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Chloroplast phylogeography of *Dipentodon* (Dipentodontaceae) in southwest China and northern Vietnam

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Abstract

The evolutionary history of plants in the southeast Tibetan Plateau might be the most complicated around the world because of the area's extremely complex topography and climate induced by strong tectonic activity in recent history. In this research, we implemented a phylogeographical study using chloroplast sequences (*psb*A-*trn*H and *trn*Q-*rps*16 intergenic spacer) on *Dipentodon*, a monotypic or ditypic genus (*D. sinicus* and *D. longipedicellatus*) distributed in southwest China and adjacent areas including Myanmar (Burma), northeast India and northern Vietnam. A total of 257 samples from 16 populations from the southeast Tibetan Plateau (*D. longipedicellatus*) and the Yungui Plateau (*D. sinicus*) were collected. The results revealed that *Dipentodon* had 11 haplotypes for the two intergenic spacers, high genetic diversity (h_T = 0.902) and high genetic differentiation (N_{ST} = 0.987 and G_{ST} = 0.948). AMOVA analysis showed that the component of among-population within region/species (55.25%) was unexpectedly larger than the among-species/region component (43.69%), which indicates that there is no justification for recognizing two species in *Dipentodon*. Correlation of pairwise genetic and geographical distances showed that *Dipentodon* populations in the southeast Tibetan Plateau may have suffered more habitat fragmentation than populations in the Yungui Plateau because of the uplift of the Tibetan Plateau than populations in the Yungui Plateau have. Nested clade analysis showed that 11 haplotypes formed two 3-level, three 2-level and seven 1-level clades, with eight clades showing significant geographical association. However, clade 2-1 and 2-2 did not cluster together, although they are distributed in the same region (Yungui Plateau) and belong to the same species (*D. sinicus*). This led not only to incongruence between haplotype network and geographical distribution of 2-level clades, but also to paraphyly of *D. sinicus* to *D. longipedicellatus*. We concluded that the incongruence and paraphyly may result from incomplete lineage sorting during the rapid and extreme tectonic events of the Tibetan Plateau. The results reported here will no doubt provide new insights into the evolution of biodiversity on the Tibetan Plateau and adjacent areas, and a historical framework for the conservation of biodiversity in this area, including *Dipentodon*.

Keywords: chloroplast DNA, *Dipentodon* Dunn, phylogeography, Tibetan Plateau

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Introduction

Elucidating the factors that determine genetic structure of plant populations has been of longstanding interest to population geneticists (Schaal *et al*. 1998; Avise 2000). Genetic diversity and population structure of plant species

are affected not only by their life histories and ecological traits (Hamrick *et al*. 1992) but also by historical events (Newton *et al*. 1999; Hewitt 2004). Within a historical framework, Quaternary climate oscillations may have had the most profound effects on the present genetic structure of plants (Hewitt 2000; Hewitt 2004). However, Quaternary climate oscillation is just one facet of the recent history of the earth; other historical events such as orogenesis have been proved to be equally important in shaping the genetic structure of many plants. A case in point is *Hygrophila pogonocalyx* (Acanthaceae) in Taiwan (Huang *et al*. 2005), in which strong differentiation was found between eastern and western populations of the species. This genetic structure was then attributed to physical isolation since the formation of the Central Mountain Range about 5 million years ago.

The Tibetan (Qinghai-Xizang) Plateau is the highest and youngest plateau in the world, with an average elevation of approximately 4500 m and an area of 2.5×10^6 km^2 (Zheng 1996). Although there are still disputes about the chronology of its elevation process, it is believed that the most recent uplift of the Plateau as well as its adjacent areas (e.g. the Yungui Plateau) took place 3.4 million years ago (Sun & Zheng 1998; Cheng *et al*. 2001). The current 4500-m elevation of the Tibetan Plateau was assumed to be not reached until as recently as the Quaternary (Sun & Zheng 1998). The extremely complex topography and climate were formed during the uplift, especially in the southeast part of the Tibetan Plateau, where large mountains and river systems in deep gorges occur in parallel. The significant increase in geological and ecological diversity that accompanied such an uplift promoted rapid divergence and speciation in small and isolated populations (Liu *et al*. 2006), which has been assumed to be one of the reasons for high plant diversity in this region (Axelrod *et al*. 1996). With the greatest number of endemic temperate flora in the world, many of which are endangered species, the southeast part of the Plateau and its adjacent areas has been listed as one of the world's biodiversity hotspots (Wilson 1992; http://www.biodiversityhotspots.org/xp/Hotspots; Myers *et al*. 2000). However, because of the complicated topography and limited access, this region is among the areas where biodiversity studies have been limited. Previous studies in this area mainly focused on botanical inventory and taxonomic treatment, and phylogeny-based species radiation (Sun & Zheng 1998; Su *et al*. 1999; Luo *et al*. 2004; Guo *et al*. 2005; Liu *et al*. 2006). Relatively few investigations have been made on the population divergence and phylogeography of plants in this region (Ge *et al*. 2005; Zhang *et al*. 2005).

The genus *Dipentodon* Dunn (Dipentodontaceae) has been treated as a monotypic genus by most taxonomists, comprising only *D. sinicus* Dunn (Peng *et al*. 2003). However, another species (*Dipentodon longipedicellatus* Cheng et Liu) was previously proposed based on its longer peduncles and thin leaves (Liu & Cheng 1991). Currently, this genus is locally scattered in isolated patches in southwest China and adjacent areas including Burma, northeast India and northern Vietnam (Liu & Cheng 1991; Peng *et al*. 2003). In China, its distribution range covers the southeast of Tibetan Plateau (SETP) and most parts of the Yungui Plateau (YGP) (Fig. 1). The two distribution regions correspond well to the two species previously proposed by Liu & Cheng (1991), namely, *D. longipedicellatus* in SETP and *D. sinicus* in YGP. *Dipentodon* is a semi-evergreen shrub or small tree and primarily occurs in montane habitats (from 800 to 2800 m) with a mild humid climate. It has leathery or papery leaves, yellow-green flowers and wide ellipsoid or ovoid capsules, each bearing one seed. The genus is pollinated by bees and flies, and its seeds can be ejected mechanically from capsules to a distance of several metres. Over the past 20 years, the genus has suffered rapid population declines, and thus was listed as a vulnerable species in the China Species Red List (Wang & Xie 2004). However, no information is available about the level and pattern of genetic diversity of this vulnerable genus.

Phylogeography uses genealogical and geographical information to infer the demographic and historical processes that shaped the evolution of populations and species (Avise 2000; Kuchta & Meyer 2001). As temporal and spatial dimensions are considered simultaneously, phylogeography forms the conceptual framework for studying intraspecific historical processes (Schaal *et al*. 1998; Avise 2000). Because chloroplast DNA (cpDNA) is transmitted only through seeds in most angiosperms and often shows a more highly geographical structure than the nuclear genome (Schaal *et al*. 1998; Petit *et al*. 2003), cpDNA markers have been successfully employed to detect phylogeographical patterns in numerous plant species (see reviews in Avise 2000; Hewitt 2001; Petit *et al*. 2005). In this study, we used chloroplast sequences of *psb*A-*trn*H and *trn*Q-*rps*16 intergenic spacers to examine the phylogeographical pattern of 16 populations of *Dipentodon* across the southeast Tibetan Plateau and the Yungui Plateau of China and northern Vietnam. Our specific objectives were to address the following questions: (i) How is the cpDNA variation hierarchically apportioned? Is there sufficient genetic differentiation between the two regions so that classification as two species (*D. sinicus* and *D. longipedicellatus*) is justified? (ii) Have SETP populations experienced more habitat fragmentation, thus resulting in more pronounced genetic structure, than those from the YGP region because of the complex geomorphological configuration in the southeast part of the Tibetan Plateau? (iii) What are the main historical factors that shaped the phylogeographical structure of this genus? Does the phylogeographical structure relate to recent tectonic events? Such information will not only shed light on the evolutionary history of this genus, but also facilitate understanding of the historical and ongoing evolutionary forces for maintaining the extraordinarily high biodiversity of this region.

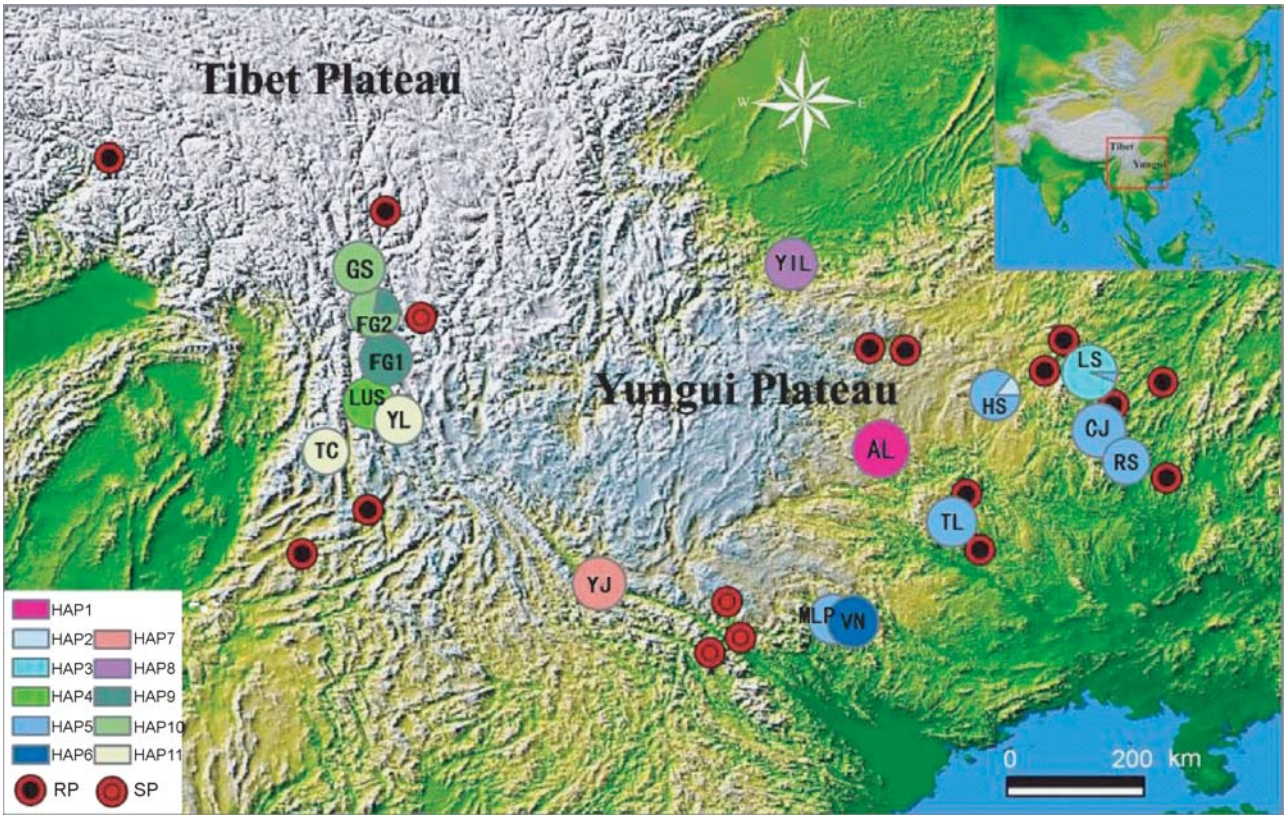

Fig. 1 The geographical distribution of *Dipentodon* in China and northern Vietnam and haplotype frequency within and among populations. Population abbreviations are the same as Table 1, RP represents recorded populations which have not been surveyed, SP represents surveyed populations which nowadays might be extinct and thus cannot be sampled. The pie sizes of sampled populations are proportional to their sample sizes.

Materials and methods

Population sampling

For this research, we conducted extensive field investigations from May 2004 to October 2005. Some populations of *Dipentodon* recorded in herbaria such as those in Weixi (voucher specimen: IBSC 612306), Jinping (KUN 759765), Pingbian (KUN 668321) and Mengzi (M.B.G. 28551), have become extinct probably because of deforestation and other human activities in recent decades (those marked as 'SP' in Fig. 1). A total of 257 *Dipentodon* individuals were collected from 16 populations covering the entire distribution of this genus in SETP and YGP. Of them, all six populations in SETP (GS, FG1, FG2, LUS, YL, TC) belong to *Dipentodon longipedicellatus*, whereas the populations in YGP belong to *Dipentodon sinicus* according to the criteria of Liu & Cheng (1991) (Fig. 1 and Table 1). Twelve to 19 individuals about 100 m apart were sampled for each population. Fresh leaves were collected from each individual and dried in silica gel.

DNA extraction, polymerase chain reaction protocol and sequencing

Genomic DNA was extracted using a modified cetyltrimethyl ammonium bromide (CTAB) protocol (Doyle & Doyle 1987). Screening for variation of cpDNA fragments used the universal primers described in Hamilton (1999) and Sang *et al*. (1997), and some primers designed from *Oryza sativa* chloroplast genome. After preliminary screening of eight fragments, we chose *psb*A-*trn*H and *trn*Q-*rps*16 intergenic spacers for the full survey because they contained the most polymorphic sites. The primers of *psb*A-*trn*H spacer were described in Sang *et al*. (1997). The primers of *trn*Q-*rps*16 spacer were originally designed according to the chloroplast genome of *O. sativa*. After successful amplification and sequencing in several *Dipentodon* individuals, two new primers were designed for *Dipentodon*. The sequences of forward and reverse primers were 5′-ATAGTCATTGGTT CGGTCGG-3′ and 5′-CGAAGGTAGCTTTGGTACTG-3′, respectively. DNA amplification was performed in a T1 thermocycler (Biometra), programmed for an initial 240 s

Table 1 Details of sample locations and sample sizes and cpDNA haplotype frequencies in 16 populations of *Dipentodon*

					cpDNA haplotype										
Population	Lat. (N)	Long. (E)	Alt. (m)	*N*	Hap1	Hap2	Hap3	Hap4	Hap5	Hap6	Hap7	Hap8	Hap9	Hap10	Hap11
YGP															
Congjiang (CJ)	25°37.123′	108°18.464′	1093	16					16						
Leishan (LS)	26°22.638′	108°11.158′	1577	17			16		1						
Huishui (HS)	26°04.525′	106°57.085′	1090	14		2			12						
Anlong (AL)	25°23.017′	105°27.299′	1761	18	18										
Malipo (MLP)	23°08.226′	104°49.040′	1907	14					14						
Yiliang (YIL)	27°48.566′	104°16.032′	1912	15								15			
Yuanjiang (YJ)	23°36.620′	101°44.562′	2295	16							16				
Rongshui (RS)	25°13.392′	108°40.172′	965	12					12						
Tianlin (TL)	24°25.429′	106°22.785′	1309	14					14						
Vietnam (VN)	23°06.527′	105°04.968′	1370	15						15					
Subtotal				151	18	2	16		69	15	16	15			
SETP															
Tengchong (TC)	25°21.252′	98°08.377′	1823	15											15
Yunlong (YL)	25°45.846′	99°06.020′	2450	17											17
Lushui (LUS)	25°58.967′	98°42.450′	2729	18				18							
Fugong1 (FG1)	26°33.045′	98°55.774′	2527	19									19		
Fugong2 (FG2)	27°09.917′	98°46.783′	2576	18									4	14	
Gongshan (GS)	27°45.045′	98°34.731′	2666	19										19	
Subtotal				106				18					23	33	32
Total				257	18	2	16	18	69	15	16	15	23	33	32

Abbreviations: Lat., latitude; Long., longitude; Alt., altitude; *N*, number of sampled individuals; YGP, Yungui Plateau; SETP, southeast Tibetan Plateau.

at 94 °C, followed by 30 cycles of 45 s at 94 °C, 30 s at 58 °C (*psb*A-*trn*H) or 54 °C (*trn*Q-*rps*16), 90 s at 72 °C, and a final 4 min at 72 °C. Reactions were carried out in a volume of 20 μL containing 2.0 mM/L $MgCl_2$, 0.5 μM/L dNTP, 10× buffer, 2.5 μM/L primer, 1 U *Taq* DNA and 20 ng DNA template.

Sequencing reactions were conducted with the forward or reverse primers of the amplification reactions using the DYEnamic ET Terminator Kit (Amersham Pharmacia Biotech), following the manufacturer's protocol. Sequencing was performed on a MegaBACE 1000 automatic DNA sequencer (Amersham Pharmacia Biotech) after the reaction product was purified through precipitation with 95% ethanol and 3-sodium acetate (pH 5.2).

Data analysis

Sequences were aligned using CLUSTAL_X version 1.81 (Thompson *et al*. 1997), and all indels were coded as substitutions following Caicedo & Schaal (2004). All individuals were characterized for cpDNA haplotype and haplotype distribution was plotted on a relief map of southwest China and northern Vietnam using ARCMAP 8.3 (ESRI, Inc.). We calculated within-population diversity (h_S), total diversity (h_T) and level of population differentiation (G_{ST}) at species and regional levels. To incorporate the relationships between haplotypes, an estimate of population subdivision for phylogenetically ordered alleles (N_{ST}) was obtained, and the test statistic U, comparing the values of N_{ST} and G_{ST}, was calculated. A higher N_{ST} than G_{ST} usually indicates the presence of phylogeographical structure (Pons & Petit 1996). All aforementioned parameters were calculated using the program HAPLONST, which is available at www.pierroton.inra.fr/genetics/labo/Software/. To estimate the distinction between the two putative species in *Dipentodon*, genetic structure was also evaluated by AMOVA analysis, partitioning the genetic diversity into three levels: among putative species/regions (*D. longipedicellatus*/SETP and *D. sinicus*/YGP), among-population within regions and within-population.

Because of the complex topography in SETP, populations in this region are expected to be more isolated from each other, showing stronger genetic drift and less gene flow, thus resulting in a more pronounced genetic structure. However, traditional F_{ST} (or G_{ST}) estimates cannot distinguish different genetic structures with similar F_{ST} values because F_{ST} is a compound product of gene flow and drift (Hutchison & Templeton 1999). Hutchison & Templeton (1999) proposed a method to evaluate the relative historical influences of gene flow and drift on regional population structure by constructing regional scatterplots of F_{ST} on geographical distances and calculating the correlation coefficients describing the relationship between them. This kind of analysis enables the consideration of various historical (such as crustal uplift in this study) and ecological factors characteristic of a region. It also allows the comparison of patterns between regions to determine how gene flow and drift have influenced populations within one region relative to another. Following this method, F_{ST} measures were calculated between all pairwise populations within SETP and YGP using DNASP 4.00 (Rozas *et al*. 2003), respectively, as were the geographical distances (GEODIS 2.4, Posada *et al*. 2000). These data were used to construct regional scatterplots of F_{ST} on geographical distances and to calculate the correlation coefficients describing the relationship between them with a Mantel test implemented by IBDWS (Jensen *et al*. 2005).

A nested clade procedure was implemented to assess geographical associations of haplotypes and infer the phylogeographical pattern of *Dipentodon*. We estimated intraspecific relationships using TCS version 1.13 (Templeton *et al*. 1992; Clement *et al*. 2000). This method uses coalescence theory (Hudson 1990) to determine the limits of parsimony, and maximum parsimony to define a set of plausible connections among haplotypes that have a cumulative probability of > 95% of being true (Templeton *et al*. 1992). Haplotypes were then organized into a system of nested clades where a higher nesting level corresponds to longer evolutionary time (Templeton *et al*. 1992).

In the nested clade analysis, we defined nested sets of haplotypes for geographical analysis according to standard rules (Templeton *et al*. 1987). When ambiguities (closed loops or 'stranded' clades) occurred in the networks, they were resolved using published rules and predictions based on coalescence theory (Crandall & Templeton 1993; Templeton & Sing 1993). Geographical association between haplotypes was first assessed using the nested contingency test described in Templeton & Sing (1993) by permuting clade types within a nested category against sampling locations (considered as categorical variables). Using the geographical coordinates of each population, two main statistics were calculated, the clade distance (D_c), which measures the geographical spread of a clade, and the nested clade distance (D_n), which measures how a clade is geographically distributed relative to other clades in the same higher-level nesting category. Two interior-tip statistics [(I–T) D_c and (I–T) (D_n)] were also estimated within each nested category as the average interior distance minus the average tip distance. The significance of these statistics was estimated through a Monte Carlo procedure. Null distributions were constructed by randomizing the contingency data table for each clade and nesting level and estimating again the test statistics for each randomized data set. The inference key given by Templeton (2004) was then used to infer recurrent and historical events from patterns of statistically significant distance measures.

To relate the divergence of the clades defined by nested clade analysis to the tectonic events of the Tibet Plateau and adjacent areas, we estimated the divergence times between high-level clades. Net pairwise divergence per base pair

Table 2 Variable sites of the aligned sequences of two chloroplast DNA fragments in 11 haplotypes of *Dipentodon* (* and † denote two indels)

	*psb*A-*trn*H						*rps*16-*trn*Q														
	2	4	6	1	2	3	1	1	1	2	3	3	5	7	7	8	9	9	9	1	1
	0	5	5	4	8	8	0	3	4	8	9	9	4	2	8	5	4	7	8	0	0
				9	4	7		5	0	4	5	6	3	5	1	6	1	2	2	1	9
Nucleotide position																				0	3
Hap 1	T	A	G	T	C	C	T	T	G	T	T	T	—	C	—	C	A	G	G	A	T
Hap 2	T	A	C	A	A	C	—	C	T	G	—	—	—	T	—	C	C	T	A	A	G
Hap 3	T	A	C	T	A	C	—	C	T	G	—	—	—	T	—	C	A	T	A	A	G
Hap 4	T	G	C	A	C	C	—	T	G	T	—	—	*	C	†	T	A	T	G	T	T
Hap 5	T	A	C	A	A	C	—	C	T	G	—	—	—	T	—	C	A	T	A	A	G
Hap 6	T	A	C	A	A	C	—	C	T	G	T	—	—	T	—	C	A	T	A	A	G
Hap 7	T	A	C	T	C	C	T	T	G	T	T	—	—	C	—	C	A	G	G	A	T
Hap 8	T	A	C	T	C	A	T	T	G	T	T	T	—	C	—	C	A	G	G	A	T
Hap 9	G	A	C	A	C	C	—	T	G	T	T	T	—	C	—	C	A	T	G	A	T
Hap 10	T	A	C	A	C	C	—	T	G	T	T	—	—	C	—	C	A	T	G	T	T
Hap 11	T	A	C	A	C	C	—	T	G	T	—	—	—	C	†	T	A	T	G	T	T

*CTATGTTAAATATTTAACATTTAAG; †TTCCAGTTCACATAGAT.

(d_A), which is proportional to time since divergence (T) of two clades assuming homogeneity of mutation rates across lineages, was calculated using MEGA3 (Kumar *et al*. 2004) under the Kimura-2 model. Divergence time was calculated as $T = d_A/2\mu$, where μ is the rate of nucleotide substitution (Nei & Kumar 2000). An appropriate rate had not been calibrated for the chloroplast substitutions of *Dipentodon*, so we took 1.01×10^{-9} substitutions per site per year for synonymous sites of cpDNA in seed plants (Graur & Li 1999) as the approximate evolutionary rates of *psb*A-*trn*H and *trn*Q-*rps*16 intergenic spacers (Chiang *et al*. 2006) for estimating divergence time.

Results

Sequence characteristics and haplotype distribution

The aligned sequences of *psb*A-*trn*H spacer were 442 bp in length, and no length polymorphism was observed. Nucleotides A and T were rich in these sequences, with A/T contents of 72.63–72.85%, in accordance with the nucleotide composition of most noncoding chloroplast regions (Li 1997). Nucleotide substitutions occurred at six sites resulting in eight haplotypes (Table 2). The length of aligned sequences of *trn*Q-*rps*16 spacer was 1116 bp, with the sizes ranging from 1071 bp to 1113 bp because of the deletions at sites 10, 395–396, 543–567 and 781–797, respectively (Table 2). Nucleotide composition of A and T ranged from 70.68% to 71.07%, similar to that of *psb*A-*trn*H. Indels along with 10 substitutions constituted nine haplotypes (Table 2). The sequences of eight *psb*A-*trn*H and nine *trn*Q-*rps*16 haplotypes have been deposited in GenBank databases under accession nos DQ450989–DQ450996 and DQ450997–DQ451005, respectively.

A total of 11 haplotypes (hap1–hap11) were identified when *psb*A-*trn*H and *trn*Q-*rps*16 sequences were combined (Table 2). Haplotype frequencies in each population and geographical distribution are presented in Table 1 and Fig. 1. The geographical distribution of haplotypes was highly structured in SETP: population LUS was fixed for unique haplotype (hap4), populations TC and YL were both fixed for a different haplotype (hap11), and FG2 shared hap9 with GS and hap10 with FG1, respectively. Populations (YJ, YIL and AL) in the west part of YGP were also highly structured with each population possessing one unique haplotype. However, the populations of the southeastern edge of YGP were much less structured, with hap5 common in most populations (i.e. MLP, TL, HS, RS, CJ and LS) except for VN. In addition, no haplotypes were shared between SETP and YGP.

Genetic diversity and genetic structure

Population subdivision of all 16 populations was very high ($G_{ST} = 0.948$, $h_S = 0.047$, $h_T = 0.902$). When taking into account the relationships between haplotypes, the genetic

structure was even higher (N_{ST} = 0.987, v_S = 0.011, v_T = 0.905, where v_S and v_T represent within-population and total genetic diversity of ordered alleles, respectively). The *U*-test showed that N_{ST} was significantly larger than G_{ST} (U = 1.79, P < 0.01). AMOVA analysis showed that a large portion of chloroplast variation in *Dipentodon* occurred between species/regions (43.69%, P < 0.0001). However, the component of among-population within regions (55.25%, P < 0.0001) was unexpectedly larger than the among-species/region component. The results of AMOVA analysis were consistent with the value of within-population diversity (h_S) that only 1.06% of the total variation resided within populations. The genetic differentiation within the two areas, SETP (G_{ST} = 0.930, h_S = 0.061, h_T = 0.867; N_{ST} = 0.949, v_S = 0.044, v_T = 0.870) and YGP (G_{ST} = 0.951, h_S = 0.038, h_T = 0.784; N_{ST} = 0.994, v_S = 0.005, v_T = 0.788), was similarly high.

Correlation of pairwise genetic and geographical distances

The scatterplot for YGP region (Fig. 2a) shows a positive and linear relationship across all the pairwise F_{ST} values and geographical distances separating the populations of the region. The correlation coefficient obtained from the respective matrices indicated this positive association was marginally significant (r = 0.3110, $P \leq 0.0486$). The relationship between genetic and geographical distances for YGP region was consistent with the Case I proposed by Hutchison & Templeton (1999), which describes a scenario of regional equilibrium between gene flow and drift, that is isolation by distance. In contrast, although the scatterplot of the pairwise F_{ST} values and geographical distances for SETP region (Fig. 2b) also showed a positive and linear relationship, the association between them was insignificant (r = 0.2832, $P \leq 0.1525$), which indicated that populations in SETP lack regional equilibrium. If taking the large F_{ST} values between most pairwise populations in this region into consideration, populations in SETP fit well with Case III of Hutchison & Templeton (1999), a scenario where drift is much more influential than gene flow.

Intraspecific cladogram and phylogeographical inferences

A nested cladogram was constructed for cpDNA haplotypes (Fig. 3) using a TCS network by linking the haplotypes in a hierarchical manner. Seven 1-step, three 2-step and two 3-step clades were unveiled, with eight clades that could be subjected to the geographical association test. No alternative connections between haplotypes ('loops') were observed, which indicates that no homoplasy is involved in the network. Clade 1-5 was the only observed haplotype symmetrically stranded and then was grouped with the nesting category (clade 2-3) that had the smallest sample size (Templeton & Sing 1993). Clade 3-1 and clade 3-2 were connected by eight mutations suggesting their long independent evolutionary

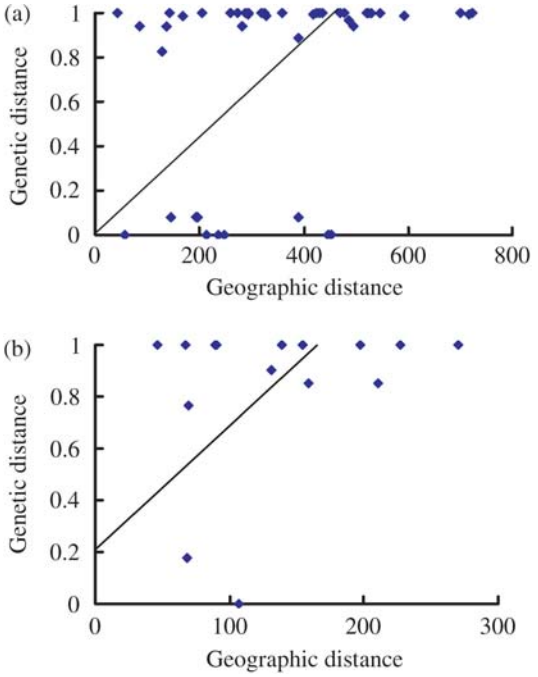

Fig. 2 Scatterplots of F_{ST} estimates against geographical distances (kilometres) separating each pairwise combination of populations within YGP (a) and SETP (b).

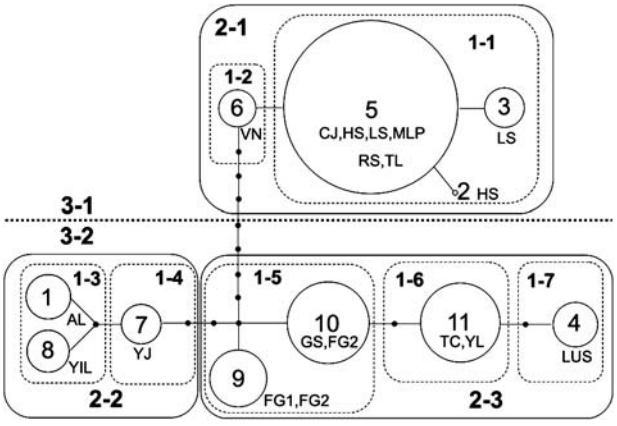

Fig. 3 Nested cladogram of 11 chloroplast haplotypes in *Dipentodon*. Circles with numbers denote haplotypes. Dots represent putative haplotypes. Each branch represents one mutation. The distribution of a certain haplotype is marked around/in the circles. The size of each circle is proportional to the haplotype frequency.

histories. Note that the two 2-step clades of YGP, 2-1 and 2-2, were not clustered together, but were the most distant 2-step clades in terms of genetic distance.

Nested contingency analysis recovered a strong overall association between clades and their geographical distributions (Table 3). All clades that could be subjected to the test

Table 3 Nested contingency analysis of geographical associations and phylogeographical inferences made from a nested haplotype analysis of *Dipentodon*. Numbers in parentheses indicate choice made in the dichotomous key given in Templeton (2004)

Clade	Permutational chi-squared statistic	Probability	Clade key	Inferences
Clade 1-1	90.96	0.0000	(1,2,3,4) No	Restricted gene flow with isolation by distance
Clade 1-3	33.00	0.0000	(2,19) No	Allopatric fragmentation
Clade 1-5	43.14	0.0000	(1,2,3,4) No	Restricted gene flow with isolation by distance
Clade 2-1	1.00	0.0000	(1,2,11) Yes	Range expansion
Clade 2-2	49.00	0.0000	(2,19) No	Allopatric fragmentation
Clade 2-3	212.00	0.0000	(2,19) No	Allopatric fragmentation
Clade 3-2	1.00	0.0000	(2,19) No	Allopatric fragmentation
Total cladogram	1.0000	0.0000	(1,2,11,17,4) No	Allopatric fragmentation

Table 4 Results of nested clades and geographical distance analysis for *Dipentodon* based on the nested design of Fig. 2. For each clade and interior-tip clade comparison (I-T), the clade distance (D_c), and nested clade distance (D_n) are reported. Significantly small (S) or large (L) values are indicated for each clade and I-T status. Tip (T) or interior (I) clades (haplotypes) are also indicated

Haplotype	D_c	D_n	1-step clade	D_c	D_n	2-step clade	D_c	D_n	3-step clade	D_c	D_n
Hap2 (T)	0	107.03^S	1-1 (T)	164.79^S	175.49^S	2-1 (T)	—	—	3-1 (T)	188.59^S	419.44
Hap3 (T)	0^S	167.85									
Hap5 (I)	168.20	165.89									
I-T	168.20^L	4.79									
Hap6 (I)	—	—	1-2 (I)	0^S	274.57^L						
			I-T	164.79^S	99.08^L						
Hap1 (T)	0^S	147.21^L	1-3 (T)	147.09^S	207.41^S	2-2 (I)	239.92	416.07^L	3-2 (T)	267.40^S	398.75^S
Hap8 (T)	0^S	146.96^S									
Hap7 (I)	—	—	1-4 (I)	0^S	304.95^L						
			I-T	–147.09^S	97.53^L						
Hap9 (T)	20.78^S	57.14^L	1-5 (I)	46.78^S	85.41	2-3 (T)	86.94^S	193.06^S			
Hap10 (I)	33.81^S	39.65^S									
I-T	13.03	–17.50^S									
Hap11 (I)	—	—	1-6 (I)	53.25^S	107.92^L						
Hap4 (T)	—	—	1-7 (T)	0^S	49.58^S						
			I-T	49.13^L	44.01^L	I-T	–152.98^S	–223.01^S			

of geographical association showed significant geographical association. Distance measures and probability values for clades further showed that a scenario of allopatric fragmentation could describe most clades and the total cladogram of *Dipentodon* (Table 4), except for clades 1-1, 1-5 and 2-1. For clades 1-1 and 1-5, isolation by distance was recovered by distance analyses, which could be detected by visual inspection of the haplotype map. Range expansion from northern Vietnam along the southeastern edge of Yungui Plateau was detected for clade 2-1.

Divergence time between the high-level clades of Dipentodon

Estimation of divergence time only used substitutions in *psb*A-*trn*H and *trn*Q-*rps*16 intergenic spacers. Therefore, between the two 3-level clades (3-1 and 3-2), the genetic distance (d_A) was 0.006, which indicated that clades 3-1 and 3-2 separated approximately 2.97 million years ago. The genetic distances (d_A) between three pairs of 2-level clades were 0.006 (2-1 vs. 2-2), 0.003 (2-2 vs. 2-3), 0.006 (2-1 vs. 2-3),

which suggested that divergence between these clades occurred over approximately 2.97, 1.48 and 2.97 million years ago, respectively.

Discussion

Two species (Dipentodon sinicus *and* Dipentodon longipedicellatus) *are not justified in the genus* Dipentodon

Given the patterns of morphological variation described by Liu & Cheng (1991), one would suppose that genetic variation between the two species (*Dipentodon sinicus* and *Dipentodon longipedicellatus*) would be much higher than that within the two species. However, AMOVA analysis indicated that the opposite was true, with the among-population variance (55.25%) within the two species being higher than their among-species component (43.69%). This suggests that the classification proposed by Liu & Cheng (1991) is not justified by our results. Furthermore, nested clade analysis showed that clades of the same species (*D. sinicus*) did not form a monophyletic clade, with clade 2-2 of *D. sinicus* in west YGP (YIL, AL and YJ) clustering with clade 2–3 of *D. longipedicellatus* in SETP, rather than with clade 2-1 of *D. sinicus* in southeast YGP, that is *D. sinicus* is paraphyletic to *D. longipedicellatus*. Therefore, the most likely explanation to the phenotypic similarities between west and southeast YGP *Dipentodon* populations may be the retention of ancestral polymorphisms in the paraphyletic group due to incomplete lineage sorting (Avise 2004). Furthermore, the similarities may also be ascribed to pollen-mediated gene flow among geographically proximate populations in YGP and/or phenotypic convergence under similar selection schemes in the same region. Because of the reasoning above, all 16 populations analysed are hereafter referred to as *D. sinicus*, and thus without considering any further intrageneric taxonomic subdivision.

Genetic diversity and genetic structure of D. sinicus

The mean value of cpDNA diversity (h_T) detected by various cpDNA markers is 0.67 in 170 plant species compiled by Petit *et al.* (2005). The cpDNA diversity in *D. sinicus* ($h_T = 0.902$) was high relative to those plants. This result is consistent with the high morphological variation within the species (Liu & Cheng 1991). The high cpDNA diversity of *D. sinicus* may be due to the long evolutionary history of this archaic monotypic genus (Thorne 1999). The long evolutionary history allowed this species to accumulate mutations (Chiang & Schaal 1999; Huang *et al*. 2001). Furthermore, the highly diverse habitats of *D. sinicus* caused by rapid and extreme orogenesis since the late Pliocene (Sun & Zheng 1998) might have created a wide spectrum of habitats to accommodate new mutations.

Although a high level of cpDNA diversity was detected at the species level, very low diversity was uncovered within populations ($h_S = 0.047$). Therefore, the population differentiation within *D. sinicus* is very high ($N_{ST} = 0.987$ and $G_{ST} = 0.948$), placing it among the plant species with the highest cpDNA differentiation (Petit *et al*. 2005). The haplotype distribution, AMOVA analysis and phylogeographical inferences also showed that cpDNA variation in *D. sinicus* was highly structured. Two factors are the most likely reasons for the high population subdivision within *D. sinicus.* First and foremost, an inefficient seed dispersal mechanism may account for most population differentiation. This species disperses its seeds by ejecting them from capsules when fruits ripen, probably confining seed flow mainly within populations. Petit *et al*. (2003) demonstrated that seed dispersal mechanism plays an important role in shaping the plant genetic structure of maternally inherited cpDNA. Generally, genetic differentiation is positively related to the dispersal ability of seeds. The other factor may be related to the rapid and extreme orogenesis since the late Pliocene (Sun & Zheng 1998). Isolation caused by the orogenesis and subsequent genetic drift, together with adaptation to local environments could lead to genetic differentiation and further speciation (Rieseberg *et al*. 2003; references therein). This issue will be fully discussed in the following section.

Influence of recent tectonic events on genetic and phylogeographical structure of D. sinicus

As the richest temperate flora in the world with a high number of endemic and endangered species, the southeast part of the Tibetan Plateau and its adjacent areas has been listed as one of the world's biodiversity hotspots (Wilson 1992; www.biodiversityhotspots.org/xp/Hotspots; Myers *et al*. 2000). After a molecular phylogenetic survey of more than 200 Tibetan Plateau-endemic species of the *Ligularia–Cremanthodium–Parasenecio* complex of the Tussilagininae (Asteraceae: Senecioneae), Liu *et al*. (2006) hypothesized that rapid divergence and speciation, promoted by significant increases in geological and ecological diversity that accompanied the uplift of the Tibetan Plateau, could be one of the reasons for high plant species diversity in this region (Axelrod *et al*. 1996). The distribution pattern of *Dipentodon* (SETP vs. YGP) provides an excellent model to test this hypothesis at intraspecific level, because the much less complex geomorphological configuration of the Yungui Plateau can act as a control, although the Plateau itself was also deeply affected by the uplift of the Tibetan Plateau (Cheng *et al*. 2001). Consistent with the hypothesis of Liu *et al*. (2006), the results of this study showed that populations of the extremely uplifted region, SETP, contained more haplotype diversity (0.867) than those (0.784) of the much less uplifted region, YGP. Also, populations in SETP displayed very similar genetic differentiation ($G_{ST} = 0.930$)

to those in YGP (G_{ST} = 0.951), although YGP covers a much larger geographical area than SETP does.

Furthermore, correlation of pairwise genetic and geographical distances in YGP showed that populations of YGP exhibited regional equilibrium between gene flow and drift. This indicates that *Dipentodon* populations may have existed in YGP region for a long enough period of time with relatively stable conditions for localized gene flow to have interacted with drift to produce a pattern of isolation by distance across the region (i.e. regional equilibrium). On the contrary, *Dipentodon* populations in SETP may have suffered more habitat fragmentation due to the rapid and extreme uplift of the Tibetan Plateau, because a scenario of genetic drift being more influential than gene flow was revealed in this region. It is well known that genetic drift is a driving force for genetic differentiation and even for speciation, especially in isolated and small populations (Barton & Charlesworth 1984), thereby promoting biodiversity in the regions of concern, such as the southeast part of the Tibetan Plateau of this study.

Rapid and extreme uplift of the Tibetan Plateau and adjacent areas not only influenced the genetic structure of *D. sinicus*, but also is the possible reason for an interesting phylogeographical pattern. Specifically, the haplotype network did not reflect the geographical distribution of 2-step clades, as geographically proximate clades 2-1 and 2-2 were the most strongly differentiated, and the westernmost clade 2-3 was interior to clades 2-1 and 2-2 (Fig. 3). As mentioned above, the paraphyletic status of *D. sinicus* to *D. longipedicellatus* may have resulted from incomplete lineage sorting. The lack of association between the genealogical relationships of haplotypes and their geographical distribution may also be a product of the incomplete lineage sorting of polymorphisms, caused by fragmentation of an ancestral population during the uplift of the Tibetan Plateau and adjacent areas and subsequent climate changes. Lineage sorting is a kind of stochastic process randomly allocating ancestral polymorphisms into different populations or species (Doyle & Gaut 2000). It has been proved to be a major factor for incongruence between gene trees and species trees (Wendel & Doyle 1998), disassociation between chloroplast and mitochondrial lineages (e.g. Chiang 2000), as well as lack of association between the genealogical relationships of haplotypes and their geographical distribution (e.g. Caicedo & Schaal 2004). It is most likely that chloroplast polymorphisms existed across the whole range of *Dipentodon* before, or at the beginning of, the rapid and extreme uplift of the Tibetan Plateau and adjacent areas. Afterwards, population fragmentation predominated the range of *Dipentodon* as evidenced by nested clade analysis, lineage sorting randomly allocated clade 3-1 to southeast YGP, but clade 3-2 to west YGP and SETP. With the advance of the uplift of the Tibetan Plateau, populations of SETP likely became separated from those of west YGP by large gorges and mountains, then clade 3-2 diverged into two 2-step clades (2-2 and 2-3). Thus, it can be seen that incomplete lineage sorting leads to the incongruence between the genealogical relationships of haplotypes and their geographical distribution as well as the paraphyly of *D. sinicus* to *D. longipedicellatus* as discussed above.

Incomplete lineage sorting in *Dipentodon* caused by fragmentation during the rapid and extreme uplift of the Tibetan Plateau and adjacent areas can be further justified by the ages of clades in question. By taking 1.01×10^{-9} substitutions per site per year for synonymous sites of cpDNA in seed plants (Graur & Li 1999) as the approximate evolutionary rates of *psb*A-*trn*H and *trn*Q-*rps*16 intergenic spacers (Chiang *et al*. 2006), the ages represented by the mutations between two 3-step clades are about 2.97 million years. This indicates that ancestral polymorphisms of *Dipentodon* existed at the beginning of the rapid and extreme uplift of the Tibetan Plateau and Yungui Plateau, which began 3.4 million years ago. Furthermore, clade 2-3 diverged from clade 2-2 only about 1.48 million years ago, suggestive of there has not been enough time for polymorphisms within YGP populations to be sorted into a monophyletic clade with respect to clade 2-3.

The extremely complex topography and climate in the Tibetan Plateau, especially in the southeastern edge, has led this area to become a world biodiversity hotspot (Myers *et al*. 2000). As suggested in this study, the active tectonics may have had fundamental influences on plant evolution in this area. The results of this study will shed additional light on the evolution of biodiversity on the Tibetan Plateau and adjacent areas and provide a historical framework for the conservation of biodiversity in this area, undoubtedly including *Dipentodon*.

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