東海大學生物學系碩士論文

以粒線體細胞色素 *b* 區域序列探討臺灣地區短尾鼩族群的地理親緣變異

Phylogeographic Variation in Mitochondrial Cytochrome *b* Region of Formosan Burrowing Shrew, *Anourosorex squamipes yamashinai* (Mammalia: Insectivora)

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中華民國九十二年七月

Contents

摘要

短尾鼩（*Anourosorex squamipes*）為一屬一種的食蟲目動物，分佈在由越南至緬甸到中國大陸的四川省，以及臺灣的中高海拔山區，呈現斷續型分佈。臺灣的短尾鼩為一亞種，即山階氏鼩鼱（*Anourosorex squamipes yamashinai*）。本研究的目的在探討臺灣島內各地的短尾鼩族群之遺傳分化現象，以及臺灣和中國大陸短尾鼩族群呈現不連續分佈，其遺傳組成的變化，藉此推估二大族群分離的時間以及可能原因。

本研究中共收集到臺灣 19 處山區中短尾鼩 78 隻，並取得中國大陸四川省南充市 8 隻以及綿陽市 2 隻短尾鼩的肝組織。以自行設計之引子進行 PCR 反應增幅粒線體細胞色素 *b* 片斷後定序，得到全部樣本之部分序列 737 bp。此外，為解釋臺灣和大陸族群的演化關係，選取合歡山地區 5 隻個體和中國大陸綿陽市 2 隻個體定出其細胞色素 *b* 區域完整序列 1140 bp。部分序列之親緣分析結果顯示，臺灣地區短尾鼩粒線體細胞色素 *b* 序列分屬於 28 種單型（haplotype），單型間具有 43 個多型性位點。親緣關係樹之分析結果顯示臺灣地區族群與大陸四川地區族群的遺傳距離有很大的差距；且臺灣短尾鼩族群被分成南、北二大群，此二群距大陸四川族群的遺傳距離幾乎相等。有 7 個辨識位點（diagnostic site）可用以分隔此二群。此外，臺灣島內族群的單型多樣性指數（*h*）與族群分化指數（F_{ST}）均相當高，顯示臺灣

短尾鼩族群的分化顯著，AMOVA（analysis of molecular variation）檢定亦支持此一結果。

完整序列的分析中，臺灣島內族群和中國大陸四川族群的遺傳距離約 6%，屬於種內的差異。若以分子時鐘理論推估分群的時間點，顯示臺灣族群約在 72 萬年前與大陸族群分離，而臺灣島內南北二族群則約在 19 萬年前分離。此一結果顯示臺灣短尾鼩族群應屬一次入侵，可能在古德冰期海退時由大陸進入臺灣，於西部平原開始廣泛分佈，後因冰河期結束，族群量銳減，經歷瓶頸效應後，二不同小族群在中高海拔山系快速擴散，因山脈的阻隔而形成南北二群並獨立演化。

Abstract

Anourosorex squamipes, which is the monotypic species in the genus *Anourosorex*, is distributed from Tonkin, through Burma to Szechuan, China and Taiwan, showing a disjunct distribution pattern. The Taiwanese form was described as a subspecies *A. squamipes yamashinai* and occurs in the mountainous areas of Taiwan. The purpose of my study is to investigate the genetic variation within Taiwan population and compare the divergence with disjunct population in Mainland China. Moreover, I will try to explain the possible process and divergence time that caused the isolation.

Liver tissues were obtained from 78 individuals who were collected at 19 localities in Taiwan. Those of 8 individuals from Nan-Chung city and 2 individuals from Main-Yang City in Szechuan province were prepared also. The cytochrome *b* fragment was amplified using PCR with two primers designed newly. The partial sequences (737 bp) of all specimens from Southwestern China and Taiwan were successfully determined. The complete sequences (1140 bp) of cytochrome *b* gene were also determined from 5 specimens of Mt. Houhuan in Taiwan and 2 specimens of Main-Yang City in Szechuan province. Those complete sequences were used to explain the evolutionary relationship between two populations of Taiwan and Southwestern China. In a result of phylogenetic analysis of partial sequences, 28 haplotypes were identified with 43 variable sites of Taiwan population. The results of phylogenetic trees were indicating that Taiwan population distinctly diverged from Southwestern China population and 28 haplotypes in Taiwan were

clustered into two groups (Northern and Southern groups). In addition, there were 7 diagnostic sites could be used to distinguish both groups of Taiwan. The genetic distances between Northern group in Taiwan and Southwestern China population was almost similar to that between Southern group in Taiwan and Southwestern China population. Furthermore, the haplotype diversity (h) and fixation index (F_{ST}) were revealed that the divergence would be significantly high within Taiwan population. This result was supported by AMOVA test also.

In a result of analysis of complete cytochrome *b* gene sequences, an intraspecies variation (6%) was indicated from the genetic distance between Taiwan and Southwestern China population. Analysis of divergence time based on molecular clock theory was revealed that Taiwan and Southwestern China population was separated at 0.72 Mya and the two groups of Taiwan population were separated at 0.19 Mya. From these data, the invasion of *A. squamipes* from continent to Taiwan might have been occurred once. The population of *A. squamipes* might have reached at the western area of Taiwan during the Günz glacial period and then been distributed into Taiwan. After glacial period, the population could have been reduced, and took refuge to the middle-elevation of mountainous area, with the "bottleneck effect". So, the two groups could have been isolated by topography of Taiwan and evolved independently.

Introduction

The mole-shrew (*Anourosorex squamipes*), which is the monotypic species in the genus *Anourosorex*, is distributed from Tonkin, through Burma to Szechuan and Taiwan, showing a disjunct distribution pattern. The mole-shrew in Taiwan was described as a subspecies *A. squamipes yamashinai* by Kuroda (1935), based on its smaller size and tail length. The mole-shrew is a semi-fossorial animal which could build specific tunnel system and is living under the ground, but is seeking invertebrates as food on the ground (Alexander *et al*. 1987, Corbet and Hill 1992). In the previous records, it is known that *A. squamipes* is distributed at altitude of 500 – 3200 m in mountainous area of Taiwan, and the most abundant area is 1000 – 2500 m of moisture forest or grassland (Yu 1994). Moreover, Alexander *et al*. (1987) suggested that this species had high habitat preference for digging and hiding.

Avise (2001) demonstrated that time and geography are two major parameters producing the isolation with the barrier among populations cooperatively. In recent studies, the geographic isolation has been found in terrestrial animals of Taiwan, based on genetic variations (Creer *et al.* 2001, Hsu *et al.* 2000, Hsu *et al.* 2001, Wang *et al.* 2000 and Yang *et al.* 1994). Most of the results revealed a differentiation had occurred in animal population of Taiwan. For the lowland species (Taipei tree frog, *Rhacophorus taipeianus* and bamboo viper, *Trimeresurus stejnegeri*), the isolation pattern is associated with the presence of the Central Range. Those populations are separated into western and eastern lineages with the Central Range. However, for the midland and highland species

(cyprinid fish, *Acrossocheilus paradoxus* and Formosan wood mouse, *Apodemus semotus*), the isolation pattern seems to be affected by mountainous ridges and drainage systems. They are separated into northern and southern lineages in mountainous area of Taiwan. It is considered that highland species invaded from continent to Taiwan in glacial periods and took refuge in highland environment of Taiwan during the interglacial periods. Therefore, the phylogeographic variation of highland species is not only related to the topology of mountainous area of Taiwan but also affected by the geographic history during the Quaternary. According to Maldonado *et al*. (2001), shrews with semi-fossorial habit is predicted to be highly isolated by geographic barriers. Therefore, the population of *A. squamipes* could be possibly isolated by the specific topology of Taiwan. However, the geographic features of those highland species do not be examined completely especially for the order *Insectivora* in Taiwan.

For those of disjunct animal populations, the long-term isolation may be produced deep genealogical structure and concordant with differentiation in other characters (Avise 2001). Motokawa and Lin (2002) examined 18 cranial morphometric characters of 112 specimens from several localities of Mainland and Taiwan of *A. squamipes*. They found that there were three allopatric forms (Taiwan population was one of them). However, the phylogeographic variation and phylogenetic relationships based on molecular data are still unknown among and within populations of *A. squamipes*.

Thus, the purpose of this study is to clear phylogeographic variation of *Anourosorex squamipes* population of Taiwan and examine phylogenetic

relationship between two disjunct populations of Taiwan and Mainland China inferred from the variation of mtDNA cytochrome *b* region of *A. squamipes*.

Materials and Methods

1. Sampling

In this study, a total of 88 mole-shrews (*Anourosorex squamipes*) were examined. Seventy-eight samples were collected at an altitude of 480m to 3580m in mountainous areas of Taiwan, including two samples provided from the National Museum of Natual Science (NMNS), Taiwan (Fig. 1, Table 1). Ten samples, which had been captured in Mian-Yang and Na-Chung city of Mainland China were provided from Dr. Wu of Guangzhou University, China and Dr. Harada of Osaka City University, Japan (Fig. 1). In each locality, several transparent lines were set with a total number of 20 to 100 sherman traps for collecting mole-shrews using baked sausages as the bait. For DNA extraction, the liver tissue or muscle tissue was preserved in 98.9% ethanol at room temperature.

2. DNA extraction, amplification, and sequencing

Total DNAs were extracted according to the phrnol/ proteinase K/ sodium dodecyl sulfate method of Sambrook *et al*. (1989). The tissues (100 mg) were incubated in 455 μl of STE buffer (100mM Tris-HCl, pH

8.0/ 10mM EDTA/ 100mM NaCl) containing a final concentration of 0.5% SDS and 1 μg proteinase K at 37°C overnight, and treated twice with 500 μl of phenol/chloroform isoamyl alcohol (25:24:1) and once with chloroform/ isoamyl alcohol (24:1). The DNA was precipitated in cold absolute ethanol with sodium chloride (5M) at 4°C and finally recovered by centrifugation at 12000g for 15 min. The DNA was resuspended in TE buffer (10mM Tris-HCl, pH 7.5/ 1mM EDTA) and stocked in 4°C. Based on the complete mitochondrial DNA sequence of *Soriculus fumidus* (Lin 2002), two primers were designed newly for amplification of mitochondrial DNA cytochrome *b* gene: L14182 5'-CATCGTTGTTATTCAACTATAGGAAC-3' and H15445 5'-GAATATCAGCTTTGGGTGTTGATA-3'. Polymerase chain reaction (PCR) mixture of 50 μl contained approximately 50 ng of genomic DNA, 7.5 pmol of each primer and 0.2 mM of dNTP mix in reaction buffer including 2.0 mM Tris-HCl (pH 8.0), 0.01 mM EDTA, 0.1 mM DTT, 0.1% Triton X-100, 5% glycerol, and 1U of *Taq* DNA polymerase (Viogene). Amplifications were performed in a GeneAmp® PCR System 2400 thermal cycler employing 35 cycles as follows: denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 2 min.

The final extension was 72℃ for 10 min. The PCR products were loaded onto 1.2% agarose gel, and then stained with ethidium bromide. To identify PCR products, the gel was illuminated with UV-light. Approximately 100 ng of the purified PCR product was used for sequencing with PCR primer L14182 and another internal primer: 5'-GTAATAGCCACCGCCTTTATAGG-3'. Additionally, two internal primers were also designed for complete sequence of cytochrome *b* gene: 5'-CTAGTCCTGTTCTCCCCAGACC-3' and 5'-CCTATAAAGGCGGTGGCTATTAC-3'. The sequencing process was used the BigDye terminator kit and determined directly with an ABI 3730*xl* DNA analyzer (Applied Biosystems).

3. Phylogenetic analysis

The partial sequences (737 base pairs) of central domain of cytochrome *b* gene were aligned by computer software ClustalW in BioEdit 5.0.9 (Hall 1999) and corrected by eyes. Phylogenetic analyses were conducted via neighbor-joining (NJ) and maximum parsimony (MP) methods, and minimum spanning network. In NJ analysis, genetic distances were corrected by the Kimura's (1980) two-parameter model

using the MEGA program version 2.1 (Kumer *et al*. 2001) . Maximum parsimony analysis with un-weighted characters was performed using heuristic search algorithm with tree bisection-reconnection (TBR) in the PAUP program version 4.0 b10 (Swofford 1998). Confidence in estimated relationships was determined using 1000 bootstrap pseudoreplicates (Felsenstein 1985) for both of NJ and MP analyses. The minimum spanning network (Excoffier *et al*. 1994) was constructed to estimate the evolutionary parsimony relationship between haplotypes using computer program Arelequin version 2.001 (Schneider *et al*. 2000).

4. Population genetics

For understanding the significant geographic differentiation among certain of groups within population, the AMOVA (analysis of molecular variance) was used in this study by the computer program Arelequin version 2.001 (Schneider *et al*. 2000). AMOVA is a hierarchical approach to analysis of variance in which haplotype distances compared at various hierarchical levels are used as F-statistic analogus, designated as ψ statistics (Excoffier *et al*. 1992). Furthermore, the genetic polymorphism and diversity were described by haplotype diversity (*h*) (Nei 1987) and

nucleotide diversity (π) (Nei 2000). The equation of haplotype diversity is as follows: $h = n(1- \Sigma x_i^2)(n-1)$, where n is the number of individuals sampled and x_i is the frequency of number x haplotype. The nucleotide diversity indicates the number of nucleotide differences per site between two sequences which can be estimated by: $p = \frac{q}{q-1}\sum_{ij} x_i x_j d_{ij}$, where q is the total number of different haplotypes, x_i is the population frequency of the i-th haplotype, and d_{ij} is the number of nucleotide differences or substitutions per site between the i-th and j-th haplotypes. If the genotypes deviations occurred in a subdivided population, the degree of genetic differentiation can be measured by fixation indices (F_{st}) (Hudson *et al*. 1992) that is defined as: $F_{st} = 1 – (H_w/H_b)$, where H_w is the mean number of differences between different sequences sampled from the same subpopulation (group), and H_b is the mean number of differences between different sequences sampled from the two different subpopulations (groups) sampled. The F_{st} value could be ranged from zero to one, the larger value indicates a larger differentiation between subpopulations (groups). Gene flow was estimated from F_{st}. The equation as follows: $Nm = \frac{1}{2}\left(\frac{1}{Fst} - 1\right)$ for mitochondrial DNA. Computer program DNASP version 3.53 (Rozas and Rozas 1999) was used for all indices

represented above.

5. Estimation of divergence

To estimate the divergence time of different populations, the complete cytochrome *b* gene sequence (1140 bp) was used. The p-distance (Nei and Kumar 2000) was used to correct the genetic disances of amino acid sequeces. The divergence time between different populations was estimated, based on the evolutionary rate at third-position transversion in shrews (1.36%/Myr with a 95% confidence interval of 1.08 - 1.64%/Myr) of cytochrome *b* gene (Fumagalli *et al*. 1999).

Results

1. Sequence analysis of Taiwan and China populations

1) Sequence variation

All of the 88 specimens were successfully sequenced, and the partial sequences of cytochrome *b* gene (737 bp) were analysed. In Taiwan population, 28 haplotypes were found. Among the haplotypes, 43 polymorphic sites were identified with transitions at 34 sites and transversions at 9 sites, showing 28 parsimony informative polymorphic sites (Table 2). The total haplotype diversity was 0.932% (SD 0.013%) and nucleotide diversity was 0.936% (SD 0.062%) (Table 3). The percentage divergences among haplotypes were 0.13 – 2.44% and the genetic distances (Kimura's two parameter distance) were 0.14 – 2.50%, averages of which were 1.10 and 1.12%, respectively (Table 4).

In the population of Southwestern China, three haplotypes with two transitions were obtained from 10 specimens of two different localities. The haplotype and nucleotide diversities of Southwestern China population were 0.711% (SD 0.086% and 0.121% (SD 0.023%), respectively. Total of 75 polymorphic sites were identified in whole data

from Taiwan and Southwestern China populations, and overall haplotype and nucleotide diversities were 0.943% (SD 0.010%) and 1.946% (SD 0.250%).

2) Molecular phylogeny and phylogeography

In NCBI GenBank, the four species (*Sorex araneus*, *Soriculus fumidus*, *Sorex shinto*, and *Mogera insularis*) that are closely related with *A. squamipes* were chosen as an outgroup in phylogenetic analysis. Parsimony analysis with all characters weighted equally produced one most-parsimonious tree of 434 steps, consistency index (CI) of 0.726, and retention index (RI) of 0.768. The Taiwan population of *A. squamipes* was subdivided into two major lineages (Fig. 2) corresponding to geographic regions with 100% bootstrap values: Northern group contained most of specimens from the Snow Range (see Fig. 1, Nos. 1 - 4), and Southern group contained most of specimens from the Central Range and the Ali Range (see Fig. 1, Nos. 5 - 17). Seven diagnostic sites between both populations were recognized (Table 5). In addition, in Mt. Houhuan, two specimens belonging to northern group and three specimens belonging to southern group were identified, suggesting the

presence of contact area between both populations. Also, in NJ tree, the same two major lineages as MP tree was found with high nodal supports (100% bootstrap values) (Fig. 3).

Phylogenetic relationships between Taiwan and Southwestern China populations demonstrated the significant divergence in NJ and MP tree with 100% bootstrap values (Figs. 2, 3). The genetic distances estimated by Kimura's two-parameter model between northern group of Taiwan and Southwestern China population were almost similar to those between southern group of Taiwan and Southwestern China population (Fig. 4).

3) Divergence within Taiwan population

Unique haplotypes occurred in most of sampling localities suggesting that genetic subdivision had taken place in population of *A. squamipes* of Taiwan (Table 6). Moreover, two common haplotypes were observed in the total of 28 haplotypes of Taiwan population (T2 and T16; Table 6). Nine specimens from five localities (Lidungshan, Guanwu, Wulin, Suyuanyakou, Mt. Houhuan) of northern Taiwan had "T2". The "T16" was recognized in ten specimens from six localities (Guanyuan, Mt. Houhuan, Tsufung, Meifung, Nenggau, Aowanda) of central Taiwan.

Minimum spanning network revealed the parsimonious evolutionary relationships of haplotypes in both of northern and southern groups (Fig 5) and two haplotypes of T2 and T16 were carried ancestral characters in the result of network.

Overall, the southern group had higher nucleotide and haplotype diversities (*h*: 0.901 and π: 0.499%) than northern group (*h*: 0.789 and π: 0.17%). The F_{st} and *Nm* were 0.80 and 0.13 respectively, indicating of the significant genetic differentiation between two groups of Taiwan population. In the hierarchical analysis by AMOVA, northern group was contrasted with southern group with or without samples from Mt. Houhuan as the third group. The result suggested a statistically significant divergence between two groups of Taiwan ($P<0.001$) (Table 7).

2. The estimation of divergence time among Taiwan and China populations

The complete sequence (1140 bp) of mitochondrial cytochrome *b* gene of *A. squamipes* was determined from five samples from Mt. Houhuan of Taiwan (specimen No. H1, H2, H3, H4 and H5) and two samples from Mian-Yang city of Southwestern China (specimen No. 8403 and 8405)

using two additional sequencing primers. In the sequence alignment, 23 polymorphic sites were found. Each of sequence was unique and 78 sites were variable including 79 substitutions with 7 non-synonymous substitutions at first-codon positions, 3 non-synonymous substitutions at second-codon positions, and 3 non-synonymous substitutions at third-codon positions. Average of nucleotide usage was thymine: 32.9%, cytosine: 24.0%, adenine: 30.8%, and guanine: 12.4%. The pairwise comparison based on Kimura's two parameter method among Taiwan and Southwestern China populations was 5.7% to 6.3% and the mean value was 6.02% (SE 0.68%) (Table 8).

The topology of phylogenetic trees constructed from complete sequence of cytochrome *b* gene were identical to those constructed from partial sequence in both NJ and MP methods (Figs. 6, 7). Furthermore, the NJ tree based on p-distance of amino acid sequence supported that the subdivision had occurred in Taiwan population and that the obvious divergence existed between Taiwan and Southwestern China populations (Fig. 8). A plot of transitional and transversional substitutions against pairwise genetic distances suggested that substitutional saturation did not appear within and between populations (Fig. 9). The divergence time

between Taiwan and Southwestern China populations was estimated to be approximately 0.72 Mya and that between two groups of Taiwan population was approximately 0.19 Mya (Fig. 10).

Discussion

1. Phylogeographic variation of *Anourosorex squamipes* of Taiwan and Southwestern China populations

Recently, most of the phylogeographic studies suggested that the geographic isolation had occurred to populations of vertebrates of Taiwan, due to the specific topography of Taiwan Island (Table 9). The present result revealed a remarkable phylogeographic differentiation within the population of *A. squamipes* of Taiwan, showing that two geographic groups (northern and southern groups) were identified with high nodal supports, and both groups seemed to contact in Mt. Houhuan.

The F_{st} was 0.80 and *Nm* was 0.13 between two groups, indicating that the genetic flow between groups was significantly low. Ohdachi *et al*. (2001) revealed that the nucleotide diversity of six *Sorex* shrews were ranged from 0.759% to 2.04%. Comparing with this report, a lower π (<0.5%) and higher *h* (>0.5) were observed in Taiwan (Table 3). Grant *et al*. (1998) reported that the population under this combination may be attributed to expansion after a period of low effective population size and the following rapid population growth enhanced the retention of

mutations. Therefore, it was suggested that the population of Taiwan had been experienced bottleneck effect before. Moreover, in the minimum spanning network, ancestral haplotypes (T2 & T16) were found in northern and southern groups, respectively (Fig. 5). This relationship was conformed to the category IV intraspecific pattern in Avise (2001), whose suggested the population numbers were increased explosively from ancestral-like haplotypes but had not been isolated by long-term biogeographic barriers. Network relationships of southern group were more complicated than those of northern group, indicating that two groups probably differ in the evolutionary history. Wang *et al*. (2000) reported the origin of cyprinid fish population might have been located at central Taiwan by network relationship. In the study on spatial pattern of *Cyclobalanopsis glauca* in Taiwan, Huang *et al*. (2002) reported that the highest genetic diversity was found in three localities: two of them were at central and southern Taiwan. In the allozyme analysis of *Michelia formosana*, Nantou County (central Taiwan) was considered as a glacial refugium for this species which contained highest genetic variations (Lin 2001). Thus, the evidences of those refugia sites suggested that the environments in southern Taiwan (the Central Range and the Ali Range)

might have maintained more genetic diversities in glacial periods for plant populations that provided more habitats, and might have affected on genetic structure of animal populations.

Recently, the glacial refugium theory is regarded as the most possible scenario for geographic isolation of animals in Taiwan (Creer *et al*.2001, Hsu *et al*. 2000 2001, Huang *et al*. 2001, Lin 2001, Wang *et al*. 2000). It was reported that the connection between Taiwan and Mainland had occurred during the four major glacial periods (Lin 1963; 1974, Tsukada 1966 and Voris 2000) (Table 10). The land bridge of Taiwan Strait in glacial periods had provided terrestrial animals to migrate from continent to Taiwan (Lee 1989, Lin 1974, Tsukada 1966). According to the molecular data of *A. semotus*, Hsu *et al*. (2001) also supposed that the ancestral stock of this species had invaded about 0.24 – 0.15 Mya.

The divergence time estimated in the present study based on cytochrome *b* gene sequence indicated northern and southern groups of Taiwan had diverged about 0.19 Mya; it fell into the Riss glacial periods (Table 10 and Fig. 10). Moreover, the pariwise genetic distance (K2P) between northern group of Taiwan and Southwestern China population was almost similar to that between southern group of Taiwan and

Southwestern China population. Therefore, assuming that the divergence between two groups had occurred in Taiwan, the invasion of *A. squamipes* might have taken place once before Riss glacial periods.

2. Molecular phylogeny of *Anourosorex squamipes* between Taiwan and Southwestern China populations

In the previous study, Taiwan population of *A. squamipes* was treated as a subspecies *A. s. yamashina* based on morphological characters (Kuroda 1935). Using cranial morphometric characters, Motokawa and Lin (2002) discovered three geographic forms and assigned them to subspecific status: *A. s. squamipes*, *A. s. assamensis*, and *A. s. yamashinai.* The result in the present study revealed a significant differences between Taiwan and Southwestern China populations with high nucleotide and haplotype diversities, indicative of a long evolutionary history of these two populations. The divergence among populations was similar to that of other northestern Asiatic *Sorex* shrews (Ohdachi *et al*. 2001). The genetic distances (K2P) between Taiwan and China samples was 5.7 – 6.3% (average: 6.02%) (Fig. 7 and Table 8). It was estimated that Taiwan and Mainland populations had been separated at approximately 0.59 – 0.90

(average: 0.72) Mya in the middle of Pleistocene (Fig. 10). Thus, it could be hypothesized that this species had been wildly distributed at the south of Mainland in the interglacial period of early Pleistocene and one population had gone across the Taiwan Strait to Taiwan island during the longest glacial period (Günz: 0.55 – 1 Mya) (Table 10). After the galical period, both populations could have been reduced due to environmental change and then expanded again in the next glacial period. However, the glacial periods in middle and late Pleistocene would not have been long enough for population of *Anourosorex* to secondary immigrate from continent to Taiwan. Fossil records of *Anourosorex* were reported in Szechwan of China (*Anourosorex kui*) and Japan (*Anourosorex japonicus*) from middle Pleistocene (He 1984, Shikama *et al*. 1958). These evidence lends possibility to the migration of *Anourosorex* from Asia continent to Japan in middle Pleistocene that coincided with the estimation in the present study.

Different level of mtDNA cytochrome *b* variation was also found between Taiwan and Southwestern China populations. From the two localities that were about 200 km apart, three haplotypes with two polymorphic sites were observed in Southwestern China population. On

the other hand, in Taiwan population, more variable polymorphic sites were observed from a shorter geographic distance among sampling localities (ex. northern group has nine polymorphic sites in 70 km, Table 3). This observation may imply that the environment of Taiwan was more unstable, and induced more variations in Taiwan population of *A. squamipe*s. Lin (1974) suggested that the topography of Taiwan Island was extremely different from that of Mainland China. This island is 67.4% occupied with the mountain area, so that the Taiwan has more undulating land. The topography of Taiwan could have affected the genetic structure of populations.

In the mammals, the intraspecific genetic distance of cytochrome *b* gene sequences was varying from 0 to 5.21±0.75% (Fumagalli *et al*. 1999). The present distance data between Taiwan and Southwestern China populations (6.02%) were regarded near to the interspecific differences. Moreover, Bradley and Baker (2001) examined 11 species of bats and 4 species of rodents to test the genetic species concept from mitochondrial DNA cytochrome *b* gene. Their result suggested that genetic distance values below 2% were indicative of intraspecific variation, that values between 2 and 11% had a high probability of being

indicative of conspecific populations or valid species, and that values above 11% were indicative of specific recognition. Motokawa *et al*. (personal communication) analyzed the karyological variation of *A. squamipes*, the result revealed the subspecies in Taiwan (Kuroda 1935) was differed in karyotypes than China population. So, they suggested the mole-shrew from Taiwan to the separated species. However, in the further study, to provide reliable evidence for systematic problem, more specimens throughout East, Southeast, and South Asia was needed.

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Table contents

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Table 1. Sampling localities and specimens of *Anourosorex squamipes* examined in this study.

Sampling locality	Code	Elevation	Date	Specimen number	Note
1. Li-dung-shan (Hsin-chu county)	Li	1500m	2002/07/19	10	
2. Guan-wu (Miao-li county)	Ku	2030m	2001/08/23	1	
3. Wu-lin farm (Tai-chung county)	Wu	1960m	2001/07/25	5	
4. Su-yuan-ya-kou (Yi-lan county)	S	1900m	2002/11/19	2	
5. He-ping (Hua-lian county)	P	1100m	2000/01/18	1	
6. Guan-yuan (Hua-lian county)	Ky	2480m	2002/05/09, 2002/08/08	4	
7. Mountain Hou-huan (Hua-lian county)	H	3210m	2001/03/28, 2001/07/26	5	
8. Mei-fung (Nan-tou county)	Me	2120m	2001/08/15	5	
8. Tsu-fung (Nan-tou county)	Ts	2309m	2001/08/15	2	
9. Neng-gau (Nan-tou county)	N	3000m	2002/07/25	2	
10. Ao-wan-da (Nan-tou county)	O	1100m	2002/01/14	1	
11. Wan-rong (Hua-lian county)	W	480m	2001/05/07, 2001/07/01	8	
12. Dan-da (Nan-tou county)	D	2380m	2002/02/08	5	
13. Shi-tou (Nan-tou county)	Xi	1000m	2000/12/22	3	
13. Sha-lin-shi (Nan-tou county)	Sh	1600m	1996/09/20,1997/08/26	2	NMNS T1791, T1790
14. A-li-shan (Jia-yi county)	A, Ac	2160m	2001/08/20	15	
15. Tataka (Nan-tou county)	Yu	2600m	2001/08/27, 2002/09/01	4	
16. Tian-chi at southern cross island highway (Kao-hsiung county)	Ti	2280m	2001/07/20	2	
17. Mt. Guan (Kao-hsiung county)	G	3580m	2002/04/15	1	

Table 2. Sequence variation of partial mtDNA cytochrome *b* gene in Taiwan population

Dot indicate identical nucleotides with those of haplotype T1.

Number of Haplotypes	43 Polymorphic sites
T1	TTTTTCTCACTTGTTACTCTAATGCCTCCAGTGTGTCATTTTT
T2	.......T.....................................
T3	.......T.....................................G..
T4	.......T.....................................A.
T5	.......T................A.....................
T6	.......T.......G..............................
T7	...C...T......................................
T8	.......T...C..................................
T9	.......T..C........C..........................
T10	..C.C..T.....C....T.G...TTC.T.A.A...T......
T11	....C..T.....C..T.T.G...TTC.T.A.A...T......
T12	....C..T.....C....T.....TTC.T..C....T......
T13	C...C..T.....C....T.....TTC.T..C....T......
T14	....C..T.....C...CT.....TTC.T..C....T......
T15	..C.C..T.....C....T.....TTC.T..C..........
T16	..C.C..T.....C....T.....TTC.T..C....T......
T17	..C.C..T.....C....T.....TTC.TC.C....T......
T18	..C.C..T.....C....T.....TTC.T..C....T..C...
T19	..C.C..T.....C....T.....TTC.T..C...GTC....G
T20	..C.C..T....AC....T.....TTC.T..C....T......
T21	..C.C.AT.....C....T.....TTC.T..C....T......
T22	..C.C..T.A...C....T...C.TTC.T..C.CA.T.C....
T23	..C.C..T.....C..T.T.....TTCTT..C....T.C....
T24	..C.CTGTC....C..T.T.....TTCTT..C....T.C....
T25	..C.C........CC...T.....TTCTT..C....T.C....
T26	..C.C..T.....C....T.....TTCTT..C....T.C....
T27	.CC.C..T.....C....T..T..TTCTT..C....T.C....
T28	..C.C..T.....C....T..T..TTCTT..C....T.C....

Table 3. Haplotype diversity (*h*) and nucleotide diversity (π) indices of Taiwan population. The numbers in brackets are the standard deviations.

	Sample size	Number of polymorphic site	*h*	π
Northern Taiwan	20	9	0.789 (0.086)	0.00170 (0.00033)
Southern Taiwan	58	28	0.901 (0.020)	0.00499 (0.00044)
Total Taiwan	78	43	0.932 (0.013)	0.00936 (0.00062)

Table 4. Pairwaise comparision of partial mtDNA cytochrome *b* sequence between 28 haplotypes of Taiwan population

	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13	T14	T15	T16	T17	T18	T19	T20	T21	T22	T23	T24	T25	T26	T27	T28
T1		0.14	0.27	0.27	0.27	0.27	0.27	0.27	0.41	1.80	1.80	1.38	1.52	1.52	1.38	1.52	1.65	1.66	1.93	1.66	1.65	2.22	1.94	2.36	1.80	1.80	2.08	1.93
T2	0.14		0.14	0.14	0.14	0.14	0.14	0.14	0.27	1.66	1.66	1.24	1.38	1.38	1.24	1.38	1.51	1.52	1.79	1.52	1.51	2.08	1.80	2.21	1.94	1.66	1.93	1.79
T3	0.27	0.14		0.27	0.27	0.27	0.27	0.27	0.41	1.79	1.79	1.37	1.51	1.51	1.37	1.51	1.65	1.65	1.93	1.65	1.65	2.21	1.93	2.35	2.08	1.79	2.07	1.93
T4	0.27	0.14	0.27		0.27	0.27	0.27	0.27	0.41	1.79	1.79	1.37	1.51	1.51	1.37	1.51	1.65	1.65	1.93	1.65	1.65	2.21	1.93	2.35	2.08	1.79	2.07	1.93
T5	0.27	0.14	0.27	0.27		0.27	0.27	0.27	0.41	1.80	1.80	1.38	1.52	1.52	1.38	1.52	1.65	1.66	1.93	1.66	1.65	2.22	1.94	2.36	2.08	1.80	2.08	1.93
T6	0.27	0.14	0.27	0.27	0.27		0.27	0.27	0.41	1.80	1.80	1.38	1.52	1.52	1.38	1.52	1.65	1.66	1.93	1.66	1.65	2.22	1.94	2.36	2.08	1.80	2.08	1.93
T7	0.27	0.14	0.27	0.27	0.27	0.27		0.27	0.41	1.80	1.80	1.38	1.52	1.52	1.38	1.52	1.65	1.66	1.93	1.66	1.65	2.22	1.94	2.36	2.08	1.80	2.08	1.93
T8	0.27	0.14	0.27	0.27	0.27	0.27	0.27		0.41	1.80	1.80	1.38	1.52	1.52	1.38	1.52	1.65	1.66	1.93	1.66	1.65	2.22	1.94	2.36	2.08	1.80	2.08	1.93
T9	0.41	0.27	0.41	0.41	0.41	0.41	0.41	0.41		1.94	1.94	1.52	1.66	1.66	1.52	1.66	1.79	1.80	2.07	1.80	1.79	2.36	2.08	2.50	2.22	1.94	2.22	2.08
T10	1.76	1.63	1.76	1.76	1.76	1.76	1.76	1.76	1.90		0.27	0.68	0.82	0.82	0.68	0.55	0.68	0.68	0.96	0.68	0.68	1.23	0.96	1.37	1.10	0.82	1.10	0.96
T11	1.76	1.63	1.76	1.76	1.76	1.76	1.76	1.76	1.90	0.27		0.68	0.82	0.82	0.96	0.82	0.96	0.96	1.23	0.96	0.96	1.51	0.96	1.37	1.38	1.10	1.37	1.23
T12	1.36	1.22	1.36	1.36	1.36	1.36	1.36	1.36	1.49	0.68	0.68		0.14	0.14	0.27	0.14	0.27	0.27	0.54	0.27	0.27	0.82	0.55	0.96	0.68	0.41	0.68	0.55
T13	1.49	1.36	1.49	1.49	1.49	1.49	1.49	1.49	1.63	0.81	0.81	0.14		0.27	0.41	0.27	0.41	0.41	0.68	0.41	0.41	0.96	0.68	1.09	0.82	0.55	0.82	0.68
T14	1.49	1.36	1.49	1.49	1.49	1.49	1.49	1.49	1.63	0.81	0.81	0.14	0.27		0.41	0.27	0.41	0.41	0.68	0.41	0.41	0.96	0.68	1.09	0.82	0.55	0.82	0.68
T15	1.36	1.22	1.36	1.36	1.36	1.36	1.36	1.36	1.49	0.68	0.95	0.27	0.41	0.41		0.14	0.27	0.27	0.54	0.27	0.27	0.82	0.55	0.96	0.68	0.41	0.68	0.55
T16	1.49	1.36	1.49	1.49	1.49	1.49	1.49	1.49	1.63	0.54	0.81	0.14	0.27	0.27	0.14		0.14	0.14	0.41	0.14	0.14	0.68	0.41	0.82	0.55	0.27	0.55	0.41
T17	1.63	1.49	1.63	1.63	1.63	1.63	1.63	1.63	1.76	0.68	0.95	0.27	0.41	0.41	0.27	0.14		0.27	0.54	0.27	0.27	0.82	0.55	0.96	0.68	0.41	0.68	0.54
T18	1.63	1.49	1.63	1.63	1.63	1.63	1.63	1.63	1.76	0.68	0.95	0.27	0.41	0.41	0.27	0.14	0.27		0.54	0.27	0.27	0.82	0.55	0.96	0.68	0.41	0.68	0.55
T19	1.90	1.76	1.90	1.90	1.90	1.90	1.90	1.90	2.04	0.95	1.22	0.54	0.68	0.68	0.54	0.41	0.54	0.54		0.54	0.54	1.09	0.82	1.23	0.96	0.68	0.96	0.82
T20	1.63	1.49	1.63	1.63	1.63	1.63	1.63	1.63	1.76	0.68	0.95	0.27	0.41	0.41	0.27	0.14	0.27	0.27	0.54		0.27	0.82	0.55	0.96	0.68	0.41	0.68	0.55
T21	1.63	1.49	1.63	1.63	1.63	1.63	1.63	1.63	1.76	0.68	0.95	0.27	0.41	0.41	0.27	0.14	0.27	0.27	0.54	0.27		0.82	0.55	0.82	0.68	0.41	0.68	0.54
T22	2.17	2.04	2.17	2.17	2.17	2.17	2.17	2.17	2.31	1.22	1.49	0.81	0.95	0.95	0.81	0.68	0.81	0.81	1.09	0.81	0.81		0.82	1.23	0.96	0.68	0.96	0.82
T23	1.90	1.76	1.90	1.90	1.90	1.90	1.90	1.90	2.04	0.95	0.95	0.54	0.68	0.68	0.54	0.41	0.54	0.54	0.81	0.54	0.54	0.81		0.41	0.41	0.14	0.41	0.27
T24	2.31	2.17	2.31	2.31	2.31	2.31	2.31	2.31	2.44	1.36	1.36	0.95	1.09	1.09	0.95	0.81	0.95	0.95	1.22	0.95	0.81	1.22	0.41		0.82	0.54	0.82	0.68
T25	1.76	1.90	2.04	2.04	2.04	2.04	2.04	2.04	2.17	1.09	1.36	0.68	0.81	0.81	0.68	0.54	0.68	0.68	0.95	0.68	0.68	0.95	0.41	0.81		0.27	0.55	0.41
T26	1.76	1.63	1.76	1.76	1.76	1.76	1.76	1.76	1.90	0.81	1.09	0.41	0.54	0.54	0.41	0.27	0.41	0.41	0.68	0.41	0.41	0.68	0.14	0.54	0.27		0.27	0.14
T27	2.04	1.90	2.04	2.04	2.04	2.04	2.04	2.04	2.17	1.09	1.36	0.68	0.81	0.81	0.68	0.54	0.68	0.68	0.95	0.68	0.68	0.95	0.41	0.81	0.54	0.27		0.14
T28	1.90	1.76	1.90	1.90	1.90	1.90	1.90	1.90	2.04	0.95	1.22	0.54	0.68	0.68	0.54	0.41	0.54	0.54	0.81	0.54	0.54	0.81	0.27	0.68	0.41	0.14	0.14	

Data above diagonal present percentage genetic distance (K2P). Data below diagonal present percentage substitutions.

Table 5. The diagnostic sites between northern and southern groups of Taiwan population.

	Diagnostic sites						
	18616	31262	46828	57474	61848	63161	66919
Northern group	T	T	C	C	C	T	C
Southern group	C	C	T	T	T	C	T

Table 6. The frequences of 28 haplotypes in 19 sampling localities of *Anourosorex squamipes* of Taiwan

	Li	Ku	Wu	P	S	Ky	H	Ts	Me	N	O	W	D	Xi	Sh	A	Ac	Yu	Ti	G
T1	1																			
T2	4	1	1		2		1													
T3			1																	
T4	1																			
T5	3																			
T6	1																			
T7							1													
T8			1																	
T9			2																	
T10							1													
T11							1													
T12									1			8	1							
T13													1							
T14													3							
T15								1												
T16						2	1	1	4	1	1								1	
T17				1																
T18						1														
T19										1										
T20																			1	1
T21						1														
T22																6	2	2		
T23														1			1	1		
T24																	1			
T25																1				
T26																1	3	1		
T27														2						
T28															2					

Table 7. Analysis of molecular variance (AMOVA) among northern and southern groups of Taiwan population

Group	Among regions				Among populations within regions				Among individuals within popualtions			
structure	Va	%	P	ψ_{CT}	Va	%	P	ψ_{SC}	Va	%	P	ψ_{ST}
1	4.77181	74.86	0.0005	0.74861	0.72207	11.33	<0.0001	0.86190	0.88030	13.81	<0.0001	0.45063
2	3.77578	68.13	0.0005	0.68134	0.66054	11.92	<0.0001	0.80054	1.10537	19.95	<0.0001	0.37405

Group structure: 1. without specimens from Mt. Houhuan, two groups (regions)
2. with specimens from Mt. Houhuan, three groups (regions)

Va: variance components; %: percentage of variation; P: possibility of the significance test of ψ-statistics;
ψ_{CT}, ψ_{SC} and ψ_{ST}: fixation indices of ψ-statistics (Excoffier 1992)

Table 8. Pairwise comparision of mtDNA cytochrome *b* gene complete sequence between 7 haplotypes from *Anourosorex squamipes*

	H1	H2	H3	H4	H5	8405	8403
H1		2/0	13/3	15/1	15/2	57/7	57/8
H2	0.2		13/3	15/1	15/2	55/7	55/8
H3	1.4	1.4		8/2	4/3	58/8	58/7
H4	1.4	1.4	0.9		4/1	59/6	59/7
H5	1.5	1.5	0.6	0.4		60/7	60/8
8405	5.9	5.7	6.1	6.0	6.2		0/1
8403	6.0	5.8	6.0	6.1	6.3	0.1	

Above diagonal: Numbers of nucleotide substitutions (transitions / transversions)

Below diagonal: Kimura's two parameter distance (%)

H1 to H5: Specimens collected from Mt. Houhuan of Taiwan

8403, 8405: Specimens collected from Mian-Yang City of Southern China

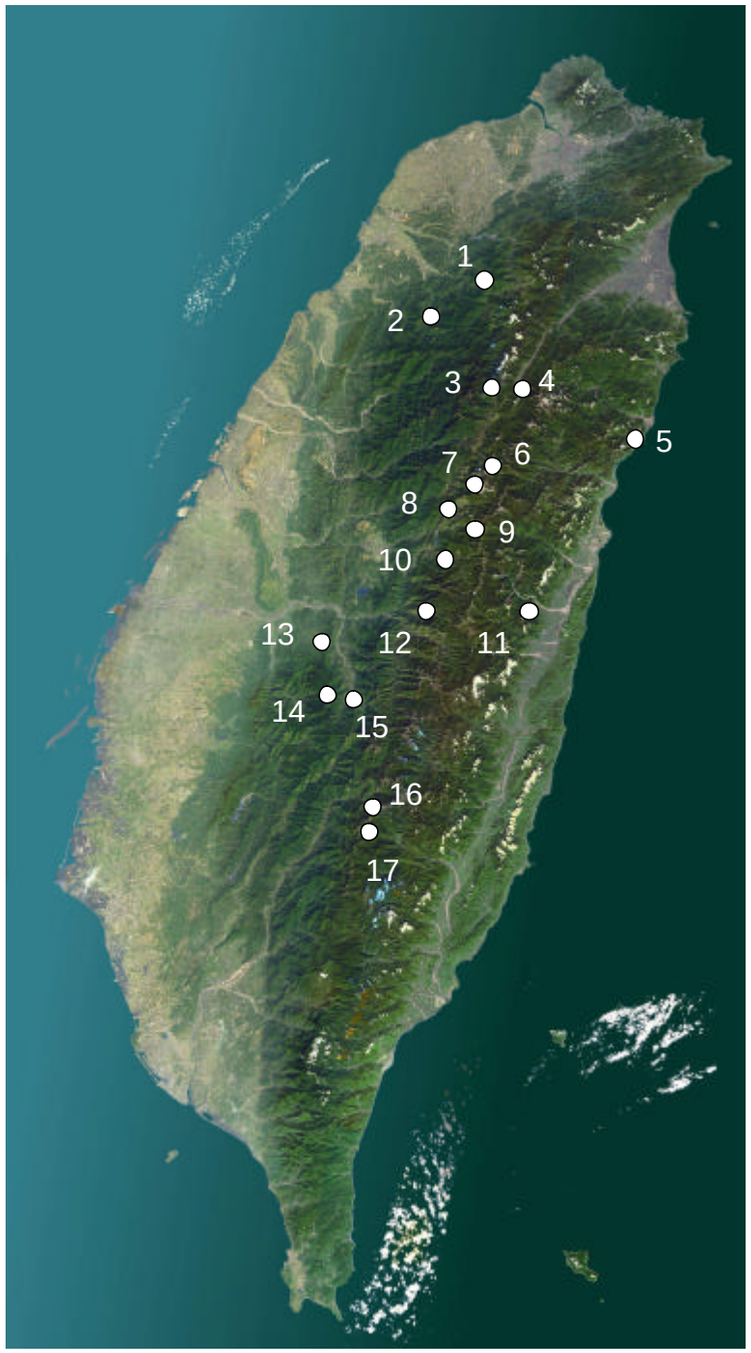
Table 9. A review of phylogeographic studies of animals in Taiwan

Species	Genetic marker	Localities / Sampling size	Phylogeographic pattern	References
Acrossocheilus paradoxus	Nucleolar DNA: Hind III (Cloning)	7 / 14	Middle+South / North	Wu W. L. (1999)
Acrossocheilus paradoxus	mtDNA: cytb+tRNA+ D-loop+12s rRNA	12 / 168	Geo. Dis. vs. Var. Middle+South / North	Wang J. P. (2000)
Apodemus semotus thomas	mtDNA: cytb+D-loop	23 / 271	Middle+South / North	Hsu F. H. (2001)
Niviventer culturatus	mtDNA: cytb+D-loop 12s+16s rRNA	20 / 128	—	Hsu F. H. (2000)
Rhacophorus taipeianus	mtDNA: cytb+D-loop	6 / 200	North / Middle	Yang Y. J. (1994)
Trimeresurus stejnegeri	mtDNA: cytb	10 / 201	North+East / All but North	Creer S. (2001)

※The "/" indicates the isolated pattern in each study

Table 10. Sea level changes and major galcial periods in the geohistory of Quaternary (modified from Lin 1963)

Period	Epoch	Mya	Glacial period	Sea level(m)
Quaternary	Holocene	0.01		+10~15
	Pleistocene	0.06~0.01	Würm	-100~130
		0.15~0.06		+6~+30
		0.24~0.15	Riss	-100
		0.38~0.24		+30~+40
		0.45~0.38	Mindel	-100
		0.55~0.45		+30~+60
		1~0.55	Günz	-100



A. Mian-Yang City, Szechuan, China

B. Na-Chung City , Szechuan, China

Names of sampling localities of Nos. 1 - 17 list in Table 1.

Fig. 1. Sampling localities of *Anourosorex squamipes* in Taiwan and Southwestern China

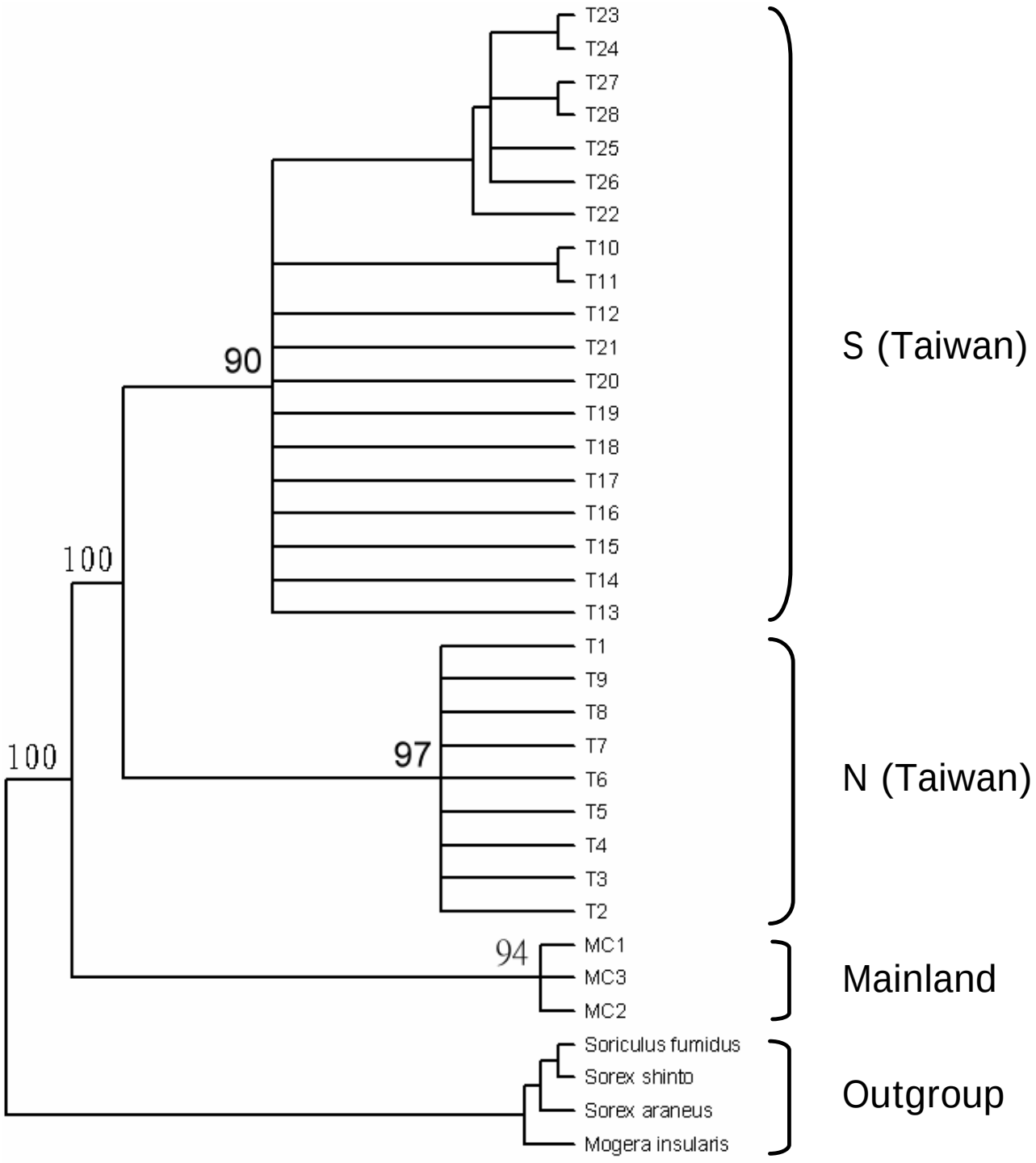


Fig. 2. Maximum parsimony tree constructed from 31 haplotypes of *Anourosorex squamipes*. Numbers at the nodes represent over 50% supporting value by 1000 bootstrap replicates. (CI=0.726, RI=0.768)

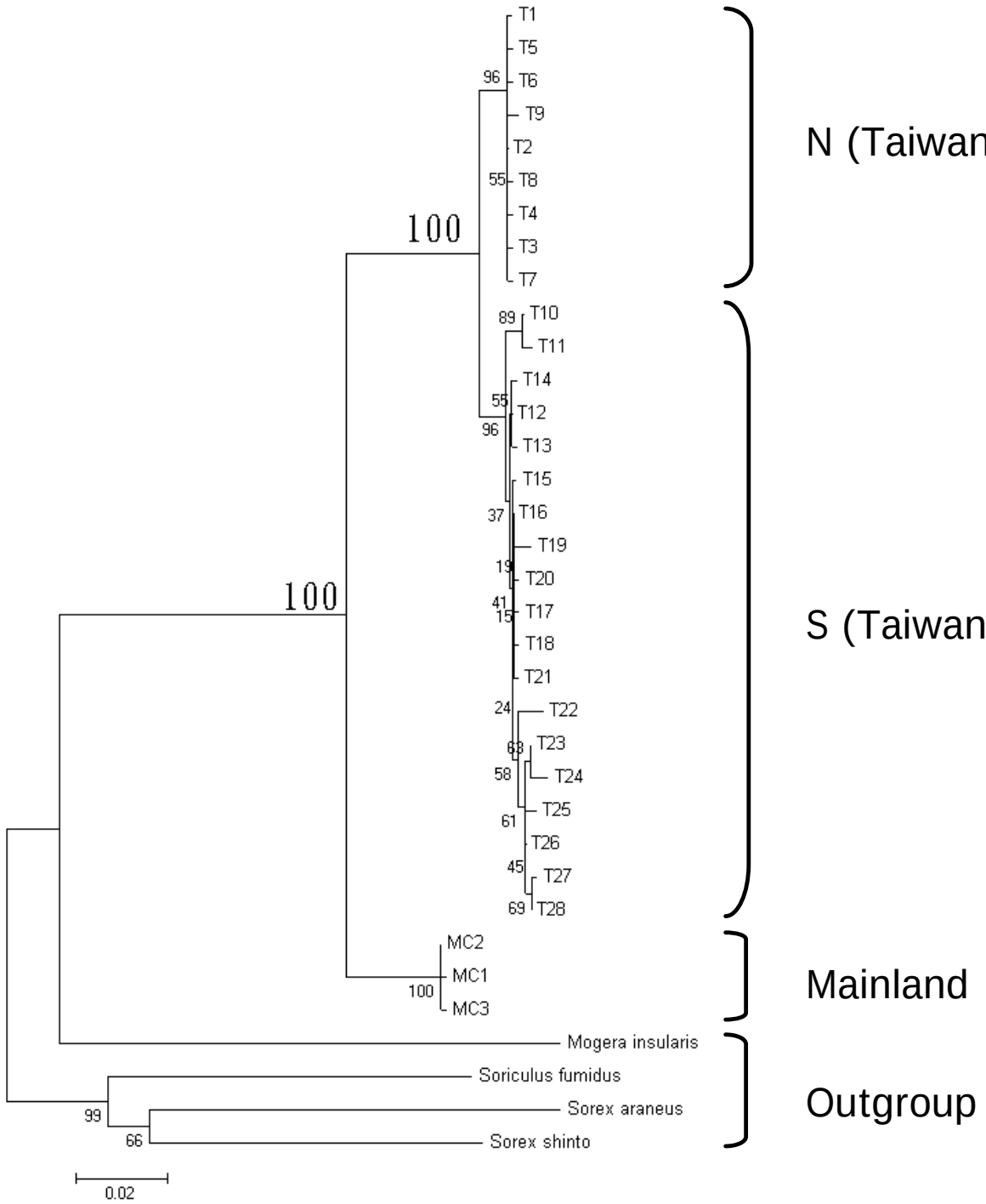


Fig. 3. The neighbor-joining tree constructed from 31 haplotypes of *Anourosorex squamipes.* Numbers at the nodes represents supporting value by 1000 bootstrap replicates. The scale bar indicates branch lengh in terms of nucleotide substitution.

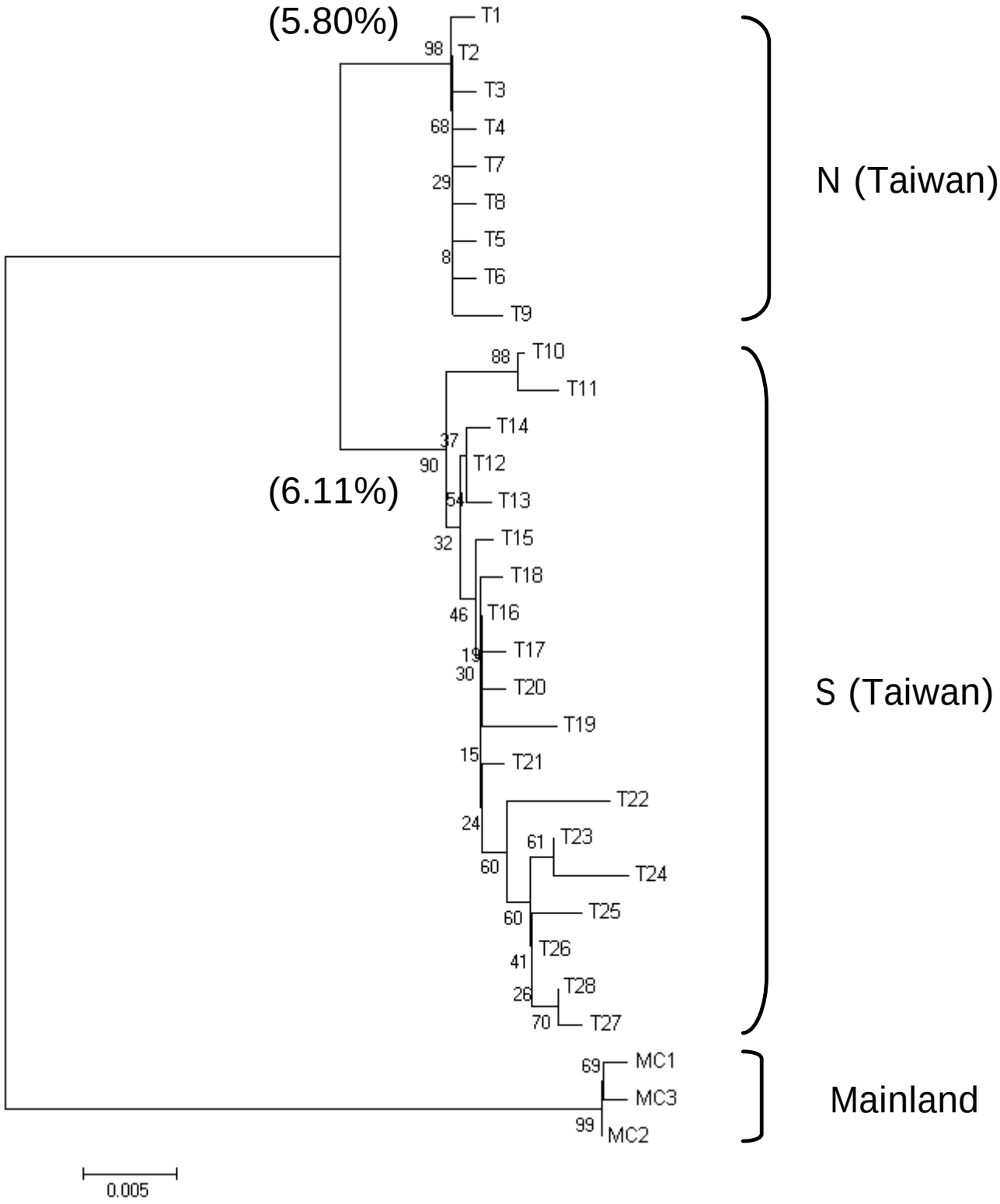


Fig. 4. The un-rooted neighbor-joining tree constructed from 31 haplotypes of *Anourosorex squamipes*. Numbers at the nodes represents supporting value by 1000 bootstrap replicates. The scale bar indicates branch lengh in terms of nucleotide substitution. Percentages reveals the average genetic disance based on Kimura's two-parameter model between two groups of Taiwan and Mainland China.

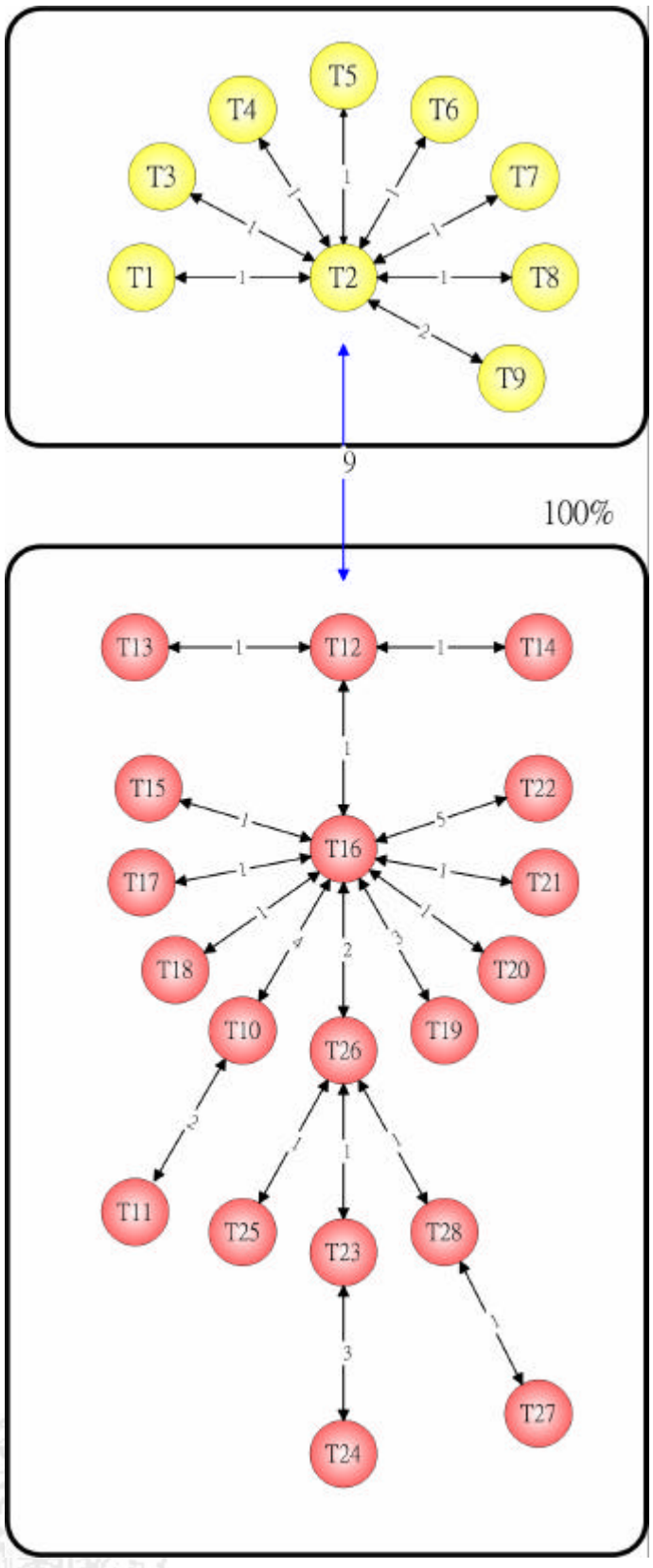


Fig. 5. The phylogenetic relationships of 28 haplotypes reconstructed by minimum spanning network. Numbers at the arrows indicate the number of substitutions between haplotypes. Yellow and red circles represent the northern and southern groups, respectively. The bootstap values from NJ and MP method were also displayed among two groups as 100%.

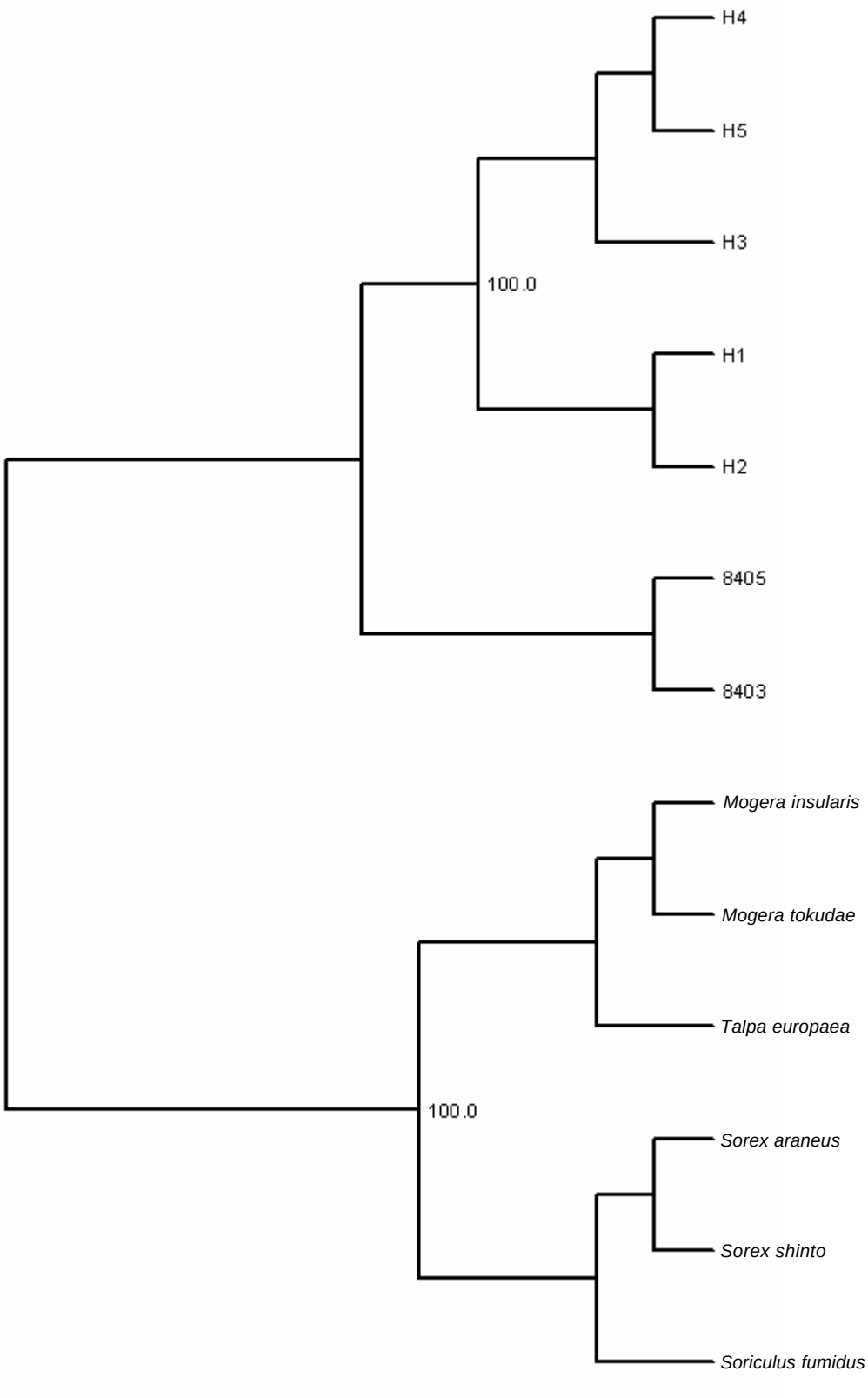


Fig. 6. Maximum parsimony tree constructed based on mtDNA cytochrome *b* gene complete sequence of *Anourosorex squamipes*. Numbers at the nodes represent supporting values by 1000 bootstrap replicates. (CI=0.6651, RI=0.6926)

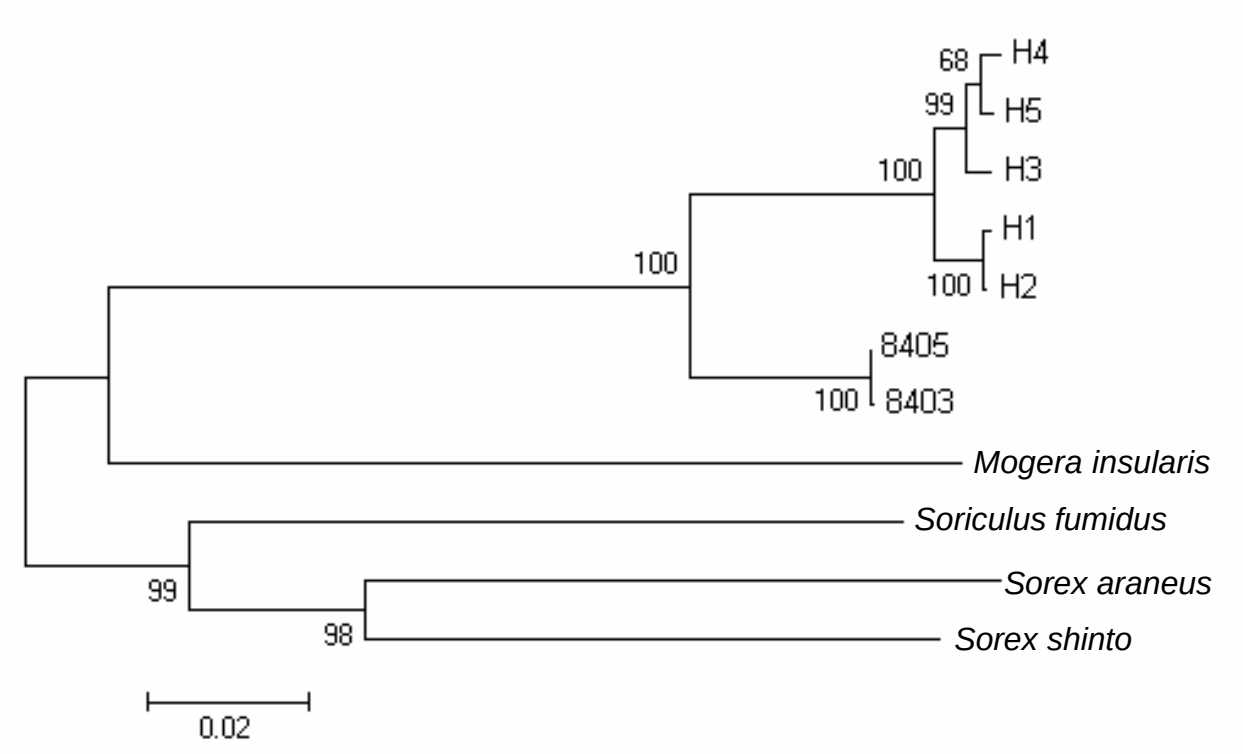


Fig. 7. The neighbor-joining tree based on cytochrome *b* gene complete sequence of *Anourosorex squamipes*. Numbers at the nodes represent supporting values by 1000 bootstrap replicates. The scale bar indicates branch lengh in terms of nucleotide substitution.

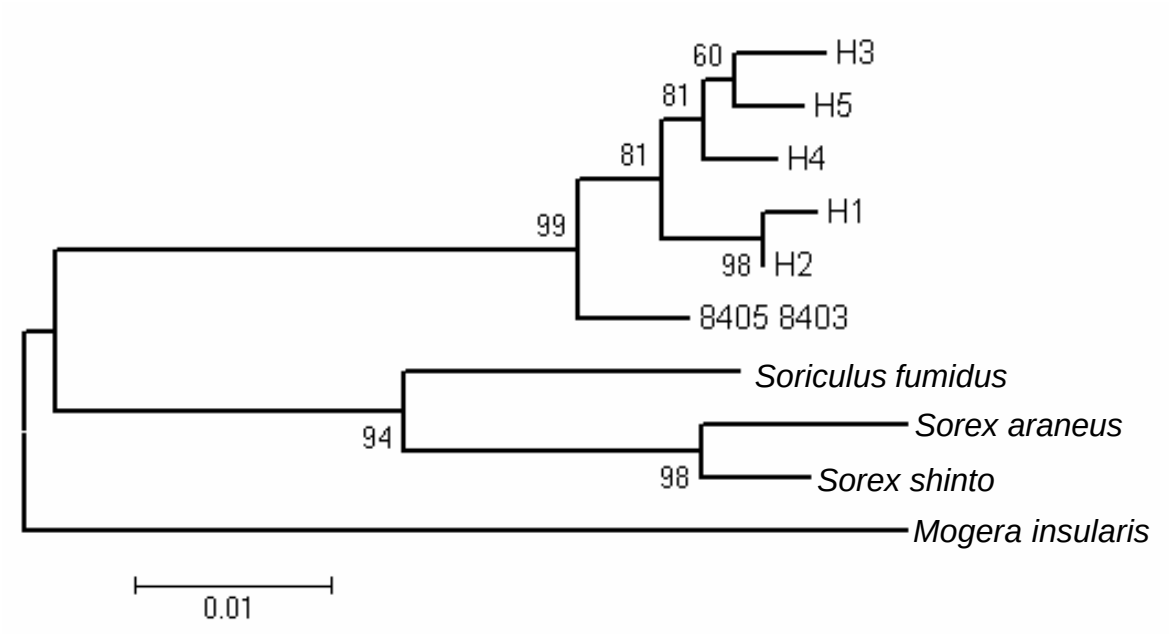


Fig. 8. The neighbor-joining tree based on mtDNA cytochrome *b* amino acid sequence of *Anourosorex squamipes*. Numbers at the nodes represent supporting values by 1000 bootstrap replicates. The scale bar indicates branch lengh in terms of nucleotide substitution.

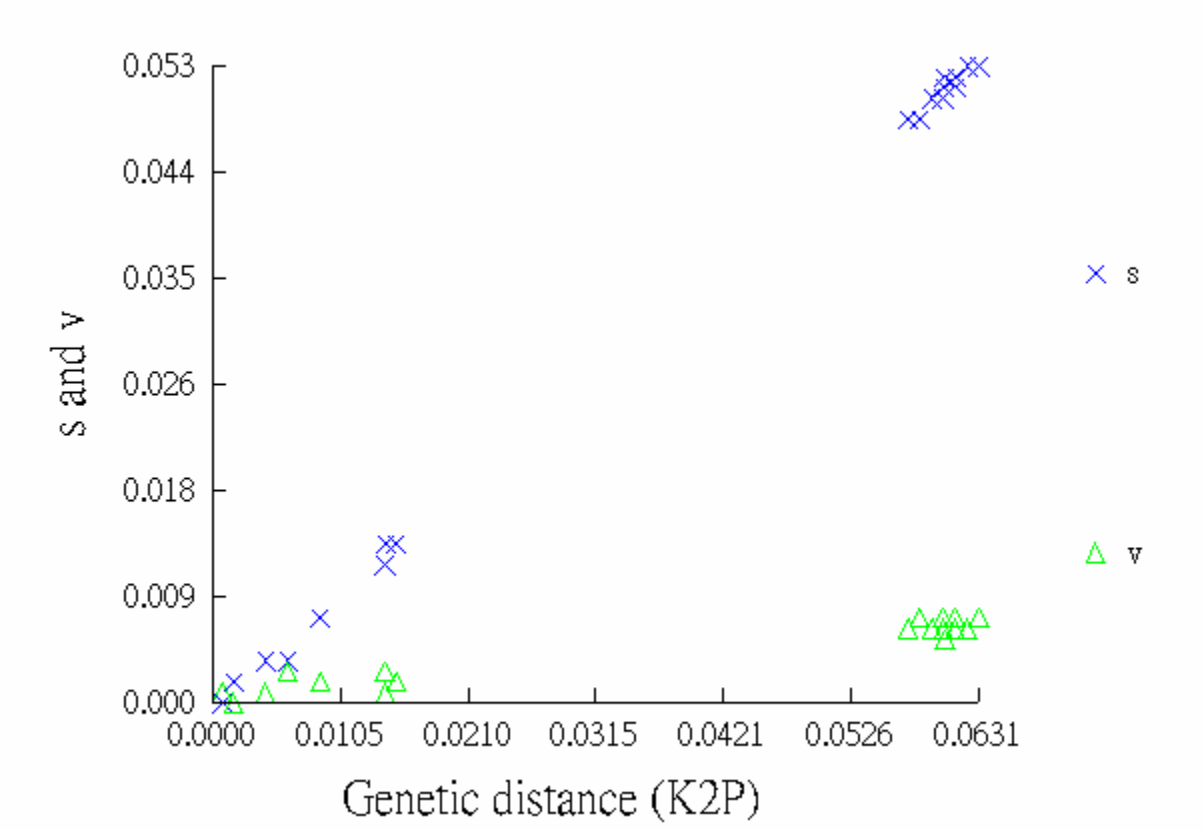


Fig. 9. Plot of transitions (triangles) and transversions (X) in pairwise comparisons against kimura 2 parameter distances for cytochrome *b* sequences of *Anourosorex squamipes*.

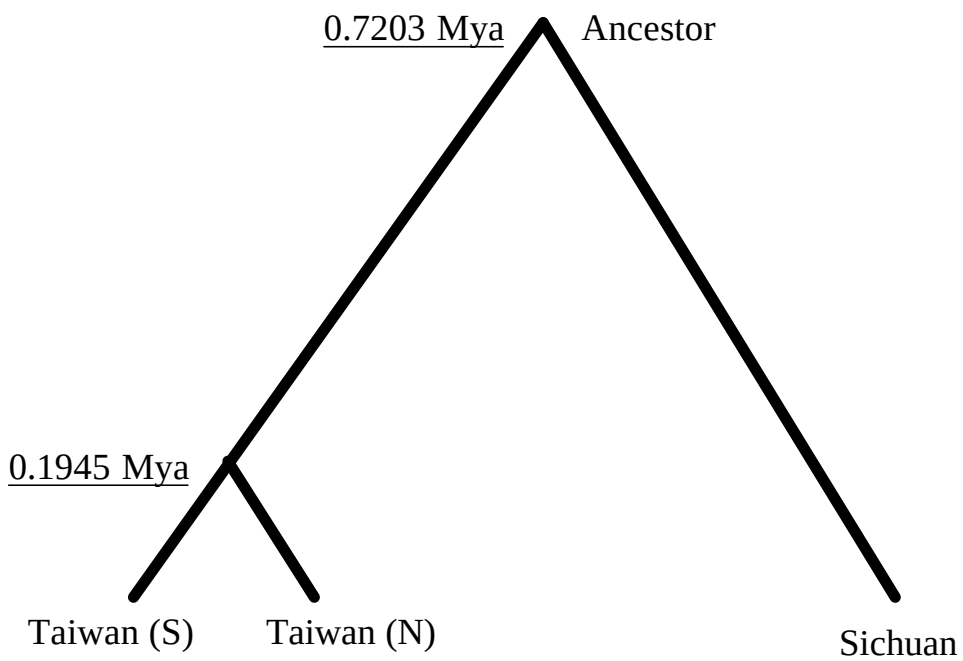


Fig. 10. Divergence of *Anourosorex squamipes* populations estimated from cytochrome *b* sequences.