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Molecular Phylogeny of the *Ficus auriculata* Complex (Moraceae)

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Abstract

The closely related species of the genus *Ficus* with continuous variation have been confusing taxonomists, co-evolutionary researchers, and other related scientists. The boundary between species in the complex of *F. auriculata*, *F. oligodon*, *F. hainanensis*, *F. beipeiensis*, and *F. variegata* is still unclear. To clarify this problem, the nuclear loci ITS and *G3pdh*; chloroplast loci *trn*H-*psb*A, *trn*L-*trn*F, *trn*S-*trn*G, and *psb*K-*psb*I; and 15 pairs of SSR markers were used to reconstruct the phylogenetic relationship and clarify the species boundaries. The results of the present study indicated that *F. variegata* should be an independent species in *Ficus* sect. *Sycomorus* subsect. *Neomorphe*, which together with *F. auriculata*, *F. oligodon*, *F. hainanensis*, and *F. beipeiensis* compose a monophyletic group. The last four species of this complex are with small genetic distances, shared haplotypes, and overlapped geographic distribution, and should be treated as a single species.

Kew words: Phylogenetics, Species Delimitation, Microsatellite, Subsection *Neomorphe*

Introduction

The genus *Ficus* L. (figs) belongs to the family Moraceae, which includes about 735 species (Berg & Corner, 2005). Plants of this genus present syconium (enlarged, fleshy, hollow receptacles with multiple male, female, and/or gall flowers on the inner surfaces) and have a specific symbiotic relationship with their pollinators (Galil *et al.* 1973). The classification and nomenclature of *Ficus* have been significantly revised by Corner (1960a, 1960b, 1961, 1962, 1965), Berg (2003), and Pederneiras *et al.* (2015a). Molecular methods have been widely used in phylogenetic studies on *Ficus* (e.g. Herre *et al.* 1996; Weiblen, 2000; Jousselin *et al.* 2003; Rønsted *et al.* 2005, 2008; Xu *et al.* 2011; Cruaud *et al.* 2012; Li *et al.* 2012; Bruun-Lund *et al.* 2017). Many studies have focused on fig/fig-wasp relationships (Ramírez, 1974, 1977; Wiebes, 1979; Cruaud *et al.* 2012; Moe & Weiblen, 2012; Suleman *et al.* 2013; Borges, 2015).

The morphological characteristics, especially the leaves and syconia, of some *Ficus* species vary widely within the given species and allies, which makes it difficult to verify the boundaries of the related species group, such as in *F. hirta* Vahl and allies (*F.* subgen. *Ficus* sect. *Eriosycea* (Miq.) Miquel), *F. erecta* Thunberg and allies (*F.* subgen. *Ficus* sect. *Ficus*), and *F. auriculata* Loureiro and allies (*F.* subgen. *Sycomorus* Rafinesque sect. *Sycomorus* (Raf.) Miquel subsect. *Neomorphe* (King) C.C. Berg) (Zhou & Gilbert, 2003; Berg & Corner, 2005). Based on the sequences of ITS, ETS, and *trn*H-*psb*A; and SSR analysis, Lu *et al.* (2016) reported that the four species within the *F. hirta* complex have a close relationship between each other, and therefore were treated as one (*F. simplicissima* Loureiro). Lu *et al.* (2017) studied the *F. erecta* complex, which contained 17 taxa, with methods similar to those of Lu *et al.* (2016), confirmed that just five of the included species can be identified properly, whereas the rest of the taxa were still difficult to identify by morphological or molecular approach.

Ficus auriculata and its allies (*F.* subsect. *Neomorphe*) include about eight species (*F. hainanensis* Merrill & Chun and *F. oligodon* Miquel were recognized temporarily considering the opinion of Wei *et al.* (2014)). Among them, *F. hainanensis* is distributed in Thailand, Vietnam, and Southern China (Berg & Corner, 2005); *F. beipeiensis* S. S. Chang has the narrowest distribution, recorded only in the limestone region of Beipei, Chongqing, China (Chang, 1984; Zhou & Gilbert, 2003; Deng *et al.* 2014); *F. variegata* Blume is widely distributed from East Asia to Northern Australia (Berg & Corner, 2005; Berg *et al*. 2011); *F. auriculata* and *F. oligodon* are distributed from Pakistan to

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Southwest China, Indochina, Thailand, and Peninsular Malaysia (Berg & Corner, 2005; Berg *et al*. 2011) (Fig. 1); the other three species (*F. nodosa* Teijsmann & Binnenddijk, *F. robusta* Corner, and *F. semivestita* Corner) are distributed in the southernmost regions, from the Moluccas to Solomon Islands and Queensland, mainly in New Guinea (Berg & Corner, 2005).

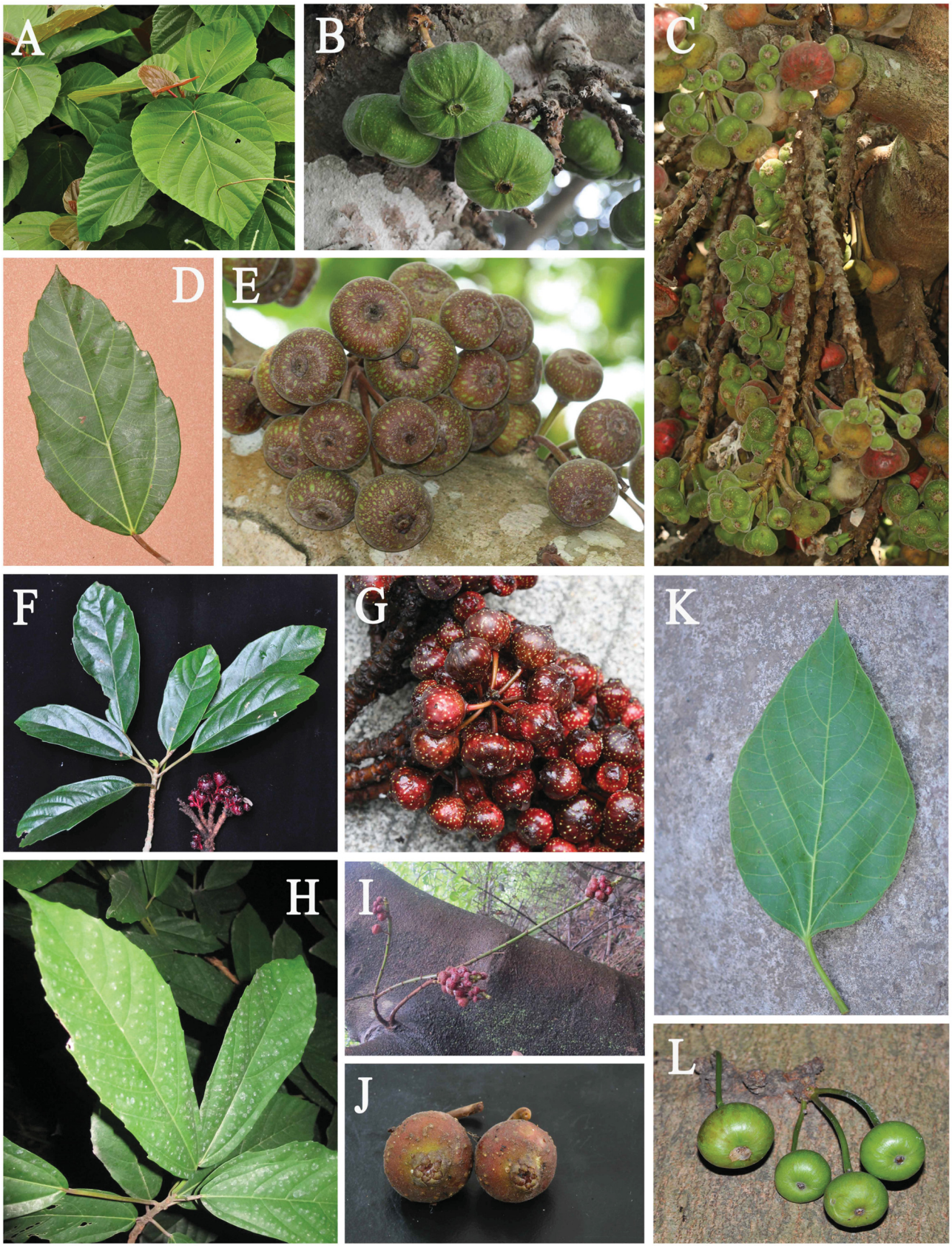

FIGURE 1. Selected typical morphology of *Ficus auriculata* complex. A–C: *F. auriculata* Lour. *s. s.*; D–E: *F. oligodon* Miq.; F–G: *F. hainanensis* Merr. et Chun; H–J: *F. beipeiensis* S. S. Chang; and K–L: *F. variegata* Blume.

The species in *Ficus* subsect. *Neomorphe* (except the three southernmost species) exhibit significant variation in leaf size, shape of perianth in female flowers, and length of stoloniflorous branches, and therefore its classification is difficult (Berg, 2007; Berg *et al.* 2011). Corner (1965) treated *F. hainanensis* as the synonym of *F. oligodon*, while Berg (2004) transferred *F. oligodon* to *F. auriculata* based on the features of female perianth; however, *F. hainanensis* was treated as an independent species. The subsequent year, Berg reported that *F. auriculata* represents the northern group with heart-shaped to round leaves, while *F. oligodon* represents the southern group with ovate to elliptic leaves, i.e., *F. oligodon* is the southern geographical type of *F. auriculata* (Berg & Corner, 2005). Individuals between these two types might be due to hybridization (Corner, 1978; Berg & Corner, 2005). As *F. hainanensis* is distinguished from *F. oligodon* and *F. auriculata* by longer stoloniflorous branches and narrower leaves, Berg and Corner thought it might be a subspecies of *F. auriculata* (Berg & Corner, 2005). However, due to the lack of clear boundary among *F. auriculata*, *F. oligodon*, and *F. hainanensis*, Berg (2007) merged both *F. oligodon* and *F. hainanensis* into *F. auriculata* (synonyms *F. macrophylla* Roxburgh & Buchanan-Hamilton ex Smith, *Tremotis cordata* Rafinesque, *F. roxburghii* Wallich, *F. sclerocarpa* Griffith, *F. regia* Miquel, and *F. pomifera* Wallich ex King) and divided *F. auriculata* into three forms, namely, the cauliflorous, stoloniflorous, and intermediate form.

Wei *et al*. (2014) made different observations after studying the genetic structure of *F. auriculata*, *F. oligodon*, and *F. hainanensis* using seven SSR markers, and referencing the size and length to width ratio of leaves, length of stoloniflorous branches, and color of perianths. They divided these three species into four groups, in which *F. oligodon* was divided into northern and southern forms. The population sampling strategy (more than 80% of the irregularly collected populations limited at 22 sites along the southwest borderline of Yunnan, China) might have affected the result of clustering, because continuous widespread taxon generates genetic gradients and spatial autocorrelation under long-term neighbor mating (Schwartz & McKelvey, 2009). The same problem was encountered by Wang *et al.* (2016), in which all the samples were collected from seven sites. Considering the gradually varying characters within and among these taxa (Berg, 2007; Berg *et al.* 2011), it is not enough to divide them into four taxa according to the biased population samples only.

Ficus beipeiensis is a member of *F.* sect. *Sycomorus* (Raf.) Miquel ser. *Auriculata* Corner and could be distinguished from *F. hainanensis* by smaller syconia; longer stoloniflorous branches; and characters of male, gall, and female flowers (Chang *et al*. 1984; Zhou & Gilbert 2003). Deng *et al.* (2014) considered that *F. beipeiensis* shows visible differences with other related species in terms of size and number of flowers, and it has distinct habitat and distribution region, although the characters of leaves and syconia are similar to those of *F. hainanensis* and *F. oligodon*. However, its genetic background is still unclear and its species identity is doubtful and needs to be verified.

Ficus variegata has relatively obvious features, such as glabrous laminae and stipules, umbilicate outer apical bracts, and brown ovaries. Corner reported that *F. variegata* can be divided into several varieties (Corner, 1965); however, Berg denied some of them according to the shape, dimensions and venation of leaves, and color of syconia (Berg & Corner, 2005). The molecular results of Xu *et al.* (2011), Harrison *et al.* (2012), and Cruaud *et al.* (2012) indicated that *F. variegata* has relatively far relationship with *F. auriculata* and *F. oligodon*, even out of the *F.* subsect. *Neomorphe*. While, Li *et al.* (2012) found that *F. variegata* was sister to the branch of *F. auriculata* and *F. oligodon*. However, samples of these studies were insufficient with respect to the widely distributed species of *F.* subsect. *Neomorphe*. Therefore, the taxonomic status of *F. variegata* and its relationship with other species of the subsection are still inconclusive.

The classification status and genetic relationship of the five species—*F. auriculata*, *F. oligodon*, *F. hainanensis*, *F. beipeiensis*, and *F. variegata*, named as the *F. auriculata* complex here, an expanded concept based on Wei *et al*. (2014) and Wang *et al.* (2016)—are far from being solved. In the present study, new samples and molecular data were used to analyze the genetic background of this complex. The present study aimed to: 1) verify if the *F. auriculata* complex composed of one or more species and 2) what the results suggest when dealing with species concept under different sampling strategies.

Material & Methods

Sampling and DNA extraction

A total of 132 samples, representing *Ficus auriculata* (51), *F. oligodon* (36), *F. hainanensis* (16), *F. beipeiensis* (5), and *F. variegata* (24), were collected from South China, Cambodia, Vietnam, and Malaysia. The first four species also included some individuals from their type localities. Sampling interval was usually above 80 km for each species,

including some cultivated samples. A few samples from same sites represented different forms, except the samples from Xizang and Chongqing. Additional 32 samples out of *F. auriculata* complex in this genus were also collected for phylogenetic analysis. Identification of samples was based on the type—*F. oligodon*: Mont Khasia, India, syntype (P-00710813); *F. hainanensis*: Hainan, China, syntype (IBSC-0001241); *F. beipeiensis*: ♀ S. S. Chang 2, holotype, ♂ S. S. Chang 1, Chongqing, China, paratype (HGAS); *F. variegata*: Indonesia, isotype (P-00756623); and *F. auriculata*: Annam, Vietnam, candidate neotype (P-06826446). Detailed information of all the samples has been provided in Table S1. Voucher specimens have been deposited in HSNU and/or KUN herbaria. Fresh young leaves were collected from the field and dried in silica gel. The CTAB method was followed for DNA extraction (Doyle & Doyle, 1987).

Phylogenetic analysis

Four chloroplast regions (*trn*H-*psb*A, *trn*L-*trn*F, *trn*S-*trn*G, and *psb*K-*psb*I), nuclear ribosomal internal transcribed spacers (ITS), and single copy nuclear gene coding glyceraldehyde 3-phosphate dehydrogenase (*G3pdh*) were selected for the phylogenetic study. Primers, protocols and systems used for the amplification of the six loci have been listed in Table S2. Polymerase chain reaction (PCR) was run on a TaKaRa TP600 thermocycler. The PCR products were bidirectionally sequenced by HuaGene Biotech Co. Ltd. (Shanghai, China) after inspecting on 1% TAE agarose-gels. Sequences were assembled and edited by SeqManII (DNA STAR package, Madison, WI, USA) (Burland, 1999). In addition, 78 sequences were downloaded from the Genbank based on previous studies (Weiblen, 2000; Azuma *et al.* 2010; Li *et al.* 2012; Cruaud *et al.* 2012; Pederneiras *et al.* 2015b). Alignment was performed using MUSCLE from the MEGA 5 package (Tamura *et al.* 2011) with manual adjustments when necessary. The six separate matrixes of each DNA region and two combined matrices (ITS+*G3pdh* and *trn*H-*psb*A+*trn*L-*trn*F+*trn*S-*trn*G+*psb*K-*psb*I) were then analyzed using Bayesian inference (BI) and maximum likelihood (ML). Matrix parameters and models have been listed in Table 1.

TABLE 1. Matrix parameters and models of individual and combined datasets.

Matrix	ITS	*G3pdh*	ITS+*G3pdh*	*trn*H-*psb*A+*trn*L-*trn*F+ *trn*S-*trn*G+*psb*K-*psb*I
Amount of samples	147	143	142	141
Aligned length	643	708	1344	1964
Variable sites	152	56	203	108
Parsimony informative sites	100	24	121	66
Model of Bayesian	GTR+G	GTR+ G	SYM+I+G	GTR+I+G
Model of ML	GTR+G	GTR+G	GTR+I+G	GTR+I+G

Bayesian inference (BI) was carried out using MrBayes 3.2.6 (Ronquist *et al.* 2012). Detailed protocols of model selection and phylogenetic tree reconstruction followed Lu *et al.* (2017). RAxML 8.2.0 (Stamatakis, 2014) was used to perform ML and associated bootstrapping (1000 replicates). The models for ML were in accordance with BI (except GTR+I+G for nuclear combined datasets). *Ficus maxima* Miller and *F. insipida* Willdenow were selected as outgroups. Bayesian trees were chosen to show the phylogenetic topology with Bayesian posterior probability (PP) values over 0.50 and maximum likelihood bootstrap support (MLBS) over 50 mapped on the trees. The bootstrap support values were defined as weak (50–70), moderate (71–89), and high (90–100); and posterior probability were weak (0.50–0.80), moderate (0.81–0.94), and high (0.95–1), respectively.

To investigate the geographic differentiation, haplotype network based on four chloroplast loci (*trn*H-*psb*A, *trn*L-*trn*F, *trn*S-*trn*G, and *psb*K-*psb*I) were conducted on 116 accessions of *Ficus auriculata*, *F. beipeiensis*, *F. hainanensis*, *F. oligodon*, and *F. variegata*. Long indel fragments were treated as single base variable sites, structure of ploy A/T were treated as single base invariable site, and both jagged ends were deleted. Haplotype network was constructed by TCS 1.21 (Clement *et al.* 2000) with confidence interval as 95%. Gaps were treated as missing data.

SSR analysis

One hundred and twenty-nine accessions representing the five species were subjected to SSR analysis. The primers were selected according to Giraldo *et al.* (2005) and Zhang *et al.* (2011). The reaction mixture (15 μL) contained 7.5 μL of 2X Taq MasterMix, 0.5 μL of each primer, 1 μL of template DNA, and 5.5 μL of double distilled water. The PCR was carried out under the following temperature profile: initial denaturation for 4 min at 94°C, followed by 35 cycles for 30 s at 94°C, annealing for 45 s at 55°C, elongation for 60 s at 72°C, and extension for 5 min at 72°C. The amplified products were electrophoresed on 1% agarose gel stained with goldview. Fifteen polymorphic microsatellite loci, which exhibited clear and bright amplification pattern, were selected for further SSR analysis (Table S3). Under the same

reaction condition, the reverse primers of each pair were labeled with fluorescent dye 5′ HEX, 5′ TAMRA, 5′ ROX, or 5′ 6-FAM (Sangon, Shanghai, China). The PCR products were divided into four pools according to the labeled dyes, viz. FP435+LMFC14+LMFC15+LMFC35, FP213+LMFC20+LMFC30+LMFC32, FP328+LMFC17+LMFC22, and LMFC19+LMFC24+LMFC26+LMFC36, and then scanned by Sangon Biotech (Shanghai, China). All the loci were inspected using GeneMarker V2.2.0 (Applied Biosystems), adjusted manually, and translated into codom-genotypic data (Table S4).

Principal coordinate analysis (PCoA) was conducted in GenALEx 6.5 (Peakall & Smouse, 2012). Furthermore, unweighted pair group method with arithmetic mean (UPGMA) analysis was carried out. The codom-genotypic SSR data were analyzed to obtain an individual-by-individual genetic distance matrix using GenALEx 6.5. For UPGMA, the genetic distance matrix was translated into MEGA format and analyzed in MEGA 5.

To further investigate the genetic cluster in the *F. auriculata* complex, a model-based bayesian software STRUCTURE version 2.3.4 (Hubisz *et al.* 2009) was used. According to the results of phylogenetic analyses, 107 samples representing the four closest allies (*F. auriculata*, *F. oligodon*, *F. hainanensis*, and *F. beipeiensis*) were implemented. For $K = 1$ to $K = 8$, each K performed 15 independent runs with a burnin of 200000 and 2000000 iterations. All the parameters were set the default values. The best K value evaluated by ΔK (Evanno *et al.* 2005) was generated under Structure Harvester (Earl & von Holdt 2012). The result was summarized using the Greedy algorithm in CLUMMP (Jakobsson & Rosenberg 2007) and visualized with DISTRUCT (Rosenberg 2004).

Results

Phylogenetic analysis

In the present study, a total of 844 sequences were obtained. Among them, 768 were new (Table S1). The phylogenetic trees of ITS and *G3pdh* (Fig. S1, S2) were faintly similar. Considering that the conflict mainly originated from the lack of resolution (soft incongruence), these two nuclear loci were combined for a detailed analysis (Fig. 2). It showed that the three southmost species, *F. robusta*, *F. semivestita*, and *F. nodosa*, had a distinct relationship with the other five species in *F.* subsect. *Neomorphe* (Fig. 2, S1, S2). The latter, *F. auriculata*, *F. oligodon*, *F. hainanensis*, *F. beipeiensis*, and *F. variegata*, formed a monophyletic branch (clade A in Fig. 2) with moderate to high support (PP = 0.98, MLBS = 64). Within clade A, samples of *F. variegata* composed a high support secondary branch (clade B, PP = 1, MLBS =65) and the rest samples in clade A were scattered and did not form a clear monophyletic branch. The phylogenetic trees of each of the four chloroplast loci (*trn*H-*psb*A, *trn*L-*trn*F, *trn*S-*trn*G, and *psb*K-*psb*I) were of low resolution (trees not shown). Therefore, the phylogenetic tree of the four chloroplast loci combined analysis was selected (Fig. 3). It showed that the species of the *F. auriculata* complex combined and formed a paraphyletic group with *F. racemosa* Linnaeus, *F. semicordata* Buchanan-Hamilton ex Smith, and *F. tikoua* Bureau, which contradicts that of nuclear loci. Simultaneously, incongruence length difference (ILD) test (Farris *et al.* 1994) also showed that there are significant differences between chloroplast loci and nuclear loci ($p = 0.01$). Therefore, the nuclear and chloroplast loci combined analysis was not conducted. However, both the trees had a special branch (clade B in Fig. 2, clade A in Fig. 3) that included all the samples of *F. variegata*.

The haplotype network (Fig 4, bottom right) showed that 33 haplotypes from the four chloroplast loci. There were no shared haplotypes among all the five species. The original haplotype 01 was shared by *F. auriculata*, *F. oligodon*, *F. hainanensis*, and *F. beipeiensis*; haplotype 26 was shared by *F. auriculata*, *F. oligodon*, and *F. hainanensis*; and haplotype 11 was shared by *F. auriculata*, *F. hainanensis*, and *F. beipeiensis*. No haplotype was shared by the cultivated samples. Five haplotypes (29–33) related to *F. variegata* was far from other haplotypes. The distribution map of haplotype network (Fig. 4) showed that the haplotypes 01 to 10 were mainly distributed in Southwest Yunnan, while the haplotype 07 was endemic to Southern Xizang. The haplotypes 14 and 19–25 were mainly distributed in Guangxi and Southeast Yunnan, and haplotypes 26–28 concentrated in the Hainan Island. Furthermore, the cultivated samples in Fujian possessed three haplotypes (01, 08, and 20), and they might have originated from Xizang or Guangxi.

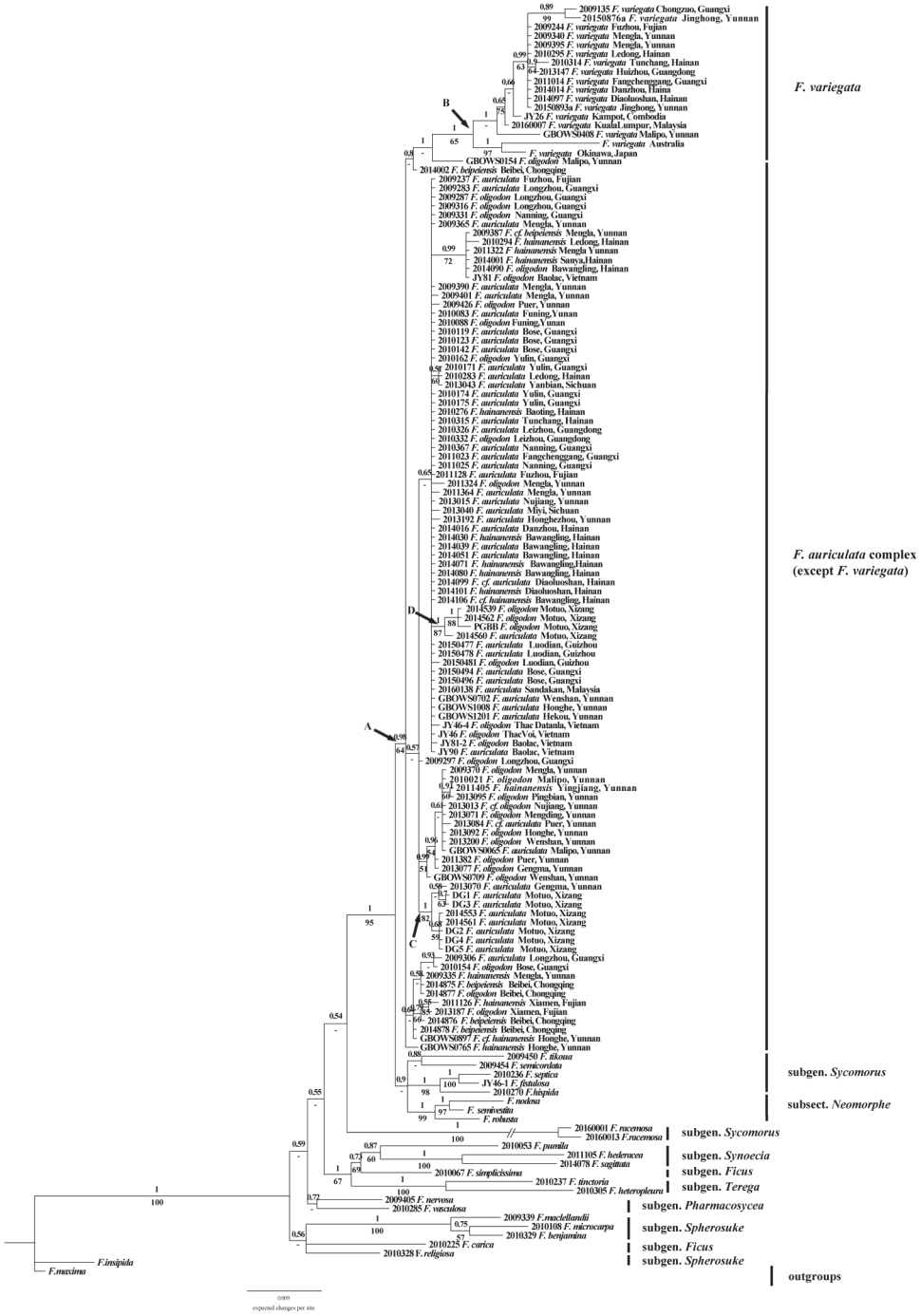

FIGURE 2. Bayesian majority consensus tree based on ITS+*G3pdh* combined datasets. The posterior probability (PP) values listed above the branches; the maximum likelihood bootstrap support (MLBS) below the branches; –, branches not support, PP < 0.50 or MLBS < 50. Localities showed following the collection numbers.

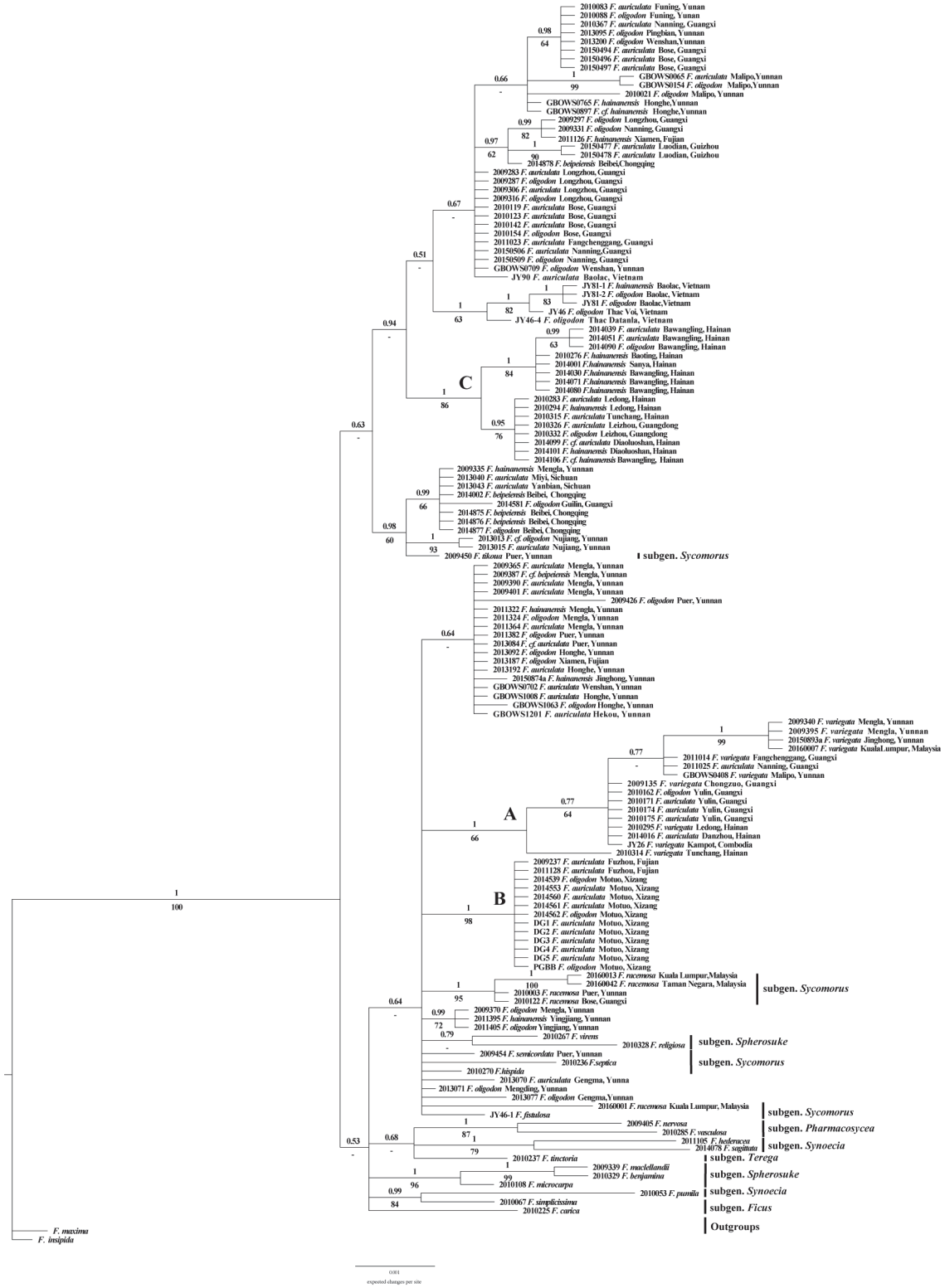

FIGURE 3. Bayesian majority consensus tree based on *trn*H-*psb*A+*trn*L-*trn*F+*trn*S-*trn*G+*psb*K-*psb*I combined datasets. The posterior probability (PP) values listed above the branches; the maximum likelihood bootstrap support (MLBS) below the branches; –, branches not support, PP < 0.50 or MLBS < 50. Localities showed following the collection numbers.

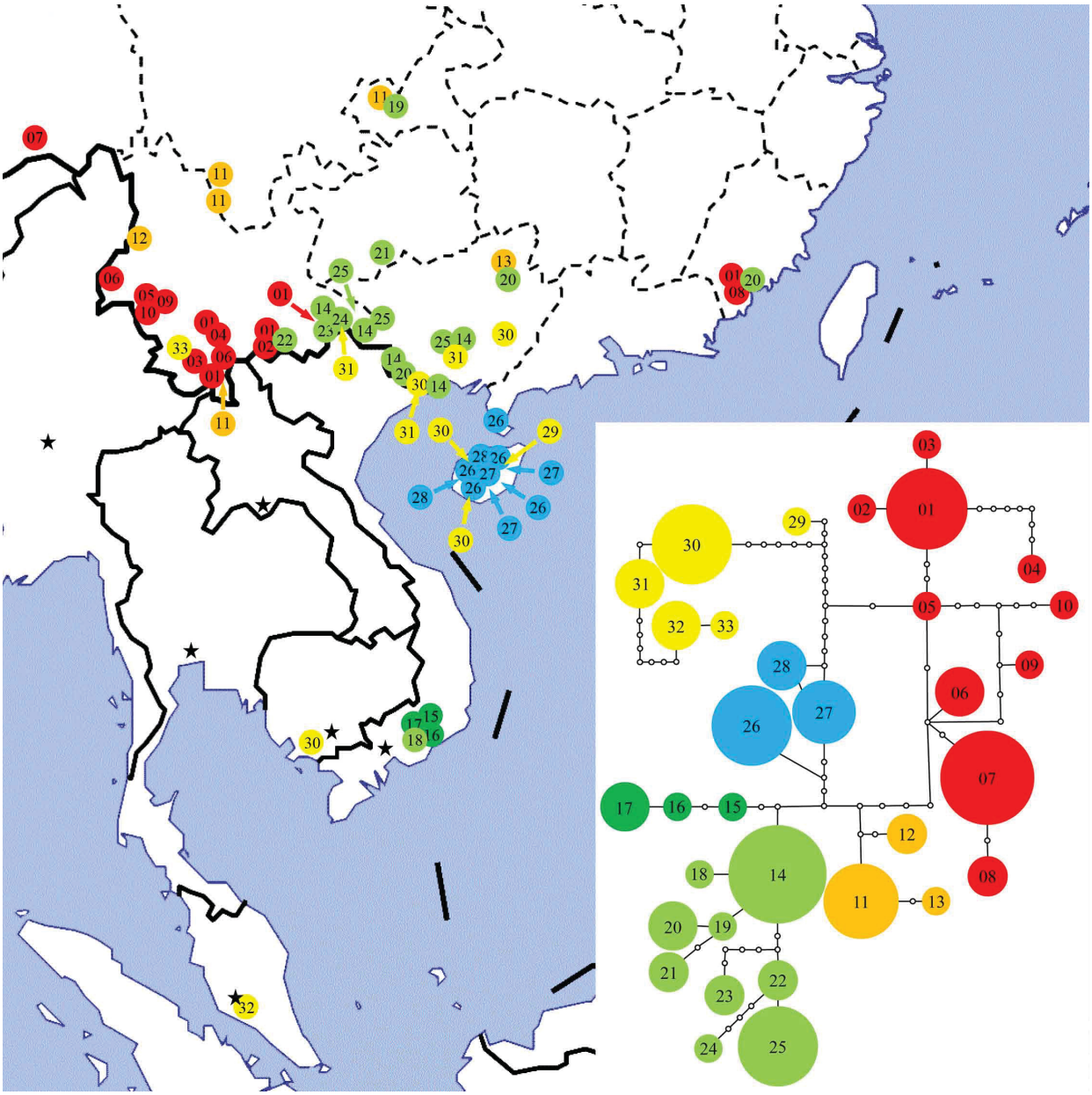

FIGURE 4. Sampling locations and chloroplast DNA haplotype distributions of *Ficus auriculata* complex. Haplotypes 1–33 are distinguished by serial number and closely related haplotypes are marked with same color. Small white circles represent missing or unsampled haplotypes.

SSR analysis

In the present study, 129 samples of the *Ficus auriculata* complex were included. The UPGMA analysis (Fig. 5) revealed that *F. auriculata*, *F. oligodon*, *F. hainanensis*, and *F. beipeiensis* were clustered and formed clade A. Furthermore, they could be separated from *F. variegata* (clade B). The cultivated samples were scattered among clade A. The principal coordinate analysis (PCoA) also revealed the same results, and these samples of clade A in Fig. 5 were scattered in a narrow area (Fig. 6), and *F. auriculata* and *F. oligodon* from different geographic regions were of no obvious aggregation.

For the STRUCTURE analysis (Fig. 7), the optimal K was 2 with very large ΔK value of 977.08, whereas neither of the clusters composed of distinct species, and vice versa. The clusters are also indistinct when $K = 3$ or 4. As $K = 2$ is the lowest possible number of clusters under Evanno's method (Evanno *et al.* 2005), there is no obvious subpopulation structure detected among the four closely related species of the *F. auriculata* complex. This is consistent with the results of UPGMA (Fig. 5) and PCoA (Fig. 6). Owing to the widespread and scattered sampling scheme, the result (to some extent) of the present study avoided the effects of high degree of association between spatial blocks and STRUCTURE clusters that occurred in the study of Wei *et al.* (2014).

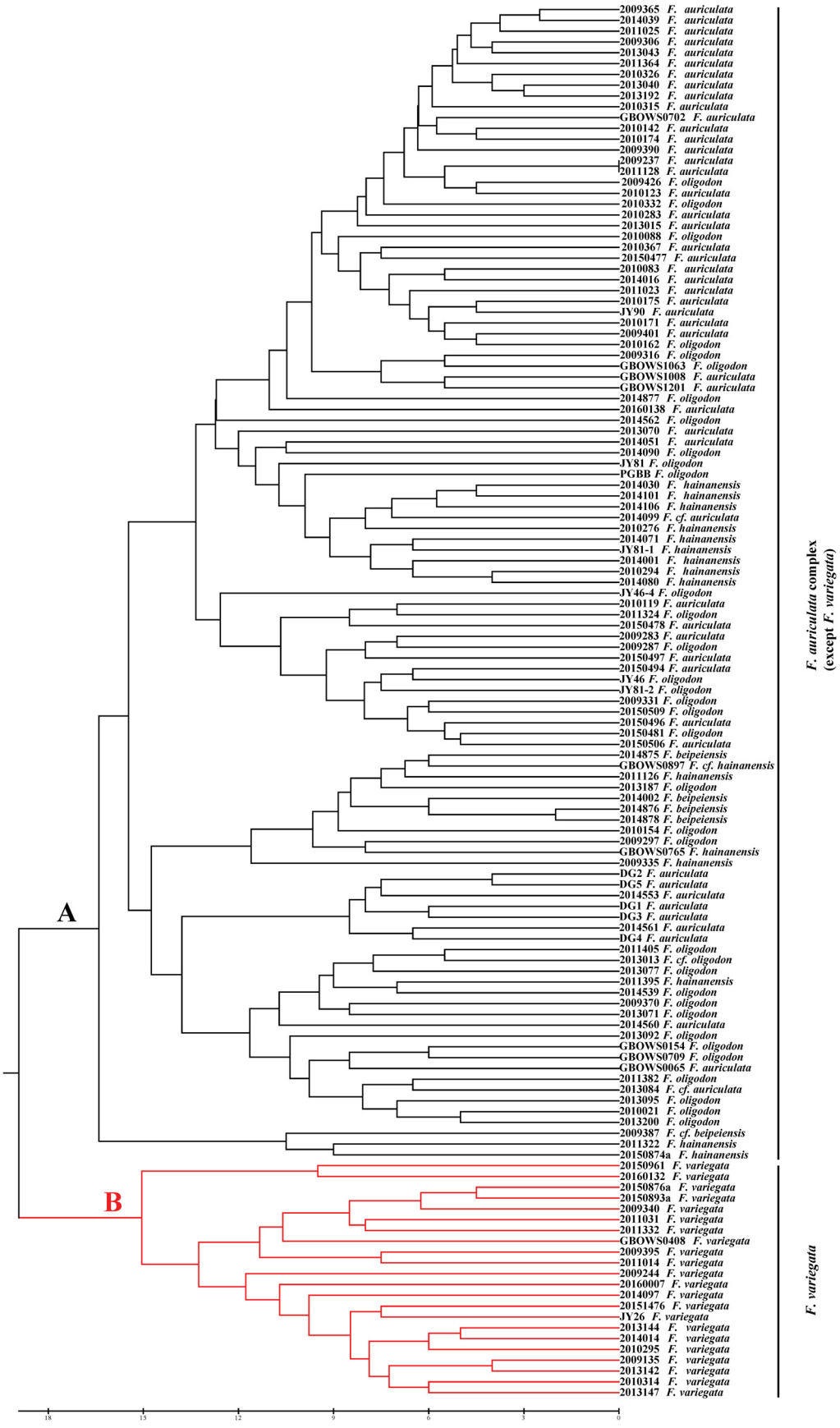

FIGURE 5. Genetic distances shown by UPGMA clustering tree based on codom-genotypic SSR data. Scale at the bottom is genetic distance generated by GenALEx software. Two clades were uncovered: clade A comprises all the samples of *Ficus auriculata*, *F. oligodon*, *F. hainanensis* and *F. beipeiensis*, and clade B comprises all the samples of *F. variegata*.

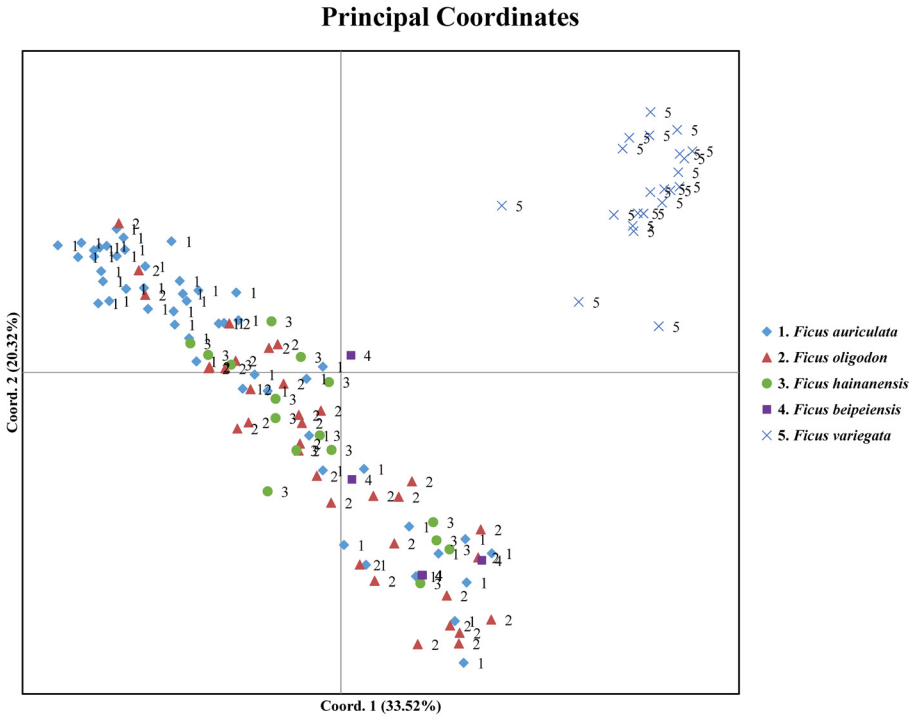

FIGURE 6. Genetic distances shown by principal coordinate analysis based on codom-genotypic SSR data. Two clusters were detected, of which the upper right one corresponds to *Ficus variegata*, and the other one corresponds to *Ficus auriculata*, *F. oligodon*, *F. hainanensis* and *F. beipeiensis*.

Discussion

Criteria for identifying samples of F. auriculata complex

Yang *et al.* (2012) treated *F. auriculata* (including *F. oligodon*) as *auriculata*-form and *oligodon*-form, mainly according to the length of fig-bearing branchlets, diameter of syconia, and the northern latitude limit. Wei *et al.* (2014) and Wang *et al.* (2016) treated *F. auriculata*, *F. oligodon*, and *F. hainanensis* as separate species; however, divided *F. oligodon* into northern and southern types using a set of quantitative morphological criteria. This is good for fig-wasp co-evolutionary analysis under population sampling strategy, and also for taxonomy when transitional characters are occasional and ineffective. Their identification results are acceptable considering their specific situation. However, for comprehensive taxonomy, blade shape, length of fig-bearing branchlet, color of perianth, and blurred latitude line for dividing northern and southern samples formed abundant transitional types, which indicates that these criteria are not adequate. The present study verified the types of *F. oligodon*, *F. hainanensis*, *F. beipeiensis*, and *F. variegata*, and thus identification of these species is sensible academically. As failed to find the type of *F. auriculata*, the specimen deposited in P (P-06826446) as candidate neotype (unpublished data) was chosen to identify the associated materials, abiding by the International Code of Botanical Nomenclature. It was collected from type locality Quảng Nam, Annam, Vietnam, and is morphologically similar to the protologue of Loureiro (1790). The obvious transitional characters usually could be seen within a population and within a tree, which might bring about unidentifiable specimens or misidentification, especially when verifying specimens from herbaria. To address this issue, the present study evaluated as many type and non-type localities as possible and observed the variations of each population in the field. Variant samples ensured taxonomic assessment of most of the samples in this complex taking full advantage of the taxonomic types and rules of nomenclature. As most of the samples shown in Fig. 2, 5 and 6 could not be visibly clustered (except *F. variegata*), the transitional samples (or even misidentified samples if exist) have little negative influence on the phylogenetic results.

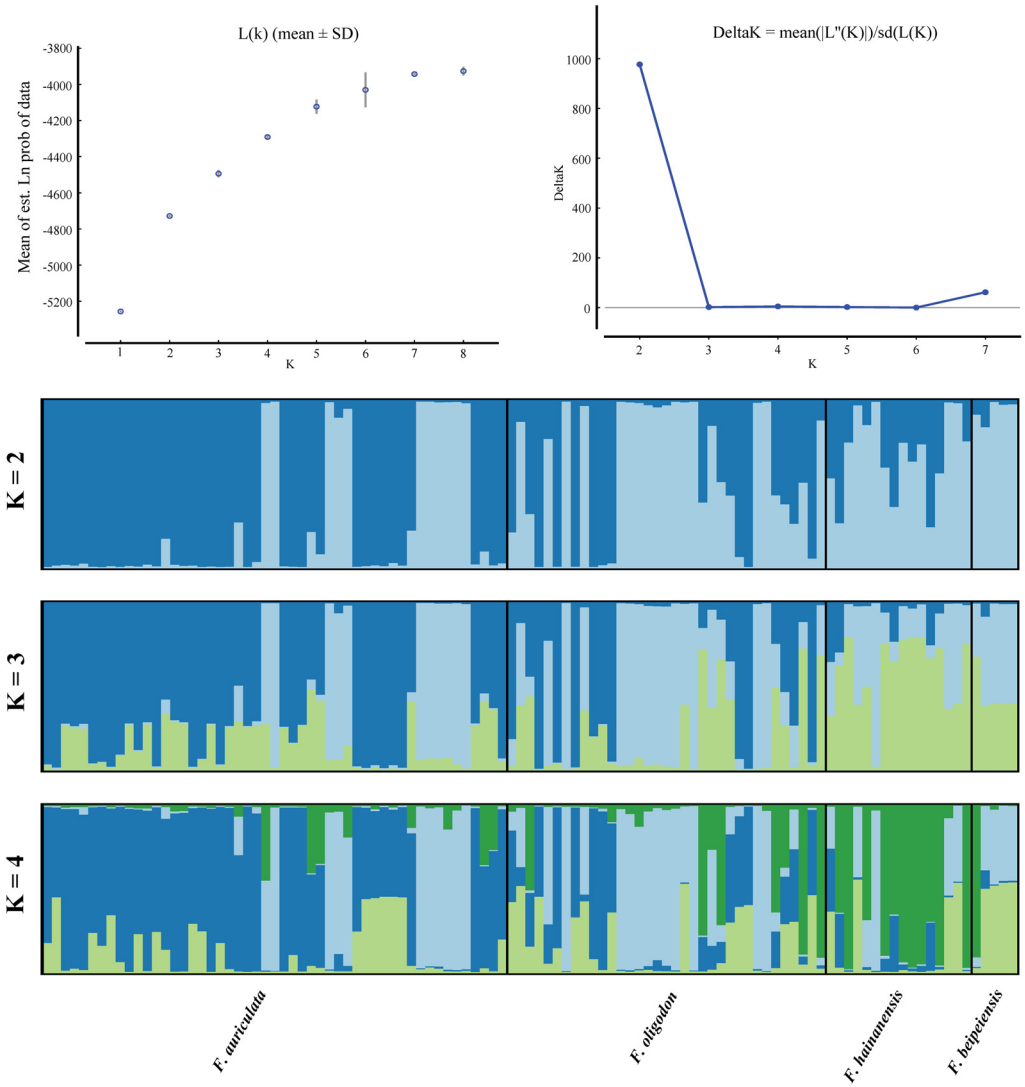

FIGURE 7. STRUCTURE analysis for *Ficus auriculata* complex. The relationships between ln(*K*), Δ*K* and *K* are shown on the top; subpopulation structures for the 107 individuals of the four species are shown in the bottom (*K*=2, 3, 4), for every *K*, each color represents one genetic cluster; each individual is delegated by a single vertical line. For every *K*, there is no exclusive cluster in certain species, which indicates that no obvious population structure exists in the complex.

Taxonomic status of Ficus beipeiensis

Samples of *Ficus beipeiensis* were mainly collected from the type location Beipei Chongqing. The morphological characters of these samples are basically the same as described by Chang (1984): leaf blade oblong-elliptic, 12–22 × 5–9 cm, papery, base cuneate; figs pendulous on specialized 15–60 cm stoloniflorous branches (voucher number: 2014876, 2014878, in HSNU), pear-shaped, 1–2 cm in diameter, densely covered with short rust-colored pubescence, and sparsely globose tuberculate. They were similar to those of *F. hainanensis*. No male trees of *F. beipeiensis* were found in the location where Chang (1984) collected the types. It is noteworthy that morphologically, the male tree of Deng *et al.* (2014, 2015) is similar to *F. oligodon*, because its figs are obviously bigger than that of *F. beipeiensis*.

The phylogenetic trees showed that the samples of *F. beipeiensis* imbedded in branches including *F. auriculata*, *F. oligodon*, and *F. hainanensis* (Fig. 2, 3), instead of a separate branch. Further, the UPGMA and PCoA analyses indicated that there were no identifiable genetic differences between *F. beipeiensis* and related species (Fig. 5, 6). This was also concordant with the results of STRUCTURE analysis (Fig. 7), which demonstrated that *F. beipeiensis* contains mostly one genetic cluster shared by other three species. The samples of *F. hainanensis* (Voucher numbers:

2011126, 2014001, and GBOWS0765, deposited in HSNU and/or KUN) with long stolon-like branches (> 50 cm) bearing red figs with red perianths were not different from that of *F. beipeiensis* with long thin stoloniflorous branches. Furthermore, long stoloniflorous branches were also found in *F. oligodon*. The genetic data of *F. beipeiensis* samples did not reveal obvious tendency of cluster (Fig. 2, 5). This indicated that the length of stoloniflorous branches and width of leaves, and molecular data cannot distinguish *F. beipeiensis* from other species.

Combination of Ficus auriculata*, *F. oligodon*, *F. hainanensis*, and *F. beipeiensis

A previous study reported that *Ficus auriculata* and *F. oligodon* (shown as *auriculata*-form and *oligodon*-form of *F. auriculata*) presented synchronous fruiting period and the hybrid seeds could germinate (Yang *et al.* 2012). These fig trees also shared the pollinator *Ceratosolen emarginatus* Mayr (Yang *et al.* 2012). Further, the pollinators of *F. auriculata* could reproduce in the syconia of *F. hainanensis* and pollinators of *F. hainanensis* (*Ceratosolen sp.*) can breed their next generation in the syconia of *F. auriculata* (Yang *et al.* 2012). According to Deng *et al.* (2015), wasps from *F. oligodon* (misidentified as *F. beipeiensis*) can pollinate female flowers of *F. beipeiensis* and resulting hybrid seeds. The phylogenetic trees based on Cytb and EF1α confirmed that it was common to see mutual pollination in the sympatric *F. auriculata*, *F. oligodon*, and *F. hainanensis* (Wei *et al.* 2014). It is clear that the non-strict specific pollinators in the co-evolution system limited the differentiation of *F. auriculata* complex and blurred the interspecific boundaries.

Within these four species, a few sub-branches with high support (Fig. 2, C and D, Fig. 3, B and C) and regional haplotypes were detected (Fig. 4); however, they were not monophyletic and without specialized morphological characters. The samples collected from Motuo in Xizang appeared clustered. However, this might be attributed to intensive sampling from this area. The population sampling strategy of Wei *et al*. (2014) confirmed the existence of four genetic groups (excluding *F. beipeiensis*), which is beneficial for detailed co-evolutionary and infraspecific researches. Considering the existence of morphological and genetic continuity, and the influence of diverse speciation modes (Baker, 2005), it is wise to regard these four traditional species as parts of a biological species. Before obtaining comprehensive evidences for identifiable subgroups, subspecies/varieties taxonomic treatments will not be carried out.

Relationship between Ficus variegata and other species in F. auriculata complex

A study on phylogenetic and biogeographic history of the genus *Ficus* (Xu *et al.* 2011) presented a chronogram, which showed that *F. variegata* might not be a member of *F.* subsect. *Neomorphe*. This observation was partly supported by Harrison *et al.* (2012) and Cruaud *et al*. (2012). However, including just one or two samples of the related species in such studies might not provide sufficient representation of the phylogeny of *F.* subsect. *Neomorphe*. In the present study, the combined analysis of ITS and *G3pdh* sequences showed that *F. variegata* is close to other species of the *F. auriculata* complex. They formed a monophyletic group (Fig. 2, clade A) with moderate to high support (PP = 0.98, MP = 64), which is sister to the clade including the southmost species *F. robusta*, *F. semivestita*, and *F. nodosa*. The results also showed that all the *F. variegata* samples were clustered together (Fig. 2, clade B) with moderate to high support (PP = 1, MP = 65). The combined chloroplast analysis showed a paraphyletic clade (Fig. 3, clade A) containing samples of *F. variegata, F. auriculata*, and *F. oligodon*, which implies that they shared common ancestral chloroplast gene fragments, and showed low genetic differentiation. The haplotype network analysis also showed that *F. variegata* has unique differentiation routes (Fig. 4, haplotypes 29–33 belong to *F. variegata*). The SSR analysis indicated that the genetic independence of *F. variegata* is significant when compared with that of other species (Fig. 5, 6). This, to some extent, is in agreement with the treatment of Corner (1965) and Berg *et al.* (2005). These studies agreed that *F. variegata* should belong to *F.* subsect. *Neomorphe* and it should be regarded as an independent species sharing common origin with other species of the *F. auriculata* complex. However, this deduction needs further validation considering the limited representativeness of *F. variegata* samples, especially the lack of samples from Malesia region and the type location (Java Island).

Conclusions

The present study suggests that the four traditional species *F. auriculata*, *F. oligodon*, *F. hainanensis*, and *F. beipeiensis* should be treated as one, i.e., *F. auriculata s. l.*, considering that they showed continuous variability in the shape of leaf and fruit, color of perianth, and length of stoloniflorous branch. Furthermore, they genetically formed a unit with vague

interspecific differentiation, shared haplotypes, less obvious genetic distance, and overlapped geographic regions. As there are some hybridization cases and genetic clades in this ecologically important complex, a detailed study on the speciation history will be valuable.

The present study confirmed the close relationship between *F. variegata* and *F. auriculata s.l.* (both members of the subsect. *Neomorphe*) based on phylogeny of nuclear loci, haplotype analysis of chloroplast makers, and cluster results based on microsatellite data. However, a comprehensive population sampling among the distribution range of *F. variegata* is necessary for population genetic analysis in the future.

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