CYTOGENETICS OF THE RARE AND ENDANGERED *TRIGONOBALANUS DOICHANGENSIS* (FAGACEAE) FROM NORTHERN THAILAND

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CHOKCHAICHAMNANKIT P & ANAMTHAWAT-JÓNSSON K. 2015. Cytogenetics of the rare and endangered *Trigonobalanus doichangensis* (Fagaceae) from northern Thailand. *Trigonobalanus doichangensis* is rare and endangered in Thailand. The species is found in northern Thailand, in hill-evergreen forests at high elevations. In this study, *T. doichangensis* was identified for the first time from Khun Mae Kuong Forest in Chiang Mai Province, and this discovery has extended the species distribution eastwards significantly. Samples were collected from trees of this species: twigs with fully grown leaves, fruits and flowers were used for taxonomic identification, whereas shoot and flower buds were used in the chromosome preparation. Results confirmed that *T. doichangensis* was diploid with chromosome number 2n = 2x = 14 (base number x = 7 confirmed with meiosis), whereas other Fagaceae species from the same forest (*Castanopsis*, *Lithocarpus* and *Quercus*) were diploid with 2n = 2x = 24. The karyotype analysis indicated a common genomic origin. The chromosomal mapping of highly repetitive 18S–25S and 5S ribosomal genes was performed by fluorescence in-situ hybridisation. *Trigonobalanus doichangensis* showed two pairs of 18S–25S rRNA loci (subtelomeric and paracentromeric) and one pair of 5S paracentromeric rRNA loci. The presence of linked 5S and 18S–25S ribosomal sites indicated an ancestral state of the genome of *T. doichangensis*.

Keywords: Chromosomes, fluorescence in-situ hybridisation, karyotype, ribosomal genes

INTRODUCTION

Fagaceae (beech family) includes 7–12 genera and 600–900 species distributed worldwide (Soepadmo 1972, Nixon 1997). Fagaceae dominates forests in the temperate and seasonally dry regions of the northern hemisphere, with a centre of diversity in tropical south-east Asia (Soepadmo 1972, Manos et al. 2001). In Thailand, this family comprises four genera with 119 indigenous species: *Castanopsis* (chinkapin chestnut, mostly evergreen, 33 species, ca. 28% of the world's estimate for this genus), *Lithocarpus* (stone oak, mostly evergreen, 56 species or about 19% of the world's estimate), *Quercus* (oak, mostly deciduous, 29 species or about 5% of the world's estimate) and *Trigonobalanus* (evergreen, one species) (Phengklai 2008). The Fagaceae family in Thailand is both abundant and diverse. South-East Asia, in particular Indo-China and northern Thailand, maintains the greatest assemblage and most primitive forms of *Castanopsis*, *Lithocarpus* and *Quercus* (subgenus *Cyclobalanopsis*) compared with other regions of the world (Soepadmo 1972).

Trigonobalanus is the smallest Fagaceae genus as it comprises only three species, two in south and south-east Asia and one in South America (Soepadmo 1972, Nixon & Crepet 1989, Phengklai 2008). The south Asian species *T. verticillata* is relatively diverse in its distribution. The species is found at medium to high altitudes in the Malay peninsula and Sumatra, on Mount Kinabalu in Sabah and in the Hose Mountains in Sarawak, Borneo. In addition, the species was recently identified in Hainan Island, South China (Ng & Lin 2008). On the other hand, the south-east Asian species *T. doichangensis* has very limited distribution and is found in a few locations in southern China (Yunnan) and in the northernmost part of Thailand, namely,

in the provinces of Mae Hong Son (including Doi Chang from where the species was first described in 1933 (Phengklai 2008) and Chiang Mai (e.g. Doi Inthanon, which is one of the Ultra Prominent peaks in South-East Asia). The South American species *T. excelsa* was described from wet montane Andean forests in Columbia as recently as 1979 (Manos et al. 2001). The discovery of *T. excelsa* raised the question of how such taxonomically related species could exist in both Asiatic and American subtropical floras. Van der Hammen and Cleef (1983) therefore investigated the phytogeographical history of this genus using paleobotanical and palynological evidence and were able to conclude that *Trigonobalanus* was a tropical amphi-pacific element in the north Andean forest, and had migrated to South America via the Panamanian land bridge of the Cenozoic.

All members of the genus *Trigonobalanus* have limited distribution and this raises concerns about the survival of the species. Global climate change is going to have serious impact on genetic resources in tropical forest trees and, due to the long-standing and ongoing human activities and urbanisation, tropical forests have been drastically reduced and fragmented (Bawa & Dayanandan 1998, Pautasso 2009). The Colombian *T. excelsa* has been declared a vulnerable species facing high risk of endangerment in the wild (Calderon 1998). As for *T. doichangensis*, Sun et al. (2006) considered it an endangered plant as the Chinese collections showed high genetic differentiation, low level of genetic diversity and poor gene flow. This species is rare and endangered to Thailand (Phengklai 2008). The species can be found in only a few locations, mainly among other Fagaceous trees and pines in hill-evergreen forests at high elevations above 1000 m. Its seedlings are restricted in distribution and acorns are always destroyed by fungi and insects. Germination of Fagaceae seeds ex-situ is always very poor.

The objective of the present study was to gain better understanding about the Thai *T. doichangensis* for conservation purposes, as no information about this species from Thailand other than the taxonomic description (Phengklai 2008) is available. We investigated 30 Fagaceae species in the genera *Castanopsis*, *Lithocarpus* and *Quercus* from Khun Mae Kuong Forest in Doi Saket District, Chiang Mai Province, using molecular and cytogenetic approaches (Chokchaichamnankit et al. 2007, 2008a, b). During the investigation, two *Trigonobalanus* trees were identified for the first time in this forest and became the subject of the study reported here. In the present study, 18S–25S and 5S rDNA were localised on mitotic chromosomes of *T. doichangensis* and then compared with other Fagaceae species from the same forest (Chokchaichamnankit et al. 2007, 2008a). Ribosomal fluorescence in-situ hybridisation (FISH) markers can be used for differentiating taxonomically related species and inferring evolutionary relationships among species of Fagaceae (Zoldos et al. 1999, Chokchaichamnankit et al. 2008a, Ribeiro et al. 2008, 2011).

MATERIALS AND METHODS

Study site and sample collection

The study site is Khun Mae Kuong Forest in Doi Saket District, Chiang Mai Province, one of the northernmost provinces of Thailand. The forest is an expansive, mountainous forested area of about 550 km^2 and is situated at approximately 18.93^o N and 99.21^o E. Geographically, the area lies in the westernmost part of Khun Tan Range, a mountain range that occupies the central position in northern Thailand and is considered part of the Phi Pan Nam Mountain System. In the forestry context, however, the area is considered the easternmost part of the Thanon Thong Chai Range (Doi Inthanon Range), which is the southernmost prolongation of the Shan Hills or Shan Highland that extends through Yunnan (southern China) to Myanmar and Thailand, linking to the Himalayas. The forests of Shan Highland are rich in Fagaceous species.

Our investigation began in 2003 when we studied the species and genetic diversity of Fagaceae in Khun Mae Kuong Forest (Chokchaichamnankit et al. 2007, 2008a, b). Six locations, representing three types of habitats, were selected, namely, hill-evergreen forest and hill-evergreen forest with pine, both at relatively high elevations (1000–1800 m), and dry-deciduous forest at altitudes below 800 m. From this work we identified 30 species out of

146 trees randomly selected: 12 *Castanopsis*, 11 *Quercus* and 7 *Lithocarpus* species. Towards the end of this investigation, we found *Trigonobalanus doichangensis* for the first time in Khun Mae Kuong Forest. New visits to the area were made between 2007 and 2010 and in the end we selected two trees for the present study. The trees, assigned with codes RD17 and RD30, were about 1 km apart and located at the same hill-evergreen forest with pine at an elevation above 1000 m. Leaves, flowers and acorns were used for taxonomic identification following Flora of Thailand (Phengklai 2008). For chromosome isolation, leaf buds and flower buds were collected from these two trees.

Chromosome preparation

In the field, samples of leaf buds were placed in iced water (4 °C) for 23–27 hours to arrest metaphases. After this, the samples were fixed in 3:1 mixture of absolute ethanol and glacial acetic acid. The samples were kept at -20 °C in the fixative until use. Chromosomes were prepared from fixed samples according to the protoplast dropping protocol of Anamthawat-Jónsson (2003). Each sample was digested for at least 3–4 hours at room temperature in 100 µL of enzyme mixture. Ten mL of this enzyme mixture contained 500 units of Cellulase Onozuka R10 and 280 units of pectinase from *Aspergillus niger* in a buffer (pH 4) containing 75 mM KCl and 7.5 mM EDTA. After digestion, the filtered protoplast suspension was treated with hypotonic solution (1.5 mL of cold 75 mM KCl) for 15 min at room temperature. The protoplasts were cleaned with fresh and cold fixative three to four times before being dropped onto microscopic slides, one drop on each slide. The slides were kept dry until use. After staining with fluorochrome 4, 6-diamidino-2-phenylindole (DAPI) the chromosome number was determined under 1000× magnification in an epifluorescence microscope. Images for karyotype analysis were captured with a digital camera using maximum resolution of 12 megapixels. Chromosome pairs were identified and arranged on the basis of chromosome length and arm-ratio (Levan et al. 1964). Karyotypes were constructed from at least five metaphases in each sample.

Fluorescence in-situ hybridisation (FISH)

FISH was performed according to Chokchaichamnankit et al. (2008a). Two ribosomal DNA probes were used for double-target FISH, namely, (1) clone pTa71, a 9-kb fragment from wheat, which contained part of 18S and the entire 5.8S and 25S coding region, together with non-transcribed spacers (Gerlach & Bedbrook 1979), was used as an 18S–25S rDNA probe and (2) clone pTa794, which contained a complete 410-bp *Bam*HI fragment of the 5S rRNA gene and spacer regions from wheat (Gerlach & Dyer 1980), was used as a 5S rDNA probe. The rDNA probes were labelled by nick translation using linearised cloned fragments as templates and with red or green fluorochrome-conjugated nucleotides in the labelling reactions. The red label used in this study was SpectrumRed-dUTP and the green label was Fluorescein-12-dUTP. The labelled probes were cleaned through Illustra ProbeQuant G-50 Micro Columns following the manufacturer's protocol.

Before performing FISH experiments, chromosome preparations were treated in fresh fixative for 10 min at room temperature, washed twice with 95% ethanol and air dried. The preparations were then treated with RNase-A (5 µg mL^{-1}) for 1 hour at 37 °C, Proteinase-K (4–10 µg mL^{-1} depending on the amount of cytoplasms) for 20 min at 37 °C and paraformaldehyde (4% w/v) for 20 min at room temperature. In FISH experiments, 50 ng of each of the 5S and 18S–25S rDNA probes, which were labelled in different fluorescent colours, were applied to a chromosome preparation together with 50% formamide, 20% dextran sulphate, 2× saline sodium citrate buffer (SSC) and 0.5% sodium dodecyl sulfate. The probe and slide were denatured together at 89 °C for 20 min in a PTC-100 FISH thermocycler after which hybridisation was allowed to take place overnight at 37 °C. Post-hybridisation washing steps included a stringent wash in 0.1× SSC at 60 °C for 15 min. Chromosomes were stained again with DAPI and examined in an epifluorescence microscope using appropriate filters. Images were captured at 1000× magnification. Fluorescent signals of rDNA FISH on metaphase chromosomes were analysed both in terms of number of sites and physical position on chromosomes.

RESULTS

Trigonobalanus doichangensis from Khun Mae Kuong Forest

The discovery of this rare Fagaceae species *T. doichangensis* from Khun Mae Kuong Forest in Doi Saket District, Chiang Mai Province, revealed for the first time that the natural distribution of *T. doichangensis* is farther east than that is currently recorded in floras, at least 100 km farther north-east of Doi Inthanon, the peak which is about 70 km north-east of Doi Chang. Khun Mae Kuong Forest has been protected as a national forest reserve since 1982 as part of the Huai Hong Khrai Royal Development Study. One of the main objectives of this project is the conservation of watersheds and reforestation by natural means. Today the forest has very much recovered. The area is among the richest in natural species diversity with regard to Fagaceae (e.g. Chokchaichamnankit et al. 2008b).

Fagaceous plants in Thailand and Malaysia can be identified to genus based essentially on flowers and acorns, while leaves and other vegetative characteristics confirm the identification (Soepadmo 1972, Phengklai 2008). Acorns of *Castanopsis* are usually covered by spiny cupules, whereas acorns of *Lithocarpus* and *Quercus* are mostly or partly covered by non-spiny cupules (Chokchaichamnankit et al. 2008b). Acorns of *Trigonobalanus* are unique in that they are strongly longitudinally trigonous, as the name implies. One of the Thai common names for *T. doichangensis* is ko sam liam which means triangular nut.

Based on the two trees identified in the present study, *T. doichangensis* from Khun Mae Kuong Forest (Figure 1) is an evergreen tree, 10–15 m high, with dark brown and scaly bark (Figure 1a). The leaves are spirally arranged, entire and elliptic (Figure 1b), 6–12 × 2–5.5 cm, base cuneate, apex acute, green and shiny on the upper surface but pale on the lower, midrib and veins prominent on the lower surface. Inflorescences (Figures 1b–d) are 10–12 cm long, simple in axils of leaves, male inflorescences suberect and puberulous throughout, female inflorescences with catkin erect. Male flowers are sessile and in clusters, perianth campanulate, 6-lobed, stamens 6, anthers ovoid. Female flowers are often in clusters of 3, perianth as male, adnate to ovary at base, staminodes 6 and styles 3, stigmata capitate. Fruit (Figure 1d) when mature is about 1.5 cm long and 1 cm in diameter. The cupule is sessile, saucer-shaped, irregularly lobed, completely covered with brown hairs, normally bearing one to three nuts. The nut is prominently triangular ridged. Botanical features of *T. doichangensis* from Khun Mae Kuong Forest are in accordance with the species description in *Flora of Thailand* (Phengklai 2008).

Chromosome number, karyotype and ribosomal gene mapping

The somatic chromosome number of both trees of *T. doichangensis* from Khun Mae Kuong Forest was 14 (Figures 2a and b). From the male flower bud, 7 bivalents were formed during the first meiotic division (Figure 2c), indicating that the base number of this species is 7. The species is therefore diploid with 2n = 2x = 14. All species in the genera *Castanopsis*, *Lithocarpus* and *Quercus* from this forest are also diploid but have the base number 12 (forming 12 bivalents in meiosis), hence 2n = 2x = 24 (Chokchaichamnankit et al. 2007).

Metaphase chromosomes of *T. doichangensis* were relatively small, from about 3 µm in size for the smallest pair of chromosomes up to 6 µm for the largest homologous pair (Figure 2d). The karyotypic arrangement (Figure 2d) revealed notable similarity in the chromosome morphology within the chromosome complement in this species. All chromosomes were considered metacentric, based on the arm ratios data (Table 1). The karyotype of *T. doichangensis* in the present study is highly symmetrical (asymmetry type 1A) according to Stebbins (1971), in contrast to the karyotypes constructed from 18 species of *Castanopsis*, *Lithocarpus* and *Quercus* from Khun Mae Kuong Forest (Chokchaichamnankit et al. 2007). This asymmetry sorting revealed to some extent a genus-specific pattern, in which *Quercus* was essentially type 2A and *Lithocarpus* 2B, though *Castanopsis* was variable in this respect.

The FISH mapping of ribosomal genes on chromosomes of *T. doichangensis* from Khun Mae Kuong Forest revealed four sites (two pairs) of 18S–25S rRNA genes and two sites (one pair) of 5S rRNA genes (Figure 2e). One of the two pairs of 18S–25S rDNA sites was paracentromeric or intercalary on homologous chromosomes no. 2,

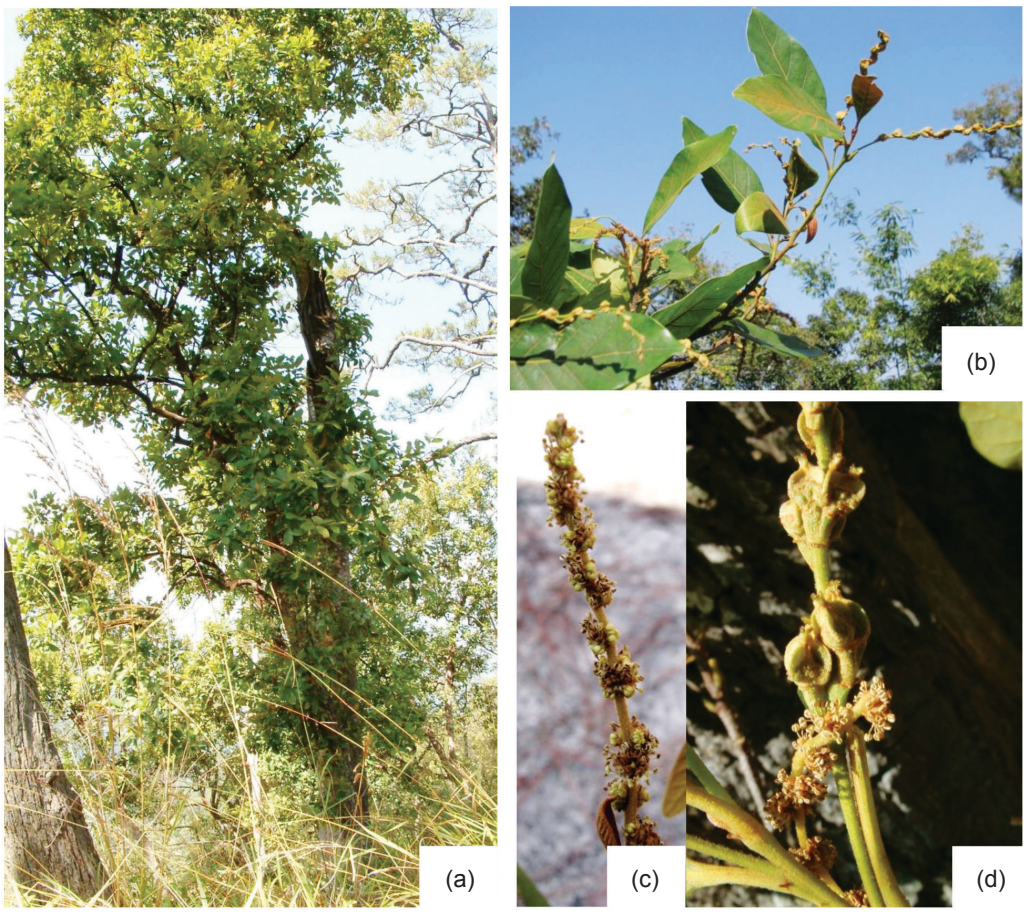

Figure 1 *Trigonobalanus doichangensis*—(a) Tree ID no. RD 17 from Khun Mae Kuong Forest, 10–15 m in height; (b) leaves single, elliptic, 10 cm × 5 cm, base cuneate, apex acute, female inflorescences apparent; (c) male inflorescence 10–12 cm long; (d) fruits, trigonous, 1.5 cm long and ca. 1 cm in diameter

whereas the other pair was sub-terminal (sub-telomeric) on chromosomes no. 3 (Figure 2f and Table 1). This subtelomeric locus coincided with the nucleolar organising region of satellite chromosomes (results not shown). The pair of 5S rDNA was paracentromeric, adjacent to the 18S–25S locus on chromosomes no. 2 (Figures 2e and 2f).

Most of the 15 Fagaceae species in the genera *Castanopsis*, *Lithocarpus* and *Quercus* from this forest also show four 18S–25S rDNA sites (two pairs, one of which is subtelomeric on the satellite chromosome pair) and two 5S rDNA sites (one pair, paracentromeric locus) (Chokchaichamnankit et al. 2008a). In this respect, the ribosomal gene map of *T. doichangensis* is similar to those of other Fagaceae species. The ribosomal gene maps among Fagaceae species, however, differed in the occurrence and position of the second 18S–25S locus, which was expected as the ribosomal genes in the second locus are usually inactive, and hence are more predisposed to evolutionary changes. Based on the second 18S–25S locus, the genome of *T. doichangensis* was significantly more similar to those of *Quercus* and *Lithocarpus* than to *Castanopsis*. In *Castanopsis*, the 18S–25S and the 5S genes were localised on three different chromosome pairs, i.e. the second 18S–25S locus was often found as a minor site on a different chromosome pair from that which contained the 5S locus.

DISCUSSION

Trigonobalanus doichangensis from Khun Mae Kuong Forest was diploid with 2n = 2x = 14, whereas other Fagaceae species from this forest

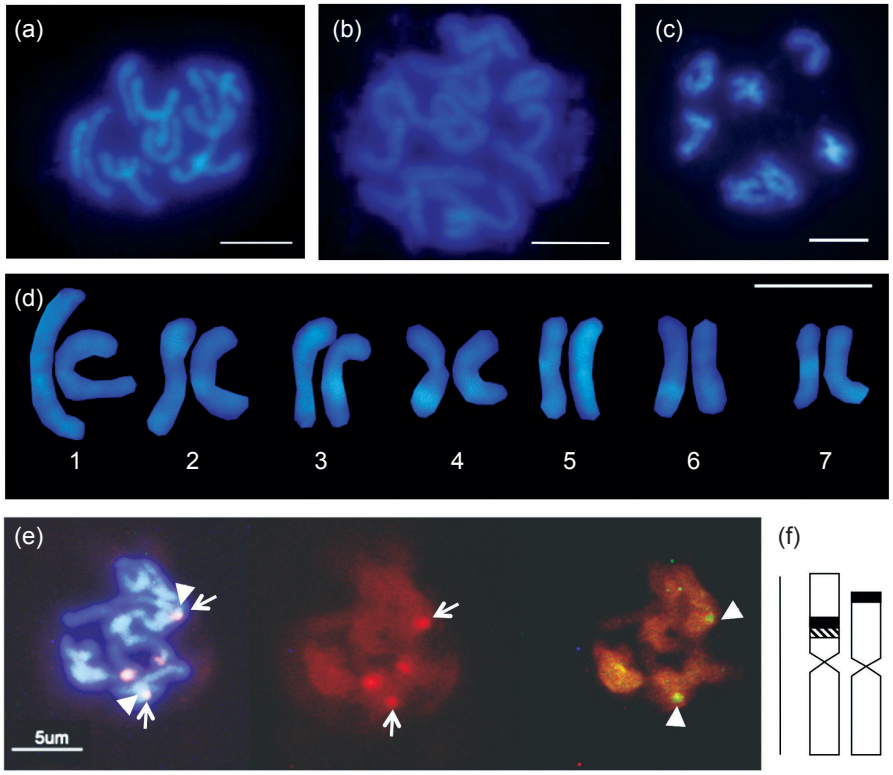

Figure 2 Cytogenetics of *Trigonobalanus doichangensis*—(a and b) mitotic metaphases (c) meiotic metaphase showing 7 bivalents, (d) karyotype showing the chromosome number 2n = 2x = 14, (e) fluorescence in-situ hybridisation (FISH) of mitotic metaphase chromosomes showing two pairs of 18S–25S rDNA sites (arrow) and one pair of 5S rDNA sites (arrowhead) and (f) ideogram showing relative positions of the 18S–25S rRNA loci (black) and the 5S rRNA locus (hatch box); all scale bars = 5 µm

Table 1 Karyomorphology, chromosome arm ratios and location of 5S and 18S–26S ribosomal genes in *Trigonobalanus doichangensis*

Chromosome pair no.	Arm ratio	Chromosome type	rDNA localisation
1	1.07	m	
2	1.40	m	5S & 18S–26S
3	1.25	m	18S–26S (SAT)
4	1.25	m	
5	1.00	m	
6	1.00	m	
7	1.33	m	

Ratio to determine chromosome type:1.00–1.70 = metacentric chromosome, 1.71– 3.00 = submetacentric chromosome, 3.01–7.00 = acrocentric chromosome and 7.01–α = telocentric chromosome; m = metacentric, SAT = satellite and is the nucleolar organising region bearing chromosome

(*Castanopsis*, *Lithocarpus* and *Quercus*) were diploid with 2n = 2x = 24. The difference was in the base numbers. Seed samples of *T. doichangensis* collected from four locations in Yunnan, southern China and from one unknown location in Chiang Rai Province, northern Thailand, produced root tip metaphases from which 2n = 14 was obtained (Chen et al. 2007). The same 2n number was reported for *T. doichangensis* from China (Han & Sun 2005). The base number x = 7 was proposed for *T. verticillata* (Chen & Sun 2010), although for a long time the base number for this species was accepted as x = ca. 21 because chromosome numbers of *T. verticillata* were reported as 2n = 40, 42 and 44 (Hou 1971). The cytogenetic study showed that seedlings of *T. verticillata* that was grown in a botanical garden from the collection in Hainan, China, turned out to be diploid with 2n = 2x = 14, whereas the material from Fraser's Hill in Malaysia produced hexaploid seedlings with 2n = 6x = 42 (Chen & Sun 2010). No report on the chromosome number of the Colombian species *T. excelsa* can be found. The conclusion based on the two species of *Trigonobalanus* investigated thus far is that its base number is 7, and in this respect *Trigonobalanus* is unique among Fagaceae genera worldwide.

Fagaceae is one of the families of woody angiosperms that maintain high stability in their chromosome numbers. Numerous chromosome number searches (via www.tropicos.org) have produced single common chromosome number for Fagaceae, i.e. 2n = 2x = 24 (base number x =12), and this chromosome number characterises all major genera including *Fagus*, *Quercus*, *Castanea*, *Castanopsis* and *Lithocarpus* (Mehra et al. 1972, Ohri & Ahuja 1991, D'Emerico et al. 1995, Chokchaichamnankit et al. 2007). The only exception in Fagaceae is evidently *Trigonobalanus*, which has the 2n number 14 (x = 7). Base numbers of modern woody genera have most probably evolved by ancient polyploidy, and the original base numbers of angiosperms, both woody and herbaceous, are x = 6 and 7 (Stebbins 1971). Therefore, the base number x = 7 in *Trigonobalanus* is likely a relic of ancient base numbers.

Analysis of karyotypic symmetry points in the same direction, i.e. that *Trigonobalanus* may be an ancestral genus of Fagaceae. In the plant kingdom as a whole, symmetrical karyotypes are usually primitive and the predominant trend is from symmetry to greater asymmetry (Stebbins 1971). The most common mechanism towards asymmetry or heterogeneous karyotype is a shift in centromere position, for example by centromeric inversion. The present study showed that the karyotype of *T. doichangensis* from Khun Mae Kuong Forest was highly symmetrical (asymmetry type 1A) and that the complement consisted of only metacentric chromosomes. Karyotypes of *T. doichangensis* from Yunnan are of similar types, mainly 1A (Chen et al. 2007). Compared with other Fagaceae species, karyotypes of *T. doichangensis* are more symmetrical, or less asymmetrical, than those of other species, implying that *T. doichangensis* is more primitive or ancestral in its chromosome evolution.

The ribosomal gene map of *T. doichangensis* from Khun Mae Kuong Forest is more similar to those of *Quercus* and *Lithocarpus* than to *Castanopsis* from the same forest. The difference lies in the 18S–25S rRNA gene maps. The chromosomal location of 5S rRNA genes, on the other hand, is the same in all four genera—it is always paracentromeric and usually on the second largest metacentric chromosome pair (compared with Chokchaichamnankit et al. 2008a). This is to be expected, as it has been shown by numerous studies that 5S ribosomal gene maps tend to be highly conserved and are therefore useful as FISH markers for comparative identification of plant groups at the genus or higher taxa levels, for example in the studies of forest tree genera and families (Ribeiro et al. 2008, Goryachkina et al. 2013). The 5S ribosomal gene map established in the present study also appeared to be the same as that identified among Fagaceous plants in the genera *Quercus* and *Castanea* from Europe, North America and east Asia (Zoldos et al. 1999, Ribeiro et al. 2011). Based on the 5S rDNA localisation by FISH, *Trigonobalanus* and other Fagaceae genera probably have the same genome origin.

However, ribosomal FISH maps of the 18S–25S rRNA genes have been found to be variable, which can be expected. The genes encoding the 18S-5.8S-25S ribosomal RNA are highly repetitive and consist of thousands of copies in ample excess of that required to sustain ribosomal synthesis (Gerlach & Bedbrook 1979). Due to this reduced constraint, the rDNA copy number in a given genome can change in a short evolutionary time and such drive could

generate species–specific patterns of ribosomal gene maps. FISH maps of the 18S–25S rDNA can be even more specific when co-localising with the 5S rDNA (Taketa et al. 2001, Ribeiro et al. 2008, Barros e Silva et al. 2013). This is the case with *T. doichangensis* and species of *Lithocarpus* and *Quercus* (subgenus *Cyclobalanopsis*) from Khun Mae Kuong Forest, whereas in *Castanopsis* from this forest and most other Fagaceae species from Europe and North America all three 5S and 18S–25S rDNA loci are on separate homologous pairs of chromosomes (Zoldos et al. 1999, Ribeiro et al. 2011). The 5S and 18S–25S co-localisation had probably occurred via some form of chromosomal rearrangement, e.g. translocation and paracentric inversion, or by transposition and amplification. Linked 5S and 18S–25S rDNA sites are known to be highly conserved (Barros e Silva et al. 2013), implying an ancestral state. A breakdown of such linkage could also happen, e.g. by duplication of one of the sites and posterior deletion of the linked site, and this implies a derived state (Carvalho et al. 2005). FISH mapping of rDNA in other plant groups has revealed an association between karyotypic divergence and geographical distribution of species, with Asiatic karyotypes being ancestral or primitive whereas European and American species tend to possess more derived, evolutionarily younger or phylogenetically more advanced karyotypes, for example in wild barley (*Hordeum*) (Taketa et al. 2001), orchids (*Paphiopedelum*) (Lan & Albert 2011), fruit and forest trees (*Citrus*) (Carvalho et al. 2005) and *Larix* (Goryachkina et al. 2013).

The present study has provided karyotypic and molecular cytogenetic evidence which supports that *Trigonobalanus* is phylogenetically ancestral to other Fagaceae genera. The characters of *Trigonobalanus* are common to the other genera in the family, but there are unique characters that may have diverged much earlier than the other Fagaceous genera (Forman 1966, Nixon & Crepet 1989). The great antiquity and relictual nature of *Trigonobalanus*, which is based on cladistic analyses of morphological characters, reproductive traits, molecular and genomic data, is well documented (Manos et al. 2001, Li et al. 2004, Kremer et al. 2012). On the other hand, information about genetic relationships among individual species of *Trigonobalanus* and in relation to their closest relatives is very limited.

CONCLUSIONS

From this investigation of the molecular cytogenetics of *Trigonobalanus* and, together with the karyotype analysis, we have obtained insights into the relationship between *T. doichangensis* and other Fagaceae from the same forest in northern Thailand. The results support the notion that *Trigonobalanus* is phylogenetically ancestral in the family Fagaceae and indicate that the species *T. doichangensis* is closely related to Fagaceae species from tropical South-East Asia, including the Asian *Lithocarpus* and the *Quercus* subgenus *Cyclobalanopsis*. *Trigonobalanus doichangensis* is a rare and endangered species in Thailand and China and, therefore, needs urgent monitoring and protection.

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