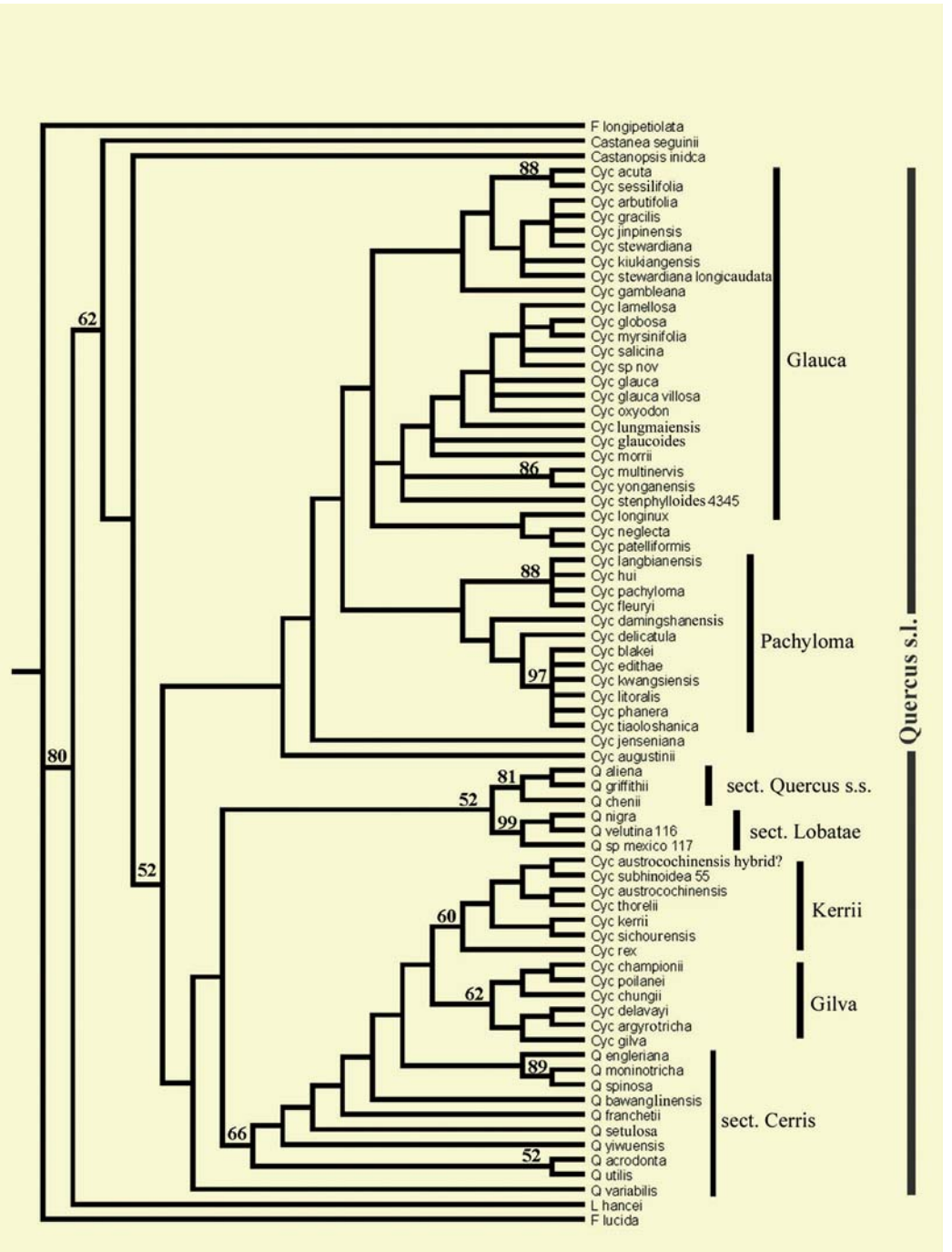

Figure 4/ Strict consensus tree of 600 MP cladograms from ITS (CI = 0.545; RI = 0.803). Bootstrap proportions using MP are indicated above branches (discussion, p. 55).

Taxonomy and Systematics of *Quercus* Subgenus *Cyclobalanopsis*

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ABSTRACT

Quercus subgenus *Cyclobalanopsis* is one of the dominant woody plant groups in E and SE Asia, but comprehensive studies on its systematics and taxonomy are limited. In this study, we compared the leaf epidermal and acorn features of 52 species in subgenus *Cyclobalanopsis* and 15 species from *Quercus* subgenus *Quercus*. We also studied molecular phylogeny using DNA sequences from the nuclear ribosomal internal transcribed spacer (ITS) region and the chloroplast *psbA–trnH* and *trnT–trnL* regions. Both the leaf epidermal and acorn features indicated five morphologically distinct groups in *Cyclobalanopsis*: 1) Gilva group (fused stellate trichomes with compound trichome base); 2) Kerrii group (with fasciculate trichomes, and radicles emerging from the basal seed scar; 3) Pachyloma group (papillae on epidermal cells, but glabrous when mature and densely yellowish woolly on the cupule walls); 4) Jenseniana group (lamellae mostly fused to the cupule wall with only the rims free); 5) Glauca group (appressed-lateral-attached trichomes). Molecular phylogeny suggested 4 major clades in *Cyclobalanopsis* corresponding approximately to the morphological groups: Kerrii clade, Gilva clade, Pachyloma clade and Glauca clade; but the phylogenetic relationship of the 4 main clades is not resolved, nor is monophyly of *Cyclobalanopsis*. The stable morphological features that can be applied to delimit the species in *Cyclobalanopsis* are discussed.

Keywords: *Quercus* subgenus *Cyclobalanopsis*, oak phylogeny

Introduction

The genus *Quercus* s.l. is the biggest genus in *Fagaceae* with about 500 species worldwide (Frodin and Govaerts, 1998). The genus is widely distributed throughout the Northern Hemisphere (Nixon, 1993) and is one of the most economically important in temperate and subtropical areas (Camus, 1934-1954). The taxonomy and systematics of genus *Quercus*, especially for the species distributed in North and South America and Europe, have been well studied both at species level (Nixon, 1993; Nixon, 1997; Nixon, 1997; Manos et al., 1999; Manos and Stanford, 2001; Bellarosa et al., 2005; Oh and Manos, 2008; Denk and Grimm, 2010) and population level (Huang et al., 2002; Petit et al., 2002; Petit et al., 2002; Ainsworth et al., 2003; Lin et al., 2003; Shih et al., 2006; Neophytou et al., 2008). Based on cladistic analysis of morphological data, two subgenera are recognized: *Cyclobalanopsis* and *Quercus*. *Cyclobalanopsis* has also been recognized as an independent genus in some local floristic studies (Hsu and Sun, 1983; Hsu et al., 1985; Huang et al., 1999). Within subgenus *Quercus*, cladistic analysis of morphological data imply three sections: *Lobatae* (red oaks), *Protobalanus* (intermediate oaks), and *Quercus* (white oaks, including section *Cerris*) (Nixon, 1989; Nixon, 1993). This subdivision of *Quercus* s.l. was generally accepted in many taxonomical works (Nixon, 1997). However, molecular phylogenetic studies based on ITS have suggested that section *Cerris* forms a clade sister to all other oaks in subgenus *Quercus* (Manos et al., 1999; Manos and Stanford, 2001; Manos et al., 2001; Pearse and Hipp, 2009). Alternatively, phylogenetic reconstructions based on single-copy nuclear genes *CRABS CLAW* and ITS plus the nuclear ribosomal intergenic (IGS) spacer region suggested two major clades in *Quercus* s.l.: a New World clade that includes section *Protobalanus*, section *Lobatae* and section *Quercus* s.s.; and an Old World clade, comprising subgenus *Cyclobalanopsis* and section *Cerris* (Oh and Manos, 2008; Denk and Grimm, 2010). However, phylogenetic relationships within *Quercus* s.l. are still not well resolved since the bootstrap value of the node of the main sections and subgenus is generally missing or very low (54–66) (Oh and Manos, 2008; Denk and Grimm, 2010), and less is known about the tropical and subtropical oak lineages from E Asia and SE Asia.

Subgenus *Cyclobalanopsis* is restricted to subtropical and tropical regions in SE Asia, with 90–150 species (Luo and Zhou, 2000; Luo and Zhou, 2001; Deng, 2007). Based on cupule and vegetative features, subgenus *Cyclobalanopsis* was divided into 27 series (groups) (Camus 1934–1954). Later, Menitsky (1984) established 8 sections in subgenus *Cyclobalanopsis* based on features of the style and leaf architecture. However, neither taxonomic system for subgenus *Cyclobalanopsis* has been generally accepted, as they are based largely on variable characters rather than stable, discrete character states (Frodin and Govaerts, 1998). On the other hand, reproductive structures in *Fagaceae* exhibit high homoplasy (Manos et al., 2008; Oh and Manos, 2008) and thus offer few clues on the phylogeny of subgenus *Cyclobalanopsis*. Molecular data provide an independent test of morphological homologies to gain insight into the evolutionary history of this oak group. In the present study we investigated the phylogeny of subgenus *Cyclobalanopsis* and of other taxa in *Quercus* s.s. to: 1) explore the phylogeny of subgenus *Cyclobalanopsis* using two genomes (nuclear and plastid); and 2) evaluate the consistency between the molecular phylogenetic trees of cpDNA and ITS and the morphologically-based subdivisions of oak species in subgenus *Cyclobalanopsis*.

Materials and Methods

Materials

Leaf materials were collected from both wild populations and cultivated plants. 45 species from *Quercus* subgenus *Cyclobalanopsis* and 15 species from subgenus *Quercus* (with 9 from section *Cerris*, 3 from section *Quercus* s.s. and 3 from section *Lobatae*) were studied. One species each from *Lithocarpus*, *Castanopsis* and *Castanea* and two species from *Fagus* were included in phylogenetic construction. *Fagus* was used as an outgroup to root the tree on the basis of previous phylogenetic studies within *Fagaceae* (Manos et al., 1999, 2001, Oh and Manos, 2008).

Methods

1. Molecular phylogeny

DNA extraction and experiment setting. Leaf materials were dried in silica gel in the field. Genomic DNA was extracted following standard plant CTAB protocols (Doyle and Doyle, 1987) with slight modifications. Chloroplast *psbA-trnH* and *trnT-trnL* regions and the internal transcribed spacer (ITS) regions were selected as molecular markers. All PCR reactions were conducted using Takara rTaq DNA polymerase (Takara, China) in a Bio-Rad T100 thermal cycler (Bio-Rad, USA). The ITS region was amplified using primer sets ITS1 and ITS2 (Bellarosa et al., 2004) following Manos et al., (1999). ITS fragments for sequencing were obtained either directly from purified PCR products or by cloning. Purified PCR products of ITS regions that could not be sequenced directly were immediately cloned using the TA Cloning Kit (Takara, China). Plasmids were purified using a modified alkaline lysis method. Transformation efficiency was assessed by PCR using the ITS1 and ITS2 primers. Five positive clones for each sample were sequenced. Methods for sequencing *trnT-trnL* and *psbA-trnH* followed Huang et al., (2002) and Pei et al., (2011) respectively. Purified clones and PCR products were all sent to a professional laboratory (Sangon, Shanghai, China) for sequencing. Double-stranded sequences were assembled and edited using SEQUENCHER 4.01 (Gene Codes Corp., Ann Arbor, MI, U.S.A.).

2. Morphological features

Morphological data from leaf epidermal materials and acorn features were obtained from Deng (2007) and Luo and Zhou (2001). Taxonomically significant features were compared.

3. Data analysis

Data filtering. Several previous studies have reported the existence of nonfunctional, paralogous ITS sequences in *Fagaceae* (Coleman, 2003; Goertzen et al., 2003; Bellarosa et al., 2005; Ma and Zhou, 2006). Since our aim was to understand general differentiation patterns in *Quercus* s.l., all potentially pseudogeneous sequences were filtered prior to the analysis. Three criteria were applied to identify functional ITS copies: 1) minimal-length variation across the spacers and high levels of sequence conservation in the 5.8S gene; 2) modest amounts of sequence divergence with clades and among the entire sample, and 3) general “taxonomic sense” of preliminary results.

For multiple different ITS clones obtained from the same sample, we first ran the cladistic analysis. If those clones from the same sample were clustered in a clade with high bootstrap value then one clone would be chosen randomly to represent the ITS sequences of this sample. The clones from the same samples not clustered in a clade were excluded from phylogenetic tree construction.

Outgroups and rooting. *Fagus* species were chosen as outgroup taxa on the basis of previous phylogenetic studies within *Fagaceae* (Oh and Manos, 2008). *Castanea*, *Castanopsis* and *Lithocarpus* were included in the analysis (Manos et al., 2001; Oh and Manos, 2008)

Alignment and phylogenetic analysis. Optimal multiple alignment of ITS, *psbA–trnH* and *trnT–trnL* was obtained with CLUSTAL W in MEGA5 (Tamura et al., 2007), adjusted manually and checked by eye. Secondary structure models were helpful to resolve ambiguous alignments, as already reported in previous studies (Štorchová and Olson, 2007; Hao et al., 2010). To increase the homology of the matrix, we applied the phylogenetic information from the folding structure (the arms and loops) of ITS2 and *psbA–trnH* to improve the alignment. Ambiguous polystructures were excluded from further analysis.

Parsimony analyses were performed with PAUP*4.0b1 (Swofford, 2002). Heuristic searches were run with default options. All characters were weighted equally. The gaps were excluded for analysis. In regions where demonstrably different gaps showed partial overlap, the character was scored as missing in the appropriate cells of the supplemental binary matrix. Bootstrap (Felsenstein, 1985) resampling was performed (1000 replicates), using TBR branch-swapping on 100 random taxon-addition replicates per bootstrap replicate and MULTREES option in effect under parsimony criterion.

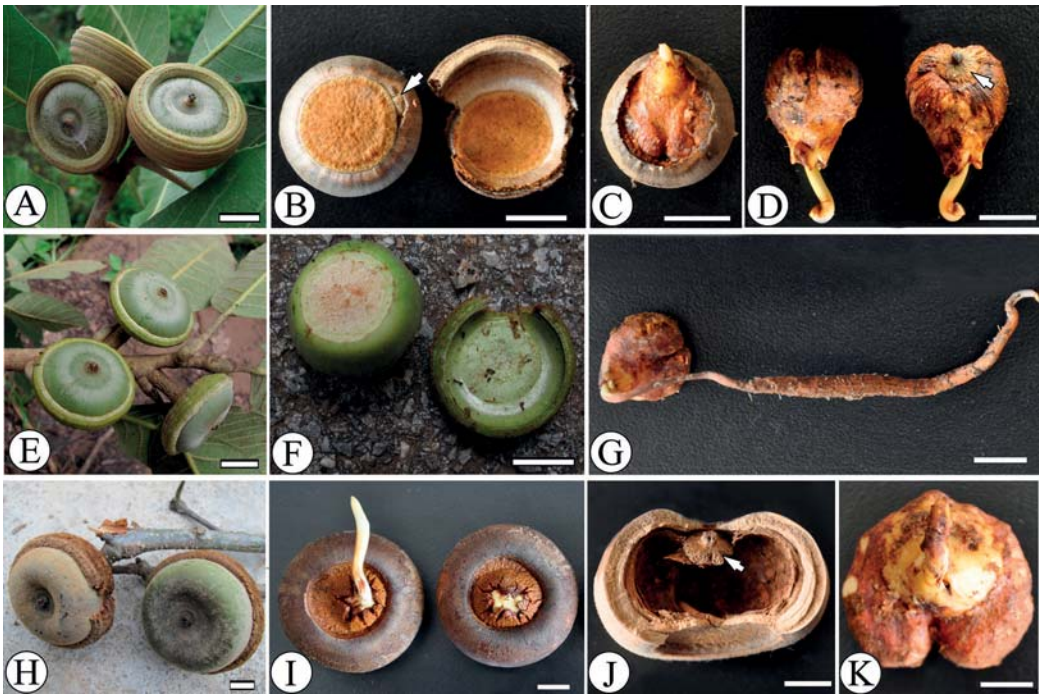

Figure 1/ Acorns of species in the Kerrii group.
(A-D) *Quercus austrocochinchinensis* bar=1 cm: (A) Fresh acorns; (B) Acorns and cupule; (C) Basal germination; (D) Elongated radicle / (E-G) *Quercus kerrii*, bar=1 cm: (E) Fresh acorns; (F) Acorn and cupule; (G) Germinated acorn showing the heart-shaped cotyledon and elongated radicle / (H-K) *Quercus rex*, bar=2 cm: (H) Fresh acorns; (I) Basal germination; (J) Transverse section of the acorn; the arrow shows the thick, fibrous, plug structure beneath the style base; (K) Germinated acorn showing the heart-shaped cotyledon.

Results

Morphological comparison

The acorn and cupule features offered limited information for grouping species in subgenus *Cyclobalanopsis*. In most species of the subgenus no discrete features from reproductive structures were found except for the heart shaped cotyledon, the radicle emerging from the basal seed-scar and the fibrous plug structure at the style base in species from tropical regions, e.g., *Q. austrocochinchinensis* Hick. & Camus (Figs. 1A-D), *Q. kerrii* Craib (Figs. 1E-G) and *Q. rex* Hemsl. (Figs. 1H-K).

A wide variation was found in leaf epidermal features. A total of ten trichome types were found in subgenus *Cyclobalanopsis*. Uniseriate solitary trichomes (Figs. 2A-B), fasciculate trichomes (Fig. 2C) generally present in *Quercus* subgenus *Quercus* and other genera of *Fagaceae*, indicating that it is a plesiomorphism. Fused stellate (Fig. 2D), multiradiate (Fig. 2E), stellate (Fig. 2F), and rosulate trichomes (Fig. 2G). Appressed-lateral-attached trichomes (ALA) (Figs. 2I-J) reported in *Quercus* subgenus *Quercus* were also found in subgenus *Cyclobalanopsis*. Jellyfish-like trichomes (Fig. 2H), and papillae on epidermal cells (Fig. 2L) were only detected in subgenus *Cyclobalanopsis* in the species used for comparison in this study. Simplified stellate trichomes (Fig. 2K) were only found in *Q. arbutifolia* Hick. & Camus. This trichome type is composed of

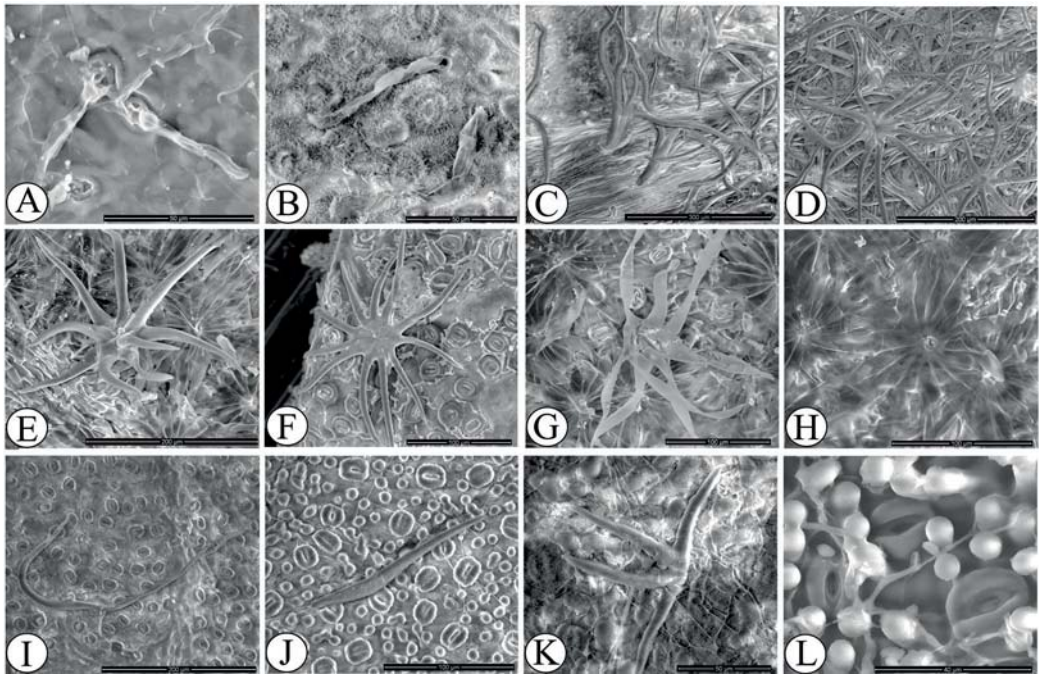

Figure 2/ Trichome types found in subgenus *Cyclobalanopsis*.
(A) Solitary trichome, *Quercus austrocochinchinensis*, bar=50 ηm; (B) Solitary trichome, *Quercus glauca*, bar=50 ηm; (C) Fasiculate trichome, *Quercus kerrii*, bar=300 ηm; (D) Fused stellate trichome, *Quercus delavayi*, bar=200 ηm; (E) Multiradiate trichome, *Quercus sichourensis*, bar=200 ηm; (F) Stellate trichome, *Quercus patelliformis*, bar=100 ηm; (G) Rosulate trichome, *Quercus sichourensis*, bar=100 ηm; (H) Jellyfish-like trichome, *Quercus sichourensis*, bar=100 ηm; (I) ALA trichome, *Quercus schottkyana*, bar=200 ηm; (J) ALA trichome, *Quercus myrsinifolia* and papillae on epidermal cells, bar=100 ηm; (K) Clustered ALA forming a stellate-like structure, *Quercus arbutifolia*, bar=50 ηm; (L) Papillae, *Quercus langbianensis*, bar=40 ηm.

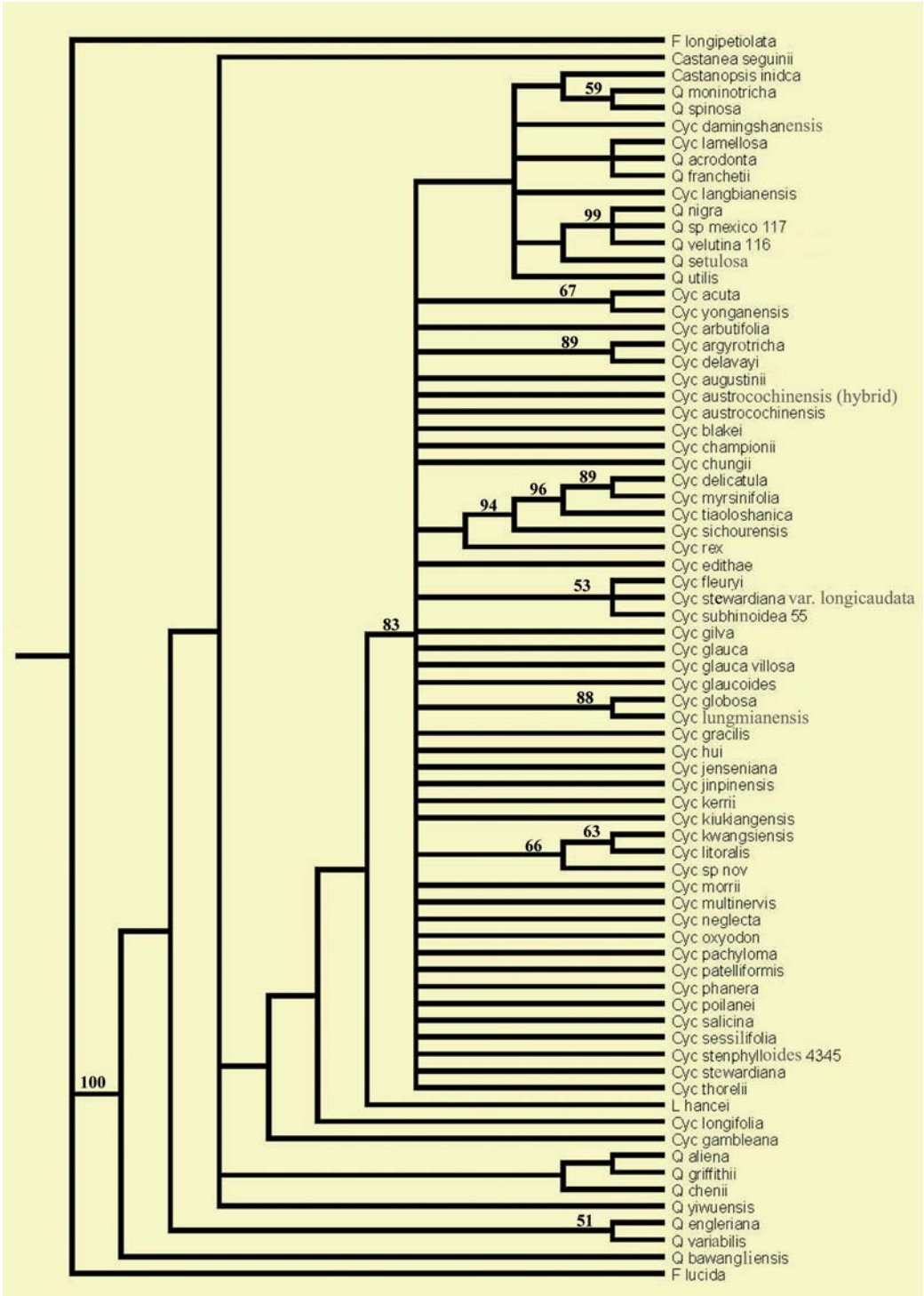

Figure 3/ Strict consensus tree of 2300 MP cladograms based on chloroplast psbA-trnH and trnT-trnL (CI = 0.729; RI = 0.446). Bootstrap proportions using MP are indicated above branches.

2-5 rays, however individual ray morphology was consistent with ALA that were also distributed in the species. Therefore, we still recognize this trichome type as a special form of ALA.

Based on epidermal and acorn features, five distinct groups in subgenus *Cyclobalanopsis* were detected:

1) Kerrii group: with fasciculate trichomes, radicle emerging from the basal seed scar, heart-shaped cotyledons with the placenta passing through to the style base, dense fiber plug at style base.

2) Gilva group: with stellate, fused stellate and multiradiate trichomes with compound trichome bases.

3) Pachyloma group: epidermal cells flat or with papillae; mature leaves glabrous except for scattered uniseriate trichomes, seed scar convex, acorn with 4-6 styles, cupule usally large with the wall densely covered by yellowish woolly or silky procumbent trichomes.

4) Jenseniana group: lamellae mostly fused to the cupule wall with only the rims free, no trichomes were detected on mature leaves, leaf epidermal cells with papillae.

5) Glauca group: with ALA trichomes or ALA clustered into a stellate-like structure with a simple trichome base.

Molecular phylogeny

In the matrix of final alignments, the length of the ITS, *psbA–trnH* and *trnT–trnL* regions for 52 taxa is 678 base pairs (bp), 563 bp and 889 bp long. The size of *psbA-trnH* ranged from 501 to 559, including a large number of poly-TA repeat regions in the middle. The phylogenetic information of the *psbA–trnH* folding structure was used to improve the alignment. However, it is still difficult to align the sequences of this region due to a few poly-A and poly-T structures and insertions/deletions. 116 ambiguous alignment sites were excluded from further analysis. The length of the *trnT–trnL* region of species from subgenus *Cyclobalanopsis* and section *Cerris* varies from 821 to 880 bp, with final alignment 889 bp long. 82 ambiguous sites were not counted in the analysis. Of the remaining 808 sites, 57 characters were parsimony-informative. 2300 MP trees were recovered in heuristic search. Strict consensus trees based on the combined dataset of *trnT–trnL* and *psbA–trnH* are shown in Fig. 3 with bootstrap annotated on the nodes. Retention index (RI) of the MP tree is equal to 0.803 and consistency index (CI) is 0.545. Combined analysis of *psbA–trnH* and *trnT–trnL* offered very little resolution within *Quercus* s.l., even at genus, section or subgenus level. Therefore, several major clades collapse in the strict consensus tree, the bootstrap only presented at the terminal and we even failed to discriminate *Lithocarpus* from *Quercus* using chloroplast DNA sequence.

ITS sequences were obtained either directly from purified PCR products or by cloning. Six samples from the subgenus *Cyclobalanopsis* each have a few distinct clones which distributed to different species clades supported by moderate bootstraps. These samples were excluded from phylogenetic analysis. The size of the ITS region ranges from 590 to 678 bp. The length variation between taxa is mainly derived from indels in intron areas. The poly-G- and poly-C-rich region has been detected in all ITS sequences from *Quercus* in this study and also previous molecular phylogenetic approaches (Manos et al., 1999, 2001), which could account for the difficulties in DNA sequencing and alignment. 108 ambiguous sites were excluded for analysis. Of the remaining (included) 572 sites, 332 are constant, 79 are variable but parsimony-uninformative, and 161 are parsimony-informative. Strict consensus trees from 600 MP trees are shown in Fig. 4 (p. 48) with bootstrap annotated above the nodes. MP tree scores were RI = 0.803

and CI = 0.545. Three major lineages in *Quercus* s.l.. section *Cerris*, Gilva group and Kerrii group formed an independent clade with 66 bootstrap support; section *Quercus* and section *Lobatae* formed a clade with 55 bootstrap support, Glauca group and Pachyloma group clustered together but without bootstrap support ([(section *Cerris* + Kerrii Group+Gilva Group (with bootstrap = 66) + (section *Quercus* + section *Lobatae*) (with bootstrap=52)]+(Glauca group+Pachyloma group). The four major clades formed by species from subgenus *Cyclobalanopsis* are weakly to moderately supported by ITS based on bootstrap resampling, and they correspond to the morphological groups: Glauca group, Pachyloma group, Kerrii group and Gilva group. Interestingly, the Kerrii and Gilva groups cluster with species from section *Cerris* with low bootstrap support, while the other species from *Cyclobalanopsis* form a clade but without strong bootstrap support (Fig. 4; p. 48). cpDNA data failed to reveal such a phylogenetic pattern.

Discussion of the systematics of subgenus Cyclobalanopsis *and its affinities*

The present study is a molecular phylogenetic study that includes representative oaks from subgenus *Cyclobalanopsis*. Previous phylogenetic studies using *ITS* and *CRABS CLAW* (Manos and Stanford, 2001; Oh and Manos, 2008; Denk and Grimm, 2010) revealed two major clades corresponding to geographic distribution: an Old World clade and a New World clade of *Quercus* s.l. However, in this study, the phylogeny based on ITS data revealed 3 major clades in *Quercus* s.l.: 1) an Old World clade comprising section *Cerris* and the Gilva and Kerrii groups of *Cyclobalanopsis*; 2) a New World clade comprising sections *Lobatae* and *Quercus* s.s.; and 3) a Cyclo oak clade, comprising the Pachyloma and Glauca groups, which falls sister to the Old World and New World clades. However, the bootstraps of the major clades are extremely low. Previous phylogeny studies of *Quercus* s.l. by Manos et al., (1999, 2001), Oh and Manos (2008) and Denk and Grimm (2010) as well as the present study could not provide high resolutions on the phylogeny of *Quercus* s.l. Although ITS strict consensus trees revealed some topology structures including the 4 major clades pattern in *Cyclobalanopsis*, these topologies need further assessment with other molecular markers, such as AFLP (Pearse and Hipp 2009) and DNA sequences (CRC gene, Oh & Manos, 2008) to provide a better solution for the phylogeny of these oaks. The phylogenetic relationships between these clades is still uncertain and section *Cerris* failed to cluster into a clade on a strict consensus tree. This indicates that ITS could not offer enough informative sites to resolve the phylogenetic relationships of *Quercus* s.l. Even the monophyletic status of subgenus *Cyclobalanopsis* and subgenus *Quercus* is questionable. Future in-depth studies need to include more species from subgenus *Cyclobalanopsis* for phylogenetic construction to obtain a better understanding of the evolutionary scenario in *Quercus* s.l.

The four main clades of *Cyclobalanopsis* were united by a series of synapomorphic morphological features, especially trichome types and epidermal cells which were useful to delimit the species in different groups: ALA trichomes in the Glauca group; uniseriate only with flat or papillose epidermal cells in the Pachyloma group; rosulate, jellyfish-like, fasciculate (including stipitate fasciculate) with compound trichome base in the Kerrii group and the dense stellate, fused stellate, multiradiate trichomes with a compound trichome base in the Gilva group can be applied as diagnostic features for each group. The heart-shaped cotyledon, basal germination and thick fiber plug structure at the style base were only found in the Kerrii group. The distribution of the Kerrii group is restricted

to tropical SE Asia. These unique features plus the narrow distribution of the Kerrii group indicates that it is a natural monophyletic clade, although this clade does not have very strong bootstrap support.

The trichome types can vary within a species depending on the growth stage and habitats. For example, dense hairs were present on young leaves of most species, but these hairs have a tendency to shed when the leaves mature in most species of *Cyclobalanopsis*. Generally, the trichomes on the mature leaves are far fewer or altogether lacking, but the more or less scattered trichome bases on both leaf surfaces are evidence of their existence. Luo and Zhou (2002) studied leaf architecture variation in subgenus *Cyclobalanopsis* and their results indicated that the marginal teeth type and secondary vein pattern also show stable and rich variation among the species. Although these features are mainly derived from convergent features, they are also very useful in identifying the species of *Cyclobalanopsis*. When comparing the leaf epidermal features of *Q. austrocochinchinensis*, *Q. kerrii* and their suspected hybrids, it can be seen that the anticlinal wall of the lower epidermal cells of the hybrids is a sinus, which is similar to that of *Q. austrocochinchinensis*. However, the leaf teeth are obtuse, which is close to those of *Q. kerrii* (our unpublished data). Therefore, the leaf epidermal features and leaf architecture features are informative to identify species from subgenus *Cyclobalanopsis* and even could assist with identifying hybridized individuals.

Remarkably, fasciculate, rosulate and multiradiate trichomes have also been reported in some species of section *Cerris* (Luo and Zhou, 2001; Tschan and Denk, 2012). Scanning electron microscope (SEM) of oak pollen also shows the tectum of the pollen grain in the Kerrii group (Deng 2007) and some species from section *Cerris* ("Ilex group") (Denk and Grimm, 2009) with a plesiomorphic stage with rod-like ([micro] rugulate) to (micro) verrucate scattered elements. This morphological evidence indicates that the Kerrii group and some species from section *Cerris* may represent primitive stages in *Quercus* s.l.

Most interestingly in this study, ITS data recovered a clade with low bootstrap support (62%) formed by the closely related Kerrii group, Gilva group and section *Cerris*. Another clade comprising the remaining species from *Cyclobalanopsis* collapses in the strict consensus tree. Remarkably, both the Kerrii group and the Gilva group have the compound trichome base as in species from section *Cerris*. However, Kerrii group + Gilva group + section *Cerris* pattern were not detected in *psbA–trnH* and *trnT–trnL* combined analysis (Fig. 3). As in this study, previous work on phylogenetic and population genetics in *Quercus* s.l. revealed conserved nucleotide variations in chloroplast genomes (Huang et al., 2002; Lin et al., 2003), but great variations were found in nuclear rDNA (ITS, IGS, ETS) regions (Manos et al., 1999; Manos and Stanford 2001; Manos et al., 2008; Denk and Grimm, 2010) and nuclear low-copy sequences G3pdH (Shih et al., 2006) and 11S (original data downloaded from Pubmed and compared by ourselves). Furthermore, multiple different copies of rDNA sequence and G3pdH were found in many oak species. The different evolutionary pattern between the chloroplast and nuclear genomes indicated a scenario of recent species radiation events, followed by the dramatic genome re-assembling which may be caused by frequent hybridization of different genetic resources in *Quercus* s.l.

For many years, cupule ornamentation was regarded as an important characteristic to subdivide species in *Fagaceae* genera (Camus 1934-1954; Forman, 1966). However, this may be a trait derived from ecological adaptation rather than having a true phylogenetic basis (Kaul, 1985; Kaul, 1986; Kaul 1988). The new Kerrii group + section *Cerris* pattern

revealed by ITS data may offer us another view to examine the evolutionary pattern of cupule ornamentation in *Fagaceae*.

ITS is problematic for phylogenetic use because of multiple copies (Baldwin et al., 1995; Alvarez and Wendel, 2003; Coleman, 2003). Orthologous locus or DNA sequences are crucial to using gene trees to construct the phylogenetic relationship among the species. Based on the study of rDNA FISH patterns in *Fagaceae*, species from subgenus *Cyclobalanopsis* have different rDNA loci patterns (Chokchaichamnankit, et al., 2008; Alves et al., 2012). The 5S rDNA were conserved with only one pericentromeric locus in *Q. sessilifolia* Blume, *Q. glauca* Thunb. (Alves et al., 2012), *Q. brandisiana* Kurz and *Q. kerrii* (Chokchaichamnankit et al., 2008), but 18S-25S rDNA loci were variable both in chromosomal location and in the chromosomes bearing these genes, e.g., *Q. sessilifolia* (Glauca group), *Q. glauca* (Glauca group), *Q. brandisiana* (Kerrii group) and *Q. kerrii* (Kerrii group) with 5, 2, 2, 4 loci of 18s-25s rDNA gene, respectively (Chokchaichamnankit et al., 2008; Alves et al., 2012). Alves et al., (2012) suggested that unequal crossing over and transposition events contributed to diversified rDNA loci and multiple copies in oak species. As a result, it is difficult to distinguish orthologous from paralogous rDNA loci in oaks. Based on ITS and 5S-IGS data, Denk and Grimm (2010) concluded that subgenus *Cyclobalanopsis* shares a common ancestor with the *Cerri*s and *Ilex* groups, and at the same time it is closer to other *Fagaceae* than to the rest of the species of *Quercus*. Oh and Manos (2008) constructed the phylogeny of *Quercus* based on the nuclear *CRABS CLAW* gene, but only limited samples of subgenus *Cyclobalanopsis* were analyzed. However, none of these studies offered a robust resolution in *Quercus* s.l. since bootstrap values of the nodes of section *Cerris* + and *Cyclobalanopsis* were also very low (54,70). No species from Kerrii group and Gilva group were ever sampled for analysis. In preliminary experiments, we tested 15 cpDNA universal primer sets (*psbA-trnH, trnT-trnL, atpB-trnH, trnC-trnG, ndhF, matK, rbcL, petG-trnH, atpF-atpH, trnS-trnM, rps16, ycf6-psbM, trnD-trnT, trnT-psbCr, rpoC1-trnCr*), but most of the nucleotide variations are too low to offer phylogenetic information. Therefore, more single (or low) copy genes from nuclear or DNA fingerprint-based molecular markers and more taxa should be added to future approaches and testing of *Quercus* s.l. phylogeny.

Numerous studies have reported hybridization between oak species, especially within *Quercus* subgenus *Quercus* (Hardin, 1975; Jensen et al., 1984; Bacilieri et al., 1996; Abrahamson et al., 1998; Graham, 2000; Marchelli and Gallo, 2001; Craft et al., 2002; Ishida et al., 2003; Schnitzler et al., 2004). In this study, we also detected distinct ITS sequences in predicted parental species; these ITS sequences are easily obtained from purified PCR products. In the predicted hybrids, two types of ITS clones were found, however, one of the parental haplotypes of *psbA-trnH* and *trnT-trnL* were found in the predicted hybrids, such as in *Q. kerrii*, *Q. austrocochinchinensis* and their suspected hybrid, *Q. glauca*, and in *Q. multinervis* (W.C. Cheng & T. Hong) Govaerts and suspected hybrids. Hybridization was proved to be occurring in those species. Considering the large number of plastic morphological features between the sympatric oak species from subgenus *Cyclobalanopsis* (Deng et al., 2007), the high conservation of karyotypes, the dramatic changes in the number of the 18s-25s rDNA loci and the locations on chromosomes (Ribeiro et al., 2011), and multiple ITS copies, hybridization-caused genome restructuring may be an important factor in the evolution of oak species from subgenus *Cyclobalanopsis*. Comprehensive further studies on introgression at population level can offer us better opportunities to understand the evolution of oak groups.

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