

Karyomorphology of the endangered *Trigonobalanus doichangensis* (A. Camus) Forman (Fagaceae) and its taxonomic and biogeographical implications

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Cytological studies were carried out for the first time on five populations of *Trigonobalanus doichangensis* in China and Thailand. In all populations, the pattern of interphase nuclei was of the simple chromocentre type, the mitotic prophase were of the proximal interstitial type and chromosome numbers were $2n = 2x = 14$. Two B chromosomes were commonly observed at prophase and prophase–metaphase, but rarely at metaphase. Karyotype variation among the populations at the diploid level was limited, but there were some distinguishing cytological characters. Based on the comparison of all the available data on cytology, taxonomy, phytogeography and molecular systematics related to the genus *Trigonobalanus*, we recommend that the three species of *Trigonobalanus* comprise the subfamily Trigonobalanoideae and that the genus should not be segregated into three monotypic genera. © 2007 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2007, 154, 321–330.

ADDITIONAL KEYWORDS: B chromosomes – China – cytotaxonomy – *Trigonobalanoideae*.

INTRODUCTION

Trigonobalanus Forman, a genus in Fagaceae, includes three species: *T. verticillata* Forman from Sulawesi, Borneo and Malaysia, *T. doichangensis* (A. Camus) Forman from Thailand and southern China, and *T. excelsa* Lozano, Herno Camo & Henao from wet montane forests in Colombia, South America (Forman, 1964; Lozano, Hernandez & Henao, 1979; Hsu, Wang & Li, 1981). The genus has long generated considerable interest because of its hemispheric disjunction and its morphological characters, some of which are unique in Fagaceae. So far, the taxonomy and related botanical aspects of *Trigonobalanus* have been studied intensively, and there have been several recent studies on the conservation biology of *T. doichangensis* (Han

& Sun, 2004; Zeng & Sun, 2004; Sun, Zhou & Zhao, 2004; Sun *et al.*, 2006) and *T. verticillata* (Kamiya *et al.*, 2002).

The pollen morphology (Wang, Pu & Zheng, 1998), wood and seed anatomy (Liao, Gou & Ye, 1998), taxonomic status and phylogenetic relationships (Forman, 1964; Abbe, 1974; Lozano *et al.*, 1979; Hsu *et al.*, 1981; Jones, 1986; Nixon & Crepet, 1989; Jenkins, 1993; Li, 1996; Takhtajan, 1997; Zhou, 1999; Manos, Zhou & Cannon, 2001; Chen *et al.*, 2004), palaeobotany (Crepet & Nixon, 1989) and biogeography (Raven & Axelrod, 1974; Melville, 1982; Nixon & Crepet, 1989) of *Trigonobalanus* have been studied widely, but the systematic position of the genus is still disputed. This paper addresses the phylogenetic relationships and systematics of *Trigonobalanus* and other allied genera such as *Fagus* and *Quercus*, and the delimitation of Fagaceae (summarized in Table 1) in order to compare the three main taxonomic schemes. These are:

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Table 1. Comparison of the three main classification schemes of Fagaceae

Forman (1964)	Jones (1986)	Nixon & Crepet (1989)
Fagaceae	Fagaceae	Fagaceae
Fagoideae	Fagoideae	Fagoideae
<i>Fagus</i>	<i>Fagus</i>	<i>Fagus</i>
<i>Nothofagus</i>	Quercoideae	<i>Quercus</i>
Quercoideae	<i>Quercus</i>	<i>Trigonobalanus</i>
<i>Quercus</i>	Trigonobalanoideae	<i>Colombobalanus</i>
<i>Trigonobalanus</i>	<i>Trigonobalanus</i>	<i>Formanodendron</i>
Castaneoideae	Castaneoideae	Castaneoideae
<i>Lithocarpus</i>	<i>Lithocarpus</i>	<i>Lithocarpus</i>
<i>Castanea</i>	<i>Castanea</i>	<i>Castanea</i>
<i>Castanopsis</i>	<i>Castanopsis</i>	<i>Castanopsis</i>
<i>Chrysolepis</i>	<i>Chrysolepis</i>	<i>Chrysolepis</i>

Table 2. Localities, altitudes and voucher numbers of the investigated *Trigonobalanus doichangensis* populations

Population code	Locality	Altitude (m a.s.l.)	Voucher (KUN)
ML	China, Yunnan, Menglian	1100	SWB02T01-20
LC	China, Yunnan, Lancang	1500	SWB02T21-40
XM	China, Yunnan, Ximeng	1040	SWB02T41-60
CY	China, Yunnan, Cangyuan	1730	SWB02T61-80
CR	Thailand, Chiang Rai	1237	SWB02T081-100

1. *Trigonobalanus* and *Quercus* under Quercoideae of Fagaceae (Forman, 1964; Hutchinson, 1967; Abbe, 1974)
2. *Trigonobalanus* under Trigonobalanoideae of Fagaceae (Jones, 1986; Li, 1996; Takhtajan, 1997)
3. Three monotypic genera; *Colombobalanus* (*C. excelsa*), *Formanodendron* (*F. doichangensis*) and *Trigonobalanus* (*T. verticillata*) under Fagoideae of Fagaceae (Nixon & Crepet, 1989)

Owing to its rarity and its scientific importance in the evolution of Fagaceae, *T. doichangensis* has been regarded in China as a protected tree (Fu, 1992). In recent years, the floristic elements of community (Li, 1994), seed biology and seed germination strategy (Zhou, 2003; Zhou, Sun & Li, 2003), the natural population attributes, biological characters and conservation considerations (Sun *et al.*, 2004), reproductive biology (Zeng & Sun, 2004), and genetic diversity (Han & Sun, 2004; Sun *et al.*, 2007) have been studied in this species. Kamiya *et al.* (2002) reported genetic variability in 75 individuals of three *T. verticillata* populations in Malaysia. All of the above reports have stressed the great importance of the species and their biodiversity conservation.

Cytological data are essential in studies of plant evolution and diversity and in the solution of taxo-

nomic problems (Stebbins, 1971; Hong, 1990; Stace, 2000). Of the three species of *Trigonobalanus*, chromosome numbers are known only for *T. verticillata*, of which the basic chromosome number has been reported as $x=c. 21$ and the somatic numbers $2n = 40, 42$ and 44 (Hou, 1971). It is certain that further cytological data on *T. doichangensis* are essential to solve the taxonomic debate about the genus and also to provide valuable information for species conservation.

This paper will focus on: (1) counting chromosome numbers of the five extant *T. doichangensis* populations, (2) analysing the asymmetry of the karyotype in the different populations, and (3) discussing the systematic position of the genus *Trigonobalanus* based on all the available cytological, phytogeographical and molecular data.

MATERIAL AND METHODS

Seeds of the five *Trigonobalanus doichangensis* populations were collected in China and Thailand (Table 2; Fig. 1). All cytological observations were made from cells of root tips obtained from germinated seeds. Vouchers of all the individuals from which seeds were collected and the permanent slides are deposited in

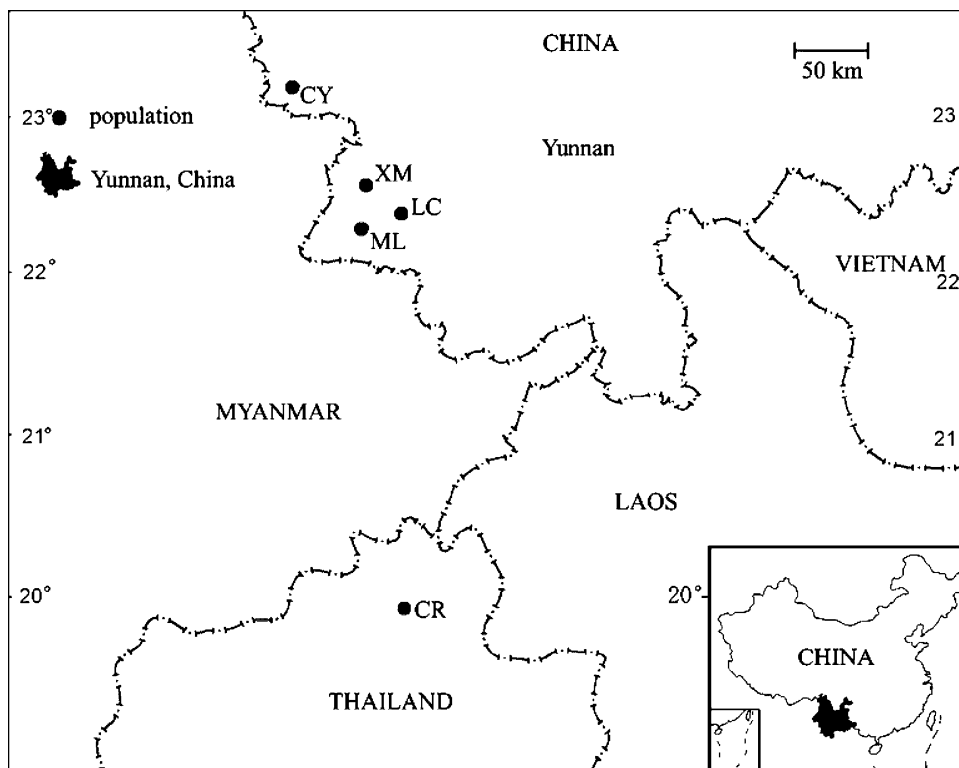


Figure 1. Map of the seed-collection sites of *Trigonobalanus doichangensis*. CR, Chiang Rai population; ML, Menglian population; LC, Lancang population; XM, Ximeng population; CY, Cangyuan population.

the Herbarium of the Kunming Institute of Botany (KUN). Root tips were pretreated in 0.002 mol L⁻¹ 8-hydroxyquinoline solution at 25 °C for 120 min, then fixed with Carnoy's fluid (absolute ethanol: glacial acetic acid = 3:1 by volume) at 4 °C for at least 30 min. The fixed roots were hydrolysed in a 50/50 mixture of 1 N HCl and 45% acetic acid at 60 °C for 1 min, stained with 1% aceto-orcein for 1 h and squashed for cytological observation. Slides were made permanent using the standard liquid nitrogen method.

Karyotypes of somatic chromosomes at metaphase were determined from at least ten well-spread metaphases in three or more different root tips from each population. The karyomorphological classification of the mitotic interphase nuclei and prophase chromosomes followed Tanaka (1971; 1977), the designation of the centromere position as median (m) and submedian (sm) followed Levan, Fedga & Sandberg (1964) and the asymmetry of the karyotype followed Stebbins (1971).

RESULTS

INTERPHASE NUCLEI

The interphase nuclei of all the five populations had the same pattern of chromatin distribution and,

according to Tanaka (1971, 1977), this could be categorized as the simple chromocentre type (Fig. 2).

PROPHASE CHROMOSOMES

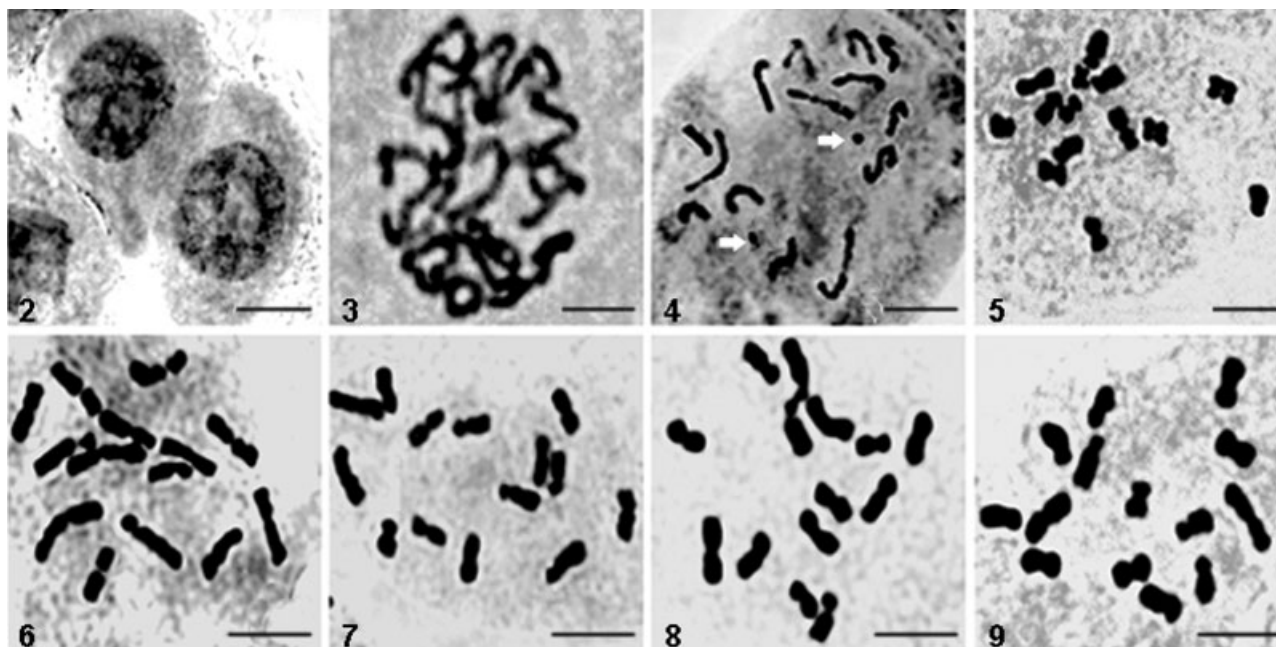
Heterochromatin and euchromatin segments were clearly visible at mitotic prophase in individuals of all populations. The heterochromatic segments were located in the deeply stained proximal regions, which indicated early condensation, while the euchromatic segments in the distal regions of chromosomes were lightly stained and extended, indicating late condensation (Fig. 3). Two B chromosomes were commonly observed at prophase and prophase–metaphase (Fig. 4), but rarely at metaphase. The prophase chromosomes of all the populations belonged to the proximal interstitial type.

CHROMOSOME COUNTS AND KARYOMORPHOLOGY

The base chromosome number of $x = 7$ ($2n = 14$) was observed in all of the populations. The karyomorphological characteristics (Table 3; Figs 5–11) are described as follows.

CR population

The karyotype formula was $2n = 14 = 12m(2SAT) + 2sm + 2Bs$. One pair was sm in centromere position



Figures 2–9. Cytological features of the five *Trigonobalanus doichangensis* populations. Fig. 2. Interphase nuclei of simple chromocentre type. Fig. 3. Prophase chromosomes. All populations in this study were of the proximal interstitial type. Fig. 4. Two B chromosomes at prophase–metaphase (arrows). Figs 5–9. Mitotic metaphases, all with $2n = 14$. Fig. 5. CR population. Fig. 6. LC population. Fig. 7. XM population. Fig. 8. ML population. Fig. 9. CY population. Scale bars = 10 μm .



Figure 10. Karyotypes of the five *Trigonobalanus doichangensis* populations, all with $2n = 14$. A, CR population; B, LC population; C, XM population; D, ML population; E, CY population.

(pair 6) and the other six were m. Both chromosomes of the first pair had satellites on the terminal regions of the short arms. The ratio of the longest to the shortest chromosome was 1.56. The karyotype asymmetry was classified as type 1A (Figs 5, 10A, 11A).

LC population

The karyotype formula was $2n = 14 = 14m(2SAT) + 2Bs$. All seven pairs were m in centromere position.

The first pair had satellites on the terminal regions of the short arms. The ratio of the longest to the shortest chromosome was 1.76. The karyotype asymmetry was classified as type 1A (Figs 6, 10B, 11B).

XM population

The karyotype formula was $2n = 14 = 12m(2SAT) + 2sm + 2Bs$. One pair was sm in centromere position (pair 5) and the others were m. The first pair had

Table 3. Karyomorphological parameters of the five *Trigonobalanus doichangensis* populations ($2n = 14$)

Pair no.	RL	AR	CI	Type	Pair no.	RL	AR	CI	Type
CR population					LC population				
1	8.99	1.27	44.05	m*	1	9.07	1.18	45.87	m*
2	8.27	1.30	43.48	m	2	8.58	1.69	37.17	m
3	7.91	1.20	45.45	m	3	7.35	1.31	43.29	m
4	6.83	1.11	47.39	m	4	6.86	1.55	39.22	m
5	6.12	1.13	46.95	m	5	6.62	1.45	40.81	m
6	6.12	1.83	35.34	sm	6	6.37	1.36	42.37	m
7	5.76	1.43	41.15	m	7	5.15	1.62	38.17	m
XM population					ML population				
1	8.81	1.07	48.31	m*	1	8.29	1.27	44.05	m*
2	7.95	1.15	46.51	m	2	8.29	1.25	44.44	m
3	7.39	1.36	42.37	m	3	7.61	1.30	43.48	m
4	6.82	1.40	41.67	m	4	7.61	1.56	39.06	m
5	6.82	2.32	30.12	sm	5	7.28	1.44	40.98	m
6	6.25	1.18	45.87	m	6	5.96	1.57	38.91	m
7	5.96	1.10	47.62	m	7	4.98	1.60	38.46	m
CY population									
1	11.07	1.21	45.25	m*					
2	8.21	1.09	47.85	m					
3	6.79	1.50	40.00	m					
4	6.43	2.17	31.54	sm					
5	6.07	1.13	46.95	m					
6	6.07	1.43	41.15	m					
7	5.36	1.33	42.92	m					

Abbreviations: RL, relative length; AR, arm ratio (L/S); CI, centromeric index (100S/TL); m, median region; sm, submedian region. *Chromosomes with satellites.

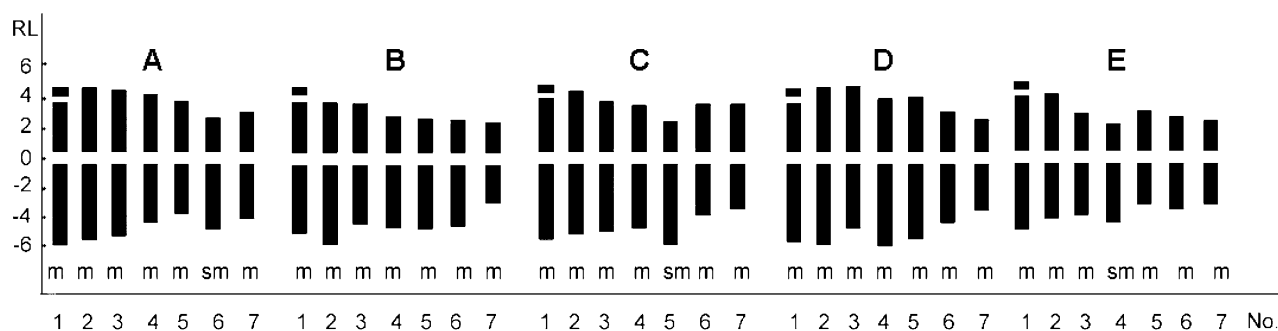


Figure 11. Karyotype ideograms of the five *Trigonobalanus doichangensis* populations. A, CR population; B, LC population; C, XM population; D, ML population; E, CY population. RL, Chromosome relative length; m, Median region; sm, Submedian region.

satellites on the terminal regions of the short arms. The ratio of the longest to the shortest chromosome was 1.48. The karyotype asymmetry was classified as type 2A (Figs 7, 10C, 11C).

ML population

The karyotype formula was $2n = 14 = 14m(2SAT) + 2Bs$. All chromosomes were m in centromere position.

The first pair had satellites on the terminal regions of the short arms. The ratio of the longest to the shortest chromosome was 1.66. The karyotype asymmetry was classified as type 1A (Figs 8, 10D, 11D).

CY population

The karyotype formula was $2n = 14 = 12m(2SAT) + 2sm + 2Bs$. One pair was sm in centromere position.

(pair 4) and the others were m. Two chromosomes of the first pair had satellites on the terminal regions of the short arms. The ratio of the longest to the shortest chromosome was 2.06. The karyotype asymmetry was classified as type 2B (Figs 9, 10E, 11E).

DISCUSSION

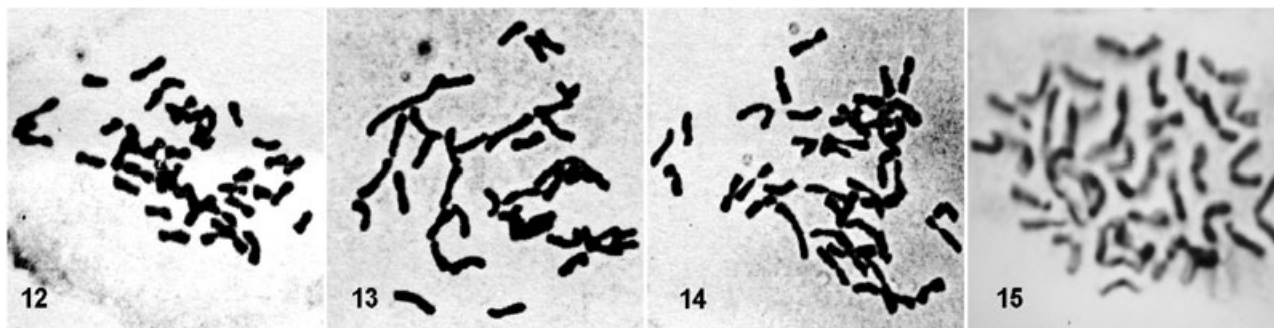
SYSTEMATIC RELATIONSHIPS WITHIN *TRIGONOBALANUS*

Hou (1971) reported that the base chromosome number of *Trigonobalanus verticillata* is $x = c. 21$ ($2n = 40, 42, 44$), and this has been cited widely in discussions considering the cytology of the genus. However, our observations on the five populations of *T. doichangensis* all indicated that the base chromosome number of the species is $x = 7$ ($2n = 14$). After carefully studying Hou's original article and the photographs therein (his chromosome numbers are dubious as a consequence of unclear illustrations; Figs 12–15) and comparing all records and references of *T. verticillata* stored in the Herbarium of the Royal Botanic Gardens, Kew, we propose that the mitotic number of $2n = 42$ for *T. verticillata* is likely the correct one. We may therefore conclude that *T. verticillata* is a hexaploid ($2n = 6x = 42, x = 7$) derived from the ancestral *T. doichangensis*. Certainly, the chromosome number of *T. verticillata* needs to be re-counted and *T. excelsa* should also be investigated if material can be obtained.

Unequivocal macrofossils of Trigonobalanoids (the possible ancestors of the modern genus *Trigonobalanus*) have been recorded from the Upper Eocene Baltic Amber, Upper Eocene of Europe, Palaeocene/Eocene and Oligocene of Tennessee (Crepet & Nixon, 1989). The distribution of the modern *Trigonobalanus* relative to putative fossil ancestors from Europe and eastern North America suggests that tropical amphipacific biogeographical connections spanned the Northern Hemisphere, with subsequent extinction

throughout most of this range in the Quaternary (Crepet & Nixon, 1989; Walter, 2000). Manos & Stanford (2001) suggested that the continuous distribution of *Trigonobalanus* was achieved most probably via the North Atlantic land bridges during the Palaeocene before the complete formation of the Turgai Strait (Tiffney & Manchester, 2001). Tiffney (2000) supported a Bering Land Bridge (BLB) exchange and the resulting amphipacific disjunctions involving thermophilic Fagaceae (Fig. 16).

Stebbins (1971) and Stace (2000) considered that almost all polyploids ultimately come from diploids. As all the five *T. doichangensis* populations are invariably diploid ($2n = 14$) and *T. verticillata* is hexaploid ($2n = 42$), we suspect that *T. doichangensis* might represent the basal lineage of the genus *Trigonobalanus*, which may have originated in south-eastern Asia (Zhou, 1999; Manos & Stanford, 2001). From there it possibly spread in a tri-directional emigration (Fig. 16): southwards to Celebes, Borneo and Malaya, generating the hexaploid *T. verticillata*; north-eastwards via the Bering land bridge to North America, then along the Rockies/Andes to Colombia of South America and generating *T. excelsa*; north-westwards to Europe and then via North Atlantic land bridges to North America. *Trigonobalanus* was probably widely distributed (perhaps over the whole northern hemisphere) during the Tertiary, or even earlier (Zhou, 1992). However, extreme climate changes in the late Tertiary period brought about habitat disappearance and a mass extinction of species. Certainly, such global changes could cause species of *Trigonobalanus* to be diminished or even extinguished in their distribution ranges. In this way *T. doichangensis* became a relict, currently restricted to a few scattered populations in South Yunnan and North Thailand (Sun *et al.*, 2006). Nixon and Crepet (1989) divided the genus *Trigonobalanus* into three monotypic genera, based mainly on the lack of synapomorphic characters of pollen morphology, cupule arrangement, staminate catkins, phyllot-



Figures 12–15. Mitotic metaphase chromosomes of *Trigonobalanus verticillata*. Fig. 12. $2n = 40$; Fig. 13. $2n = 42$; Fig. 14. $2n = 44$; Fig. 15. $2n = c. 42$. Figures 12–14 are from Hou (1971) and Fig. 15 is from an original photograph stored in the Herbarium of the Royal Botanic Gardens, Kew (photographed by W.B. Sun, 2004).

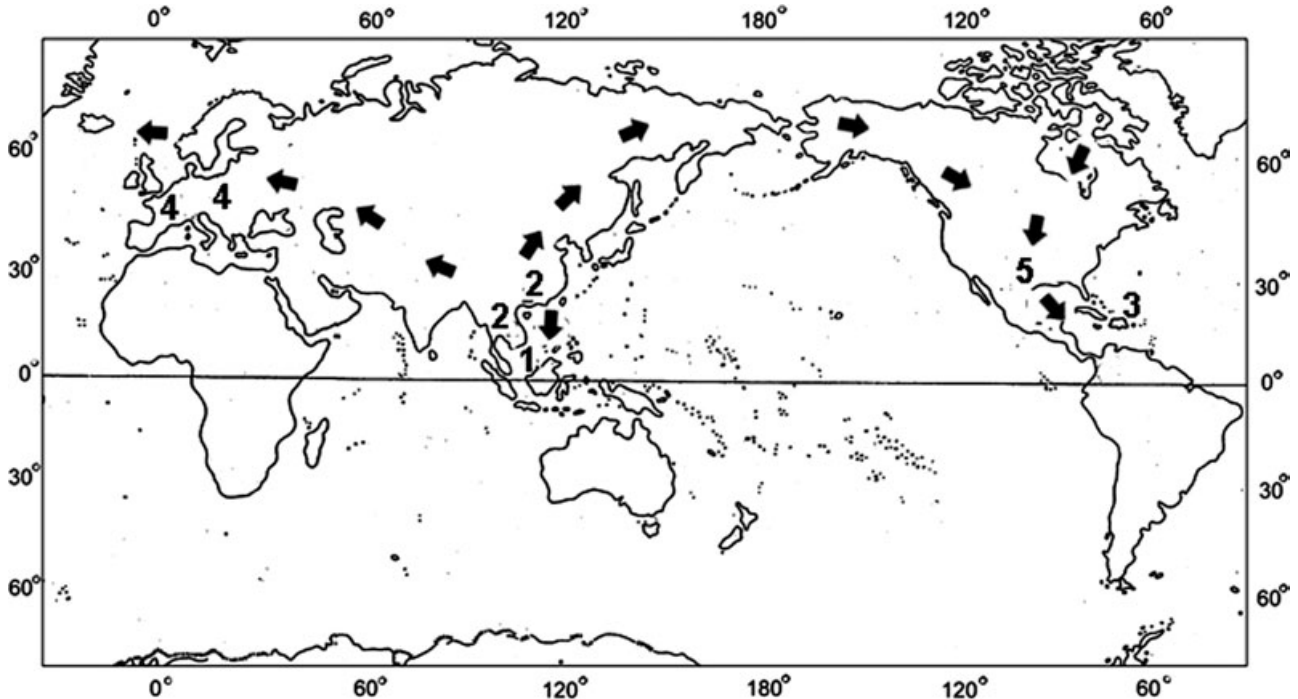


Figure 16. Distribution patterns of *Trigonobalanus* and its fossil relatives. 1. *Trigonobalanus verticillata*; 2. *T. doichangensis*; 3. *T. excelsa*; 4. *Trigonobalanopsis exacantha* and *Tr. rhamnoides*, the fossil species in Europe; 5. *Trigonobalanoidea americana* in America (cited and modified from Crepet & Nixon, 1989). Arrows indicate probable dispersal routes of the genus *Trigonobalanus*.

axy, buds and chromosome number (Table 4). However, the naked-bud character of *T. excelsa* may not be taxonomically important because it is related probably to its tropical habitat. In addition, Hou (1971) emphasized that leaf scars were alternate in the basal part of *T. verticillata*. Meanwhile, molecular data (Manos & Stanford, 2001) also indicated that *T. verticillata* was sister to *T. doichangensis* in Asia (Fig. 17A).

In conclusion, we consider that the three species of *Trigonobalanus* should not be treated as monotypic genera, on the basis of phytogeographical, cytological, fossil and molecular data (Crepet & Nixon, 1989; Manos *et al.*, 2001; Chen *et al.*, 2004), and other synapomorphy characters (Table 4) discussed in this article.

CHROMOSOME ASYMMETRY WITHIN *T. DOICHANGENSIS*

Stebbins (1971) stated that the formation of B chromosomes might represent a plant's adaptation to an extreme environment and their accumulation might diminish reproductive fertility. Undoubtedly, further studies on B chromosome formation are needed to confirm this hypothesis. However, as *T. doichangensis* is a relict species, its B chromosomes might have arisen as a result of the strong climate changes of the late Tertiary (Zhou, 1999). B chromosomes might also contrib-

ute to the low production of fertile nuts by the species (fewer than 10%, as reported by Sun *et al.*, 2006).

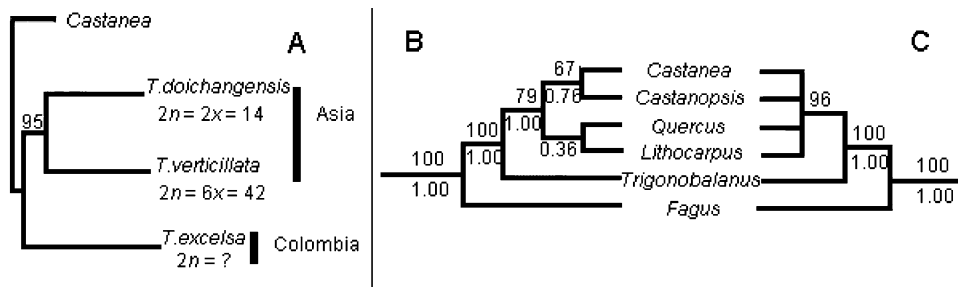
The karyotypes of the five *T. doichangensis* populations are all similar, comprising a uniform somatic chromosome number of $2n = 14 + 2B$ and having the same interphase nuclear and prophase chromosome morphology. Despite these similarities, there were some distinguishable cytological characters; for example, the asymmetry of karyotypes of the CR, LC and ML populations were of Stebbins' 1A type, while the populations of XM and CY were of Stebbins' 2A and 2B type, respectively. Furthermore, the LC and ML populations had only m chromosomes, while the CR, XM and CY populations had sm chromosomes in different positions (pair 6 in CR, pair 5 in XM; pair 4 in CY). Stebbins (1971) deduced that karyotype asymmetry and the original base chromosome numbers in angiosperms ($x = 7, 8, 9$) might be related to plant evolution and it seems that our study on *T. doichangensis* supports this hypothesis.

SYSTEMATIC POSITION OF *TRIGONOBALANUS* IN FAGACEAE

Fagaceae has been divided into four subfamilies: Fagoideae (*Fagus*), Quercoideae (*Quercus*), Trigonobalanoideae (*Trigonobalanus*), Castaneoideae

Table 4. Characters of the three species in the genus *Trigonobalanus* (modified from Nixon & Crepet, 1989). Asterisks indicate differences

Character	<i>T. verticillata</i>	<i>T. excelsa</i>	<i>T. doichangensis</i>
Cupules	Valvate	Valvate	Valvate
Valves	Free	Free	Free
Fruit number	(1-)3-7(-15+)	(1-)3-7(-15+)	(1-)3-7(-15+)
Cupule appendages	Scaly	Scaly	Scaly
Appendage arrangement	Transverse	Transverse	Transverse
Pistillate inflorescence	Branched	Branched	Branched
Staminodia	Present	Present	Present
Stigmas	Capitate	Capitate	Capitate
Fruit shape	Trigonous	Trigonous	Trigonous
Pistillode	Trichome tuft	Trichome tuft	Trichome tuft
Staminate flowers	Dichasial clusters	Dichasial clusters	Dichasial clusters
Germination type	Epigeal	Epigeal	Epigeal
Abortive ovules	Apical	Apical	Apical
Endocarp	Tomentose	Tomentose	Tomentose
Cupule arrangement	*Opposite or whorled	*Alternate	*Alternate
Pollen morphology	*Prolate	*Prolate	*Peroblate
Bud	*Scaly	*Naked	*Scaly
Staminate catkins	*Erect	*Lax (pendent)	*Lax
Phyllotaxy	*Whorled	*Alternate	*Alternate
Chromosome no.	* $2n = 6x = 42, x = 7$	Unknown	* $2n = 2x = 14, x = 7$

**Figure 17.** Molecular data of *Trigonobalanus* available in the literature. A, Cladogram for disjunct taxa within *Trigonobalanus*, Bootstrap values are based on 1000 pseudoreplicates, showing that *T. verticillata* is sister to *T. doichangensis* in Asia (cited and modified from Manos & Stanford, 2001). B, C, Single most parsimonious trees for Fagaceae, based on conglomerate-taxa matrix (cited from Chen *et al.*, 2004). B, Strict consensus tree of 1215 shortest trees based on molecular data; C, Bootstrap percentages are above branches, and the values below are Bayesian posterior probability. Trees B and C indicate that *Trigonobalanus* can be separated distinctly from other branches (genera) in Fagaceae.

(*Lithocarpus*, *Castanea*, *Castanopsis* and *Chrysolepis*), based on morphological observations (Jones, 1986). Nixon and Crepet (1989) divided Fagaceae into two subfamilies: subfamily Castaneoideae (*Castanea*, *Castanopsis*, *Chrysolepis* and *Lithocarpus*) and subfamily Fagoideae (*Fagus*, *Quercus* and *Trigonobalanus*, including *Colombobalanus* and *Formanodendron*) (Table 1). However, the base chromosome number of $x = 7$ (genus *Trigonobalanus*) has not been reported in any of the previous chromosome counts within Fagaceae ($x = 12$) (Sax, 1930; Duffield, 1940; Jaynes, 1962; Morawetz & Samuel, 1989; Nixon & Crepet, 1989; D'Emerico *et al.*, 1995; Cao & Zhou,

2000). Molecular data (Manos & Steele, 1997; Manos & Stanford, 2001; Manos *et al.*, 2001; Chen *et al.*, 2004) have indicated that there are three basic lineages in Fagaceae: *Fagus*, *Trigonobalanus*, and the remaining genera (Fig. 17). Therefore, our study supports the recognition of *Trigonobalanus* as a separate subfamily (Trigonobalanoideae) of Fagaceae.

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