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Status and conservation of *Trigonobalanus doichangensis* (Fagaceae)[#]

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Abstract. *Trigonobalanus doichangensis* is a national rare and endangered plant of China. It is restricted to 4 sites in southwest Yunnan, China and 1 site in Chiang-Rai, northern Thailand. Investigations revealed that 4 community types are currently extant in Yunnan: *isolated individuals*, *sprouting woods*, *mono-dominant forest* and *co-dominant forest*. The habitats have been severely damaged and the populations there are facing a high risk of extinction. The adult phase of *T. doichangensis* is reached when a tree attains a height of about 4 m. The flowering and fruiting time varies slightly among populations and/or across the micro-habitats. In comparison with other fagaceous plants in the community, *T. doichangensis* has an inverse flowering and fruiting period from October through to May. Although microspore genesis and the development of male gametes are normal, the pollen does not germinate until at least 8 h culture. The highest germination rate was 37.8%. In addition, only 9.8% of the nuts contain well-developed seeds. Genetic variation analyzed with random amplified polymorphic DNA indicated that its total genetic diversity was 0.1600 and genetic diversity within populations was 0.0749, the coefficient of gene differentiation was 0.5320. Therefore, *T. doichangensis* has high genetic differentiation, a low level of genetic diversity and a poor gene flow compared to the other fagaceous species. It seems that habitat degradation, over exploitation and reproductive barriers, are most likely to be the factors threatening the species. However, it may be a combination of geographical catastrophic events, habitat deterioration, declining genetic diversity and physiological stress. Therefore, a practical conservation strategy for *T. doichangensis* is urgently needed.

Introduction

The fagaceous genus *Trigonobalanus* Forman shows many ancestral features of the family (Forman 1964; Crepet and Nixon 1989; Nixon and Crepet 1989) and was probably widely distributed (perhaps over the whole northern hemisphere)

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during the tertiary period or even before (Zhou 1992). The genus has only three extant species and *Trigonobalanus doichangensis* is the only one distributed in China (Hsu et al. 1981). *T. doichangensis* is most closely related to the genus *Quercus* L. (Zhou 1992) and has a high scientific value for studies on Fagaceae phylogeny, continental drift theory and global environmental changes.

Due to heavy exploitation for fuel wood, vegetation destruction in China and its restricted distribution, *T. doichangensis* is seriously threatened and is being pushed to the verge of extinction (Fu 1992). Hence, it was listed as a national rare and endangered plant in 1984 and has also been proposed as a second-ranked plant for national protection in China (Anon 1999). So far, its phylogeny (Wang and Zhang 1988; Nixon and Crepet 1989; Wu and Xiao 1989; Liao et al. 1998; Wang et al. 1998) and the community floristic elements (Li 1994) have been comprehensively studied. However, there are only a few recent reports on its conservation biology (Zhou et al. 2003; Zhou, 2003; Sun et al. 2004).

It is well known that a practical conservation strategy for an endangered plant is based on an understanding of the threats it faces. In this study we describe the status of the current distribution, population ecology, reproductive biology and genetic variation of *T. doichangensis*. Our goal was to provide comprehensive information relevant to the conservation and restoration of this species.

Methods

Trigonobalanus doichangensis is restricted to Thailand and south Yunnan in southwest China (Fu 1992). In this study all localities from recorded herbarium specimens, both from Thailand and China were used to determine its current distribution (including collection of vouchers and research materials). Based on this, the 4 known localities of *T. doichangensis* in Yunnan, China, Menglian, Lancang, Ximeng and Cangyuan, were regarded as 4 populations on which to carry out the comprehensive study. These 4 populations are situated in areas bordered by southeast Myanmar, within the southern part of the Tropic of Cancer. The climate belongs to the south subtropics type (EBYV 1987) and the soil is mainly red acid (Xiong 1987). Dai, Wa and Laku are the three indigenous ethnic groups in the region.

Threat category and the characteristics of population ecology

Data analysis from the general surveys and ecological plotting studies, determined the threat category by assessment of current distribution and population size following to the IUCN Red List Categories and Criteria (IUCN 2001). Investigations were performed in the flowering and fruiting season. Plot sizes of 10×20 m, 20×20 m, 30×30 m and 50×60 m were applied in accordance with

the population size. The characteristics recorded were: (1) Site conditions, (2) Plant height and crown size (m×m), trunk base diameter and diameter at breast height of all *T. doichangensis* in the plot, (3) Habitat quality, (4) The main accompanying higher plants and their abundance, and (5) Regeneration capacity of *T. doichangensis* from seeds.

Analysis of genetic diversity

The random amplified polymorphic DNA (RAPD) technique was applied in this study. Each locality was taken as a population, which, including that in Thailand, made a total of 5 populations. Young but fully expanded leaves of 19 or 20 individuals within each population were selected and harvested, dried quickly with silica gel and stored in a refrigerator at 4 °C.

DNA extraction

Total DNA was extracted from dried leaves using a modified CTAB method (Doyle and Doyle 1987). Modifications included incubation in CTAB at 65 °C for 1.5 h; a second extraction in chloroform–isoamyl alcohol; repeated washings in 70% ethanol without ammonium acetate; a final washing in 100% ethanol and the addition of RNase to the TE buffer. DNA was visualized on 1.0% agarose gels.

RAPD PCR amplification

A mixed DNA from 5 randomly selected individuals was used to screen 137 primers (Operon Technologies, Alameda, CA, USA). The primers that generated clear bands with high polymorphism were determined for PCR amplification on a T3 thermocycler (Biomotra, Goettingen, Germany) using a final volume of 20 μl. The reaction mix consisted of 2 ng of template DNA, 2.0 mmol/L $MgCl_2$, 0.5 μmol/l dNTP, 10×buffer, 2.5 μmol/L of primer and 2 units of Taq DNA polymerase. The reaction included 4 min of initial denaturation at 94 °C, followed by 45 cycles of 15 s of denaturation at 94 °C, 45 s of annealing at 36 °C, 90 s of extension at 72 °C, and a final extension step of 4 min at 72 °C. Subsequently 10 μl amplification products were visualized on 1.5% agarose gels after electrophoresis in TAE at pH 8.0 for 3 h. The gels were photographed with a GekDoc2000 (BIORAD) image analyzer (BIORAD, Hercules, CA, USA).

Data analysis

The individuals were scored for the presence (1) or absence (0) of amplified bands. The data matrix of RAPD bands was statistically analyzed using POPGENE (Yeh et al. 1997). The parameters of the genetic diversity and genetic structure are the percentage of polymorphic loci *P*(%), Shannon's index of diversity *(I)*, Nei's gene diversity *(h)*, the coefficient of gene differentiation (Gst) and an estimate of gene flow *(Nm)*.

Reproduction biology

Flowering and fruiting time of the 4 *T. doichangensis* populations in China were observed and compared. The floral organs at different developmental phases were fixed with FAA (50% ethanol/acetic acid/formaldehyde = 90/6/4 by volume) and preserved. The conventional paraffin slice thickness was 5–7 μm. These were stained with iron alum–haematoxylin, dyed with chrysoidine G, then observed and photographed using an Olympus BX-51 optical microscope.

Various levels of agar, sucrose, calcium nitrate and boric acid were selected and combined for pollen germination tests. Fresh pollen collected from each population was germinated at 25 ± 1 °C under darkness. The germination process was observed every 2 h and the final germination rate (%) was measured at hour 24.

Seeds (nuts) were harvested between April and June from each population and stored in a refrigerator at 4 °C. Seed structure and embryo development were observed and recorded under the ZS-PT Olympus anatomical lens. Seed germination was tested in the LRH-250-GS climatic box made in China. A conventional seed sowing was also conducted under Kunming climatic conditions in Spring.

Results

Current distribution and threat status

When it was originally described, *T. doichangensis* was only known from northern Thailand (Forman 1964). Later it was also found to occur in China (Hsu et al. 1981). Since it was recorded in China some field surveys and specimen identifications showed that it occurs in Menglian, Lancang, Ximeng and Mengla,Yunnan, China (Fu 1992). Recent investigations revealed that *T. doichangensis* still exists in fragmented populations of various sizes in Menglian (Vouchers: SWB 02T001∼020), Lancang (Vouchers: SWB02T021∼040), Ximeng (Vouchers: SWB02T041∼ 060) and Chiang-Rai Province in northern Thailand (Vouchers: SWB02T081∼100). In addition, the new localities in Cangyuan (Vouchers: SWB02T061∼080) were recorded, and it was shown that the species does not occur in the previously recorded locality in Mengla. Therefore, it can be concluded that the natural distribution of *T. doichangensis* is in the south and southwest Yunnan, China and in northern Thailand, and currently only 5 populations are extant.

Trigonobalanus doichangensis has been listed as one of the national second-ranked protected plants in the *No.4 Order of the Forest and Agricultural Ministry of China* (Anon 1999) because of its threatened status in the wild. It has also been recognized as a seriously threatened species because of the amount of vegetation destruction and cutting for fuel-wood (Fu 1992). A case study from the township market in Lancang, showed that the annual trading of

fuel-wood of *T. doichangensis* was about 1000–1400 m^3 . Also a natural stand close to the township is still cut continuously (Sun et al. 2004). At present the indigenous ethnic people of Dai, Wa and Laku are still causing great destruction to the habitats and using the wood resources, and as a result the populations are fragile and fragmented. *T. doichangensis* in China is facing a high risk of extinction.

Attributes of the communities

The populations in Yunnan, China showed different characteristics in their tree-age structure and floristic composition (Table 1). Four community types were recognized during the recent investigations.

(1) Type IsI (*Isolated individuals*): Individuals of *T. doichangensis* were often found in secondary woods, by roadsides, in mixed woods or farmland, and occasionally within the evergreen broadleaf forests. The forests surrounding

Table 1. Population characteristics of *Trigonobalanus doichangensis* in different communities.

Communities	SW	MDF	CDF		
Localities	Menglian	Lancang	Cangyuan		
			BTS	RBS	MDS
Plots					
Altitude (m)	1020	1450	1550	1590	1730
Slope	N, 30 degrees	W, 40 degrees	SE, 40 degrees	S, 30 degrees	S, 30 degrees
Coverage (%)	>90	>90	>95	>95	>95
Size (m^2)	800	400	3000	600	400
TD no. in the plot	195	140	41	101	118
TD no. per 100 m^2	24	35	~2	17	30
TD Avg. height (m)	5.1	5.6	17.9	6.8	14.4
Hst/Shst of TD (m)	13.5/0.2	9.5/0.3	35/0.4	32/0.28	20/0.2
% of TD ≥20 m	/	/	51	6	0.8
% of TD ≥4–20 m	59	73	44	55	92
% of TD 2–3 m	15	21	/	23	5
% of TD ≤ 1.0 m	26	6	5	16	3
% of dead TD	/	/	/	17	15
Avg. height of DP (m)	/	/	/	1.5~10	3.5~18
Accompanier no.	45	30	25	27	25
Main companions (TP)	①②③	②③	④⑤	④⑤	④⑤
Evaluation	HAD	PR	RSS	USD	MDSD

Notes: SW = Sprouting wood; MDF = Mono-dominant forest; CDF = Co-dominant forest; HTS = Huge-tree structure; RBS = Relatively balance structure; MDS = Mono-dominant structure; No. = numbers; TD = *Trigonobalanus doichangensis*; Hst = Highest; Shst = Shortest; Avg. = Average; DP = dead plants; TP = top layer; ① = *Vaccinium bracteatum*; ② = *Castanopsis echinocarpa*; ③ = *Castanopsis hytrix*; ④ = *Castanopsis calathiformis*; ⑤ = *Schima wallichii*; *HAD = heavily disturbed by human activities*; *PR = Population in recovery*; *RSS = relatively stable structure*; *USD = unstable structure in developing*; *MDSD = mono-dominant structure in developing*.

these individuals were mostly replaced by other native plant species, alien plant invaders, such as *Eupatorium adenophorum* (*Ageratina adenophora*) and *Eupatorium odoratum* (*Chromolaena odorata*), and agricultural crops. Occasionally, some seedlings or young trees appeared around scattered individuals inside the evergreen broadleaf forests. However, other plants such as *Castanopsis calathiformis*, *Castanopsis echinocarpa*, *Lithocarpus fenestratus*, *Schima wallichii* and *Anneslea fragrans* had already dominated in the vegetation. The formation of Type IsI is due to heavy cutting and vegetation destruction. The isolated individuals are found in the most endangered habitat and their genetic diversity is easily reduced by further human activities, grazing animals, farming and further biotic invasion.

(2) Type SW (*Sprouting woods*): Type SW is the result of fuel wood cutting by indigenous people. Investigations show that the indigenous ethnic groups of Dai, Wa and Laku are familiar with *T. doichangensis* and have realized that the tree can sprout easily after top-cutting and thinning and thus they have adopted the methods of 'alternate cutting or thinning cutting' for the primitive sustainable use of the tree as fuel wood. As a result of these practices, plants of *T. doichangensis* in these woods showed some unique characteristics in tree shape, tree height structure and associated floristic composition (Table 1). In this community the tallest *T. doichangensis* was about 13 m and the average height was around 5 m. Some 60% of *T. doichangensis* reached the reproductive phase and about 25.6% of the individuals were below 1 m in height. *T. doichangensis* was the dominant species in woods, and 50 species of accompanying higher plants were present. The most important trees were *Vaccinum bracteatum*, *Castanopsis hystrix, Lithocarpus fenestratus, Craibiodendron stellatum, Anneslea fragrans*, *Ternstroemia gymnanthera* and *Schima wallichii*. *Vernonia parishii, Arthraxon lanceolatus, Carex baccans* and *Zingiber striolatum* were the typical herbaceous plants. Among epiphytes, *Phymatodes lucida*, *Vanda coerulea* and *Eria pannea* were also commonly found on the trunk. As ontogenesis of *T. doichangensis* was prevented by cutting, most trees could not complete their natural growth and their reproductive capacity was relatively restrained.

(3) Type MDF (*Mono-dominant forest*): Plants of *T. doichangensis* in type MDF are found as small mono-dominated patches scattered in the secondary evergreen broadleaf forest. The tallest plant of *T. doichangensis* was about 10 m and the average height was around 5–6 m. Approximately 70% of the trees in the plots were mature with a height of ≥4 m. Around 20% of the plants were 2–3 m in height, while seedlings and young trees represented only 6% of the total population. Accompanying higher plants were represented by some 30 species and most of the woody species were the same as in Type SW, but there were far fewer herbaceous plants and epiphytes. *Osyris wightiana, Viburnum cylindricum, Broussonetia papyrifera* and *Phyllanthus emblica* were present in the community. *T. doichangensis* in Type MDF showed vigorous growth and a strong regenerative ability.

(4) Type CDF (*Co-dominant forest*): Plants of *T. doichangensis* in Type CDF often formed a mosaic of mono-dominant patches in the primitive evergreen

broadleaf forest. In this type of community, *T. doichangensis* grows naturally without destructive disturbance from human activities. Based on the tree height-grade Type CDF can be divided into 3 ranks. These are Big Tree Structure (BTS), Relatively balanced structure (RBS) and Mono-dominant structure of mature trees (MDS) (Table 1). In rank BTS over 50% of *T. doichangensis* were trees with a height of ≥20 m and approximately 90% of the plants were in the reproductive phase. Compared with the other 2 ranks BTS had a lower percentage of young trees and seedlings. In rank *RBS* only 6% of *T. doichangensis* were trees with a height of ≥20 m, while some 17% of the total were dead, and almost 60% of the plants were in the flowering and fruiting stage. Rank RBS had almost equal number of flowering and non-flowering individuals of *T. doichangensis*. Compared with the ranks of BTS and RBS, rank MDS had the highest number of flowering individuals and nearly 90% of them were in the reproductive phase. The percentages of trees over 20 m tall or less than 1 m in the MDS were the lowest, and around 15% of all trees with a height of 3–18 m were dead. Both ranks of RBS and MDS had more individuals per 100 m^2, however, both of them also had a certain percentage of plants that had died naturally. It can be inferred that *T. doichangensis* in the co-dominant forest is dynamic, and both types of RBS and MDS would develop into BTS with some individuals dying gradually because of strong competition for resources. The interrelationship of MDS ↔ RBS ↔ MDS may be the main characteristic for *T. doichangensis* in the primitive evergreen broadleaf forest. About 25 species of accompanying higher plant were found in CDF. Of these, *Castanopsis calathiformis*, *Lithocarpus echinotholus* and *Lithocarpus fenestratus*, were the most common in the upper canopy and *Castanopsis calathiformis* was the most competitive species with *T. doichangensis*. Other woody plants, such as *Olea rosea*, *Eriobotrya cavaleriei*, *Cinnamomum bejolghota*, *Phoebe macrophylla*, *Helicia nilagirica*, *Ternstroemia gymnanthera*, *Pyrenaria diospyricarpa*, *Pithecellobium clypearia*, and *Eurya groffii*, were also commonly found.

Genetic diversity and population genetic structure

One hundred and fifty seven bands from 100 to 2900 bp were generated by the 16 selected primers, with each primer producing 6–13 bands with an average of 9.81 bands. Eighty three of the total bands were polymorphic and accounted for 52.87% (Table 2). These findings show that genetic diversity at the species level was abundant. Table 2 also indicates that the percentages of polymorphic bands in both populations of Lancang and Chiang-Rrai were far lower than those in the 3 other populations of Cangyuan, Menglian and Ximeng. Therefore, the genetic variation within both populations of Lancang and Chiangrai was lower that that in others.

Table 3 shows that the effective number of alleles *(ne)*, Shannon' s index of diversity *(I)* and Nei' s gene diversity *(h)* were 1.2646, 0.2431 and 0.1595,

Table 2. The sequences of random oligonucleotide primers and the number of polymorphic bands.

Primers	Sequences	Total bands	Polymorphic bands
OPC05	TCGTCTGCCC	9	4
OPC14	TGCGTGCTTG	10	5
OPC15	GACGGATCAG	11	6
OPJ 09	TGAGCCTCAC	11	6
OPM02	ACAACGCCTC	6	0
OPM06	CTGGGCAACT	8	5
OPM13	GGTGGTCAAG	10	4
OPM15	GACCTACCAC	12	5
OPM16	GTAACCAGCC	13	7
OPM20	AGGTCTTGGG	10	4
OPN14	TCGTGCGGGT	12	9
OPN20	GGTGCTCCGT	7	3
OPS03	CAGAGGTCCC	11	6
OPV06	ACGCCCAGGT	10	9
OPV10	GGACCTGCTG	9	4
OPV15	CAGTGCCGGT	8	6

Table 3. The genetic diversity and genetic structure of *T. doichangensis*.

Population	*na*	*ne*	*H*	*I*	*P*/%	*Ht*	*Hs*	Gst	*Nm*
LC	1.1019	1.0538	0.0311	0.0472	10.19				
CHR	1.1911	1.0804	0.0513	0.0812	19.11				
CY	1.3185	1.1675	0.0985	0.1502	31.85				
ML	1.2930	1.1469	0.0911	0.1403	29.30				
XM	1.3312	1.1696	0.1024	0.1568	33.12				
Mean	1.5287	1.2646	0.1595	0.2431	52.87	0.1600	0.0749	0.5320	0.4398

Notes: *na*, observed number of alleles; *ne*, effective number of alleles Kimura and Crow (1964); *h*, Nei's (1973) gene diversity; *I*, Shannon's Information index; *P*, the percentage of polymorphic loci; *Ht*, gene diversity of species; *Hs*, gene diversity within populations; *Nm*, gene flow; Gst, coefficient of gene differentiation.

respectively, and the genetic variation of *T. doichangensis* is rather low (the effective number of alleles < 1.5000). As the species gene diversity (*Ht*) was 0.1600 and the gene diversity within populations (*Hs*) was 0.0749, the genetic variation within populations was lower than that between populations. Table 3 also indicates that *T. doichangensis* shows a very strong genetic differentiation between its populations because the coefficient of gene differentiation (Gst) reached 0.5320, more than 10 times that of other fagaceous plants (0.020–0.065) (Chen et al. 1997).

The analysis also indicated that *T. doichangensis* had a lower gene flow (*Nm* = 0.4398) (Table 3) compared with other fagaceous plants, such as *Castanopsis fargesii* (*Nm = 11.2645*) (Zhu et al. 2002), *Cyclobalanopsis glauca* (*Nm = 20.49*) (Zhu et al. 2002) and *Quercus aquifolioides* (*Nm = 2.66*) (Li et al. 1997). Thus, there is less gene movement between *T. doichangensis* populations compared to other plants in Fagaceae.

Microspore genesis and development of male gametes

Some 400 floral organs of *T. doichangensis* were sampled at different development stages from the 4 populations. The anther is 4-sporangiate and the anther wall formation conforms to the dicotyledonous type. The tapetum is of the glandular type and most cells are 2-nucleate. Cytokinesis at meiosis of microspore mother cells is simultaneous and tetrads are tetrahedral, occasionally decussate. Mature pollen grains are 2-celled. These characteristics are consistent with the result of Stairs's results on *Quercus* (Johri et al. 1992). The meiosis process of microspore mother cell in *T. doichangensis* is the same as the common angiosperms (Hu 1982).

These observations also showed that different anthers or different anther sacs of the same anther in the meiosis phase of the microspore-mother-cell showed asynchronism in *T. doichangensis*. Various authors (Whelan 1974; Li and Cao 1986; Huang et al. 2001) have given different opinions of this asynchronism. However, it seems certain that the asynchronism of the meiosis phase in the microspore-mother-cell is a universal phenomenon in angiosperms, and it does not relate to the normal development of anthers and the formation of fertile pollen. Therefore, the observations did not find any abortion or other abnormal phenomena in anther wall development, microspore genesis and male gamete formation in *T. doichangensis*.

Pollen germination in vitro and its abortion

Pollen germination started after 8 h of culture, and was completed at 24 h. Different combinations of agar, sucrose, boric acid and calcium nitrate could affect pollen germination, but the highest germination rate was only 37.8% (Table 4) and more than 63% of the pollen of *T. doichangensis* was aborted.

Pollen germination has rarely been reported in Fagaceae, but some major chestnut cultivars were tested (Xia et al. 1989). The pollen germination rate of *Castanea mollissima* was lower (31%) than that of its cultivars (60–78%), and the lower pollen germination of *C. mollissima* was one of the causes of its poor fruiting (Xia et al. 1989). The cause of pollen abortion in *T. doichangensis* may be complicated, and yet its lower pollen germination rate and the delayed germination (8 h after culture) may contribute to the lower fruiting percentage (9.8%) (Sun et al. 2004).

Ovule abortion and embryo development

In *T. doichangensis* the ovary has 3 locules with 2 ovules per locule. Observation on the ovary in transverse section showed that 6 ovules in each ovary were generated at an early stage, and at the developmental anaphase 1 of 2 ovules per chamber were well developed, while another one was aborted. Meanwhile, both axile and parietal placentas were observed, and thus

Table 4. Pollen germination of *T. doichangensis* on different media.

Medium code	Germination rate (%)
①	1.9
②	8.1
③	18.3
④	22.9
⑤	22.1
⑥	22.4
⑦	22.2
⑧	20.2
⑨	20.9
⑩	21.8
⑪	37.8
⑫	37.2
⑬	22.7
⑭	21.4
⑮	18.3

Notes: ① agar 7; ② agar 7 + sucrose 100; ③ agar 7 + sucrose 150; ④ agar 7 + sucrose 200; ⑤ agar 7 + sucrose 250; ⑥ agar 7 + sucrose 200 + boric acid 100; ⑦ agar 7 + sucrose 200 + boric acid 150; ⑧ agar 7 + sucrose 200 + boric acid 200; ⑨ agar 7 + sucrose 200 + boric acid 300; ⑩ agar 7 + sucrose 200 + boric acid 400; ⑪ agar + sucrose 200 + calcium nitrate 100; ⑫ agar 7 + sucrose 200 + calcium nitrate 200; ⑬ agar 7 + sucrose 200 + calcium nitrate 300; ⑭ agar 7 + sucrose 200 + calcium nitrate 400; ⑮ agar 7 + sucrose 200 + calcium nitrate 500 (All units are g/L).

T. doichangensis may show a transition trend from axile to parietal placentation. The ovule is anatropous and is enclosed by outer and inner integuments.

The following 3 phenomena were also observed in the young fruits: (1) Only 1 of the 6 ovules per ovary developed into an embryo and the others were aborted. The percentage of the one-ovule developed nuts was less than 10%; (2) In some fruits all the 6 ovules in the ovary were aborted and the ovary was lignified; (3) The ovules in some fruits seem to be developed but the embryos were membranous. It can be concluded that considerable embryo abortion occurs in *T. doichangensis*.

Flowering and fruiting

Investigations in the wild indicated that *T. doichangensis* becomes of reproductive age once its height reaches 4 m. The flowers are unisexual and plants are monoecious. Staminate flowers are often clustered on the rachis forming axillary catkins or occur terminally on leafless branchlets. Pistillate inflorescences are unbranched in the axils of the upper (younger) leaves and bear 1–3 flowers on distal parts of rachis.

The pollination mechanism in Fagaceae is complex. Flower characters in *Quercus* Subgen. *Cyclobalanopsis* show a transition from entomophily to anemophily (Kaul and Abbe 1989; Zhou 1992), and the upright or curved staminate inflorescence in genus *Trigonobalanus* may also represent such a

transition (Hsu et al. 1981). The staminate inflorescences of *T. verticillata* are erect and often branched, and are reminiscent of inflorescences found in subfamily Castaneoideae. And this, along with reports of odor in flowers (Soepadmo 1972) suggests the possibility that *T. verticillata* is an entomophilous species, as are the species of Castaneoideae such as *Lithocarpus densiflorus*, *Castanea sativa*, *C. mollissima* etc (Nixon and Crepet 1989). *T. doichangensis* and *T. excelsa* have lax staminate inflorescences like those of *Quercus*, which is generally accepted as anemophilous (Nixon and Crepet 1989). However, our observation on fresh male flowers of *T. doichangensis* under the stereo-anatomical lens, found that tiny beetles frequently visited the anthers to collect pollen. Therefore, we infer that these tiny beetles may also participate in the pollination of *T. doichangensis*.

The flowering time of *T. doichangensis* varies slightly between different populations and micro-habitats. However, the period of flowering and fruiting is generally from October to the following May, a period in which the weather is cool and wet (YWB 1983). This phenology is unique compared to most other plants in the community, which flower in Spring and fruit in Autumn; a factor that may also contribute to its low fruiting percentage.

Seeds and seed germination

The nut of *T. doichangensis* is normally called a seed and has a kilo-grain weight of 12.7 g. In fact, the seed is exoendosperm enclosed by a semi-transparent episperm and endocarp. *T. doichangensis* fruits well, but only 9–11% of the nuts contain well-developed seeds (with fertile embryos).

Nuts containing well-developed seeds germinated 4 d after culturing at various temperatures under darkness, and after 11 d all germination was completed. Lighting slightly stimulated the germination and the highest germination rate was around 63–73%. Epigeal germination has been reported in *T. doichangensis* (Nixon and Crepet 1989; Liao et al. 1998) and this was confirmed by sowing nuts in Spring at Kunming,Yunnan, China. Observations indicated that hypocotyls grew rapidly at 8 d and at 11 d, 2 or occasionally 3 cotyledons emerged from the compost. Germination in compost lasted around 20 d and the germination rate was close to that found in tests in the LRH-250-GS climatic box. Transplanted young plants have been growing well at Kunming (where the recorded minimum absolute temperature is −5.4 °C) for 3 years. And thus, *T. doichangensis* may have a broad climatic adaptability.

Discussion

Global threat category of T. doichangensis

Trigonobalanus doichangensis in China has been considered as an endangered plant for more than 10 years (Fu 1992). However, it was ranked as DD (Data

Deficient) in *The World List of Threatened Trees* (Oldfield et al. 1998) and its threat category has not yet been evaluated by using any version of IUCN Red List Categories and Criteria.

Based on our observations and data analyses, the threat category of *T. doichangensis* in China is proposed as EN (Endangered) [EN, B1a, B1b(i,ii,iii)] (IUCN 2001). It is therefore considered to be facing a very high risk of extinction in the wild, because: (1) Its extent of occurrence is estimated to be less than 5000 km^2, (2) Its populations are severely fragmented and (3) There is a continuing decline in the extent of occurrence, area of occupancy and quality of habitat. Judging by the limited distribution of the species in northern Thailand, and its only postulated occurrence in Myanmar, it is likely that EN will also be the Global threat category.

Identifying threats

Rarity and endangerment of a plant species may be due to intrinsic (related to the biology of the species) or extrinsic (environmental) factors (Rabinowitz 1981; Fiedler and Ahouse 1992) and anthropogenic habitat fragmentation has been widely cited as a major threat to biodiversity (Simberloff 1988). Destruction and disappearance of habitat are the major extrinsic factors threatening biodiversity (Pang et al. 2003; Jiang et al. 1997). Some remnant plant species, such as *Metasequoia glyptostroboides*, *Cathaya argyrophylla* and *Liriodendron chinense*, may maintain a present population in the 'endangered habitats' because large parts of their populations have disappeared due to historical events such as climate change (He et al. 1996). As a remnant species *T. doichangensis* may also be in an 'endangered habitat'. Its present habitat destruction and disappearance, caused by expansion of agricultural land and over cutting, are threatening its survival. Therefore, a very low gene flow among populations in *T. doichangensis* may certainly be due to its habitat degeneration and population isolation.

Reproductive barriers are among the main factors to threaten a plant species (Pan et al. 2003). *T. doichangensis* does not show any abnormal phenomena in anther wall development, microspore genesis and male gamete formation. It did, however, show serious ovule development abortion, the causes of which need to be studied. The low pollen germination, as well as the length of time for pollen to germinate and the inverse flowering and fruiting behaviour compared to other fagaceous plants in the population, show that *T. doichangensis* exhibits a reproductive barrier in seed development. In addition, its seed (nut) germination and seedling establishment need a moist environment and the establishment of young trees from seedlings needs comparatively sunny conditions (Sun et al. 2004). Evidently, the environment in the severely fragmented habitat is not ideal for seed germination and plant development into the reproductive phase.

Some rare species that existed in glacial refuges, have long survived in certain restricted habitats even though their populations are isolated and the habitats

are already fragmented (Li et al. 2003). However, long-term species survival depends on the maintenance of sufficient genetic variability within and among populations to accommodate new selection pressures brought about by environmental changes (Elena et al. 2003), and populations with low genetic variation have a high risk of extinction (Li et al. 2003; Ledig et al. 2002).The decline of seed viability of *Metasequoia glyptostroboides*, is caused by its poor genetic base and inbreeding, and its effective seed germination relies on suitable conditions (Liao and Zhou 1989). Accordingly, lower levels of genetic diversity and intense genetic differentiation in *T. doichangensis* will not be able to respond as well to changes in abiotic or biotic environmental conditions as can other fagaceous species. The low seed germination and plant establishment may also partly contribute to its present genetic status caused by habitat fragmentation and population isolation.

We may conclude that endangerment of *T. doichangensis* is not caused by a single factor. It seems that habitat destruction, over-exploitation and reproduction barriers, are the most likely factors. However, it may be a combination of historic factors such as climate changes, habitat degeneration, a poor genetic base and physiological stress. Therefore, a practical conservation strategy for *T. doichangensis* is urgently needed.

Conservation considerations

Protection and restoration of natural habitats is the best and cheapest method of preserving the biological diversity and stability of global ecosystem (Lande 1988). Undoubtedly, the long-term survival of *T. doichangensis* is dependent on habitat conservation. However, only 1 of the 4 extant populations of *T. doichangensis* in China has been legally preserved by government ownership and others are facing a high risk of habitat disappearance. Perhaps more than most endangered plant species in China, the unique characteristics of *T. doichangensi*s, population isolation and the complex community interaction, exemplify the importance of habitat preservation and *in-situ* conservation. Nevertheless, both *in-situ* and *ex-situ* measures are needed for preserving *T. doichangensis* and its genetic diversity, and following aspects should be particularly considered:

(1) Preservation of the habitat and population of *T. doichangensis* in Canyuan must be reinforced. The Canyuan population is the only 1 of the 4 populations in China which has been well protected inside a national natural reserve. Therefore, the population is a vital resource for further research into the origin and evolution, eco-biological characteristics and genetic realities of *T. doichangensis*. Furthermore, the population also has a great value in population restoration research.

(2) As *T. doichangensis* populations in Menglian, Lancang and Ximeng are still exposed to high habitat destruction and cutting for fuel wood, it is essential that new *in-situ* conservation sites in these area should be urgently planned.

These new sites will play an important role in habitat recovery and population restoration.

(3) Although cross-planting is often controversial in plant conservation planning, it is proposed that seedling cross-planting between different populations of *T. doichangensis* should be considered. At least, the potential impacts of this measure should be studied to determine if low gene flow would be enhanced and if overall diversity would be increased.

(4) As young *T. doichangensis* plants propagated from seeds can tolerate temperatures below −2 °C at Kunming (Sun et al. 2004), it may be cultivated for fuel wood and as a landscaping plant in northern parts of the Tropic of Cancer. However, the mixed planting of trees propagated from various populations is essential.

(5) *Ex-situ* efforts need to be undertaken to preserve genetic diversity and multiply specimens. This applies in particular to those populations outside of the well-protected national nature reserve. Ideally, *ex-situ* sites will be close to nature reserves or to botanical gardens. Propagation from seeds is preferred, since it would be the least detrimental to the extant populations and would include the widest range of genetic diversity. Optimally, seeds should be taken from many individuals from each of the populations.

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