Journal of Tropical Resources and Sustainable Science

journal homepage: jtrss.org

Phylogenetic Analysis of Mitochondrial Genes of Malaysian Tupaia Reveals Composite Species in Tupaia Glis

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Available online 28 June 2015

Keywords:
New species, Tupaia, mtDNA, taxonomy.

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Abstract

Recent morphometric analysis on T. glis in Peninsular Malaysia indicates that there were more than one morphotypes in this species. Thus this study attempts to examine this phenomenon using mitochondrial DNA sequences of Cyt b and CO1 genes. A total of 74 DNA sequences for both genes were generated using available universal primers. Samples from Southern Thailand were found to be misidentified as T. glis when in fact these samples clustered with T. belangeri while one T. tana from Borneo was miss-identified as T. minor. The phylogenetic trees showed that there are at least one confirmed morphotype of Tupaia (new Tupaia sp.) that have yet to be described. The results also showed that the separation of T. glis morphotype 1 and 11 were visible in the combined genes tree, congruent with the morphometric phylogeny but had poor phylogenetic support.

1. Introduction

The unique and endemic tree shrews of the Indo-Malayan region are most intriguing creatures. These small mammals are very much like squirrels but has long snout that resembles shrews. When Diard (1820) first described a tree shrew he found in Penang Island, he placed it in the genus Sorex (S. glis). Such confusion was the beginning of a long history of taxonomic uncertainty with many taxonomic revisions along the way. The situation was further confounded by the high morphological variation in this taxa. Currently, these small mammals are place in the Order Scandentia and consists of two families namely Tupaiidae and Ptilocercidae. Helgen (2005) recognized 20 species of tree shrews, a stark contrast to the 11 recognized species in Corbet and Hill (1992). In recent years efforts have been made to better understand the taxonomy of these mammals. Although the inter-ordinal relations Scandentia have been rigorous examined (see Sargis et al., 2004; Sargis et al., 2007 and Sargis et al., 2013a) and most inter-species have been resolved (see Roberts et al., 2011) there are still gaps of knowledge in the taxonomy of this taxon that needs to be addressed.

In Malaysia, there are 11 species of tree shrews, with just three species occurring in Peninsular Malaysia. Borneo holds higher diversity of tree shrews, with nine species of tree shrews distributed throughout the island. However, wide spread species within this group of mammals have been known to have high morphological variation. These morphotypes have been a source of confusion on the taxonomy of the species in Scandentia. A number of recent taxonomic studies focusing on wide spread, morphologically diverse and geographically isolated tree shrews have revealed some new species on the basis of morphometrics and genetic data (Sargis et al., 2013a,

ISSN Number: 2289-3946

b). Recent morphometric examination by Ahmad-Tahir et al. (2013) using 23 morphological characters suggested that T. glis may probably be a composite species, with at least one un-described form that exist in Peninsular Malaysia. Up to date there are 54 published synonyms of T. glis, and historically the taxonomy of T. glis have been revised many times as new species have been described based on populations of T. glis. Thus this study further attempts to look at the possibility of finding genetically diverge Tupaia from T. glis populations in Peninsular Malaysia based on Ahmad-Tahir et al. (2013) findings.

2. Materials and Methods

Samples of this study came from two sources, museum deposits and field collection. Museum samples were obtained from Zoological Museum of Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, Malaysia and *Princess* Maha Chakri Sirindhorn Natural History Museum, Prince Songkla University, Thailand. For field sampling, tree shrews were captured using cage traps baited with banana. Upon capture, treeshrews were transferred in to cloth bags and their standard measurements including weight were taken. Tree shrews were then identified based on descriptions by Payne *et al.* (1985) and Francis (2008). Next the tree shrews were euthanised using a high dose of chloroform and approximately 5 mg of liver tissue were removed and preserved in absolute ethanol. The whole carcass was then preserved in 70% ethanol and was kept as museum vouchers in Zoological Museum at Faculty of Earth Science, Universiti Malaysia Kelantan, Malaysia. Figure 1 outlines all the sampling sites and localities of samples and Table 1 lists out all the museum samples that were obtained in this study.

Table 1. Museum samples of *Tupaia* used in this study

No	Species	Field No	Location	Region	Museum
1	*T. glis*	PL003	Langkawi	Pen. Malaysia	UNIMAS
2	*T. longipes*	UNIMAS0001879	Kapit	Borneo	UNIMAS
3	*T. splendidula*	3T	NA.	Borneo	UNIMAS
4	*T. gracilis*	NA016	NA.	Borneo	UNIMAS
5	*T. minor*	UNIMAS T. minor	NA.	Borneo	UNIMAS
6	*T. minor*	S011	Sematan	Borneo	UNIMAS
7	*T. tana*	WC01	Wind Cave	Borneo	UNIMAS
8	*T. tana*	UNI A035	Arboretum UNIMAS	Borneo	UNIMAS
9	*T. glis*	126.2	Hat-Yai	Thailand	PSU
10	*T. glis*	126.3	Hat-Yai	Thailand	PSU

DNA was extracted using Modified C-TAB protocol descibed in Grewe *et al.* (1993). Universal PCR primers targeting mtDNA partial Cytochrome *b* (450 bps; Palumbi *et al*. 1991) and Cytochrome Oxidase 1 (658 bps; Folmer *et al.*, 1994) genes were used for PCR amplification. A 50 µl PCR master mix for each reaction was prepared as follow: 28.5µl of dH2O, 5µl of 10× reaction buffer, 1µl of dNTP mix (10mM), 3µl of MgCl2, 0.5 µl of *Taq* polymerase, 2.5 µl of each primers (forward and reverse with probes) and 2µl of template DNA.

The PCR conditions and primers are as follow: 1) Cyt *b* primer sequences GluDG-L (5'-TGACT TGAAR AACCA YCGTT G- 3') and CB2-H (5'-CCCTC AGAAT GATAT TTGTC CTCA- 3'):- initial denaturation of 94ºC for 2 min followed by 30 cycles of 94ºC denaturation for 30 sec, 51.7ºC annealing for 1 min and 72ºC of extension for 30 sec. A final extension of 72ºC for 2 min were added after the 30 cycles of PCR described earlier; 2) CO1 primer sequences LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') :- initial denaturation of 94ºC for 2 min followed by 30 cycles of 94ºC denaturation for 30 sec, 43.6ºC

ISSN Number: 2289-3946

annealing for 1 min and 72ºC of extension for 1min. A final extension of 72ºC for 2 min were added after the 30 cycles of PCR. PCR products were then sent for DNA purification and sequencing at MyTAGG Genomic Bioscience and Tech, Malaysia.

Figure 1: Map of Peninsular Malaysia indicating sampling sites of this study.

Chromatogram of DNA sequences were then checked for errors, BLASTed and aligned using MEGA 6.1 (Tamura *et al*., 2013). Aligned DNA sequences with poor peak reads were then trimmed (at the front and the end) and the DNA sequences were then translated into amino acid sequences to check for the presence of stop codons. Any stop codons that were present were referred back to the original chromatogram for corrections. DNA sequences with the NCBI accession no. AY321643 (*T. glis* Cyt *b* gene sequence) and JF459875 (*T. glis* CO1 gene sequence) were used as the guide for amino acid translation during stop codon checking. DNA sequences from both genes were then concatenated to form DNA sequences for phylogenetic analysis. Saturation plots were used to assess the level of saturation using DAMBE 5.374 (Xia, 2013).

Neighbor-joining tree (NJ), Maximum-Likelihood (ML) and Bayesian tree were generated using TOPALi 2.5 (Milne *et al*., 2008) and Maximum-Parsimony (MP) tree and Minimum-Evolution tree (ME) was generated using MEGA 6.1. A quick NJ tree was generated using F4+G substitution model (1000 bootstrap replicates) to have an overview of the phylogeny. Next, a Maximum Parsimony tree were generated using tree-bisection-reconnection heuristic search option with random addition of 10 initial trees and 1000 bootstrap replicates. Subsequently, Modeltest was performed to find the best fit model for ML, ME and BA trees and the models that best fit the data was used to generate the MP, ME and BA trees. TrNef + G model was used to generate the ML and ME trees based on the Modeltest results and 1000 bootstrap replication was included to show phylogenetic confidence. For BA tree, K80+G parameter was implemented and trees were sampled every 100 generations with burn in-value of 1,000. Bootstrap values of more than 80% and Bayesian posterior probabilities of ≥0.95 were considered as statistically significant. The genetic divergence table was generated using K2P parameter to show the genetic divergence values of the groups in this study.

3. Results

Both Cyt *b* and CO1 genes from 74 tree shrew specimens were successfully sequenced in this study and produced unequivocal DNA alignments without stop codons. The aligned DNA sequences of Cyt *b* gene were 405bps in length and for CO1 it was 618 bps. A BLAST in NCBI revealed that two samples (126.2 and 126.3) were misidentified, both gene sequences were congruent in identifying Sample ID 126.2 and 126.3 as *T. belangeri*. UNIMAS0001879 which was identified as *T. glis* from Kapit when BLASTed was found to be *T. longipes*, congruent with Helgen's (2005) classification. Post concatenation of DNA sequences produced 1023bps length sequences and there were 544 conserved sites from the 1023 bps length data. The remaining 479 variable sites had 357 parsimony-informative sites and 122 autoapomorphic sites. Saturation plots indicate that there were no saturation present (Figure 2). The initial neighbor-joining tree (Figure 3) has strong support in most major branches and declined at the internal nodes. The MP tree generated a single most- parsimonious tree with the

ISSN Number: 2289-3946

tree length of 1427 with a consistency index of 0.51 and Retention index of 0.78. The single optimal Maximum-likelihood tree had a likelihood value of -8504.16, whereas for the ME tree, the least-evolved tree had a score of 1.47. The BA tree generated high support values for most major clades but were low at lower branches.

The average genetic divergence between the samples ranged from 2.8% to 21.9% (Table 2). However within species the average genetic distance was very low ≈1.4%. The phylogenetic trees generally produced three major clades which consist of i) various *Tupaia* species in Malaysia; ii) new *Tupaia* sp. and; iii) a second large clade of *Tupaia glis*. This large clade was further separated into two smaller clades (*T. glis* morphotype 2 and 3). All major clades produced strong to moderate monophyletic clades, however the division between *T. glis* morphotype 2 and 3 was poorly supported. In terms of genetic divergence, *T. glis* morphotype 2 and 3 were 2.8% divergent. Post phylogenetic analysis revealed that sample ID UNIMAS T. minor was missidentified and the sample was in the *T. tana* clade.

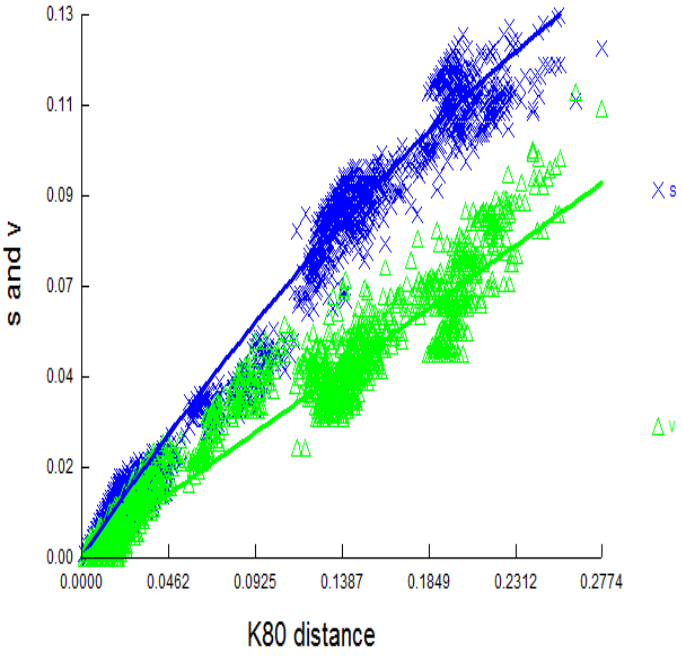

Figure 2: Kimura-2 parameter Saturation plot (transition versus transversion)

Table 2: Average genetic divergence among samples used in this study

Taxa			Clade 1				Clade 2	Clade 3(1)	Clade 3(11)
T. belangeri	0								
T. gracilis	0.220	0							
T. splendidula	0.196	0.208	0						
T. minor	0.206	0.209	0.134	0					
T. tana	0.189	0.194	0.195	0.077	0				
T. longipes	0.191	0.219	0.210	0.176	0.118	0			
New *Tupaia* sp.	0.202	0.216	0.130	0.133	0.189	0.210	0		
T. glis morphotype 1	0.199	0.225	0.158	0.142	0.203	0.211	**0.141**	0	
T. glis morphotype 11	0.196	0.223	0.155	0.139	0.201	0.205	**0.140**	**0.028**	0

ISSN Number: 2289-3946

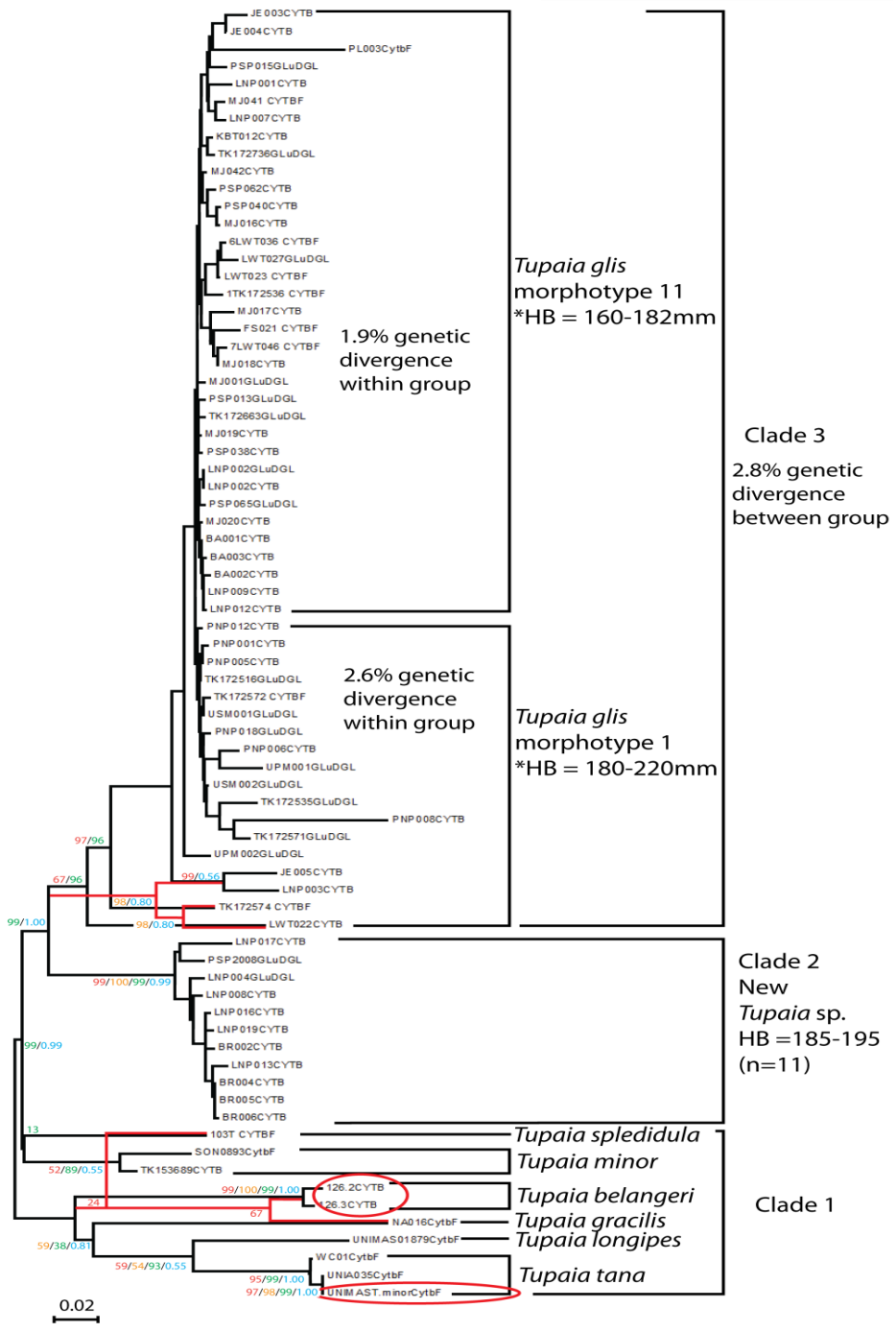

Figure 3: Combined MP, ML, ME and BA trees of *Tupaia* spp. in this study. red colored branches are different topology on the phylogenetic trees. Colored values on branches refer to bootstrap support (Red for MP, orange for ME and Green for ML) and Bayesian posterior probabilities (Blue). Values lower than 50% for bootstrap and >0.70 for Bayesian posterior probabilities were removed from the branches.

ISSN Number: 2289-3946

4. Discussion

The phylogenetic trees revealed several points in this study that needs to be further explored. There is one new Tupaia sp. that is yet to be described and needs to be addressed promptly(Figure 3). The high bootstrap and Bayesian support shows that this new morphotype is distinct and it is not associated with the other 20 species of tree shrews listed in Helgen (2005). The high average genetic divergence between species (17.9%) for this Tupaia sp. was higher than the other Tupaia spp. (Table 2). in this study (15.3%). A check on head body length shows that this Tupaia sp. (185-195 mm) overlaps with T. glis (135-205mm) while the other distinctive features of T. glis such as the reddish tinge and the number of mammae (2 pairs) are the same. Habitat wise a check on the locality of the sampling sites reveal that this morphotype was only found in forests unlike the other T. glis in this study and its distribution was restricted to southern Peninsular Malaysia. We have yet to discover any distinct morphological features that separates this new species with its sympatric kin T. glis, thus there is a need to run a morphometric analysis to find key features that can identify this new species.

The morphological divergence of T. glis morphotype 1 and 11 was clear (see Ahmad-Tahir et al., 2013), however the poor bootstrap and Bayesian values does not statistically support the division of these two morphotypes in the combined genes tree (Figure 3). The average genetic divergence between these two clades was 2.8% and according to Baker and Bradley's (2008) Genetic Species Concept, these two morphotypes would only qualify as populations within a species. However there were previous studies that indicate the 2% rule (representing different populations only) does not apply on Macroglossus spp. M. sobrinus and M. minimus is only 2.5% divergent (Khan, 2008; Jayaraj, 2009). The average genetic distances within both morphotypes was also low (2.6 in T. glis morphotype 1 and 1.9% in T. glis morphotype 11). According to the DNA barcoding concept by Herbert et al. (2003), these two morphotypes are divergent based on the average divergence within (1.9 and 2.6%) and between morphotypes (2.8%). Based on the sampling data, T. glis morphotype 11 was more widespread as compared to all the other Tupaia in this study and were found in most sampling sites except Penang Island. T. glis morphotype 1 distribution were restricted to the west coast of Peninsular Malaysia and both species were collected in a wide range of habitats especially in areas with moderate anthropogenic activities.

The implications of this study may have an impact on the conservation of tree shrews in Peninsular Malaysia as T. glis is regarded as widespread and Least Concern in IUCN Red List of Threatened Species (2008). The existence of at least one undescribed species (new Tupaia sp.) which its distributional range is restricted to forests may require more data on their geographic distribution and abundance in order to assess their conservation status. As there is a rapid decline in forest, the loss of habitat may drive this species to extinction. The remaining two morphotypes were more successful and were found to be able to tolerate moderate levels of anthropogenic disturbance and may not require immediate conservation needs.

On another note, T. minor and Ptilocercus lowii once had a more wide distribution in Peninsular Malaysia but are currently Vulnerable under PERHILITAN (2010). Previously these species was widely distributed in Malaysia (Corbet and Hill, 1992), however the current distribution is restricted to Krau Wildlife Reserve (PERHILITAN, 2010). Our sampling efforts up to date of approximately 3600 cage trapping days (10 sampling sites; 15 sampling stations), did not capture any T. minor and Ptilocercus lowii, further confirming PEHILITAN's (2010) review

Acknowledgement

We are very grateful to the Department of Wildlife and National Parks, Peninsular Malaysia for granting us permit to conduct this 3 year study. We are also thankful to Zoological Museum of Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, Malaysia and Princess Maha Chakri Sirindhorn Natural History Museum, Prince Songkla University, Thailand for providing us museum samples taht were used in this study. This study was a part of an on-going Project entitled ‘DNA taxonomy and evolution of tree shrews in Peninsular Malaysia and Borneo’ led by Jayaraj Vijaya Kumaran (UMK) and was supported by three grants namely Malaysian

ISSN Number: 2289-3946

Torray Science Foundation Science and Technology Grant (R/MTSF/A03.00/00481A/003/2012/000071), Ministry of Higher Education Research Acculturation Collaborative Effort (RACE) grant R/RACE/A07.00/00525A/002/2012/000085 awarded to the project leader together with Assoc. Prof. Dr. Seri Intan Mokhtar (UMK), Assoc. Prof. Dr. Ahmad Sofiman Othman (USM), Assoc. Prof. Dr. Shahrul-Anuar Mohd Sah (USM) and Prof. Dr. Mohd Tajuddin Abdullah (UNIMAS) and NAGAO Natural Environmental Foundation Grant to Jayaraj Vijaya Kumaran.

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ISSN Number: 2289-3946