Genetic diversity within and among populations of the fulvous fruit bat *Rousettus leschenaulti* using RAPD analysis

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The genetic diversity of 14 *Rousettus leschenaulti* populations was examined using the random amplified polymorphic DNA marker technique. Samples were collected from various districts of southern Tamil Nadu, India. A total of 266 bands were identified in the bat genome using a polymerase chain reaction, of which 189 were found to be polymorphic. The range between 0.05 and 0.70 in the dendrogram represented the relative genetic similarities between the populations. According to principal coordinate analysis, the first two components were accountable for 50.3% of the overall variation. The species exhibited a significant level of genetic variability across populations. This might continue as a result of population gene flow, which primarily results through extra-colony copulations without permanent dispersal from the parent colony. Also, greater vagility is an adaptation associated with cave roosting in this species. Our preliminary results indicate that all 14 populations should be considered to maintain genetic diversity. The development of effective management measures for their conservation depends on knowledge of genetic variation within and between populations.

Keywords: Anthropogenic disturbances, cave roosting, genetic diversity, RAPD analysis, *Rousettus leschenaulti*.

BATS represent one of the most speciose and ubiquitous animals belonging to the order Chiroptera, which follows the mammalian order Rodentia[1], with approximately 1300 species divided into two extant suborders: Megachiroptera (Old World fruit bats) and Microchiroptera (laryngeally echolocating bats)[2]. More than 20% of all mammalian species are bats[1]. They play a crucial role in seed dispersal, plant pollination and structuring forest ecosystems[3].

The fulvous fruit bat, *Rousettus leschenaulti* is grey–brown in colour, frugivorous and belongs to the suborder Yinpterochiropteran bat; it is distributed all over India and most of southeast Asia[1]. These bats live in caves, historical monuments, wells, mines, deserted buildings, temples and unused tunnels[1,4,5]. A mixed-sex colony of *R. leschenaulti* can range from as low as 2–3 individuals up to several thousand bats[4,5]. The behaviour of post-partum pregnancy[6], development of the vomeronasal organ[7], postnatal growth, age estimation with development of foraging behaviour[8], wing morphology and flight performance[5], entrainment and phase shifts[9], echolocation signals[10], olfaction and vision[11], food and foraging preferences[12], roosting habits and seasonal variation in the diet[13], morphological characters[14], roosting ecology and distribution[15] are extensively studied aspects in this species. Until now, studies on genetic variations within and among the populations of *R. leschenaulti* are meagre. This is certainly due to their unique features. The study of bats is particularly challenging when using conventional behavioural techniques; molecular approaches have proven to be more useful.

In the declining populations of *R. leschenaulti*, it is important to know their genetic structure for suitable conservation measures. Several molecular genetics techniques are widely used to study population biology[16]. Random amplified polymorphic DNA (RAPD) method of DNA fingerprinting was used in this study due to its quick results, low cost and reproducibility. Since this technique does not require prior knowledge of the DNA sequence[16], its use in population and evolutionary biology is now commonplace. Studies on a wide variety of animal and plant taxa, including bats[17–19], adopt this approach. Understanding the genetic diversity of the *R. leschenaulti* population might provide useful guidelines for conservation methods, and as such, it should be a crucial component of effective conservation management. The objective of the present study was to examine the degree of genetic diversity within and among populations of *R. leschenaulti* in southern India.

Material and methods

Sample collection

R. leschenaulti samples were collected during February–April 2016, August–October 2017 and January–March 2018 from different locations in southern Tamil Nadu, India. The colony size ranged from 100 to 3000 bats. The minimum and maximum distance between the roosting sites were 10

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Table 1. Fourteen natural populations of *Rousettus leschenaulti* collected from southern Tamil Nadu, India

Population no.	Location	District	Sample size	Colony size	Sex
1	Tirupparankundram	Madurai	6	>1000	2M; 4F
2	Vengadachalapuram	Theni	9	>2500	4M; 5F
3	Tiruppudaimaruthur	Tirunelveli	5	>3000	1M; 4F
4	Kallidaikurichi	Tirunelveli	5	>450	3M; 2F
5	Arikesavanallur	Tirunelveli	7	>100	5M; 2F
6	Cheranmahadevi	Tirunelveli	6	>1000	2M; 4F
7	Nanguneri	Tirunelveli	7	>250	3M; 4F
8	Palayamkottai	Tirunelveli	9	>400	6M; 3F
9	Srivaikundam	Tuticorin	7	>450	5M; 2F
10	Seydunganallur	Tuticorin	8	>250	3M; 5F
11	Manapadu	Tuticorin	5	>200	2M; 3F
12	Poothapandi	Kanyakumari	7	>500	2M; 5F
13	Aralvaimozhi	Kanyakumari	5	>200	1M; 4F
14	Agastheeswaram	Kanyakumari	6	>100	2M; 4F

and 270 km respectively (Table 1). Using a hoop net with an extensible aluminium pole, bats were captured during their emergence from the temples and caves. In addition, they were captured using nylon mist nets measuring 9 m × 2.6 m with a mesh size of 38 mm (Avinet-Dryden, New York, USA) from the above-mentioned locations. Using gloves, the bats were removed immediately from the mist nets and placed in cloth bags[20]. Only adult bats were selected to excise tissue utilizing a medical punch (approximately 4 sq. mm). To prevent injury, the punch was carefully positioned between the blood vessels (wing membranes healed within 3–4 weeks). The punched hole and the punch were cleaned with 70% ethanol following each sampling. Until DNA extraction, the tissue samples were placed in ice, transferred to the laboratory and maintained at –20°C (refs 18, 19). We followed the Institutional Ethics Committee Guidelines (Internal Research Review Board, ethical clearance, Biosafety and Animal Welfare Committee approval to one of us (T.K.) dated 22 June 2015, Madurai Kamaraj University (MKU)).

Genomic DNA extraction and primer screening

Genomic DNA was extracted from wing-membrane biopsy samples using the phenol : chloroform extraction technique and routine proteinase K digestion[21]. Using spectrophotometric measurements at A260 and A280 nm and 0.7% agarose gel electrophoresis, the quality and quantity of the isolated DNA were evaluated (Hitachi U-2000, Tokyo, Japan). In this study, RAPD-PCR was carried out using three series of primers, each consisting of 10 primers: OPA (OPA1-OPA10), SK (SK1-SK10) and A (A-01-A-10) (Microsynth, Switzerland). Variable concentrations of the PCR master mix, primer and template DNA were used to optimize the PCR conditions. All 30 primers were used for the first screening, which involved DNA from four populations. The RAPD-PCR analysis was performed at least three times and all 14 samples were analysed using primers that produced prominent and distinct bands.

Polymerase chain reaction

PCR was performed in a total volume of 25 µl reaction comprising 100 ng of template DNA, 12.5 µl of 2X MyTaq mix (Bioline Inc, USA), 1 µl of 20 µM primer and 9.5 µl of H_2O. PCR amplification was performed using an Eppendorf PCR machine (PCR program with a temperature gradient in the annealing step of each cycle). The first step was denaturation for 5 min at 94°C followed by 35 cycles of denaturation for 30 sec, annealing for 2 min at a temperature gradient of 38–50°C (annealing temperature varied with the primers) and an extension for 2 min at 72°C. The final step was an extension for 10 min at 72°C. The amplified products were electrophoresed using 2% agarose gel and observed under ultraviolet light and analysed using gel documentation system (Biorad, USA, model 2000; Quantity One software).

Data analysis

NTSYS-pc version 2.0 computer package was used to analyse the RAPD data[22]. Jaccard's coefficient of similarity was used to determine the genetic similarity (GS). Each RAPD fragment was evaluated as either 1 (present) or 0, and was considered a unit character (absent). All fragments that were scored were put into a 1/0 matrix, and the information was utilized to calculate Jaccard's similarity coefficients for RAPD bands[23]. The resulting Jaccard's coefficients were then used to construct a dendrogram utilizing the unweighted pair group technique and arithmetic averages (UPGMA). The distribution of the genotypes was then displayed in a scatter plot using the software MVSP, version 3.22 (http://www.kovcomp.com/mvsp), which was run using the Jaccard's similarity matrix as the base.

Table 2. Primers with their sequences used for RAPD analysis of *R. leschenaulti*, and the total number of bands, polymorphic amplification products, percentage of polymorphism and polymorphic information content (PIC) yielded by each primer

Primer code	Primer sequence	Total no. of bands	No. of polymorphic bands	Percentage polymorphism	PIC
OPA2	AGTCAGCCAC	10	9	90.0	0.497
OPA3	CATCCCCCTG	16	14	87.5	0.498
OPA4	GGTGACGCAG	11	7	63.6	0.475
OPA5	TGCGCCCTTC	11	9	81.8	0.495
OPA6	TGCTCTGCCC	10	6	60.0	0.467
OPA7	GAAACGGGTG	16	16	100.0	0.500
OPA8	GTGACGTATG	8	6	75.0	0.489
OPA10	GTGATCGCAG	5	4	80.0	0.494
SK1	GTGTCTCAGG	7	4	57.1	0.463
SK3	GTCCATGCCA	15	12	80.0	0.494
SK4	ACATCGCCCA	13	9	69.2	0.484
SK5	GTGGTCCGCA	12	7	58.3	0.465
SK6	TCCCGCCTCA	10	7	70.0	0.484
SK7	AACGCGTCGG	15	8	53.3	0.454
SK8	AAGGGCGAGT	19	14	73.7	0.489
SK9	GGAAGCCAAC	7	6	85.7	0.497
A01	CAGGCCCTTC	8	5	62.5	0.473
A02	TGCCGAGCTG	11	9	81.8	0.495
A03	AGTCAGCCAA	10	3	30.0	0.355
A04	AATCGGGCTG	10	7	70.0	0.484
A05	AGGGGTCTTG	9	7	77.8	0.492
A06	GGTCCCTGAC	10	7	70.0	0.484
A07	GAAACGGGTG	13	6	46.2	0.432
A08	GTGACGTAGG	10	7	70.0	0.484
Total		266	189	70.6	
Mean per primer		11.1	7.9		

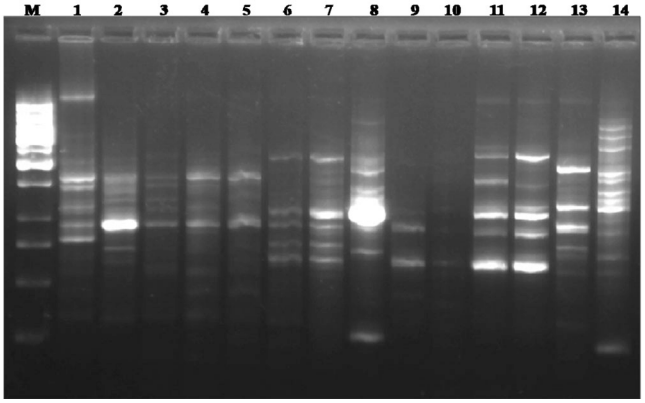

Figure 1. RAPD profile of 14 population samples of *Rousettus leschenaulti* using random decamer primer OPA7. Lane M, 100 bp DNA ladder. Lane numbers correspond to the population number given in Table 1.

Results

DNA fingerprinting

In this study, 14 population samples were examined for their RAPD-PCR patterns. RAPD-PCR was performed using three primer series, namely OPA (OPA1-OPA10), SK (SK1-SK10) and A (A01-A10), each comprising 10 primers. Out of 30 primers screened, 24 were selected based on their reproducibility, robustness of amplification and scorability of banding patterns. These 24 primers were employed for the diversity analysis of 14 populations. There were numerous DNA amplification products from each polymeric primer. To increase the number of characters that may be scored and reduce statistical error, both intense and faint bands were scored when determining the similarity index values. The 24 chosen decamer oligonucleotide primers produced banding profiles that yielded 266 amplification products, of which 189 (70.6%) were polymorphic bands. The average was 11.1 bands per primer, with individual band counts ranging from 5 (OPA10) to 19 (SK8). The average number of polymeric bands per primer was 7.9, with a range of 3 (A03) to 16 (OPA7). Among the 24 polymorphic primers, the highest polymorphic information content (PIC of (0.50)) was observed for primer OPA7 and the lowest value (0.35) for primer A03, which indicated that the primers could detect-RAPD polymorphism among the 14 populations (Table 2). Figure 1 shows a representative RAPD gel pattern generated using the OPA7 primer.

Genetic similarity, cluster analysis and principal coordinate analysis

Based on RAPD data, the genetic similarity matrix of the pairwise Jaccard's coefficients was generated. All 14 population samples had genetic similarity coefficients that ranged

Table 3. Similarity matrix for Jaccard's coefficients of the 14 *R. leschenaulti* populations

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	1.00													
2	0.50	1.00												
3	0.16	0.35	1.00											
4	0.40	0.38	0.42	1.00										
5	0.18	0.31	0.33	0.36	1.00									
6	0.31	0.47	0.42	0.60	0.50	1.00								
7	0.29	0.44	0.29	0.42	0.60	0.70	1.00							
8	0.30	0.35	0.29	0.24	0.33	0.31	0.38	1.00						
9	0.27	0.25	0.50	0.40	0.30	0.56	0.36	0.12	1.00					
10	0.29	0.35	0.38	0.42	0.33	0.55	0.38	0.22	0.50	1.00				
11	0.24	0.16	0.21	0.07	0.15	0.14	0.31	0.31	0.17	0.06	1.00			
12	0.16	0.10	0.38	0.13	0.23	0.13	0.20	0.38	0.25	0.29	0.55	1.00		
13	0.30	0.35	0.29	0.40	0.18	0.31	0.22	0.37	0.19	0.38	0.05	0.22	1.00	
14	0.21	0.20	0.12	0.20	0.13	0.29	0.19	0.15	0.23	0.19	0.13	0.12	0.35	1.00

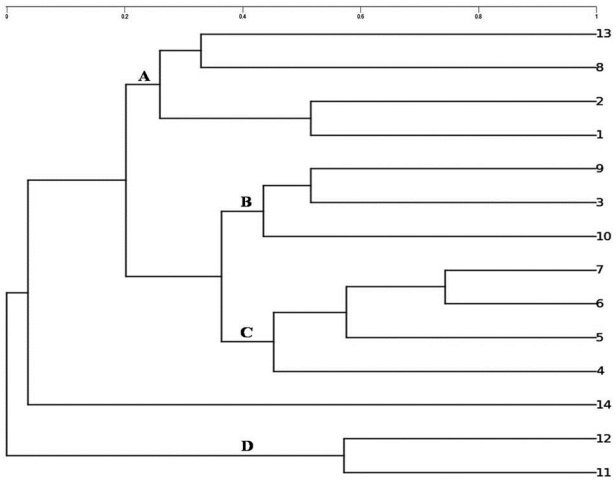

Figure 2. Dendrogram showing diversity of geographical populations of *R. leschenaulti* obtained from RAPD analysis using UPGMA. Bar on the top represents similarity index based on Jaccard's coefficients. Numerals indicate population numbers given in Table 1.

from 0.05 (between population nos 11 and 13) to 0.70 (between population nos 6 and 7; Table 3). RAPD data were subjected to cluster analysis using UPGMA, revealing the general genetic links between the *R. leschenaulti* populations (Figure 2). The genetic relationship among the populations was determined using principal coordinate analysis (PCoA). The populations were plotted on primary coordinates 1 and 2, which jointly explained 50.3% of the variation and accounted for 32.4% and 17.9% of the variation respectively (Figure 3). Four distinct groups of the 14 *R. leschenaulti* populations were found using PCoA and UPGMA clustering with RAPD data. Four primary clusters, viz. A, B, C and D of the 14 populations were identified through dendrogram analysis. The two populations (11 and 13 and 6 and 7) did not show considerable similarity, as indicated by the similarity coefficients, which ranged from 0.05 to 0.70.

Cluster A comprised populations from four districts, i.e. Madurai, Theni, Tirunelveli and Kanyakumari (populations nos 1, 2, 8 and 13). This cluster represents populations nos 1 and 2 from Tirupparankundram (Madurai) and Vengadachalapuram (Theni) respectively, and population nos 8 and 13 from Palayamkottai (Tirunelveli) and Aralvaimozhi (Kanyakumari) respectively. It is interesting that population nos 8 and 13 are included in this cluster since they are geographically different from population nos 1 and 2, but similar at the genetic level (Figure 2). Greater similarities were observed between population nos 1 and 2 with a value of 0.50, while population nos 1 and 8, 1 and 13 showed the lowest similarity value of 0.30 (Table 3). The representation of cluster A is mostly from the central (Madurai) rain-shadow region of the Western Ghats (Theni), southern (Tirunelveli) and the coastal belt (Kanyakumari) of Tamil Nadu. Cluster B consisted of populations from two districts in the southern (Tirunelveli) and coastal belt (Tuticorin) of Tamil Nadu (population nos 3, 9 and 10). In this cluster, population no. 3 belonged to Tiruppudaimaruthur (Tirunelveli) and population nos 9 and 10 were from Srivaikundam and Seydunganallur (Tuticorin) respectively (Figure 2). Population nos 3 and 10 showed the least similarity, with a value of 0.38, whereas population nos 3 and 9, 9 and 10 showed maximum similarity with a value of 0.50 (Table 3). Unlike cluster A, cluster B did not show much geographical distance. Unsurprisingly, these populations are genetically close as they belong to two different districts that are closely located. Cluster C comprised populations from only one district, i.e. Tirunelveli, with population nos 4, 5, 6 and 7 from Kallidaikurichi, Arikesavanallur, Cheranmahadevi and Nanguneri respectively (Figure 2). Maximum similarity was observed between population nos 6 and 7 with a value of 0.70, while population nos 4 and 5 showed minimum similarity (0.36; Table 3). Since these populations belong to the same region, they are both geographically and genetically close.

Cluster D consisted of populations from two districts, i.e. Tuticorin and Kanyakumari. This cluster represented population no. 11 from Manapadu (Tuticorin), and population nos 12 and 14 from Poothapandi and Agastheeswaram

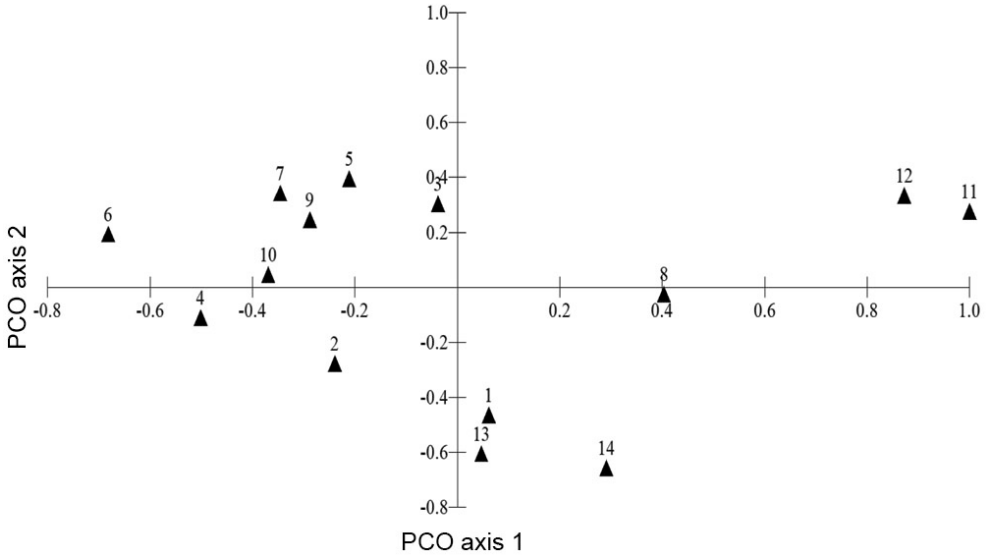

Figure 3. Principal coordinate analysis of 14 *R. leschenaulti* populations based on Jaccard's similarity matrix. Numerals represent population numbers listed in Table 1.

(Kanyakumari) respectively (Figure 2). Maximum similarity was observed between population nos 11 and 12 with a value of 0.55, while population nos 12 and 14 showed minimum similarity (0.12; Table 3). Since these populations belong to two different districts that are closely located, they are geographically and genetically close and this cluster is similar to cluster B. Considerable level of genetic similarity is expected among *R. leschenaulti* populations in the central and southern part of Tamil Nadu due to the same geographical area. Also, there are populations as close as 0.70 in terms of similarity index, even though they belong to geographically distinct locations. Populations nos 1 (Tirupparankundram) and 13 (Palayamkottai) appeared closely related at the genetic level, although geographically they belonged to highly distinct locations in Tamil Nadu (Madurai and Tirunelveli). Cluster analysis and PCoA support the above results (Figures 2 and 3). This indicates that RAPD markers are well suited to determine the genetic diversity and differentiation in *R. leschenaulti* populations.

Discussion

Genetic variation is among the most fundamental information for the conservation of bats. In this study, we found considerable levels of genetic diversity for all 14 populations of *R. leschenaulti* using RAPD-PCR analysis. A wide and diverse genetic base from different populations could be inferred due to the moderate level of polymorphism identified in the study. The percentage of polymorphic bands in RAPD (70.6) was similarly greater in the species. The degree of dispersal (when resulting in gene flow) that occurs among them as well as the formation of new social groups significantly impact the genetic make-up of discrete populations[24]. Majority of the mammalian species display a social structure that is defined by polygynous mating and female philopatry[25].

The high level of genetic diversity observed in *R. leschenaulti* can be described using four factors: (i) extra-colony copulations without permanent dispersal from the parent colony, (ii) absence of substructure, (iii) greater vagility of the species (an adaptation associated with cave roosting) and (iv) seasonal migration. These factors are the consequences of higher levels of gene flow among colonies[26]. In spite of coming from geographically different locations in Tamil Nadu, populations from Tirupparankundram and Vengadachalapuram as well as Palayamkottai and Aralvaimozhi, appeared genetically closely connected to each other. Previous studies suggest that *R. leschenaulti* migrates seasonally, with maternity colonies reaching populations of up to 20,000 individuals in summer and declining during winter[27]. Therefore, depending on whether mating occurs prior to or during migration, it may offer additional chances to encourage gene flow among colonies. Additionally, one of the adaptations displayed by this species is greater vagility which is associated with cave roosting[26].

From RAPD analysis, the genetic differentiation coefficient of *R. leschenaulti* was found to be 70.6%, which suggests a considerable level of genetic diversity among populations. For example, even when separated by large geographical distances (up to 4000 km), a high level of gene flow was observed in the migratory bats[28]. Similar results have been reported in *Myotis daubentonii*[29], *Myotis brandtii*, *Myotis mystacinus*[30], *Myotis myotis*[31], *Hipposideros speoris*[32] and *Megaderma lyra*[33]. Although gene flow between populations generally appears to be more constrained than in migratory species, the pattern of population structure and gene flow in the species that do not experience

seasonal migration is not well understood. Without permanent dispersal from the natal colony, the gene flow primarily occurs by extra copulation between the colonies[33]. However, gene flow may be constrained by the recent population fragmentation, distance or availability of mating sites. It is interesting to note that neither sex experienced natal dispersal despite extra-colony copulation having been documented in other animal species[34]. Among migratory species, a wider range of genetic differentiation was found. Also, in numerous species, a significant correlation between geographic and genetic distances has been reported. For instance, a high degree of structure was discovered in the Australian ghost bat *Macroderma gigas*, with a substantial correlation between geographic and genetic distances[35]. Similar results have also been reported in *Plecotus auritus*[36], *Myotis bechsteinii*[37], *Rhinolophus affinis*[38] and the non-migratory island population of *Eidolon helvum*[39]. Population nos 6 and 7, 4 and 6, 6 and 9 and 11 and 12 had the highest level of similarity in this study, with values of 0.70, 0.60, 0.56 and 0.55 respectively. These populations were also the closest to one another. Therefore, it is expected that gene flow will be larger when populations remain close together. With an increase in distance between two or more populations, there will be less gene flow[18]. As a result, at the neutral loci, the closest population is more comparable[40]. This relationship refers to geographical isolation and assumes a stepping-stone model of gene flow, which will provide the population enough time to achieve equilibrium conditions[41]. However, levels of gene flow are influenced by the environment of the surrounding landscape between populations, in addition to the distance between populations[18,42].

Seasonal movement is expected to have the biggest impact on the populations of migratory species since the genetic structure often seems to be low. The level of genetic structure in migratory species can only be low when the individuals mate during their migration. In migratory species, if mating and conception take place before migration, patterns of genetic structure for both migratory and non-migratory species may be similar[28]. If the population distribution is continuous, gene flow may also be larger than the ability of an individual species to disperse. These results suggest that *R. leschenaulti* mating before or during migration may offer additional possibilities to encourage gene flow[26]. Due to the adaptability of *R. leschenaulti* to cave roosting and extra-colony copulations without permanent dispersal from the natal colony, the majority of genetic variation distributed within populations is not surprising and may be due to significant levels of gene flow. Our results are consistent with those of other studies linking social structure to genetic differentiation and with predictions that colonial cave-roosting bats exhibit greater gene flow and dispersal than tree-roosting species[43]. Our understanding of genetic diversity within and among *R. leschenaulti* populations can be improved by future research using advanced molecular markers (microsatellites and mitochondrial DNA) in larger samples and distant populations. This will also help determine the conservation status of this species in the Indian subcontinent.

Conclusion

In this study, we followed a molecular approach to describe the genetic diversity of *R. leschenaulti.* Despite the high level of fragmentation and anthropogenic disturbances, the *R. leschenaulti* population sustains high levels of genetic diversity. The results suggest that this species has greater vagility and can migrate between caves more easily. The current bat populations in many caves and historical monuments may continue to decline. Our findings on day-roost features and roost diversity also indicate a steady fall in the natural population size of *R. leschenaulti*. The genetic baseline information provided by this study will be useful for future research. According to our results, high levels of genetic variation were found in *R. leschenaulti* in southern India. These results will help develop a method to effectively and meaningfully conserve this species. Moreover, our findings suggest that *R. leschenaulti* conservation plans may be based on the regional pattern of landscape structure, as well as safeguarding natural caves and historical sites to boost population genetic diversity and connectivity.

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