Phylogenetic Reconstruction Among Species of *Chiritopsis* and *Chirita* Sect. *Gibbosaccus* (Gesneriaceae) Based on nrDNA ITS and cpDNA *trnL-F* Sequences

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Abstract. Sequences from the internal transcribed spacer region of nuclear DNA (ITS) and the *trnL-F* regions of chloroplast DNA for fifteen species of *Chirita* and seven species of *Chiritopsis* were used to assess phylogenetic relationships between *Chiritopsis* and *Chirita* section *Gibbosaccus*. Parsimony and Bayesian inference (BI) analyses were conducted using separate nuclear and chloroplast data sets, as well as a combined data set. Phylogenetic trees resulting from separate analyses proved highly congruent and the combined analysis of the two data sets produced a well-supported topology of the species and sections examined. Section *Gibbosaccus* proved paraphyletic and *Chiritopsis* polyphyletic in all analyses. Our results provide evidence that the species of *Chiritopsis* are embedded in section *Gibbosaccus* and were derived at least twice from within section *Gibbosaccus*. Finally, we reconsider the morphological evolution and adaptation between and within the two genera. The present analyses indicate that nomenclatural changes will be needed to reflect more accurately relationships in the *Gibbosaccus-Chiritopsis* complex. Only about 10% of the species in *Chirita* have been sampled and further data are required before any taxonomic changes can be suggested.

Keywords: *Chirita* sect *Gibbosaccus*, *Chiritopsis*, Gesneriaceae, ITS, phylogeny, *trnL-F*.

Chiritopsis (Gesneriaceae-Cyrtandroideae-Didymocarpeae), a genus endemic to China, was described by Wang (1981), who put the greatest weight on ovary and capsule features in separating this genus from other Old World Gesneriaceae. All members of *Chiritopsis* are perennial herbs that are uniform in gross morphology, and have small plant bodies and flowers, ovoid ovaries that are shorter than the styles, and capsules that are straight ovoid to ellipsoid. Later, Wang (1992) split the nine species into two sections, section *Chiritopsis* (with undivided leaves) and section *Schistophyllos* (with irregularly pinnatifid leaves). Using the current circumscription, *Chiritopsis* consists of nine species and two varieties with the center of species distribution and diversity in southern China (Wang 1992). Eight species and two varieties of *Chiritopsis* occur in northeast Guangxi and northwest Guangdong. Only *Chiritopsis xiuningensis* is disjunctly distributed in Anhui province. All species of *Chiritopsis* occur exclusively on limestone.

The vegetative characters of *Chiritopsis* are quite similar to those of *Chirita* sect. *Gibbosaccus*, with stout rhizomes and leaves crowded in basal rosettes. Furthermore, the geographic distributions of the two taxa are largely overlapping in southern China. Thus, Wang (1992) considered *Chiritopsis* to be potentially sister to *Chirita*. *Chiritopsis* and *Chirita* share a distinctive combination of morphological characters that indicate not only affinities to

each other but differences from the other Old World genera by having a lamellate, usually bilobed or deeply bifid stigma. *Chirita* was originally described by D. Don (1822) for a small group of Himalayan herbs. At present, *Chirita* has swelled to at least 140 species and the description of new species, especially of sect. *Gibbosaccus* in southern China, is still in progress (Wang 2004). The most comprehensive examination of *Chirita* was done by Wood (1974) who recognized three sections: (1) sect. *Chirita*, (2) sect. *Gibbosaccus* C. B. Clarke, and (3) sect. *Microchirita* C. B. Clarke. A fourth section, (4) sect. *Liebigia* (Endl.) C. B. Clarke, was recently revived by Hilliard (2004) to accommodate *C. asperifolia* and allies. Because of its comprehensiveness and great practical value, Wood's classification has been the authoritative work on the genus and has been the most widely followed by later authors (Weber 1975, 2004; Burtt 1977; Wang 1985, 1992; Wang 2004). Wang (1985) divided sect. *Gibbosaccus* into three subsections and Wang's elaborate system has received palynological support (Yan and Li 2003).

Wood's sections of *Chirita* are readily distinguished on the basis of morphological characters. In sect. *Microchirita*, members typically show a characteristic pattern of inflorescence peduncles adnate to petiole, anthers joined by an apical ligature, and monocarpic habit (annual in areas with seasonal climate). These characters serve as synapomorphies to unite these species that are

found predominantly in Thailand, Vietnam, the Malay Peninsula, Java, and Kalimantan, with the northern limit on the southern flank of Yunnan and Guangxi in China.

Section *Chirita* exhibits a rich diversity of morphological characteristics and comprises a broad range of habits including caulescent perennial or annual herbs or even small shrubs. One distinctive characteristic of this section is that the calyx lobes are more or less fused into a tube. Section *Chirita* has the widest geographic distribution, ranging from Sri Lanka to southeast Asia and southern China.

Section *Gibbosaccus* is the largest section, comprising more than seventy percent of the *Chirita* species. It has a wide geographical distribution, ranging from southern China to northern Vietnam. Like *Chiritopsis*, the distribution of most species is local and endemic, and their range at each site is small. They are found in karst terrains, usually on naked and exposed limestone surfaces. Morphologically, sect. *Gibbosaccus* can easily be recognized by the rosulate perennial habit with stout rhizomes and free calyx lobes.

Species of sect. *Gibbosaccus* and *Chiritopsis* are difficult to separate based only on their vegetative characters. Both *Chirita* and *Chiritopsis* species are well known ornamentals and medicinals, yet most recent studies are limited to the description of new species (Liu and Guo 1989; Fang et al. 1993). To date, our knowledge of morphology, floral development, molecular data, and phylogenetic relationships is deficient. Recently, molecular phylogenetic approaches helped resolve many longstanding controversies and nurtured a better understanding of the evolutionary processes that have shaped the evolution of closely related pairs of genera (Möller and Cronk 1997; Compton et al. 1998; Bräuchler et al. 2004). Previous molecular data for *Chiritopsis* and *Chirita* seemed to indicate that *Chiritopsis* is closely related to *Chirita* and that sect. *Gibbosaccus* is paraphyletic (Mayer et al. 2003), but the phylogenetic relationships between the two taxa have not been worked out in depth due to limited sampling. Thus, our study represents the first phylogenetic investigation to focus specifically on sect. *Gibbosaccus* and *Chiritopsis*.

In this paper, we have followed the detailed *Chirita* classification of Wang (1985) to investigate phylogenetic relationships using nuclear ITS and plastid *trnL-F* sequences. The aims of this work are: (1) to test the monophyly of *Chiritopsis*; (2) to explore the phylogenetic relationship of sect. *Gibbosaccus* with *Chiritopsis*, particularly to test previous systematic treatments and the classification of Wang (1985); (3) to evaluate the evolution of the morphological characters used to circumscribe genera and sections of *Chirita* and *Chiritopsis*.

MATERALS AND METHODS

Ingroup Selection. Twenty-two species were sampled from field-collected materials, including fifteen species of *Chirita*, representing all three of Wood's (1974) and Wang's (1985) sections, and seven species of *Chiritopsis*, representing both of Wang's sections (1992). Voucher specimens are deposited in the Herbarium of the Institute of Botany, Chinese Academy of Sciences (PE). The material studied and details of voucher specimens are shown in Appendix 1.

Outgroup Selection. Ten genera from other Didymocarpeae, two from Trichosporeae that may be a close relative of Didymocarpeae (Smith 1996, 1997; Wang and Li 1998), one from Ramondieae, and two from Epithemateae were included. *Rehmannia henryi* and *Rehmannia glutinosa* (Scrophulariaceae) were used as outgroups in preliminary analyses of *trnL-F* to determine which of these fifteen genera were most appropriate for rooting the final trees (Appendix 1). *Ornithoboea wildeana* and *Paraboea rufescens* were chosen as the outgroups for the subsequent analyses of both data sets (data not shown). Despite possible close relationships, the two final outgroups used here are clearly distinct from *Chirita* and *Chiritopsis* in both morphological and molecular characters, and so there seems to be no risk that any one of them is nested within the ingroup.

DNA Isolation. DNA was extracted from silica-dried (Chase and Hills 1991) or fresh leaf material using the method of Rogers and Bendich (1988) modified by adjusting the concentration NaCl, Tris-HCL and EDTA.

DNA Amplification. The entire ITS region, comprising ITS1, 5.8SrDNA and ITS2 from nuclear DNA and *trnL-F* region from chloroplast DNA were chosen for phylogenetic analysis. The markers were amplified from total DNA via the polymerase chain reaction (PCR), using the primer pairs ITS1 and ITS4 (Wendel et al. 1995) and the *trnL-F* primer pairs c and f of Taberlet et al. (1991), respectively. All PCR amplifications were carried out on PTC 200. For ITS the following program was chosen: 94°C for 5 min, 35 cycles at 94°C for 1 min, 52°C for 1 min, 72°C for 1 min, and a terminal extension phase at 72°C for 10 min. For *trnL-F* each cycle consist of 94°C for 1 min, 52°C for 1 min. 72°C for 2 min, the other steps remained unchanged according to amplification of ITS. The PCR products were purified with a Uniq-10 PCR Purification kit (Sangon Inc., Shanghai, China).

Sequencing. All *trnL-F* and ITS sequences were

obtained directly using MegaBACE™ 1000 DNA Analysis Systems (Amershan Pharmacia Biotech Inc.) following manufacturer's protocol. The marker *trnL-F* was sequenced bidirectionally using the same primer pairs as for amplification. The ITS1 and ITS4 primers were used to sequence the ITS region in both directions, with additional sequences from internal primers CITS2 (5' GCATTTCGCTACGTT CTTCA' 3) and CITS3 (5' CCATCGAGTCTTTG AACGCA' 3) designed, based on ITS sequence of *Chirita* for taxa in which ITS1 and ITS4 sequences did not provide sufficient overlap.

Phylogenetic Analysis. The sequences were aligned using CLUSTAL W version 1.83 (Thompson et al. 1997) and adjusted manually to minimize indels in BioEdit 5.0.9.1. Identification of the start of ITS1 and the end of ITS2 were determined by comparison with various published sequences available in GenBank (Möller and Cronk 2001). The resulting ITS and *trnL-F* data sets were subsequently analyzed separately with both parsimony and Bayesian inference (BI) methods. For parsimony analysis, using PAUP*4.0b10 (Swofford 2003), gaps were treated as missing. Heuristic searches were performed with 1,000 replicates of random addition, one tree held at each step during stepwise addition, tree- bisection-reconnection (TBR) branch swapping, MulTrees in effect, and steepest descent off. Characters and character-state changes were weighted equally. To examine the robustness of various clades, bootstrap analysis was performed with 1,000 replicates of bootstrapping using heuristic search with 1,000 replicates of random sequence addition and TBR branch swapping.

Modeltest and Bayesian Inference. BI was conducted using MrBayes version 3.0b4 (Ronquist and Huelsenbeck 2003). Modeltest 3.06 (Posada and Crandall 1998) was employed to adopt the appropriate model of sequence evolution for each DNA data set from a comparison of 56 models. The posterior probabilities (PP) of the phylogenetic model were estimated using Markov Chain Monte Carlo (MCMC) simulations by sampling trees from the PP distribution. Four chains, three heated and one cold, were run, each for 1,000,000 generations, and were sampled every 1,000 generations, starting with a random tree. After the analysis was complete, likelihoods were graphed against generation number in Excel and the ''burn-in'' was visually determined to be the initial 50,000 generations for each run. Trees from these generations were excluded from the analysis (Miller et al. 2004).
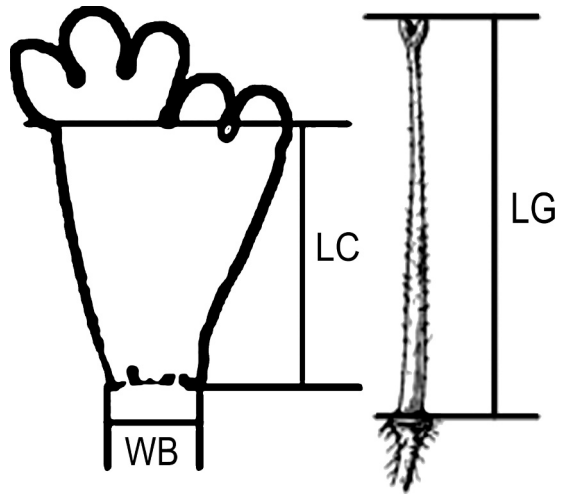
Incongruence Test and Combined Data Analysis.

FIG. 1. Delimiters used in morphometric measurements: WB: width below middle part of corolla tube; LC: Length of corolla tube; LG: length of gynoecium.

To assess character congruence between ITS and *trnL-F*, the incongruence length difference (ILD) test (Farris et al. 1994) as implemented in PAUP*4.0b10 (Swofford 2003) was performed with 100 replicates, each with 10 random additions with TBR branching swapping. The resulting p value was used to determine whether the two data sets had significant incongruence (0.05). Both parsimony and BI analyses for the combined data set were conducted using the same methods as those used for ITS and *trnL-F*.

Morphological Data. Flowers of thirty-three species were sampled from PE or fresh floral material cultivated in the Greenhouses at the Institute of Botany, Chinese Academy of Sciences (Table 1). Each of the parameters is the average of the measurement from at least four to seven flowers per species. Care was taken to ensure that measurements were recorded consistently (Figure 1). Two ratios calculated from them are provided for all taxa investigated. The independent t-test was used to test for differences of the ratios between sect. *Gibbosaccus* (including *Chiritopsis*) and sect *Chirita* in SPSS.

RESULTS

Analysis of ITS. The ITS sequences varied in size from 617 bp to 666 bp within *Chirita* and 634–639 bp within *Chiritopsis*. The aligned sequences had 708 bp, of which 339 (47.88%) were constant, 122 (17.23%) were variable but uninformative and 247 (34.89%) characters were parsimony-informative. Modeltest indicated HKY + I + G as the best-fit model for the ITS sequence data. Parsimony analyses resulted in three trees of equal length (L

TABLE 1. Parameters of floral morphology of *Chirita* and *Chiritopsis* measured for this study. Species are arranged by sections and genera according to the systems of Wang (1985, 1992). C = *Chirita*, Cs = *Chiritopsis*; WB: width below middle part of corolla tube (mm); LC: Length of corolla tube (mm); LG: length of gynoecium (mm).

Taxon	WB	LG	LC	WB/LC	LG/LC
***Chirita* sect. *Gibbosaccus* C. B. Clarke**					
C. fimbrisepala Hand.-Mazz.	9.67	35	32.17	0.3006	1.0880
C. fordii var. *dolichotricha* W. T. Wang	10.6	29.6	24.2	0.4380	1.2231
C. eburnea Hance	8.17	33.17	26.83	0.3045	1.2363
C. heterotricha Merr.	11	30.8	30.4	0.3618	1.0132
C. laxiflora W. T. Wang	7.25	19.5	19	0.3816	1.0263
C. linearifolia W. T. Wang	3.5	16	14	0.25	1.1429
C. longgangensis W. T. Wang	4.5	20	17	0.2647	1.1765
C. medica D. Fang ex W. T. Wang	3	17.5	15.5	0.1935	1.1290
C. mollifolia D. Fang, Y. G. Wei et J. Murata	4	30	27	0.1481	1.1111
C. obtusidentata W. T. Wang	5.75	30.25	28.25	0.2035	1.0708
C. ophiopogoides D. Fang et W. T. Wang	3	15	10	0.3	1.5
C. pinnata W. T. Wang	6.75	28	25	0.27	1.12
C. pinnatifida W. T. Wang	6	28.71	25.75	0.2330	1.1167
C. pteropoda W. T. Wang	5.6	24.7	24.7	0.2267	1
C. sclerophylla W. T. Wang	6.667	29.667	25	0.26668	1.7867
C. sinensis Lindl.	9.4	17	14	0.6714	1.2143
C. tennituba W. T. Wang	2.9	19.8	16.6	0.1747	1.1928
C. tribracteata W. T. Wang	9.8	25.3	22	0.4455	1.1500
C. wentsaii D. Fang et L. Zeng	6	32	30	0.2	1.0667
Chirita* sect. *Chirita					
C. anachoreta Hance	3	25.67	27.5	0.1091	0.9334
C. dielsii B. L. Burtt	3.9	34.4	41.4	0.0942	0.8309
C. fasciculiflora W. T. Wang	2.5	50	55	0.0455	0.9091
C. macrophylla Wall.	4	27.67	36	0.1111	0.7686
C. pumila D. Don	3.2	27.4	33.8	0.0947	0.8107
C. shuii Z. Y. Li	4.125	34.25	42.5	0.0969	0.8056
C. speciosa Kurz	4	35	39.5	0.1013	0.8861
C. urticifolia D. Don	4	33.67	41.33	0.0968	0.8147
Chiritopsis* sect. *Chiritopsis					
Cs. cordifolia D. Fang et W. T. Wang	3.667	9	10	0.3667	0.9
Cs. mollifolia D. Fang et W. T. Wang	3	6	4.667	0.6428	1.2856
Cs. repanda var. *guilinensis* W. T. Wang	2	6	7	0.2857	0.8571
***Chiritopsis* sect. *Schistophyllos* W. T. Wang**					
Cs. bipinnatifida W. T. Wang	2.5	9	7	0.3571	1.2857
Cs. glandulosa D. Fang, L. Zeng et D. H. Qin	3.45	8.75	8.525	0.4047	1.0264
Cs. subulata W. T. Wang	2	8.5	7.5	0.2667	1.3333

= 828, CI = 0.673, RI = 0.6903). The topologies of the MP and Bayesians trees are congruent. The ITS strict consensus tree can be primarily divided into three clades (Fig. 2). Clade I consists of a monophyletic *Chirita* sect. *Microchirita* with maximum support and is sister to the remaining taxa with low to high support (Bootstrap [BS] value = 62%, Posterior probability [PP] = 99%). Four species of sect. *Chirita* including *C. urticifolia*, *C. pumila*, *C. dielsii*, and *C. anachoreta*, form Clade II with high support (BS = 95%, PP = 100%). Within Clade III, *C. heterotricha* and *C. peteropoda* are separated with maximum support (BS = 100%, PP = 100%) and are sister to the remaining taxa which are composed of *Chiritopsis mollifolia* and four other well-supported lineages. The first includes *Cs. sp.* 054271 and *Cs. cordifolia*. The second lineage (BS = 99%, PP = 100%) demonstrates a close relation among four species in *Chiritopsis*, i.e. *Cs. bipinna-*

tifida, *Cs. sp.* 001, *Cs. repanda* var. *guilinensis*, and *Cs. glandulosa*. Four species of sect. *Gibbosaccus* in *Chirita*, *C. minutimaculata*, *C. ophiopogoides*, *C. wentsaii*, and *C. spinulosa*, cluster together with high support (BS = 99%, PP = 100%). The fourth group (BS = 86%, PP = 99%) encompasses *C. mollifolia*, *C. linearifolia* and *C. longgangensis*.

Analysis of **trnL-F.** The *trnL-F* matrix included the same set of taxa as the ITS analysis (Fig. 3). Prior to phylogenetic analyses, all ambiguous positions at the start and end of each *trnL-F* sequence were excluded from the data matrix due to poor sequencing. The aligned *trnL-F* region encompassed 893 positions, of which 778 (87.12%) were constant, 61 (6.8%) were variable but uninformative, including 54 (6.05%) parsimony-informative characters. Modeltest indicated TVM as the best-fit model for *trnL-F*. Maximum parsimony analysis yielded two trees (L = 133, CI = 0.9399, RI
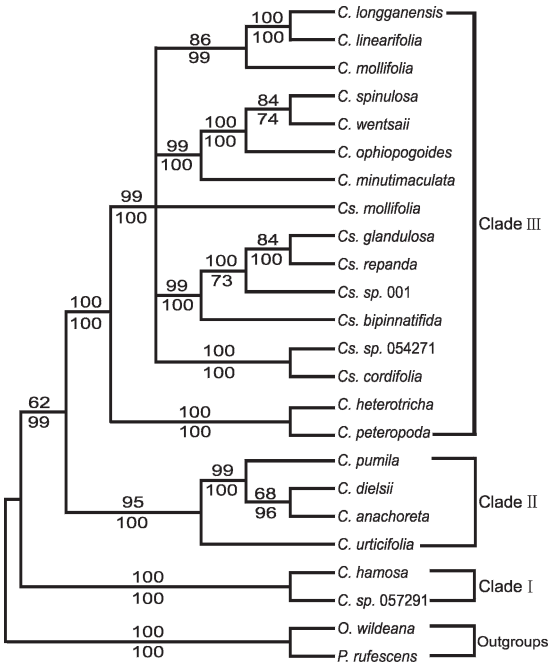
FIG. 2. Strict consensus of 3 MPTs generated from the ITS data which is congruent with the majority rule consensus from the Bayesian analysis. The bootstrap values are shown above the branches, and the Bayesian posterior probabilities are indicated below the branches. *C* = *Chirita*, *Cs* = *Chiritopsis*, *O* = *Ornithoboea*, *P* = *Paraboea*.
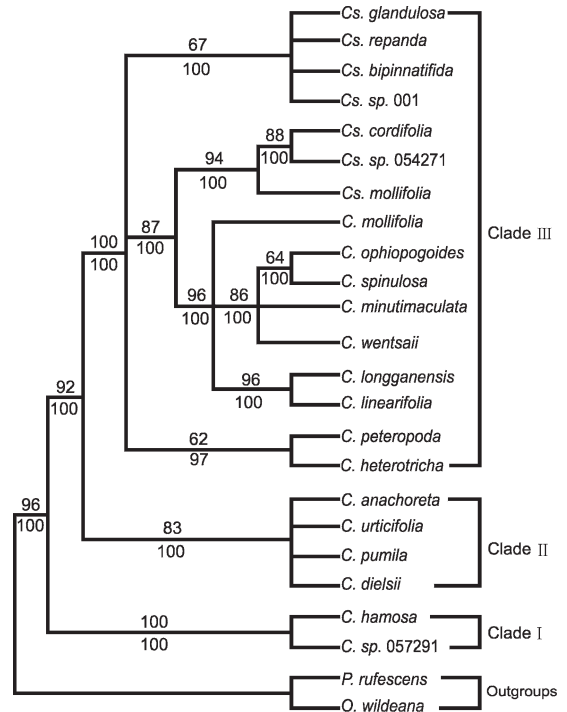
FIG. 3. Strict consensus of 2 MPTs generated from the *trnL-F* data which is congruent with the majority rule consensus from the Bayesian analysis. The bootstrap values are shown above the branches, and the Bayesian posterior probabilities are indicated below the branches. *C* = *Chirita*, *Cs* = *Chiritopsis*, *O* = *Ornithoboea*, *P* = *Paraboea*.

= 0.9509). The topologies of the MP and Bayesians trees for *trnL-F* are also congruent. The topology of the strict consensus tree based on *trnL-F* is largely congruent with the ITS topology (Figs. 2–3). Differences between the two trees were detected in Clade III. Although *C. peteropoda* and *C. heterotricha* also cluster in a clade (BS = 62%, PP = 97%) in *trnL-F* data, they are not sister to *Chiritopsis* and other species of sect. *Gibbosaccus* as was resolved in the ITS tree. Within the largest clade (BS = 87%, PP = 100%), *Cs. cordifolia*, *Cs. sp.* 054271 and *Cs. mollifolia* are separated by high support (BS = 94%, PP = 100%) and are sister to the remainder of sect. *Gibbosaccus* (excluding *C. peteropoda* and *C. heterotricha*) which forms a branch with strong support (BS = 96%, PP = 100%). The latter is composed of *C. mollifolia* and two strongly supported clades (Fig. 3). Within the *Cs. mollifolia* clade (BS = 94%, PP = 100%), the *trnL-F* data placed *Cs. mollifolia* as sister to the *Cs. cordifolia* and *Cs. sp.* 054271 clade (BS = 88%, PP = 100%) which was unresolved in the ITS tree (Fig. 2). The data also resolved *C. mollifolia* as part of a clade that includes the *C. spinulosa* and the *C. longgangensis* clades of Fig. 3, although there is less resolution within these clades than with ITS data alone. The third group includes *Cs. glandulosa*, *Cs. repanda* var. *guilinensis*, *Cs.*

bipinnatifida and *Cs. sp.* 001 revealing almost identical *trnL-F* sequences with low to maximum support (BS = 67%, PP = 100%).

Combined Matrix. The ILD test gave a value of *p* = 0.214, indicating that the data from two distinct marker regions were congruent, thereby justifying the combined analysis of both loci. Modeltest suggests that GTR + I + G best fit the combined data for BI analysis. The combined matrix included the identical set of taxa as single marker matrices, consisted of 1,601 positions, 183 (11.43%) of which were variable but uninformative and 301 (18.80%) parsimony-informative. The parsimony analysis produced a single tree that was 964 steps long with CI = 0.707 and RI = 0.728 (Fig. 4).

The combined data parsimony and BI trees are a hybrid between the ITS and cpDNA topologies (Figs. 2–4). Highly supported nodes based on only one data set are generally present and strongly supported in the combined analysis (Clade II, Figs. 2–4). In general, the combined analysis represents the strongly supported nodes of the individual analyses and there are no contradictions between the topology of the trees obtained from the combined analysis and those from analyses of
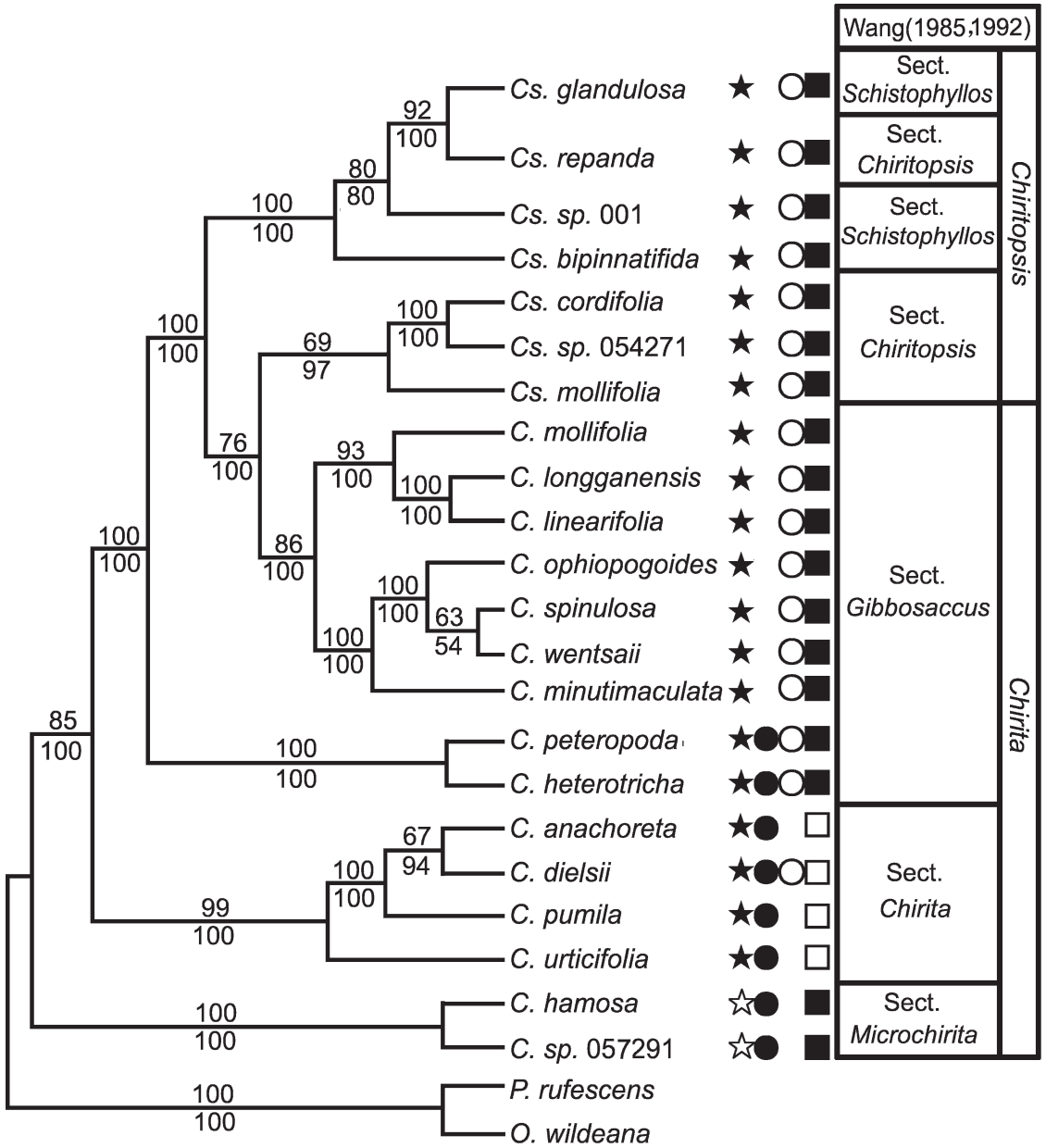
Fig. 4. Single MPT generated from the combined ITS and *trnL-F* data which is congruent with the majority rule consensus from the Bayesian analysis. The bootstrap values are shown above the branches, and the Bayesian posterior probabilities are indicated below the branches. *C* = *Chirita*, *Cs* = *Chiritopsis*, *O* = *Ornithoboea*, *P* = *Paraboea*. Notes of the following symbols: ☆ anthers joined by an apical ligature; ★ anthers fused face to faces; □ tubular calyx; ■ free calyx lobes; ○ rhizomes; ● caulescent.

the separate matrices. The only difference is better and more strongly supported internal branches in Clade III.

   Morphological Data.   Apart from differences in floral form, three characters are phylogenetically informative (Table 1). Interestingly, an increase in corolla width corresponds to a shorter corolla length in *Chirita* sect. *Gibbosaccus* and *Chiritopsis*.

Another character separating *Chirita* sect. *Gibbosaccus* and *Chiritopsis* from sect. *Chirita* is the presence of a whole gynoecium longer than the corolla tube. The ratios between the three characters are the most variable. In *Chirita* sect. *Gibbosaccus* and *Chiritopsis*, the proportions of the corolla below the middle and corolla mouth width are prominently increased as compared to section *Chirita* (t-

test, $p < 0.001$). Similar proportional correlations are found between whole gynoecium and corolla tube length (t-test, $p < 0.001$).

DISCUSSION

Phylogenetic Analyses. In all trees, Clade I consisted of species of sect. *Microchirita* that showed maximum support and was sister to the remainder of the species in the analysis. This is not surprising as sect. *Microchirita* bears a series of synapomorphies such as monocarpic-annual habit, peduncles adnate to or arising from the petiole, or crested inflorescences with peduncle fused to the short petiole, and anthers fused apically. Section *Microchirita* is traditionally considered isolated from other *Chirita* species (Wood 1974).

Section *Chirita* traditionally has been considered closely related to sect. *Gibbosaccus*, sharing the habit of perennial herbs, peduncle not adnate to petiole, and anthers fused face to face (Wood 1974; Wang et al. 1990). However, plants of sect. *Chirita* are usually caulescent, sometimes annual, and their calyx is often tubular. These characters are distinctively different from those of sect. *Gibbosaccus*. The monophyly of sect. *Chirita*, i.e. Clade II, is strongly supported by both ITS and *trnL-F* trees in our study. The phylogenetic trees presented here suggest that the traditional circumscriptions of sects. *Chirita* and *Microchirita* represent monophyletic groups. However the limited sampling (four species of sect. *Chirita*, and two of sect. *Microchirita*), the widespread geographic distribution and great morphological diversity in these two sections limit the extent to which we can make inferences regarding the monophyly of these sections.

Clade III is comprised of representative species of *Chiritopsis* and *Chirita* sect. *Gibbosaccus*. Morphologically, the species of Clade III can be distinguished from sect. *Chirita* by the acaulescent perennial habit, the presence of rhizomes, leaves often fleshy, and calyx divided to the base. *Chiritopsis* differs from *Chirita* species by having an ovoid ovary shorter than the style and the straight capsule ranging from ovoid to ellipsoid in shape (Wood 1974; Wang et al. 1990). In sect. *Gibbosaccus*, *C. peteropoda* and *C. heterotricha* are sister to the remaining members of Clade III. Concomitantly, they exhibit a morphological transitional form between sect. *Gibbosaccus* and sect. *Chirita*, such as a relatively long corolla tube and a long vertical rhizome similar to the short aerial stems in some species of sect. *Chirita*. Furthermore, even though the remaining members of sect. *Gibbosaccus* are well supported as monophyletic, they are clustered with some species of *Chiritopsis* sect. *Chiritopsis* (Fig. 4). Thus, in sect. *Gibbosaccus*

there are, in addition to *C. peteropoda* and *C. heterotricha*, two further monophyletic groups. The first group consists of *C. mollifolia*, *C. longgangensis*, and *C. linearifolia*. These are compact species with narrow, densely hairy and fleshy leaves, often produced on a long rhizome, a kind of trunk-like "neck". *C. spinulosa*, *C. ophiopogoides*, *C. wentsaii*, and *C. minutimaculata* form another well supported monophyletic group. The first three species were placed in subsect. *Spinulosae* on account of their linear and finely spiny denticulate leaves (Wang 1985; Fang et al. 1993). In the *trnL-F* tree, *C. minutimaculata* is nested in but not sister to subsect. *Spinulosa* which was resolved in ITS and combined tree (Figs. 2–4). *C. minutimaculata* with its oblong leaves without spiny teeth, does not fit into Wang's (1985) concept of this subsection. However, the narrowly elliptical, leathery leaves are shared between *C. minutimaculata* and the species of subsect. *Spinulosae*.

In the combined trees, the division of *Chiritopsis* into two clades shows that its species belong to two distinct lineages. The first clade, with *Cs. cordifolia*, *Cs. sp.* 054271 and *Cs. mollifolia*, corresponds to sect. *Chiritopsis* and is sister to sect. *Gibbosaccus* (excluding *C. peteropoda*, and *C. heterotricha*). These species are characterized by rather narrow, densely hairy, undivided leaves and are distributed in the northwest of Guangxi. The second is relatively isolated as the sister lineage to the remainder of Clade III (excluding *C. peteropoda* and *C. heterotricha*). In all trees, *Cs. repanda* var. *guilinensis* is embedded in sect. *Schistophyllos* (Figs. 2–4), as is morphologically supported by the plants having broader, glabrous or sparsely hairy leaves. In addition, they are all distributed in northeastern Guangxi.

The corolla tubes in both *Chiritopsis* and sect. *Gibbosaccus* are relatively short and broad (Table 1). In *Chiritopsis*, the proportion of corolla width and length ranges from 0.25 to 0.65 and the ratio of width between lower part and mouth of corolla tube varies from 0.66 to 0.84. In sect. *Gibbosaccus*, the proportion of width and length of corolla tube is from 0.14 to 0.65 and the ratio of width between lower part and mouth of corolla tube ranges from 0.5 to 0.8. In contrast, in Clade II, the proportions are 0.04–0.12 and 0.17–0.3, respectively, which are statistically different ($p < 0.001$). The morphological character of short and broad corolla tube is further pronounced in Clade III. The consistency of corolla form between *Chiritopsis* and *Chirita* section *Gibbosaccus* indicates that the two taxa are closely related. Wang (1985) suggested that sect. *Gibbosaccus* and sect. *Chirita* should be combined and upgraded to the level of subgenus. Further studies will be required to offer a more conclusive answer

for this intriguing matter. Our data indicate that these two sections are sister to each other and that the floral morphologies are distinctly different between the clades. Based on our small sampling of species from section *Chirita* no further statements can be made.

Some Aspects of Morphological Evolution and Adaptation. The present molecular phylogeny represents the first major step toward understanding the evolution of *Chirita* and *Chiritopsis*. However, mapping morphological characters onto the molecular phylogeny, and further analyzing their biological and evolutionary significance under particular geographical and ecological backgrounds, would enhance our understanding of morphological diversity in relation to the evolutionary history of these clades. In particular, the changes in morphological diversity seen in sect. *Gibbosaccus* and *Chiritopsis* may be the result of their special habitat. Southern China and northern Vietnam, where sect. *Gibbosaccus* and *Chiritopsis* are concentrated, has undergone a slow epeirogenic uplift as a result of the upthrust of the Himalayas after the collision of the Indian subcontinent with the mainland of Asia, commencing about 50 million years ago (Axelrod et al. 1998). The successive elevation has exposed broad plateaus of gently dipping to horizontal carbonate strata (Sweeting 1978). After each uplift event a renewed phase of karstification occurred which resulted in a karst topography composed of various types of carbonate rocks (Yuan et al. 1991). Geological changes were intimately connected with climatic changes resulting in a humid, subtropical monsoon climate, characterized by sharply contrasting dry and rainy seasons. Therefore, southern China and northern Vietnam, the largest area in the world covered with pure carbonate substrate, provided various ecological niches, such as numerous caves, bare rocks with crevices and pockets, and diverse climatic and ecological environments for plants and wildlife (Wu 1980; Xu 1993, 1995). The severe erosion in the rainy season leaves soil only in crevices and pockets of rocks. When a long dry season occurs, it is extremely strenuous for the growth of plants (Wu 1980; Xu 1995). In addition, the forests on limestone are usually isolated and distributed in a mosaic pattern, mixed with bare areas and acid-soil forests. According to Ying and Zhang (1984) and Xu (1995), the limestone areas in southern China are characterized by enormous plant diversity, with high rates of endemism. The heterogenous and variable ecological environments apparently have promoted the isolation of species and exerted strong selection pressure, resulting in a rapid adaptive evolution. This may also explain the great diversity of sect. *Gibbosaccus*, since about 90% of the species in sect. *Gibbosaccus* are endemic to the limestone areas (Wen et al. 1998; Wang 2004).

FLORAL ORGANS. The shift in floral form that is seen between the species of Clade II (sect. *Chirita*) and those of Clade III (sect. *Gibbosaccus/Chiritopsis*) may be the result of selection by pollinators. In sect. *Chirita* the stigma is usually located just below the corolla mouth and the stamens are held below the stigma (Wood 1974; Wang 2004). When a visiting insect pushes into the flowers, its back becomes dusted with pollen (Wood 1974, pers. obs.). When the insect leaves the corolla, it pushes against the lower surface of the stigmatic lamella pressing the receptive upper surface of the lamella against the roof of the corolla. This reduces the chance of self-pollination. In sect. *Gibbosaccus* and *Chiritopsis*, there is an evolutionary trend for the corolla tube to become shorter and broader than in sect. *Chirita* (t-test, $p < 0.001$). The stigma in sect. *Gibbosaccus* and *Chiritopsis* is usually placed at the mouth of the corolla tube or completely exserted from the corolla tube while the stamens are included. Stigmas and anthers are widely separated and pollinators may contact only one set of sex organs or touch them with different body parts while visiting flowers (Barrett et al. 1996). This placement of the stigma presumably further reduces the chance of self-pollination as compared to sect. *Chirita*. Fenster (1991) suggested, reduction of corolla tube length might be accompanied by increasing taxonomic diversity of pollinators and reduced specificity of pollen placement on pollinators' bodies. The short and broad corolla tube with exserted stigma may enable the flowers to be more frequently visited by different pollinators while self-pollination is effectively avoided. Thus the flowers of species in Clade III (sect. *Gibbosaccus* and *Chiritopsis*) seem to have switched to a generalist pollinators, while minimizing self-pollination.

AERIAL STEM AND RHIZOME. In addition to the relatively short and broad corolla tube with calyx divided to base, the species of Clade III (sect. *Gibbosaccus* and *Chiritopsis*) are characterized by an acaulescent perennial rhizomatous habit. However, in some species of Clade II various transitional forms from aerial stems to rhizomes can be observed. For example, in *C. dielsii* the aerial stem is short and stout, with leaves borne on its upper part caused by internode condensation. On the other hand, *C. peteropoda* and *C. heterotricha* of Clade III, being sister to the remaining members of Clade III in ITS and combined cladograms, have long vertical rhizomes morphologically similar to the short aerial stems in some species of sect. *Chirita*. Apparently, there is an evolutionary trend

in plant habit leading from caulescent perennial herbs to acaulescent perennial herbs with rhizomes. As with the shifts in floral morphology, the shift in vegetative form between the two clades may be the result of selection in the Karst habitat. The species of Clade III are able to revive after a drought in the cracks of naked and exposed limestone surfaces or areas covered by thin soil. For example, the leaves of *C. heterotricha* produce a wax layer on the surface that inhibits water-loss and seem to have a greater capability to retain water in the early stages of dehydration, since the relative water content remains at around 80% after 2 days of dehydration (Deng et al. 2003). As noted by Wei et al. (2004), the fleshy leaves of some species of sect. *Gibbosaccus* are able to store up enough water to survive through the dry season. Even if the leaves perish, the rhizomes can shoot out new leaves and resume growth as soon as the first rain falls in the spring recur.

Parallelisms, continuous variation and environmental plasticity all plague the use of morphological characters in systematics. Comparisons of adult morphologies often can be misleading because unrelated taxa may arrive at an apparently similar adult form through different developmental processes. Conversely, organisms may share similar developmental patterns and evolutionary histories, but look quite different as adults because of one or a few divergences in the developmental pattern (Kellogg et al. 2004). The reconstruction of phylogenies from DNA sequence data over the last decade has provided more robust phylogenies that stimulate more rigorous interpretation of morphology (Endress et al. 2000). In this study, sect. *Gibbosaccus* (including *Chiritopsis*) proved sister to sect. *Chirita* in all trees. The close alliance between the two sections had also been recognized by Wang (1985) due to many shared morphological characters such as peduncle not adnate to petiole, anthers coherent face to face and perennial herbs with rhizome, rarely annuals. Furthermore, *Chiritopsis* is split into two different clades within sect. *Gibbosaccus*. Even though species of *Chiritopsis* are uniform in gross morphology and display the simplest architecture in the present alliance, i.e. small plants, and flowers with ovoid ovary (Wang 1981, 1992), these characters cannot serve as synapomorphies to isolate these species as a monophyletic and to separate them from section *Gibbosaccus*. The inclusion of all taxa in a single redefined genus appears to be the best solution (Möller and Cronk 1997; Compton et al. 1998; McNeill 2000; Bräuchler et al. 2004). It is, therefore, recommended to include *Chiritopsis* in sect. *Gibbosaccus* rather than to retain them in a separate

genus. However, future sampling may also lead to a better understanding of sects. *Chirita* and *Microchirita* and additional nomenclatural changes may be needed.

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APPENDIX 1. List of taxa are presented in the following sequence: Groups (in bold) arranged by sections, genera or tribes according to the systems of Wang et al. (1990), taxon, GenBank accession numbers for ITS and *trn*L-F, locality, collector, voucher. Previously published GenBank accessions are noted with an asterisk. — indicates data not shown at present. PE = the Herbarium of the Institute of Botany, Chinese Academy of Sciences. The species (excluding *Chirita*, *Chiritopsis*, *Ornithoboea* and *Paraboea*) were used only in a preliminary analysis to resolve that *Ornithoboea* and *Paraboea* were sister to *Chirita*.

***Chirita* sect. Gibbosaccus** C. B. Clarke: *Chirita heterotricha* Merr.; DQ872826, DQ872816; Guangxi, Yin-Zheng Wang, 067311 (PE); *Chirita linearifolia* W. T. Wang; DQ872834, DQ872810; Guangxi, Jia-Mei Li, 11121 (PE); *Chirita longgangensis* W. T. Wang; DQ872833, DQ872809; Guangxi, Jia-Mei Li, 1183 (PE); *Chirita minutimaculata* D. Fang et W. T. Wang; DQ872828, DQ872815; Guangxi, Jia-Mei Li, 067134 (PE).*Chirita mollifolia* D. Fang, Y. G. Wei et J. Murata; DQ872832, DQ872811; Guangxi, Jia-Mei Li, Ljm-04-42 (PE); *Chirita ophiopogoides* D. Fang et W. T. Wang; DQ872829, DQ872814; Guangxi, Yin-Zheng Wang, 067134 (PE); *Chirita pteropoda* W. T. Wang; DQ872827, DQ872817; Guangxi, Yin-Zheng Wang, 067312 (PE); *Chirita spinulosa* D. Fang et W. T. Wang; DQ872830, DQ872813; Guangxi, Yin-Zheng Wang,

067133 (PE); *Chirita wentsaii* D. Fang et L. Zeng; DQ872831, DQ872812; Guangxi, Jia-Mei Li, 11630 (PE). **Chirita sect. Chirita:** *Chirita anachoreta* Hance; DQ872837, DQ872820; Yunnan, Jia-Mei Li, 1022 (PE); *Chirita dielsii* (Borza) B. L. Burtt; DQ872838, DQ872818; Yunnan, Jia-Mei Li, 058132 (PE); *Chirita pumilia* D. Don; DQ872836, DQ872819; Yunnan, Jia-Mei Li, 05833 (PE); *Chirita urticifolia* Buch.–Ham. ex D. Don; DQ872835, DQ872821; Yunnan, Jia-Mei Li, 05851 (PE). **Chirita sect. Microchirita C. B. Clarke:** *Chirita hamosa* R. Br.; DQ872839, DQ872822; Guangxi, Jia-Mei Li, 1181 (PE); *Chirita sp.* 057291; DQ872840, DQ872823; Yunnan, Jia-Mei Li, 057291 (PE). **Chiritopsis sect. Chiritopsis:** *Chiritopsis cordifolia* D. Fang et W. T. Wang; DQ872845, DQ872803; Guangxi, Jia-Mei Li, 05561(PE); *Chiritopsis mollifolia* D. Fang et W. T. Wang; DQ872847, DQ872802; Guangxi, Jia-Mei Li, 054281(PE); *Chiritopsis repanda* var. *guilinensis* W. T. Wang; DQ872846, DQ872808; Guangxi, Jia-Mei Li, 05523 (PE); *Chiritopsis sp.* 054271; DQ872844, DQ872807; Guangxi, Jia-Mei Li, 054271(PE). **Chiritopsis sect. Schistophyllos W. T. Wang:** *Chiritopsis glandulosa* D. Fang, L .Zeng et D. Qin; DQ872841, DQ872804; Guangxi, Jia-Mei Li, 054291 (PE); *Chiritopsis sp.* 001; DQ872843, DQ872805; Guangxi, Jia-Mei Li, 067136 (PE). **Other Didymocarpeae:** *Briggsia mihieri* (Franch.) Craib; —, —; Guizhou, Jia-Mei Li, 2003014 (PE); *Didymocarpus purpureobracteatus* W. W. Smith; —, —; Yunnan, Jia-Mei Li, Ljm-04-50 (PE); *Gyrocheilos retrotrichum* var. *oligolobum* W. T. Wang; —, —; Guangxi, Jia-Mei Li, 10232 (PE); *Hemiboea subcapitata* C. B. Clarke; —, —; Guangxi, Jia-Mei Li, 1128 (PE); *Oreocharis benthamii* var. *reticulata* Dunn; —, —; Guangdong, Jia-Mei Li, Ljm-04-89(PE); *Ornithoboea wildeana* Craib; DQ865197, DQ872824; Guangxi, Jia-Mei Li, Ljm-04-44 (PE); *Paraboea rufescens* (Franch.) B. L. Burtt; DQ865196, DQ872825; Guangxi, Jia-Mei Li, 0185 (PE); *Petrocosmea nervosa* Craib; —, AJ492299*; —, —; *Primulina tabacum* Hance; —, AJ492300*; —, —; *Pseuochirita guangxiensis* var. *glauca* Y. G. Wei et Y. Liu; —, —; Guangxi, Jia-Mei Li, 1163 (PE). **Trichosporeae:** *Aeschynanthus buxifolius* Hemsl.; —, —; Yunnan, Jia-Mei Li, 10410 (PE); *Loxostigma cavaleriei* (Lev. et Van.) B. L. Burtt; —, —; Guangxi, Jia-Mei Li, 10292 (PE). **Ramondieae:** *Conandron ramondioides* Sieb. et Zucc.; —, —; Zhejiang, Nan Xiao, Xn-03-39 (PE). **Epithemateae:** *Rhynchoglossum obliquum* var. *hologlossum* Blume; —, AY423133*; —, —; *Whytockia tsiangiana* (Hand.-Mazz.) A. Weber; —, AJ492289*; —, —. **Scrophulariaceae:** *Rehmannia glutinosa* Libosch.; —, —; Beijing, Zhi Xia, XZ-04-05 (PE); *Rehmannia henryi* N. E. Brown; —, —; Hubei, Zhi Xia, XZ-04-02 (PE).