Molecular phylogeny of rediscovered Travancore flying squirrel (*Petinomys fuscocapillus*) and its conservation implications

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***Petinomys fuscocapillus* (Travancore flying squirrel), Jerdon 1847, is a near threatened species, native to India and Sri Lanka. Deforestation, wood plantation, infrastructure development, poaching and natural predators are major threats to the species. This study reports for the first time the molecular phylogenetic position and level of genetic divergence of *P. fuscocapillus* among the flying squirrel species of South and Southeast Asia, based on two mitochondrial genes. The phylogenetic analysis confirms that the *P. fuscocapillus* and *Petinomys setosus* (Temminck's flying squirrel) are sister taxa and share most recent common ancestry. Phylogenetic position of other flying squirrels obtained in the present study was also supported by the previous studies. We also emphasize on the extensive survey for population sampling, need for plantations to maintain a continuous canopy and enforcement of strict laws at the potential geographical distribution of the species in two countries.**

Keywords: Conservation, molecular phylogeny, *Petinomys fuscocapillus*.

THERE is global interest in the rediscovery of extinct species[1,2]. An estimated 351 species have been rediscovered, mostly from the tropics over the last 122 years[3]. Rediscovery of previously thought extinct species requires new conservation efforts to preserve such species and helps to understand the reason for their population decline as a consequence of human disturbance[4,5].

Genus *Petinomys* consists of eight species[6], distributed in South and Southeast Asia. The genus is represented by only one species, *Petinomys fuscocapillus* Jerdon, 1847 from India and Sri Lanka. *P. fuscocapillus* was considered as extinct in India, but after 100 years it was rediscovered in 1989 in a coconut grove in Kerala, India[7] and in Sri Lanka, it was rediscovered after a gap of 78 years in Knuckles mountain range[8]. *P. fuscocapillus* is differentiated from other species of genus due to the peculiar honeycombed bones in their ear. Two subspecies are known, i.e. *P. f. fuscocapillus* Jerdon, 1847 found in Western Ghats of southern India and *P. f. layardi* Kelaart, 1850 from Sri Lanka. In India, the species is distributed in Brahmagiri Wildlife Sanctuary and Makutta, Coorg in Karnataka[9] and in the states of Tamil Nadu[10–12] and Kerala[7,13] (Figure 1). In Sri Lanka, it has been reported from Central Provinces and Sabaragamuwa Provinces[14,15]. The species is arboreal (lives in tree canopy) and nocturnal and it occurs in evergreen, deciduous and montane forest. Kumara and Suganthasakthivel[16] predicted the potential distribution of *P. fuscocapillus* using Genetic Algorithm for Rule Set Prediction (GARP) and their result indicates that the potential distribution of *P. fuscocapillus* in India is restricted to the narrow band on the western slope of the Western Ghats; and in Sri Lanka, the distribution is predominant in the lowlands of wet and intermediate zones.

Koprowski and Nandini[17] mentioned that there is a lack of knowledge on distribution, population and conservation status of flying squirrels in the tropical countries and tropical flying squirrels are at a high risk of extinction due to high deforestation rate (mainly through expansion of agriculture, small-scale logging, small wood plantation, infrastructure development and harvesting for local consumption), natural predators and poaching[15]. According to IUCN[18], the species is Near Threatened because its level of occurrence could be approximately 30,000 sq. km and its habitats are probably declining and it occurs as a severely fragmented population, thus making the species close to qualifying as vulnerable.

Wildlife conservation programmes usually integrate molecular techniques for the ecological studies of species of concern. Not much is known about the ecology of *P. fuscocapillus*; therefore, it is very difficult to develop an efficient conservation programme. Mitochondrial genes were extensively used for the molecular phylogenetics of rediscovered and possibly extinct species[19,20]. This study is the first earnest attempt to construct the phylogenetic relationship and level of genetic divergence of *P. fuscocapillus* with other flying squirrels from South and Southeast Asia (Table 1) and to know the utility of markers for identification of the species. We hope that identification of *P. fuscocapillus* closest relative through phylogeny for which ecological data could be available would help in its conservation.

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Materials and methods

Ethical statement

Sample used in the study was obtained from specimens deposited in the Mammals collection of Zoological Survey of India, Kolkata, India with the permission of the Director, Zoological Survey of India. Our sampling did not violate any law, rule or regulation thus required no ethical approval.

Sampling

In this study, we used one museum skin sample. The specimen was collected by D. R. Sugathan from Idukki, Kerala, India on 22 March 2005 (Registration no. 25793). All tools were flame sterilized prior to sample collection. A small fragment of skin (approx. 0.5 cm × 0.5 cm) was sliced in such a way that there was no significant loss of skin that could compromise further studies. Additionally, 12s rRNA and cytochrome *b* available sequences of South and Southeast Asian Sciuridae were obtained from Genebank (Table 1). Thus, in the present study, the phylogenetic position of *Petinomys fuscocapillus* was searched among the species of flying squirrels (9 and 15 species of flying squirrel for 12s rRNA and cytochrome *b* analysis, respectively) of South and Southeast Asian countries.

DNA preparation and sequencing

Fur from the skin sample was removed using a sterile scalpel. Then, the sample was washed with sterile milliQ water and ethanol 70% (v/v) respectively. The skin sample was hydrated before digestion by incubating the dried skin sample for 24 h in 1 ml TE solution (Tris 10 mM and EDTA 1 mM, pH 7.6)[21]. After 24 h of hydration, the DNA was isolated from the skin sample using HiPur A^{TM} Forensic Sample Genomic DNA Purification

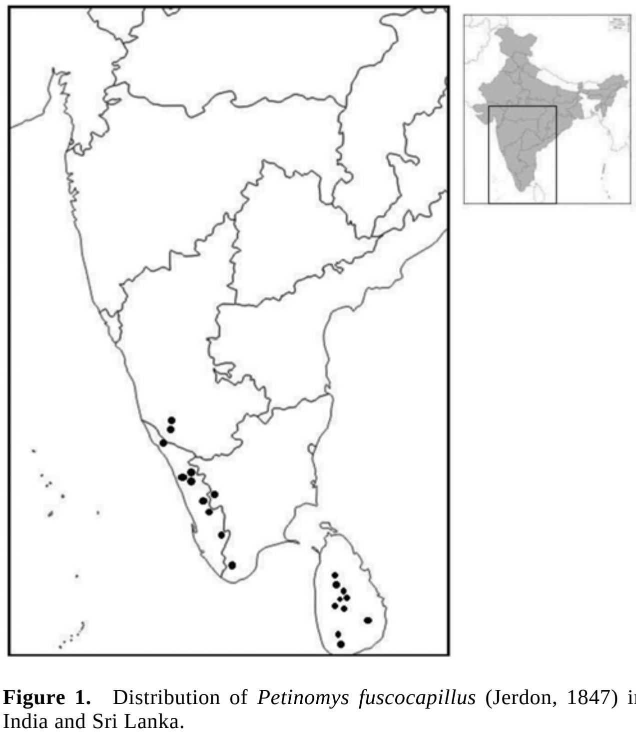

Figure 1. Distribution of *Petinomys fuscocapillus* (Jerdon, 1847) in India and Sri Lanka.

Table 1. Sciuridae species used for phylogeny reconstruction with their accession numbers of respective sequences obtained from Genbank

	Accession number	
Taxon	12s rRNA	Cytochrome *b*
Flying squirrels		
Petaurista alborufus	AY227541	AB092614
Petaurista elegans	–	AB092610
Petaurista xanthotis	–	DQ072111
Petaurista philippensis	–	JQ928697
Petaurista petaurista	D50282	AB092608
Petaurista leucogenys	D50280	AB433269
Belomys pearsonii	AY227537	AB126245
Eoglacomys fimbriatus	AY227562	AB126248
Petinomys fuscocapillus	KP973561*	KP973562*
Petinomys setosus	AY227544	AB030260
Eupetaurus cinereus	AY227538	AY331668
Hylopetes alboniger	–	DQ093187
Hylopetes spadiceus	–	DQ093189
Hylopetes phayrei	AY227539	–
Hylopetes nigripes	–	DQ093190
Hylopetes Lepidus	–	AB126251
Petaurillus kinlochii	AY227542	–
Non-flying squirrels		
Ratufa affinis	AY227547	–
Ratufa bicolour	AY227548	–
Callosciurus nigrovittatus	–	AB499917
Callosciurus inornatus	–	AB499907
Callosciurus finlaysonii	–	AB499911
Callosciurus caniceps	–	AB499919
Callosciurus erythraeus	–	AB499909
Callosciurus notatus	AY227510	AB499913
Callosciurus prevostii	–	AB499915
Tamiops mcclellandii	–	EF539333
Tamiops maritimus	–	HQ698387
Tamiops rodolphii	–	HQ698400
Tamiops swinhoei	AY227522	EF539334
Dremomys rufigenis	AY227511	EF539341
Dremomys lokriah	–	EF539335
Dremomys pernyi	–	EF539336
Dremomys pyrrhomerus	–	EF539342
Dremomys gularis	–	EF539339
Funambulus layardi	FJ861245	–
Funambulus sublineatus	FJ861259	–
Funambulus palmarum	FJ861251	–
Funambulus pennantii	FJ861254	–
Marmota himalayana	NC_018367	GQ329721
Menetes berdmorei	AY227516	–
Rhinosciurus laticaudatus	AY227519	JF417972
Outgroup		
Rattus norvegicus	AY012115	AB033713

*Novel DNA sequence data from this study. –, Sequence not available.

Kit (HIMEDIA) following the manufacturer's protocol. The quantity of isolated DNA was estimated using Invitrogen, Qubit® 2.0 Fluorometer. 12s rRNA sequences were amplified using a set of primer pair, L1091 and H1478, and a primer set of L14841 and H15149 was used to amplify cytochrome *b* gene[22]. The PCR reaction was performed in Q-cycler, Quanta Biotech, in a total volume of 25 μl of reaction mixture (10X PCR with $MgCl_2$, 2.5 μl; 10 mM dNTPs, 2.5 μl; 5 pmol primer, 0.45 μl each; 15 ng of DNA template; 1.5 U *Taq* enzyme). Polymerase chain reaction consisted of initial denaturation of 94°C for 4 min and each cycle of denaturation for 1 min at 94°C, hybridization for 1 min at 55°C (50°C for cytochrome *b*) and extension for 1 min at 72°C followed by final elongation for 10 min at 72°C. The cycle was repeated 35 times. The PCR products were sequenced using ABI's AmpliTaq FS dye terminator cycle sequencing chemistry on an automated ABI 3100 Genetic Analyser. All experiments were performed in a PCR workstation (Bangalore GeNei™). Negative controls were used in all DNA extractions and PCR amplifications to control for potential contamination.

Mitochondrial DNA analysis

Nucleotide sequences were proofread using MEGA5.0 (ref. 23) and aligned using ClustalW[24]. To cross-check the quality of sequence generated, query sequences were compared with NCBI/GenBank (http://www.ncbi.nlm.nih.gov/) database using BLAST tool. Quantitative pairwise comparisons between the species in study were performed, and the uncorrected percentage differences (*p*-distance) between phylogenetic clades were calculated using Kimura's[25] 2-parameter (K2P) method.

To elucidate the phylogenetic position of *P. fuscocapillus* among the Southeast and South Asian species, phylogenetic analyses was performed to assess maximum likelihood with RAxML 7.4.2 (ref. 26) and implemented in ra × mlGUI 1.3 (ref. 27) and Bayesian inference (BI) using MrBayesv3.2.2 (ref. 28). The best-fit evolutionary model was calculated using jModeltestv2.1.3 (ref. 29) and determined using Bayesian information criterion (BIC). The chosen models were GTR + I + G for 12s rRNA sequence and TPM3uf +I + G for cytochrome *b* data. The selected model, i.e. TPM3uf + I + G for cytochrome *b* data cannot be implemented in RaxmlGUI and MrBayes. So, the TPM3uf model was replaced by closed parameterized model, GTR model[30,31].

ML tree calculation was performed using the GTRGAMMA (general time-reversible model with gamma distribution) substitution model[32], and a rapid bootstrap analysis and search for a best-scoring ML tree (ML + rapid bootstrap) was carried out[26] with 1000 repetitions.

Bayesian analyses were executed using a random starting tree and program's default distribution for model parameter. The analyses were repeated twice and each analysis included 3 million generation. The results were sampled every 1000th generation. Convergences were assessed by calculating the effective sample sizes (ESS) using Tracer v1.6 (ref. 33). Conservatively, the first 25% of the sampled trees were discarded as 'burn in' and the remaining 75% of the sampled trees were used to calculate the Bayesian posterior probabilities (BPP). The 12s rRNA and cytochrome *b* gene sequence of *Rattus norvegicus* was used as an outgroup for rooting the trees[34].

Result and discussion

Genes

Partial sequences of the 12s rRNA gene (≈410 bp) and cytochrome b gene (≈ 370 bp) of *Petinomys fuscocapillus* were determined. Both generated sequences (12s rRNA and cytochrome b) showed 96% and 95% identity with their congeneric sequences of accession numbers AY227544 and AB030260 respectively. Thus, BLAST search confirms the specific identity of the studied specimen of *P. fuscocapillus*. These generated sequences were analysed with previously published sequences (Table 1). The average nucleotide composition of the 12s rRNA gene was 35% A, 22.6% C, 17.7% G and 24.6% T. The aligned sequences included 471 variable sites along with 273 parsimony informative sites (25.41% of the entire sequence). Cytochrome *b* gene sequences shows a deficiency in guanine (12.8% G), while the remaining three nucleotide were more balanced, i.e. 27.8% A, 29.3% C and 30.1% T. The alignment of cytochrome *b* gene sequence gives 566 variable sites and 476 parsimony informative sites (41.21% of the entire sequence). These sites depicted the overall variability among the species examined leading to evolution of the species.

Phylogenetic analyses of flying squirrels

On the basis of fossil records, Mein[35], Black[36] and De Brujin[37] mentioned that the diverse fauna of flying squirrels were dominant in Eurasian continents during the Miocene and Oligocene. Fossil records and molecular data[38] indicate that the flying squirrels diverge from Europe and their distribution may have shifted to Southeast Asia and North America. In this study, which is based on the 12s rRNA and cytochrome b data, the flying squirrels are clustered in a separate clade from the non-flying squirrels (ground and tree squirrels).

Maximum likelihood and Bayesian inference analysis generated congruent tree topologies for both 12s rRNA and cytochrome *b* genes (Figures 2 and 3). ML and Bayesian analysis of cytochrome *b* gene generated two major clades, i.e. the flying squirrels represent a separate phylogroup from giant squirrels, stripped squirrels and

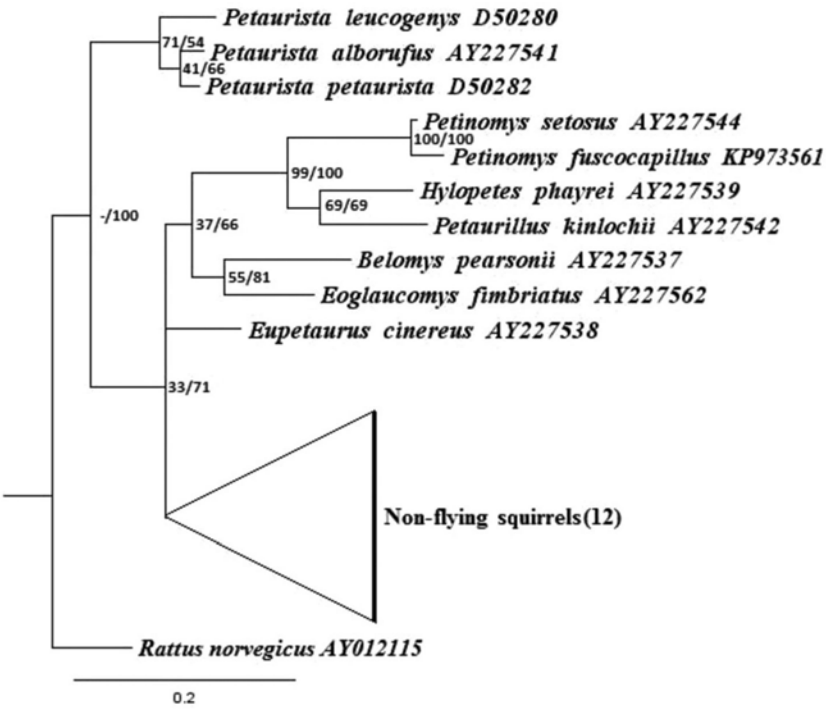

Figure 2. Maximum likelihood (ML) and Bayesian Inference (BI) phylogram based on 12s rRNA sequence (ML and BI analyses generated the same tree topology). Significance values are listed in the order ML (bootstrap support)/BI (Bayesian posterior probability). Terminal triangle represents the non-flying squirrels used in tree generation (detailed topology of non-flying squirrels is not shown as it is not necessary for this study). Number in parentheses is the number of non-flying squirrel species. *Rattus norvegicus* was used as an out-group.

Table 2. Pairwise comparison of partial 12s rRNA sequence of *Petinomys fuscocapillus* with 10 flying squirrels. Data above the diagonal represents nucleotide substitution. Data below the diagonal are uncorrected percentage difference (*p*-distance)

		1	2	3	4	5	6	7	8	9	10
Petaurista alborufus	(AY227541)		9	19	43	35	37	31	48	46	43
Petaurista petaurista	(D50282)	0.024		20	42	37	40	28	49	44	47
Petaurista leucogenys	(D50280)	0.051	0.054		41	38	38	34	47	49	45
Belomys pearsonii	(AY227537)	0.116	0.114	0.111		35	44	36	46	46	50
Eoglaucomys fimbriatus	(AY227562)	0.095	0.100	0.103	0.095		39	30	35	42	43
Petinomys setosus	(AY227544)	0.100	0.108	0.103	0.119	0.105		34	36	39	10
Eupetaurus cinereus	(AY227538)	0.084	0.076	0.092	0.097	0.081	0.092		37	39	42
Hylopetes phayrei	(AY227539)	0.130	0.132	0.127	0.124	0.095	0.097	0.100		36	39
Petaurillus kinlochii	(AY227542)	0.124	0.119	0.132	0.124	0.114	0.105	0.105	0.097		49
Petinomys fuscocapillus		0.116	0.127	0.122	0.135	0.116	0.027	0.114	0.105	0.132	

other ground squirrels (non-flying squirrels) (Figure 3). All the nodes were supported by moderate Bayesian posterior probability (BPP) while low Bootstrap support (BS) was observed at some nodes. The phylogram obtained from ML and BI analysis of 12s rRNA sequence (Figure 2), generated three main clades, i.e. Clade I is represented by the genus *Petaurista* (giant flying squirrels), Clade II is represented by other flying squirrels and Clade III comprises non-flying squirrels (ground and tree squirrels). The genus *Petaurista* (giant flying squirrels) includes a group of diverse species that are adapted for arboreal life and are distributed from Western Himalayas to East Asia, North Indo-China and Southeast Asia[39–42]. In this study, this genus was noted to form a separate clade from other flying squirrels, i.e. genus *Belomys* Thomas, 1908; *Eupetarus* Thomas, 1888; *Eoglaucomys* Howell, 1915; *Hylopetes* Thomas, 1908; *Petinomys* Thomas, 1908 and *Petaurillus* Thomas, 1908 (Figure 2). The phylogenetic position of Genus *Petaurista* with other flying squirrels is noted to be in accordance with previous studies by Oshida *et al*.[34,38]; Li *et al.*[43]; Thorington *et al.*[44] and Mercer and Roth[45].

Phylogeny obtained from the analysis of cytochrome *b* gene showed an early divergence of *Marmota himalayana* from rest of the squirrel species and is supported by high BPP value (100% BPP, Figure 3). Additionally, the genera *Belomys* and *Eupetaurus* also show an early divergence from rest of the flying squirrels (BS = 66%, BPP = 100%, Figure 3). This kind of early divergence of *B. pearsonii* and *E. cinereus* has also been reported in earlier studies

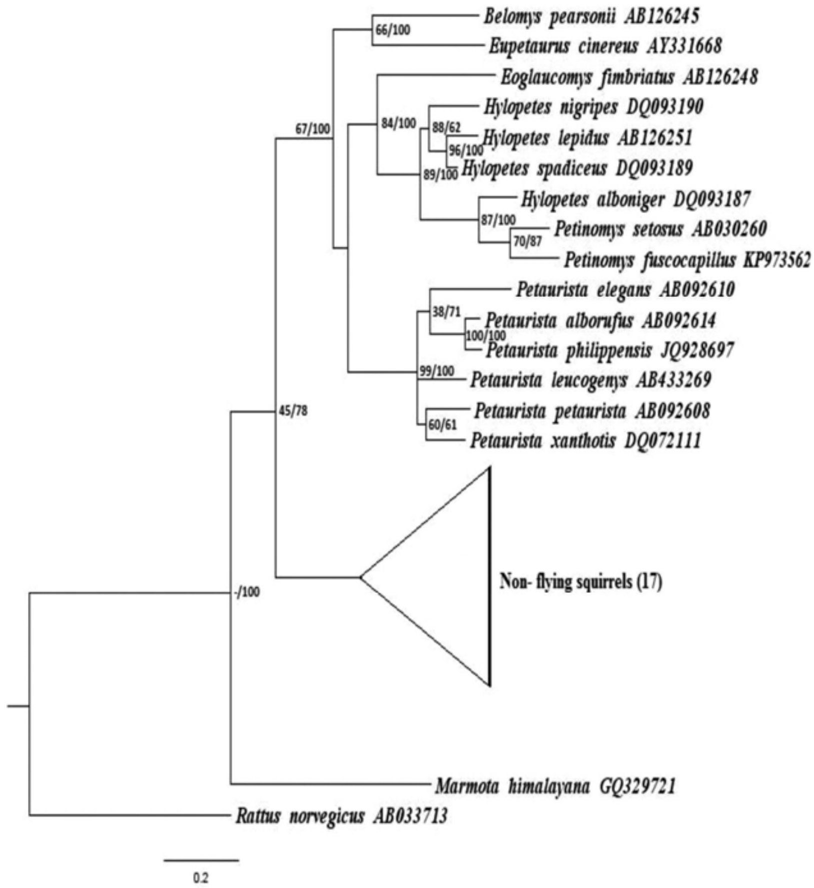

Figure 3. Maximum likelihood (ML) and Bayesian Inference (BI) phylogram based on cytochrome *b* gene sequence (ML & BI analyses generated the same tree topology). Significance values are listed in the order ML (bootstrap support)/BI (Bayesian posterior probability). Terminal triangle represents the non-flying squirrels used in tree generation (detailed topology of non-flying squirrels is not shown as it is not necessary for this study). Number in parentheses is the number of non-flying squirrel species. *Rattus norvegicus* was used as an out-group.

on flying squirrels by Oshida *et al.*[38,46]; Li *et al.*[43] and Yu *et al.*[47]. On the other hand, our 12s RNA phylogenetic tree (Figure 2) and previous studies by Thorington *et al.*[44] and Mercer and Roth[45] do not show the early divergence of these two species from other flying squirrels.

Phylogenetic position of P. fuscocapillus

This is the first study ever conducted on the molecular phylogeny of *P. fuscocapillus* based of two mitochondrial sequences. Travancore flying squirrel is known to be an example of discontinuous distribution of mammals in India and adjacent countries[48]. Its distribution is restricted to Western Ghats and Sri Lanka. Based on 12s rRNA and cytochrome *b* data analysis, *P. fuscocapillus* and *P. setosus* (Temminck's flying squirrel), which are distributed in Malaysia, Myanmar and Thailand[44], are clustered together and their phylogenetic relationship was strongly supported with high BS and BPP in 12s rRNA tree (BS and BPP = 100, Figure 2); while moderate BS and BPP was observed in cytochrome *b* tree (BS = 70% and BPP = 87%; Figure 3). The *p*-distance of *P. fuscocapillus* was found to be the highest in *Belomys pearsonii* (0.135, Table 2) and *Eoglaucomys fimbriatus* (0.210, Table 3) and lowest in *P. setosus* (0.027 and 0.095, Tables 2 and 3). Thus, the topology of the phylogenetic tree and low genetic divergence between *P. fuscocapillus* and *P. setosus* strongly supports that these two species are monophyletic and share recent common ancestry.

In this study, *P. fuscocapillus* and *P. setosus* together form a sister clade with *Hylopetes phayrei* (from China, Myanmar, Thailand and Vietnam) (Figures 2 and 3). So, our result strongly supports the Corbet and Hills[40] hypothesis that *Petinomys* is closely related to *Hylopetes* and the two genera are distinguished by number of septa in the auditory bullae. In addition to the Corbet and Hills hypothesis, our study also supports the phylogeny provided Oshida *et al.*[48] and Herron *et al.*[49] based on Cytochrome *b* gene in which they reported that genus *Petinomys* is closely related to genus *Hylopetes*. Moreover, in a study by Mercer and Roth[45], using three genes (IRBP, 12s and 16s rRNA sequence) found that genus *Petinomys* is phylogenetically closely associated with genus

Table 3. Pairwise comparison of partial cytochrome *b* gene sequence of *Petinomys fuscocapillus* with 15 flying squirrels. Data above the diagonal represents nucleotide substitution. Data below the diagonal are uncorrected percentage difference (*p*-distance)

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Petaurista philippensis	(JQ928697)		24	55	46	49	52	61	57	59	55	53	61	60	61	59
Petaurista alborufus	(AB092614)	0.069		56	42	46	45	59	63	56	58	52	56	57	63	59
Petaurista elegans	(AB092610)	0.172	0.176		47	61	51	67	64	63	63	60	56	61	68	60
Petaurista xanthotis	(DQ072111)	0.139	0.125	0.142		40	37	56	62	61	59	51	59	59	56	53
Petaurista petaurista	(AB092608)	0.149	0.138	0.192	0.118		50	61	68	67	63	59	69	62	71	65
Petaurista leucogenys	(AB433269)	0.161	0.136	0.157	0.108	0.152		52	55	62	66	56	57	52	55	57
Belomys pearsonii	(AB126245)	0.187	0.180	0.210	0.171	0.188	0.156		61	57	53	59	51	57	57	59
Eoglaucomys fimbriatus	(AB126248)	0.173	0.194	0.199	0.191	0.213	0.166	0.187		68	55	60	62	59	63	67
Eupetaurus cinereus	(AY331668)	0.180	0.170	0.196	0.187	0.208	0.191	0.173	0.212		60	58	48	58	61	66
Hylopetes alboniger	(DQ093187)	0.166	0.176	0.195	0.180	0.196	0.206	0.160	0.166	0.185		47	47	49	43	43
Hylopetes spadiceus	(DQ093189)	0.159	0.156	0.184	0.153	0.181	0.170	0.182	0.186	0.178	0.142		44	30	44	40
Hylopetes nigripes	(DQ093190)	0.188	0.170	0.170	0.181	0.219	0.173	0.153	0.193	0.143	0.143	0.134		42	60	55
Hylopetes lepidus	(AB126251)	0.184	0.173	0.188	0.181	0.192	0.156	0.174	0.182	0.177	0.149	0.087	0.126		47	52
Petinomys setosus	(AB030260)	0.187	0.194	0.214	0.170	0.225	0.166	0.174	0.195	0.189	0.130	0.132	0.189	0.142		33
Petinomys fuscocapillus	(KP973562)	0.180	0.180	0.183	0.159	0.203	0.173	0.183	0.210	0.206	0.128	0.118	0.169	0.158	0.095	

Petaurillus. Similar findings were obtained in our study, where genus *Petinomys* formed a sister clade with *Petaurillus kinlochii* (Figure 2).

Species of genus *Petinomys* is distributed in South and Southeast Asia. Seven among the eight species of the genus is distributed across the Maritime and Mainland Southeast Asian countries[6,15]. *Petinomys fuscocapillus* is the only species representing the genus in the South Asia and it appears that it has become endemic to two regions of Western Ghats, India and Sri Lanka because of geographical barriers as well as adaptations to climatic conditions.

This study has indicated that the species is forming a separate lineage from other species of flying squirrel and can be identified by using the two molecular markers, i.e. cytochrome b and 12S rRNA. Thus, the two markers used in this study are useful in providing scientific proof for wildlife forensic cases as well as for status survey and will thus strengthen the conservation efforts.

Implications for conservation

Ashraf *et al*.[10]; Umapathy[50] and Karanth[51] reported the presence of this squirrel, but unfortunately there were no sight records available. Few decades later, Kurup[7], Kumara and Singh[52] sighted this small flying squirrel from Makut Reserve Forest, Karnataka, India and Jayasekara *et al*.[8] sighted the squirrel in Sinharaja tropical rain forest, Sri Lanka.

This study provides the species-specific gene sequence by using two markers, useful for generating scientific proof for wildlife forensic cases and status survey. Since half of the potential distribution of *P. fuscocapillus* lies in human inhabited areas[16], strict laws should be enforced in these areas to control hunting in the forest. Kumara and Suganthasakthivel[16] modelled the potential distribution of Travancore flying squirrel in the two countries thus by using this study for identification of the species as well as by applying conservation plans such as afforestation for continuous canopy with height of the forest profile around 25 m as suggested by Koprowski and Nandini[17] and Nanayakkara *et al*.[53], the population of the species can be restored. Moreover, an extensive survey is pivotal at these potential distributions for a thorough population sampling to determine the probable reason behind the presence of a genetic bottleneck that reduced the genetic richness.

Conclusion

This study was designed to determine the molecular phylogenetic position of rediscovered Travancore flying squirrel (*P. fuscocapillus*) using two mitochondrial genes, i.e. 12s rRNA and cytochrome *b*. With respect to the phylogenetic position of the species, tree topology of both genes provided the same result. Thus, both markers can be used for identification of the species. The Travancore flying squirrel was found to be monophyletic with the Temminck's flying squirrel (*Petinomys setosus*). According to IUCN (2008), the population of *P. fuscocapillus* was continuously decreasing; so, there is a strong need for some conservation action plans for the species. Conservation plans may include extensive sampling, captive breeding, maintenance of continuous forest canopy and enforcement of strict laws to control hunting.

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