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

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ABSTRACT. A migratory history of *Trochodendron aralioides* was postulated by integrating the published fossil records and the data sets of chloroplast (cp) DNA and allozyme. The populations from Taiwan, the Ryukyus, and Japan were investigated. The haplotype network of *Trochodendron* was constructed by the computer program TCS by taking *Tetracentron* as outgroup to direct the haplotype network. The paper shows that Japan's populations were clearly distinguished from those of the Ryukyus and Taiwan, with Japan's being more primitive. Nine substitutions were found between *Tetracentron* and Japan's population of *Trochodendron*. Based on fossil evidence, taking 50 My (million years ago) as divergence time between *Tetracentron* and *Trochodendron*, the date of separation between Japan and Taiwan's populations can be dated to 5.5 My, and the standard error is ± 2.8 My. Given a likely temperate origin and no extant or fossil plants discovered in eastern China, the migratory route that led *Trochodendron* to move into Taiwan was likely from Japan via the Ryukyus. However, the ancient populations in the Ryukyus should have vanished at least once as the Ryukyus were submerged around 1.0 My. As a result, the populations now in the Ryukyu Islands were derived from Taiwan, which is exemplified by the shared haplotype of cpDNA between Taiwan and the Ryukyus and less heterozygosity in the Ryukyus compared to Taiwan based on allozyme data.

Keywords: Migration; petG-trnP; Taiwan; *Trochodendron aralioides*.

INTRODUCTION

Historical biogeography is the study of taxa in space and time including their origin, migration, and diversification (Myers and Giller, 1988). Explaining how geological events and/or fluctuations in climate have shaped the distribution of extant taxa is one of the main aims of historical biogeography. However, when the hypotheses of historical events are uncertain, the distribution pattern of the genetic polymorphisms of the focused taxa may provide another line of evidence with which to test their soundness.

Taiwan is a continental island adjacent to mainland China, the Ryukyus, and Japan. Migration of plants through oceanic barriers to Taiwan may be due to former landbridges or to long distance dispersal. Landbridges are the direct connection between landmasses that make the migration of taxa possible. Three hypotheses on landbridges between Taiwan, the Ryukyus and Japan since the late Miocene have been proposed (Ota, 1998). They can be summarized as follows: Hypothesis I, proposed by Kizaki and Oshiro (1977), postulated that a landbridge connecting southeastern China, Taiwan, and the Ryukyus was formed in the early Pleistocene about 1.5 My (million years ago) and might have lasted to the middle Pleistocene about 1 My. Hypothesis II, developed by Ujiie and his colleague (Ujiie, 1990; Ujiie et al., 1991; Ujiie and Nakamur, 1996), postulated (1) that one landbridge connected the Asian continent, Taiwan, the central and northern Ryukyus, and Japan in the late Miocene and that the connection between Taiwan, the Ryukyus, and Japan was broken during the Pliocene; (2) that another landbridge connected southeastern China, Taiwan, and the Ryukyus (excluding the islands of southern Ryukyus) during the late Pleistocene about 20000 years ago; (3) that another landbridge might have connected Taiwan and the islands of the southern Ryukyus about 3800 years ago. Hypothesis III was proposed by Kimura (2000). It postulated (1) that one large landbridge connected the Asian continent, Taiwan, the Ryukyus, and Japan during the period of 1.6-1.3 My; (2) that the other landbridge connected southeastern China and Taiwan, and extended in one direction to eastern China and in the other direction to the Ryukyus during the period of 1.3-1.0 My; (3) that the other landbridge connected the Asian continent, Taiwan, and the Ryukyus and might have extended to Japan about 0.2 My. After 0.2 My, only the southern Ryukyus connected with Taiwan.

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In summary, Taiwan might have been connected with the Asian continent several times in different geological periods. The landbridges connecting Taiwan and Japan through the Ryukyus were proposed in three different time periods. (1) Ujiie and his colleague think they occurred from the late Miocene perhaps to the early Pliocene, about 5 My. (2) Kimura believes they occurred in the Pleistocene about 1.6-1.3. (3) It occurred about 0.2 My proposed by Kimura. The landbridges connecting Taiwan and the Ryukyus were proposed in four different time periods. (1) Kizaki and Oshiro placed it in the Pleistocene 1.5-1.0 My, and Kimura 1.6-1.0 My. (2) It occurred during 0.2-0.04 My proposed by Kimura, but this landbridge only connected Taiwan and the southern Ryukyus. (3) It occurred about 0.02 My proposed by Ujiie and his colleagues, but this landbridge excluded the southern Ryukyus. (4) It occurred about 3800 years ago proposed by Ujiie and his colleague, but this landbridge only connected Taiwan and the southern Ryukyus.

A calibration of divergence time may be performed from a molecular substitution rate because genetic mutation accumulates over time (Li, 1997). Thus, molecular phylogeny provides us information about the timing of historical events and reduces our reliance on the timing of geological events and/or fossil records. However, molecular phylogeny provides only weak information about taxa in space, as extinction events are not reflected in the gene tree with extant taxa. As a result, to reconstruct a historical biogeography of a certain taxon requires the incorporation of fossils and gene trees (Manchester and Tiffney, 2001; Tiffney and Manchester, 2001). Fortunately, fossil records related to *Trochodendron* have been reviewed recently (Pigg et al., 2001). Thus, integrating fossil data and gene tree information to better understand the phytogeography of *Trochodendron* is now possible.

Trochodendron is characterized by a tree habit with ves-selless wood, alternate leaves in a 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). Phylogenetically, it is closely 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 grouped as the family Trochodendraceae or as separate families and were considered as primitive in Hamamelidae (Lu et al., 1993). *Trochodendron aralioides* is the only extant member of *Trochodendron* and is distributed in Japan, the Ryukyus, and Taiwan (Wu et al., 2001). Allozyme data suggest that Japan's populations are differentiated from those in the southern Ryukyus and Taiwan (Wu et al., 2001), as indicated by cpDNA data (Huang et al., 2004). Huang et al. (2004) investigated the genetic structure of all sampled populations and inferred a possible refuge in the north-central part of the west of the Central Mountain

Range in Taiwan during the last glaciation.

In this study, we used *Tetracentron* as outgroup to direct the gene genealogy of *Trochodendron aralioides* and infer the migratory events involving it in Taiwan and its adjacent areas by integrating the data of fossil records and the genetic information of allozymes and cpDNA.

MATERIALS AND METHODS

Sampling

A total of 24 populations were sampled, including 20 from Taiwan and two each from the Ryukyus and Japan as described before (Huang et al., 2004: Table 1), and each population was represented by four individuals at least 50 meters apart. An outgroup, *Tetracentron sinense*, was collected from one tree in the botanical garden of the Kunming Institute of Botany, Chinese Academy of Science, which originated from Kaoligongshan, Yunnan. Fresh leaves were collected from each individual tree, and they were either desiccated with silica gel and stored in a freezer (-30°C) permanently after complete dryness, or they were stored in the freezer (-70°C) directly.

DNA sequencing

The DNAs were extracted from the sample leaves using the protocol of Murray and Thompson (1980). The DNA extracting solution was then used to amplify the markers for detecting the variation by polymerase chain reaction (PCR). One marker was used in this study, i.e. the intergenic spacer of *pet*G-*trn*P. The primers for *pet*G-*trn*P were 5´-GGT CTA ATT CCT ATA ACT TTG GC-3´ forward and 5´-GGG ATG TGG CGC AGC TTG G-3´ in reverse. The initial denaturing temperature was 95°C for 3 min and then 30 s for each thermal cycle. Thirty four thermal cycles were given for amplification with the annealing temperature of 55°C for 30 s, and the extension temperature of 72°C for 45 s. The last extension time was set for 10 min after the completion of 34 thermal cycles. 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 sequence of *Tetracentron sinense* (GenBank accession number AY835400) was aligned with 95 sequences of *Trochodendron aralioides* (GenBank accession number, AY294754-AY294848) by eye. The computer program TCS (Templeton et al., 1992) was used to reconstruct the gene genealogy by taking gaps as missing data.

Fossil records of *Trochodendron*

A possible distribution pattern in the past and divergent history of *Trochodendron* was described by reviewing the papers of Jin and Shang (1998), Manchester (1999), and Pigg et al. (2001).

Molecular dating

Tests of the existence of a constant substitution rate have been developed, such as the relative rate test (Sarich and Wilson, 1973) and likelihood ratio test (Goldman and Yang, 1994) although estimation of constant rate has been relaxed for different taxa (ref. Near and Sanderson, 2004). These tests either require three taxa or a phylogenetic tree for testing or assessing. In this paper, two taxa, *Trochodendron* and *Tetracentron*, were dealt with so that a test for the existence of a local molecular clock is out of question. In consequence, such a clock was assumed to exist in the *Tetracentron* and *Trochodendron* group. The equation K=2RT can then be applied, where K is the predicted average substitution per site between two homologous sequences, R is the substitution rate, and T is the divergence time (Li, 1997). Using the simplest substitution model (Jukes and 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 base pairs (Li, 1997). In practice, D is represented by N/L, where N is the observed number of substitution, and L is the total length of base pairs. The substitution rate R can be calculated by applying known D and T. D is obtained through observation, and T may be estimated from fossil records. Once R is estimated, the divergence time between any two isolated groups can be calculated when the D between them is observed.

RESULTS AND DISCUSSION

Gene genealogy of *Trochodendron*

A total of 482 base pairs and 96 sequences between genes *pet*G and *trn*P were aligned for *Trochodendron* and *Tetracentron*. Thirteen variable sites were detected (Table 1). A cpDNA gene genealogy was reconstructed (Figure 1). There are nine substitutions between *Tetracentron*, denoted O in Figure 1, and the closest *Trochodendron* haplotype of Aishu, Japan (denoted H in Figure 1). From haplotype H, haplotype I in Japan and haplotype A in Taiwan and the Ryukyus are derived. From haplotype A,

haplotypes C and D in Taiwan are derived.

Fossil records of *Trochodendron*

Fossils related to *Trochodendron*, namely *Nordenskioldia* Heer and *Trochodendroides* E.W. Berry, can be traced back to the late Cretaceous in the higher latitudes in the Northern Hemisphere, including North America, Siberia, Japan and northeastern China (Lu et al., 1993). *Nordenskioldia* became widespread in the Paleocene in higher latitudes in North America, Europe, and Asia (Manchester, 1999; Pigg et al., 2001). The immediate ancestor of *Trochodendron*, the fruit of which is similar to extant
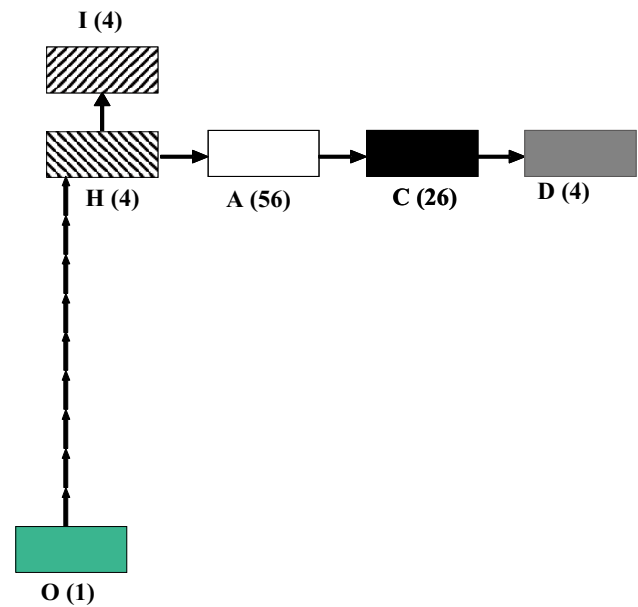

Figure 1. The gene genealogy of *Trochodendron aralioides* (A, C, D, H, I) and *Tetracentron sinensis* (O) based on a sequence between genes *pet*G and *trn*P of chloroplast pDNA reconstructed by TCS, Version 1.03 by taking gaps as missing data. Sampling individuals are in parentheses. Haplotypes H and I are restricted to Japan; haplotype A includes populations from Taiwan and the Ryukyus; and haplotypes C and D are restricted to central-north Taiwan. O is the outgroup. One arrow represents one substitution.

Table 1. Haplotypes of *Trochodendron* and *Teracentron*, based on variation of sequence between genes *pet*G and *trn*P of chloroplast DNA and their distribution.

Haplotype	Variable site 0000122222333 0669756779059 3384258292082	Distribution
O (*Tetracentron*)	TCCCGTTTTTGGG	Yunnan (China)
A (*Trochodendron*)	CATTGCTGGCATA	Taiwan, the Ryukyus
C (*Trochodendron*)	CATTGCCGGCATA	Taiwan
D (*Trochodendron*)	CATTGCCGGCATA	Taiwan
H (*Trochodendron*)	CATTGTTGGCATA	Aishu (Japan)
I (*Trochodendron*)	CATTCTTGGCATA	Chomonkyo (Japan)

Trochodendron and the leaves of which are intermediate between *Trochodendron* and *Tetracentron* (Pigg et al., 2001), appeared in the early Middle Eocene (49-50 My) in western North America. Plants of *Trochodendron* were also recorded in the Oligocene in Liaoning, northeastern China (Jin and Shang, 1998), and in the Miocene in western North America, Kamchatka, and Japan (Manchester, 1999). Manchester (1999) suggested that the migration of this genus might have occurred through Beringia in an intercontinental exchange of flora because the occurrence of the fossil *Trochodendron* in Europe has been excluded (Pigg et al., 2001).

Molecular dating: colonization of Taiwan's *Trochodendron* population

The fossil pollen records of *Trochodendron* in Taiwan were found from the peat of the Quaternary between 38000 and 4500 years before the present (BP) (Chung and Huang, 1972a, b). Since the *Trochodendron* pollen was recovered near the bottom of the peat, it was estimated to have inhabited northern Taiwan for at least 30000 years. This suggestion seems too recent to give any insight into the migratory history of *Trochodendron*. Instead, a molecular dating is used to estimate the possible timing of *Trochodendron's* first move into Taiwan. Because the direct ancestor of *Trochodendron*, the leaves of which were intermediate between *Trochodendron* and *Tetracentron*, may be traced to the early Middle Eocene about 49-50 My (Pigg et al.), we may use 50 My as the divergence time between *Trochodendron* and *Tetracentron*. Nine substitutions between *Tetracentron* and Japan's *Trochodendron* and one substitution between Japan and Taiwan's populations were detected based on *pet*G-*trn*P cpDNA (Figure 1). The divergence time between Taiwan and Japan, thus, would be about 5.5 My ($\doteqdot 50*10^6*\ln(1-4*(1/482)/3))/\ln(1-4*(9/482)/3))$, and the standard error would be ± 2.8 My ($\doteqdot 50*10^6*(((1/482)*(1-1/482)/(482*(1-4*(1/482)/3)^2))/((-3/4)*\ln(1-4*(9/482)/3))$. The average of this dating is consistent with the landbridge connecting the Asian continents and Japan, the Ryukyus and Taiwan during the late Miocene and Pliocene proposed by Ujiie and his colleague, and the timing corresponds to the incipient emergence of Taiwan.

How did *Trochodendron* migrate into Taiwan?

Considering that Taiwan is the southern limit of the distribution of the extant *Trochodendron*, and the fossil records were only reported in north-eastern China, Japan, Kamchaka, and North America; the populations of *Trochodendron* in Taiwan should have migrated from the north instead of from south-western China as suggested by Lu et al. (1993). This was proposed on the basis of the higher diversity of the extant Hamamelidae in southwestern China but was not supported by the fossil records of *Trochodendron*. There are two possible migratory routes for the temperate plants moving into Taiwan from the North: (1) from the North via continental China to Taiwan and (2) from Japan via the Ryukyus to Taiwan.

The extant species is only distributed in Japan, the Ryukyus, and Taiwan while the fossils on the Asian continent are only recorded in Liaoning and northeastern China (Jin and Shang, 1998). No fossils have ever been reported in eastern China. Thus, the first route via continental China to Taiwan seems unlikely. The likelier route is thus from Japan via the Ryukyus to Taiwan, which traces the distribution pattern of the extant species. Moreover, about 5% of the flora of Taiwan is restricted to Japan, the Ryukyus and Taiwan (Hsieh, 2002), which implies that some of these species may have experienced the same historical events as *Trochodendron*.

Ryukyu's extant populations were derivatives of Taiwan

As the migratory route of *Trochodendron* is from Japan via the Ryukyus to Taiwan, the population from the Ryukyus should be older than Taiwan's. However, the Ryukyus' haplotype of cpDNA is the same as Taiwan's and different from Japan's (Table 1; Huang et al., 2004). As mentioned by Kimura (2000), the Ryukyus were submerged around 1 My. As a result, the ancient populations in the Ryukyus were completely wiped out at least once. As the haplotype of cpDNA of Amami of the central Ryukyus is also the same as Taiwan's (Huang et al., 2004), a direct connection between them must exist. The connection between Amami and Taiwan proper existed until 25000 years ago (Kimura, 2000) during the last glaciation, or around the 20000 years ago proposed by Ujiie and his colleague. According to the allozyme data (Huang et al., 2004: Table 5), the genetic diversity in Iriomote Island of the southern Ryukyus is the smallest (H_0=0.076) in contrast with 0.133 in Erkeshan, 0.131 in Yuanyang Lake, and 0.127 in Taipingshan of northern Taiwan. The appearance of a population with higher heterozygosity is either due to being in a distribution center or being in an area to which individuals have migrated from two or more distribution centers. The case of being in a distribution center is more likely as the populations are located in northern Taiwan, where a geographical barrier occurs in the north. Higher heterozygosity in Taiwan's population suggests a colonization event from Taiwan to the Ryukyus.

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昆欄樹 (*Trochodendron aralioides*) 在台灣及其鄰近地區之遷移歷史

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　　本文利用已發表之化石及族群遺傳資料，包括同功異構酶及葉綠體 DNA 片段序列變異，來推測昆欄樹遷移到台灣及其鄰近地區之可能歷史。昆欄樹之葉綠體 DNA 變異型 (haplotype) 經由比較外群植物 — 鐵青樹 (*Tetracentron sinensis*)，來認定原始型及其衍生型，日本族群之變異型是最原始的，而且與台灣及琉球之變異型不同。分子時鐘顯示，日本與台灣之族群是在 5.5 ± 2.8 百萬年前分化出來的，這個時間也可能是昆欄樹最初遷移到台灣之時間。至於遷移路線最有可能是由日本經由琉球而移入台灣。由於近昆欄樹之化石在亞洲只有在中國東北，日本及堪察加群島發現，推測由中國大陸遷移入台灣之可能性不高。至於現生之琉球族群，推測是由台灣遷移過去的，因為琉球在一百萬年前曾沉入到海面下，故早期移入之族群隨之消失。同時，台灣及琉球之族群具有相同之葉綠體 DNA 變異型，而且同功異構酶之岐異度北台灣之族群平均比琉球的族群高一倍左右，顯示出遷移方向是由台灣移向琉球。

關鍵詞：葉綠體 DNA；親緣地理；台灣；昆欄樹。