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Phylogeography of *Trochodendron aralioides* (Trochodendraceae) in Taiwan and its adjacent areas

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Abstract

Aim We suspect that the phylogeographical history of temperate trees is different from that of lowland forest in subtropical Taiwan. To investigate this question we use a wide-spread temperate tree species for comparison. In this paper, we describe current geographical patterns of chloroplastic DNA variation of *Trochodendron aralioides* and infer its phylogeographical history in subtropical Taiwan and East Asia.

Location A total of 24 populations were sampled including 20 from Taiwan, two each from the Ryukyus and Japan.

Methods A haplotype network was constructed, various parameters of genetic diversity were calculated and neutrality was tested. To examine the similarity of genetic structure among populations, a neighbor-joining tree was reconstructed. Results of isozyme and ITS nuclear ribosomal DNA study of *T. aralioides* from a previous publication and sequence data from the GenBank, respectively, were incorporated into this study to infer the phylogeoraphical history.

Results In this study, two cpDNA intergenic spacer fragments (*pet*G-*trn*P and *pet*A-*psb*J) were selected to reveal the spatial genetic structure of *T. aralioides*. Nine haplotypes according to six substitutions, two indels and one inversion were recognized. The genetic structure was differentiated among populations revealed by G_{st}=0.700 and N_{st}=0.542, and the population genetics was clearly spatially structured. Two population groups were recognized in Taiwan. The first group was distributed islandwide and extended to the Ryukyus. The second group contained five of the seven known haplotypes, and was restricted to the area between latitude 24°46'N and

24°06'N.

Conclusions Two population groups according to cpDNA sequences were recognized in Taiwan. One group confining to the area between latitude 24°46'N and 24°06'N is potentially a refugium in Taiwan during the last glaciations as genetic diversity was higher there on the basis of cpDNA and allozyme. Integrating the data sets of cpDNA, allozyme and ITS nuclear ribosomal DNA demonstrated that Japan's populations were clearly distinguished from those of the Ryukyus and Taiwan. On the basis of two transitions of cpDNA, the diverging time between these two groups was estimated to be 900,000±320,000 years which might represent the time of initial colonization of Taiwan. By *Trochodendron*, the migratory route taken to Taiwan was possibly from Japan via the Ryukyus by stepping stone events. The population in Amami Island of central Ryukyu shared the same cpDNA haplotype with its southern relatives but was closer to Japan's relatives with ITS, suggesting a migration pathway of seed from Taiwan to Amami Island while of pollen from Japan to Amami.

Keywords

cpDNA, phylogeography, Taiwan, *Trochodendron aralioides*

INTRODUCTION

Phylogeography (Avise et al., 1987) is a subdiscipline of historical biogeography that involves determining the history of taxa in space and time by integrating the phylogenetic and geographical patterns. This is done especially at the level of species complex or intraspecific populations to reveal how the present distribution patterns of

taxa have been shaped by geological events or other factors. Since the taxon is at species level, the scale in time and space is much shorter and smaller than the study of generic or higher taxonomic levels, and is suitable for revealing the taxon's history from the Quaternary, less than 2 million years.

The origin of the island Taiwan can be traced to the Pliocene, about 4-5 million years ago (Ma), when it began to emerge. Taiwan quickly became the present shape at about 2 Ma through mountain building (Ho, 1982; Shaw, 1996). It is thus suitable to study Taiwan's species based on phylogeography. Taiwan hosts more than 4,000 species of vascular plants distributed from the sea level to 3,500 meters in elevation (Hsieh, 2002), and most of them are distributed within one of three floristic regions delimited altitudinally (Hsieh et al., 1994).

Trochdendron aralioides, the only extant member of *Trochodendron*, is distributed in Japan, the Ryukyus and Taiwan (Wu *et al*., 2001). It is mostly related to *Tetracentron* based on DNA markers including 5.8S nuclear ribosomal (nr)DNA, *trn*L intron chloroplast (cp)DNA and *rbc*L-*atp*B intergenic spacer cpDNA (Wu *et al*., 1999; Wu, 2001). These two genera are either grouped as the family Trochodendraceae or recognized in the separate families Trochodendraceae and Tetracentraceae, and have been considered as primitive in Hamamelididae (Lu *et al*., 1993). Fossils related to *Trochodendron*, namely *Nordenskioldia* Heer and *Trochodendroides* E.W. Berry, can be traced back to the late Cretaceous in the higher latitude in the Northern Hemisphere including North America, Siberia, Japan and northeastern China (Lu *et al*., 1993; Serbet, 1997). *Nordenskioldia* became widespread in the Paleocene in higher latitude in North America, Europe and Asia (Manchester, 1999; Pigg *et al*., 2001). The earliest representative of the genus *Trochodendron* may be traced back to the Eocene of western North America to plants with fruit similar to extant *Trochodendron* while

leaves (*T. nastae*) are intermediate between extant *Trochodendron* and *Tetracentron* (Pigg *et al*., 2001). Plants of *Trochodendron* were also recorded in the Oligocene in Liaoning, northeastern China (Jin & Shang, 1998), and in the Miocene in western North America, Kamchatka and Japan (Manchester, 1999; Pigg *et al*., 2001). Manchester (1999), therefore, suggested that the migration of this genus was most likely to be through Beringia route, since Europe lacks fossil evidence of *Trochodendron* (Pigg *et al*., 2001). Ignoring the existing fossil evidence due to scanty fossil records in Asia, Lu *et al*. (1993) suggested that southeastern Asia including northern Vietnam and southwestern China could be the center of origin of *Trochodendron* based on the fact that the extant primitive groups of Hamamelididae were mainly distributed there. Wu *et al*. (2001) also adopted this view. Considering that Taiwan is the southern limit of the distribution of extant *Trochodendron*, and the fossil records were only reported in the middle and high latitudes, it seems more likely that the populations of *Trochodendron* in Taiwan should have migrated from the north, rather than the southwest. According to its present latitudinal range the center of origin of *Trochodendron* would be more probably in middle to higher latitudes, and either in Asian Angara (Budantsev, 1992) or North America based on fossil data. Since the earliest *Trochodendron* fossils were found in North America (Manchester, 1999; Pigg *et al*., 2001), North America would be the most plausible candidate.

The species *T. aralioides* is characterized by tree habit with vesselless wood, alternate leaves in pseudowhorled arrangement, flowers without sepals and petals, stamens in three to four whorls, and many fused carpels with free stigmas. The flowers are dichogamous, self-incompatible and obligatorily xenogamous (Chaw, 1992). Two main population groups were distinguished in *T. aralioides* based on the analyses of genetic variation of allozyme and sequence of ITS nrDNA (Wu, 2001; Wu

et al., 2001). The first group includes populations from Japan and Amami Island, central Ryukyu, and the second group includes the populations from Taiwan and the Iriomote Island, southern Ryukyu. In Taiwan, *T. aralioides* has a wide distribution from north to south and across a range of elevations. It is distributed up to 3,000 meters in the Central Mountain Ridge in the cloud-foggy zone of relatively cold mountain forest. In the north and south, it inhabits 400-1,000 meters in elevation in subtropical evergreen broad-leaved forest. Two population groups of *T. aralioides* were recognized in Taiwan based on the allelic frequencies of allozyme, i.e., north, and southcentral (Wu *et al*., 2001). The results of the analyses are summarized as follows: (1) the genetic diversity is higher in central Taiwan, and (2) the genetic variation comes from within areas (F_{st}=0.100) rather than among them.

An integration of the history of species representative of different elevations may give insight to understanding the consensus history of the vascular plants in Taiwan. So far, a low altitude species, *Cyclobalanopsis glaua*, Fagaceae, has been studied by Huang *et al*. (2002) based on cpDNA. Here we present evidence from cpDNA by surveying the genetic variation of *T. aralioides* in Taiwan and its adjacent areas, and we also try to integrate the data available including allozyme and ITS nrDNA to address the genetic distribution patterns and their possible evolutionary history. We found that the data of cpDNA provided insights that were not detected in allozyme study (Wu *et al*., 2001). We postulate that north to central mountain area might be the major refugium of *T. aralioides* in Taiwan during the last glaciations. These results also indicate that the phylogeographical history of *T. aralioides*, a temperate tree species, did not conform to that of *Cyclobalanopsis glauca*, a low land tree species (Huang *et al*., 2002).

Materials and methods

Sampling

A total of 24 populations were sampled including 20 from Taiwan, two each from the Ryukyus and Japan (Fig. 1; Table 1). For the analysis of cpDNA, each population was represented by four individuals that were at least 50 meters apart. Fresh leaves were collected from each individual tree, and they were either desiccated with silica gel and then stored in a freezer (-30 ^{o}C) permanently after complete dryness, or they were stored in the freezer (-70 ^{o}C) directly.

DNA Sequencing

The DNAs were extracted from the sample leaves by using the protocol of Murray and Thompson (1980). The DNA extracting solution was then used to amplify the markers for detecting the variation in polymerase chain reaction (PCR). Ten universal primer pairs had been screened to detect the variation among the population; only two markers were taken in this study, i.e., intergenic spacer of *pet*G-*trn*P and *pet*A-*psb*J. The primers for *pet*G-*trn*P are 5'-GGT CTA ATT CCT ATA ACT TTG GC-3' in forward and 5'-GGG ATG TGG CGC AGC TTG G-3' in reverse; and the primers for *pet*A-*psb*J are 5'-GGA GAT GCA GAG ATA GTA C-3' in forward and 5'-CTC TTT GGT TGA TAG GTA CTG-3' in reverse. Thirty four thermal cycles were given for amplification. The annealing temperature is 55 ^{o}C for 45 seconds for *pet*G-*trn*P and 50 ^{o}C for 90 seconds for *pet*A-*psb*J. The extension temperature is 72 ^{o}C for 60 seconds for *pet*G-*trn*P and 72 ^{o}C for 90 seconds for *pet*A-*psb*J. The PCR products were then purified with the commercial kit and then sequenced with a sequencing machine ABI3100 using Big Dye terminator.

Sequence analysis

The DNA sequences were aligned by eye. Construction of haplotype network was then performed by TCS (version 1.06), as described by Templeton *et al*. (1992). Haplotype diversity (*h*), nucleotide diversity (π) (Nei, 1987), nucleotide diversity (θ) (Watterson, 1975), tests of neutrality including Tajima's *D* (Tajima, 1989), Fu and Li's *D** and Fu and Li's *F* (Fu & Li, 1993), and the determination of their associated significance were performed using the DnaSP program (Rozas & Rozas, 1999).

Analyses of population structure

Two measures of diversity and differentiation, G_{st} and N_{st}, were analyzed by HaploNst (Pons & Petit, 1996). G_{st} depends only on haplotype frequencies while N_{st} is influenced by haplotype frequencies and genetic distance between haplotypes.

The SGS software (Degen *et al*., 2001) was used to test if there is geographical genetic structure in *T. aralioides*. For the preparation of a data matrix, haplotype frequencies from different populations were calculated. While executing the program, a genetic distance measure, *Dg* (Gregorius, 1978), was selected, and classes and size were selected to make sure that the sampling size within each class was over 30.

To examine the similarity of genetic stricture among populations, a neighbor-joining tree was reconstructed. A data matrix was made by taking population in each locality as an OTU (operational taxonomic unit) and haplotypes and haplotype lineages as characters. Character state codes 0 when haplotype or lineage was absent, and codes 1 to 4 depending on how many individuals hold such haplotype (lineage) in this population. The data matrix was then performed with PAUP* version 4.0b10 (Swofford, 2000) with characters being set in order, and the minimum evolution being

selected for generating a distance of neighbor-joining tree. Resampling was performed with bootstrap for 1,000 replicates.

Results

Sequence analysis

Among the sequences of *pet*G-*trn*P (GenBank accession numbers AY294754-AY294848), no indels (insertion-deletion) but four substitutions were detected at position 162 for GC transversion, 239 for TC transition, 252 for TC transition, and 376 for GA transition (Table 2). Among the sequences of *pet*A-*psb*J (GenBank accession numbers AY294659-AY294753), two substitutions were detected at position 143 for CT transition, and 570 for AC transversion. The other mutations include one inversion and many short indels. Among indels, polyA and polyT were excluded as polymorphic sites. The following are positions of polyA and polyT that show indels: 80 and 520-521 for polyA and 152-153 for polyT. The other indels are detected as follows: 154-157 for ATTT, 329 for A, and 332-335 for CTAT. The inversion due to intramolecular recombination event occurred at positions 684-694 for GAACAAACAAA and TTTGTTTGTTC. The phenomenon of intramolecular recombination was similar as described in oak trees by Dumolin-Lapègue *et al*. (1998) by forming a 13 bp stem-loop hairpin (Fig. 2).

Haplotypes and their distribution

On the basis of 95 sequences with 1106 sites, a total of nine haplotypes were detected (Table 2; Fig. 1), and the relationship among these haplotypes is shown in Fig. 3. Type A is the most common and widely distributed in the Ryukyus, the northern tip of Taiwan including Yangmingshan and Shihting, and central and southern Taiwan.

Type C is the next common haplotype restricted to the northern Taiwan including Hsueshan Range and to the northern Central Mountain Ridge (Fig. 1). Type D is derived from type C due to a substitution and this type spreads in Nanchatienshan, Chilanshan and Piluchi. Type E is derived from type D due to an inversion and this type is a singleton at Ssuyuan. Type B is derived from type A by an inversion and this type is found in the populations of Iriomote Island (Ryukyu) and Hakannishan (Taiwan). Type F is also derived from type A due to a deletion at position 329-335 of petA-psbJ and this type is entirely restricted to Tuona. Type G is a singleton and is derived from type A due to a substitution and this type is only found in Tanta. Japanese populations are quite different from others, and they are represented by types H and I; type H is restricted to Asiu, whereas type I is confined at Chomonkyo. Type H is considered ancestral because (1) fossil Trochodendron was only recorded in the middle and high latitudes in North Hemisphere, and (2) the nucleotide position 239 of petG-trnP, separating H, I from the remaining types, of *Cyclobanalopsis glauca* (GenBank accession number AY091648) is of H type. *Cyclobanalopsis glauca* may be considered as an outgroup so far we can get.

Diversity of haplotype and nucleotide

The haplotype diversity (h) is 0.658 and the nucleotide diversity (π) is 0.00088 for all populations while h and π for the population in Taiwan are 0.595 and 0.00052, respectively (Table 3). Nucleotide diversity (θ) is 0.00123 for all populations, 0.00027 for Japan's populations, 0.00029 for Ryukyus' populations and 0.00071 for Taiwan's populations. Parameters of Japan are underestimated due to low representatives in this study. Regarding local populations, only Ryukyus' Iriomote Island, and Taiwan's Nanchatienshan, Chilanshan, Hakannishan, Ssuyuan, Piluchi,

Meifeng and Tanta are polymorphic, whereas the remaining local populations are monomorphic. Selection was not detected to play a role in shaping the genetic structure because the sequence variation did not deviate from the expectation of neutral selection when neutrality tests were performed using Tajima's *D* test, Fu and Li's *D** and *F* tests (Table 3).

Analyses of population structure in Taiwan

Population differentiation in Taiwan was 0.700 and 0.542 for G_{st} and N_{st}, respectively, and both the values were significantly higher than zero (Table 4). This suggests that the distribution of haplotypes is significantly structured among populations in Taiwan. The test of spatial genetic structure for Taiwan's populations gave further support for the significant structure as it shows that the genetic structure occurs at classes of 40 km, 120 km and 160 km (Fig. 4) while the genetic structure over 160 km cannot be tested because the sampling size is too small.

A neighbor-joining tree revealing similarity of genetic structure among populations is shown in Fig. 5. Japan's populations can be separated from the populations of Ryukyus and Taiwan, and this is supported by bootstrap value of 89. In Taiwan, two major groups of populations can be recognized. One is restricted to northern Taiwan between latitudes 24 ^{o}46' N and 24 ^{o}06'N, and the other is distributed widely. The restricted group shares the haplotype C and its derivatives, D and E (Fig. 3). The other group shares haplotype A and its derivatives, B, F and G (Fig. 3). However, Meifeng's population is collapsed in bootstrap majority consensus tree (Fig. 5), indicating an overlapping position of Meifeng between these two groups as it contains both haplotype A and C equally. The populations of Ryukyus are nested inside the group that is distributed widely in Taiwan.

Discussion

Intramolecular recombination

Among the polymorphic sites that distinguish haplotypes, substitutions and indels are associated without any redundancy while inversion due to intramolecular recombination (Fig. 2) appears redundantly in types B and E that belong to two different lineages (Fig. 3). This implies that the loop inversion in the secondary structure is likely to occur in chloroplastic DNA repeatedly, as in the case of mitochondrial DNA in oaks (Dumolin-Lapègue *et al*., 1998).

Center of genetic diversity of T. aralioides *in Taiwan*

Genetic diversity in a population is accumulated either from newly evolved alleles and/or from other sources that join the population. Thus an area showing higher genetic diversity could imply that (1) this area could have been a refugium: an area with a stable ecological habitat during the fluctuation of environmental change that led to the accumulation of genetic diversity (Tzedakis *et al*., 2002), or (2) this area could be an intermediate zone that received organisms from different sources and resulting in higher genetic diversity than in each of the original sources. In the first case, haplotypes within this area are closely related, whereas in the latter case haplotypes may include those that are distantly related. According to cpDNA polymorphism, higher genetic diversity in *T. aralioides* in Taiwan is located in the latitudes between 24°06'N and 24°46'N where five haplotypes out of seven were found, and haplotypes C, D and E were restricted there. Moreover, π and θ are higher in Hakannishan, Piluchi and Ssuyuan (Table 3) areas within the range of this area. Thus this area is a center of genetic diversity and was possibly a shelter in Taiwan for

T. aralioides during the Quaternary glacial period.

A primary refugium in central Taiwan below latitude 24 °06'N was proposed by Lin (2001) according to the expected heterozygosity of allozyme data of eight distantly related species including *T. aralioides,* although the possibility of the occurrence of other refugia in Taiwan was not excluded. Regarding allozyme data, genetic diversity measures are mainly described by allele richness and expected heterozygosity (El Mousadik & Petit, 1996; Comps *et al*., 2001; Widmer & Lexer, 2001). Allele richness deals with rare alleles while heterozygosity deals with common alleles. It is hypothesized that a population first colonizes a place from a source area (refugium) by a few individuals (founder event). During the colonization, rare alleles occur in few individuals and are easily drifted away while the common alleles are many and hence have more chance to colonize this place. Thus it is predicted that allele richness decreases with distance from the source area (El Mousadik & Petit, 1996; Comps *et al*., 2001). In contrast, a place may receive individuals from different sources resulting higher heterozygosity than expected (Comps *et al*., 2001). Thus inference of a possible refugium can be proposed by the measure of allele richness and expected heterozygosity, but attention should be paid to expected heterozygosity.

According to the data of allozymes, allele richness of *T. aralioides* in Taiwan is the highest in Meifeng, followed by Hsiangyang and Yuanyang Lake (near Chilanshan) while the expected heterozygosity is the highest in Chitou followed by Nantou, Alishan, Rhkeshan (near Shihting) and Lalashan (near Nanchatienshan) (Table 5). According to the allele richness and expected heterozygosity of allozymes, we may conclude that there are two centers of diversity of the Nantou area and the Lalashan-Yuanyang Lake area. It seems in conflict with the proposed center of genetic diversity based on cpDNA. The possible causes are as follows. Firstly, high genetic diversity in

Nantou area including Meifeng may be due to the accumulation of mutations after the last glaciations, as in the case of *Cyclobananopsis glauca* examined by Huang *et al*. (2002). Secondly, the highest expected heterozygosity in Nantou area may be due to its intermediate position between other centers of diversity such as Hsiangyang and Lalashan-Yuanyang Lake.

Is Central Mountain Ridge a barrier between eastern and western populations of T. aralioides?

Taiwan has more than 100 mountain peaks over 3,000 meters and most of them are located in the Central Mountain Range that extends from north to south. Thus it is likely that the Central Mountain Range could serve as a barrier between eastern and western populations. This is manifested by the study of *Cyclobalanopsis glauca* (Huang *et al*., 2002), a subtropical species distributed in the elevation from 50 to 1,200 meters in Taiwan. However, the barrier is not problematic to *T. aralioides* whose distribution is considerable higher in elevation, as indicated by the presence of haplotype C on both sides of the Central Mountain Ridge. Although many high mountain peaks are located in the Central Mountain Ridge, saddles allow for dispersal to occur across the range and let populations penetrate through the barrier. Additionally, the temperature was 2^{o}C higher than today about 5,000 years ago (Tsukada 1966), so the distribution of *T. aralioides* in Taiwan would be distributed higher in elevation and have a better chance to transcend the barrier.

Temperate species has a different evolutionary history from subtropical species

It is an interesting observation that subtropical tree species, like *Cyclobalanopsis glauca* (Huang *et al*., 2002) has different phylogeographical pattern from temperate

species, like *T. aralioides*. As stated above, *T. aralioides* has higher genetic diversity in north to central mountainous area, and a possible refugium also occurring in this area, but *Cyclobalanopsis* has many unique haplotypes in the east of the Central Mountain Range, and a possible refugium in southeastern part of Taiwan. The distinct effect of the Central Mountain Range on gene flow was also observed between east and west populations of these two species. It could be temperate species occurring in present habitat between 1,500m and 2,500m would find an optimal growth habitat in lower elevations but not in the south part where subtropical climate prevailed in glaciation stage. Further species will be needed to test the hypothesis that temperate vegetation and subtropical vegetation have different evolutionary history.

How did Trochodendron migrate into Taiwan?

Since fossil *Trochodendron* was recorded from the middle to high latitudes, Taiwan's population is definitely derived from the North. There are three possible ways for the temperate plants to migrate into Taiwan from the North: (1) from the North via continental China to Taiwan when the sea level was low during glacial cycles, (2) from the North by ways of Japan via the Ryukyus to Taiwan by a land bridge that connected them (Ujiie, 1990 cited by Ota, 1998; Kimura, 2000), (3) from Japan via the Ryukyus to Taiwan due to stepstone events when there was no land bridge as direct connection or no suitable habitat as well. If the first hypothesis is correct, there should be fossils or extant sister lineage in China. If the second hypothesis is correct, there should be many species in Taiwan that show the same distribution pattern, and such species are not inclined to be long-distance dispersed, especially for species inhabiting forest or woodland. If the third hypothesis is correct, species that show the same distribution pattern as *Trochodendron* is relatively few and all such species are

inclined to be long-distance dispersed.

The extant species is only distributed in Japan, the Ryukyus and Taiwan while the fossils in the Asian continent is only recorded in Liaoning, northeastern China (Jin & Shang, 1998) and no fossil evidence has been reported in eastern China. Thus the first route via continental China seems unlikely according to the present knowledge. Land bridge connected Taiwan and Japan was proposed but inconsistent in timing. The earliest land bridge connected Japan, the Ryukyus and Taiwan during the late Miocene more than 6 million years ago, with Japan separating from the others in the early Pliocene (Ujiie, 1990 cited by Ota, 1998). A second possible one was formed during 1.6-1.3 Ma with Japan separating from the others after 1.3 Ma (Kimura, 2000). If Japan was separated from the others in the early Pliocene, Taiwan was still under water (Shaw, 1996) and thus no plants could invade Taiwan. If *Trochodendron* colonized Taiwan during 1.6-1.3 Ma through land bridge, many species should have shown the same pattern as *Trochodendron*. However, forest species in Taiwan showing the *Trochodendron*'s distribution pattern are few. Besides, if *Trochodendron* colonized Taiwan during this period, the cpDNA average substitution rate per site per year is estimated as 6.23×10^{-10}, much lower than $1\text{-}3 \times 10^{-9}$ based on previous estimates (Wolfe *et al*., 1987). Thus it is less likely that a direct connection existed allowing *Trochodendron* in Japan to migrate into Taiwan via the Ryukyus. The only way left for migration to Taiwan is by long-distance dispersal via stepstone events. Seeds of *T. aralioides* are 3-4 mm in length and about 0.1 mg in mass (Wu, 2001). Such condition is suitable for wind dispersal since winter monsoon from northeast is long and sometimes strong.

When did Trochodendron colonize Taiwan?

The fossil pollen record of *Trochodendron* in Taiwan was found from the peat of the Quaternary between 38,000 and 4,500 years before present (BP) (Chung and Huang, 1972a,b). Since *Trochodendron* pollen occurs near the bottom of the peat core, it is estimated that it has inhabited northern Taiwan about 30,000 BP. As the major landmass of Taiwan proper emerged after the mountain building in the middle Pleistocene about 2 Ma (Ho, 1982; Shaw, 1996), and plants of *Trochodendron* require a wet forest habitat in the late stage of succession, Taiwan's population could not be older than 2 million years. Two transitions and one insertion were detected in the spacer of *pet*G-*trn*P and *pet*A-*psb*J between Japan's populations and those of Ryukyus and Taiwan. When there is a molecular clock between two lineages, the equation K=2RT can be applied, where K is the predicted average substitution per site between two homologous sequences, R is substitution rate and T is diverging time (Li, 1997). Using the simplest substitution model (Jukes & Cantor, 1969), K= (-3/4)ln(1-4D/3) where D is the observed average substitution per site between two homologous sequences, and the variance of K is $D(1-D)/[L(1-4D/3)^2]$ where L is the total length of the base pairs (Li, 1997). If we take 1.45 Ma as the timing that *Trochodendron* colonized Taiwan because a land bridge between Japan and Taiwan occurred about 1.6-1.3 Ma (Kimura, 2000), the substitution rate will be 6.23×10^{-10} average substitution per site per year. However, this substitution rate is much slower than that estimated ($1\text{-}3 \times 10^{-9}$) previously (Wolfe *et al*., 1987). Besides, this land bridge is not preferred as suggested in the previous section. Taking the substitution rate as 1×10^{-9} average substitution per site per year because the synonymous substitution rate for cpDNA is about $1\text{-}3 \times 10^{-9}$, the diverging time for two transitions is 904,000±319,000 years. Thus Taiwan's population could be derived then.

Inconsistency in genealogy between ITS nrDNA and intergenic spacer cpDNA in Amami population in central Ryukyu

By comparing the data sets of cpDNA, allozyme and ITS nrDNA, Japan's populations are well differentiated and they can be separated from the remaining populations (Figs. 3, 5 and 6). Two populations are represented in the Ryukyus. The southern population in Iriomote Island is geographically near to Taiwan and shows affinity with those from Taiwan according to cpDNA and ITS. However, the northern population in Amami Islands is geographically near to Japan and shows affinity with Japan's population according to the ITS (Fig. 6; Wu, 2001) while it shows affinity with its southern populations according to cpDNA (Table 1).

Since the ITS is a nuclear gene fragment, the genetic variation in this fragment can be accumulated not only due to mutation but also by the recombination from both parents via pollination, whereas DNA fragments from chloroplasts mainly accumulate genetic variation through mutation due to maternal inheritance. Two possible scenarios may explain why the northern Ryukyu's population shows the same cpDNA haplotype with southern populations while shows similarity with Japan's ITS. The first scenario is as follows. Amami's population was colonized from the south during the glacial period while the pollen from the north might have had the chance to fertilize the plants there, so the population shows cpDNA haplotype of the south due to seed dispersal while showing similar ITS cytotype due to recombination. The second scenario is as follows. Amami's population was derived from Japan and then accumulated mutations due to long period of isolation. It then migrated to the south without further divergence, because the mutation rate of cpDNA was slower (Wolfe *et al*., 1987). Gene flow between Japan and Amami Island through pollination either via

insects or even wind should also have happened during the separation of these populations. Of these two scenarios, the first is more likely. The reason is as follows. Since the cpDNA haplotype of Amami's population is the same as Taiwan's, it is not likely that *Trochodendron* migrated from Japan to Amami Island before 1.3 Ma when a land bridge connected them (Ota, 1998; Kimura, 2000) because it would require at least two substitutions when a molecular clock is considered. In contrast, there was a direct connection between Taiwan and Amami Island lasted to 25,000 years ago (Kimura 2000) when the temperature was the coldest about 10°C below than today about 50,000 years ago (Tsukada, 1966). Since the genetic diversity in Iriomote Island is the smallest (H_0=0.076 in contrast with 0.133 in Erkeshan, 0.131 in Yuanyang Lake, and 0.127 in Taipingshan, Table 5), this suggests a colonization from Taiwan. This kind of situation may also apply to Amami's population. Therefore in recent glaciations the population in Amami Island could have been wiped out and the present population is derived from Taiwan as described in the first scenario.

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Table 1. Sampling localities chosen for the population study of *Trochodendron aralioides* accompanied with sample sizes and their haplotypes.

Locality and population no.	Latitude-longitude	Altitude (meter)	Sample size	Haplotype (sample no.)
Japan				
1 Asiu	35°00'N-135°42'E	700	4	H(4)
2 Chomonkyo	34°23'N-131°20'E	150	4	I(4)
Ryukyus				
3 Amami	28°24'N-129°42'E	500	3	A(3)
4 Iriomote	24°25'N-123°45'E	100	4	A(2)B(2)
Taiwan				
5 Yangmingshan	25°10'N-121°34'E	700	4	A(4)
6 Shihting	24°59'N-121°40'E	400	4	A(4)
7 Nanchatienshan	24°46'N-121°24'E	1600	4	C(3)D(1)
8 Chilanshan	24°41'N-121°20'E	1550	4	C(3)D(1)
9 Hsiakeluoshan	24°34'N-121°12'E	2000	4	C(4)
10 Hakannishan	24°32'N-121°02'E	1600	4	B(1)C(3)
11 Taipingshan	24°30'N-121°32'E	1800	4	C(4)
12 Ssuyuan	24°24'N-121°21'E	1900	4	C(3)E(1)
13 Hoping	24°18'N-12 1°39'E	1200	4	C(4)
14 Piluchi	24°13'N-121°17'E	2400	4	A(3)D(1)
15 Meifeng	24°06'N-121°11'E	2000	4	A(2)C(2)
16 Tanta	23°46'N-121°07'E	2450	4	A(3)G(1)
17 Chitou	23°40'N-120°47'E	1700	4	A(4)
18 Zueshui	23°32'N-121°16'E	1500	4	A(4)
19 Tatachia	23°30'N-120°52'E	2500	4	A(4)
20 Takuanshan	23°17'N-120°56'E	2500	4	A(4)
21 Hsiangyang	23°15'N-120°58'E	2350	4	A(4)
22 Tuona	22°53'N-120°46'E	1800	4	F(4)
23 Tahanshan	22°25'N-120°43'E	1500	4	A(4)
24 Lilongshan	22°10'N-120°43'E	900	4	A(4)

Table 2. Nine haplotypes of *Trochodendendron aralioides* were recognized based on the distribution of polymorphic sites in the intergenic spacers of *pet*G-*trn*P and *pet*A-*psb*J *cp*DNA.

	*pet*G-*trn*P				*pet*A-*psb*J					
Haplotype	162	239	252	376	143	154-157	329-335	570	684-694	Distribution
A	G	C	T	G	T	----	AATCTAT	A	GAACAAACAAA	Taiwan, Ryukyu
B	G	C	T	G	T	----	AATCTAT	A	TTTGTTTGTTC*	Taiwan, Ryukyu
C	G	C	C	G	T	----	AATCTAT	A	GAACAAACAAA	Taiwan
D	G	C	C	A	T	----	AATCTAT	A	GAACAAACAAA	Taiwan
E	G	C	C	A	T	----	AATCTAT	A	TTTGTTTGTTC*	Taiwan
F	G	C	T	G	T	----	-AT----	A	GAACAAACAAA	Taiwan
G	G	C	T	G	T	----	AATCTAT	C	GAACAAACAAA	Taiwan
H	G	T	T	G	C	ATTT	AATCTAT	A	GAACAAACAAA	Japan
I	C	T	T	G	C	ATTT	AATCTAT	A	GAACAAACAAA	Japan

* Inversion due to intramolecular recombination

Table 3: Populations of *Trochodendron aralioides* with haplotype and nucleotide diversity associated with the results of neutrality tests based on intergenic spacers of *pet*A-*psb*J and *pet*G-*trn*P cpDNA

Populations	Haplotype diversity (h)	Nucleotide diversity (π)	θ (per site)	Tajima's D (P value)	Fu and Li's D* (P value)	Fu and Li's F (P value)
Total	0.658±0.040	0.00088±0.00012	0.00123	-0.72(P>0.1)	0.58(P>0.1)	-2.07(P>0.1)
Japan	0.571±0.0089	0.00040±0.00007	0.00027	1.44 (P>0.1)	0.89(P>0.1)	0.97(P>0.1)
Asiu	0.000	0.00000	0.00000			
Chomonkyo	0.000	0.00000	0.00000			
Ryukyu	0.476±0.171	0.00033±0.00012	0.00029	0.56 (P>0.1)	0.95 (P>0.1)	0.59(P>0.1)
Amami	0.000	0.00000	0.00000			
Iriomote	0.667±0.204	0.00047±0.00014	0.00038			
Taiwan	0.595±0.040	0.00052±0.00006	0.00071	-0.59(P>0.1)	0.02 (P>0.1)	-2.49 (P>0.1)
Nanchatienshan	0.500±0.265	0.00035±0.00019	0.00038			
Chilanshan	0.500±0.265	0.00035±0.00019	0.00038			
Hsiakeluoshan	0.000	0.00000	0.00000			
Hakanishan	0.500±0.265	0.00070±0.00037	0.00076			
Taipingshan	0.000	0.00000	0.00000			
Ssuyuan	0.500±0.265	0.00070±0.00037	0.00076			
Hoping	0.000	0.00000	0.00000			
Piluchi	0.500±0.265	0.00070±0.00037	0.00076			
Meifeng	0.667±0.204	0.00047±0.00014	0.00038			
Yangmingshan	0.000	0.00000	0.00000			
Shihting	0.000	0.00000	0.00000			
Chitou	0.000	0.00000	0.00000			
Tanta	0.500±0.265	0.00035±0.00019	0.00038			
Zueshui	0.000	0.00000	0.00000			
Tatachia	0.000	0.00000	0.00000			
Takuanshan	0.000	0.00000	0.00000			
Hsiangyang	0.000	0.00000	0.00000			
Tuona	0.000	0.00000	0.00000			
Tahanshan	0.000	0.00000	0.00000			
Lilongshan	0.000	0.00000	0.00000			

Table 4. Analysis of population structure of *Trochodendron aralioides* in Taiwan.

Diversity parameter	Value (SD)	Test value (U)	P value
H_s	0.183 (0.0579)		
H_t	0.611 (0.0673)		
G_{st}	0.700 (0.0876)	7.99	P<0.01
V_s	0.279 (0.1492)		
V_t	0.607 (0.2074)		
N_{st}	0.542 (0.1929)	2.809	P<0.01
N_{st}-G_{st}	-0.158(0.1646)	-0.96	P>0.05

Table 5: Allele richness and heterozygosity of *Trochodendron aralioides* in each locality modified from the allozyme study of Wu et al. (2001: Table 3).

Populations in allozyme study	Sample size	Alleles/locus Observed	*$R_{(40)}$	Heterozygosity Observed	Expected	Populations in this study
Asiu	20	1.71	1.71	0.188	0.226	Asiu
Chomonkyo	25	1.76	1.70	0.111	0.124	Chomonkyo
Yangmingshan	25	1.59	1.55	0.093	0.123	Yangmingshan
Erkeshan	23	1.59	1.59	0.133	0.153	Shihting
Lalashan	25	1.76	1.74	0.146	0.152	Nanchatienshan
Yuanyang Lake	27	2.00	1.90	0.131	0.130	Chilanshan
Taipingshan	25	1.88	1.82	0.127	0.130	Taipingshan
Iriomote Island	20	1.35	1.35	0.076	0.099	Iriomote Island
Ssuyuan	24	1.53	1.51	0.100	0.104	Ssuyuan
Anmashan	30	1.88	1.81	0.110	0.135	
Pilu	31	1.88	1.82	0.131	0.125	Piluchi
Meifeng	24	2.12	2.01	0.115	0.106	Meifeng
Nantou	21	1.88	1.88	0.144	0.162	Tanta
Chitou	20	1.71	1.71	0.135	0.166	Chitou
Alishan	20	1.89	1.87	0.150	0.154	Tatachia
Kuaiku	23	1.76	1.74	0.132	0.135	Takuanshan
Hsiangyang	20	1.94	1.94	0.129	0.125	Hsiangyang
Tahanshan	27	1.76	1.69	0.102	0.091	Tahanshan
Lilongshan	27	1.71	1.64	0.092	0.086	Lilongshan

*$R_{(40)}$: Allele richness of each population when sampling size of all populations is equal to 20 individuals and the values of allele richness are obtained by performing the program rarefaction through the internet web site:

www.pierroton.inra.fr/genetics/labo/Software

Figure Legends

Fig. 1. Distribution of haplotypes (A-H) of *Trochodendron aralioides* in Taiwan and its adjacent areas. 1. Asiu; 2. Chomonkyo; 3. Amami; 4. Iriomote; 5. Yangmingshan; 6. Shihting; 7. Nanchatienshan; 8. Chilanshan; 9. Hsiakeluoshan; 10. Hakannishan; 11. Taipingshan; 12. Ssuyuan; 13. Hoping; 14. Piluchi; 15. Meifeng; 16. Tanta; 17. Chitou; 18. Zueshui; 19. Tatachia; 20. Takuanshan; 21. Hsiangyang; 22. Tuona; 23. Tahanshan; 24. Lilongshan.

Fig. 2. Intramolecular recombination leading to inversion occurs in intergenic spacer of *pet*A-*psb*J cpDNA of *Trochodendron aralioides* in a stem-loop hairpin with 13 pair's nucleotide as stem and 11 nucleotides as loop. The symbol * indicates the fragment that shows inversion.

Fig. 3. Genealogy of haplotypes (A-I) of *Trochodendron aralioides* modified from the results of TCS program. Each arrow stands for one mutation including substitutions and indels between haplotypes. Types H and I are restricted to Japan while the other types are confined in the Ryukyus and Taiwan. Numbers of Individuals containing such haplotype are in parentheses.

Fig. 4. Genetic distogram of genetic distance D of 20 populations of *Trochodendron aralioides* in four geographical distance classes in Taiwan. The average genetic distance D is in the middle with 95% confidence interval between the boundaries. The confidence intervals of D were computed by means of 500 permutations over populations with redistributed geo-coordinates. The observed D in each distance class is in the Empty Square.

Fig. 5. The neighbor-joining tree showing similarity of populations of *Trochodendron aralioides* generated from computer program PAUP* version 4.10 based on intergenic spacers of *pet*A-*psb*J and *pet*G-*trn*P cpDNA with minimum evolution criterion. Bootstrap values that were obtained by resampling for 1000 replicates are on branches where * is less than 50%. Shaded horizontal line shows two major groups of population can be differentiated in Taiwan.

Fig. 6. A simplified maximum likelihood gene tree of *Trochodendron aralioides* based on ITS sequences downloaded from the GenBank by lumping the clade of southern Ryukyu's and Taiwan's samples. The ITS sequences were aligned by eye. The data matrix was performed with PAUP* version 4.0b10. The HKY model with invariable sites and gamma distribution estimated from the empirical data was chosen to generate this tree. Bootstrap values are on branches generated from resampling of 1000 replicates using minimum distance as criterion.

Fig. 1

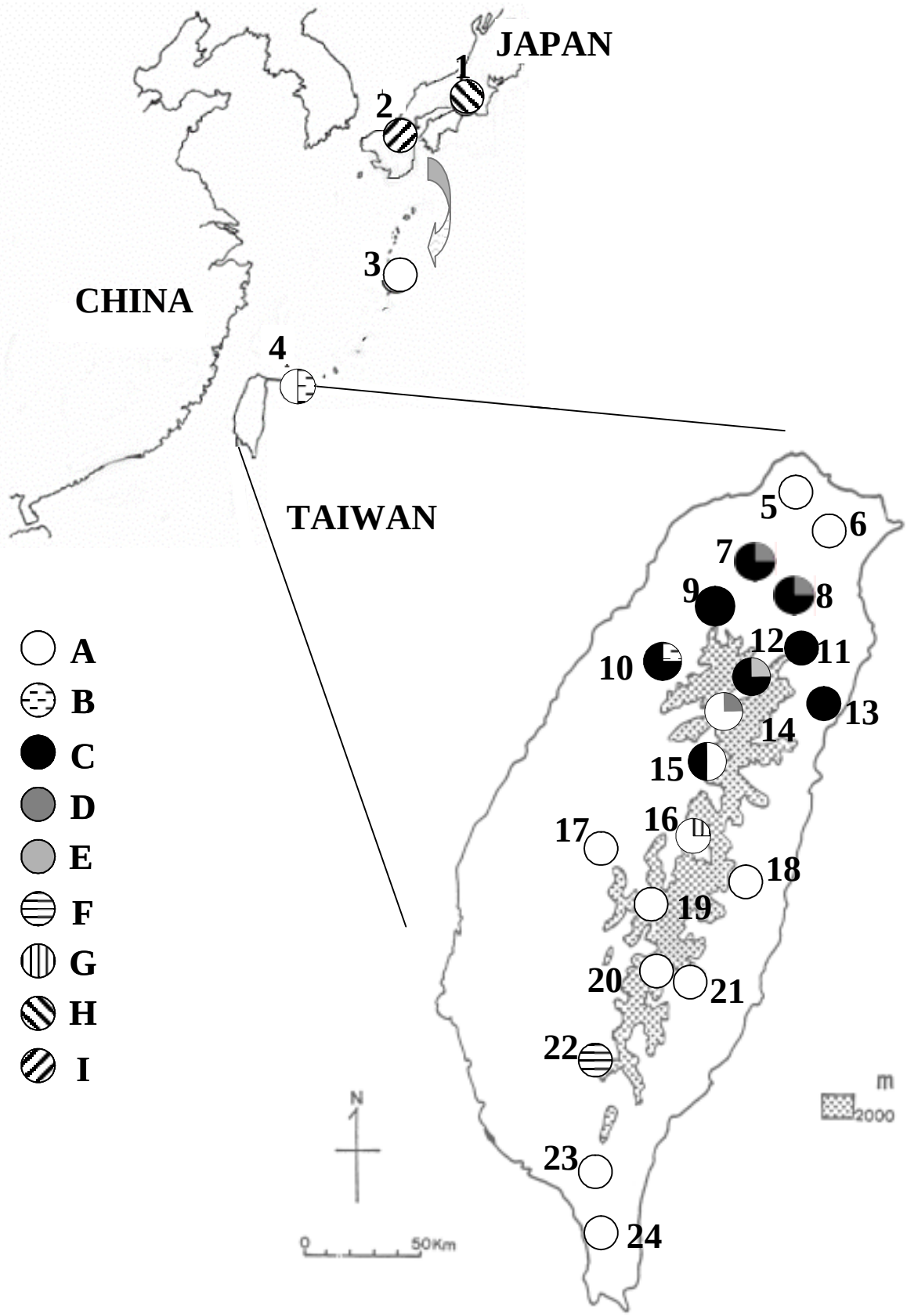
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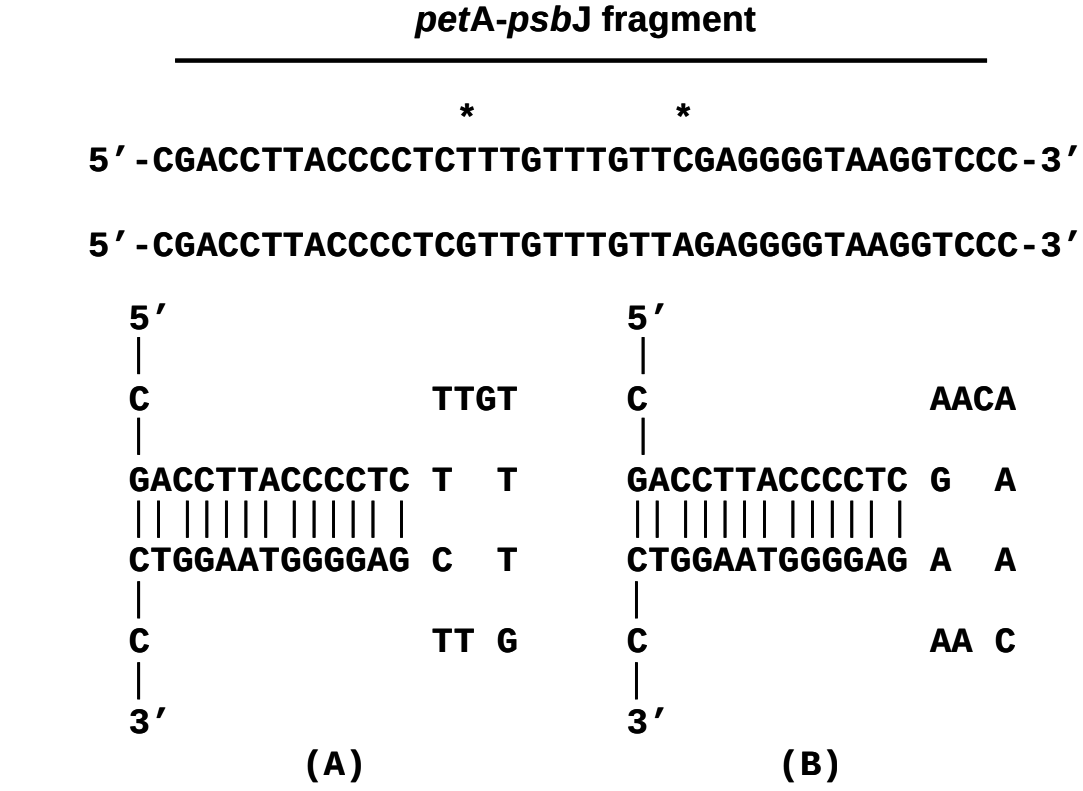
Fig. 2


petA-psbJ fragment
*
*
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5′-CGACCTTACCCCTCGTTGTTTGTTAGAGGGGTAAGGTCCC-3′
5′
C
GACCTTACCCCTC
CTGGAATGGGGAG
C
3′
TTGT
T T
C T
TT G
(A)
5′
C
GACCTTACCCCTC
CTGGAATGGGGAG
C
3′
AACA
G A
A A
AA C
(B)

Fig. 3

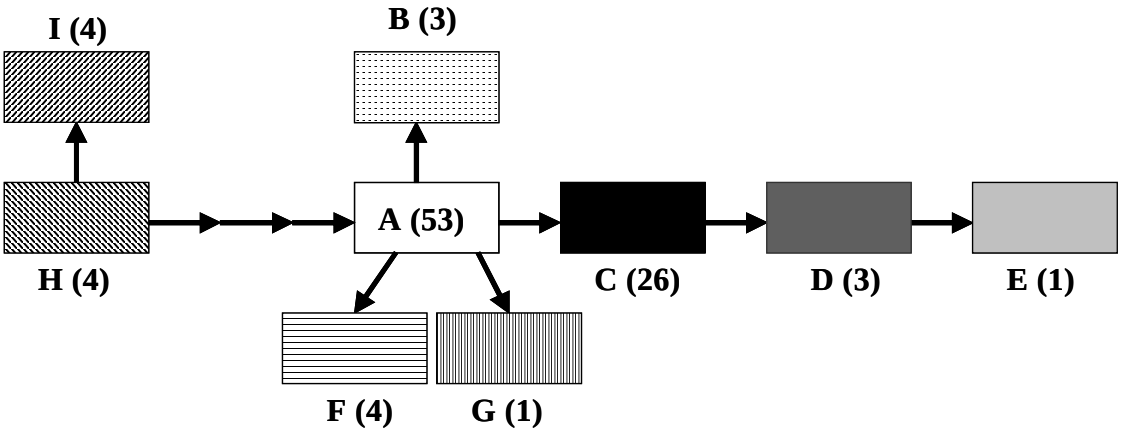


Fig. 4

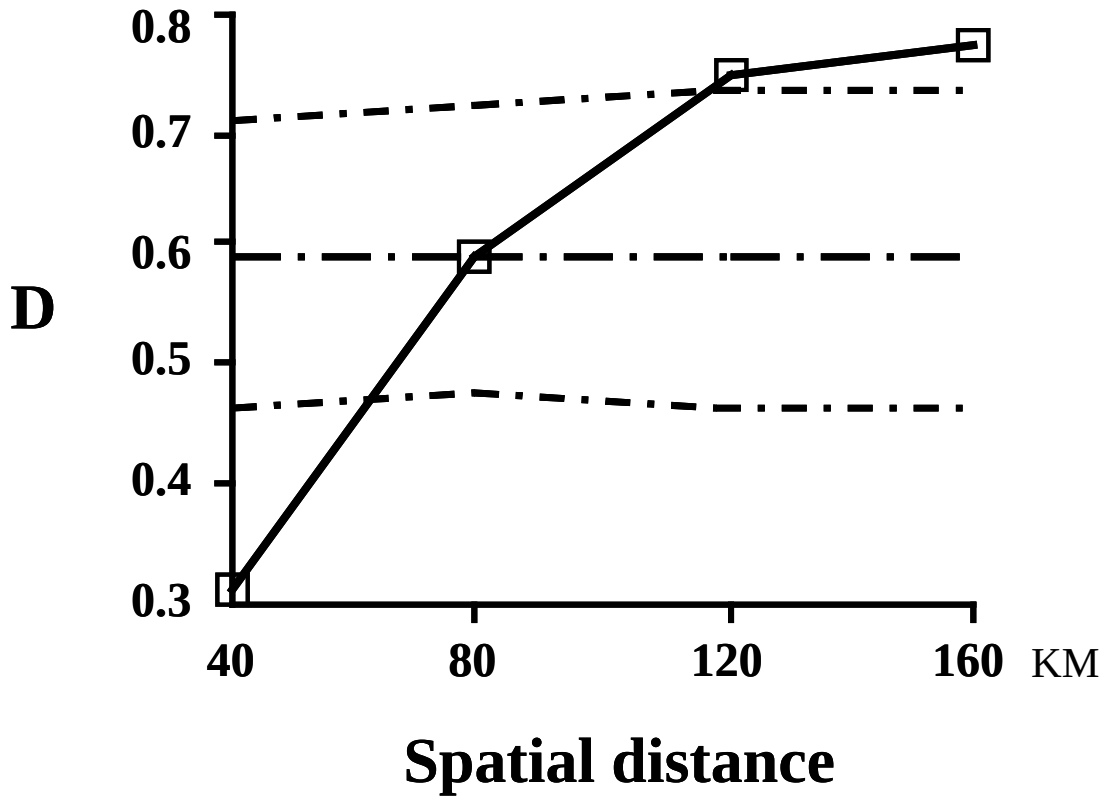
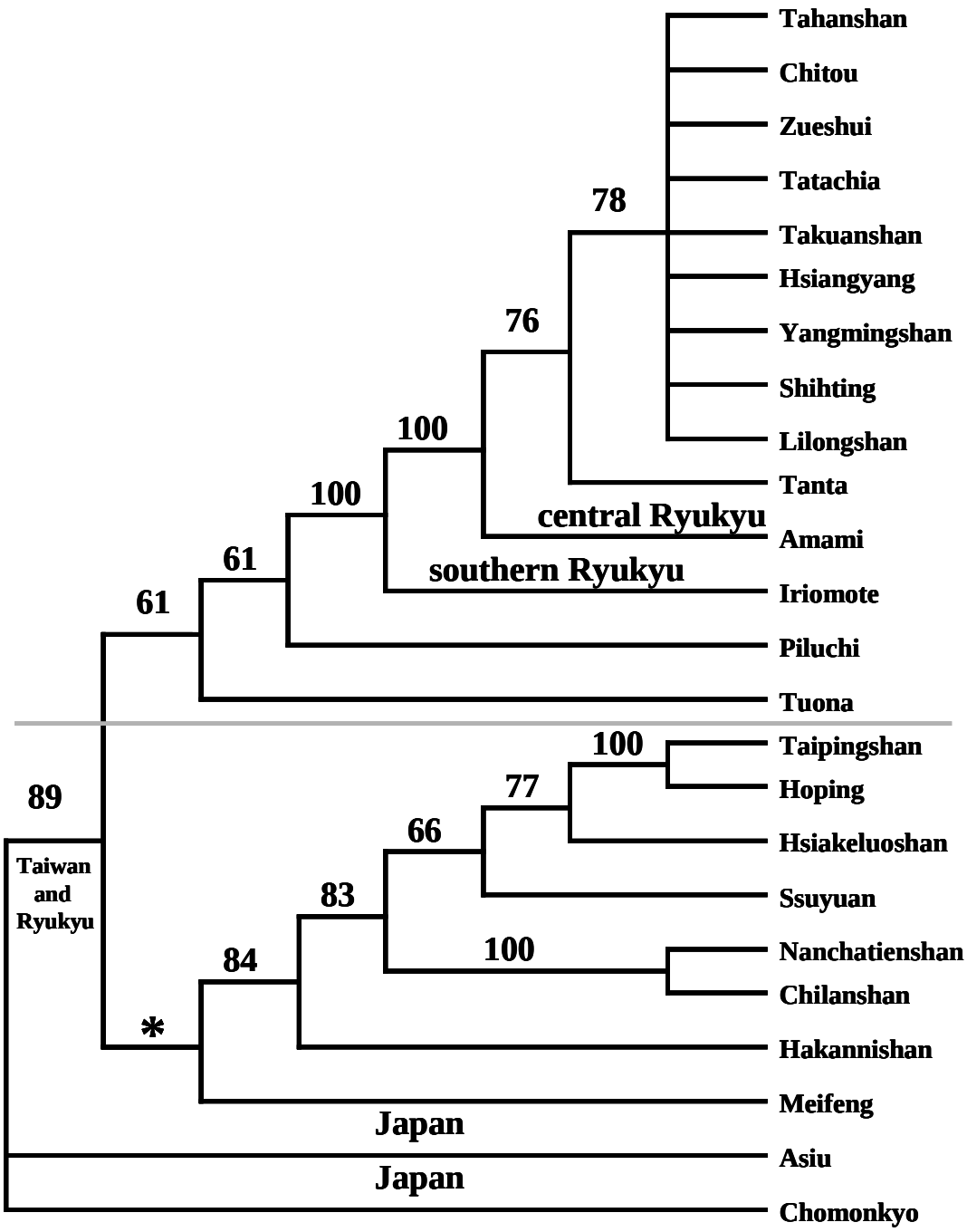


Fig. 5


Tahanshan
Chitou
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Shihting
Lilongshan
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Amami
Iriomote
Piluchi
Tuona
Taipingshan
Hoping
Hsiakeluoshan
Ssuyuan
Nanchatienshan
Chilanshan
Hakannishan
Meifeng
Asiu
Chomonkyo
78
76
100
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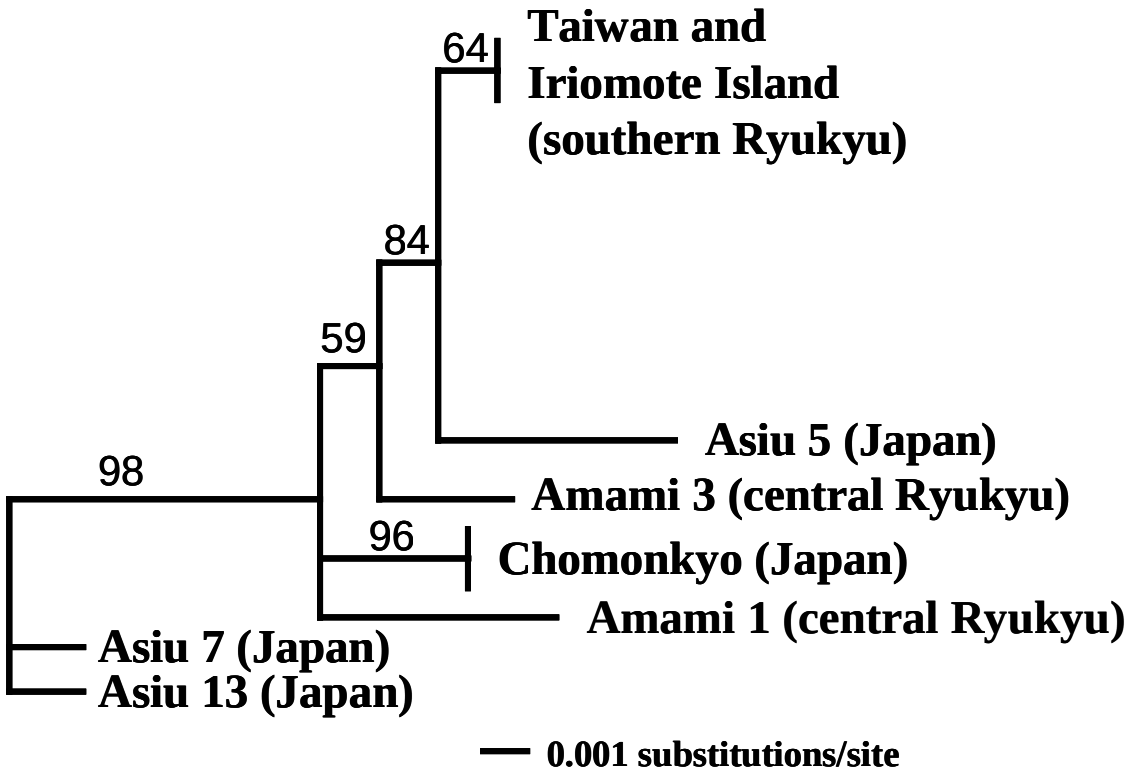
*
central Ryukyu
southern Ryukyu
Taiwan and Ryukyu
Japan
Japan

Fig. 6


Taiwan and
Iriomote Island
(southern Ryukyu)
64
84
59
Asiu 5 (Japan)
98
Amami 3 (central Ryukyu)
96
Chomonkyo (Japan)
Amami 1 (central Ryukyu)
Asiu 7 (Japan)
Asiu 13 (Japan)
0.001 substitutions/site