國立中山大學生物科學系

碩士論文

台灣產標蛇屬之親緣地理

Phylogeography of Odd-scaled Snake *Achalinus* in Taiwan

研究生：謝佳蓉 撰

指導教授：張學文 博士

中華民國 九十五 年 七月

摘要

標蛇(*Achalinus niger*)爲台灣特有種，在分類上屬於蛇亞目(Serpentes)，黃頷蛇科(Colubridae)，閃皮蛇亞科(Xenodermatinae)中的標蛇屬(*Achalinus*)。標蛇分布在台灣的中、高海拔山區，海拔分布由 1000 公尺到 3000 公尺。由於生物族群的遺傳結構與地理分布，容易受到地理環境的影響，造成族群之間分群的現象，尤其是山脈以及被河流切割的河谷，提供了一個阻擾生物的擴散及遷移的環境。本研究欲利用分子生物的方法，來檢視標蛇族群內與族群之間的基因變異程度，並去釐清標蛇族群間的親緣地理關係，進一步去探討標蛇族群的分布，是否與台灣的山脈分布有關。研究樣本來自台灣北、中、南各山區共 18 個採集點，萃取組織中粒線體 DNA 中的 ND2 片段來進行序列分析。結果得知本研究分析 ND2 基因序列的長度爲 994 bp，核苷酸歧異度平均值爲 3.5%，平均基因距離爲 3.87%。其中，梅峰地區的族群遺傳結構較爲特殊，分析其他資料後，推測標蛇爲多次入侵的物種。親緣關係方面，標蛇族群分爲三個主要的系群，且系群的分布與地理位置大致相同，系群Ⅰ爲分布在台灣北部的雪山山脈；系群Ⅱ爲分布在台灣中南部的山區；系群Ⅲ則爲中部的梅峰地區。從 AMOVA 得到標蛇族群的變異來自各系群之間，並非來自族群內的個體，因此推測標蛇族群的分布與山脈的隔離有程度上的相關。

關鍵字：標蛇，親緣關係，多次入侵

Abstract

Achalinus niger is an endemic species of Taiwan. It distributes from 1000 to 3000 m in altitude. The effect of geological isolation, especially mountains and valleys may influence the population genetic structure of the species. The ND2 gene was used as genetic marker to analyze the genetic structure of populations and phylogeography of *A. niger*. The mean genetic distance is 3.87％ and the mean nucleotide diversity 3.5％. The Meifeng population was distinct in genetic structure from other populations. The data suggest that *A. niger* had more than one incursion events. In phylogenetic analyses, there were three major lineages in *A. niger*, and these lineages were correlated to geographic distribution. Lineage Ⅰ was sampled from northern Taiwan, lineage Ⅱ from southern Taiwan, while lineage Ⅲ from Meifeng only. The result of AMOVA indicated that there were high genetic variations among groups. The genetic isolation and geographic distribution of these groups probably resulted from the geographic barriers.

Key words：*Achalinus niger,* genetic structure, phylogeography, phylogenetic analyses

Contents

Introduction **1**

Materials and Methods **5**

Results **11**

Discussion **16**

Literature Cited **22**

Figure and Table Contents

Figure 1 28
Figure 2 29
Figure 3. 30
Figure 4 31
Figure 5 32
Figure 6 33
Figure 7 34
Figure 8 35
Figure 9 36
Figure 10 37
Figure 11 38
Figure 12 39
Figure 13 40

Table 1. 41
Table 2. 42
Table 3. 43
Table 4. 44
Table 5 44
Table 6 45
Table 7 46
Table 8 47
Table 9 47
Table 10 48
Table 11 48

Introduction

Studying phylogenetic relationship among populations of a species elucidates a number of important issues on population biology. First, we could know the genetic structure among populations (Avise, 2000; Ashton, 2001). Second, such studies revealed the geographic distribution of lineages and we could inferenced the historical biogeography among populations (Avise, 2000). Third, these studies provided a framework for other evolutionary inquiries about a species, as well as provide the means to identify phylogenetically independent data points for comparative analysis (Ashton, 2001).

As we know, the genetic structure and geographic distribution of a species were influenced by many factors, such as ecology, demography, dispersal of a species, and vicariance events. However, variation of gene accompanies with some degrees of geographic structure among populations in a species. And these factors of the natural selection, gene flow and genetic drift will be able to influence the variation and distribution of a gene.

The study of intraspecific phylogeography researches the relationship between genetic structure of species and fauna in a specific area. There are five categories about the genetic divergence and geographic distribution of species, (Avise, 1987) (Ⅰ) the genetic divergence is discontinuous, but the geographic distribution of species is not the same with the genetic divergence. (Ⅱ) the genetic divergence is discontinuous, and the species could co-occur geographically.(Ⅲ) the genetic divergence is continuous, and the distribution of species in geography is similar to the gene type. (Ⅳ) the genetic divergence is continuous, and the divergence in gene and geography is discordant. (Ⅴ) the genetic divergence is continuous, but some gene type distribution over some specific regions. (Fig.1)

In Taiwan, two-thirds of the island are mountains more than 1000 meters in altitude and it supposes to form a potential barrier of dispersal of lowland species. Another factor of causing the differentiation of species is the basins or the valleys formed by rivers, because some species are unable to adapt the low altitude environment or their competition abilities with other species will be limited if they are in this kind of environment. Therefore the gene flow is limited and these populations are apart from different areas.

Many studies about the phylogeography of species in Taiwan mentioned an common model of origin of species. During the Pleistocene, some species moved to Taiwan from other continental Asia through the land bridge then the mountain rise which was caused by orogeny in Taiwan made the species differentiation. There are two population distributions of low altitude species, such like Gecko, *Gekko hokouensis* (Tsai 1999), Rice field frog, *Rana limnocharis* (Toda 1998), House Mice, *Mus musculus* (Yang 1997) *and* Swinhoe's japalura, *Japalura swinhonis* (Liu 1995) etc., apart from east and west of Taiwan is associated with the Central Mountain Range. But for the middle and high altitude species, such like Short-legged japalura, *Japalura brevipes Gress* (Xiang 1997), Siberian weasel, *Mustela sibirica* (Wu 2004), Sauter's brown frog, *Rana sauteri* (Chen 1994), and Formosan Wood Mouse, *Apodemus semotus* (Hsu 2001) etc., their distributions aren't associated with mountains because the mountains stretch along the entire island from north to south, therefore they are divided into northern, central and southern clades. Otherwise, the distributions of the other species have no relationship with the mountains or other geography factors, e.g. Green bamboo viper, *Trimeresurus stejnegeri* (Creer 2001), Farmland green treefrog, *Rhacophorus arvalis* (Liu 2000) and Formosan white-bellied rat, *Rattus culturatus* (Hsu 2001) etc.

The sequence diversity reveals the evolution of nucleotides which are

between individuals and populations, and shows the evolution of populations at the same time. Due to some advantages of mitochondrial DNA, we use it to do phylogenetic study. The first advantage is that the evolutional rate of mitochondrial DNA (mtDNA) is five to ten times faster than nucleus DNA (Futuyma, 1998). The second advantage is that mtDNA is maternal inheriteance, so there is no recombinant event when it reproduces its generations. The third advantage is that mtDNA is a close loop structure, and it is less complex than nucleus DNA in the structure. The forth advantage is that mtDNA has no repair system, so it has a high mutation rate. MtDNA analysis has been used to be an especially powerful tool for investigating underlying biogeographic patterns of many taxa and it is particularly well suited for providing a beneficial marker at the intraspecific level (Bernatchez and Wilson, 1998).

DNA sequence data are used for constructing phylogeny and for the evaluation of genetic divergence. I choose the NADH dehydrogenase subunit 2 (ND2) fragments from mitochondrial genome for sequencing. ND2 gene is preferable for examining intra-specific relationship (Ashton, 2001) and it evolves at a high rate (Otto, 1996), so ND2 gene has been effective in discerning relationships among the closely related species (Duvemell, 1998; Macey *et al*, 1998). This fragment has been used extensively in amphibians and reptiles (Macey *et al*, 1998)

The odd-scaled snakes belong to genus *Achalinus*, of family Colubridae, of subfamily Xenodermatinae, and there are two endemic species in Taiwan. One is *Achalinus niger* and the other is *Achalinus formosanus formosanus*. The habitats of these species are similar. Both of the habitats are under the forest and the distributed ranges are from north to south of Taiwan. The distribution of these snakes is restricted to middle and high altitudes which is about one thousand to three thousand meters. Except the habitats, their appearances are alike as well. The

only difference in their appearance is variation of scales.

Based on the above statements, we aim at four purposes about this study:
(1) to understand the genetic variability among individuals and intraspecifics of *A. niger*.
(2) to realize the phylogeographic patterns of *A. niger* based on the molecular biology
(3) to use a statistical approach to know what historical events can be invoked to explain this biogeographic pattern
(4) to take one step ahead to get the phylogenetic relationships between *A. niger* and *A. f. formosanus* and also to compare the method with traditional taxonomy.

Materials and Methods

Sampling

From July 2004 to September 2005, I collected 70 samples from 18 locations in Taiwan. (Fig. 2; Table1). Sixty samples were collected in mountain areas at an altitude from 430m to 2500m, and ten samples were obtained from Endemic Species Research Institute in Taiwan and Taipei Zoo.

The live snakes to the laboratory by collecting bag. If the snake died on road, the samples were preserved in 70％ ethanol before they were taken to the laboratory for extracting DNA. I extracted DNA from all samples which were including Meigu burrowing snake (*Achalinus meiguensis*) and Red Bamboo snake (*Elaphe poryphyracea*). These snakes were chosen as outgroups for phylogenetic analysis.

species identification

To recognize *A. niger* and *A. f. formosanus* from external, the identifiable standards were based on original papers (Maki, 1931; Boulenger, 1908). The diagnostic character which are between *A. niger* and *A. f. formosanus* differ from: (1) the dorsals of *A. niger* which were counted to 25 rows in the middle of body, but *A. f. formosanus*was counted to 27 rows; (2) the dorsals of *A. niger* were smooth but *A. f. formosanus*had keels on the dorsals; (3) the ventrals of *A. niger* were counted to 181-184 rows, but *A. f. formosanus*was counted to 175 rows; (4) the subcaudals of *A. niger* were counted to 52 rows, and *A. f. formosanus*was counted to 64 rows.

DNA Extracted

Genomic DNA was extracted from the livers and the muscles which were in the tissue digestion buffer overnight, and then I used a standard phenol chloroform/ proteinase K method (Palunbi *et al*,1991) or Blood ＆ Tissue

Genomic DNA Extraction Miniprep System Kit (Viogene). The protocol of Phrnol-Chloroform method was described below:

1. Cut 0.1g tissue and ground it, then put it into 1.5ml sterile eppendorf tube with 94 μL TE buffer, 5 μL 10% SDS and 1μL proteinase K. Mixed and incubated at 55℃ overnight.
2. After the reaction done, added 200 μL PCI (phenol: chloroform: isoamyl alcohol=25:24:1) into the tube and inverted the tube to make the solution well-mixed, and centrifuged for 10 minutes at 12K rpm.
3. Preserved the upper stratum solution and added 100 μL CI (chloroform: isoamyl alcohol=24:1) into the tube, then inverted the tube to make the solution well-mixed, and centrifuged for 10 minutes at 12K rpm.
4. Preserved the upper stratum solution and added 400 μL absolute Ethanol to reduce the solubility of DNA. Refrigerated the DNA under -80℃ at 30 min, then centrifuged in a low temperature for 15 minutes at 12K rpm.
5. Discarded Ethanol and dried the DNA, and eluted DNA using 40μL double-distilled water (ddH_2O).

PCR amplification, DNA purification

The entire mitochondrial NADH dehydrogenase subunit 2 (ND2, 1030 bp) was amplified by using the polymerase chain reaction (PCR) (Saiki et al, 1988). The ND2 primers were L4774:5'-AAGCTTCGGGCCCATA-3' and H5877:5'-GG CTTTGAAGGCTACCTAGTTT-3' (Fig. 3). Polymerase chain reaction (PCR) was conducted in 50μl volumes with 5.0μl 10X PCR buffer (100mM Tris-HCl,500Mm KCl,15mM $MgCl_2$), 4.0μl dNTP, 1.0μl each primer, 2.0μl template, 0.3μl taq DNA polymerase and 36.7μl double-distilled water. I used GeneAmp PCR System 2400 to perform the reactions, with the amplification conditions which were an initial predenaturation for 3 min at 95℃, then followed by 35 cycles of denaturation for

40 seconds at 94℃, annealed for 1 minute at 51℃, extended for 90 seconds at 72℃, the final extension lasted 10 minutes at 72℃ and cooled to 4℃. I ran the PCR products by electrophoresesing in 1.0% agarose gals and 5X tris boric EDTA (TBE) at 100V for 30 minutes, then visualized by stained ethidium bromide under UV light. PCR products were purified by using the Viogene PCR-M^{TM} CLEAN-UP System Kit or Gel-M^{TM} GEL Extraction System Kit.

DNA autosequencing

All nucleotides were obtained by cycle sequencing which was performed by DIVERSITAS Research Center in National Sun Yat-sen University. The ND2 gene sequenced on an ABI 3100 DNA Sequencer with the same primers which were used for amplification and sequences were double checked with forward and reversed strands.

Sequence test

Every sequence blasted with NCBI to confirm the sequences to see if they were ND2 gene. Gaps resulting from the alignment were treated as missing data. The saturation was analyzed by p-distance versus the percentage of transitions and transversions and by using DAMBE 4.2.13 (Xia, 2000). I computed the number of transitions and transversions over each codon position for all pairwise comparisons for ND2 gene by using MEGA3.1. To confirm the evolution did not effected by natural selection and tested the molecular maker is neutrality on evolution, I made Tajima's test of neutrality by using MEGA3.1.

Sequence structure

Then I check sequence by eyes and aligned by using the BioEdit 7.0.4.1 (Hall, 1999). The nucleotide frequency of all sequences was calculated by MEGA3.1 (Kumar *et al*, 2001). The nucleotide diversity (π) indicated the numbers of

nucleotide differences per site between two sequences (Nei, 2000), and the π could be estimated: $\pi=(n/n-1)\ \Sigma x_i x_j \pi_{ij}$. The symbol n indicates the number of samples, symbol x_i and x_j indicates the frequency of the type i and type j DNA in population, symbol π_{ij} indicates the differential numbers of nucleotide. Nucleotide substitution per site (p-distance) and genetic distances used Tamura-Nei model which were between any two nucleotides. We use modeltest3.7 to select the model of nucleotide substitution that best fits the data.

Population genetics

The nucleotide diversity between any populations is dxy, and the equation of dxy was $dxy=\Sigma_{ij} x_i y_j d_{ij}$. The symbol y_j indicates the frequency of type j DNA in population y. The genetic polymorphism and diversity were described by haplotype diversity (*h*)(Nei, 1987), and the equation of *h* was $h=n(1-\Sigma x_i^2)(n-1)$. The degree of genetic differentiation could be measured by Fixation indices (Fst) (Hudson *et a*, 1992), and the definition of Fst was $Fst=1-(H_w/H_b)$. If the Fst value was higher than 0.25, it expressed a extr-highly differentiation. If the value was smaller than 0.05, it expressed a extr-low differentiation. Another index was gene flow (Nm), and the equation was $Nm=(1-Fst)/2Fst$. The higher Nm value indicated that the gene interflowed smoothly between populations. All diversity indexes above between populations and individuals were calculated by DnaSP 4.1 (Julio Rozas *et al*, 2005). The genetic distances between haplotypes and populations were also computed in MEGA3.1 and used a Tamura-Nei model with gamma distribution of the substitution rates. Then I calculated the regression about the Fst and geographic distances to test if the “distances” and “genetic differentiation” have a correlation in statistics.

Phylogenetic analyses

For phylogenetic reconstruction, I used one distance method: Neighbor-Joining (NJ) and two character-based methods: maximum parsimony (MP) and

maximum likelihood (ML) methods. The Neighbor-Joining tree was executed with 1000 replicates of bootstrap by using MEGA3.1. For maximum parsimony method, I used branch and bound algorithm to find the most parsimonious tree, because the number of taxa was more than 20 in our data, and it was large to permit by using this method. In additional, I calculated the values of consistency index (CI) and rescaled consistency index (RC) about maximum parsimony tree. Bootstrapping was applied to assess support for individual nodes with 1000 replicates. When two or more parsimonious trees were produced, a 50% majority-rule consensus tree was constructed. The maximum likelihood tree was constructed by GTR+I+Γ model with heuristic search and 100 replicates. Both of maximum parsimony and maximum likelihood phylogenetic trees were estimated by using PAUP 4.0 (Swofford, 1998).

Population genetics analyses

To further resolve relationships within major haplotype clusters, a statistic parsimony approach was used (Tempeleton *et al*, 1992), and it was carried out using TCS 1.21 (Posads, 2004). Using this, I could resolve the haplotypes are more likely to be connected to common than to rare haplotypes or haplotypes are more likely to be connected to interior than to exterior haplotypes (Templeton and Sing, 1993).

Samples were grouped as a population and subjected to a hierarchical analysis of variance (AMOVA)(Excoffier *et al*,1992) to ascertain whether the population structure observed is significant. AMOVA was conducted to test the relative of variance within populations and among populations by using Arlequin 3.01. The fixation index was computed and the genetic variation was estimated by the pairwise distances.

Divergence time

To estimate the divergence time of different populations in *A. niger*, I used the molecular dating to gain an idea of the age of these populations. The pairwise distance (Nei and Kumar, 2000) was used to correct the genetic distance of nucleotide sequences. Because no calibration point was available in this genus, I estimated the divergence times were based on the rate comprised between 1.5％ ~ 2.5％ divergence per million years (Myr) for reptilian (Crochet, 2004).

Results

I got twenty-eight individuals of *A. niger* from five isolated mountain localities (Fig4). Sequences of ND2 were obtained for the twenty-five individuals of *A. niger*, and two outgroups specimens (*Achalinus meiguensis* and *Elaphe poryphyracea*).

I. Tests

The saturated-graph showed the rate of transversion was slower than transition (Fig.5), and the saturated test indicated the sequences among these individuals of *A. niger* did not reach saturation (Table.2). Besides, Tajima's D value was -1.079 and Fu and Lis D^* test value was -1.014；these values did not differ significantly from expected values of neutrality test. According to these two tests, these sequences were calculated for further analyses.

II. Nucleotide sequences

The entire length of ND2 is 1024 bp, and I analyzed 994 bp and there are 156 variable sites with 88 phylogenetically informative sites after aligning. The base frequencies are C: 36.03％, A: 31.76％, T: 21.14％, G: 11.06％. A+T=52.9％ >C+G=47.1％. The average of Transition / Transversion is 6.2, it's similar to *Sphenomorphus taiwanensis*, but higher then Gecko (*Gekko hokouensis*). The plot of entropy of the variable site was showed in Figure 6.

III. Individuals

The average of genetic distance of individuals is 0.0387. The minimum distance is 0.00 (which is distribution in Shanlinhsi). The maximum distance is 0.1152 (which is distribution between Meifeng and Dasheishan). Nucleotide diversity of individuals on average is 3.5％, and the range was from 0.0％ (which is distribution in Shanlinhsi) to 10.12％ (which is distribution between Meifeng

and Dasheishan) (Table.3).

IV. Populations

I set the samples from the same collected location to a population, but except Lichia and Szuyuanyakou where I got only one sample. So I analyzed seven populations of *A. niger*. The range of the nucleotide diversity (dxy) among populations is 0.2％ (which is distribution between and, Tatachi and) to 9.1％ (which is distribution between Meifeng and Dasheishan) and the average value is 4.19％. The minimum value of Fst is 0.048 (which is distribution between Tatachia and Alishan), and the maximum value is 0.961 (which is distribution between Shanlinhsi and Dasheishan) (Table.4).

I calculated the geographic distance between any two sites by using MapSource 6.9.1 (Table.5) and tested the correlation of the Fst and geographic distance values, only the geographic distance values are normal distribution, therefore I calculated the Spearman's correlation about Fst and geographic distance. The result showed the values of r and Z are 0.668 and 2.989. There is a significant correlation between Fst and geographic distance ($p=0.003<0.05$) (Fig.7).

V. Haplotypes

According to the polymorphic (variable) sites, the ND2 gene indicats twenty-one unique haplotypes which are found in *A. niger* (Table.6). The haplotype diversity of all samples is 0.983. Nucleotide substitution per site (p-distance) and genetic distance of haplotypes and outgroups is presented in Table 7. The average genetic distance of haplotypes is 0.037. The minimum value is 0.001 (H4 and H5、H7 and H8、H7 and H9、H7 and H12、H7 and H13、H9 and H10、H9 and H11) and the maximum is 0.011 (H6 and H20). The least distance of Haplotypes and outgroup is 0.220 (MG and H21), the high value is 0.499 (D1 and

H1). The nucleotide diversity of haplotypes range is from 0.1％ (H4 and H5、H7 and H8、H7 and H12、H7 and H13) to 10.12％ (H6 and H20), and the average value is 3.36％.

VI. Phylogenetic analyses

Parsimony analysis with all characters weighted equally produced one most-parsimony tree of 184 steps, and the consistency index (CI) was 0.87, and the retention index (RI) was 0.912. The log-likelihood score for the maximum likelihood tree obtained is LnL=-3887.67823. The ND2 data of both NJ tree, MP and ML trees showed the same topologies (Fig.8; Fig.9). The phylogenetic trees are subdivided into three lineages, and the main clades was supported by high bootstrap values (>99％).

Then I constructed the phylogenetic tree for 21 haplotypes, it could also divided into three clades. The first clade includes the individuals of Shei-Pa National Park, Da Sheishan and Szuyuanyakou, and the second clade includes the individuals of Alishan, Tatachia, Shanlinhsi, South-Cross Highway-Tienchi and Lichia, and the third clade was made of Meifeng alone. However the first clade contained samples from the Snow Range, and the second clade contained samples from the Central Range and the Ali Range. I put outgroups into phylogenetic tree, and did not affect ingroup topologies. And the populations of outgroups and *A. niger* shows significant divergence in each phylogenetic trees (Fig.10). The analysis of the ND2 gene resulted in a topology indicating *A. chalinus* to be monophyletic.

VII. Groups

Based on the phylogenetic tree, I set the first clade as a group (Northern group), the second clade as another one (Southern group), and the third clade as the other one (Meifeng). The nucleotide and haplotype diversity of the first

group are 0.6％ and 0.952, the second group are 0.5％ and 0.971 and third group are 4.0％ and 0.667 respectively (Table.8). Besides, the nucleotide diversity between any two groups is 0.038 to 0.090. The minimum and maximum of Fst are 0.725 (southern and Meifeng groups) and 0.842 (northern and southern groups) and the values of Nm are 0.093 to 0.189 (Table.9).

VIII. Population genetics analyses

The relationships within haplotypes showed in Figure11. Haplotype1, haplotype2, haplotype3, haplotype5 and haplotype6 don't cluster with other haplotypes. haplotype4 and haplotype7 are ancestry types and these two clusters represent different region. One is the northern group, another is the southern group. As it shows, there are many open circles between haplotype19 and haplotype7, haplotype11 and haplotype18_haplotype17, haplotype4 and haplotype3. These empty circles represent haplotypes that are necessary intermediates but are not present in the samples. Besides, haplotype20 and haplotype21 are included in southern cluster but there are not connected with other haplotypes.

The groups determined from phylogenetic trees were examined by AMOVA (Table.10; Tab.11). First, I analysed the variations within and among these groups of *A. niger*, and the result reveals the most of the variations among groups (83.72 ％), while 16.27％ of the variation is assigned to differences within groups. Then I analysed the variations within and among the populations in three groups, but the populations don't include Szuyuanyakou and Lichia. The result showed there are 83.44％ variation among populations, and 16.56％ variance within populations.

IX. Divergence time

I used the pairsiwe distances to estimate the divergence time among haplotypes in *A. niger*. The pairwise distance between meifeng groups and other haplotypes in *A. niger* was 0.076, therefore the divergence time was probably 2.53

~1.52 Ma. The distance between northern group and southern group is 0.034, therefore the divergence time is probably 1.13~0.68 Ma.

X. Genus *Achalinus*

I combined the samples of *A. niger* and *A. f. formosanus* to construct the phylogenetic trees. Both of NJ tree and MP tree showed *A. niger* appear as non-monophyletic with *A. f. formosanus*. And the main clades are highly supported by their bootstrap values (99%)(Fig.12).

Discussion

In this study, the adenine (A) and cytosine (C) had higher base frequencies (>30%), while the guanine (G) had least frequency (11.0%). This result is similar to that of *Sphenomorphus taiwanensis* (G=16.1%) and *Gekko hokouensis* (G=16 %), also conformed with the studies of other reptiles (John and Avise, 1998; Tasi, 1999; Michael, 2001; Gue, 2002). The intraspecific genetic distance is 0.0% to 11.52% in *A. niger*, it is similar to *Sphenomorphus taiwanensis* (0.0% to 10.06 %), it is higher than other species in Taiwan. The high variation in this species, I infer the species could be an ancient species or there were complex temporal and spatial factors in speciation.

When comparing the geography with the phylogeny, creating an area-cladegram (Fig.13). I got three genetically and biogeographically distinct clades of *A. niger* in Taiwan. The northern clade consisted of populations located Snow Range. A second clade consisted of population of Meifeng only. The third clade included the remaining populations examined, the middle-southern of Central Range. Each clade is supported by high bootstrap value.

Phylogeography

For species which living in dissimilar altitude, mountain offers different isolated model. The populations in low altitude are subdivided into Eastern and Western Taiwan by Central Range and the populations in middle-high altitude form northern and southern clades, even it does not correlate with distribution in geography. In this study, *A. niger* populations form the Northern group and Southern group. Compare with other species living in middle-high altitude in Taiwan, burrowing shrew (*Anourosorex swuamipes yamashinai*), Siberian weasel (*Mustela sibirica*), Short-legged japalura (*Japaluraa brevipes gress*) and Formosan

wood mouse (*Apodemus semotus*) have the same model with *A. niger*. But there are some species (White-bellied rat, *Niviventer culturatus* and *Sphenomorphus taiwanensis*) having no obvious isolation on phylogeography.

In the five patterns of intraspecific phylogeography, the relationships between phylogeny and geography of *A. niger* is similar to category Ⅰ. As the data show, the genetic divergence is discontinuous, and these populations separate from geographic regions within the range of species. I infer the population may undergo a long term geography barriers, and have extrinsic barriers to gene flow, or it has extinctions of intermediate genotypes in species with limited gene flow. Species have different category in Taiwan, even these species distribute similar habitat or altitude (*Sphenomorphus taiwanensis,* category Ⅰ+Ⅱ; *Niviventer coxingi*, category Ⅴ; *Squmipes yamashinai*, category Ⅳ).

What reasons caused the difference from these species?

Due to the various evolution rate of mtDNA and ecologically diverse in species, maybe these species undergo geological events of different period and effect by dispersal factors, to cause the diversity of genetic structure, and lead to the difference of distribution in geography in every species (Avise 1992, Hsu 2000).

Meifeng population

All populations have low nucleotide diversity (0.10％~0.30％) except Meifeng population. It has high nucleotide diversity (π=4.05％) and genetic distance, it represents the large diversity among haplotypes. The high nucleotide diversity between Meifeng population and there are higher values of nucleotide diversity and genetic distance between other populations and Meifeng population (8.1％~9.1％). However Meifeng is located at the middle of Central Range, so the diversity with other populations don't cause by distance. In addition most

individuals have unique haplotype in *A. niger* and they don't share the same haplotype with other populations, and the network of haplotype indicates the haplotypes of Meifeng don't connect with others. So I have no confidence to support the Meifeng is original place, then spread northern and southern. Contrast with other populations, there are unusual degree of genetic variation and gene distance in the population of Meifeng.

What reasons caused the special Meifeng population?

Based on the above mentioned, I guess *A. niger* populations have at least one incursion events from other place. In the phylogenetic tree, the Meifeng population is first branched, then followed with other groups. So the original *A. niger* population enter into Taiwan, and the population was isolated in Meifeng, then second incursion events happened, and another populations formed northern and southern groups. However we need comprehensive sampling of mainland Chinese, Ryukyu Islands and Japan of genus *Achalinus*, would be able to unequivocally support or refute either hypothesis.

Populations

The nucleotide diversity between populations (dxy) is larger than it within population (π), and there is a reasonable assumption that they represent genetically distinct non-overlapping populatios. There is a kind of obvious isolated mechanism between populations (Gravlund, 2002) and the outcome is supported by AMOVA.

The result of AMOVA indicates that only 16.5％ of variation within groups and 16.2％ within populations. The result also supported the main genetic variation was among groups and populations. What is the reason causing the high variation within *A. niger* population? I guess the mountain blocks the gene flow between populations, so there are many types of haplotype in population. Additional the different of environment of mountain in Taiwan and tectonic

evolution of Taiwan, hence *A. niger* has high degree of gene diversity.

The genetic distance between populations increase by the geographic distance. Base on the correction between Fst and geographic distance, the geographic distance is farther, the higher gene differentiation in *A. niger.* And it is correspond with the "isolation by distance" model. If I deleted the Meifeng population, there is significant correction between nucleotide diversity and geographic distance ($r= 0.933$; $p<0.01$)

The topology of phylogenetic trees shows that *A. niger* divided into the Snow Range (northern group) and the Central Range (southern group) approximately, and Meifeng area of in the middle of Taiwan forms another lineage. In southern group, there are the less degree of genetic differentitation among the populations of Alishan and Shanlinhsi in the Ali Range and Tatachia in the Yushan Range. In *Sphenomorphus taiwanensis*, there are the same haplotypes in Tatachia and other place around it, author thinks the area of Tatachia is a passageway between Central Range and Yushan Range, so the speices can raise the genetic interflow through the area. But this phenomenon doesn't appear in this study, although the degree of genetic differentiation is low among these areas, I still think there is a weaker degree of genetic interflow among populations of *A. niger*.

These two species live in similar environment and altitude, but the form of gene flow is different. What kinds of mechanism about genetic interflow exist in *A. niger* and *Sphenomorphus taiwanensis*? Compare with other vertebrate, reptiles have weaker dispersal ability (Gravlund, 2002), but the activities capability of lizard is more excellent than snake, so the lizard has wider home range than snake (Roger, 1969; John, 1983). In specially, *A. niger* lives under the forest, and its preys are mainly invertebrate, hence they doesn't need to chase preys and the size of the home range is limited. I guess the dispersal ability is a factor to cause the high genetic diversity among populations of *A. niger*.

Snow Range and Central Range

In *A. niger*, most individuals have unique haplotype and the high haplotype diversity within populations. The Fst is higher than 0.7 and the high nucleotide diversity between Northern group and southern group, indicating that the genetic flow is significantly low between them. I guess there are some mechanisms between the Snow Range and the Central Range to limit the gene flow in *A. niger*.

It shows the lower genetic flow within in populations and they don't share the same haplotype. *Achalinus niger* population subdivided into three groups and supported by the result of relationship among haplotypes. The ancestry haplotypes arose from the Snow Mountain Range and the Central Mountain Range respectively. It means *A. niger* spreaded population from different sites in Taiwan, and the populations was separated by distance and the more diversity in gene pass by time.

The birthplace of Taichia River lie between Snow Mountain and Nanhudashan, and the highest altitude of Taichia River is high than 3500 meters, so the special valley topography separated the connection between the Snow Range and Central Range for some species. For snake, maybe it is impossible to cross over the river to the other side. However we have not many samples from Szuyuanyakou, so I cann't judge.whether this place is ecological corridor for *A. niger* in this study. But according to the above mentioned, I guess the reasons of vicariance are the special barrier caused by Taichia River and weaker dispersal ability.

I have the phylogenetic analyses and nested work for *A. niger*, but it is unable to clarify the palaeoclimate and tectioic within aspects of the population historical events of *A. niger* in Taiwan. Besides, there are few studies about biogeography of living in middle-high altitude species in Taiwan, so I can not refer complete explanation about the evolution progress and divergence model of *A. niger*. For these reasons, I need more data of tectioic about Taiwan and phylogeography study about this kind species of living in high altitude to establish founded data.

According to the phylogenetic tree of the genus *Achalinus* living in Taiwan, it's obvious indicated that each of these two species were not monophyletic, and some individuals of *A. niger* and *A. f. formosanus* gathered in one cluster. Hence, there were some undefined about the taxonomy of molecular biology and morphology. Future analysis of both more genetic data and morphological data will reveal more about the taxanomic status of these isolated populations.

Literature Cited

Alfaro, M. E. and J. A. Steven. 2001. Molecular systematics and evolution of *Regina* and the thamnophiine Snakes. Molecular Phylogenetics and Evolution 21: 408-423.

Ashton, K. G. and D. Q. Alan. 2001. Molecular systematics of the western rattlesnake, *Crotalus viridis* (viperidae), with commenets on the utility of the D-Loop in phylogenetic studies of snakes. Molecular Phylogenetics and Evolution 21: 176-189.

Avise, J. C. 1992. Molecular population structure and the biogeographic history of a regional fauna: a case history with lessons for conservation biology. Oikos 63:62-76.

Avise J. C. 2000. Phylogeography: the history and formation of species. Harvard University Press, Cambridge, Massachusetts, U.S.A.

Avise, J. C., J. Arnold and R. Martin. 1987. Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. Annual Review of Ecology and Systematics 18: 489-522.

Bernachez, L. and C. C. Wang. 1998. Comparative phylogeography of Nearctic and Palearctic fish. Molecular Ecology 7:431-452.

Boulenger. 1908. The annals and magazine of Natural History 8:2:222

Cann, R. L., M. Stoneking and A. C. Wilson. 1987. Mitochondrial DNA and human evolution. Nature 325: 31-36.

Catharine, E. P., W. Wuster and S. T. Roger. 2000. Hisorical biogeoeraphy of the western rattlesnake (Serpentes: Viperidae: *Crotalus viridis*), inferred from mitochondrial DNA sequence information. Molecular Phylogenetics and Evolution 15: 269-282.

Chang, C. H. 2005. Systematics, biogeography, and evolution of the earthworms in the *Metaphire formosae* species group inferred from morphology and mitochondrial DNA sequences. Master's thesis, National Taiwan University,

Taipei.

Chang, X. C. 2002. Phylogeny and Biogeography of the genus *Capricornis* (Artiodactyla: Bovidae) based on mitochondrial DNA sequences and cranial morphometrics. Master's Thesis, National Sun Yat-sen University, Kaohsiung.

Chen, H. C. 1994. Preliminary study on mitochondrial DNA sequence and population variation of *Rana sauteri*. Master's Thesis, National Taiwan Normal University, Taipei.

Chen, M. H., H. W. Yuan and Y. S. Lin. 2004. Genetic diversity and population genetic structure of the Ring-necked Pheasant (*Phasianus colchicus*) in Taiwan. Journal Experiment Forest National Taiwan University 18(2):65-75.

Creer, S. A., R. S. Malhotra and W. H. Chou. 2001. Multiple causation of phulogeographical pattern as revealed by nested clade analysis of the bamboo viper (*Trimeresurus stejnegeri*) within Taiwan. Molecular Ecology 10: 1967-1981.

Crochet, P. A., O. Chaline and S. G. Yang. 2004. Speciation in mountains: phylogeography and phylogeny of the rock lizards genus *Iberolacerta* (Reptilia: Lacertidae). Molecular Phylogenetics and Evolution 30: 860-866.

Duvall, D., and S. J. Beaupre. 1998. Sexual strategy and size dimorphism in rattlesnakes: Integrating proximate and ultimate causation. American Zoologist 38:152-165.

Excoffier, L., P. E. Smouse and J. M. Quattro. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131: 479-491.

Fredric, J. J., G. K. James and S. H. Tamara. 2002. Molecular phylogeography of common garter snakes (*Thamnophis sirtalis*) in western north America: implications for regional historical forces. Molecular Ecology 11: 1739-1751.

Futuyma, D. J. 1998. Evolutionary Biology, 3rd ed. Sinauer, Sunderland, MA.

Gravlund, P. 2002. Molecular phylogeny of Tornier's cat snake (*Crotaphopeltis*

tornieri), endemic to east African mountain forests: biogeography, vicariance events and problematic species boundaries. Journal of Zoological Systematics and Evolutionary Research 40: 46-56.

Guo, C. H. 2002. The population genetic structure of *Sphenomorphus taiwanesis*. Master's Thesis, National Taiwan Normal University, Taipei.

Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Window 95/98/NT. Nucleic Acid Research Symposium Series 41: 95-98.

He, M. G. 2000. Phylogenetic relationship between *Papilio paris* Linnaeus and *P. hermosanus* Rebel. Master's Thesis, National Taiwan University, Taipei.

Hsu, F. H., F. J. Lin and Y. S. Lin. 2001a. Phylogeographic structure of the formosan wood mouse, *Apodemus semotus* thomas. Zoological Studies 40: 91-102.

Hsu, F. H., F. J. Lin and Y. S. Lin. 2001b. Phylogeographic variation in mitochondrial DNA of formosan white-bellied rat *Niviventer culturatus*. Zoological Studies 39:38-46

Hsu, K. C., K. C. Tsai and H. D. Lin. 2005. Phylogeography and population genetic structure of Sinibrama macrops based on mtDNA. BioFormosa 40(2):58-67.

Hsu, Y. T. 1999. The evolutionary relationship of Chinee bulbul (*Pycnonotus sinensis*) and Taiwan bulbu (*P. taivanus*) revealed by their population genetic structure. Master's Thesis, National Taiwan University, Taipei.

Javier A. R., F. D. Dale and E. S. Richard. 1999. Phylogeography of the California mountain kingsnake, *Lampropeltis zonata* (cilobridae). Molecular Ecology 8: 1923-1934.

Kumar, S., K. Tamura, I. B. Jakobsen and M. Nei. 2001. MEGA2: Molecular evolutionary genetics analysis software, Arizona State University, Arizona, USA.

Lee, J. K. 2003. Phylogeography of the red-bellied tree squirrel *Callosciurus*

erythraeus of taiwan and reexamination of its sunspecific. Master's Thesis, Tunghai University, Taichung.

Lin, S. M. 2003. Phylogeny and phylogeoeraphic studies of *Takydromus* in taiwan and adjacent regions (Squamata: Lacertidae). Doctor's Thesis, National Taiwan Normal University, Taipei.

Liu, K. C. 1995. Phylogeographic relationship of *Japalura swinhonis* based on analysis of mtDNA. Master's Thesis, National Sun Yat-sen University, Kaohsiung.

Liu, Y. E. 2000.Analysis of population genetic structure of genus Rhacophorus in Taiwan using RAPD markers. Master's Thesis, National Taiwan University, Taipei.

Macey, J. R., A. S. James and L. Allan. 1998. Phylogenetic relationships of toads in the *bufo bufo* species group from the escarpment of the Tibetan plateau: a case of vicariancd and dispersal. Molecular Phylogenetics and Evolution 9: 80-87.

Maki, M. 1931. A Monograph of The Snakes of Japan. P60-62. Tokyo.

Michael P. C., S. P. Otto and W. John. 1999. Genes and other Sampled of DNA Sequence Data for Phylogenetic Inference. Biological Bulletin 196: 245-250.

Nei, M and S. Kumar. 2000. Molecular Evolution and Phylogenetics. Oxford University Press, New York, USA.

Nei, M. 1987. Molecular Evolutionary Genetics. Columbia University Press, New York.

Otto, S. P., M. P. Cummings, and J. Wakeley. 1996. Inferring phylogenies from DNA sequence data: the effects of sampling. In "New Uses for New Phylogenies". Oxford Univ. Press, Oxford.

Palumbi, S., R. A. Martin, S. Romano, W. O. Mcmillan. 1991. The simple fool's guide to PCR, Version 2. University of Hawaii Zoology Department, Honululu.

Pedro, G., P. Francisco and N. Manuel. 1996. Phylogenetic relationships of the

canary islands endemic lizard genus *Gallotia* (sauria: *Lacertidae*), inferred from mitochondrial DNA sequences. Molecular Phylogenetics and Evolution 6: 63-71.

Posada, D. 2004. TCS, version 1.18. Computer program distributed by the author.

Saiki. R. K., D. H. Gelfand, S. Stoffel, S. Scharf. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 39: 487-491.

Swofford, D. L. 1998. PAUP. Phylogenetic analysis using parsimony. Version 4.0 beta. Sinauer, Massachusetts, USA.

Tasi, C. Y. 1999. Population phylogeny of *Gekko hokouensis* based on mitochondrial 12S rRNA and cytochrome b sequences. Master's Thesis, National Sun Yat-sen University, Kaohsiung.

Templeton, A. R., K. A. Crandall and C. F. Sing. 1992. A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data Ⅲ. Cladogram estimation. Genetics 132:619-633.

Toda, M. M. N. and M. Matsui. 1998. Genetic variation in the Indian rice frog, *Rana limnocharis* (Amphoboa: Anura) in Taiwan, as revealed by allozyme data. Herpetologica 54: 73-82.

Wu, M. J. 2004. Phylogeography variation of siberian weasel (*Mustela sibirica*) in taiwan, based on control region sequences of mitochondrial DNA. Master's Thesis, National Sun Yat-sen University, Kaohsiung.

Xia, X and Z. Xie. 2000. DAMBE: data analysis in molecular in molecular biology and evolution. Journal and Heredity 92: 371-373.

Xiang, G. S. 1997. Phylogenetic relationships and biogeography in the genus *Japalura* of taiwan based on the variation of mtDNA sequences. Master's Thesis, National Sun Yat-sen University, Kaohsiung.

Yoshinori, K., H. Ota and M. Nishida. 1998. The complete nucleotide sequence of a snake (*Dinodon semicarinatus*) mitochondrial genome with two identical

control regions. Genetics 150: 313-329.

Yuan, S. L. 2003. Phylogeographic variation in mitochondrial cytochrome b region of formosan burrowing shrew, *Anourosorex squamipes yamashinai* (Mammalia:Insectivora). Master’s Thesis, Tunghai University, Taichung.

CATEGORY	GENETIC DIVERGENCE PATTERN	GEOGRAPHIC DISTRIBUTION		
		REGION 1	REGION 2	REGION 3
I	discontinuous	A B C	L M N	X Y Z
II	discontinuous	A B C / L M N / X Y Z		
III	continuous	L	M	N
IV	continuous	L / M / N		
V	continuous	N	L	M

Figure 1. General phylogeographic patterns (relationships between phylogeny and geography) theoretically observable in mtDNA surveys. (Avise, 1987)

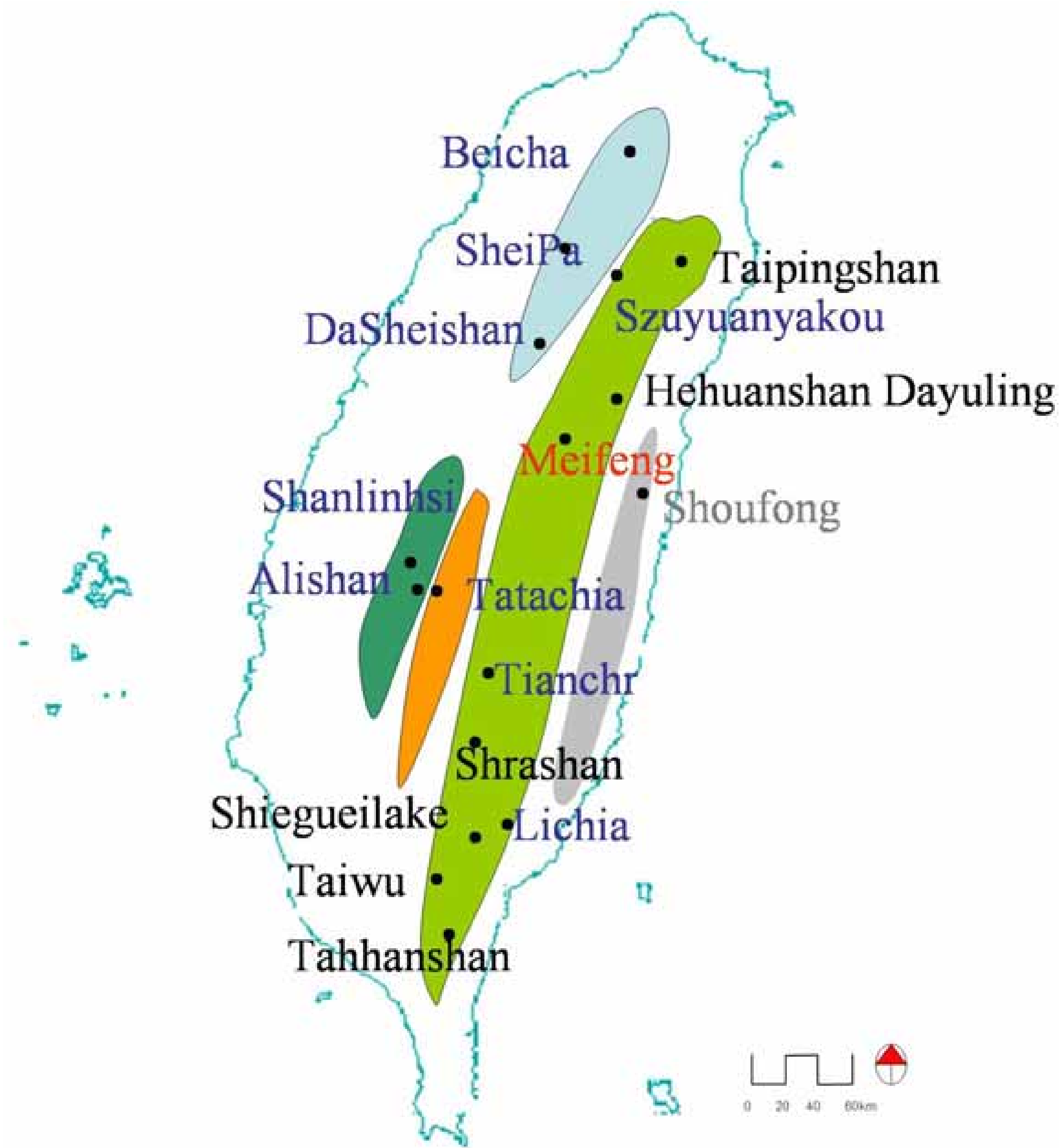

Figure 2. Sampling localities of species of the genus *Achalinus* in Taiwan. These five range represent the five mountain range in Taiwan.

Figure 3. The location of ND2 and primers on the mtDNA.

Figure 4. Sampling sites of *Achalinus niger* in Taiwan.

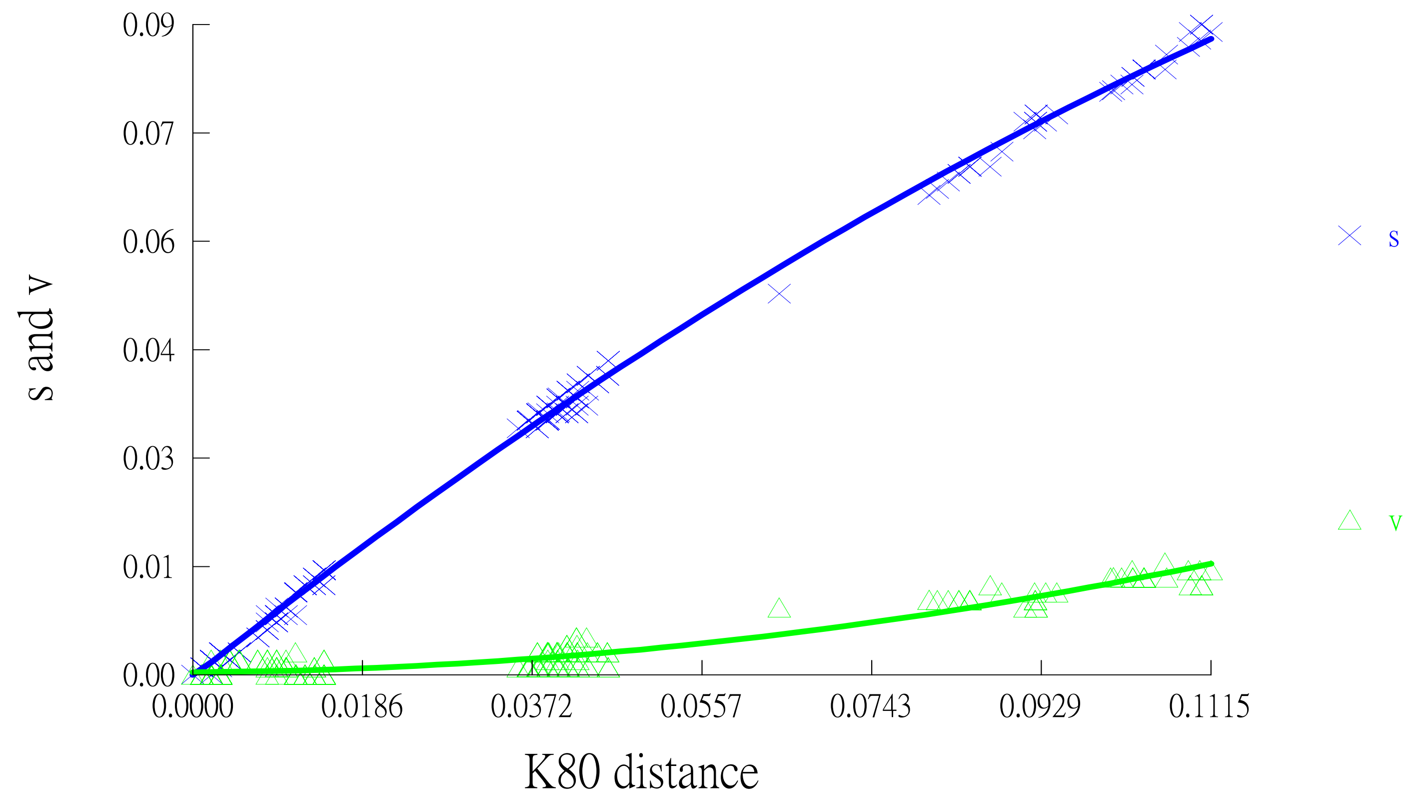

Figure 5. The curve of saturation about ND2 gene. The above curve is transition. The below curve is transversion.

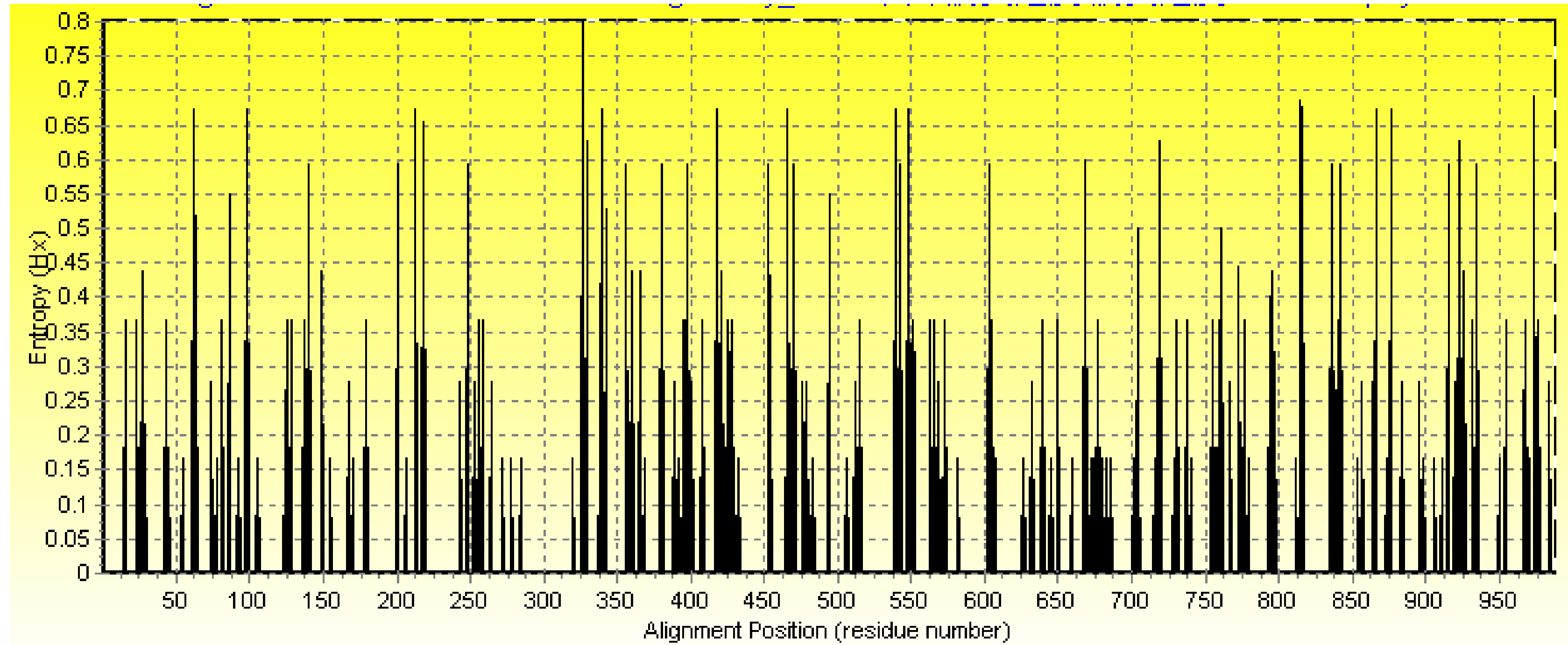

Figure 6**. The entropy of variant sites in ND2 of *Achalinus niger*.**

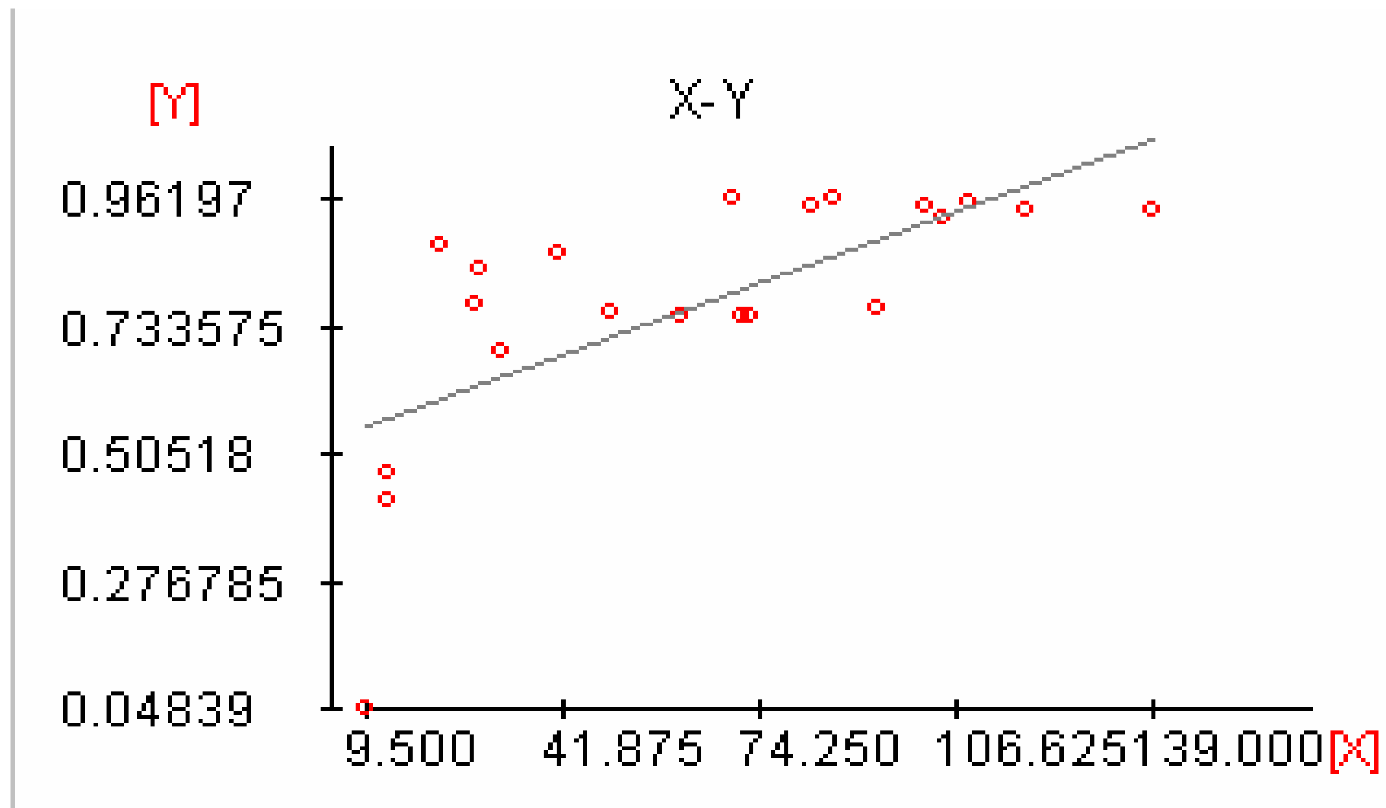

Figure 7 **. The graph of the correlation of geographic distance and Fst between populations of *Achalinue niger*. (p=0.022<0.05; r=0.518). The X represents geographic distance and the Y represents the value of Fst.**

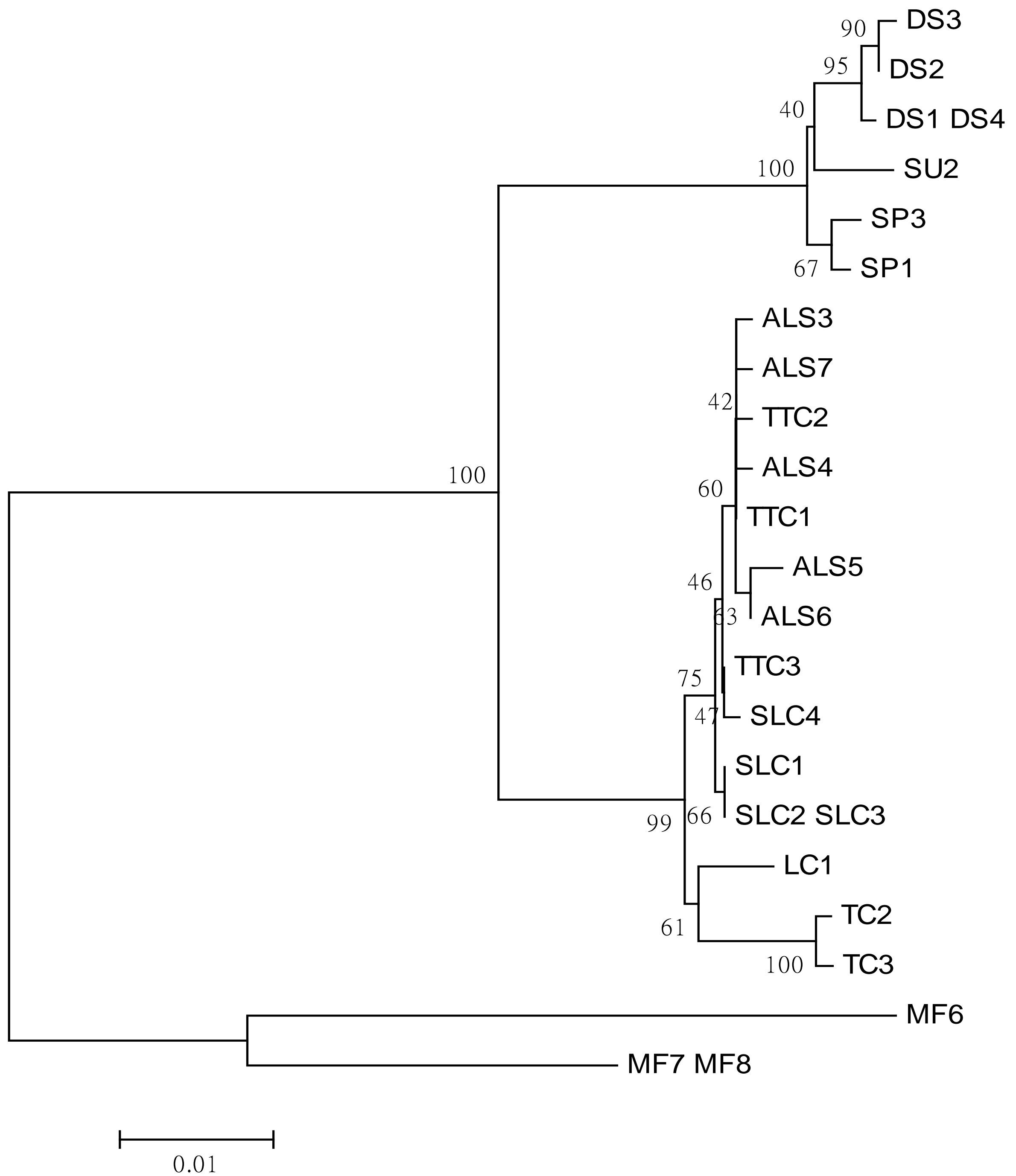

Figure 8. Neighbor-Joining tree constructed from 25 samples of *Achalinus niger* by using ND2. The scale below the figure showed the genetic distance. The numbers on the nodes are bootstrap values drived from 1000 replications.

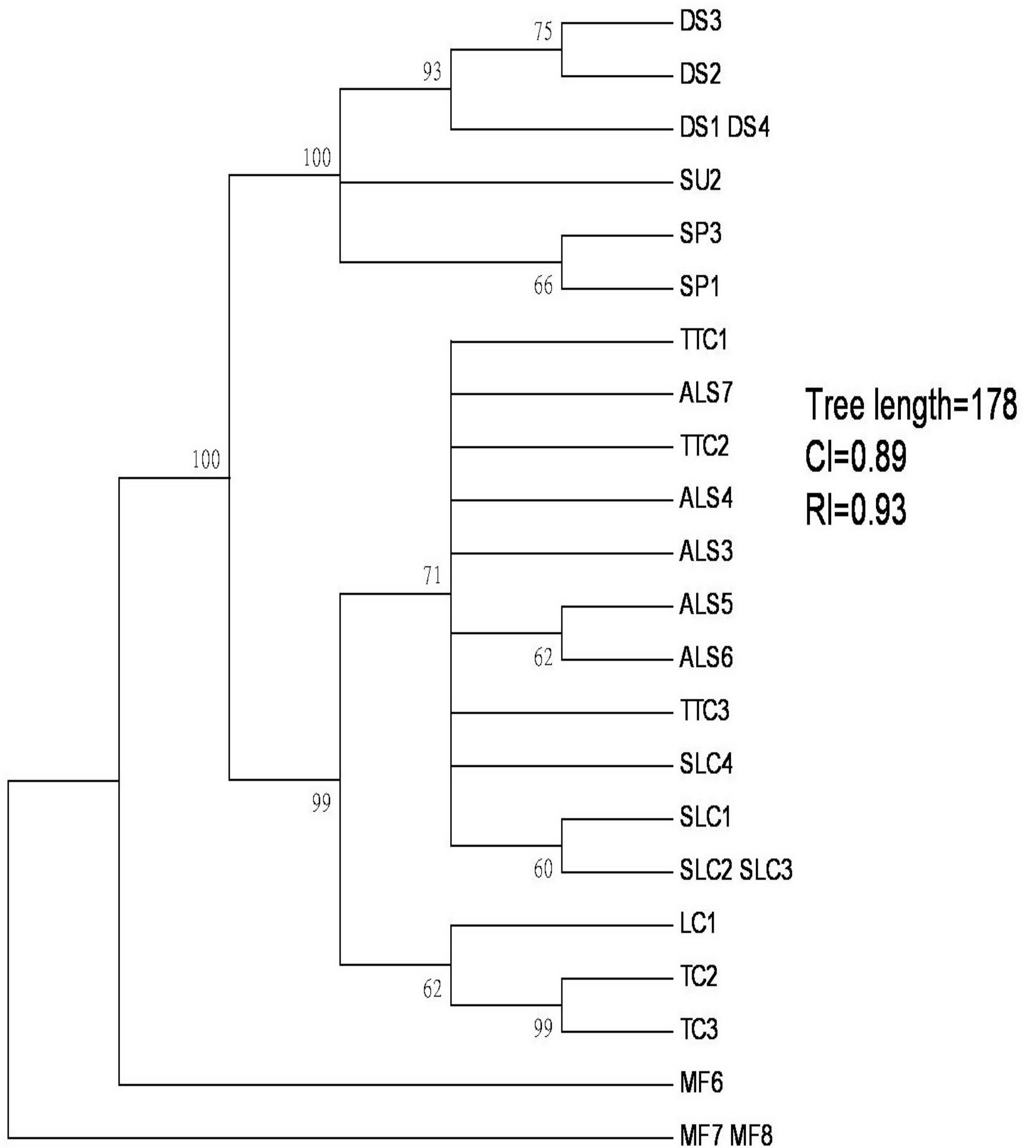

Figure 9 . A most parsimony tree constructed with ND2 gene data of *Achalinus niger*. The numbers aboveor under the branches are bootstrap values from 1000 replications.

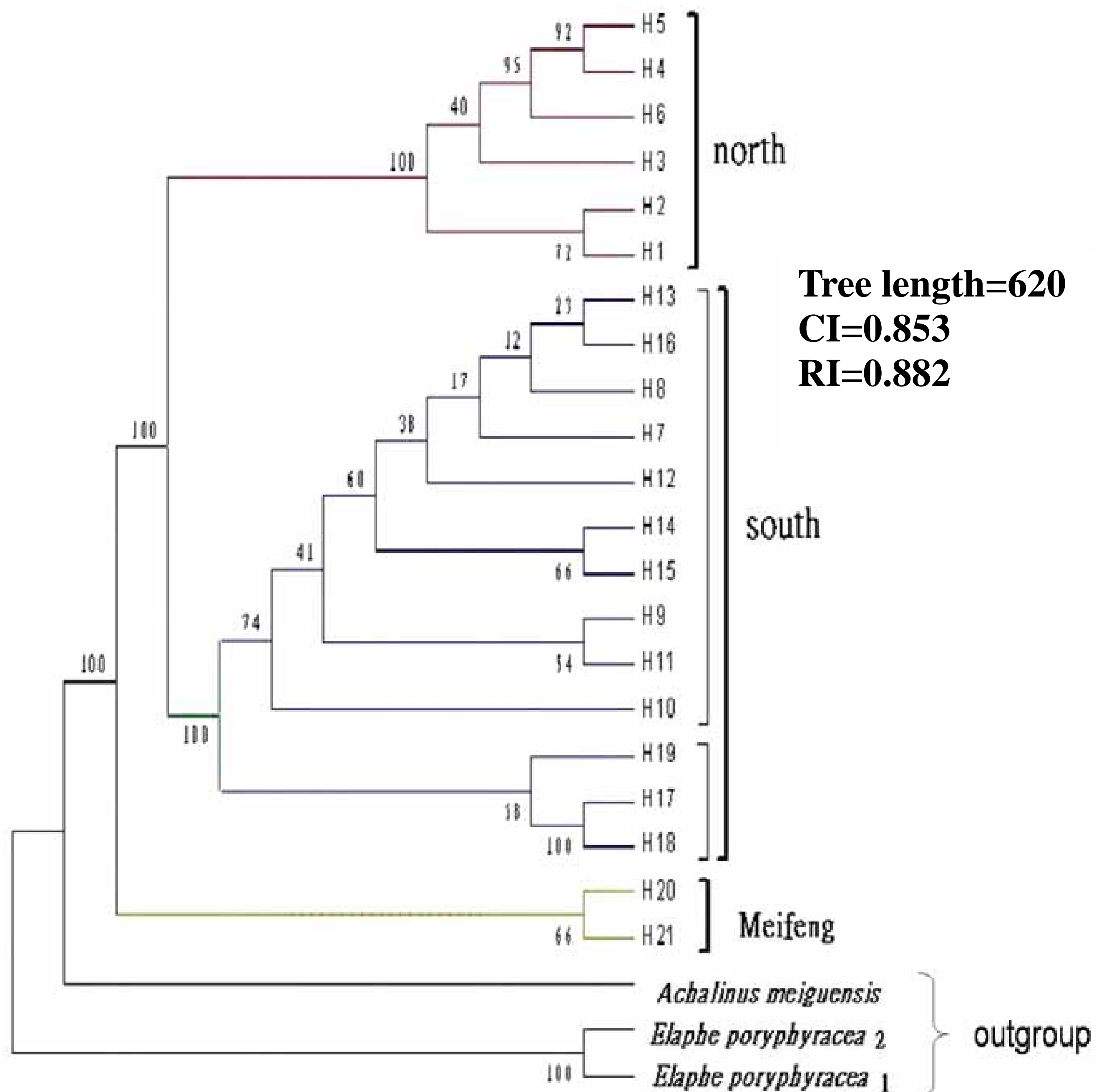

Figure 10**. A most parsimony tree based 21 haplotypes based ND2 gene data of *Achalinus niger*. Numbers above the branches are bootstrap values from 1000 replications. The three main clades are supported by bootstrap values of 100.**

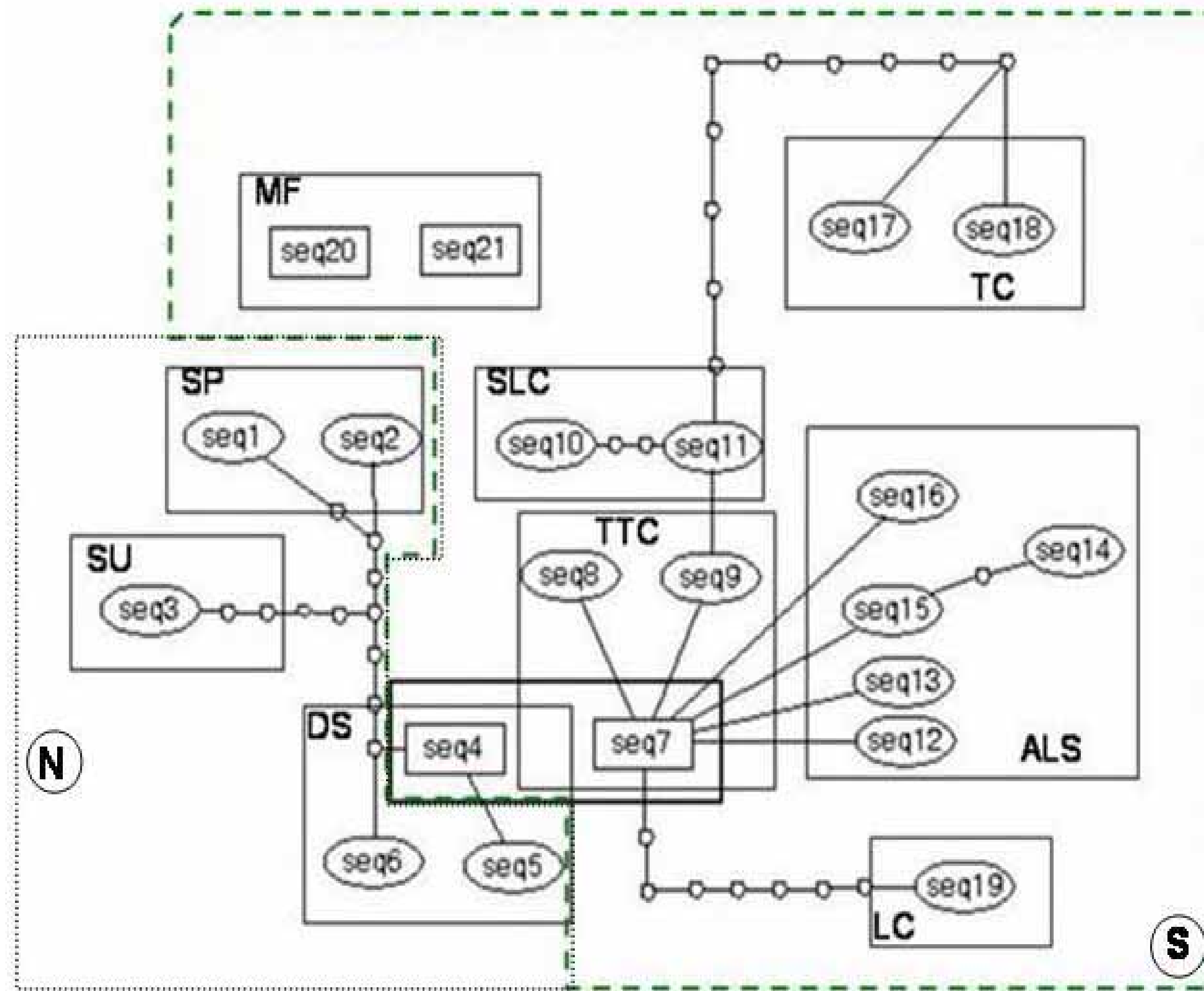

Figure 11. **The network of haplotypes was generated by a statistical parsimony with TCS. Seq n represents haplotype n. The thick square indicated the original haplotypes, and empty circles represent haplotypes that are necessary intermediates but were not present in the samples.**

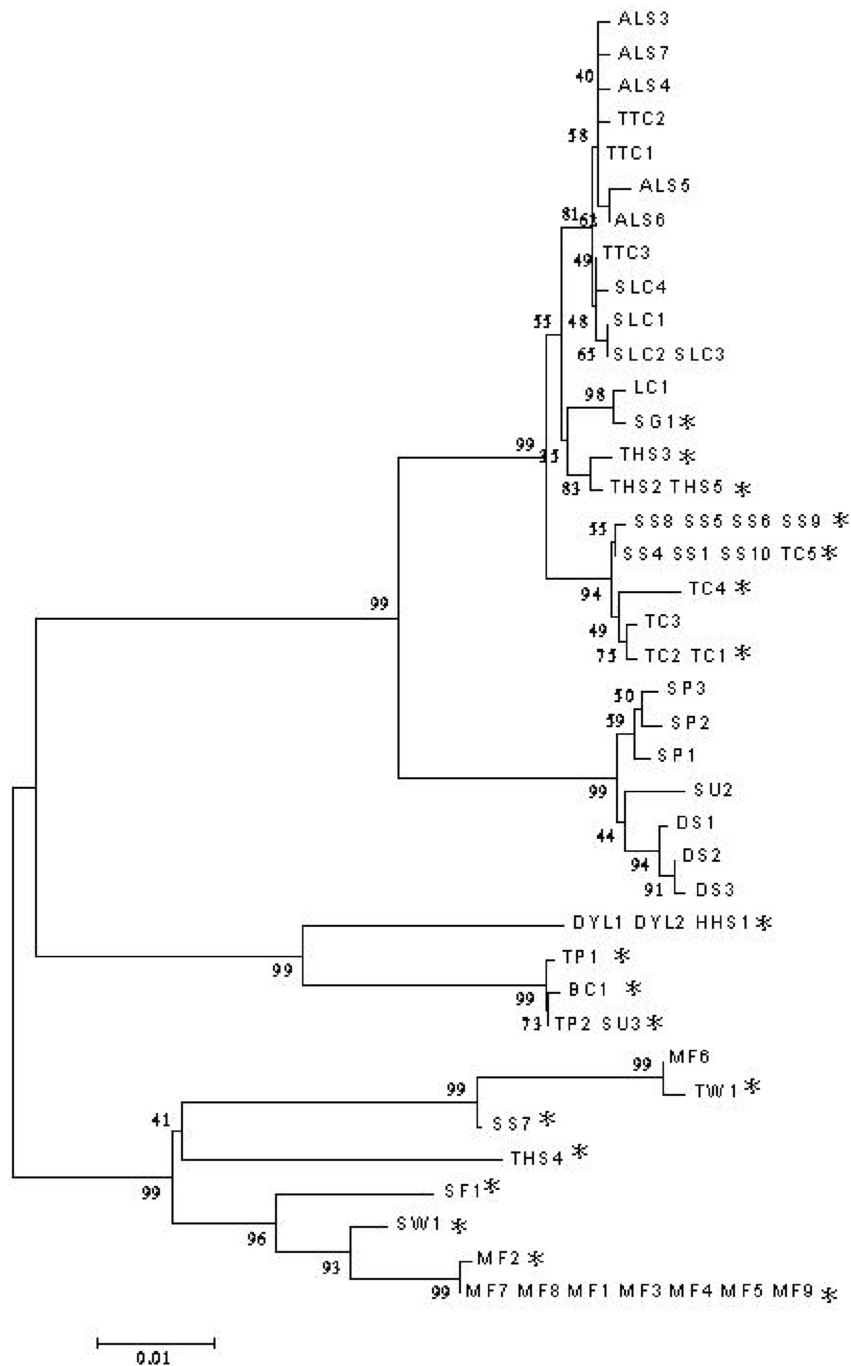

Figure 12. Neighbor-Joining tree constructed from samples of genus *Achalinus* in Taiwan. The numbers on the nodes are bootstrap values drived from 1000 replications. The star mark represents *Achalinus formosanus formosanus* except MF7、MF8、TC2.

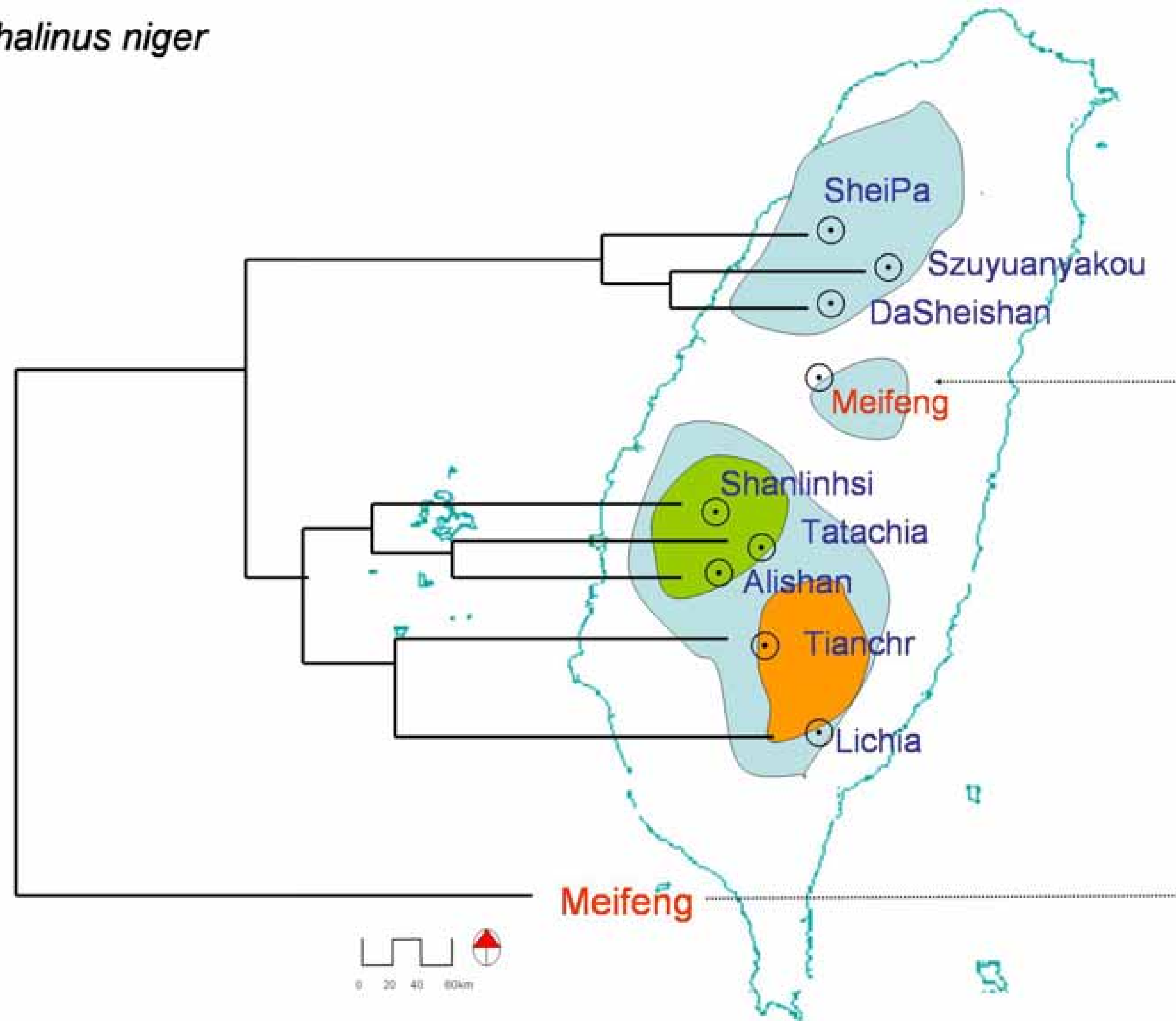

Figure 13**. Area cladogram with the phylogeny resulting from the analyses imposed on the geography of the sample localities.**

Table 1. Location code of samples of genus *Achalinus*.

	Collected location English name	Collected location English name	Collected location code
1	SheiPa	雪霸國家公園	SP
2	Beicha	北插天山	BC
3	Taipingshan	太平山	TPS
4	Szuyuanyakou	思源啞口	SU
5	Dasheishan	大雪山	DS
6	Dayuling	大禹嶺	DYL
7	Hehuanshan	合歡山	HHS
8	Meifeng	梅峰	MF
9	Shanlinhsi	杉林溪	SLS
10	Alishan	阿里山	ALS
11	Tatachia	塔塔加	TTC
12	Tianchr	南橫天池	TC
13	Shrshan	石山林道	SS
14	Lichia	利嘉林道	LC
15	Taiwu	泰武鄉	TW
16	Tahanshan	大漢山	THS
17	Shiegueilake	小鬼湖	SG
18	Shoufong	花蓮縣壽豐鄉	SF

	English name	Scientific name	code
Outgroup	Meigu burrowing snake	Achalinus meiguensis	MG
	Red bamboo snake	Elaphe poryphyracea	E

Table 2. T-test between the observed saturation index and the expected value assuming full saturation

Prop. invar. Sites	0.0000
Mean H	0.0763
Standard Error	0.0064
Hmax	1.7764
Iss	**0.0430**
Iss.c	**0.7587**
T	111.5622
DF	987
Prob (One-tailed)	0.0000
95% Lower Limit	0.0304
95% Upper Limit	0.0555

∗Testing whether the observed Iss is significantly lower than Iss.c. And the result is Iss < Iss.c, indicated there are no saturation.

Table 3 . The genetic distance (below diagonal) of individuals.The genetic distance were calculated using Tamura-Nei model.

	SP1	SP3	SU2	DS1	DS2	DS3	DS4	TTC1	TTC2	TTC3	SLC1	SLC2	SLC3	SLC4	YLS3	YLS4	YLS5	YLS6	YLS7	TC2	TC3	LC1	MF7	MF6
SP1																								
SP3	0.003																							
SU2	0.009	0.008																						
DS1	0.008	0.007	0.009																					
DS2	0.008	0.007	0.009	0.002																				
DS3	0.009	0.008	0.010	0.003	0.001																			
DS4	0.008	0.007	0.009	0.000	0.002	0.003																		
TTC1	0.037	0.039	0.041	0.040	0.040	0.041	0.040																	
TTC2	0.039	0.040	0.042	0.041	0.041	0.043	0.041	0.001																
TTC3	0.036	0.037	0.039	0.038	0.038	0.040	0.039	0.001	0.002															
SLC1	0.038	0.039	0.041	0.040	0.040	0.041	0.040	0.002	0.003	0.001														
SLC2	0.038	0.039	0.041	0.040	0.040	0.041	0.040	0.002	0.003	0.001	0.000													
SLC3	0.038	0.039	0.041	0.040	0.040	0.041	0.040	0.002	0.003	0.001	0.000	0.000												
SLC4	0.037	0.039	0.041	0.040	0.040	0.041	0.040	0.002	0.003	0.001	0.002	0.002	0.002											
YLS3	0.039	0.040	0.042	0.041	0.041	0.043	0.041	0.001	0.002	0.002	0.003	0.003	0.003	0.003										
YLS4	0.039	0.040	0.042	0.041	0.041	0.042	0.041	0.001	0.002	0.002	0.003	0.003	0.003	0.003	0.002									
YLS5	0.041	0.042	0.044	0.043	0.043	0.045	0.043	0.003	0.004	0.004	0.005	0.005	0.005	0.005	0.004	0.004								
YLS6	0.039	0.040	0.042	0.041	0.041	0.043	0.041	0.001	0.002	0.002	0.003	0.003	0.003	0.003	0.002	0.002	0.002							
YLS7	0.039	0.040	0.042	0.041	0.041	0.043	0.041	0.001	0.002	0.002	0.003	0.003	0.003	0.003	0.002	0.002	0.004	0.002						
TC2	0.043	0.045	0.047	0.043	0.046	0.047	0.044	0.014	0.015	0.012	0.011	0.011	0.011	0.014	0.015	0.015	0.015	0.012	0.015					
TC3	0.044	0.045	0.047	0.043	0.046	0.048	0.044	0.014	0.015	0.013	0.011	0.011	0.011	0.014	0.015	0.015	0.015	0.013	0.015	0.002				
LC1	0.040	0.041	0.043	0.042	0.042	0.044	0.042	0.008	0.009	0.009	0.010	0.010	0.010	0.010	0.009	0.009	0.011	0.009	0.009	0.014	0.014			
MF7	0.095	0.094	0.095	0.097	0.095	0.096	0.098	0.086	0.088	0.085	0.084	0.084	0.084	0.086	0.088	0.087	0.090	0.088	0.088	0.096	0.096	0.092		
MF6	0.114	0.113	0.114	0.115	0.112	0.114	0.115	0.106	0.107	0.104	0.103	0.103	0.103	0.106	0.107	0.107	0.109	0.107	0.107	0.107	0.110	0.106	0.066	
MF8	0.095	0.094	0.095	0.097	0.095	0.096	0.098	0.086	0.088	0.085	0.084	0.084	0.084	0.086	0.088	0.087	0.090	0.088	0.088	0.096	0.096	0.092	0.000	0.066

Table 4. The Fst (below diagonal) and nucleotide diversity (dxy) (above diagonal) among populations of *Achalinus niger*.

	SP	DS	TTC	SLC	ALS	TC	MF
SP		0.007	0.035	0.035	0.037	0.041	0.089
DS	0.688		0.037	0.037	0.039	0.041	0.091
TTC	0.938	0.957		0.002	0.002	0.013	0.083
SLC	0.943	0.961	0.416		0.003	0.011	0.081
ALS	0.924	0.942	0.048	0.470		0.013	0.084
TC	0.938	0.953	0.871	0.860	0.830		0.088
MF	0.757	0.767	0.748	0.746	0.745	0.760	

Table 5. The Fst (below diagonal) and geographic distance (above diagonal) among populations of *Achalinus niger*.

【Km】

	SP	DS	TTC	SLC	ALS	TC	MF
SP		31.8	118	102	105	139	49.8
DS	0.688		86.6	70.5	83.1	109	27.6
TTC	0.938	0.957		13.1	9.5	21.6	73
SLC	0.943	0.961	0.416		13.1	41.3	61.4
ALC	0.924	0.942	0.048	0.470		28.2	71.8
TC	0.938	0.953	0.871	0.860	0.830		93.7
MF	0.757	0.767	0.748	0.746	0.745	0.760	

Table 6. The comparative table of haplotypes and samples of *Achalinus niger*.

Haplotype	Location	Sample size	Sample number	Mountain
H1	SP	1	SP1	Snow Range
H2	SP	1	SP3	Snow Range
H3	SU	1	SU2	Snow Range
H4	DS	1	DS2	Snow Range
H5	DS	1	DS3	Snow Range
H6	DS	2	DS1、DS4	Snow Range
H7	TTC	1	TTC1	Yushan Range
H8	TTC	1	TTC2	Yushan Range
H9	TTC	1	TTC3	Yushan Range
H10	SLC	3	SLC1、SLC2、SLC3	Ali Range
H11	SLC	1	SLC4	Ali Range
H12	ALS	1	ALS5	Ali Range
H13	ALS	1	ALS6	Ali Range
H14	ALS	1	ALS7	Ali Range
H15	ALS	1	ALS8	Ali Range
H16	ALS	1	ALS9	Ali Range
H17	TC	1	TC2	Central Range
H18	TC	1	TC3	Central Range
H19	LC	1	LC1	Central Range
H20	MF	1	MF6	Central Range
H21	MF	2	MF7、MF8	Central Range

Table 7. The P-distance (below diagonal) and genetic distance (above diagonal) of individuals. The genetic distance were calculated using Tamura-Nei mode.

	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17	H18	H19	H20	H21	E2	E1	MG
H1		0.003	0.009	0.008	0.009	0.008	0.038	0.039	0.037	0.038	0.038	0.039	0.039	0.041	0.039	0.039	0.044	0.044	0.040	0.114	0.095	0.498	0.499	0.228
H2	0.003		0.008	0.007	0.008	0.007	0.039	0.040	0.038	0.039	0.039	0.040	0.040	0.042	0.040	0.040	0.045	0.045	0.041	0.113	0.094	0.491	0.492	0.223
H3	0.009	0.008		0.009	0.010	0.009	0.041	0.042	0.040	0.041	0.041	0.042	0.042	0.044	0.042	0.042	0.047	0.047	0.043	0.114	0.095	0.489	0.489	0.227
H4	0.008	0.007	0.009		0.001	0.002	0.040	0.041	0.039	0.040	0.040	0.041	0.041	0.043	0.041	0.041	0.046	0.046	0.042	0.112	0.095	0.489	0.489	0.227
H5	0.009	0.008	0.010	0.001		0.003	0.041	0.043	0.040	0.041	0.041	0.043	0.042	0.045	0.043	0.043	0.047	0.048	0.044	0.114	0.096	0.486	0.486	0.229
H6	0.008	0.007	0.009	0.002	0.003		0.004	0.041	0.039	0.040	0.040	0.041	0.041	0.043	0.041	0.041	0.044	0.044	0.042	0.115	0.098	0.489	0.489	0.227
H7	0.036	0.037	0.039	0.038	0.039	0.038		0.001	0.001	0.002	0.002	0.001	0.001	0.003	0.001	0.001	0.014	0.014	0.008	0.106	0.086	0.487	0.488	0.224
H8	0.037	0.038	0.040	0.039	0.040	0.039	0.001		0.002	0.003	0.003	0.002	0.002	0.004	0.002	0.002	0.015	0.015	0.009	0.107	0.088	0.485	0.485	0.226
H9	0.034	0.036	0.038	0.037	0.038	0.037	0.001	0.002		0.001	0.001	0.002	0.002	0.004	0.002	0.002	0.012	0.013	0.009	0.104	0.085	0.485	0.485	0.226
H10	0.036	0.037	0.039	0.038	0.039	0.038	0.002	0.003	0.001		0.002	0.003	0.003	0.005	0.003	0.003	0.011	0.011	0.010	0.103	0.084	0.485	0.485	0.224
H11	0.036	0.037	0.039	0.038	0.039	0.038	0.002	0.003	0.001	0.002		0.003	0.003	0.005	0.003	0.003	0.014	0.014	0.010	0.106	0.086	0.485	0.485	0.228
H12	0.037	0.038	0.040	0.039	0.040	0.039	0.001	0.002	0.002	0.003	0.003		0.002	0.004	0.002	0.002	0.015	0.015	0.009	0.107	0.088	0.485	0.485	0.224
H13	0.037	0.038	0.040	0.039	0.040	0.039	0.001	0.002	0.002	0.003	0.003	0.002		0.004	0.002	0.002	0.015	0.015	0.009	0.107	0.087	0.487	0.488	0.226
H14	0.039	0.040	0.042	0.041	0.042	0.041	0.003	0.004	0.004	0.005	0.005	0.004	0.004		0.002	0.004	0.015	0.015	0.011	0.109	0.090	0.493	0.494	0.229
H15	0.037	0.038	0.040	0.039	0.040	0.039	0.001	0.002	0.002	0.003	0.003	0.002	0.002	0.002		0.002	0.012	0.013	0.009	0.107	0.088	0.490	0.490	0.226
H16	0.037	0.038	0.040	0.039	0.040	0.039	0.001	0.002	0.002	0.003	0.003	0.002	0.002	0.004	0.002		0.015	0.015	0.009	0.107	0.088	0.490	0.490	0.224
H17	0.041	0.042	0.044	0.043	0.044	0.041	0.013	0.014	0.012	0.011	0.013	0.014	0.014	0.014	0.012	0.014		0.002	0.014	0.107	0.096	0.492	0.488	0.231
H18	0.041	0.042	0.044	0.043	0.044	0.041	0.013	0.014	0.012	0.011	0.013	0.014	0.014	0.014	0.012	0.014	0.002		0.014	0.110	0.096	0.492	0.488	0.234
H19	0.038	0.039	0.041	0.040	0.041	0.040	0.008	0.009	0.009	0.010	0.010	0.009	0.009	0.011	0.009	0.009	0.013	0.013		0.106	0.092	0.495	0.495	0.231
H20	0.100	0.099	0.100	0.099	0.100	0.101	0.094	0.095	0.093	0.092	0.094	0.095	0.095	0.097	0.095	0.095	0.095	0.097	0.094		0.066	0.464	0.459	0.220
H21	0.085	0.084	0.085	0.085	0.086	0.087	0.078	0.079	0.077	0.076	0.078	0.079	0.079	0.081	0.079	0.079	0.085	0.085	0.082	0.061		0.468	0.468	0.220
E2	0.352	0.349	0.348	0.348	0.347	0.348	0.347	0.346	0.346	0.346	0.346	0.346	0.347	0.350	0.348	0.348	0.349	0.349	0.350	0.335	0.336		0.009	0.484
E1	0.352	0.349	0.348	0.348	0.347	0.348	0.347	0.346	0.346	0.346	0.346	0.346	0.347	0.350	0.348	0.348	0.347	0.347	0.350	0.333	0.336	0.009		0.481
MG	0.187	0.184	0.186	0.187	0.188	0.187	0.185	0.186	0.186	0.185	0.187	0.185	0.186	0.188	0.186	0.185	0.190	0.192	0.190	0.180	0.180	0.343	0.341	

Table 8. The base analyses within the three groups of *Achalinus niger*.

Groups	Samples size	Distance(average)	Polymorphic site	Haplotype diversity(h)	Nucleotide Diversity(π)	Fst(average between populations)
Northern	7	0.006	16	0.952	0.006	0.688
Southern	15	0.006	25	0.971	0.005	0.445
Meifeng	3	0.044	60	0.667	0.040	x
Total	25	0.038	156	0.983	0.035	0.566

※The Fst don't calculate in Meifeng group because there is only one population.

Table 9. The index of genetics interflow among the three groups of *Achalinus niger*.

Group x	Group y	dxy	Nm	Fst
Northern	Southern	0.038	0.093	0.842
Northern	Meifeng	0.090	0.174	0.741
Southern	Meifeng	0.084	0.189	0.725

Table 10. **AMOVA results for testing genetic subdivision between clades of *Achalinus niger*.**

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation
Among groups	2	333.38	23.69	83.72
Within groups	22	101.37	4.60	16.27
Total	24	434.75		

Table 11. **AMOVA results for testing genetic subdivision between populations of *Achalinus niger*.**

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation
Among populations	6	350.31	17.02	83.44
Within populations	16	54.03	3.37	16.56
Total	22	404.34	20.39	