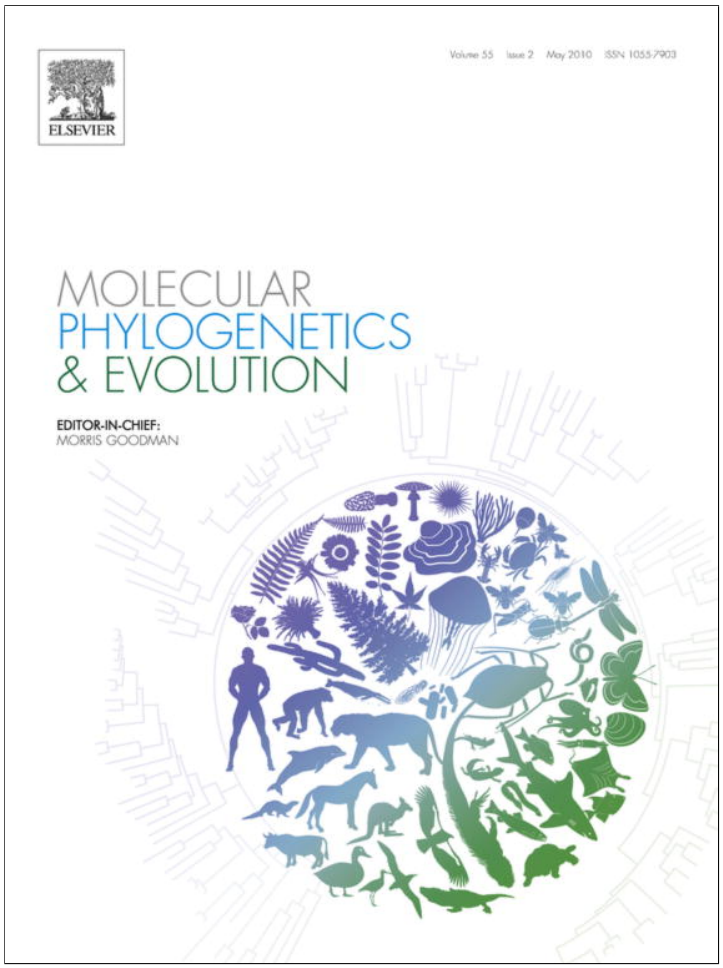

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Speciation dynamics in the SE Asian tropics: Putting a time perspective on the phylogeny and biogeography of Sundaland tree squirrels, *Sundasciurus*

Robert-Jan den Tex [a], Richard Thorington [b], Jesus E. Maldonado [b,c], Jennifer A. Leonard [a,c,d,*]

[a] Department of Evolutionary Biology, Uppsala University, Norbyvägen 18D, 752 36 Uppsala, Sweden
[b] Department of Vertebrate Zoology National Museum of Natural History Smithsonian Institution, MRC 108, Washington, DC 20013-7012, USA
[c] Center for Conservation and Evolutionary Genetics, National Zoological Park, Smithsonian Institution, Washington, DC 20008, USA
[d] Estación Biológica de Doñana-CSIC, Avd. Americo Vespuccio s/n, 41092 Sevilla, Spain

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ABSTRACT

Tropical rainforests are well known for their extraordinarily high levels of biodiversity. The origin of this species richness is still debated. For instance, the museum hypothesis states that over evolutionary time more and more species will accumulate with relatively few extinctions. In contrast, the Pleistocene diversification model argues that during the last 2 million years, climatic factors (glaciations) caused environmental changes that drove isolation and vicariant speciation events. In this study, we construct a molecular phylogeny of the Sundaland (Malay Peninsula, Sumata, Borneo, Palawan) and Greater Mindanao (Mindanao, Samar, Leyte) tree squirrels (genus *Sundasciurus*). Our results show that most speciation events in this forest dependent taxon occurred before the Pleistocene and that even the timing of intraspecific splits among populations from different landmasses are relatively old. Additionally, we found unexpectedly high divergence within and between highland populations of *S. tenuis* on Sumatra and the Malay Peninsula, highlighting the importance of Pliocene events in both speciation and within species divergences in this region.

1. Introduction

The tropics are the most speciose regions of the world, suggesting that speciation occurs more rapidly or extinction more slowly there, or both, than in temperate zones. The reasons for this have been much debated, and are still unknown. This biodiversity is threatened by urban development and logging, agriculture and mining, with many of the extracted materials exported for use outside the region.

Sundaland is the region of southeast Asia which encompasses the Malay Peninsula and the islands on the Sunda shelf, including Sumatra and Borneo (Fig. 1). This region is largely covered by tropical forest, although historically it has been heavily impacted by human development. The region shows high levels of species richness and endemism (Myers et al., 2000), which is partly attributed to its dynamic and complex climatic and geological history (Sodhi et al., 2004). Squirrels follow this pattern- there are 18 genera and 46 species of tree squirrels and flying squirrels in this area (Corbet and Hill, 1992).

During the Pleistocene (2 million years ago–10,000 years ago), sea level fluctuations exposed large regions of the shelf thereby connecting these islands to each other and to the mainland (Bintanja et al., 2005; Bird et al., 2005; Hall, 1998; Heaney, 1991) and then isolating them again multiple times. These connections lasted for tens of thousands of years, and the islands last became isolated only 10,000–12,000 years ago when sea level rose to less than 50 m below present levels (Voris, 2000; Hanebuth et al., 2000). Sea level remained below −50 m for approximately 40% of the time in the last 250,000 years (Voris, 2000), so the islands were not nearly as isolated from one another and the mainland as they are at present.

This history has led to a 'Pleistocene pump' hypothesis to explain the diversity in the area. Under this scenario, populations diverge and speciate in allopatry when sea level is high, and then expand their range when sea levels are low and the islands are connected by land (Gorog et al., 2004; see also Steppan et al., 2003). When repeated several times throughout the Pleistocene, this system has the potential to generate a lot of diversity. This hypothesis

* Corresponding author. Address: Department of Evolutionary Biology, Uppsala University, Norbyvägen 18D, 752 36 Uppsala, Sweden. Fax: +46 18 471 6310.
E-mail addresses: JLeonard@ebd.csic.es, Jennifer.Leonard@ebc.uu.se (J.A. Leonard).

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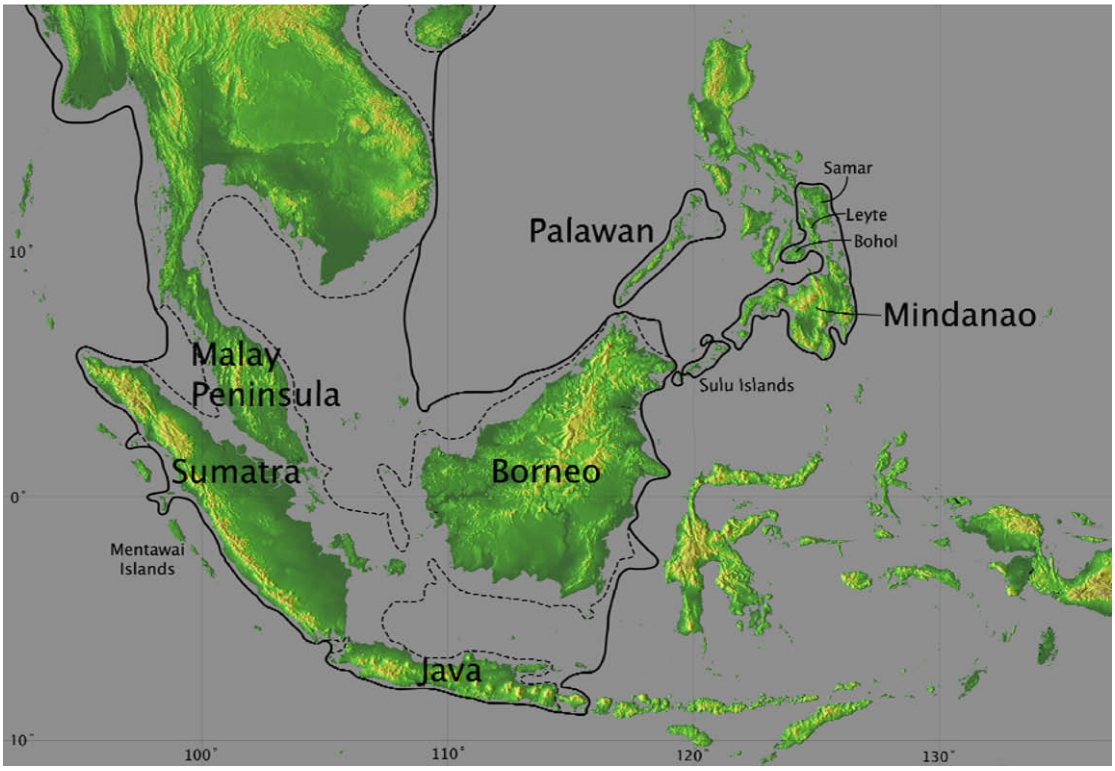

Fig. 1. Map of Southeast Asia showing Sundaland (Malay Peninsula, Sumatra, Java, Borneo and Greater Palawan) and the oceanic Philippine Islands that make up Greater Mindanao (Mindanao, Samar, Leyte, Bohol and smaller adjacent islands) and the Sulu archipelago. Lighter color land areas are more than 1000 m above sea level. The black lines show the maximum exposure of land during the Last Glacial Maximum (LGM) around 18,000 years ago when sea levels were 120 m below their current level (after Voris, 2000). The dashed line shows the contour line of exposed land when sea levels were around 40 m below current. For Palawan, Greater Mindanao and the Sulu Islands the 40 m contour line coincides with the current coastline. The basic map was obtained from http://www.ngdc.noaa.gov/mgg/topo/pictures/ASIAcolshadezw.tif.

has been supported by low levels of genetic divergence between populations of some Sunda shelf mammals (Campbell et al., 2004, 2006; Gorog et al., 2004; Lucchini et al., 2005; Roos et al., 2003). However, the vegetation during the Pleistocene glacial periods was undoubtedly very different from the present, and the interactions between climate and sea level and their effects on the distribution of the fauna and flora is complex (van den Bergh et al., 2001). For example, Cranbrook (2000) interpreted the large mammal fauna of the Pleistocene of coastal Borneo as being more adapted to savannas than to the rainforests of the present. Gorog et al. (2004) adduced evidence that populations of rainforest rats on the Sunda Islands and Malay Peninsula (*Maxomys* and *Leopoldamys*) became isolated from one another in the mid- to late Pliocene, perhaps 2 million years ago. This evidence may support the museum or stasis hypothesis, which states that the high diversity in the tropics was generated over a much longer time period due to high ecological stability (Gathorne-Hardy et al., 2002; Morley, 2002).

Many Sunda taxa have also colonized the southwestern Philippine Islands (Heaney, 1986), particularly the Palawan Islands and Greater Mindanao (including Mindanao, Samar, Leyte and smaller nearby islands, Fig. 1). The Philippines lie just to the east of the Sunda shelf. It is possible that the Palawan Islands were connected to Borneo by land in the middle and/or late Pleistocene (Bird et al., 2005; Heaney, 1986). The islands of Greater Mindanao were connected to each other during Pleistocene low sea level stands (Heaney et al., 2005), but were separated from the Sunda shelf by a series of narrow straits of deep water and small islands (Musser and Heaney, 1985; Jones and Kennedy, 2008; Steppan et al., 2003). It is possible that the Sunda fauna colonized the Philippines via a land bridge to Palawan and then spread through the main Philippine Islands to Greater Mindanao. Alternatively, they could have colonized Palawan and Greater Mindanao separately, as suggested by geographic distance from Borneo. Both of these hypotheses require squirrels to have colonized and then gone extinct on some islands.

Tree squirrels in the genus *Sundasciurus* are a good model system in which to test these biogeographic hypotheses. They are forest dependent squirrels distributed across the Sunda shelf, the Palawan Islands and Greater Mindanao (Fig. 1). The genus includes both narrowly and widely distributed species (Corbet and Hill, 1992). However, they are poor over-water dispersers (Mercer and Roth, 2003). The alpha taxonomy is relatively well known and the group is not too species rich (Thorington and Hoffmann, 2005).

We use near complete mitochondrial cytochrome *b* sequences from most species from the genus *Sundasciurus*, and as many major populations as possible for widely distributed species, to construct a phylogeny of the genus. We use this phylogeny to test the Pleistocene pump and the museum/stasis hypotheses and to determine the colonization history of the Philippine Islands.

2. Materials and methods

2.1. Materials

Fourteen of the 15 species of the genus *Sundasciurus* recognized by Thorington and Hoffmann (2005) are included in this study. The missing species is *S. davensis*, which is only known from its type locality on Mindanao, Philippines (Thorington and Hoffmann 2005) and considered a synonym of *S. philippinensis* by Corbet and Hill (1992) and Heaney et al. (1998). A minimum of two individuals per species were included, and more for the widely distrib-

uted species (*S. tenuis*, *S. lowii* and *S. hippurus*). In total 79 individuals were included, the majority of which ($n = 61$) came from museum specimens (Table 1). The remaining 18 samples were obtained from fresh tissue (Table 1). Cytochrome *b* sequences from the squirrel genus *Callosciurus* were selected as the most appropriate outgroup based on Steppan et al. (2004) and Mercer and Roth (2003) and sequences were downloaded from GenBank (Oshida et al. 2001; AB043875, AB043879, AB043880 and AB043882).

2.2. Molecular methods

The museum samples were extracted in a special dedicated laboratory for low quality DNA that is physically separated from the main laboratory. DNA was extracted by phenol–chloroform and concentrated in Centricons as in Leonard et al. (2005) or by DNeasy animal tissue and blood extraction kit (Qiagen, Hilden, Germany) following the animal tissue protocol. To monitor for contamination, negative extractions were included in every batch of extractions. DNA from the fresh tissue samples was extracted by phenol–chloroform extraction and alcohol precipitated (Sambrook et al., 1989). DNA samples were quantified by taking absorbance readings using a Nanodrop ND-1000 Spectrophotometer (Thermo Scientific) at 260 nm wavelength.

We amplified the whole cytochrome *b* gene in one fragment for the high quality samples and in four to nine fragments from the historic specimens (Table 2). Seven nuclear intron fragments were screened in high quality tissue samples. They were *Fib6* (RodFib6Fwd: 5′-GAA GAA GTA CTG GCT TGC CAG; RodFib6Rev: 5′-CAT TGC CAA GCC AAT ATT C) designed from mouse, rat and human sequences from GenBank (NP_862897.1, NP_064456.1, NP_005132.2); *AP5* (DeBry and Seshadri, 2001); *c-myc* (cmycFwd: 5′-AAT TTT CTT GCC TGT GCC ATA ACC; cmycRev: 5′-CTG TGA GGA GGT TTG CTG TGG CTT) based on sequences from Steppan et al. (2003); and *FTH1*, *POR*, *GBG* and *HMG1* from Jiang et al. (1998).

Fragments were amplified in 25 μl reactions that included 1× Gold Buffer (Applied Biosystems, Foster City, CA, USA), 2.5 mM $MgCl_2$, 0.8 mM dNTPs (0.2 mM each), 1 μM of each primer, 1.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems) and between 10 and 50 ng of DNA. The PCR program started with an initial denaturation step of 95 °C for 10 min followed by 36 cycles of 95 °C for 30 s, annealing of 50–60 °C for 30 s and extension of 72 °C for 45 s; with a final extension of 72 °C for 10 min. In all cases negative controls were included to identify possible contamination.

The PCR products were checked on a 2% agarose gel stained with ethidium bromide. Successful amplifications were purified in 25 μl reactions containing 21 μl PCR product, 16.8 U of Exonuclease I (New England Biolabs, Ipswich, MA, USA) and 1.68 U of Shrimp Alkaline Phosphatase (USB Corporation, Staufen, Germany) incubated at 37 °C for 15 min followed by 80 °C for 15 min. Both strands of each PCR product were sequenced with BigDye (Applied Biosystems) according to manufacturer's recommendations with the same primers as used for amplification. Sequencing reactions were separated and analyzed on an automated ABI 3730xl DNA Analyzer (Applied Biosystems).

In order to control for possible apparent mutations caused by DNA damage, each fragment from all historical specimens was amplified in at least two independent reactions and sequenced. The sequence fragments were checked, edited and concatenated in Sequencher 4.6 (Gene Codes Corporation, Ann Arbor, MI, USA) into one sequence that was used in phylogenetic and molecular dating analyses.

2.3. Phylogenetic analyses

The Akaike Information Criterion (AIC), as implemented in the program Modeltest version 3.7 (Posada and Crandall, 1998), was used to infer the most appropriate models of sequence evolution for both the whole data set and the codon-partitioned data set. The base composition among the variable sites of the ingroup was tested with a χ^2 test as implemented in PAUP* version 4.10b (Swofford, 2002). To investigate if saturation affects our data set, the Maximum Likelihood corrected distances were plotted against both the 3rd codon uncorrected transversion distances and 3rd codon uncorrected transition distances.

Different methods of phylogenetic inference were used to construct phylogenies: maximum parsimony (MP), maximum likelihood (ML) and Bayesian analyses. The MP and ML analyses were conducted with PAUP* version 4.10b. We used a heuristic search algorithm with a 1000 additional sequence replicates with random sequence addition and tree bisection and reconnection (TBR) for the MP analysis. The MP bootstrap (1000 replicates) analysis was performed using a heuristic search with 1000 additional sequence replicates with random sequence addition and TBR for each bootstrap replicate.

The ML analysis was performed with a heuristic search and 100 additional sequence replicates with random sequence addition and TBR and the model based on the whole data set selected by Modeltest. ML bootstrap (100 replicates) used 10 additional sequence replicates with random sequence addition and TBR for each bootstrap replicate.

MrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003) was used for the Bayesian inference. Bayesian analyses were performed both with and without codon partitioning and for the partitioned data set it was replicated twice. Four heated chains were used that ran for 5 million generations, at which point the average standard deviation of split frequencies approached zero (<0.0001). Trees were sampled every 100 generations and the first 25% were discarded as burnin.

2.4. Molecular dating

We first tested if the cytochrome *b* sequences evolved under a molecular clock by comparing the ML scores of the unconstrained tree versus the ML tree constrained with a molecular clock. No significant difference was found. Therefore, branch lengths of the molecular clock were estimated with the baseml package in PAML version 4.1 (Yang, 2007). Age estimates for the different nodes were calculated by calibrating the root of the tree with an age estimate of 10 million years ago (MYA) that corresponds with the approximate split between the outgroup *Callosciurus* and the ingroup *Sundasciurus* (Mercer and Roth 2003). Age estimates were also calculated in a Bayesian framework as implemented in the mcmctree package included in PAML version 4.1, using an age interval between 7 and 11 MYA for the root of the tree.

Irwin et al. (1991) have shown that generally in mammals 3rd codon transversions in cytochrome *b* evolve at around 0.5% per million year and Harrison et al. (2003) found the same value for ground squirrels. However, substitution rates in rodents may be much higher than other mammals (e.g. Spradling et al., 2001). To compare our rate estimates with other rate estimates in rodents, cytochrome *b* sequence data from two different studies in which rates were calibrated based on fossil information, were downloaded from GenBank (*Apomys*, Steppan et al., 2003 and *Rattus*, Robins et al., 2008). In both cases the data evolved under a (nearly) molecular clock. The age estimates in millions of years of the different nodes were plotted against the 3rd codon transversion distances and linear regression lines were plotted through the data points and forced through the origin. The slope values constitute an estimate of the rate of 3rd codon transversions per million year and these values were used to obtain age estimates based on the 3rd codon transversion distances from our study.

3. Results

Twenty-one of the 61 museum specimens did not produce a positive PCR amplification, 19 yielded partial sequences and 21

Table 1
List of all specimens that were used in this study with their museum numbers, location, type of tissue used and whether they produced complete, partial or no cytochrome *b* sequence data.

#	Genus	Species	Subspecies	Location	Museum ID[a]	Tissue	Sequence
1	*Sundasciurus*	*steerii*		Balabac Island	USNM 477964	Skin	Partial
2	*Sundasciurus*	*steerii*		Palawan	USNM 477948	Skin	Complete
3	*Sundasciurus*	*steerii*		Palawan	RMNH 38321	Skin	Partial
4	*Sundasciurus*	*moellendorffi*		Culion Island	USNM 477947	Skin	Complete
5[b]	*Sundasciurus*	*moellendorffi*		Culion Island	USNM 477946	Skin	Complete
6[b]	*Sundasciurus*	sp.		Ikadwang Banwa Island	FMNH 195286	Fresh	Complete
7[b]	*Sundasciurus*	sp.		Ikadwang Banwa Island	FMNH 195287	Fresh	Complete
8	*Sundasciurus*	sp.		Ikadwang Banwa Island	FMNH 195288	Fresh	Complete
9	*Sundasciurus*	sp.		Ikadwang Banwa Island	DSB 5065	Fresh	Complete
10	*Sundasciurus*	*juvencus*		Palawan	USNM 477865	Skin	Partial
11	*Sundasciurus*	*juvencus*		Palawan	USNM 477866	Skin	Complete
12	*Sundasciurus*	*hoogstraali*		Busuanga Island	USNM 477851	Skin	Complete
13	*Sundasciurus*	*hoogstraali*		Busuanga Island	USNM 477855	Skin	Partial
14	*Sundasciurus*	*rabori*		Palawan	FMNH 195285	Fresh	Complete
15[b]	*Sundasciurus*	*rabori*		Palawan	USNM 477985	Skin	Complete
16	*Sundasciurus*	*rabori*		Palawan	USNM 477986	Skin	Partial
17	*Sundasciurus*	*rabori*		Palawan	USNM 477988	Skin	0
18	*Sundasciurus*	*mindanensis*		Dinagat Island	USNM 462199	Skin	Complete
19	*Sundasciurus*	*mindanensis*		Dinagat Island	USNM 462200	Skin	Partial
20	*Sundasciurus*	*philippinenesis*		Mindanao	191420	Fresh	Complete
21	*Sundasciurus*	*philippinenesis*		Mindanao	194781	Fresh	Complete
22	*Sundasciurus*	*philippinenesis*		Basilan Island	USNM 239214	Skin	Partial
23	*Sundasciurus*	*philippinenesis*		Mindanao	RMNH 38320	Skin	Partial
24	*Sundasciurus*	*samarensis*		Leyte Island	USNM 458736	Skin	Complete
25	*Sundasciurus*	*samarensis*		Samar Island	USNM 105464	Skin	0
26[b]	*Sundasciurus*	*samarensis*		?	ear 1411	Fresh	Complete
27	*Sundasciurus*	*samarensis*		?	ear 1306	Fresh	Complete
28	*Sundasciurus*	*hippurus*	*hippurus*	Malay Peninsula	USNM 488406	Skin	Complete
29	*Sundasciurus*	*hippurus*	*hippurus*	Malay Peninsula	USNM 488407	Skin	Partial
30	*Sundasciurus*	*hippurus*	*hippurosus*	Sumatra	USNM 141032	Bone	Complete
31	*Sundasciurus*	*hippurus*	*hippurosus*	Sumatra	USNM 141033	Skin	0
32	*Sundasciurus*	*hippurus*	*hippurosus*	Sumatra	USNM 143399	Skin	0
33	*Sundasciurus*	*hippurus*	*inquinatus*	Borneo	USNM 300958	Skin	Partial
34	*Sundasciurus*	*hippurus*	*inquinatus*	Borneo	USNM 300957	Skin	Partial
35	*Sundasciurus*	*hippurus*	*hippurellus*	Borneo	USNM 142272	Skin	0
36	*Sundasciurus*	*hippurus*	*hippurellus*	Borneo	USNM 142273	Bone	Complete
37	*Sundasciurus*	*hippurus*	*borneensis*	Borneo	USNM 311463	Skin	Complete
38	*Sundasciurus*	*hippurus*	*borneensis*	Borneo	USNM 154293	Skin	0
39	*Sundasciurus*	*hippurus*	*pryeri*	Borneo	USNM 19166	Skin	0
40	*Sundasciurus*	*hippurus*	*pryeri*	Borneo	USNM 19222	Skin	0
41	*Sundasciurus*	*tenuis*	*tenuis*	Malay Peninsula	USNM 488458	Skin	Complete
42	*Sundasciurus*	*tenuis*	*tenuis*	Malay Peninsula	USNM 488459	Skin	Complete
43	*Sundasciurus*	*tenuis*	*tenuis*	Sumatra	USNM 114542	Skin	0
44	*Sundasciurus*	*tenuis*	*tenuis*	Sumatra	USNM 114543	Skin	0
45	*Sundasciurus*	*tenuis*	*tenuis*	Sumatra	MVZ 192196	Fresh	Complete
46[b]	*Sundasciurus*	*tenuis*	*tenuis*	Sumatra	MVZ 192195	Fresh	Complete
47	*Sundasciurus*	*tenuis*	*parvus*	Borneo	USNM 198746	Skin	Partial
48	*Sundasciurus*	*tenuis*	*parvus*	Borneo	USNM 145386	Skin	0
49	*Sundasciurus*	*tenuis*	*parvus*	Borneo	FMNH 35967	Fresh	Complete
50	*Sundasciurus*	*tenuis*	*tahan*	Malay Peninsula	USNM 87085	Skin	Complete
51	*Sundasciurus*	*tenuis*	*tahan*	Malay Peninsula	USNM 311334	Skin	Partial
52	*Sundasciurus*	*tenuis*	*tahan*	Malay Peninsula	USNM 311333	Skin	Partial
53	*Sundasciurus*	*tenuis*	*altitudinus*	Sumatra	USNM 271038	Skin	Partial
54	*Sundasciurus*	*tenuis*	*altitudinus*	Sumatra	USNM 271037	Skin	Complete
55	*Sundasciurus*	*tenuis*	*altitudinus*	Sumatra	BMNH	Skin	0
56	*Sundasciurus*	*tenuis*	*altitudinus*	Sumatra	BMNH	Skin	Complete
57	*Sundasciurus*	*lowii*	*vanakeni*	Sumatra	BMNH	Skin	0
58	*Sundasciurus*	*lowii*	*vanakeni*	Sumatra	BMNH	Skin	0
59	*Sundasciurus*	*lowii*		Malay Peninsula	USNM 488447	Skin	Complete
60	*Sundasciurus*	*lowii*		Malay Peninsula	USNM 488451	Skin	Partial
61	*Sundasciurus*	*lowii*	*natunensis*	Sirhassen Island	USNM 104688	Skin	0
62	*Sundasciurus*	*lowii*	*natunensis*	Sirhassen Island	USNM 104689	Skin	0
63	*Sundasciurus*	*lowii*	*natunensis*	Sirhassen Island	USNM 104690	Skin	0
64	*Sundasciurus*	*lowii*		Borneo	NZP 93-434	Fresh	Complete
65[b]	*Sundasciurus*	*lowii*		Borneo	NZP 94-208	Fresh	Complete
66[b]	*Sundasciurus*	*lowii*		Borneo	NZP 96-546	Fresh	Complete
67[b]	*Sundasciurus*	*lowii*		Borneo	NZP 96-560	Fresh	Complete
68	*Sundasciurus*	*lowii*		Borneo	FMNH 35908	Fresh	Complete
69[b]	*Sundasciurus*	*lowii*		Borneo	FMNH 35935	Fresh	Complete
70	*Sundasciurus*	*jentinki*		Borneo	USNM 292576	Skin	Complete
71	*Sundasciurus*	*jentinki*		Borneo	USNM 292578	Skin	Partial
72	*Sundasciurus*	*brookei*		Borneo	USNM 488399	Skin	Complete
73	*Sundasciurus*	*brookei*		Borneo	USNM 488400	Skin	Partial
74	*Sundasciurus*	*brookei*		Borneo	USNM 300961	Skin	0
75	*Sundasciurus*	*fraterculus*	*siberu*	Siberut Island	USNM 252344	Skin	0

Table 1 (*continued*)

#	Genus	Species	Subspecies	Location	Museum ID[a]	Tissue	Sequence
76	*Sundasciurus*	*fraterculus*	*pumilus*	South Pagi Island	USNM 121628	Bone	Complete
77	*Sundasciurus*	*fraterculus*	*fraterculus*	Sipora Island	USNM 252349	Skin	0
78	*Sundasciurus*	*fraterculus*	*fraterculus*	Sipora Island	USNM 252347	Skin	0
79	*Sundasciurus*	*fraterculus*	*siberu*	Siberut Island	USNM 252339	Skin	Partial

[a] Acronyms: BMNH = British Museum of Natural History (now, the Natural History Museum), London; DSB, EAR = uncatalogued specimens from the FMNH; FMNH = Field Museum, Chicago; MVZ = Museum of Vertebrate Zoology, Berkeley; NZP = National Zoological Park (Smithsonian Institution), Washington, DC; RMNH = Rijksmuseum van Natuurlijke Historie (now, National Museum of Natural History 'Naturalis'), Leiden; USNM = US National Museum (Smithsonian Institution), Washington, DC.

[b] Individuals that differed less than 1% in uncorrected pairwise distance with the same taxon and therefore were left out in the phylogenetic analyses.

yielded the complete 1075 bp fragment of cytochrome *b* used in these analyses (Table 1). No differences were found in the sequences of the multiple replicates amplified from individual historic specimens. All fresh tissue samples were amplified and sequenced successfully. The alignment was unambiguous due to the coding nature of the cytochrome *b* gene. Within the ingroup, the distribution of variable sites over the three codon positions showed that most variable sites were at 3rd codon positions ($n = 334$ or 77.8%) followed by the 1st codon position ($n = 75$ or 17.5%) leaving the 2nd codon position with only 20 variable sites (4.7%).

Table 2
Primers used in this study with the 5′ position number that refers to the character position in the cytochrome *b* gene sequence from the complete mtDNA sequence of *Sciurus vulgaris* (cytochrome *b* from 14167–15306; GenBank Accession No. NC_002369; Reyes et al., 2000).

		Sequence 5′–3′	5′ Position
Forward primers			
1	L14724	GAT ATG AAA AAC CAT CGT TG	14126
2	Steerii fwd1	ATG ACA AAC ATT CGC AAA ACT CAC CC	14167
3	UniCB51	CCA ACA TCC GTA AAT CCC ACC C	14171
4	Phil fwd1	ATC CTA ACH GGR CTC TTC CT	14299
5	sqCB53	GGA CTC TTC CTA GCC ATA CA	14308
6	Phil fwd2	TAC ATA CAY GCC AAC GGC GC	14407
7	L15045	GCC TAT TCC TAC ATG TAG GAC G	14444
8	L15162Irw	GCA AGC TTC TAC CAT GAG GAC AAA TAT C	14555
9	L15200	GTC ATT ACC AAC CTC CTA TC	14599
10	Phil fwd3	TCA GCW ATC CCY TAY RTC GG	14617
11	Phil fwd4	GTC GAC AAA GCA ACC CTC ACA CG	14674
12	Alt fwd1	GTY CAT CTC CTA TTY CTW CAY GA	14749
13	Palawan fwd1	CTC TTT CTT CAC GAA ACC GGA TC	14758
14	L15370	CAC GAA ACA GGA TCA AAC AAC C	14767
15	Phil fwd5	AAC AAC CCA TCT GGT ATT ATC TC	14782
16	Alt fwd2	TCC GGY CTW ATC TCG GAC TC	14791
17	Rabori fwd1	TCT GGC CTA ATC TCT GAC TCC	14791
18	Phil fwd6	CTA GCC TTT ATA ATG CTA GT	14875
19	Rabori fwd2	GCC TTT ATA ATG CTA GTC CTC TTT TC	14878
20	L15506	CCT GAT CTC CTA GGA GAT CC	14905
21	L15513Ir	CTA GGA GAC CCT GAC AAC TA	14914
22	UniCb33	GGT ACT TCC TAT TTG CCT ACG	14981
23	L15878	CAC ACA TCA AAA CAA CGA AGC AT	15088
24	Phil fwd7	ACA CTW ACC TGR ATC GGA GG	15166
25	CarnCB31	GAA TTG GTG GAC AAC CAG TAG AA	15176
Reverse primers			
1	Phil rev208	GGC YGT WAG WGT YTC TGA TG	14352
2	CarnCB52	CCG TAG TTA ACG TCT CG GCA	14393
3	Steerii rev1	TCA GCC ATA GTT TAC GTC TCG GC	14397
4	Phil rev1	GCC TAC GTG GAG GAA GAG GC	14463
5	sqCB58	CCG ATG TTT CAT GTT TCG AA	14513
6	Steerii rev2	ACC CAT GAA TGC TGT GGC TAT TAC GG	14556
7	Phil rev2	TAT TTG MCC TCA TGG GAG GAC	14580
8	sqCB56	CCT CCT CAG ATT CAT TCT AC	14666
9	Phil rev3	TTC ATG AAG GAA TAG TAG GT	14772
10	Phil rev4	GTG GAA GGG AAT TTT GTC GG	14829
11	H15417	AGT AGG GGT GGA AAG GGA TTT	14836
12	Phil rev5	GTC TCC TAA GAG GTC AGG GG	14922
13	H15494	TAG TTG TCA GGG TCT CCT AG	14933
14	Alt rev1	TGT GTA ATT ATC GGG GTC TCC	14937
15	Palawan rev1	TGG CTT GAT ATG GGG AGG GGT GT	14976
16	H15683	CGA AGG ATA GCG TAG GCG AA	15011
17	Alt rev2	TCC RCC TAA TTT GTT MGG GAT TG	15036
18	Phil rev6	GAC TCC TCC CAG TTT GTT TG	15039
19	Rabori rev1	TTG TTT GGA TAA ATG GAG GGT AG	15102
20	H15509	CAT TGG CTT AGA GGT CGG AA	15134
21	CarnCB32	GGA GAA GTA TAG GAT TGA GGC TAG	15247
22	Phil rev7	TTA TAC TAT TAG GGC GTC	15321
23	Rabori rev2	CGT TAT ACT ATT AGG GCA TC	15323
24	H15910	GAT TTT TGG TTT ACA AGA CCG AG	15352

Three of the nuclear loci failed to amplify in all cases (*Fib6*, *POR* and *GBG*). It was not possible to get clean homologous sequences for three loci due to clear double signals likely due to the amplification of multiple loci in two cases (*FTH1* and *HMG1*) and possible double signal and poly G in the third (*AP5*). The locus *c-myc* was almost invariant within the genus. For these reasons no nuclear loci were amplified in all taxa or included in phylogenetic analyses.

Nuclear copies of mitochondrial DNA fragments (numts) of variable lengths were found in several species. They were identified by incongruence in base pair calling in the overlapping regions from the different PCR fragments, the presence of heterozygote bases in the electropherogram and/ or the presence of indels in the numt fragment. Further, the concatenated sequences were translated to identify inappropriate stop codons or other amino acid changes. All nuclear copies were excluded from all analyses.

In cases where multiple individuals from the same taxon and locality had an identical haplotype or an uncorrected pairwise sequence difference of less than 1%, only one individual was used in the phylogenetic analyses (see Table 1). The final dataset consisted of 29 ingroup and four outgroup sequences of 1075 base pairs (bp) of cytochrome *b*. Of these 1075 bp, 609 characters were constant, 63 variable but parsimony uninformative and 403 were parsimony informative.

The base composition of the variable sites was significantly different among the ingroup taxa ($\chi^2 = 120.55$, df = 84, $p = 0.0055$). This was solely due to differences at the 3rd codon positions. Saturation seemed to be present in our data set, especially for 3rd codon transitions (Fig. 2). To circumvent possible problems with differences in base composition and saturation, all phylogenetic analyses were also performed with only transversions at the 3rd codon position (RY coding). With this coding scheme the base composition of variable sites was not significantly different among the ingroup taxa (Chi square = 79.14; df = 84; $p = 0.63$) and also 3rd codon transversions seemed not to be saturated (Fig. 2). When using RY coding, 800 characters were constant, 72 variable but parsimony uninformative and 203 were parsimony informative. No substantial differences were observed in support values between the analyses based on the original data and the RY coded data set, so only results based on the original data are discussed further.

Three most parsimonious trees were found (tree length = 1865; CI = 0.361; RI = 0.625; and RC = 0.226). Few of the deep nodes had bootstrap support greater than 70% (Fig. 3). The ML analysis produced one tree (model used TVM + I + G with I = 0.5788 and alpha shape = 2.7626; tree score −ln = 8866.60685) and it was not in conflict with the MP bootstrap tree, although the ML tree was slightly more resolved. In the Bayesian analyses, partitioning of the data by codon position improved the posterior probabilities substan-

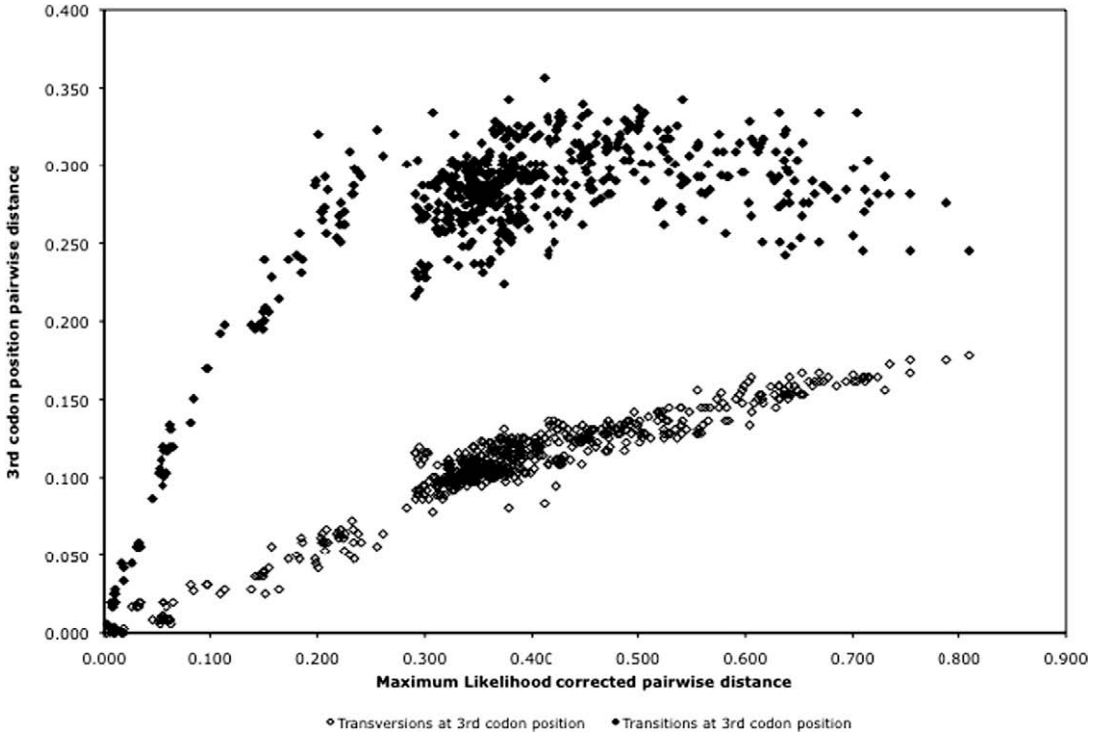

Fig. 2. Graph to illustrate the near saturation-free 3rd codon transversion distance versus the heavily saturated 3rd codon transitions. Both the uncorrected 3rd codon transversion distance and the uncorrected 3rd codon transition distance are plotted against the ML corrected distance based on the complete sequences (1075 bp). The evolutionary model used to calculate the ML distances is the same one that was used for estimating the ML tree topology.

tially, as compared with a non-partitioned data set. The three different phylogenetic methods used produced trees that were not in conflict with each other. The Bayesian analyses yielded more significantly supported deep nodes than the other analyses (Fig. 3).

3.1. Phylogenies

The subgenus *Sundasciurus*, as defined by Moore (1958) and comprising the species *S. tenuis, S. jentinki, S. brookei*, *S. lowii* and *S. fraterculus*, was not monophyletic. However, the clade including the species of the subgenus *Aletesciurus* (including *S. hippurus*, all Palawan taxa and Greater Mindanao taxa) was monophyletic. These subgenera were defined based on size and the presence of a sagittal crest.

3.2. The S. tenuis group

Together with the widespread *S. tenuis*, two Borneo endemic species, *S. brookei* and the montane *S. jentinki*, form a monophyletic clade (ML bootstrap 91%, posterior probability of 1). The relationships within this clade are poorly resolved. Only the basal placement of *S. brookei* is supported by the Bayesian analysis (posterior probability of 0.94). The relationship of *S. jentinki*, with the montane populations of *S. tenuis* or the lowland forms remained unclear.

3.3. Lowland populations of S. tenuis

Deep divergences were identified between the populations of *S. tenuis* on the Malay Peninsula, Sumatra and Borneo. The Malay samples differed by 2.5–2.8% at 3rd codon transversions from the Borneo *S. tenuis*, and the Sumatra *S. tenuis* differed by 4.85% with Malay *S. tenuis* and 5.6% with the Borneo sample. No support for monophyly of the three different lowland *S. tenuis* populations was found. A sister relationship between Borneo and Malay Peninsula *S. tenuis* was supported (MP bootstrap <50%, ML bootstrap 87% and posterior probability of 1).

3.4. Highland/lowland divergence within S. tenuis

Two highland subspecies of *S. tenuis* (*S. t. altitudinis* from Sumatra and *S. t. tahan* from the Malay Peninsula) showed considerable genetic divergence between each other and the other *S. tenuis* populations, as measured by 3rd codon TV distances (6.1–6.4% between *S. t. tahan* and *S. tenuis* from Malay Peninsula and 6.7–7.2%. between *S. t. altitudinis* and *S. tenuis* from Sumatra). Even more surprising was the large difference between the two *S. t. altitudinis* samples from North and Central Sumatra (3.9%). The two different montane subspecies (*S. t. tahan* and *S. t. altitudinis*) formed a monophyletic clade (MP bootstrap <50%, ML bootstrap 82%, posterior probability of 1).

3.5. Colonization of the Philippines

All analyses identified two well-supported and distinct Philippine clades. One included all taxa from the Palawan Islands (*S. rabori*, *S. steeri*, *S. hoogstraali*, *S. moellendorffi*, *S. juvencus* and two haplotypes derived from 4 all-white animals from Ikadwang Banwa Island) and their monophyly had strong support (MP bootstrap 100%, ML bootstrap 97%, posterior probability 1). The highland species *S. rabori* from Palawan was consistently basal to all other taxa in the clade (MP bootstrap 100%, ML bootstrap 97%, posterior probability 1). The taxa occurring in the Palawan and nearby islands (*S. juvencus*, *S. hoogstraali*, *S. moellendorffi* and the white squirrels from a previously undescribed population on Ikadwang Banwa Island of an unknown species) showed very little genetic differentiation (uncorrected 3rd codon TV 0–0.3% and uncorrected *p* distances less than 1%). The 3rd codon TV differences between *S. rabori* and the other Palawan taxa ranged from 3.6% to

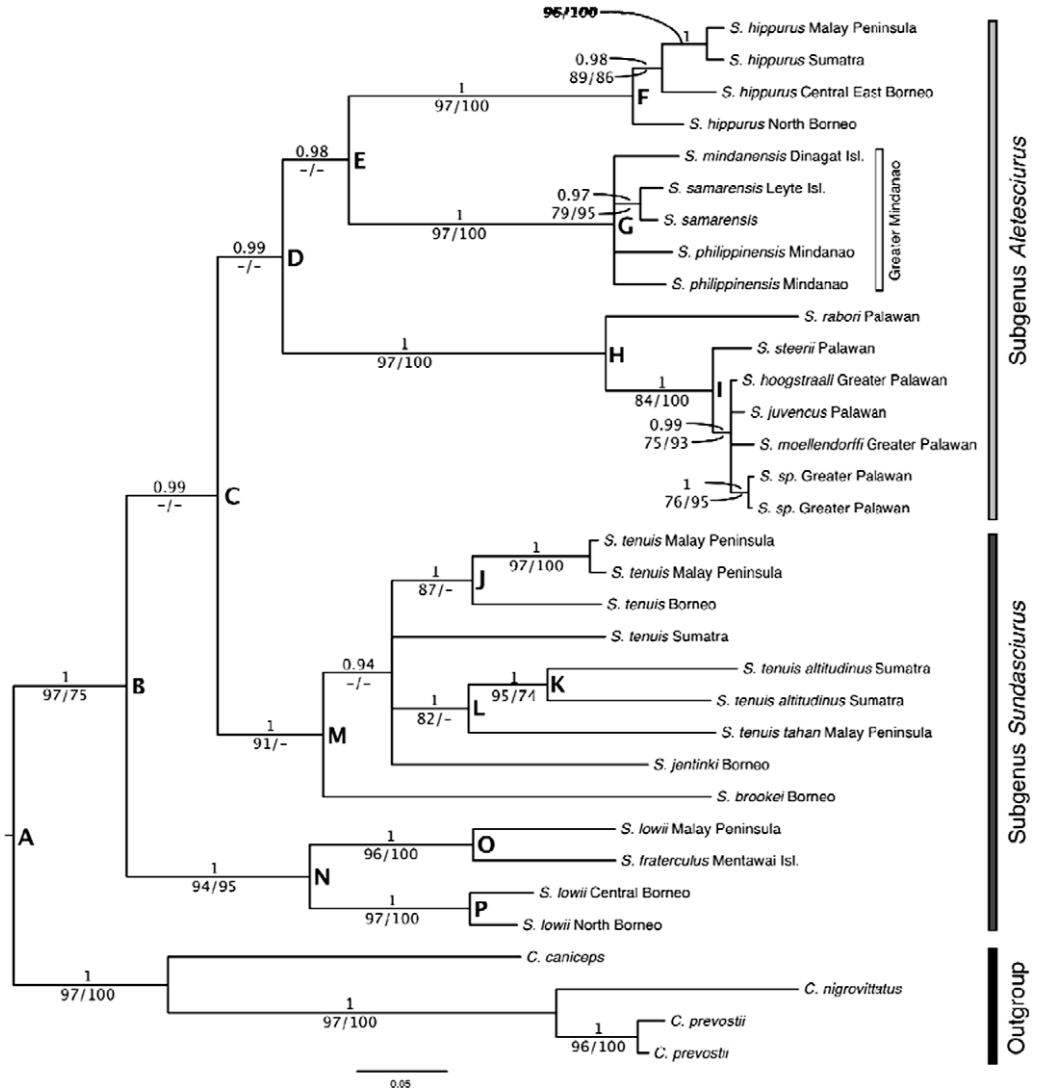

Fig. 3. Phylogenetic tree of *Sundasciurus* squirrels based on the Bayesian analysis using a codon partitioned model. Above the branches the corresponding Bayesian posterior probability is given and below the branch first the ML bootstrap value followed by the MP bootstrap value. The letters refer to the node age estimates as tabulated in Table 3.

4.2% and was 1.7% to 1.9% between *S. steeri* and the others from the Palawans.

The other Philippine clade consisted of all sampled taxa from Greater Mindanao (*S. mindanensis*, *S. samarensis* and *S. philippinensis*: MP bootstrap 100%, ML bootstrap 97% and posterior probability of 1). Within this clade, only a sister relationship between the two *S. samarensis* individuals was recovered, leaving relationships between the other taxa unresolved despite on average an uncorrected 3rd codon transversion distance of 1.4%.

The relationship between the two Philippine clades was unresolved in the MP and ML analysis; however, the Bayesian analyses showed a well-supported branching pattern in which the two Philippine clades were not sisters to each other. The Greater Mindanao clade was sister to the widespread *S. hippurus* clade (posterior probability of 0.98) and the Greater Palawan clade was basal to this group (posterior probability of 0.99).

3.6. Widespread species S. hippurus and S. lowii

The widespread species *S. lowii* is paraphyletic because *S. fraterculus* is sister to the Malay Peninsula *S. lowii* sample (MP bootstrap value 100%, ML bootstrap value 96% and Bayesian posterior probability of 1). This clade was sister to the two Borneo *S. lowii* squirrels, from Sabah and Kalimantan (MP bootstrap 95%, ML bootstrap 94%, posterior probability of 1). The Borneo samples claded together with high support (MP bootstrap 100%, ML bootstrap 97%, posterior probability 1).

Divergence within *S. lowii*, as measured by uncorrected transversions at 3rd codon positions, showed unexpectedly high values between the Malay Peninsula and Borneo samples (5.8%). Within Borneo this difference was 0.6% and *S. fraterculus* differed by 2.8% with the Malay Peninsular sample and 4.7–5.8% with the Borneo samples. Malay *S. lowii* and *S. fraterculus* are morphologically distinguishable by the color of the belly (Malayan *S. lowii* are white and *S. fraterculus* are gray).

The widespread species *S. hippurus* was monophyletic (MP bootstrap 100%, ML bootstrap 97% and Bayesian posterior probability of 1) with both Borneo samples (*S. h. borneensis* and *S. h. hippurellus*) basal to the Malay-Sumatra clade (MP bootstrap 86%, ML bootstrap 89%, posterior probability of 0.98). The divergences between the populations were also much smaller. The uncorrected pairwise TV 3rd codon difference within Borneo was 0.6% and between Sumatra and Malay 0%. Between the two Borneo and Malay-Sumatra samples this difference was on average 0.7%.

3.7. Molecular dating

No significant difference in ML tree scores from constrained and unconstrained trees was identified, so the molecular clock could not be rejected for our data ($\chi^2 = 35.18$, df = 31, $p = 0.2767$). Most speciation events predate the Pleistocene (Table 3 and Fig. 4).

The montane subspecies *S. tenuis tahan* and *S. tenuis altitudinis* show remarkably deep divergences (nodes K and L in Fig. 3). Also the populations of *S. lowii* from the Malay Peninsula and Borneo diverged in the Pliocene (node N). Only the lowland species from the Palawan Islands (node I) and Greater Mindanao taxa (node G) diverged in the Pleistocene.

The age estimate of the Palawan Islands clade (node D at 6.9 MYA) is substantially older than the geological age estimate of the Palawan Islands (5 MYA; Hall, 1998; Heaney, 1986; Steppan et al., 2003). The Bayesian age estimates using a root calibration interval of 7–11 MYA were comparable with the clock estimates with an average age estimate for the Palawan clade at 7 MYA (Table 3). The age estimates lowered overall when the clock data were calibrated with (1) a maximum age for the Palawan clade of 5 MY or (2) the Bayesian analysis calibrated with the root between 7 and 11 MYA and a maximum age for the Palawan clade of 5 MY (Table 3). Nevertheless, most divergence date estimates were pre-Pleistocene (Fig. 4). Four splits were estimated to be around the end of the Pliocene or the beginning of the Pleistocene, namely the Palawan montane species *S. rabori* from the Palawan lowland taxa; the split between the lowland populations of *S. tenuis* from the Malay Peninsula and Borneo; the endemic species *S. fraterculus* from the Mentawai Islands (west of Sumatra and formerly treated as a subspecies of *S. lowii*) versus *S. lowii* from the Malay peninsula and finally the split within the montane subspecies *S. tenuis altitudinis* from north and central Sumatra.

When estimating the node ages based on uncorrected 3rd codon transversion distances under the assumption of the molecular clock we used an average of 1.85% per MY based on the data of Steppan et al. (2003) and Robins et al. (2008). Most age estimates correspond well to estimates based on other analyses using a calibration of Palawan at 5 MYA. Only the Palawan clade (node D) is, again, older than the geological age estimate.

Table 3
Age estimates in millions of years (MY) for the different nodes using different methods and calibration points. The node letters correspond with the letters in Fig. 3. The columns labeled 'Clock' are estimates using a molecular clock with the root set at 10 MYA (root = 10) or the Palawan group set at 5 MYA (Palawan = 5). The two Bayesian estimates were made either by constraining the root to between 7 and 11 MYA or by also constraining the Palawan clade to less than MYA. Estimates based on the rate of third codon transversions using the rate of 1.85% per million years as estimated in other rodents, is labeled 1.85% per MY.

Node	Clock		Bayesian		% TV 3rd codon 1.85% per MY
	Root = 10	Palawan = 5	7 < root < 11	Palawan < 5; 7 < root < 11	
I	0.63	0.46	1.10	0.71	0.97
P	1.16	0.84	1.81	1.34	0.32
G	1.24	0.90	1.76	1.28	0.76
F	1.41	1.02	2.10	1.49	0.38
J	2.64	1.92	2.90	2.10	1.41
O	2.82	2.04	3.14	2.37	1.51
H	2.90	2.10	3.44	2.28	2.05
K	3.10	2.25	2.65	2.05	2.11
L	4.11	2.98	3.74	2.81	2.86
N	5.07	3.68	5.95	4.43	2.92
M	6.12	4.44	6.31	4.66	3.78
E	6.33	4.59	5.88	4.14	5.08
D	6.90	5.00	7.03	4.93	6.38
C	7.47	5.42	7.69	5.57	5.78
B	8.41	6.10	8.81	6.58	6.27
A	10.00	7.25	9.23	7.27	7.89

4. Discussion

4.1. Pleistocene species pump

Regardless of the molecular clock calibration, almost all speciation events predate the Pleistocene. The divergences within the widespread species *S. lowii* and *S. tenuis* also likely predate the

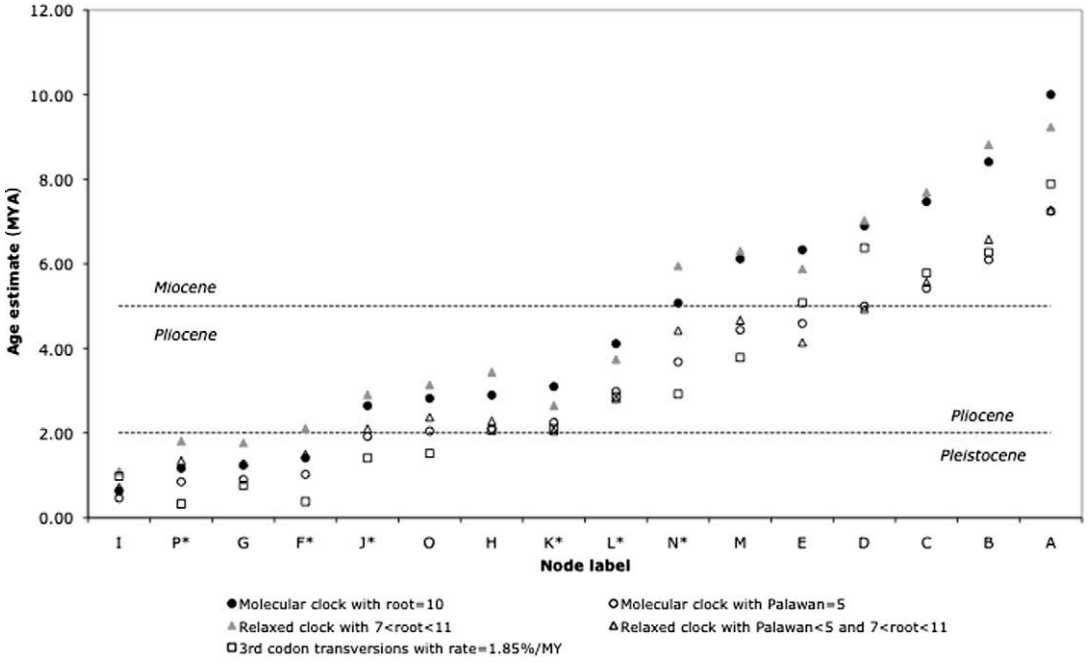

Fig. 4. Plot of age estimates for the different nodes to illustrate the effect of using different methods and calibration points. Nodes marked with an asterisk are intra-specific splits. When the root is calibrated at 10 MYA (Mercer and Roth, 2003) or around an interval of 7–11 MYA (as in the Bayesian analysis), the age estimate for the Palawan clade (node D) is in disagreement with the age estimate of Palawan based on geological data.

Pleistocene. Therefore, Pleistocene climatic and sea level fluctuations were not responsible for much of the diversification in this group. The bats, rodents and primates in which divergence estimates have been found to be consistent with Pleistocene differentiation are perhaps better able to disperse across non-forest habitat, whereas the tree squirrels are quite tightly associated with the forest. This may support the hypothesis that, although the islands and Malay peninsula were connected by land in the Pleistocene, the exposed shelf was much drier and may not have supported extensive forest habitat. The various lowland species of the Philippine Islands are the exception, and likely do have a recent origin. Pleistocene age isolation due to sea level fluctuations could have driven speciation in this system.

4.2. Philippines

The Philippine taxa did not form a monophyletic group. The taxa from the Palawan Islands and the taxa from Greater Mindanao each formed well-supported monophyletic groups. This indicates that the Palawan Islands and Greater Mindanao were independently colonized each one time. The colonization of Greater Mindanao, which was never connected to Borneo by land (Musser and Heaney, 1985), likely took place during the Pliocene. The colonization of the Palawan Islands likely occurred earlier than the colonization of Greater Mindanao. During the Pleistocene the various islands within these two areas were connected and isolated various times, leading to the possibility of a local Pleistocene species pump. All of the lowland species in both clades, including the all-white squirrels, appear to have diverged in the Pleistocene, supporting this hypothesis in these two island groups.

4.3. Highland forms

Within the widespread species *S. tenuis* several subspecies have been named (Corbet and Hill, 1992; Thorington and Hoffmann, 2005). Some of these subspecies are isolated in high altitude areas both on the Malay Peninsula (*S. t. tahan*) and Sumatra (*S. t. altitudinis*). There has been disagreement about the status of *S. t. tahan*, which was originally described as a distinct species by Bonhote (1908) but placed in the species *S. tenuis* by Chasen (1940). These data find a sister relationship between *S. t. tahan* and *S. t. altitudinis* but indicate that they may not form a monophyletic clade with the other populations of *S. tenuis* sampled. They are likely more closely related to the high attitude species *S. jentinki* from Borneo, than to the lowland populations of *S. tenuis* from the Malay Peninsula and Sumatra. The divergence of the highland forms from the lowland forms is clearly pre-Pleistocene, and the divergence between the two highland forms (*S. t. tahan* and *S. t. altitudinis*) is also pre-Pleistocene. Even the two populations of *S. t. altitudinis* sampled were highly differentiated, and probably diverged around the Pliocene/Pleistocene boundary. Despite a high level of morphological conservation across the genus, there are morphological characters that define these two high altitude taxa (Miller, 1942; Bonhote, 1908). The morphological, ecological and genetic differentiation between these taxa supports resurrecting the status of each as a valid species. A revision of the taxonomy of the group is beyond the scope of this paper, and will be dealt with in a separate publication.

Although some differentiation of the high altitude taxa was recognized by naming them as separate species and subspecies, the very deep divergences both between high and low altitude taxa and between the high altitude taxa, was unexpected. High divergence of high altitude taxa on the Sunda shelf has previously been identified in forest dependent ants (Quek et al., 2007). This may suggest a general biogeographical pattern of ancient diversity in the high altitude forests of Sundaland, and a common Pliocene origin for that diversity.

4.4. Conservation implications

The pattern of divergence between species and populations, especially the high altitude ones, was unexpectedly deep. The range of some of the taxa, especially the high altitude taxa, may be quite limited. This, in combination with rapid development, may place these taxa at risk of extinction. The high altitude taxa *S.* (*t.*) *tahan* may already be extirpated in one locality (Gunung Ulu Kali, Malaysia), as the mountain top where it was collected has since been developed into a hotel-resort, and these taxa are highly habitat sensitive.

Although more work is required to determine the specific status of some of the divergent populations identified in this study, the deep genetic divergence alone qualifies many of these populations as Evolutionary Significant Units (ESU) *sensu* Moritz (1994). This region is under particularly high population pressure (Cincotta et al. 2000), and this puts a lot of pressure on the native forests and the species dependent upon them.

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