

RESEARCH ARTICLES

Genetic diversity within and among populations of the Indian short-nosed fruit bat, *Cynopterus sphinx* assessed through RAPD analysis

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Genetic diversity was studied among 40 different zone samples in the Indian short-nosed fruit bat, *Cynopterus sphinx*. Samples were collected from 127 locations in different districts of southern Tamil Nadu, India. Polymorphism at molecular level was studied by random amplified polymorphic DNA marker technique. Polymerase chain reaction (PCR) with 30 arbitrary decamer oligonucleotide primers was applied to the 40 zone samples. Out of 30 random primers screened, 22 gave reproducible polymorphic bands. PCR product of bat genome revealed a total of 234 bands, out of which 171 were found to be polymorphic. The dendrogram displaying the relative genetic similarities between the zones ranged from 0.42 to 0.92. Principal coordinates analysis revealed that the first two components accounted for 51.4% of the total variation. The results show that considerable genetic diversity was observed in this species from different zonal populations, possibly due to complete dispersal of juveniles of both sexes from their natal groups and gene flow between the zones. The results obtained from this study suggest not only a predictive framework for future studies, but also the use of genetic data in the management and meaningful conservation of this species.

Keywords: *Cynopterus sphinx*, genetic diversity, Jaccard's coefficients, principal coordinates analysis, RAPD markers.

BATS are quintessential refuging animals, belonging to the order Chiroptera, with approximately 1100 species divided into two suborders – the Megachiroptera (often known as Old World fruit bats) with 188 species and the Microchiroptera with 917 species¹. They often form a significant proportion of the native mammalian fauna,

and their role as pollinators and seed dispersers in some ecosystems has led to their designation as 'keystone' species². The Indian short-nosed fruit bat, *Cynopterus sphinx* is a common plant-visiting bat that occurs throughout the Indo-Malayan region³, and roosts solitarily or in small groups in the foliage. It weighs about 40–70 g and lives in small clusters of about 3–30 individuals^{4,5}. These bats are known to alter different types of foliage to construct tents and attract females⁶. The behaviours of tent construction⁷, reproduction⁶ and foraging⁸ are widely studied aspects. Till date, genetic variations within and among the populations of *C. sphinx* are not well defined. Lack of genetic information is undoubtedly due, in part, to the difficulties associated with studying them in the wild. Application of molecular genetic techniques extracts valuable biological and behavioural information to document population structure of the species. In this study we used the Random Amplified Polymorphic DNA (RAPD) method of DNA fingerprinting, which is widely used in conservation biology because of the quick results, cost-effectiveness and reproducibility^{9,10}. Use of this technique in population and evolutionary biology is now almost commonplace; it is being used across an extensive range of animal and plant taxa, including bats¹⁰. PCR-based RAPD approach using single 10-mer arbitrary primers requires only nanogram quantities of template DNA, no radioactive probes, and is relatively simple compared to other techniques^{11,12}. The genetic discrimination of an individual is an important step in investigating the population biology of any species and a major contribution that conservation geneticists can make for evaluating population viability. Our understanding on genetic diversity of the *C. sphinx* population can contribute valuable guidelines for conservation strategies and should therefore be an essential part of proper conservation management. The objective of the present study was to investigate the genetic diversity within and among the populations of the short-nosed fruit bat, *C. sphinx*.

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Materials and methods

Sample collection

Extensive field trips were carried out to collect *C. sphinx* from different geographical locations in southern Tamil Nadu, India, during September–November 2003, March–June 2004 and October 2005–January 2006. The sampling strategy involved tracing several sites in different parts of the study area in order to cover as much territory as possible. Each site was sampled only once in order to avoid repeated capture of bats. Bats were captured in 127 localities grouped into 40 zones (Table 1). The term ‘zone’ was used to delimit each general location containing one or more collection sites. The criteria used to include

Table 1. Natural populations of *Cynopterus sphinx* collected across 40 zones of southern Tamil Nadu, India

Zone no.	Location	District	Sample size
1	MKU Botanical Garden	Madurai (Centre)	15
2	MKU Energy Garden	Madurai (Centre)	10
3	MKU Biodiversity Garden	Madurai (Centre)	8
4	Keelakuilkudi	Madurai (East)	12
5	Karadipatti	Madurai (East)	10
6	Puliamkulam	Madurai (West)	8
7	Chekkanoorani	Madurai (West)	12
8	Chettikulam	Madurai (South)	11
9	Sorikkampatti	Madurai (South)	7
10	Periyasorikkampatti	Madurai (South)	6
11	Mellakkal	Madurai (North)	12
12	Vikkiramangalam	Madurai (North)	10
13	Solavanthan	Madurai (North)	12
14	Thiruvudagam	Madurai (North)	14
15	Kurivithurai	Madurai (North)	15
16	Thirumanampatti	Dindigul	6
17	Alagupatti	Dindigul	8
18	Theppakulathupatti	Dindigul	10
19	Raddiarchatram	Dindigul	14
20	Kodairoad	Dindigul	12
21	Sirumalai	Dindigul	17
22	Kodangipatti	Teni	10
23	Vengadachalapuram	Teni	14
24	Boothipuram	Teni	7
25	Andipatti	Teni	8
26	Srivilliputhur	Virudhunagar	6
27	Palayamkottai	Tirunelveli	14
28	St John's College	Tirunelveli	17
29	Bishop Stowe	Tirunelveli	16
30	Veeravanallur	Tirunelveli	12
31	Karukurichi	Tirunelveli	14
32	Cheranmahadevi	Tirunelveli	15
33	Coutrallam	Tirunelveli	16
34	Srivaikundam	Tuticorin	20
35	Alwarthurinagari	Tuticorin	14
36	Kanyakumari	Nagercoil	12
37	Vivekananda Kendra	Nagercoil	10
38	Collector's Office	Nagercoil	12
39	Jeyasekaran Hospital	Nagercoil	14
40	Subramaniapuram	Nagercoil	12

different collection sites within a zone were as follows: (1) the collection sites within a given zone were closer to each other than to those from other zones, and (2) habitat continuity existed between all collection sites within a given zone. Bats were captured at the time of emergence from the foliage tents of *Polyalthia longifolia* and *Borassus flabellifer* using a hoop net with an extensible aluminium pole. In addition, the entire tree was enveloped with a 6 m × 9 m nylon mist-net (Avinet-Dryden, USA) to prevent bats from escaping. Also, bats were captured using mist-nets from various habitats such as forests, plantations, botanical gardens, orchards and villages. A small piece of wing membrane (3 sq. mm) from each bat was collected using a sterile biopsy-punch (wing membranes healed within 3–4 weeks). Tissue samples were obtained from a total of 472 bats from 40 zones, with an average sample size of 11.8 bats per zone (Table 1). After each sampling, the punched hole and the punch were disinfected with 70% ethanol. Tissue samples were stored in 70% ethanol at –20°C until DNA extraction¹³.

DNA isolation and primer screening

Genomic DNA was isolated from wing-membrane biopsy samples using standard proteinase K digestion and phenol:chloroform extraction method¹⁴. The extracted DNA quality and quantity were checked using 0.7% agarose gel electrophoresis and spectrophotometric measurement at A260 and A280 nm (Hitachi U-2000, Tokyo, Japan). In the present study RAPD–PCR was performed using a set of three series of primers, namely A (A01–A10), SK (SK1–SK10) and OPA (OPA1–OPA10), each comprising ten primers (Microsynth, Switzerland). PCR conditions were optimized by varying concentrations of template DNA, primer, MgCl₂ and *Taq* DNA polymerase. Initial screening was done with all 30 primers using DNA from five zones. PCR–RAPD analysis was repeated at least thrice and only primers producing strong and reproducible bands were used in the analysis of all the 40 zones.

Polymerase chain reaction

PCR was carried out in 20 µl reaction containing 10X PCR buffer (with 1.5 mM MgCl₂), 2 µl; 2 mM dNTP mixture, 2 µl; 2 µM primer, 5 µl; *Taq* DNA polymerase, 1U (Fermentas GmbH, Lithuania), H₂O, 10 µl, and 100 ng template DNA. All DNA amplifications were performed using an Applied Biosystems GeneAmp 2700 PCR system, with the following cycling conditions, including initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 40 s, annealing at 36°C for 2 min, extension at 72°C for 3 min and final extension at 72°C for 10 min. A negative control without the bat genomic DNA template was used for amplification along with the 40 zone samples with each primer. This was to con-

firm the quality of the primer and to avoid the scoring of bands which may arise due to primer dimers or possible contaminants. The PCR products were electrophoresed on 2% agarose gel in 1× TAE buffer, stained with ethidium bromide (0.5 µg/ml) and observed under ultraviolet light for amplification, and photographed and analysed using Gel documentation system (Biorad, USA, model 2000, Quantity One Software).

Data analysis

The RAPD data were analysed using NTSYS-pc version 2.0 computer package¹⁵. A genetic similarity (GS) was computed based on Jaccard's coefficient of similarity $GS(ij) = a/(a + b + c)$, where $GS(ij)$ is the measure of genetic similarity between individuals i and j , a is the number of polymorphic bands that are shared by i and j , b is the number of bands present in i and absent in j , and c is the number of bands present in j and absent in i . Each RAPD fragment was treated as a unit character and was scored as 1 (present) or 0 (absent). The 1/0 matrix was prepared for all fragments scored and the data were used to generate Jaccard's similarity coefficients for RAPD bands¹⁶. Jaccard's coefficients were used to construct a dendrogram using UPGMA. The Jaccard's similarity matrix was then used as the basis for ordination by principal coordinates analysis (PCoA), which was performed to show the distribution of the genotypes in a scatter plot using the software MVSP version 3.13n (Multivariate Statistical Package; <http://www.kovcomp.com/mvsp>).

Results

DNA fingerprinting

A total of 40 zone samples were examined for their RAPD-PCR patterns. Out of 30 primers screened, 22 were selected on the basis of robustness of amplification, reproducibility, scorability of banding patterns and were employed for diversity analysis. All polymeric primers yielded multiple DNA amplification products. Both intense as well as faint bands were scored for calculating similarity index values, so as to maximize the number of scorable characters and minimize statistical errors. The banding profiles yielded by the 22 selected decamer oligonucleotide primers generated 234 amplification products, out of which 171 bands (73.1%) were polymorphic. The number of bands per primer ranged from 4 (OPA5) to 21 (SK3), with an average of 10.6 bands per primer. The range of polymorphic bands per primer was 2 (OPA4) to 14 (A02), with a mean of 7.8 polymeric bands per primer (Table 2). Representative RAPD patterns generated by primers A10, SK9 and OPA7 are shown in Figure 1.

Genetic similarity, cluster analysis and PCoA

The pairwise Jaccard's coefficients genetic similarity matrix was prepared on the basis of RAPD data. The genetic similarity coefficients among all 40 zones varied from 0.25 (between zones 15 and 36) to 0.92 (between zones 3 and 9; Table 3). Cluster analysis was performed on RAPD data using UPGMA, which showed overall genetic relationships among the populations of *C. sphinx* (Figure 2). PCoA was performed in order to determine the genetic relationships among the zones. The zones were plotted on principal coordinates 1 and 2, accounting for 45.3% and 6.1% of the variation respectively and together explaining 51.4% of the total variation (Figure 3). UPGMA clustering and PCoA of RAPD data indicated that the 40 zones of the *C. sphinx* population comprise two different clusters. A dendrogram analysis indicated that the 40 zones formed two major clusters, A and B. The similarity coefficients ranged from 0.42 to 0.92, indicating that the two zones did not show 100% similarity. Cluster A comprised populations from five districts, i.e. Teni, Virudhunagar, Tirunelveli, Tuticorin and Nagercoil (zones 21–40). This cluster represents all the zones from the southern and western part of Tamil Nadu, except one (zone 21), which is a collection from Dindigul. Inclusion of zone 21

Table 2. Primers with their sequences used for RAPD analysis of *C. sphinx*, and the total number of bands, polymorphic amplification products and percentage of polymorphism yielded by each primer

Primer code	Primer sequence 5'–3'	Total no. of bands	No. of polymorphic bands	Percentage polymorphism
A01	CAGGCCCTTC	12	10	83.3
A02	TGCCGAGCTG	18	14	77.8
A04	AATCGGGCTG	11	9	81.8
A05	AGGGGTCTTG	6	4	66.7
A06	GGTCCCTGAC	8	7	87.5
A07	GAAACGGGTG	10	8	80.0
A09	GGGTAACGCC	6	5	83.3
A10	GTGATCGCAG	9	9	100
SK2	GTGGGCTGAC	8	7	87.5
SK3	GTCCATGCCA	21	6	28.6
SK4	ACATCGCCCA	13	11	84.6
SK5	GTGGTCCGCA	11	10	90.9
SK7	AACGCGTCGG	10	8	80.0
SK8	AAGGGCGAGT	13	10	76.9
SK9	GGAAGCCAAC	18	7	38.9
SK10	GGCTTGGCCT	10	9	90.0
OPA1	GTTTCGCTCC	7	7	100
OPA3	CATCCCCCTG	11	9	81.8
OPA4	AATCGGGCTG	5	2	40.0
OPA5	TGCGCCCTTC	4	3	75.0
OPA7	GAAACGGGTG	15	9	60.0
OPA9	GGTGACGCAG	8	7	87.5
Total		234	171	73.1
Mean per primer		10.6	7.8	

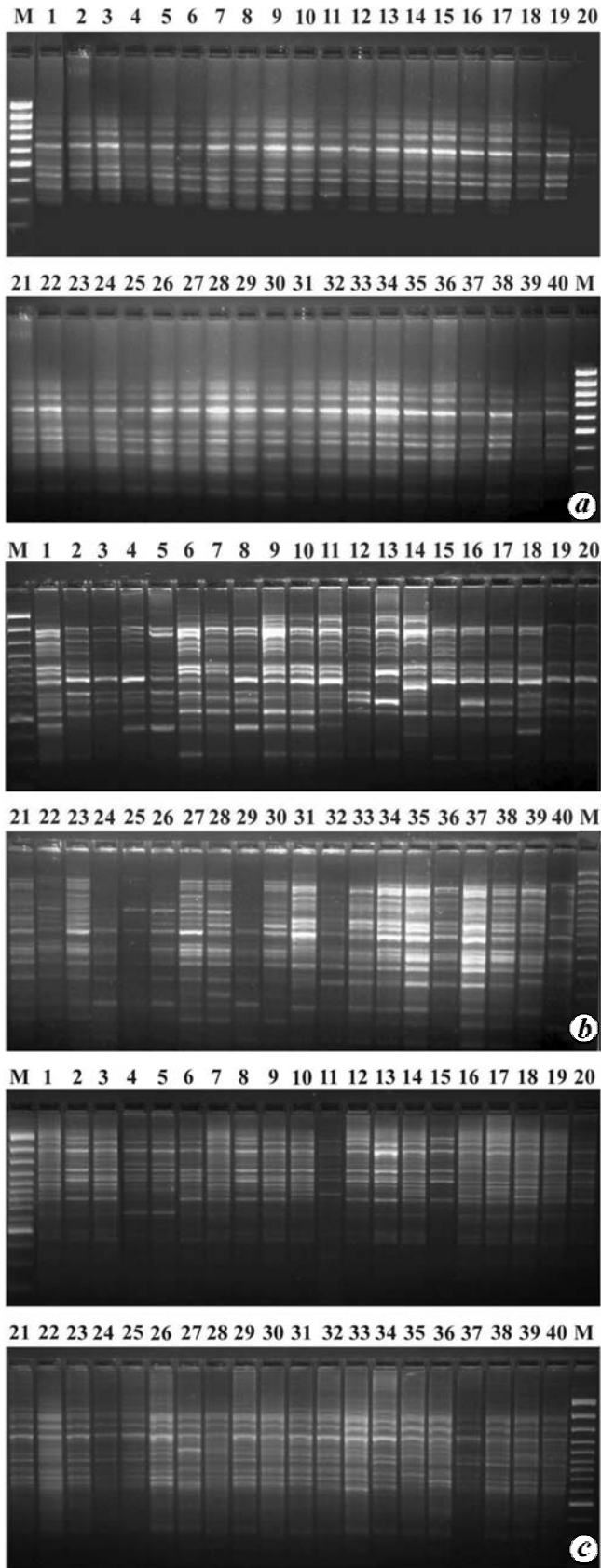


Figure 1. RAPD profile of 40 zone samples of *Cynopterus sphinx* produced using the random decamer primers (a) A10, (b) SK9 and (c) OPA7. M, 100 bp DNA ladder. Lane number corresponds to zone code given in Table 1.

(Sirumalai–Dindigul) in this cluster is interesting, since it is geographically distinct from the rest. It showed close to zones between 23 and 32 from Vengadachalapuram (Teni) and Cheranmahadevi (Tirunelveli) respectively. Zone 21 was geographically distinct, but closely related at the genetic level to zones 23 and 32. Maximum similarities were observed between zones 23 and 32 with value of 0.87, and zones 24 and 28 were found to be clustering separately and with minimum similarity value of 0.49. The cluster A representation is mostly from the rain-shadow region of the Western Ghats and the coastal belt. Cluster B is formed by the populations from two districts in the central (Madurai) and northern (Dindigul) part of Tamil Nadu. Zone 11 was distinct from the others in its cluster, with only 0.49 similarity. Zones 3 and 9 were grouped together with maximum similarity of 0.92, and zones 17 and 20 showed a minimum similarity value of 0.69 (Figure 2). High genetic similarity is expected among *C. sphinx* zones in the central and northern part of Tamil Nadu due to the same geographical location. Also, there are zones as close as 0.92 in terms of similarity index, even though they belong to geographically distinct locations. Zones 21 (Sirumalai) and 32 (Cheranmahadevi) appeared closely related at the genetic level, although geographically they are from zones of highly distinct locations in Tamil Nadu (Dindigul and Tirunelveli). Cluster analysis and PCoA of the similarity indices (Figures 2 and 3) support the above results. This indicates that RAPD markers were well suited for determining the genetic diversity and differentiation present in *C. sphinx* populations.

Discussion

Genetic variation is of vital importance in governing the potential of a species to evolve and adapt. Genetic analysis of 30 RAPD markers in the present study showed reasonably high level of diversity. The polymorphism revealed by RAPD has been problematical due to their dominance. As heterozygotes are not normally detectable, the results are not readily usable for computing Hardy–Weinberg equilibrium or Nei's standard genetic distance¹⁷. Data analysed using the Nei index need strict dominant and recessive allelic frequency. Data from RAPDs do not depend on these criteria. Therefore, in the present study, RAPD polymorphisms were analysed with a phenetic distance measure (Jaccard's coefficient) from which a dendrogram was constructed, providing an indication of the diversity present within and among the populations of *C. sphinx*. The level of polymorphism observed in the present study was relatively high, indicating a wide and diverse genetic base from different zone populations; the percentage of polymorphic bands (73.1%) of RAPD in the species was also higher. The genetic structure of discrete populations is strongly affected by the amount of

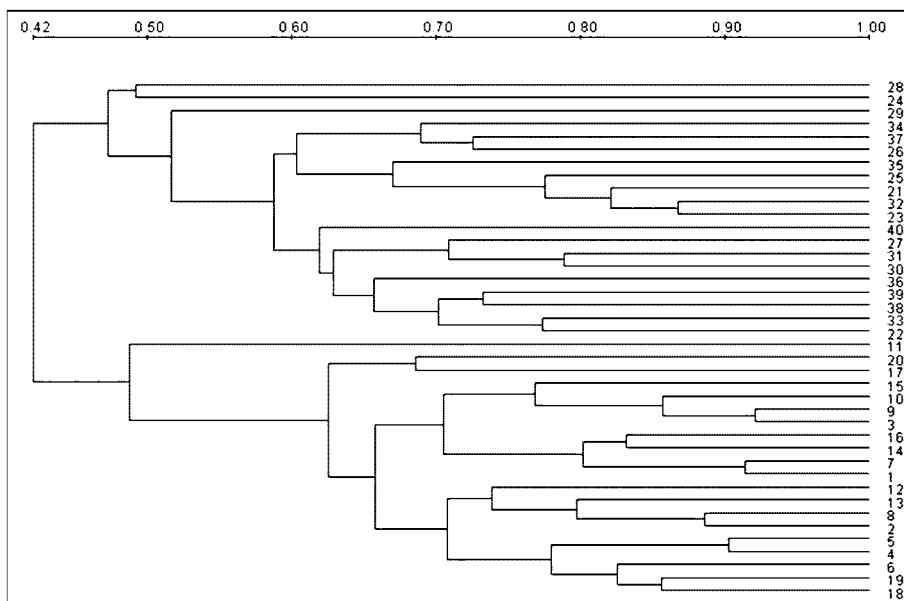


Figure 2. Dendrogram showing diversity of geographical populations of *C. sphinx* obtained from RAPD analysis using UPGMA. Bar on the top represents similarity index based on Jaccard's coefficients. Numbers indicate zone code given in Table 1.

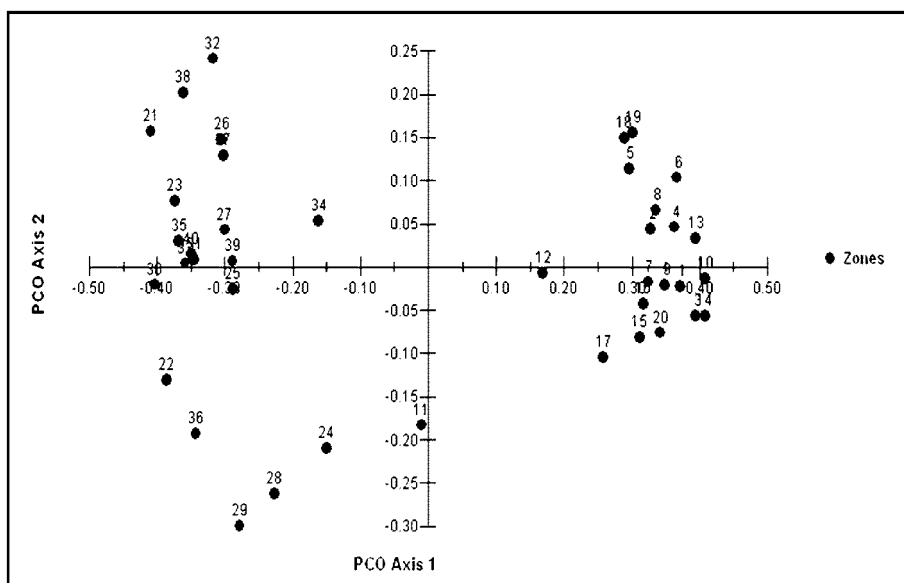


Figure 3. Principal coordinates analysis of 40 zone populations of *C. sphinx* based on Jaccard's similarity matrix. Numbers in the plot represent zones listed in Table 1.

dispersal (when resulting in gene flow) that occurs among them and formation of new social groups¹⁸. Majority of the mammalian species exhibit a social system characterized by polygynous mating and female philopatry¹⁹. *C. sphinx* is a polygynous-mating bat and both sexes were found to disperse completely from their natal harems²⁰. Moreover, young females become members of a harem much earlier than their male counterparts and they either joined established harems or formed a new harem of subadult females with an adult male. As a result, colonies

are mainly composed of unrelated or distantly related females of mixed ages²⁰. This mode of group formation enhances genetic variation. The high level of genetic diversity presently observed could be explained by three factors: (i) natal dispersal, (ii) formation of new groups, and (iii) gene flow between the zones. This is probably the reason that zones 21 (Sirumalai) and 32 (Cheranmahadevi) appeared closely related at the genetic level, although geographically they are from zones of highly distinct locations in Tamil Nadu. In addition, recent habi-

Table 3. (Contd...)

	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
1.00																				
0.59	1.00																			
0.80	0.52	1.00																		
0.54	0.39	0.62	1.00																	
0.72	0.46	0.84	0.70	1.00																
0.68	0.61	0.62	0.38	0.55	1.00															
0.76	0.50	0.67	0.55	0.57	0.55	1.00														
0.57	0.47	0.51	0.49	0.46	0.50	0.65	1.00													
0.56	0.44	0.64	0.38	0.54	0.63	0.59	0.37	1.00												
0.71	0.71	0.62	0.42	0.53	0.65	0.72	0.52	0.54	1.00											
0.65	0.70	0.61	0.47	0.53	0.57	0.70	0.62	0.42	0.79	1.00										
0.84	0.62	0.87	0.58	0.77	0.67	0.67	0.50	0.58	0.67	0.62	1.00									
0.62	0.77	0.53	0.41	0.51	0.70	0.52	0.49	0.50	0.61	0.57	0.62	1.00								
0.59	0.50	0.51	0.36	0.46	0.66	0.42	0.42	0.43	0.47	0.47	0.57	0.63	1.00							
0.71	0.63	0.65	0.48	0.58	0.63	0.57	0.49	0.50	0.66	0.59	0.74	0.59	0.59	1.00						
0.55	0.70	0.46	0.30	0.39	0.64	0.64	0.51	0.37	0.72	0.63	0.53	0.61	0.51	0.62	1.00					
0.71	0.56	0.67	0.37	0.54	0.73	0.55	0.37	0.54	0.56	0.57	0.70	0.60	0.72	0.57	0.54	1.00				
0.76	0.70	0.73	0.53	0.64	0.68	0.60	0.60	0.48	0.66	0.69	0.77	0.71	0.60	0.71	0.60	0.71	1.00			
0.58	0.72	0.50	0.35	0.46	0.60	0.60	0.47	0.39	0.72	0.74	0.58	0.67	0.51	0.57	0.72	0.53	0.73	1.00		
0.67	0.62	0.59	0.40	0.50	0.60	0.60	0.61	0.51	0.46	0.64	0.60	0.60	0.60	0.53	0.51	0.54	0.66	0.71	0.62	1.00

tat loss and degradation, which may have led to the concentration of the surviving individuals in the remaining areas and the long generation time and lifespan of the species allowed populations to retain diversity for long periods after habitat loss^{18,19}.

The 73.1% genetic differentiation coefficient of *C. sphinx* from RAPD analysis suggests that the species is of a higher genetic diversity among populations than other bat species. For example, the Brazilian free-tailed bat (*Tadarida brasiliensis*), the southwestern populations, including those occupying distinct migrational groups show low level of genetic differentiation among populations, even though banding and recapture data suggest low exchange among migratory groups²¹. Similarly, Sinclair *et al.*¹⁰ observed that the little red flying-fox, *Pteropus scapulatus* has a range exceeding 3.5 million sq. km during its seasonal migrations. The allozyme and RAPD data suggest at low degree of differentiation among populations and large amount of gene flow between all subpopulations. Similar results have been reported in *P. poliocephalus*, *P. alecto*²², *Plecotus auritus*²³, *Hipposideros speoris*²⁴ and *Megaderma lyra*²⁵. The pattern of population structure and gene flow in species that do not undergo seasonal migration is less clearly known although, in general, gene flow among populations appears more restricted than in migratory species²³. Among populations of migratory species, genetic structure appears universally low, and hence seasonal migration is likely to be the prevailing influence. However, for sedentary species an array of factors, including dispersal ability, extrinsic barriers to gene flow and historical events may determine the extent of genetic partitioning among populations²⁶. In this study maximum similarity was observed between zones 3 and 9, 1 and 7, 4 and 5, and 30 and 31 with values of 0.92, 0.91, 0.90 and 0.79 respectively, and these zones were closer to each other. Therefore, gene flow is expected to be greater when populations are closer, and, as a consequence, nearby populations should be more similar at neutral loci. This relationship is referred to as isolation by distance, and it assumes a stepping-stone model of gene flow, providing sufficient time for populations to reach equilibrium conditions²⁷. However, levels of gene flow are not only dependent on the distance between populations, but also on the nature of the surrounding landscape between populations²⁸. These findings support that *C. sphinx* is not known to undergo seasonal migrations. Moreover, it is a common plant-visiting bat that occurs throughout India and much of mainland Southeast Asia³. Therefore, most genetic variation apportioned within populations is not surprising and is possibly due to the high level of gene flow because the *C. sphinx* population distribution is continuous. Similarly, a latitudinal study of *C. sphinx* in the Indian subcontinent conducted at a geographic scale similar to that of the present study, found evidence of high gene flow and equilibrium population dynamics²⁹. Thus, maintaining gene flow may be impor-

tant for the long-term persistence of *C. sphinx* populations.

Conclusion

Using a RAPD marker, we have studied genetic diversity at the molecular level in natural populations of *C. sphinx*. We have shown that *C. sphinx* maintains relatively high levels of genetic variability, despite the increasing fragmentation of its habitat. While this may be beneficial for the conservation of bats, additional studies are necessary. This study provides baseline genetic information for future studies. In the future, microsatellite and mitochondrial DNA variation should be reassessed in these populations to investigate the long-term effects of human-induced habitat fragmentation and habitat degradation on genetic diversity and genetic structure in this species. The high levels of genetic polymorphism and genetic differentiation revealed by RAPD analysis might play a role in the dynamic evolution of *C. sphinx* in southern India. These results may help in developing an effective and meaningful conservation programme for this species. Future studies of Old World fruit bats from these areas will be of biogeographic and evolutionary interest.

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