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ORIGINAL ARTICLE

Genetic diversity and taxol content variation in the Chinese yew *Taxus mairei*

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Abstract *Taxus mairei* is an endangered plant with high medicinal value in China. In the present study, inter-simple sequence repeat markers and high-performance liquid chromatography (HPLC) were used to investigate genetic diversity, taxol content, and the inter-relationship of these two variables in 11 populations. Genetic diversity was high at the species level [percentage of polymorphic bands (PPB) = 91.73 %; Nei's gene diversity (h) = 0.2428; Shannon's information index (I) = 0.3771], but relatively lower at the population level (PPB = 54.14–67.67 %; h = 0.1809–0.2121; I = 0.2721–0.3211). Hierarchical analysis of molecular variance (AMOVA) revealed moderate genetic differentiation among populations ($\emptyset_{ST}$ = 16.13 %), in line with the low gene differentiation coefficient (G_{ST} = 0.1697) and relatively strong gene flow (N_m = 2.4480). Both UPGMA and principal coordinates analysis supported the clustering of all 11 populations into three groups. A Mantel test indicated a significant correlation between geographic and genetic distances (r = 0.405; $P < 0.005$). Taxol content varied significantly among populations, ranging from 0.0069 to 0.0127 % based on the HPLC analysis. The taxol content was not significantly associated with genetic diversity, but was significantly, negatively associated with population latitude ($r = -0.620$; $P < 0.005$). This implies that local temperature may significantly affect the taxol content, although the role of heredity cannot be neglected. Our findings provided important references for resource protection and sustainable management of this valuable plant.

Keywords *Taxus mairei* · Taxol · Genetic diversity · Inter-simple sequence repeat (ISSR) · High-performance liquid chromatography (HPLC)

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Introduction

Taxus mairei is a Tertiary relict tree within the family Taxaceae, and mainly occurs in southern China, Nepal, India and Vietnam (Poudel et al. 2012; Zhang et al. 2012). It is an evergreen plant with ornamental, medicinal and timber uses (Editorial Committee of the Flora of China 1978). This plant (especially its bark) has long been exploited as a source of taxol, a compound with anti-cancer activity. As a result, it has been seriously over-harvested. Its consequent scarcity, combined with a naturally low fertility and slow growth rate, has caused it to become very vulnerable and has led to its being listed as a rare and endangered plant in China (Yu 1999).

Following the official approval of taxol as a novel drug for curing ovarian cancer in 1992, wild *Taxus* resources have become insufficient to meet the market and clinical demands. Despite the availability of a chemical synthesis method, human-planted trees are still the primary source of taxol due to the complexity of the procedure and the resultant low yields (Shen and Wu 1997; Yang et al. 2008). Screening for strains with good genetic stability and high taxol content would contribute to the sustainable utilization of *Taxus* plants.

Previous studies demonstrated that *T. mairei* has high taxol content and is the *Taxus* species with the highest

growth rate and broadest distribution range. These characteristics offer the advantage of excellent provenance selection and make it well-suited to large-scale cultivation (Su et al. 2000; Zheng 2003). A strategy for improved development and utilization of this plant, based on the premise of reasonable protection of germplasm resources, is urgently needed. Key components in developing such a strategy include elucidating genetic diversity and taxol content differences among germplasm resources, analyzing the relationship between genetic variation and taxol content, and evaluating environmental influences on genetic variation and taxol content (Milligan et al. 1994).

Previous population genetic studies of *T. mairei* have involved only a small sample size or a limited number of molecular markers (Ru et al. 2008; Jiang et al. 2009; Zhang et al. 2009a; b; Li et al. 2011a, b), and studies of its taxol content have mainly focused on the influence of different habitats, stalk position and age (Ke et al. 2009; Li et al. 2011a, b; Yu et al. 2012; Zhang and Du 2012). Chen et al. (1999) investigated relationships among molecular characteristics, morphological features and habitats of high taxol-content plants by using random amplified polymorphic DNA (RAPD) markers and high-performance liquid chromatography (HPLC); however, no detailed effort was made to analyze the correlation between genetic variation and taxol content.

In this study, 11 *T. mairei* populations were sampled across its geographic range in China. Our major objectives were to: (1) evaluate the level and partitioning of genetic variability within/among populations using inter-simple sequence repeat (ISSR) markers; (2) determine the taxol content in each population and analyze the distribution pattern of content variability among populations by high-performance liquid chromatography (HPLC); (3) analyze the relationship between taxol content and genetic diversity, and elucidate the influence of environment on taxol content; and (4) eventually provide references for the effective conservation and sustainable utilization of this valuable plant.

Materials and methods

Plant materials

A total of 219 individuals from 11 natural populations were sampled across the major production areas of *T. mairei* in China, including Zhejiang, Jiangxi, Yunnan, Sichuan and Hunan provinces. Fresh leaves were collected, dried in ziplock bags with silica gel, transported back to the laboratory and kept in a −80 °C freezer until the ISSR analysis. For taxol content determination, three individuals were randomly sampled from each population, and branches of approximately the same age and growth conditions were collected. Voucher specimens were deposited in the herbarium of the Laboratory of Biological Resources and Application Technology, College of Life Science and Technology, Tongji University, Shanghai, China (Table 1; Fig. 1).

DNA extraction and ISSR-PCR amplification

Total genomic DNA was extracted from dried leaves using a modified CTAB method (Doyle and Doyle 1987). The DNA was dissolved in 0.1× TE buffer (10 mM Tris–HCl and 1 mM EDTA, pH 8.0), and maintained at −20 °C for long-term storage or at 4 °C for immediate use.

ISSR primers used in this study were synthesized by Shanghai Sangon Biological Engineering Technology and Service Co. (Shanghai, China) according to the primer set published by the University of British Columbia (Vancouver, BC, Canada). One hundred ISSR primers were initially screened, and 13 primers which yielded strong, discernible bands were used for assaying all 219 samples (Table 2). PCRs were performed in 20-μL reaction volumes containing 2.0 mM $MgCl_2$, 0.25 mM of each dNTP, 1.0 U Taq DNA polymerase (Takara, Dalian, China), 0.2 μM primer and 50 ng template DNA. Amplifications were performed in a Mastercycler Gradient PCR Machine (Eppendorf, Germany) using the following program: initial denaturation at 94 °C for 4 min; followed by 40 cycles of 94 °C for 45 s, an appropriate annealing temperature (see Table 2) for 45 s, and 72 °C for 1.5 min; and a final extension at 72 °C for 5 min. The amplified products were separated in 1.5 % agarose gels in 1× TAE buffer at 100 V for 1 h, stained with ethidium bromide and photographed under UV light using a UVP-GDS8000 Gel Documentation System (UVP, USA).

HPLC assays

Taxol was extracted by the methanol ultrasonic method (Liu 2008). Branches were placed in an oven at 45 °C for 24 h and then ground into powder. After sifting through five screens, 10 g of powder was mixed with 100 ml of 95 % methanol and subjected to ultrasonic extraction for 2 h. The extract was filtered, and 80 ml of 95 % methanol was added to the sediment. After ultrasonic extraction for 1 h, the extract was again filtered; 60 ml of 95 % methanol was added to the sediment, followed by ultrasonic extraction for 1 h and re-filtering. The three filtrates were combined and allowed to dry at 50 °C. The dried residue was dissolved in methanol ultrasonically and diluted to constant volume in a 10-ml volumetric flask. The diluted samples were filtered through a 0.45-μm filter membrane and used for the HPLC analysis.

Table 1 Geographic localities, sample sizes, genetic diversity and taxol contents of *Taxus mairei* populations analyzed in this study

Populations	Geographic localities	Sample size	Longitude/ latitude	Genetic diversity[a]			Taxol contents (%)[b]
				h	*I*	PPB (%)	
HNZZ	Zhuzhou, Hunan	20	113°07′E/ 27°49′N	0.2125	0.3165	59.40	0.0097 ± 0.00020 cd
HNYY	Yiyang, Hunan	19	112°12′E/ 28°19′N	0.1887	0.2849	55.64	0.0103 ± 0.00017c
JXJG	Jinggangshan, Jiangxi	20	114°51′E/ 26°19′N	0.2121	0.3211	64.66	0.0127 ± 0.00108a
JXFX	Fengxin, Jiangxi	20	115°13′E/ 28°24′N	0.2102	0.3211	67.67	0.0090 ± 0.00025de
ZJZJ	Zhuji, Zhejiang	20	120°16′E/ 29°25′N	0.2086	0.3142	61.65	0.0069 ± 0.00042f
ZJSZ	Shengzhou, Zhejiang	20	120°29′E/ 29°21′N	0.2044	0.3063	59.40	0.0085 ± 0.00005e
ZJSA	Yankou, Zhejiang	20	119°06′E/ 28°14′N	0.1973	0.2954	57.89	0.0093 ± 0.00025d
ZJSC	Miaogao, Zhejiang	20	119°09′E/ 28°21′N	0.2046	0.3105	64.66	0.0111 ± 0.00036b
YNLJ	Lijiang, Yunnan	20	100°08′E/ 26°31′N	0.1945	0.2936	58.65	0.0071 ± 0.00021f
YNSM	Simao, Yunnan	20	100°34′E/ 22°27′N	0.2028	0.3063	60.90	0.0122 ± 0.00045a
SCMY	Mianyang, Sichuan	20	104°24′E/ 31°16′N	0.1809	0.2721	54.14	0.0076 ± 0.00049f
Species-level		219		0.2428	0.3771	91.73	

[a] Three genetic diversity indices were presented, i.e., Nei's gene diversity (*h*), Shannon's information index (*I*) and the percentage of polymorphic bands (PPB, %)

[b] Values are mean ± standard error; letters following the standard error indicate statistical differences at the significance level $P < 0.05$ based on Fisher's protected least significant difference (LSD) test

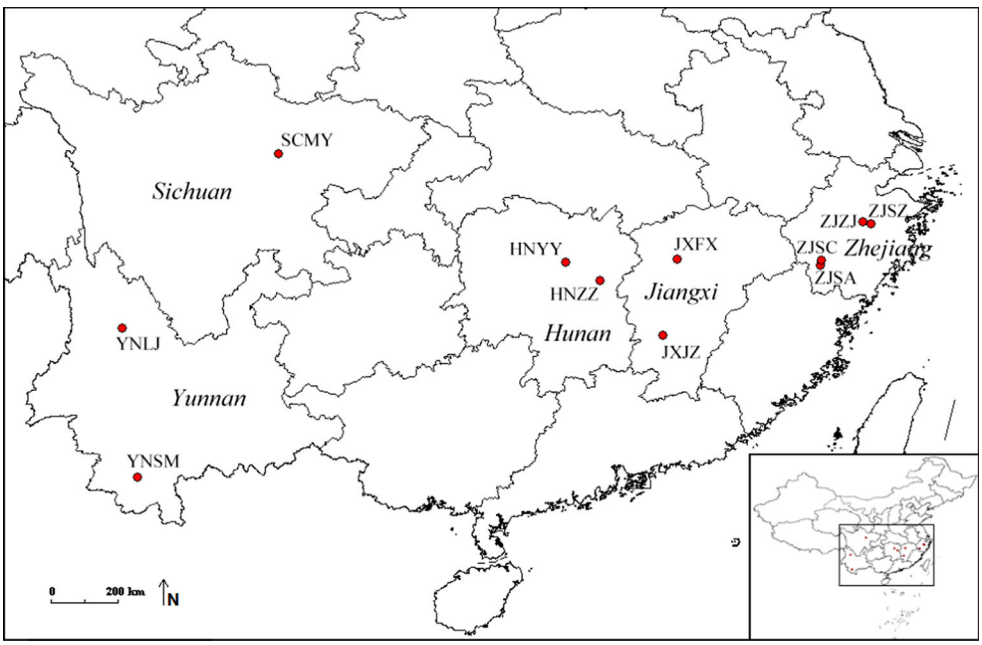

Fig. 1 Geographic distribution of the 11 sampled populations of *Taxus mairei* in China. For population abbreviations, see Table 1

HPLC was performed using an Agilent 1100 HPLC System equipped with an ultraviolet detector. Taxol extracts (20 µl) were directly injected onto a Hypersil ODS column (Agilent, Germany) of 5 µm particle size, 250 mm length and 4.6 mm diameter. A mobile phase of acetonitrile/ultrapure water (48:52 v/v) at a flow rate of 1.2 ml min^{-1} was used. The absorption wavelength was 228 nm. Three repeats were made in measuring the taxol content of each sample.

Table 2 ISSR primers used for assaying genetic diversity of *Taxus mairei*

Primer code	Sequence (5′ → 3′)	T_A (°C)	N_{PL}/N_L	PPB (%)
U807	$(AG)_8T$	52	11/11	100
U811	$(GA)_8C$	52	13/13	100
U812	$(GA)_8A$	54	11/12	91.7
U813	$(CT)_8T$	52	10/12	83.3
U814	$(CT)_8A$	52	7/9	77.8
U815	$(CT)_8G$	52	7/7	100
U822	$(TC)_8A$	52	7/10	70
U823	$(TC)_8C$	50	9/10	90
U825	$(AC)_8T$	54	10/11	90.9
U834	$(AG)_8YT$	52	8/9	88.9
U840	$(GA)_8YT$	52	11/11	100
U844	$(CT)_8RA$	52	11/11	100
U845	$(CT)_8RG$	50	6/7	85.7

Y C/T, *R* A/G, T_A, annealing temperature (°C), N_L number of loci scored, N_{PL} number of polymorphic loci scored, *PPB* percentage of polymorphic bands (%)

Statistical analyses

Because ISSR markers are dominantly inherited, each band was assumed to represent the phenotype at a single biallelic locus (Williams et al. 1990). Only reproducible and discernible fragments ranging in size from 150 to 2,000 bp were included in the statistical analysis. To construct the binary data matrix, ISSR bands were scored as presence (1) or absence (0) characters.

POPGENE v1.32 (Yeh et al. 1997) was used to calculate various genetic diversity parameters, including the percentage of polymorphic bands (PPB), Shannon's information index (*I*), Nei's gene diversity (*h*), the gene differentiation coefficient (G_{ST}) and gene flow (N_m). Nei's genetic distance (*D*) among populations were also computed using this program, and the resultant genetic distance matrix was then used to construct an UPGMA (Unweighted Pair Group Method with Arithmetic Mean) dendrogram. Grouping of populations was carried out by principal coordinates analysis (PCoA) using the software package GenAlEx v6.3 (Peakall and Smouse 2006). A Mantel test (Mantel 1967) between genetic and geographic distances was conducted using GenAlEx v6.3 to test for isolation-by-distance (IBD). This package was also employed to perform a hierarchical analysis of molecular variance (AMOVA; Excoffier et al. 1992), which statistically assessed the partitioning of genetic variability within/among populations.

HPLC data were analyzed using SPSS v17.0 (SPSS Inc., USA). A significance test of taxol content differences among populations was conducted by the single-factor analysis of variance (one-way ANOVA; $P < 0.05$). A UPGMA dendrogram was constructed and principal components analysis (PCA) was performed from the taxol content data. SPSS v17.0 was also employed to analyze possible associations of taxol content with latitude, longitude and genetic diversity. Taxol content and genetic distances were calculated and analyzed using the Mantel test.

Results

Genetic diversity

The 13 selected ISSR primers yielded 133 reproducible bands, of which 122 were polymorphic across the 219 *T. mairei* samples from 11 populations. At the population level, the percentage of polymorphic bands (PPB) varied between 54.14 and 67.67 %, with an average of 60.42 %; this estimate was substantially higher at the species level (PPB = 91.73 %) (Table 1). Nei's gene diversity (*h*) ranged from 0.1809 to 0.2125 and Shannon's information index (*I*) from 0.2721 to 0.3211 at the population level. Both parameters displayed a similar trend to PPB. As indicated by these three parameters, the highest levels of genetic diversity occurred in populations JIFX (PPB = 67.67 %; $h = 0.2102$; $I = 0.3211$) and JXJG (PPB = 64.66 %; $h = 0.2121$; $I = 0.3211$), while the lowest level in population SCMY (PPB = 54.14 %; $h = 0.1809$; $I = 0.2721$).

Genetic differentiation

The AMOVA analysis revealed a moderate level of genetic differentiation, with 16.13 % of total genetic variability residing among populations (Table 3). This pattern was further confirmed by the gene differentiation coefficient ($G_{ST} = 0.1697$) and the substantial gene flow ($N_m = 2.4480$) among populations.

Nei's genetic distances ranged from 0.0254 (YNSM vs. YNLJ) to 0.0866 (JXFX vs. SCMY), with an average of 0.0584. The UPGMA cluster analysis clustered the 11 populations into three groups. Group I comprised the single population from Sichuan Province (SCMY). Group II included eight populations from Zhejiang, Jiangxi and Hunan provinces (JXFX, JXJG, ZJSA, ZJSC, ZJZJ, ZJSZ, HNZZ and HNYY), while Group III comprised the remaining two populations from Yunnan Province (YNLJ and YNSM) (Fig. 2a). The relationship implied by the UPGMA analysis was confirmed by the PCoA plot (Fig. 2b), in which the first two factors accounted for 29.88 % (axis 1) and 22.89 % (axis 2) of the total genetic variance, respectively.

A Mantel test revealed a significant correlation between geographic and genetic distances among populations ($r = 0.405$; $P = 0.003$; 999 permutations), indicating the role of geographic isolation in shaping the present population genetic structure of *T. mairei*.

Table 3 Partitioning of genetic variability within/among populations based on the AMOVA analysis

Source of variation	*df*	SS	MS	Variance components	Ratio of variance (%)	*P* value[a]
Among populations	10	690.05	69.01	2.75	16.13	<0.001
Within populations	208	2,972.68	14.29	14.29	83.87	<0.001

SS, sum of square, *MS*, mean square

[a] Levels of significance are based on 999 permutations

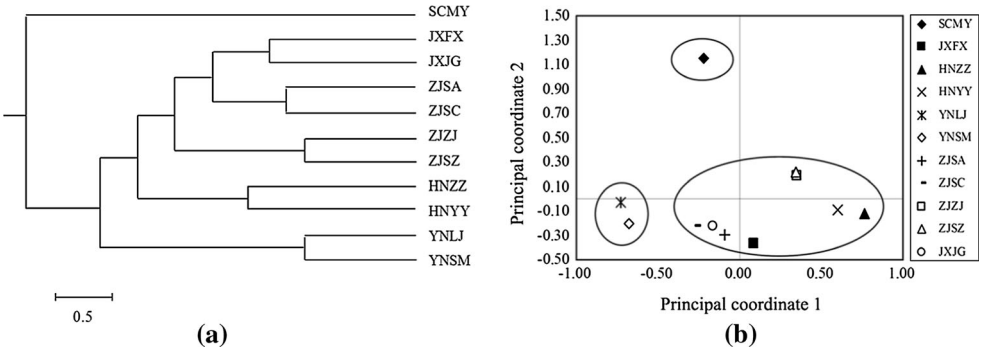

Fig. 2 UPGMA cluster analysis (**a**) and principal coordinates analysis (PCoA) (**b**) using ISSR data of the 11 populations of *Taxus mairei*

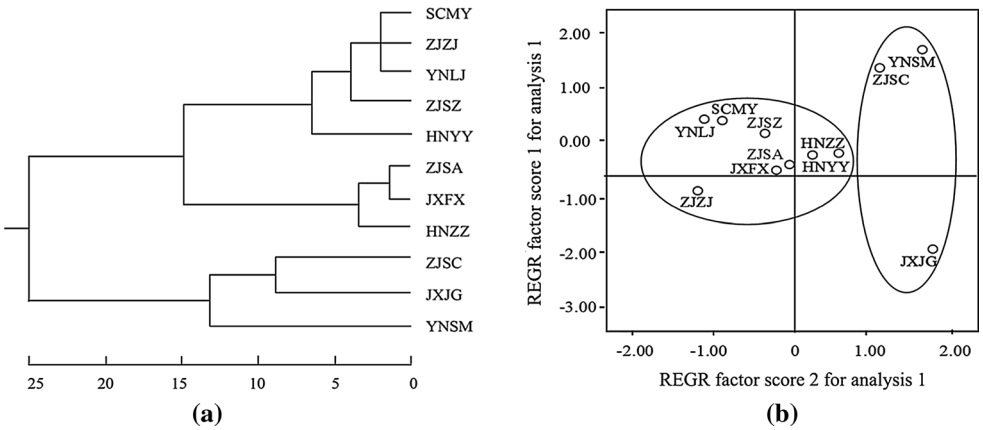

Fig. 3 UPGMA cluster analysis (**a**) and principal components analysis (PCA) (**b**) using taxol contents data for the 11 populations of *Taxus mairei*

Taxol content

The taxol content of *T. mairei* populations varied between 0.0069 and 0.0127 %. Population JXJG possessed the highest taxol content (0.0127 %), followed by populations YNSM (0.0122 %) and YNSM (0.0122 %). The lowest taxol contents were uncovered in populations SCMY (0.0076 %), YNLJ (0.0071 %) and ZJZJ (0.0069 %). A test of significance revealed significant differences in taxol content among populations (Table 1).

A UPGMA cluster analysis based on taxol content clustered the 11 populations into two groups. Group I consisted of populations sampled from Hunan (HNYY and HNZZ), Jiangxi (JXFX), Sichuan (SCMY), Zhejiang (ZJZJ, ZJSZ and ZJSA) and Yunnan (YNLJ) provinces, while Group II comprised the other three populations sampled from Jiangxi (JXJG), Yunnan (YNSM) and Zhejiang (ZJSC) provinces (Fig. 3a). This was further confirmed by the PCA plot (Fig. 3b).

ISSR and taxol content analyses revealed that populations JXJG and YNSM were highly genetically diverse and possessed high taxol contents, while populations YNLJ and SCMY were characterized by low genetic diversity and low taxol contents. Although these results suggest a possible correlation between genetic diversity and taxol content, the SPSS analysis failed to reveal significant correlations between taxol contents and PPB ($r = 0.36$; $P = 0.277$), h ($r = 0.329$; $P = 0.323$) or I ($r = 0.354$; $P = 0.286$). Analysis of the taxol content versus latitude or longitude indicated that the taxol content was significantly negatively correlated to population latitude ($r = -0.620$; $P = 0.042$). Mantel test suggested no significant correlation between taxol content differences and genetic distance ($r = 0.102$; $P = 0.275$).

Discussion

A meta-analysis of genetic diversity revealed that gymnosperms have high genetic variability, with an average PPB estimate of 70.9 % (Hamrick and Godt 1990). In our study, we uncovered an even higher species-level genetic diversity (PPB = 91.73 %), in line with previous findings of Ru et al. (2008) (PPB = 91.79 %), Zhang et al. (2009a) (PPB = 98.4 %) and Zhang et al. (2009b) (PPB = 98.89 %).

The high genetic diversity of *T. mairei* can be attributed to four major factors. Firstly, paleogeological and paleobiological studies revealed that *T. mairei* is a relict species whose origin can date back to the Tertiary glacial period. Due to its long lifespan and overlapping generations, considerable genetic variability may have been accumulated and conserved under various selection pressures during the evolutionary process. Hence, high intraspecific genetic diversity is exhibited in this plant (Tan and Chen 2006). Secondly, *T. mairei* is the most widely distributed taxon within the genus *Taxus*. The diversity of germplasm resources coupled with environmental heterogeneity could contribute to the accumulation of genetic variability (Yang et al. 2009). Thirdly, *T. mairei* is an anemophilous plant and reproduces by seed under natural conditions, both characteristics being beneficial for maintaining genetic diversity. Besides, it is a late successional species. As suggested by Hamrick (1996), competitive pressure of such species is mainly intraspecific, which may promote the formation of intraspecific genetic variability (Xi 1986).

POPGENE analysis revealed moderate genetic differentiation among *T. mairei* populations ($G_{ST} = 0.1697$); this estimate of G_{ST} was close to the average values estimated for gymnosperm species ($G_{ST} = 0.18$; Nybom and Bartish 2000), long-lived perennial herbaceous species ($G_{ST} = 0.19$; Nybom 2004) and widely distributed outcrossing species ($G_{ST} = 0.170$; Hamrick and Godt 1996). Meanwhile, the G_{ST} estimate obtained in our study approximated the value previously reported for *T. mairei* using RAPD-based data ($G_{ST} = 0.181$; Ru 2008), was slightly higher than the value for *T. mairei* using ISSR-based data ($G_{ST} = 0.1211$; Zhang 2009a), but substantially lower than the value for its congener *T. wallichiana* using cpDNA PCR–RFLP-based data ($G_{ST} = 0.694$; Gao et al. 2007).

Among-population genetic differentiation can be attributed to a variety of factors, e.g., genetic drift, breeding system, gene flow, habitat fragmentation and population isolation (Hogbin and Peakall 1999; Schaal et al. 1998; Slatkin 1987). And in our study, the moderate genetic differentiation among populations can be largely explained by gene flow, breeding system and habitat change. Typically, a homogenizing effect emerges at $N_m > 1$, in which case population differentiation is prevented (Wright 1931). In our study, strong gene flows occurred among the 11 populations ($N_m = 2.4480$), being sufficient to counteract genetic differentiation caused by genetic drift.

In general, long-distance gene flow, which occurs in plants with wind-dispersed pollen and animal-dispersed seeds, can counteract population differentiation across a wide geographical range (Loveless and Hamrick 1984). *T. mairei* is an anemophilous plant with extraordinarily high pollen dispersal ability. A steady pollen exchange occurs among populations. In addition, *T. mairei* seeds are enclosed in a clayey fleshy aril. At the seed maturity stage, the aril is red and has a sweet taste, attracting birds to eat the aril and spread the seeds. This process facilitates seed/gene flow among *T. mairei* populations (Deng et al. 2008).

As proposed by Mohapatra et al. (2009), one possible contributor to high within-population genetic differentiation coupled with low among-population genetic differentiation is the divergence from a common ancestral population. As a glacial relict species, it can be inferred that the extant populations of *T. mairei* are derived from formerly restricted ranges and may thus originate from the same source population(s). In addition, *T. mairei* has undergone habitat fragmentation in modern times; the effect of habitat fragmentation is still relatively limited, and has not resulted in substantially genetic differentiation among populations, as supported by Zhang et al. (2012).

Genetic distances between *T. mairei* populations were relatively small, but a significant correlation was found between geographic and genetic distances. UPGMA clustered the 11 populations into three groups, in line with their geographical distribution. Populations sampled from Zhejiang, Jiangxi and Hunan provinces (JXFX, JXJG, ZJSA, ZJSC, ZJZJ, ZJSZ, HNZZ and HNYY) formed one group; populations sampled from Yunnan Province (YNLJ and YNSM) formed a second group, and the third group comprised a single population from Sichuan Province (SCMY). Relatively large genetic distances were found between the third group and the former two, indicating the obvious occurrence of geographical distribution patterns among the populations. The observed patterns were similar to those of its congener *T. wallichiana* Zucc. We speculate that geographical barrier is among the major factors that contribute to the formation of the geographical distribution patterns of *T. mairei*. In contrast to the eastern regions, Yunnan Province is a relatively closed area surrounded by the Hengduan Mountains and the Himalayas. Meanwhile, the unique geographical features of the Sichuan Basin and the geographical barriers of the Daba Mountains separate Sichuan Province from the eastern region.

Taxol, a diterpenoid compound present in the bark, branches and leaves of *Taxus* plants, is a complex secondary metabolite that has been found to improve

microtubule polymerization and stabilize polymerized microtubules (Horwitz et al. 1993). The accumulation of secondary metabolites is influenced by a combination of genetic and environmental factors. Compared with primary metabolites, secondary metabolites have a stronger correlation with and correspondence to the environment. Previous studies have suggested the significant impacts of temperature, humidity and soil fertility on taxol biosynthesis (Yang et al. 2010; Su et al. 2012). Consistent with these reports, our study uncovered obvious taxol content differences among populations from different locations.

Although Mantel test revealed no significant association between taxol content and genetic variation, a significant, negative association was observed between taxol content and latitudes of populations. This result appeared to be supported by the UPGMA cluster analysis. Of the three basic geographic elements (i.e., latitude, altitude and longitude), latitude is supposed to have the greatest influence on temperature, with an average contribution of 44.4 % (Fang 1992). We thus deduce that the observed differences in taxol content among *T. mairei* populations are mainly due to temperature variations arising from latitude differences.

Considering the high intraspecific genetic diversity, loss of genetic diversity should not be a major contributor to this plant's endangered status. Other factors, such as anthropogenic destruction of natural habitats, poor seed production, low seed survival rates, poor natural population regeneration capacity and over-harvesting, may be responsible for its decline.

A combination of in situ and ex situ conservation approaches is commonly employed to protect endangered plants. Based on our findings, populations with high genetic diversity and taxol content, such as populations JXJG and ZJSC should be given a priority for in situ conservation. Meanwhile, samples from different populations should be collected for ex situ conservation. In particular, populations that are relatively genetically distant, such as populations from Sichuan and Yunnan provinces, can be used as cross parents so as to strengthen gene flow and maintain a maximum of genetic variability. By comparatively analyzing genetic diversity and taxol content, our results provide a theoretical foundation for the resource protection, utilization, cultivation and breeding of this valuable plant.

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