between china and Japan (0.07) and highest in Bangladesh and Japan (0.974) while genetic distance is low between two pair China and Bangladesh and Thailand and Bangladesh (0.026) and high between India and Bangladesh (Table 4).

Pairwise genetic differences were calculated for the Indian rhesus macaque populations which signify demographic expansion in the population. Mismatch distribution analysis shows unimodal ragged distribution (Figure 4). Neutrality test of Tajima's D and Fu's Fs were also performed within the Indian population and between different populations (Tables5 and 6). Among the Indian samples, the Uttar Pradesh samples showed significant Tajima D and Fs values. When comparing different populations, the Indian population showed significant Tajima D and Fu's Fs values when compared to the Chinese. The Japanese populations did not show any values due to the low sample size. The negative value of Tajima's D for Indian haplotypes was statistically significant indicating a recent population expansion in rhesus macaques. The Bayesian skyline plot was constructed for Indian rhesus macaque samples to show the population is recent expansion expansion (Figure 4).

Table 3 Genetic differentiation among the Rhesus macaque populations. The pairwise *FST* values (Below diagonal) and genetic distance (above diagonal) based on D-loop.

	Uttar Pradesh	Bihar	Assam	Himachal Pradesh	Punjab
Uttar Pradesh	**0.000**	0.002	0.000	0.001	0.001
Bihar	0.011	**0.000**	0.002	0.002	0.003
Assam	0.001	0.010	**0.000**	0.000	0.001
Himachal Pradesh	0.002	0.011	0.001	**0.000**	0.001
Punjab	0.004	0.013	0.003	0.004	**0.000**

Table 4. Genetic differentiation among the Rhesus macaque populations with different country. The pairwise *FST* values (Below diagonal) and genetic distance (above diagonal) based on D-loop.

	India	Thailand	Japan	China	Bangladesh
India	**0.000**	0.054	0.059	0.055	0.060
Thailand	0.800	**0.000**	0.027	0.028	0.026
Japan	0.932	0.251	**0.000**	0.034	0.035
China	0.882	0.229	0.070	**0.000**	0.026
Bangladesh	0.936	0.351	0.974	0.611	**0.000**

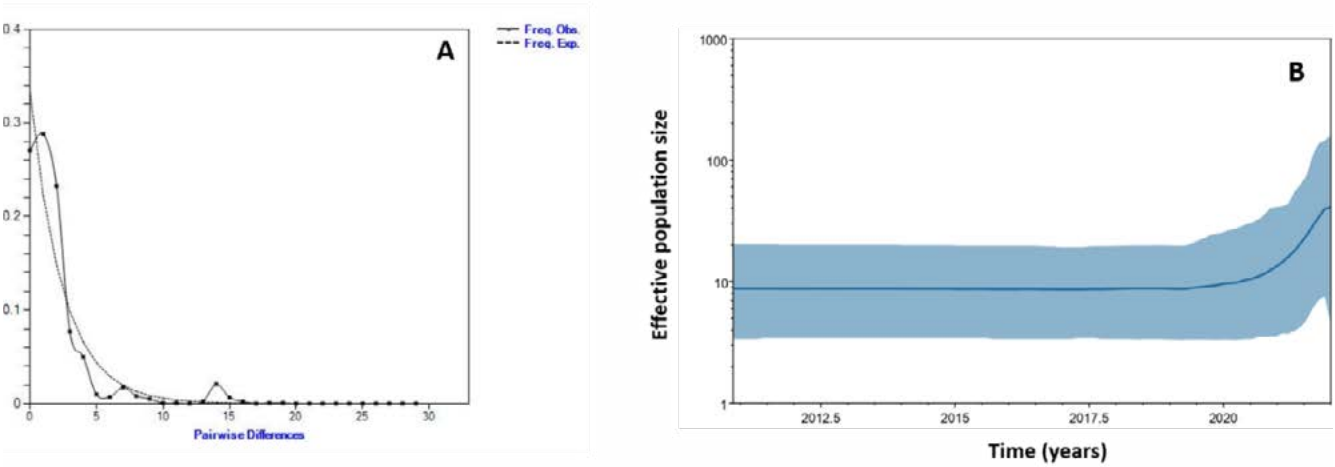

Figure 4. (A) Mismatch distribution analysis based on mtDNA (CR) sequences of Indian rhesus macaques. The black lines denote observed frequencies and the dotted lines show expected frequencies; (B) Bayesian Skyline plots showing the historical population size for the Indian Rhesus macaque populations.

Discussion

Primate populations in Asia face intense ecological pressure which is known throughout the world. The major reasons are habitat, destruction, competition for food and space, pet trade, etc. (Southwick and Siddiqi, 1994). The present study demonstrates the genetic diversity and population structure of Rhesus macaque (*Macaca mulatta*) from the five states of the country. The study was done by the mitochondrial D-loop. We processed 94 samples and generated 61 good quality sequences for analysis. Clustering patterns pattern of the haplotype network and phylogenetic analysis show no population structure in the studied samples. The median joining network with other country was formed four clusters. It shows that Indian cluster is closer to Thailand as compare to Bangladesh. This shows that the Bangladesh haplotypes were not similar to Indian haplotype samples but rather clusters with Chinese rhesus macaques. Smith & McDonough, 2005, studied that Indian haplogroup formed separate cluster that included none of the Bangladesh. This might be due to the occurrence of subspecies. The analysis of phylogenetic tree shows that Punjab and Himachal Pradesh a separate group. The sampling locations of rhesus macaques in India are separated by each other by geographic isolated distances of 30 to 300km (Hasan *et al* 2013). In some locations, the populations are separated by human settlements or natural barriers such as rivers which contribute to differences in gene flow. Hence the Chinese and the Indian rhesus macaques form two separate haplotypes due to significant genetic differences.

In the case of rhesus macaques (*Macaca mulatta*), it exhibits female philopatry and male dispersal from natal groups (Pusey and Packer, 1987), and the mtDNA contains the genetic differences among the regional populations (Smith *et al*., 2007). Previous studies have shown that the Indian and Chinese rhesus macaques were reproductively isolated during the Pleistocene, during which the Indian rhesus macaques experienced a severe genetic bottleneck, and that some gene flow westward into India was subsequently re-established. Bayesian skyline plot shows that the Indian rhesus macaques are undergoing population expansion from 2019 onwards in recent years. From our data, the phylogenetic analysis shows that the Indian rhesus forms a separate clade from other countries. The median joining network shows the Indian and the Chinese rhesus macaques form separate haplogroups. The genetic distance and the FST values also signify that they are separate populations. Groves (2001) assigned rhesus from Kashmir/Punjab region to subspecies *M. m. villosus*. The geographic range of rhesus macaques is sufficiently extensive, and the antiquity of their dispersal throughout that range is sufficiently great, that major genetic differences might have evolved in regional populations of Macaca mulatta. Smith and McDonough (2005) argued that the current geographic distribution of rhesus macaques in India resulted from a westward dispersal or redispersal, that the species dispersal took place through mainland Southeast Asia and China. Melnick and Kidd (1985) suggested that genetic similarity between Chinese rhesus macaques and cynomolgus macaques in Thailand results from the divergence of rhesus from cynomolgus macaques in Thailand during a glacial maximum, followed by the dispersal of cynomolgus macaques to the south.

The outcome of this study shows a stark difference in the rhesus macaque populations of India and China in terms of their origin and diversity. This is consistent with the phenotypic differences between the two regional populations as Chinese rhesus males tend to beheavier, longer, and taller than Indian rhesus males (Peng *et al*., 1993).The significance of both negative Tajima's D and Fu Fs, as well as the presence of low nucleotide but high haplotype diversity, shows recent expansion in the Indian population. This is because the male macaques tend to leave their natal groups and improve the gene flow. The mitochondrial haplotype network showed geographical clustering which had unique haplotypes. The mismatch distribution shows the unimodal and ragged distribution. The Bayesian skyline plot shows that the Indian rhesus macaques are undergoing population expansion from 2019 onwards in recent years. This might be due to anthropogenic factors contributing towards the expansion. It is necessary to closely monitor the wild populations of rhesus macaques to in order to maintain constant population.

Conclusion

We discussed the genetic diversity and haplogroups of various rhesus macaque (*Macaca mulatta*) populations from the five states of India and with other country. There is no much difference obtained in five states population but due to geographic barrier Punjab and Himachal population formed separate group. The Indian and Chinese rhesus macaques tend to exhibit significant differences in their mitochondrial D-loop. However, the Indian rhesus macaque populations showed expansion in population which needs to be ascertained using more sampling. The Bangladeshi macaques were not similar to the Indian macaques but showed more similarity with the macaques from Thailand.

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