Mitochondrial DNA evidence reveals genetic difference between Perny's long-nosed squirrels in Taiwan and Asian mainland

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Short communication

Mitochondrial DNA evidence reveals genetic difference between Perny's long-nosed squirrels in Taiwan and Asian mainland

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Abstract. Taiwan Island is thought to have formed around 6.0 million years ago, but, the formation of a 'proto-Taiwan' began around 9.0 million years ago. During the late Miocene, the proto-Taiwan is thought to have been a part of the Asian mainland. During the Pleistocene, Taiwan Island was again occasionally connected with the Asian mainland. Several mammal species migrated from the Asian mainland to Taiwan during Pleistocene glacial periods. Despite the connections between Taiwan and the Asian mainland, Taiwan has some endemic mammal species. Recent genetic evidence suggests that arboreal squirrels (*Petaurista alborufus*, *P. philippensis*, *Belomys pearsonii*, and *Tamiops maritimus*) adapted to Taiwan's mountainous forests are endemic species. Since these squirrels may have similarly evolved from separate ancestral stock in Taiwan, we investigated the phylogenetic relationship of Perny's long-nosed squirrel (*Dremomys pernyi*) from Taiwan and the Asian mainland by using mitochondrial cytochrome *b* gene sequences. The Taiwanese form was distantly related to the mainland form, showing deep genetic difference (10.18–10.88%). Therefore, *D. pernyi* could include at least two distinct species, with the Taiwanese form being a species endemic to Taiwan.

Key words: cytochrome *b*, *Dremomys pernyi owstoni*, molecular phylogeny, Taiwan.

Taiwan Island, located in the southern part of East Asia, is thought to have formed around 6.0 million years ago (Mya) (e.g., Yang 2001), but the beginning of the formation of a proto-Taiwan was during the late Miocene (9.0 Mya) (Sibuet and Hsu 2004). During the late Miocene, the proto-Taiwan is thought to have been a part of the Asian mainland (e.g., Kaito and Toda 2016). Taiwan Island was also sometimes connected with the Asian mainland during the Pleistocene (e.g., Huang 1984; Liu and Ding 1984). Several mammal species probably migrated from the Asian mainland to Taiwan during Pleistocene glacial periods, due to lowered sea levels (Yu 1995). Therefore, many mammal species are found in both Taiwan and the Asian mainland, such as the Pallas's squirrel *Callosciurus erythraeus*, the Asian black bear *Ursus thibetanus*, and the Siberian weasel *Mustela sibirica* (Corbet and Hill 1992). Taiwan also has endemic mammal species, such as the Formosan serow *Capricornis swinhoei* (Ci 1998).

Recent genetic evidence suggests that the three flying squirrel species occurring in Taiwan may be distinct species: the red and white giant flying squirrel *Petaurista alborufus* (Oshida et al. 2000c, 2004), the Indian giant flying squirrel *Petaurista philippensis* (Oshida et al. 2004; Yu et al. 2006), and the hairy-footed flying squirrel *Belomys pearsonii* (Oshida et al. 2015). Chang et al. (2011) also suggests that the Formosan striped squirrel *Tamiops maritimus* in Taiwan may be a distinct species,

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based on genetic data. These four arboreal squirrel species are found in the mountainous forests in Taiwan (Ci 1998). Since ancient pollen records indicated that a large part of the exposed Taiwan Strait was either grassland or barren during the last glacial maximum (Liew et al. 1998), arboreal squirrels adapted to mountainous forests are thought to have evolved in Taiwan. These squirrels could not have migrated from the Asian mainland to Taiwan across the treeless strait during the last glaciation of the Pleistocene (Oshida et al. 2015).

Taiwan has another mountainous squirrel species: the Perny's long-nosed squirrel *Dremomys pernyi* (e.g., Thorington et al. 2012). This species is distributed in northeastern India, northern Myanmar, China (Tibet, Sichuan, Yunnan, Guizhou, Hubei, Anhui, Hunan, Jiangxi, Gansu, southern Shaanxi, Zhejiang, Guangdong, and Fujian), northern Vietnam, and Taiwan (Thorington et al. 2012, Fig. 1). Currently, there are eight *D. pernyi* subspecies: *pernyi*, *calidior*, *flavior*, *howelli*, *imus*, *modestus*, *owstoni*, and *senex* (Thorington et al. 2012). The Taiwanese form, *D. pernyi owstoni*, is recognized as an endemic subspecies. It occurs in mountainous areas ranging from 1000 to 2800 m in elevation, which suggests adaptation to highland forests (Ci 1998). Like Taiwan's three flying squirrel species and *Tamiops maritimus*, *D. pernyi owstoni* may therefore be a distinct species. To test this conjecture, we examined phylogenetic relationships between *D. pernyi* from Taiwan and the Asian mainland by using mitochondrial cytochrome *b* gene sequences. Here, we include all available sequence data for different *Dremomys* species from Taiwan and the Asian mainland and discuss the distinctness of *D. pernyi owstoni* from Taiwan.

Materials and methods

Specimens

Dremomys specimens used in the present study and their collecting localities are shown in Table 1 and Fig. 1, respectively. We collected one *D. pernyi owstoni* specimen from the central part of Taiwan and three *D. rufigenis* specimens from northern Vietnam. Since Oshida et al. (2003) reported that chromosomal characteristics of *D. pernyi owstoni* are similar to those reported for *D. rufigenis* (Nadler and Hoffmann 1970), we included these *D. rufigenis* specimens in our analyses. Sequence data of *D. pernyi* and other *Dremonys* species occurring on the Asian mainland (Li et al. 2008) were obtained from the DNA Data Bank of Japan (Table 1). The specimen from Taiwan is in the Laboratory of Wildlife Biology, Obihiro University of Agriculture and Veterinary Medicine, Japan. Specimens from Vietnam are deposited in the Institute of Ecology and Biological Resources, Vietnam Academy of Sciences and Technology, Hanoi, Vietnam.

PCR and DNA sequencing

Total genomic DNA was extracted from muscle tissues using the DNeasy Blood & Tissue Kit (QIAGEN K.K., Tokyo). Complete mitochondrial cytochrome *b* gene sequences (1140 bases) were amplified with polymerase chain reaction (PCR) using a primer set: L14724 5'-GATATGAAAAACCATCGTTG-3' and H15910 5'-GATTTTTGGTTTACAAGACCGAG-3'. Former and latter primers are reported by Kocher et al. (1989) and Oshida et al. (2000b), respectively. The 50 μl reaction mixture contained about 100 ng of genomic DNA, 0.25 μM of each primer, 200 μM dNTPs, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 2.5 units of r*Taq* DNA polymerase (Takara, Tokyo). Amplification was carried out for 35 cycles. The program was 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. A final extension reaction was at 72°C for 10 min. The PCR products, purified with the PCR Clean Up-M (Viogen, Taipei, Taiwan), were directly sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit V3.1 (Applied Biosystems, CA, USA) and an automated DNA sequencer (ABI PRISM 377-96 Sequencer and ABI PRISM 3100 Genetic Analyzer, Applied Biosystem, CA, USA). For sequencing, we used the same primers used for PCR. Purification of PCR products and sequencing were performed by Mission Biotech Co. Ltd. (Taipei, Taiwan).

Sequence analyses

To root phylogenetic trees, sequences of two *Tamiops* species: *T. mcclellandii* (accession number EF539333) and *T. swinhoei* (accession number EF539334) were used as the out-group (Li et al. 2008). Since Mercer and Roth (2003) reported that *Tamiops* is more closely related to *Dremomys* than other squirrel genera, these *Tamiops* sequences were thought to be suitable out-groups for resolving phylogenetic relationships among *Dremomys* species.

All sequences were aligned with DNASIS (Hitachi, Tokyo). For maximum-likelihood (ML) and neighbor-joining (NJ) analyses and Bayesian inference (BI), the program MODELTEST 3.06 (Posada and Crandall 1998)

Table 1. *Dremomys* specimens examined in the present study

Species†	Collecting locality (locality number)	Voucher number or Identity number	Accession number
D. pernyi 1	Hehuanshan, Nantou, Taiwan (1)	TESRI-B0228	HQ698361*
D. pernyi 2	Tengzhi, Kaohsiung, Taiwan (2)	TESRI-B0291	HQ698362*
D. pernyi 3	Nan'ao, Yilan, Taiwan (3)	TESRI-B0349	HQ698363*
D. pernyi 4	Hehuanshan, Nantou, Taiwan (1)	TW12	LC150596
D. pernyi 5	Xianggelila, Yunnan, China (4)	KIZ2003012	EF539336*
D. pyrrhomerus	Pingbian, Yunnan, China (5)	KIZ84114	EF539342*
D. gularis 1	Jingdong, Yunnan, China (6)	KIZ840037	EF539339*
D. gularis 2	Jingdong, Yunnan, China (6)	KIZ84741	EF539338*
D. gularis 3	Jingdong, Yunnan, China (6)	KIZAL05485	EF539337*
D. rufigenis 1	Genma, Yunnan, China (7)	KIZ2004264	EF539341*
D. rufigenis 2	Longling, Yunnan, China (8)	KIZ206400	EF539340*
D. rufigenis 3	Tamdao, Vietnam (10)	211	LC150597
D. rufigenis 4	Tamdao, Vietnam (10)	212	LC150598
D. rufigenis 5	Tamdao, Vietnam (10)	215	LC150599
D. lokriah	Gongshan, Yunnan, China (9)	KIZ9001	EF539335*

Locality numbers correspond to those in Fig. 1.
Asterisks indicate sequences reported by Li et al. (2008) or Chang et al. (2011).
†: To identifiy each individual, we showed species name and number together for *D. pernyi*, *D. gularis*, and *D. rufigenis*.

selected the most appropriate substitution model of molecular evolution based on the Akaike information criterion (AIC). This program selected the general time reversible (GTR) model of substitution (Rodríguez et al. 1990; Yang et al. 1994), and considered proportion of invariable sites (I = 0.6360). Base frequencies were estimated as A = 0.2776, C = 0.3589, G = 0.1254, and T = 0.2381. Rate matrix was estimated as A-C = 2.1064, A-G = 17.2756, A-T = 2.3159, C-G = 6.7497, C-T = 37.4252, and G-T = 1.0000. The ML tree was constructed by a heuristic search option with a tree-bisection-reconnection. With genetic distances calculated by this model, we also conducted NJ analysis (Saitou and Nei 1987). In addition, we did an un-weighted maximum parsimony (MP) analysis. The MP tree was constructed with a branch-and-bound search option. To assess nodal supports, bootstrapping (Felsenstein 1985) was carried out with 500 replications in ML analysis, 10 000 replications in NJ analysis, and 1000 replications in MP analysis. The ML, NJ, and MP analyses were performed by PAUP* 4.0b10 (Swofford 2001). Bayesian inference reconstruction was carried out using MrBayes 3.0b4 (Huelsenbeck and Ronquist 2001). Bayesian analysis was conducted using the GTR substitution model selected by MODELTEST. Analysis involved two runs for one million iterations, using four Markov Chain Monte Carlo (MCMC) chains and sampling every 1000 generations with a burn-in of 20%. Based on remaining trees, 50% majority rule consensus trees were generated. Posterior probabilities were used to assess nodal support of the BI tree.

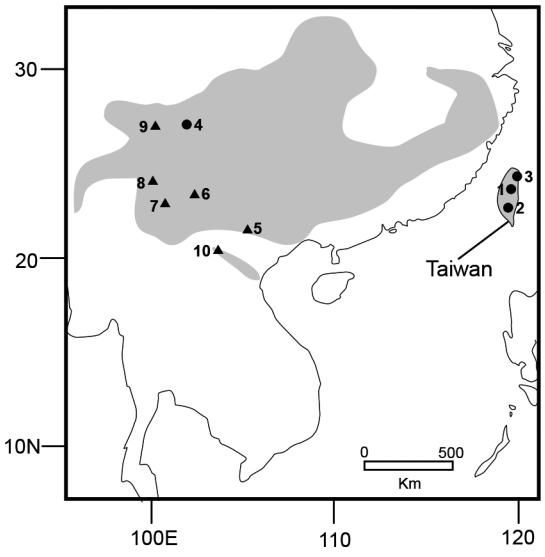

Fig. 1. Distribution of *Dremomys pernyi* (in light gray; Francis 2008; Thorington et al. 2012) and collecting localities of *D. pernyi* (closed circles) and other *Dremomys* species (closed triangles) used in the present study.

Divergence time between Taiwan and the Asian mainland populations was calculated by BEAST version 1.8.3 (Drummond et al. 2016), based on divergence time among three squirrel genera, *Dremomys*, *Callosciurus*, and *Sundasciurus*: estimated as 11.0 ± 1.2 million years

Table 2. Pairwise comparisons of cytochrome *b* nucleotide sequences (1140 bp) for 15 *Dremomys* specimens (*p*-distances in %)

Specimens	*D. pernyi* 2	*D. pernyi* 3	*D. pernyi* 4	*D. pernyi* 5	*D. pyrrhomerus*	*D. gularis* 1	*D. gularis* 2	*D. gularis* 3	*D. rufigensis* 1	*D. rufigensis* 2	*D. rufigensis* 3	*D. rufigensis* 4	*D. rufigensis* 5	*D. lokriah*
D. pernyi 1	0.61	0.70	0.26	10.18	13.77	14.12	12.98	14.30	13.07	12.37	12.53	12.46	12.37	10.53
D. pernyi 2		0.09	0.53	10.18	13.68	13.86	12.72	14.12	13.07	12.37	12.54	12.46	12.37	10.35
D. pernyi 3			0.61	10.26	13.77	13.95	12.81	14.21	13.16	12.46	12.63	12.54	12.46	10.44
D. pernyi 4				10.88	13.68	14.04	12.90	14.04	12.98	12.28	12.28	12.19	12.11	10.26
D. pernyi 5					12.28	13.33	11.14	13.33	12.46	12.19	11.14	11.05	11.14	10.00
D. pyrrhomerus						12.54	11.58	12.72	7.63	8.16	9.91	9.83	10.09	12.02
D. gularis 1							3.77	0.44	12.19	12.81	12.72	12.81	12.72	11.93
D. gularis 2								4.21	11.75	11.93	11.72	11.84	11.75	10.97
D. gularis 3									12.28	12.90	12.63	12.72	12.63	12.02
D. rufigensis 1										3.95	6.93	6.84	6.58	13.42
D. rufigensis 2											6.75	6.49	6.58	6.58
D. rufigensis 3												0.44	0.35	12.37
D. rufigensis 4													0.44	12.46
D. rufigensis 5														12.37

ago (Mya) by Mercer and Roth (2003). Instead of *T. mcclellandii* and *T. swinhoei*, we included *Callosciurus prevostii* (AB499914) and *Sundasciurus mindanensis* (AB444722) as the out-group. We applied a normal distribution as the prior model for calibration and a relaxed molecular clock to allow independence of the evolutionary rate in each branch. The MODELTEST 3.06 selected the Tamura-Nei model of substitution (Tamura and Nei 1993), considered proportion of invariable sites, and followed a gamma distribution for variable sites. The Bayesian MCMC analysis was run for ten million generations, sampling every 1000 generations.

Results

Complete sequences (1140 bases) of the cytochrome *b* gene were determined for all specimens. We deposited all sequence data in the DNA Data Bank of Japan (Table 1). Uncorrected percentage sequence divergences (*p*-distances) are shown in Table 2. Among *Dremomys* species sequences, *p*-distances ranged from 7.63 to 14.30%.

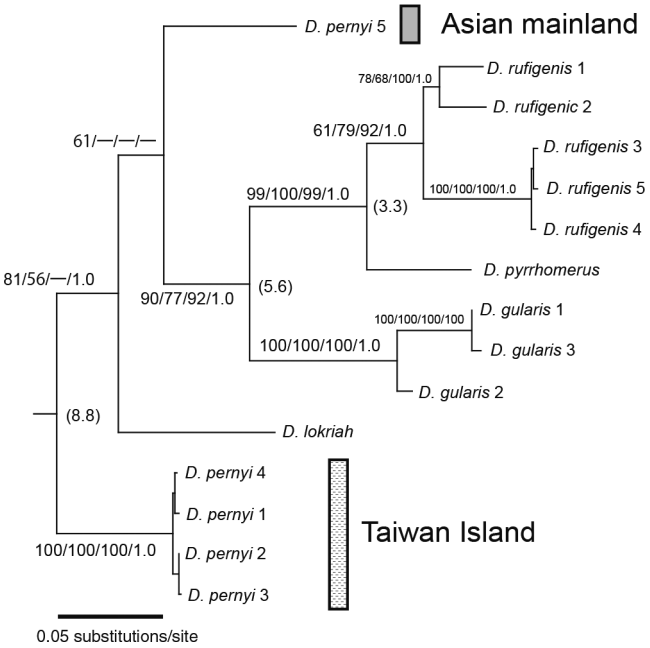

Fig. 2. Phylogeny of *Dremomys* squirrels constructed with maximum-likelihood (ML) under the GTR + I model for the mitochondrial cytochrome *b* sequences. From left, numbers above branches represent: bootstrap values from: 500 replicates of ML, 10 000 replicates of neighbor-joining (NJ), and 1000 replicates of un-weighted maximum parsimony (MP) analyses; and posterior probability supports in Bayesian analysis. Specimens defined in Table 1. Dashes mean no data, due to absence of clusters. Divergence times shown in parentheses at nodes.

An ML search, assuming the GTR + I model, produced a single tree (Fig. 2). Branching patterns of NJ, MP, and BI trees (data not shown) were essentially similar to those of the ML tree. *Dremomys rufigenis* and *D. gularis* formed separate clusters (Fig. 2). *Dremomys pernyi* did not form a single cluster (Fig. 2). All Taiwanese forms were clustered together with high nodal support (100% in ML, MP, and NJ trees and 1.0 in BI tree). The Asian mainland form was distantly related to Taiwanese forms, showing deep genetic diversities (10.18–10.88%, Table 2).

Phylogenetic relationships among the five *Dremomys* species were unclear (Fig. 2). *Dremomys gularis*, *D. rufigenis*, and *D. pyrrhomerus* were clustered together. *Dremomys rufigenis* and *D. pyrrhomerus* showed a close relationship with high nodal supports (99% in ML and MP trees, 100% in NJ tree, and 1.0 in BI tree). These results essentially correspond to those reported by Li et al. (2008). Chronological analyses estimated that Taiwanese forms diverged from other *Dremomys* species on the Asian mainland approximately 8.8 Mya.

Discussion

Dremomys pernyi owstoni of Taiwan was genetically different from the specimens from the Asian mainland. For mitochondrial cytochrome *b* sequences, Bradley and Baker (2001) reported that distance values between 2 and 11% probably indicate conspecific populations or valid species. Therefore, the genetic distances (10.18–10.88%) between the two *D. pernyi* populations suggest that they are separate species. Genetic distances between *D. rufigenis* and *D. pyrrhomerus* ranged from 7.63% to 10.09%, values less than those between the two *D. pernyi* populations. It is therefore likely that the present specific classification of *D. pernyi* includes at least two distinct species. Furthermore, Oshida et al. (2003) reported that the chromosomal constitution of *D. pernyi owstoni* is different from that of *D. pernyi flavior* (Wang et al. 1980) occurring in southern China and suggested that the karyotype of *D. pernyi owstoni* is more similar to that of *D. rufigenis* (Nadler and Hoffmann 1970). These chromosomal characteristics also reinforce our findings, although *D. pernyi owstoni* is not closely related to *D. rufigenis*.

In the present study, Taiwanese form was estimated to have diverged from the other *Dremomys* species of the Asian mainland approximately 8.8 Mya. During the late Miocene (around 9.0 Mya) [see Sibuet and Hsu (2004)], a common ancestor of *Dremomys* species would have already existed on proto-Taiwan. Before Taiwan Island completely separated from the Asian mainland (since 6.0 Mya), this squirrel may have already evolved into distinct form (*D. pernyi owstoni*). The divergence time estimated in the present study supports this evolutionary scenario. The evolutionary history of this species is similar to that of *Petaurista*, *Belomys*, and *Tamiops* occurring in Taiwan (Oshida et al. 2000a, 2000b, 2004, 2015; Chang et al. 2011), as these squirrels are clearly distinct from those occurring in Asian mainland. The Taiwanese form (*D. pernyi owstoni*) could be considered an endemic species, instead of an endemic subspecies. The type specimen of *D. pernyi owstoni* was recorded as *Zetis owstoni* (Thomas 1908). Based on the original species name, we here suggest that the Taiwanese form be classified as '*Dremomys owstoni*'. In the present study, we only examined the phylogeographical relationship between two regional *D. pernyi* populations by using a single genetic marker. If we find the haplotype of *D. pernyi owstoni* in the eastern part of Asian mainland, our tentative conclusion would need to be revised. Therefore, resolution of the taxonomic status of this species requires phylogeographical study of populations from more different areas in mainland Asia. Recently, Hawkins et al. (2016) reported that *Dremomys everetti*, which is endemic to the high mountains of Borneo Island, is more closely related to *Sundasciurus* squirrels than the other *Dremomys* species. Therefore, *Dremomys* squirrels need reclassification at the genus level.

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