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High variation and strong phylogeographic pattern among cpDNA haplotypes in *Taxus wallichiana* (Taxaceae) in China and North Vietnam

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Abstract

We studied the phylogeography of Chinese yew (*Taxus wallichiana*), a tree species distributed over most of southern China and adjacent regions. A total of 1235 individuals from 50 populations from China and North Vietnam were analysed for chloroplast DNA variation using polymerase chain reaction–restriction fragment length polymorphism of the *trn*L-F intron-spacer region. A total of 19 different haplotypes were distinguished. We found a very high level of population differentiation and a strong phylogeographic pattern, suggesting low levels of recurrent gene flow among populations. Haplotype differentiation was most marked along the boundary between the Sino-Himalayan and Sino-Japanese Forest floristic subkingdoms, with only one haplotype being shared among these two subkingdoms. The Malesian and Sino-Himalayan Forest subkingdoms had five and 10 haplotypes, respectively, while the relatively large Sino-Japanese Forest subkingdom had only eight. The strong geography–haplotype correlation persisted at the regional floristic level, with most regions possessing a unique set of haplotypes, except for the central China region. Strong landscape effects were observed in the Hengduan and Dabashan mountains, where steep mountains and valleys might have been natural dispersal barriers. The molecular phylogenetic data, together with the geographic distribution of the haplotypes, suggest the existence of several localized refugia during the last glaciation from which the present-day distribution may be derived. The pattern of haplotype distribution across China and North Vietnam corresponded well with the current taxonomic delineation of the three intraspecific varieties of *T. wallichiana*.

Keywords: China, cpDNA haplotype variation, landscape effects, phylogeography, refugia, *Taxus wallichiana*

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Introduction

During the Quaternary, plants went through repeated episodes of contraction and expansion of their geographic ranges following changes in temperature associated with glaciations (Hewitt 2004). Climate changes during Pleistocene glacial–interglacial cycles had a dramatic effect on species ranges (Pearson & Dawson 2003), causing the separation, migration and extinction of populations and an accelerated rate of evolution (Comes & Kadereit 1998; Taberlet *et al*. 1998; Hampe *et al*. 2003). The genetic structure of populations can also be affected by limited connectivity and restrictions in gene flow by seed dispersal and pollinator activity (Hewitt 2000). Subtropical mainland Asia and Taiwan, where no ice sheets formed during Pleistocene glaciation maxima, is one of the most important refugial regions for lineages that evolved prior to the late Tertiary and Quaternary glaciations that became extinct elsewhere (Axelrod *et al*. 1998). Because of its complex topography

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and climate, natural refugia have allowed the survival of many relict species in the Sino-Himalayan area (Wu 1998). Thus, the current geographic distribution of vegetation in this area is likely the result of both past and present ecological events, with the last glacial–interglacial cycle having the most profound effect on shaping the genetic structure and phylogeographic patterns of plant species there (Cheng *et al*. 2005).

Molecular techniques have provided many tools for studying the phylogeography or migratory footprints of species (Avise 2000). In plants, chloroplast DNA (cpDNA) evolves slowly, with low mutation rates and no recombination (Wolfe *et al*. 1987; Clegg & Zurawski 1992). Despite their low evolutionary rate, cpDNA RFLPs (restriction fragment length polymorphisms) are used to detect variations at the population level and for phylogeographic studies at both the interspecific and intraspecific level (Demesure *et al*. 1996; King & Ferris 1998; Dutech *et al*. 2000). They have been successfully applied to study migration routes and to identify possible glacial refugia of many plant species (Comes & Kadereit 1998; Abbott *et al*. 2000; Csaikl *et al*. 2002; Oliver *et al*. 2006).

Taxus is economically important as the source of paclitaxel, a cancer-inhibiting compound found in the bark of yew trees (Wani *et al*. 1971; Fang *et al*. 1996; Poupat *et al*. 2000; Mukherjee *et al*. 2002). This has led to an overexploitation of *Taxus wallichiana* Zucc. along the Himalaya and in China. This natural resource has thus become scarce and the plants are seriously threatened with extinction in this region. As a consequence, *T. wallichiana* was listed in Appendix II of CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora; available from http://www.cites.org/eng/app/appendices.shtml#10) in 1995 and recognized globally as LR/cd (lower risk, conservation dependent) under the old International Union for the Conservation of Nature and Natural Resources (IUCN) criteria (UNEP-WCMC 2006), although it is considered DD (data deficient) under the new criteria (IUCN 2006). *T. wallichiana* is the most widespread species of *Taxus* in China but often occurs in low numbers in widely disjunct populations. It includes three varieties, var. *wallichiana*, var. *mairei* (Lemée & H. Léveillé) L.K. Fu & Nan Li and var. *chinensis* (Pilger) Florin, according to the latest account in the Flora of China (Fu *et al*. 1999). Plants of *T. wallichiana* are small to medium size evergreen shrubs or trees, usually growing scattered in the undergrowth of broad-leaved, coniferous and mixed montane forests (Li & Fu 1997; Fu *et al*. 1999). They are dioecious, wind-pollinated, with seed dispersal by birds and mammals (Wilson *et al*. 1996; DiFazio 1996).

The taxonomy of *T. wallichiana* is controversial, partly because of morphological similarities and overlaps between the varieties and few reliable morphological diagnostic characters (Möller *et al*. in press). This taxonomic uncertainty causes problems in defining distribution ranges for the varieties in China. Thus, using a taxonomic-based sampling strategy to study the *T. wallichiana* varieties is problematic, and a biogeographic sampling approach was adopted in the present study.

Wu & Wu (1998) described diverse floristic regions in Asia and defined four kingdoms (Holarctic, Tethys, East Asiatic and Palaeotropic) and seven subkingdoms in China. Most of the country was included in the East Asiatic kingdom which was divided into three subkingdoms (Sino-Japanese Forest, Sino-Himalayan Forest and Qinghai–Xizang Plateau). *T. wallichiana* occurs mainly in the Sino-Himalayan Forest (area E in Fig. 1) and the Sino-Japanese Forest (area D) subkingdoms of the East Asiatic kingdom, with some populations reaching into the Malesian subkingdom of the Palaeotropic kingdom.

We sampled *T. wallichiana* populations across its entire distribution range in mainland China, Taiwan and North Vietnam, covering 10 regions of the three subkingdoms. Chloroplast DNA PCR–RFLPs were used to determine the level of genetic variation within and between populations. Rooted haplotype networks were reconstructed in order to elucidate phylogeographic relationships between the haplotypes to gain more detailed insight into the evolutionary history of the species, to elucidate possible Quaternary refugia and migration routes. We finally discuss the significance of our findings in the light of conservation.

Materials and methods

Materials

Leaf samples from a total of 1235 individuals belonging to *Taxus wallichiana* were collected from 50 populations covering all areas of distribution of the species in China, and two populations from North Vietnam. Fourteen populations were collected from the Sino-Himalayan Forest subkingdom, 31 from the Sino-Japanese Forest subkingdom and five from the Malesian subkingdom (Fig. 1, Table 1). For each region, at least two (except Taiwan with one) and up to 13 populations were included, reflecting the size of the respective floristic areas and their complex topography and variable climates.

The sample size of 39 populations ranged from 20 to 33 individuals, while that of seven populations ranged from 10 to 19 individuals, and for four populations, less than 10 individuals were collected (Table 1). Voucher specimens were prepared for most individuals and deposited in KUN and/or E. Young, healthy leaves were collected and immediately dried in silica gel until DNA extraction.

DNA extraction

Total genomic DNA was extracted from silica-gel-dried leaf tissue using the CTAB method of Doyle & Doyle

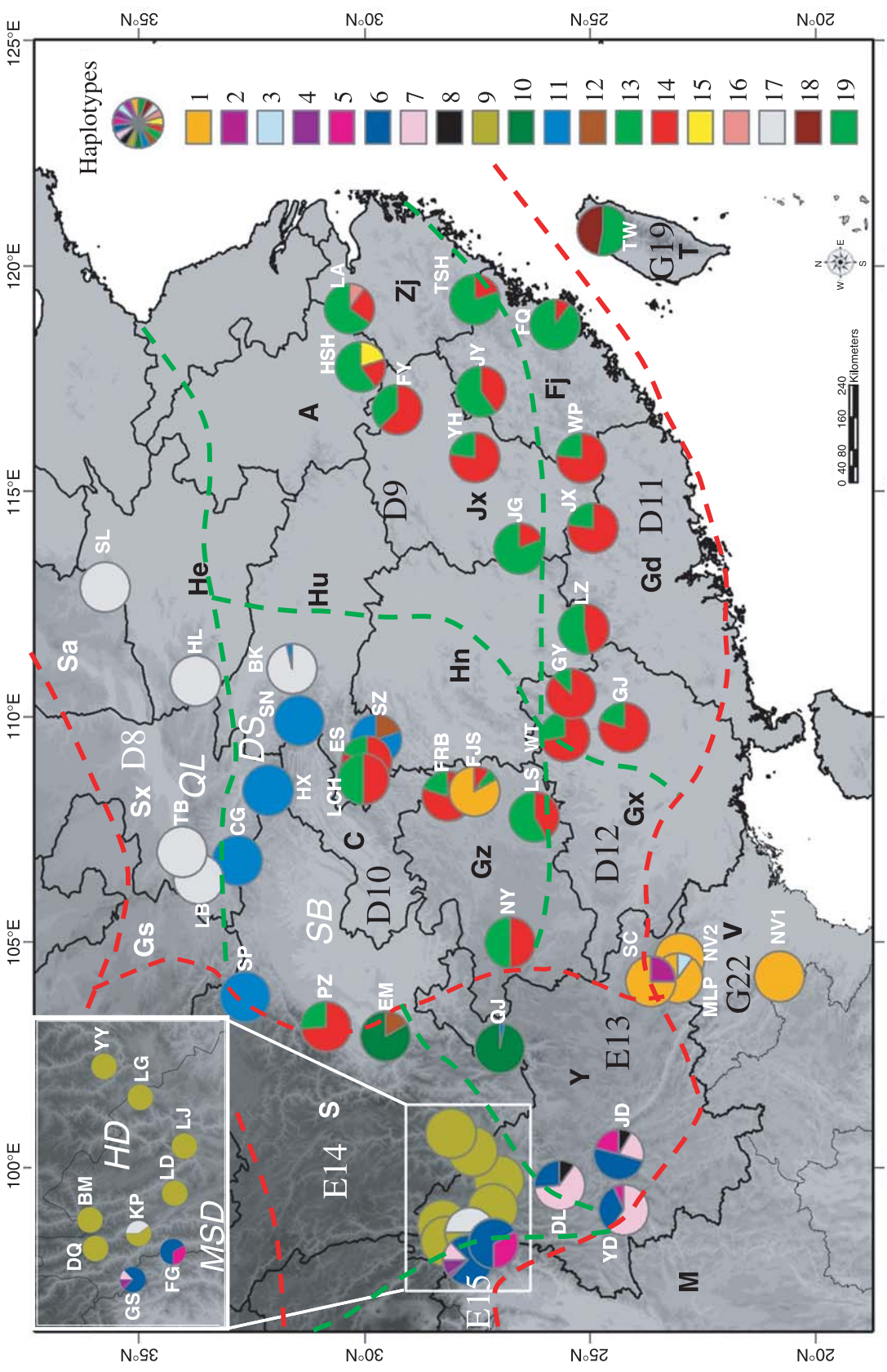

Fig. 1 Floristic regions in China according to Wu & Wu (1998), collection localities (population codes as in Table 1), and the distributions of the 19 chloroplast haplotypes found in 50 populations of *Taxus wallichiana* in China and adjacent regions. Red lines demarcate major, green lines minor, floristic boundaries. Inset in top left corner shows an enlarged view of the Hengduan Mountain and East Himalayan regions. Codes: D, Sino-Japanese Forest subkingdom; D8, North China region; D9, East China region; D10, Central China; D11, South China mountain region; D12, Yunnan. Guizhou and Guangxi limestone mountain and hill region: E, Sino-Himalayan forest subkingdom; E13, Yunnan Plateau region; E14, Hengduan mountain region; E15, East Himalayan region; G, Malesian subkingdom; G19, North Taiwan region; G22, Tonkin Bay region. *DS*, Dabashan Mountain; *HD*, Hengduan Mountain; *MSD*, Mekong-Salween Divide; *QL*, Qinling Mountain; *SB*, Sichuan Basin. **A**, Anhui; **C**, Chongqing; **Fj**, Fujian; **Gd**, Guangdong; **Gs**, Gansu; **Gx**, Guangxi; **Gz**, Guizhou; **He**, Henan; **Hn**, Hunan; **Hu**, Hubei; **Jx**, Jiangxi; **S**, Sichuan; **Sx**, Shaanxi; **Sa**, Shanxi; **Y**, Yunnan; **Zj**, Zhejiang; **T**, Taiwan; **V**, Vietnam; **M**, Myanmar.

Table 1 Collection details of accessions of *Taxus wallichiana* from China and adjacent regions used in the study. The floristic division of China and adjacent regions are based on Wu & Wu (1998)

Population	Population code	*n*	Latitude (N)	Longitude (E)	Altitude range (m a.s.l.)	Haplotype nos
Sino-Himalayan Forest subkingdom (E)						
Yunnan Plateau region (E13)						
Yunnan, Dali, Cangshan	DL	30	25°43′	100°03′	2780–2850	6,7,8
Yunnan, Jingdong, Wuliangshan	JD	24	24°22′	100°44′	2540–2730	5,6,7,8
Yunnan, Yongde, Wumulong	YD	15	24°15′	99°30′	2620–2700	5,6,7
Yunnan, Qiaojia, Xincun	QJ	31	27°13′	103°15′	2150–2600	10,11
Hengduan Mountain region (E14)						
Yunnan, Lijiang,Yulongxueshan	LJ	30	27°01′	100°09′	2910–3020	9
Yunnan, Yulong, Ludian	LD	30	27°09′	99°32′	2900–2990	9
Yunnan, Deqin, Baimaxueshan	BM	30	28°16′	99°11′	3050–3140	9
Yunnan, Deqin, Yunling	DQ	30	28°12′	98°48′	2866–2940	9
Yunnan, Ninglang, Luguhu	LG	30	27°37′	100°48′	2990–3150	9
Yunnan, Weixi, Kangpu	KP	30	27°38′	99°00′	1740–2850	9,17
Sichuan, Muli, Liziping	YY	10	28°05′	101°12′	2900	9
Sichuan, Emei, Emeishan	EM	30	29°32′	103°31′	1700–2210	10,12
East Himalayan region (E15**)**						
Yunnan, Gongshan, Gaoligongshan	GS	31	27°42′	98°23′	2960–2800	4,5,6,7
Yunnan, Fugong, Lumadeng	FG	3	27°10′	98°46′	2757	5,6
Sino-Japanese Forest subkingdom (D)						
North China region (D8)						
Shaanxi, Liuba, Taimiaozi	LB	30	33°41′	106°50′	1760–1770	17
Shaanxi, Taibai, Heping	TB	33	33°51′	107°05′	1230–1350	17
Henan, Lushi, Tanghe	HL	30	33°45′	111°14′	1180–1255	17
Shanxi, Lingchuan, Duohuo	SL	30	35°45′	113°19′	860	17
East China region (D9)						
Jiangxi, Yihuang, Huangpi	YH	22	27°15′	116°10′	740	13,14
Jiangxi, Fuliang, Jinzhushan	FY	29	29°32′	117°31′	680	13,14
Jiangxi, Jinggangshan	JG	21	26°34′	114°10′	760	13,14
Fujian, Jianyang	JY	30	27°25′	117°39′	660–685	13,14
Anhui, Huangshan	HSH	30	30°07′	118°10′	470–1630	13,14,15
Zhejiang, LinAn	LA	20	30°19′	119°30′	320–550	13,14,16
Zhejiang, Taishun	TSH	22	27°31′	119°41′	420–600	13,14
Central China region (D10)						
Sichuan, Dujiangyan, Daguan	PZ	19	30°51′	103°34′	710	13,14
Sichuan, Pingwu, Huangyangguan	SP	31	32°38′	104°13′	1820–2110	11
Guizhou, Nayong, Wangjiazai	NY	2	26°46′	105°26′	1580–1630	13,14
Guizhou, Leishan, Taojiang	LS	31	26°14′	108°13′	820–880	13,14
Guizhou, Yinjiang, Furongba	FRB	30	28°00′	108°41′	790–950	13,14
Guizhou, Jiangkou, Pinggui	FJS	30	27°45′	108°47′	500–900	1,13,14
Hunan, Sangzhi, Badagongshan	SZ	5	29°44′	109°56′	1480–1600	11,12
Hubei, Lichuan, Xingyunshan	LCH	30	30°01′	109°05′	1087	13,14
Hubei, Xuanen, Zhushan	ES	15	29°57′	109°31′	702	13,14
Hubei, Baokang, Longping	BK	29	31°40′	111°27′	1010–1440	11,17
Hubei, Shennongjia	SN	10	31°27′	110°21′	1180–1430	11
Shaanxi, Langao, Hengxi	HX	22	32°08′	108°48′	1280–1320	11
Shaanxi, Chenggu, Dapan	CG	31	32°48′	107°16′	1500–1680	11
South China mountain region (D11)						
Guangxi, Lingui, Wantian	WT	25	25°33′	110°00′	530–890	13,14
Guangxi, Guanyang, Xishan	GY	31	25°25′	110°57′	430–760	13,14
Guangxi, Jinxiu, Liuduan	GJ	30	24°14′	110°12′	1000–1080	13,14
Guangdong, Lianzhou	LZ	32	25°08′	112°23′	470	13,14
Jiangxi, Longnan, Jiulianshan	JX	31	24°50′	114°38′	680–720	13,14
Fujian, Wuping	WP	30	25°11′	116°10′	940–980	13,14
Fujian, Fuqing	FQ	30	25°46′	119°08′	535–710	13,14

Table 1 *Continued*

Population	Population code	*n*	Latitude (N)	Longitude (E)	Altitude range (m a.s.l.)	Haplotype nos
Malesian subkingdom (G)						
Taiwan (G19)						
Taiwan, Hsinchu, Chienshihshan	TW	17	24°48′	121°12′	1810–2100	18,19
Tonkin Bay (G22)						
Yunnan, Malipo, Taiping	MLP	20	23°19′	104°08′	1400	1,3
Yunnan, Xichou, Fadou	SC	24	23°22′	104°53′	1460–1525	1,2
Vietnam, Môc Châu Son La	NV1	14	20°49′	104°39′	1224–1281	1
Vietnam, Ha Giang, Du Gia Mountain	NV2	7	23°06′	105°04′	920–1450	1

m a.s.l., metres above sea level, *n*, number of plants per population.

(1987), modified by adding an ammonium acetate wash (Weising *et al*. 1995) for further purification. The DNA was dissolved in TE buffer (10 mmol/L Tris-HCl, PH 8.0, 1 mmol/L EDTA) to a final concentration of 20–40 ng/μL.

PCR amplification, RFLP and sequencing

The chloroplast DNA *trn*L-F intron-spacer region was used for RFLP, PCR-amplified using the universal primers 'c' and 'f' (Taberlet *et al*. 1991). Sequencing reactions were performed using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems). The sequencing products were analysed on an ABI 3700 automated sequencer. Contiguous DNA sequences were edited using SEQMAN (DNAStar package), complete sequences aligned using CLUSTAL_X (Thompson *et al*. 1997) and then adjusted manually. Insertion or deletion (indels) events in the alignment matrix were treated as a single mutation event, duplications scored as individual motifs.

Two to three individuals per population were randomly selected for sequencing to reveal sequence variation among *T. wallichiana*. Restriction enzyme assays were designed using the program WEBCUTTER (http://www.firstmarket.com/cutter/cut2.html). The restriction enzymes *Xba*I and *Apo*I were used for restriction digest assays on all individuals sampled, while *Mse*I was used for a further restriction digest to distinguish haplotype 11 from 12. Restriction enzyme digests were carried out following the manufacturer's instructions. The mix was incubated at 37 °C for 3 h. The digestion products were separated on 8% polyacrylamide gels, stained with ethidium bromide and visualized and photographed under UV light using the Gel Doc 2000 Gel Documentation System (Bio-Rad Laboratories).

To further confirm the haplotypes in each population, sequence data were generated for one to two individuals of each haploytype from polymorphic populations, and two to three individuals from each monomorphic population were chosen for sequencing. Each RFLP haplotype was restricted to one identical sequence, with one exception: one mutation without restriction site was detected by sequencing comparison between haplotype 6 and haplotype 7. A new primer was designed anchored on the mutation site, and PCR amplifications with 3 primers ('c', 'f' and the newly designed primer 'GSTB', sequence 5′-GGACTTGAACCCTCAC-3′) carried out to distinguish the two haplotypes within the East Himalayan populations sampled.

Data analysis

Variation within populations was estimated by dividing the number of haplotypes present by the number of individuals assayed (D_{hap}, haplotype diversity) and by calculating the gene diversity (H_E) (equivalent to the expected heterozygosity for diploid data, Weir 1996) for each population based on haplotype composition (Nei 1987).

The amount of variation among populations within regions and within populations was calculated by the hierarchical analysis of molecular variance (AMOVA) framework (Excoffier *et al*. 1992) carried out using ARLEQUIN (Schneider *et al*. 2002), with significance tested by a nonparametric permutation procedure with 1000 permutations. Estimates of average gene diversity within populations (H_S), total gene diversity (H_T) and the proportion of total diversity due to differentiations between populations (G_{ST}) were calculated for the whole data set, and with Sino-Himalayan, Sino-Japanese and Malesian populations separately, using the program PERMUT (R.J. Petit, available at http://www.pierroton.inra.fr/genetics/labo/software/Permut/) (Pons & Petit 1996). Population NY was excluded from the analysis due to a sample size of less than three individuals. Population differentiation (G_{ST}) across all populations was tested using an exact test (Raymond & Rousset 1995), while interpopulation differentiation (F_{ST}) across the whole area and within the Sino-Himalayan Forest, the Sino-Japanese Forest and

Malesian subkingdoms was evaluated by AMOVA (Excoffier *et al*. 1992). To establish the extent to which mutational differences between haplotypes contributed toward population differentiation, the program PERMUT was used to estimate N_{ST} and G_{ST}. The latter makes use only of haplotype frequencies while N_{ST} also takes into account differences between the haplotypes. A higher N_{ST} than G_{ST} usually indicates the presence of phylogeographic structure (Pons & Petit 1996) with closely related haplotypes being found more often in the same area than less closely related haplotypes (Zhang *et al*. 2005). The significance of the difference between G_{ST} and N_{ST} was tested via permutation tests.

Relationships between all chloroplast haplotypes were estimated by statistical parsimony inferring a haplotype network (Templeton *et al*. 1992) using TCS version 1.21 (Clement *et al*. 2000). The network was rooted on *Taxus baccata* (EU2; Scotland: Aberlady-4). Because each haplotype was correlated to a unique nucleotide sequence (Table 2), the network was constructed using the nucleotide characters (18 nucleotide and 10 indel characters).

The geographic distribution of the chloroplast haplotypes was mapped using ArcMap 9.1 (ESRI).

Estimation of evolutionary ages

In the absence of fossil data for *T. wallichiana*, evolutionary rates of genes were used to obtain an approximate time frame for the dispersal of the species. Sequence divergence values were calculated from the uncorrected mean pairwise distances of the *Taxus* haplotypes. The average divergence time across all previously reported rates for *trn*L-F, as summarized by Richardson *et al*. (2001), was used (Hughes *et al*. 2005), which was 0.55 (± 0.32) million years (Myr) per mutational change for *trn*L-F sequences. Evenly weighted MP branch lengths may be biased as they are not corrected for multiple hits (Sanderson 2002). However, a likelihood-based correction was not performed because of the low level of sequence divergence (Yuan *et al*. 2005).

Results

Haplotype variation

A high level of polymorphism was detected in the cpDNA *trn*L-F region in *Taxus wallichiana* (Tables 2 and 3). Seventeen chloroplast haplotypes were distinguished directly after PCR–RFLP analysis using two enzymes (*Xba*I and *Apo*I) and another haplotype distinguished by a further restriction digest (*Mse*I). The 19th haplotype was revealed by PCR using a specific haplotype primer. Among the 19 haplotypes, 16 characters were revealed throughout PCR–RFLP for all 1235 individuals. Twelve additional base changes fell outside restriction sites and were revealed through comparison of sequence data.

Population diversity and differentiation

From the 50 populations sampled, 34 were polymorphic (Tables 3 and 4). The haplotype diversities (D_{hap}) ranged from 0.03 to 1 with an overall average of 0.02, although this measure is greatly affected by the number of samples per population included. Populations GS (East Himalayan region) and JD (Yunnan Plateau region) contained four haplotypes with a relatively high haplotype diversities of 0.13 and 0.17, respectively (Tables 1 and 3).

Although a similar number of haplotypes was present in the Sino-Himalayan Forest and Sino-Japanese Forest subkingdoms, more individuals were sampled in the latter, and thus its haplotype diversity was lower ($D_{hap} = 0.01$) compared to that of the Sino-Himalayan Forest subkingdom ($D_{hap} = 0.028$) (Table 4). A relatively high haplotype diversity ($D_{hap} = 0.061$) but lower gene diversity ($H_E = 0.49$) was observed in the Malesian subkingdom. The gene diversity (H_E) at the regional level was relatively low in the Hengduan Mountain region ($H_E = 0.33$) and in the Tonkin Bay region ($H_E = 0.22$), and zero in the North China region. The highest gene diversity levels were found in the Yunnan Plateau ($H_E = 0.73$) and in the Central China regions ($H_E = 0.75$) (Table 4).

The hierarchical analysis of molecular variance (AMOVA) among populations indicated that 87.52% variation was partitioned among populations and only 12.48% of the variation within populations (all partitions were significant at $P < 0.001$). When grouping the populations according to the three subkingdoms, 44.91% of the variation was partitioned among subkingdoms, 45.38% among populations within the subkingdoms, and the remainder (9.71%) of the variation within populations (all partitions were significant at $P < 0.001$). F_{ST} values were 0.875 for the entire sample set, and for the separate subkingdoms the differentiation among populations with $F_{ST} = 0.903$ markedly higher (Table 5).

The gene diversity within population (H_S) and total diversity (H_T) among all populations and the differentiation of populations (G_{ST}) was similar for the Sino-Himalayan Forest and Sino-Japanese Forest subkingdom but slightly lower in the Malesian subkingdom (Table 6).

The test for phylogeographic structure in the haplotype variation showed that the number of substitution types (N_{ST}) was significantly higher than population differentiation over all populations (G_{ST}) ($P < 0.01$), and for the Sino-Himalayan Forest ($0.01 < P < 0.05$) and Malesian subkingdom ($P < 0.01$), but not for the Sino-Japanese Forest subkingdom (Table 6).

Haplotype distribution

The distribution of the 19 haplotypes was not random but showed strong geographic patterns (Fig. 1). Several haplotypes were distributed over multiple regions or populations

Table 2 Variable nucleotide sites of chloroplast *trn*L-F sequences of *Taxus wallichiana* in sampled population from China and adjacent regions and the GenBank sequence accession numbers. Sequences are numbered from the 5′ end to the 3′ end in the *trn*L-F region. Dot (.) indicates that the character states are the same as Haplotype 1, '–' indicates alignment gap

Haplotype	*n*	*trn*L intron 123333333 564566789 174107774	*trn*L-F spacer 455555555555555555555555555555555555555555566666666666666677778888888888888888888 926666666667777777777888888888899999999990222222225566662477111111112222222233 6 771234567890123456789012345678901234567890012345678901233012345678901234567490	GenBank accession numbers
1	82	CGCAAGTCG	GG----------GCTGGGAAATA-AAAAAAAGCGT	EU052213
2	6	....	..----------TTTTATAA------.....-.....	EU052214
3	2	A........	..----------.....-.....	EU052215
4	3	A...C.GT.	..---AAAGATCAAATATTATT---AAAGATCAAATATTATT----------.....-.....AT.	EU052216
5	8	....C.GT.	..----------...T.....-.....AT.	EU052217
6	50	....C.GT.	..AAGAAAGATCAAATATTATT----------...T.....-.....AT.	EU052218
7	37	....C.GT.	..AAGAAAGATCAAATATTATTAAGAAAGATCAAATATTATT----------...T.....-.....AT.	EU052219
8	5	....C.GT.	T.AAGAAAGATCAAATATTATT----------...T.....-.....AT.	EU052220
9	178	A...C.G..	..----------...T.....-.....G	EU052221
10	57	...T.TGT.	..----------...T.....T.....T.	EU052222
11	100	A...CTG.T	..----------TCCCAT.....-.....	EU052223
12	6	A...C.G..	..----------TCCCAT..G.....-.....	EU052224
13	232	A...C.G..	..----------.....-.....	EU052225
14	288	....C.G..	..----------.....-.....	EU052226
15	6	A.A.C.G..	..----------.G.....-.....	EU052227
16	2	....C.G..	..----------....---------...	EU052228
17	164	....C.G..	..----------.....---------...	EU052229
18	8	....C.G..	.T----------.....-.....	EU052230
19	9	.A..C.G..	.T----------T.....-.....	EU052231

n, number of individuals.

Table 3 Variation in chloroplast RFLP haplotypes among the populations of *Taxus wallichiana* in China and adjacent regions

Region	Population code	Haplotypes																			Total	D_{hap}	H_E
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19			
E. Himalayan	GS				3	1	23	4													31	0.13	0.42
	FG					1	2														3	0.67	0.44
Yunnan Plateau	DL						8	19	3												30	0.10	0.52
	JD					5	12	5	2												24	0.17	0.60
	YD					1	5	9													15	0.20	0.52
	QJ										30	1									31	0.06	0.06
Hengduan Mountain	LJ									30											30	0.03	0.00
	LD									30											30	0.03	0.00
	BM									30											30	0.03	0.00
	DQ									30											30	0.03	0.00
	LG									30											30	0.03	0.00
	KP									18								12			30	0.07	0.48
	YY									10											10	0.10	0.00
	EM										25		5								30	0.07	0.28
C. China	PZ													5	14						19	0.11	0.39
	SP											31									31	0.03	0.00
	NY													1	1						2	1.00	0.50
	LS													18	13						31	0.06	0.49
	FRB													6	24						30	0.07	0.32
	FJS	25												2	3						30	0.10	0.29
	SZ											4	1								5	0.40	0.00
	LCH													15	15						30	0.07	0.50
	ES													3	12						15	0.13	0.32
	BK											1						28			29	0.07	0.07
	SN											10									10	0.10	0.00
	HX											22									22	0.05	0.00
	CG											31									31	0.03	0.00
N. China	LB																	30			30	0.03	0.00
	TB																	33			33	0.03	0.00
	HL																	30			30	0.03	0.00
	SL																	30			30	0.03	0.00
S. China mountain	WT													7	18						25	0.08	0.40
	GY													4	27						31	0.06	0.22
	GJ													6	24						30	0.07	0.32
	LZ													17	15						32	0.06	0.50
	JX													7	24						31	0.06	0.35
	FQ													27	3						30	0.07	0.18
	WP													7	23						30	0.07	0.36
E. China	YH													5	17						22	0.09	0.35
	FY													11	18						29	0.07	0.47
	JG													17	4						21	0.10	0.31
	JY													18	12						30	0.07	0.48
	HSH													18	6	6					30	0.10	0.56
	LA													13	5		2				20	0.15	0.51
	TSH													24	6						30	0.07	0.32
Taiwan	TW																		8	9	17	0.12	0.50
Tonkin Bay	MLP	18		2																	20	0.10	0.18
	SC	18	6																		24	0.08	0.38
	NV1	14																			14	0.07	0.00
	NV2	7																			7	0.14	0.00
Total	50	82	6	2	3	8	50	37	5	178	55	100	6	231	284	6	2	163	8	9	1235	0.02	0.96

Underlined values are private haplotypes.

Table 4 Chloroplast polymorphism within regions and subkingdoms of China and adjacent regions

Subkingdom and region	No. of populations	No. of individuals	No. of haplotypes	No. of haplotypes/ individual (D_{hap})	H_E	No. of polymorphic populations	No. of private haplotypes
Sino-Himalayan forest subkingdom	14	354	10	0.028	0.72	8 (57%)	1 (14%)
Yunnan Plateau region	4	100	6	0.060	0.73	4 (100%)	0 (0%)
Hengduan Mt. region	8	220	4	0.018	0.33	2 (25%)	0 (0%)
East Himalayan region	2	34	4	0.118	0.43	2 (100%)	1 (25%)
Sino-Japanese forest subkingdom	31	799	8	0.010	0.77	23 (74%)	2 (25%)
N. China region	4	123	1	0.008	0.00	0 (0%)	0 (0%)
E. China region	7	182	4	0.022	0.52	7 (100%)	2 (50%)
C. China region	13	285	6	0.021	0.75	9 (69%)	0 (0%)
S. China mountain region	7	209	2	0.010	0.46	7 (100%)	0 (0%)
Malesian subkingdom	5	82	5	0.061	0.49	3 (60%)	4 (80%)
Taiwan region	1	17	2	0.118	0.50	1 (100%)	2 (100%)
Tonkin Bay region	4	65	3	0.046	0.22	2 (50%)	2 (66.6%)

Table 5 Hierarchical analysis of molecular variance (AMOVA) of samples of *Taxus wallichiana* based on nucleotide sequences

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	Fixation indices (F_{ST})
The whole area under study					
Among populations	49	2168.18	1.7854	87.52	
Within populations	1185	301.67	0.2546	12.48	
Total	1234	2469.85	2.0400		0.875*
Divided into three subkingdoms					
Among subkingdoms	2	776.40	1.1773	44.91	
Among populations within subkingdoms	47	1391.78	1.1895	45.38	
Within populations	1185	301.67	0.2546	9.71	
Total	1234	2469.85	2.6214		0.903*

*$P<0.001$.

Table 6 Estimates of average gene diversity within *Taxus* populations(H_S), total gene diversity (H_T), interpopulation differentiation (G_{ST}), and the number of substitution types (N_{ST}) (mean ± SE in parentheses) within the Sino-Himalayan Forest , Sino-Japanese Forest subkingdoms and all combined calculated with PERMUT, using a permutation test with 1000 permutations

Subkingdom	H_S	H_T	G_{ST}(SE)	N_{ST}(SE)†
All data	0.271 (0.0324)	0.884 (0.0170)	0.694 (0.0379)	0.883 (0.0199)**
Sino-Himalayan forest	0.267 (0.0757)	0.749 (0.0918)	0.644 (0.0702)	0.799 (0.0458)*
Sino-Japanese forest	0.280 (0.0367)	0.762 (0.0304)	0.632 (0.0604)	0.742 (0.0479)^{NS}
Malesian	0.227 (0.1076)	0.509 (0.2344)	0.554 (NC)	0.812 (NC)**

†indicates that N_{ST} is significantly different from G_{ST} at *= $0.01 < P < 0.05$, **$P < 0.01$ level; NS, not significantly different; NC, not computed due to small sample size.

while seven were unique, occurring in six populations: private haplotypes were found in population GS (haplotype 4) in the East Himalayan region (E15), in two populations in the East China region (D9), i.e. populations HSH (haplotype 15) and LA (haplotype 16), and in populations SC (haplotype 2) and MLP (haplotype 3) in the Tonkin Bay region (G22). Both haplotypes 18 and 19 from North Taiwan (G19) were unique.

The most widespread haplotypes were 13 and 14 which occurred across the Sino-Japanese Forest subkingdom,

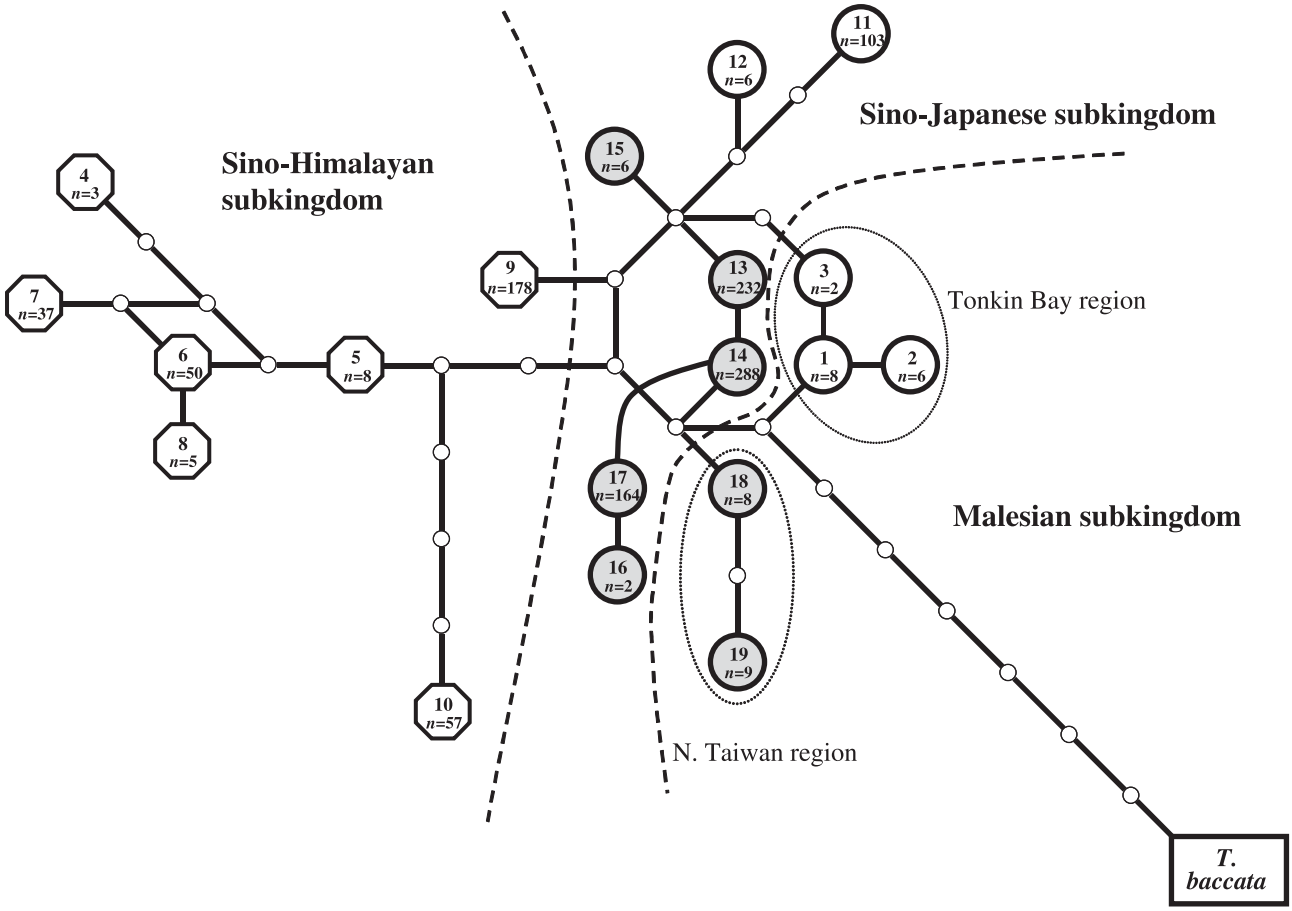

Fig. 2 Rooted haplotype network of 19 chloroplast haplotypes of *Taxus wallichiana* based on 28 characters. Bold numbers in circles indicate the haplotype identity (as in Table 1) and italic numbers represent individuals sharing this type. Small circles indicate the number of mutations between haplotypes. Floristic subkingdoms and regions are as indicated in Fig. 1, based on Wu & Wu (1998). Presently used taxonomic identities: octagon, *T. wallichiana* var. *wallichiana*; shaded circle, *T. wallichiana* var. *mairei*; open circle, *T. wallichiana* var. *chinensis*.

except for the North China region (D8) in the far north. These two haplotypes always appeared together, albeit in varying proportions.

In contrast, populations in the north of the Central China region (Dabashan Mountains, *DS*), in the North China region (Qinling Mountains, *QL*), the Southern Hengduan Mountain and the Tonkin Bay region were mainly monomorphic for haplotypes 11, 17, 9 and 1, respectively.

Haplotype relationships

The rooted network illustrates the relationships between the 19 haplotypes (Fig. 2). The network shows two large groups of haplotypes, representing the Sino-Himalayan haplotypes on one side, and haplotypes occurring in the Sino-Japanese Forest and Malesian subkingdoms on the other. A large unresolved loop, resulting from homoplasies, is present among the Sino-Japanese Forest/Malesian subkingdom haplotypes, with haplotypes 13 and 14 in a central position. Closest to the root, *T. baccata*, are the Tonkin Bay haplotypes. However, the Sino-Himalayan haplotypes are clearly in a derived position to the Sino-Japanese haplotypes. Haplotype 10 is positioned on a long branch and between the two larger subkingdoms. Haplotypes occurring in the Malesian subkingdom clustered in two groups, representing the North Taiwan and Tonkin Bay regions.

Dating results

Applying a precalibrated mutation rate to the haplotype network indicates a maximum divergence time across all sampled *T. wallichiana* populations (between the Sino-Himalayan haplotype 7 and the Sino-Japanese haplotype 11) of 6.6 (± 3.8 SE) Myr. The maximum evolutionary age of the haplotypes in the Sino-Himalayan Forest subkingdom is 2.7 (± 1.6 SE) Myr and 4.9 (± 2.8 SE) Myr when excluding or including haplotypes 9 and 10), while those of the Sino-Japanese Forest subkingdom can be dated to 3.8 (± 2.2 SE) Myr and the two Malesian subkingdom clades are both 1.1 (± 0.6 SE) Myr old (Table 7).

Table 7 Estimates of maximum evolutionary ages of *Taxus wallichiana* clades using average rates of gene evolution for *trn*L-F (Richardson *et al*. 2001)

Clade (haplotypes)	Million years
T. wallichiana (all 11 v 7)	6.58 ± 3.8
T. wallichiana var. *chinensis* (1–3 v 11 + 12)	3.84 ± 2.2
T. wallichiana var. *mairei* (13–19)	3.29 ± 1.9
T. wallichiana var. *mairei* Taiwan (18 + 19)	1.10 ± 0.6
T. wallichiana var. *wallichiana* (4–8)	2.70 ± 1.6

Discussion

Genetic diversity and population differentiation

We detected a high level of genetic variation across the 50 populations of *Taxus wallichiana* from mainland China, Taiwan and North Vietnam, with 19 different cpDNA haplotypes in the *trn*L-F intron-spacer region examined. This is high compared for example with the endemic *Juniperus przewalskii* from the Qinghai-Tibetan Plateau in which a total of six different haplotypes were detected in the combined *trn*T-*trn*F and *trn*S-*trn*G chloroplast gene region (Zhang *et al*. 2005). We also found a high level of total diversity among all *Taxus* populations ($H_T = 0.884$), which is much higher than that found for example in the *Juniperus* study ($H_T = 0.568$) (Zhang *et al*. 2005) and for *Castanopsis carlesii* ($H_T = 0.444$) (Cheng *et al*. 2005).

The high level of haplotype differentiation between populations of *T. wallichiana* was surprising. In general, widespread, outcrossing, wind-pollinated, vertebrate dispersed taxa are associated with low population differentiation (Hamrick & Godt 1989; Nybom 2004; Ge *et al*. 2005), and this has been shown to be the case for a range of conifers (cf. Petit *et al*. 2005), e.g. *Pinus flexilis* (Latta & Mitton 1997) and *Cunninghamia konishii* (Hwang *et al*. 2003). Other conifers, however, show values similar to *T. wallichiana*, for example *Juniperus przewalskii* (Zhang *et al*. 2005).

Despite its dioecy, wind-pollination and endozoochorous seed dispersal (DiFazio 1996; Wilson *et al*. 1996), populations of *T. wallichiana* seem to maintain large differentiation values between populations, probably because of its habitat as part of the undergrowth in forests. There, wind velocities are greatly reduced and the temperature inversions negatively affect pollen dispersal (Tauber 1967; Levin & Kerster 1974; Wheeler *et al*. 1995). The significant population structuring and high population differentiation recorded for *T. wallichiana* would fit a very limited range of wind-mediated pollen flow, as indicated earlier for *Taxus* by Allison (1990) who reported that the efficiency of dispersal of *T. canadensis* pollen drops significantly after a few metres under natural conditions, this is despite the small size of *Taxus* pollen (17–21.5 μM, Xing *et al*. 2000).

The scattered patchiness of *T. wallichiana* populations and individuals coupled with dioecy also seem to discourage seed dispersal specialization by birds that would allow long-distance gene flow; seed dispersals, if they happen at all, appear to be very sporadic. The dispersal capabilities of both pollen and seeds play a major role in the evolutionary trajectory of natural plant populations that have been affected by fragmentation (Lira *et al*. 2003; Ghazoul 2005), possibly including *T. wallichiana* populations. As the direction of chloroplast inheritance is paternal in *Taxus* (Anderson & Owens 1999; Collins *et al*. 2003), chloroplast markers will not distinguish between seed and pollen dispersal events. However, the high population differentiation observed among *T. wallichiana* populations strongly suggests that neither form of dispersal is very effective.

Geographic structure among chloroplast haplotypes

The strong genetic structure among the population of *T. wallichiana* observed here ($G_{ST} = 0.702$, $F_{ST} = 0.875$) was largely due to the differences in haplotype compositions between the floristic regions described by Wu & Wu (1998). There was a strong phylogeographic structure with few haplotypes shared among floristic regions or subkingdoms. Only two cases of the disjunct occurrence of haplotypes were found, involving haplotypes 1 and 17 (Fig. 1). Whether these disjunctions are the result of habitat contraction and population fragmentation, human-made origins, e.g. by transplanting, or rare long-distance dispersals, e.g. by birds, is at present uncertain and requires further investigation.

Not only lack of gene flow between regions but also geographic complexity is likely to have shaped the genetic composition among *T. wallichiana* populations. Such a landscape effect is evident in the Hengduan Mountain and East Himalayan regions, and the Qinling Mountains and Dabashan Mountains. Despite their close geographic proximity, particularly between the Hengduan Mountain and East Himalayan regions, populations occurring there possessed completely different sets of haplotypes (Fig. 1 inset). In this case, the steep mountains and deep valleys of the Mekong–Salween Divide, recognized as a geographic barrier by Kingdon Ward (1921), which runs from North to South with over 4000 m above-sea-level altitudes, appear to have effectively prevented an east–west mediated gene flow. On the contrary, there was less differentiation among populations around the Sichuan Basin that share a mix of haplotypes, especially along the west and east sides where the mountains are not as steep and high (Fig. 1).

Across the entire sampled area, the correlation between haplotype and floristic subkingdom is very strong. At the regional level, this strong geography–haplotype correlation persists and most regions possess unique sets of haplotypes, except for the Central China region that shares haplotypes with most of its surrounding regions. These local

disequilibria between geographic size and haplotype diversity may reflect the higher diversity in habitats resulting from greater geological and topological variation found in the Sino-Himalayan Forest subkingdom and the Central China region.

Phylogenetic relationships and refugia

A strong geographic structure of haplotype variation found in *T. wallichiana* ($N_{ST} > G_{ST}$, $P < 0.01$) reflected the fact different lineages occupy different geographic regions. Several distinct haplotype regions can be distinguished, with a strong underlying phylogenetic hierarchy (Figs 1 and 2).

Because of some unresolved relationships among the Sino-Japanese Forest and Malesian subkingdom haplotypes, it is difficult to interpret their evolution with respect to their ancestral position. However, the network shows a central position for haplotypes 13 and 14. These are widespread and predominant which suggests that they represent older haplotypes (Carbone & Kohn 2001), from which several smaller groups are derived, such as the North Taiwan and Sichuan Basin haplotypes. Within the entire network, the Sino-Himalayan group is derived. This is also suggested by the age estimates which indicated a younger age for the East Himalayan haplotypes, compared to the Sino-Japanese Forest and Malesian subkingdom haplotypes. Further evidence for the recent arrival of *Taxus* on the Yunnan Plateau comes from palynological data that indicates the absence of *Taxus* in the Late Pliocene there (Kou *et al*. 2006).

Our dating estimates indicate that the diversification in *T. wallichiana* started in the Late Miocene. At this point, the hitherto continuous forest in China fragmented and shrank, due to cooling and drying effects which are linked to the uplift of the Qinghai-Xizang Plateau (Shi *et al*. 1999; Guo *et al*. 2002; Dupont-Nivet *et al*. 2007). There is, however, some controversy about the timing of the uplift of the Himalayas and the Qinghai-Xizang Plateau, estimates for which range from 15 to 3 Myr ago (Wang & Ding 1998; Liu *et al*. 2001; Spicer *et al*. 2003). This time period covers the main period of diversification of *Taxus*, and the uplift would have had an effect on the evolution of *T. wallichiana*. Early effects would have included fragmentation and isolation on ancient mountains, such as the South China mountain region, which originated in the Triassic (Xie 1993). The uplift of the Himalaya created additional habitats for *Taxus* along their foothills which were occupied by a lineage from the east, from the South China Mountain region.

Climate oscillations during the Pleistocene resulted in several glacial–interglacial cycles which caused expansion and contractions of habitats (Comes & Kadereit 1998; Hewitt 2000, 2004; Abbott & Brochmann 2003). During such expansion phases, *T. wallichiana* would have had the opportunity to occupy new areas such as Taiwan, when low sea levels allowed land bridges to form (Ota 1998). Subsequent glacial–interglacial cycles are likely to have resulted in gradual isolation and divergence of haplotypes in Taiwan (cf. Juan *et al*. 2000). The presence of haplotype 9 in the Hengduan Mountain represents a further Pleistocene migration event westwards, crossing the floristic boundaries from the Sino-Japanese Forest subkingdom into the Sino-Himalayan Forest subkingdom.

From the geological and climatic history during the evolution of *T. wallichiana* and the haplotype diversity and relationships resolved, the existence of several Pleistocene refugia can be deduced, such as in the East Himalaya, the Hengduan Mountain region, around the Sichuan Basin, the East China region, the Tonkin Bay region and Taiwan. The evolutionary history of *T. wallichiana* seems to include ancient fragmentation and, because of its low vagility, localized range expansion from these refugia.

Implications for conservation

The total genetic diversity of a species is a key factor in its persistence and in conservation considerations (Rauch & Bar-Yam 2005). The maintenance of genetic diversity is critical for the long-term survival of species (Frankel & Soulé 1981), because loss of variation may largely limit the adaptability of populations to changing environments (Ge *et al*. 2005). *Taxus* populations have declined due to extensive exploitation for commercial use in recent years in East Asia; the population in northern India has, according to the World Wildlife Fund, declined by 90% over the last few decades (Behrens 2003). During fieldwork, observations suggested that human activities have caused severe reduction and fragmentation of populations which may now be vulnerable to genetic drift or complete loss. The maintenance of effective population sizes and reduction of human disturbance are thus priority requisites for conservation. Our study has shown that high levels of interpopulation differentiation exist in populations of *T. wallichiana*. Low levels were found at the intrapopulation level that may in extreme cases affect local adaptability. However, even though the levels of diversity in *Taxus* are similar between nuclear and cpDNA sequences (Shah *et al*. in press), the *trn*L-F intron-spacer region we used in the present study is likely to be selectively neutral and may not determine the adaptive or evolutionary potential of a population or a species (Holderegger *et al*. 2006). Our findings have important implications for the *in situ* conservation of the genetic diversity of *T. wallichiana*, as the protection of a single area would not capture a significant amount of the total diversity. To preserve the majority of genetic diversity, several areas possessing different haplotypes should be targeted.

For conservation purposes, a stable classification system is very helpful. In the case of *T. wallichiana*, the geographic

distribution of the chloroplast haplotypes appears to be tightly linked to the varieties currently recognized in *T. wallichiana* as delineated by a recent morphometric analysis of herbarium specimens (Möller *et al*. in press) (Fig. 2). The latter study suggests a disjunct distribution of *T. wallichiana* var. *chinensis* around the Sichuan Basin and in Southeast Yunnan and North Vietnam. In the present study, these regions are occupied by different haplotype lineages. More detailed morphological analyses are required to establish whether consistent morphological differences exist between these '*chinensis'* lineages that warrant a split.

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