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Research Article

DNA barcoding of *Gaultheria* L. in China (Ericaceae: Vaccinioideae)

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Abstract Four DNA barcoding loci, chloroplast loci *rbcL*, *matK*, *trnH-psbA*, and nuclear locus internal transcribed spacer (ITS), were tested for the accurate discrimination of the Chinese species of *Gaultheria* by using intraspecific and interspecific pairwise *P*-distance, Wilcoxon signed rank test, and tree-based analyses. This study included 186 individuals from 89 populations representing 30 species. For all individuals, single locus markers showed high levels of sequencing universality but were ineffective for species resolvability. Polymerase chain reaction amplification and sequencing were successful for all four loci. Both ITS and *matK* showed significantly higher levels of interspecific species delimitation than *rbcL* and *trnH-psbA*. A combination of *matK* and ITS was the most efficient DNA barcode among all studied regions, however, they do not represent an appropriate candidate barcode for Chinese *Gaultheria*, by which only 11 out of 30 species can be separated. Loci *rbcL*, *matK*, and *trnH-psbA*, which were recently proposed as universal plant barcodes, have a very poor capacity for species separation for Chinese *Gaultheria*. DNA barcodes may be reliable tools to identify the evolutionary units of this group, so further studies are needed to develop more efficient DNA barcodes for *Gaultheria* and other genera with complicated evolutionary histories.

Key words DNA barcoding, Ericaceae, *Gaultheria*, species discrimination, taxonomy, Vaccinioideae.

The genus *Gaultheria* L. contains approximately 135 species within the tribe Gaultherieae of Vaccinioideae, Ericaceae. *Gaultheria* occurs throughout the continental areas and islands bordering the Pacific Rim (Lu et al., 2010). As one of the centers of *Gaultheria* diversity, China possesses rich germplasm resources with approximately one quarter of the total species (ca. 34 sp.) (Fritsch et al., 2008). All species of the Chinese *Gaultheria* are endemic to Southwest China except for the varieties of *Gaultheria leucocarpa* Blume (e.g., *G. leucocarpa* var. *yunnanensis* (Franchet) T. Z. Hsu & R. C. Fang, found throughout the southern part of the Yangtze River), *G. borneensis* Stapf (Taiwan), and *G. taiwaniana* S. S. Ying (Taiwan). Chinese *Gaultheria* species have a high diversity habitat and morphologic features (e.g., leaf vein, position of bracteoles and fruit color), and are characterized by a high endemism (ca. 14 endemic species) (Fritsch et al., 2008; Lu et al., 2010). Several species within this genus have economic value as they are well-known for the presence of wintergreen oil (methyl salicylate), which is commonly used in Traditional Chinese medicine and the confection industry (e.g., *G. fragrantissima* Wall. and *G. leucocarpa*). Nevertheless, taxonomy on these taxa requires intensive studies based on the molecular phylogeny of Lu et al. (2010).

Related taxonomic treatments for Chinese *Gaultheria* have been proposed by Airy-Shaw (1941), Middleton (1991), Xu (1981, 1986a, 1986b, 1991), Fang (1999), Li et al. (2000), Fang & Stevens (2005), and Fritsch et al. (2008). Airy-Shaw (1941) named more than 30 species from the Sino-Himalayan region in his treatment. Xu (1981) recognized 24 species from China and initially investigated the classification of Chinese *Gaultheria*. On the basis of inflorescence, he divided them into three types, i.e., cymiferous type, racemiferous type and flore solitario type. Twenty-seven Chinese species were included in Middleton's (1991) classification and fell into four sections and seven series based on characters of calyx, fruit, and inflorescences. Fang & Stevens (2005) recognized 32 species (15 endemic) in the revision of *Gaultheria* for the *Flora of China*. Based on the prior treatments, Fritsch et al. (2008) modified the total number of Chinese *Gaultheria* to 34 species (14 endemic).

Based on molecular data from five genic regions, internal transcribed spacer (ITS), *matK*, *rpl16*, *trnL-trnF*, and *trnS-trnG*, Lu et al. (2010) provided the first comprehensive phylogenetic hypothesis for the

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core East Asian clade of *Gaultheria*, which was comprised of 27 out of 34 Chinese species. The current taxonomic treatments (Fang & Stevens, 2005; Fritsch et al., 2008) do not correspond to the phylogenetic relationships found in Lu et al. (2010), that is, many species were found to be non-monophyletic. Two major clades were recognized: the ser. *Leucothoides* s.l. clade; and the ser. *Trichophyllae* clade. Hybridization and introgression occur in ser. *Leucothoides* and some species (e.g. *G. notabilis* J. Anthony) appear to be hybrids. In contrast, the non-monophyletic species from ser. *Trichophyllae* seems to result from a combination of convergent character evolution and the presence of cryptic species. Species delimitation studies would benefit by a combination of molecular and morphological analyses focusing on certain characters such as pedicel length, calyx shape, and fruit shape and color. Therefore, molecular data plays a substantial role in resolving the taxonomic confusion in Chinese *Gaultheria*.

DNA barcoding involves sequencing a standard region of DNA as a tool for fast and accurate taxon identification (Hebert et al., 2003). It accelerates the pace of species discovery by allowing taxonomists to rapidly sort specimens and by highlighting divergent taxa that might represent new species (Hebert & Gregory, 2005). It is also a powerful tool for taxonomy and biogeography with utility for identifying cryptic species and biogeographic patterns, and resolving classification at the rank of genus and species (Newmaster & Ragupathy, 2009). A useful DNA barcode requires sufficient sequence variation to distinguish between species and ease of application across a broad range of taxa (Kress & Erickson, 2007). Many candidate barcode loci, including coding and non-coding regions, have been tested in different genera and families of land plants in recent years. Kress et al. (2005) proposed the nuclear ITS region and the plastid *trnH-psbA* intergenic spacer as potentially useful DNA regions for application of barcoding to flowering plants. Lahaye et al. (2007) identified a portion of the plastid *matK* gene as a universal DNA barcode for flowering plants. Analyzing >1000 species of Mesoamerican orchids, DNA barcoding with *matK* alone reveals cryptic species and has been proven useful in identifying species listed in Convention on International Trade of Endangered Species appendixes (Lahaye et al., 2007). A combination of the non-coding *trnH-psbA* spacer region and a portion of the coding *rbcL* gene are recommended as a two-locus global land plant barcode that provides the necessary universality and species discrimination (Kress & Erickson, 2007). In addition, the CBOL Plant Working Group (2009) recommended a two-locus combination of *rbcL* + *matK* as the core barcode for land plants. In this study, we selected four commonly recommended DNA loci (*rbcL*, *matK*, *trnH-psbA*, and ITS) and combinations of these loci to test their potential for species delimitation in the Chinese species of *Gaultheria*, and evaluated their value as universal plant DNA barcoding regions.

1 Material and methods

1.1 Sampling strategy

In the present study, 186 individuals from 89 populations representing 30 species were investigated (Appendix). It covered 88.24% (30/34) of the total number of *Gaultheria* species in China. Each population sampled contained one to four individuals. For some narrowly endemic species with only one population, such as *G. brevistipes* (C. Y. Wu & T. Z. Hsu) R. C. Fang, *G. dolichopoda* Airy Shaw, *G. heteromera* R. C. Fang, *G. jingdongensis* R. C. Fang, *G. notabilis*, and *G. trigonoclada* R. C. Fang, four individuals were sampled of each species. For some widespread species, such as *G. fragrantissima*, *G. griffithiana* Wight and *G. leucocarpa* var. *yunnanensis*, geographical coverage and morphological variation were considered. Four Chinese species were unavailable to us, namely, *G. longiracemosa* Y. C. Yang, *G. purpurea* R. C. Fang, *G. taiwaniana*, and *G. nivea* (J. Anthony) Airy Shaw. Leaf material was collected on silica gel in the wild or from cultivated individuals. We follow the species concepts given by Fang & Stevens (2005) and Fritsch et al. (2008) when identifying the materials.

1.2 DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from silica gel dried leaves using the CTAB method (Doyle & Doyle, 1987). The DNA was dissolved in TE buffer (10 mmol/L Tris-HCl, pH 8.0, 1 mmol/L EDTA) to a final concentration of 50–100 ng/μL. Polymerase chain reaction (PCR) amplifications were carried out on a Veriti 96-well Thermal Cycler (Applied Biosystems, Foster City, CA, USA) using 2× Taq PCR MasterMix (Tiangen Biotech, Beijing, CN) in a 25 μL reaction according to the manufacturer's instructions. The *rbcL*, *matK*, *trnH-psbA*, and ITS DNA loci were tested as barcoding markers (Table 1). Thermocycling conditions were optimized for *rbcL* and *trnH-psbA* at 95 °C for 3 min, followed by 32 cycles of 94 °C for 40 s, 53 °C for 40 s and 72 °C for 1 min, with a final extension step of 72 °C for 7 min. The PCR profiles for ITS consisted of an initial denaturation step at 94 °C for 4 min, followed by 37 cycles of 1 min at 94 °C, 45 s at 52 °C, 1 min at 72 °C, and finished with an extension step of 7 min at 72 °C.

Table 1 Polymerase chain reaction primers used in this study

Locus	Primer	Primer sequence (5′→3′)	Sources
rbcL	*1F/724R*	ATGTCACCACAAACAGAAAC/ TCGCATGTACCTGCAGTAGC	Olmstead et al., 1992; Fay et al., 1997
matK	*3F_KIM/1R_KIM*	CGTACAGTACTTTTGTGTTTACGAG/ AATATCCAAATACCAAATCC	Kim KJ, unpublished data, Korea University, Seoul, Korea
trnH-psbA	*trnH2/psbAF*	CGCGCATGGTGGATTCACAATCC/ GTTATGCATGAACGTAATGCTC	Tate & Simpson, 2003; Sang et al., 1997
ITS	ITS4/ITS5	GGAAGTAAAAGTCGTAACAAGG/ TCCTCCGCTTATTGATATGC	Swensen et al., 1998

ITS, internal transcribed spacer.

The PCR conditions for amplifying the chloroplast fragment of *matK* included an initial denaturation at 94 °C for 4 min, followed by 37 cycles of 1 min at 94 °C, 1 min 30 s at 50 °C, 2 min at 72 °C, and finished with an extension step of 7 min at 72 °C. The PCR products were checked in 1% TAE agarose gel after electrophoresis and purified using the Sangon Purification Kit (Sangon, Shanghai, China). The purified PCR products were used for sequencing directly with the PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems), according to the manufacturer's protocol. The products were run on an ABI 3730 × 1 automated sequencer (Applied Biosystems).

1.3 Data analysis

We edited and assembled the raw DNA sequences with SeqMan (DNASTAR package; DNAStar, Madison, WI, USA). Sequence alignments for each locus were initially carried out in MUSCLE (Edgar, 2004), then aligned manually. To evaluate the levels of variation within the four DNA loci, the mean intraspecific and interspecific pairwise *P*-distance for each DNA region was calculated using MEGA4 (Tamura et al., 2007). Parameters such as CG content, aligned length, parsimony-informative (PI) sites and variable sites were calculated. To access potential benefits of a multilocus barcode over a single locus barcode, we examined multiple combinations of the barcoding loci within each taxonomic group. They are *rbcL* + *matK*, *matK* + ITS, *rbcL* + *matK* + ITS, *rbcL* + *matK* + *trnH-psbA*, and *rbcL* + *matK* + *trnH-psbA* + ITS. The combinations tested included previously proposed multilocus barcode combinations (see the CBOL Plant Working Group, 2009), along with other combinations which were chosen based on the performance of an individual region (Table 2).

Wilcoxon signed rank tests were carried out to compare intra- and interspecific variability for every pair of barcodes following Kress & Erickson (2007) (Tables 3, 4). Tree-based analysis was used to evaluate species discrimination and provide a convenient method of viewing the data. Maximum likelihood (ML) tests were carried out to evaluate whether species were shown to be monophyletic with each barcode using RAxML (Stamatakis, 2006). Neighbor-joining (NJ) trees were constructed under the *P*-distance model and pairwise deletion using MEGA4 to evaluate species delimitation. Each tree contains the bootstrap values as calculated by the software with 1000 replications. Calculations assessing levels of species discrimination were only carried out when the cut-off value for condensed tree was 50% (parsimony bootstrap support >50%) of the samples for a given taxonomic group. Species resolvability at three levels (50%, 50% individuals of a species form a clade; 75%, 75% individuals of a species form a clade; and 100%, all individuals of a species form a clade) was evaluated (Table 5).

2 Results

2.1 Polymerase chain reaction and sequencing success

The coding regions *rbcL* and *matK*, and non-coding regions ITS and *trnH-psbA* used in this study were successfully amplified and sequenced. For *rbcL* and *trnH-psbA*, 186 individuals from all 30 species were successfully obtained. For ITS and *matK* loci, we sequenced 117 individuals from 26 species and 114 individuals from 26 species, respectively (the remainder of the sequences from previous work of Lu et al. (2010) were taken from GenBank). The PCR amplification and sequencing success rates of four regions were all 100%. Some sequences of *matK* were well amplified by modifying the PCR procedure and using 2× Taq PCR MasterMix.

2.2 Evaluation of DNA markers

The *rbcL* sequence was 620 bp in aligned length without indels, and included 21 PI sites and 24 variable sites. For the *matK* matrix, aligned sequence length was 809 bp with a 6-bp indel, and included 52 PI sites and 64 variable sites. The ITS matrix was 655 bp in aligned length, and included 68 PI sites,

Table 2 Summary statistics for the four barcoding DNA loci in the Chinese species of *Gaultheria*

Parameters	*rbcL*	*matK*	*ITS*	*trnH-psbA*	*rbcL* + *matK*	*rbcL* + *matK* + *trnH-psbA*	*rbcL* + *matK* + ITS	*rbcL* + *matK* + *trnH-psbA* + ITS	*matK* + ITS
Aligned length	620	809	655	475	1429	1904	2084	2559	1464
Parsim-info sites	21	52	68	17	73	388	141	456	120
Indel number (length bp)	0	1 (6)	13 (1, 2, 3)	2 (3, 20)	1(6)	3 (3, 6, 20)	14 (1, 2,3,6)	16 (1, 2, 3, 6, 20)	14 (1, 2, 3, 6)
Variable sites	24	64	75	18	88	441	163	516	139
CG content (%)	43.40	31.30	58.10	33.50	36.60	35.90	43.30	41.60	53.20
PCR success (%)	100	100	100	100	100	100	100	100	100
Sequencing success (%)	100	100	100	100	100	100	100	100	100
Species resolved† (100%)	2 (30)	8 (30)	7 (30)	2 (30)	7 (30)	5 (30)	11 (30)	8 (30)	11 (30)
Species resolved† (≥ 75%)	2 (30)	9 (30)	8 (30)	4 (30)	9 (30)	9 (30)	12 (30)	13 (30)	13 (30)
Species resolved† (≥50%)	3 (30)	12 (30)	8 (30)	4 (30)	12 (30)	12 (30)	14 (30)	15 (30)	15 (30)
Intraspecific *P*-distance, mean (range)%	0.14 (0–0.97)	0.06 (0–0.38)	0.14 (0–0.67)	0.09 (0–0.88)	0.1 (0–0.43)	0.96 (0–8.33)	0.11 (0–0.51)	0.75 (0–6.20)	0.1 (0–0.51)
Interspecific *P*-distance, mean (range)%	0.6 (0–1.6)	1.2 (0–2.8)	2.3 (0–5.6)	0.4 (0–1.3)	0.9 (0–2.0)	1.6 (0–7.8)	1.3 (0–2.9)	1.8 (0–6.6)	1.7 (0–3.9)
Kolmogorov–Smirnov *Z*	3.806	4.579	3.471	3.934	4.476	4.536	3.982	3.921	4.555
Sample species (individuals)	31 (186)	26 (114)	26 (117)	31 (186)	–	–	–	–	–

†When calculating the resolution of species discrimination, *Gaultheria discolor* and *G. prostrata* were excluded, due to only one individual sampled for each of them. –, Not applicable; ITS, internal transcribed spacer; Parsim-info, parsimony-informative; PCR, polymerase chain reaction.

75 variable sites, and 13 indels ranging from 1 to 3 bp long. For the *trnH-psbA* matrix, the aligned sequence length was 475 bp, and contained 17 PI sites, 18 variable sites, and two indels (3 bp and 20 bp). The mean CG content in different loci were 43.40% (*rbcL*), 31.30% (*matK*), 58.10% (ITS), 33.50% (*trnH-psbA*), 36.60% (*rbcL* + *matK*), 35.90% (*rbcL* + *matK* + *trnH-psbA*), 43.30% (*rbcL* + *matK* + ITS), 41.60% (*rbcL* + *matK* + *trnH-psbA* + ITS), and 53.2% (*matK* + ITS) (Table 2).

2.3 Measurement of interspecific versus intraspecific genetic divergence of various loci and combinations

To assess the degree of DNA polymorphism between DNA samples, sequence divergences between and within species were calculated by uncorrected *P*-distance. This model showed the following trend: higher average interspecific diversity and lower intraspecific distance. Eight matrices were used to characterize inter- versus intraspecific variation (Table 2). An appropriate barcode should possess a high interspecific divergence to distinguish different species and a low intraspecific divergence. For single regions, ITS and *matK* both showed significantly higher levels of interspecific discriminatory ability than *rbcL* and *trnH-psbA*. The lowest divergence between conspecific individuals was shown by *trnH-psbA*. The intraspecific differences showed a similar pattern, with *rbcL* and ITS having the largest variation and *matK* having the least. Increasing the number of loci can not only increase the aligned length, but also enhances the capacity to allow for interspecific and intraspecific divergence. The lowest intraspecific distance of combinations were both of *matK* + ITS and *rbcL* + *matK*, followed by *rbcL* + *matK* + ITS. The combined loci, *rbcL* + *matK* + *trnH-psbA* + ITS had the largest interspecific *P*-distance, followed by *matK* + ITS and *rbcL* + *matK* + *trnH-psbA*. Wilcoxon signed rank tests on combined data show that ITS is the most variable barcode at interspecific levels, followed by *matK* + ITS, whereas the lowest level of divergence is provided by *trnH-psbA* (Table 3). At intraspecific level, Wilcoxon signed rank tests show *rbcL* + *matK* + *trnH-psbA* having the highest level of divergence, whereas the lowest is provided by *matK* (Table 4).

2.4 Levels of species discrimination (at 100% resolution)

The application of the four individually evaluated DNA loci showed a range of discriminatory success by NJ analyses, with *matK* and ITS possessing relatively higher levels (Table 2). By single region analysis, *matK* was found to be the most successful at

Table 3 Wilcoxon signed rank tests of interspecific divergence among loci ($n = 435$)

W+	W−	Relative ranks/*P*-value (≤)			Result
ITS	*matK*	W+ = 85 383	W− = 9447	1.823×10^{-47}	ITS > *matK*
ITS	*rbcL*	W+ = 91 880	W− = 2515	1.76×10^{-65}	ITS > *rbcL*
ITS	*trnH-psbA*	W+ = 91 349	W− = 2612	5.03×10^{-65}	ITS > *trnH-psbA*
ITS	*matK* + ITS	W+ = 85 382	W− = 9448	1.833×10^{-47}	ITS > *matK* + ITS
ITS	*rbcL* + *matK*	W+ = 88 130	W− = 6700	2.581×10^{-54}	ITS > *rbcL* + *matK*
ITS	*rbcL* + *matK* + ITS	W+ = 88 133	W− = 6697	2.536×10^{-54}	ITS > *rbcL* + *matK* + ITS
ITS	*rbcL* + *matK* + *trnH-psbA*	W+ = 65 442	W− = 29 388	6.367×10^{-12}	ITS > *rbcL* + *matK* + *trnH-psbA*
ITS	*rbcL* + *matK* + ITS + *trnH-psbA*	W+ = 65 528	W− = 29 302	5.057×10^{-12}	ITS > *rbcL* + *matK* + ITS + *trnH-psbA*
matK + ITS	*matK*	W+ = 85 386	W− = 9444	1.793×10^{-47}	*matK* + ITS > *matK*
matK + ITS	*rbcL*	W+ = 93 096	W− = 1734	6.711×10^{-68}	*matK* + ITS > *rbcL*
matK + ITS	*trnH-psbA*	W+ = 93 963	W− = 867	1.977×10^{-70}	*matK* + ITS > *trnH-psbA*
matK + ITS	*rbcL* + *matK*	W+ = 92 213	W− = 2617	2.268×10^{-65}	*matK* + ITS > *rbcL* + *matK*
matK + ITS	*rbcL* + *matK* + ITS	W+ = 93 091	W− = 1739	6.938×10^{-68}	*matK* + ITS > *rbcL* + *matK* + ITS
matK + ITS	*rbcL* + *matK* + *trnH-psbA*	W+ = 59 542	W− = 35 288	3.794×10^{-6}	*matK* + ITS > *rbcL* + *matK* + *trnH-psbA*
matK + ITS	*rbcL* + *matK* + ITS + *trnH-psbA*	W+ = 59 946	W− = 34 884	1.785×10^{-6}	*matK* + ITS > *rbcL* + *matK* + ITS + *trnH-psbA*
matK	*rbcL*	W+ = 90 691	W− = 2837	3.327×10^{-64}	*matK* > *rbcL*
matK	*trnH-psbA*	W+ = 90 908	W− = 1327	4.767×10^{-68}	*matK* > *trnH-psbA*
matK	*rbcL* + *matK*	W+ = 90 665	W− = 2863	3.943×10^{-64}	*matK* > *rbcL* + *matK*
matK	*rbcL* + *matK* + ITS	W+ = 21 366	W− = 73 464	3.118×10^{-23}	*matK* < *rbcL* + *matK* + ITS
matK	*rbcL* + *matK* + *trnH-psbA*	W+ = 58 131	W− = 35 397	1.199×10^{-5}	*matK* > *rbcL* + *matK* + *trnH-psbA*
matK	*rbcL* + *matK* + ITS + *trnH-psbA*	W+ = 23 629	W− = 71 201	1.231×10^{-19}	*matK* < *rbcL* + *matK* + ITS + *trnH-psbA*
rbcL	*trnH-psbA*	W+ = 67 746	W− = 25 350	2.557×10^{-16}	*rbcL* > *trnH-psbA*
rbcL	*rbcL* + *matK*	W+ = 2814	W− = 90 714	2.863×10^{-64}	*rbcL* < *rbcL* + *matK*
rbcL	*rbcL* + *matK* + ITS	W+ = 1729	W− = 93 101	6.491×10^{-68}	*rbcL* < *rbcL* + *matK* + ITS
rbcL	*rbcL* + *matK* + *trnH-psbA*	W+ = 3568	W− = 89 960	3.808×10^{-62}	*rbcL* < *rbcL* + *matK* + *trnH-psbA*
rbcL	*rbcL* + *matK* + ITS + *trnH-psbA*	W+ = 1485	W− = 93 345	1.273×10^{-68}	*rbcL* < *rbcL* + *matK* + ITS + *trnH-psbA*
rbcL+ *matK*	*trnH-psbA*	W+ = 89 749	W− = 3779	1.474×10^{-61}	*rbcL* + *matK* > *trnH-psbA*
rbcL+ *matK*	*rbcL* + *matK* + ITS	W+ = 6692	W− = 88 138	2.462×10^{-54}	*rbcL* + *matK* < *rbcL* + *matK* + ITS
rbcL+ *matK*	*rbcL* + *matK* + *trnH-psbA*	W+ = 50 321	W− = 43 207	1.707×10^{-1}	*rbcL* + *matK* = *rbcL* + *matK* + *trnH-psbA*
rbcL+ *matK*	*rbcL* + *matK* + ITS + *trnH-psbA*	W+ = 6001	W− = 88 829	3.921×10^{-56}	*rbcL* + *matK* < *rbcL* + *matK* + ITS + *trnH-psbA*
rbcL + *matK* + ITS	*trnH-psbA*	W+ = 93 591	W− = 1239	2.442×10^{-69}	*rbcL* + *matK* + ITS > *trnH-psbA*
rbcL + *matK* + ITS	*rbcL* + *matK* + *trnH-psbA*	W+ = 58 055	W− = 36 775	5.002×10^{-5}	*rbcL* + *matK* + ITS > *rbcL* + *matK* + *trnH-psbA*
rbcL + *matK* + ITS	*rbcL* + *matK* + ITS + *trnH-psbA*	W+ = 57 198	W− = 37 632	1.923×10^{-4}	*rbcL* + *matK* + ITS > *rbcL* + *matK* + ITS + *trnH-psbA*
rbcL + *matK* + ITS + *trnH-psbA*	*trnH-psbA*	W+ = 94 134	W− = 696	6.184×10^{-71}	*rbcL* + *matK* + ITS + *trnH-psbA* > *trnH-psbA*
rbcL + *matK* + ITS + *trnH-psbA*	*rbcL* + *matK* + *trnH-psbA*	W+ = 65 183	W− = 29 647	1.266×10^{-11}	*rbcL* + *matK* + ITS + *trnH-psbA* > *rbcL* + *matK* + *trnH-psbA*
rbcL + *matK* + *trnH-psbA*	*trnH-psbA*	W+ = 91 197	W− = 2331	1.194×10^{-65}	*rbcL* + *matK* + *trnH-psbA* > *trnH-psbA*

ITS, internal transcribed spacer.

Table 4 Wilcoxon signed rank tests of intraspecific divergence among loci ($n = 30$)

W+	W−	Relative ranks/*P*-value (≤)			Result
ITS	*matK*	W+ = 146	W− = 25	8.419×10^{-3}	ITS > *matK*
ITS	*rbcL*	W+ = 110	W− = 61	2.86×10^{-1}	ITS = *rbcL*
ITS	*trnH-psbA*	W+ = 141	W− = 49	6.412×10^{-2}	ITS ≥ *trnH-psbA*
ITS	*matK* + ITS	W+ = 146	W− = 25	8.419×10^{-3}	ITS > *matK* + ITS
ITS	*rbcL* + *matK*	W+ = 145	W− = 45	4.421×10^{-2}	ITS > *rbcL* + *matK*
ITS	*rbcL* + *matK* + ITS	W+ = 145	W− = 45	4.421×10^{-2}	ITS > *rbcL* + *matK* + ITS
ITS	*rbcL* + *matK* + *trnH-psbA*	W+ = 134	W− = 142	9.032×10^{-1}	ITS = *rbcL* + *matK* + *trnH-psbA*
ITS	*rbcL* + *matK* + ITS + *trnH-psbA*	W+ = 135	W− = 141	9.273×10^{-1}	ITS = *rbcL* + *matK* + ITS + *trnH-psbA*
matK + ITS	*matK*	W+ = 146	W− = 25	8.419×10^{-3}	*matK* + ITS > *matK*
matK + ITS	*rbcL*	W+ = 75	W− = 115	4.209×10^{-1}	*matK* + ITS = *rbcL*
matK + ITS	*trnH-psbA*	W+ = 153	W− = 78	1.924×10^{-1}	*matK* + ITS = *trnH-psbA*
matK + ITS	*rbcL* + *matK*	W+ = 123	W− = 67	2.598×10^{-1}	*matK* + ITS = *rbcL* + *matK*
matK + ITS	*rbcL* + *matK* + ITS	W+ = 75	W− = 115	4.209×10^{-1}	*matK* + ITS = *rbcL* + *matK* + ITS
matK + ITS	*rbcL* + *matK* + *trnH-psbA*	W+ = 98	W− = 178	2.238×10^{-1}	*matK* + ITS = *rbcL* + *matK* + *trnH-psbA*
matK + ITS	*rbcL* + *matK* + ITS + *trnH-psbA*	W+ = 80	W− = 196	7.772×10^{-2}	*matK* + ITS ≤ *rbcL* + *matK* + ITS + *trnH-psbA*
matK	*rbcL*	W+ = 35	W− = 118	4.944×10^{-2}	*matK* < *rbcL*
matK	*trnH-psbA*	W+ = 58	W− = 78	6.05×10^{-1}	*matK* = *trnH-psbA*
matK	*rbcL* + *matK*	W+ = 35	W− = 118	4.944×10^{-2}	*matK* < *rbcL* + *matK*
matK	*rbcL* + *matK* + ITS	W+ = 29	W− = 161	7.908×10^{-3}	*matK* < *rbcL* + *matK* + ITS
matK	*rbcL* + *matK* + *trnH-psbA*	W+ = 40	W− = 191	8.68×10^{-3}	*matK* < *rbcL* + *matK* + *trnH-psbA*
matK	*rbcL* + *matK* + ITS + *trnH-psbA*	W+ = 40	W− = 236	2.876×10^{-3}	*matK* < *rbcL* + *matK* + ITS + *trnH-psbA*
rbcL	*trnH-psbA*	W+ = 120	W− = 51	1.329×10^{-1}	*rbcL* = *trnH-psbA*
rbcL	*rbcL* + *matK*	W+ = 117	W− = 36	5.518×10^{-2}	*rbcL* > *rbcL* + *matK*
rbcL	*rbcL* + *matK* + ITS	W+ = 115	W− = 75	4.209×10^{-1}	*rbcL* = *rbcL* + *matK* + ITS
rbcL	*rbcL* + *matK* + *trnH-psbA*	W+ = 115	W− = 116	9.861×10^{-1}	*rbcL* = *rbcL* + *matK* + *trnH-psbA*
rbcL	*rbcL* + *matK* + ITS + *trnH-psbA*	W+ = 116	W− = 160	5.034×10^{-1}	*rbcL* = *rbcL* + *matK* + ITS + *trnH-psbA*
rbcL+ *matK*	*trnH-psbA*	W+ = 131	W− = 79	3.316×10^{-1}	*rbcL* + *matK* = *trnH-psbA*
rbcL+ *matK*	*rbcL* + *matK* + ITS	W+ = 45	W− = 145	4.421×10^{-2}	*rbcL* + *matK* < *rbcL* + *matK* + ITS
rbcL+ *matK*	*rbcL* + *matK* + *trnH-psbA*	W+ = 90	W− = 141	3.754×10^{-1}	*rbcL* + *matK* = *rbcL* + *matK* + *trnH-psbA*
rbcL+ *matK*	*rbcL* + *matK* + ITS + *trnH-psbA*	W+ = 71	W− = 205	4.157×10^{-2}	*rbcL* + *matK* < *rbcL* + *matK* + ITS + *trnH-psbA*
rbcL + *matK* + ITS	*trnH-psbA*	W+ = 181	W− = 72	7.681×10^{-2}	*rbcL* + *matK* + ITS ≥ *trnH-psbA*
rbcL + *matK* + ITS	*rbcL* + *matK* + *trnH-psbA*	W+ = 132	W− = 144	8.552×10^{-1}	*rbcL* + *matK* + ITS = *rbcL* + *matK* + *trnH-psbA*
rbcL + *matK* + ITS	*rbcL* + *matK* + ITS + *trnH-psbA*	W+ = 131	W− = 145	8.314×10^{-1}	*rbcL* + *matK* + ITS = *rbcL* + *matK* + ITS + *trnH-psbA*
rbcL + *matK* + ITS + *trnH-psbA*	*trnH-psbA*	W+ = 225	W− = 51	8.143×10^{-3}	*rbcL* + *matK* + ITS + *trnH-psbA* > *trnH-psbA*
rbcL + *matK* + ITS + *trnH-psbA*	*rbcL* + *matK* + *trnH-psbA*	W+ = 134	W− = 142	9.032×10^{-1}	*rbcL* + *matK* + ITS + *trnH-psbA* = *rbcL* + *matK* + *trnH-psbA*
rbcL + *matK* + *trnH-psbA*	*trnH-psbA*	W+ = 178	W− = 53	2.983×10^{-2}	*rbcL* + *matK* + *trnH-psbA* > *trnH-psbA*

ITS, internal transcribed spacer.

Table 5 Species discrimination using the four loci and corresponding combinations based on tree-based analysis (neighbor-joining trees)

DNA locus	Resolution		
	100%	75%	50%
ITS	*G. codonantha*, *G. dolichopoda*, *G. leucocarpa*§, *G. longibracteolata*, *G. pseudonotabilis*, *G. suborbicularis*, *G. trigonoclada*	*G. codonantha*, *G. dolichopoda*, *G. leucocarpa*, *G. longibracteolata*, *G. pseudonotabilis*, *G. suborbicularis*, *G. trigonoclada*, *G. nummularioides*	*G. codonantha*, *G. dolichopoda*, *G. leucocarpa*, *G. longibracteolata*, *G. pseudonotabilis*, *G. suborbicularis*, *G. trigonoclada*, *G. nummularioides*
rbcL	*G. codonantha*, *G. suborbicularis*	*G. codonantha*, *G. suborbicularis*	*G. codonantha*, *G. suborbicularis*, *G. griffithiana*
trnH-psbA	*G. suborbicularis*, *G. dolichopoda*	*G. suborbicularis*, *G. dolichopoda*, *G. hypochlora*, *G. straminea*	*G. suborbicularis*, *G. dolichopoda*, *G. hypochlora*, *G. straminea*
matK	*G. cardiosepala*, *G. codonantha*, *G. dolichopoda*, *G. jingdongensis*, *G. leucocarpa*, *G. pyrolifolia*, *G. straminea*, *G. suborbicularis*	*G. cardiosepala*, *G. codonantha*, *G. dolichopoda*, *G. jingdongensis*, *G. leucocarpa*, *G. pyrolifolia*, *G. straminea*, *G. suborbicularis*, *G. dumicola*	*G. cardiosepala*, *G. codonantha*, *G. dolichopoda*, *G. jingdongensis*, *G. leucocarpa*, *G. pyrolifolia*, *G. straminea*, *G. suborbicularis*, *G. dumicola*, *G. griffithiana*, *G. longibracteolata*, *G. notabilis*
rbcL + *matK*	*G. codonantha*, *G. dolichopoda*, *G. jingdongensis*, *G. leucocarpa*, *G. pyrolifolia*, *G. straminea*, *G. suborbicularis*	*G. codonantha*, *G. dolichopoda*, *G. jingdongensis*, *G. leucocarpa*, *G. pyrolifolia*, *G. straminea*, *G. suborbicularis*, *G. dumicola*, *G. notabilis*	*G. codonantha*, *G. dolichopoda*, *G. jingdongensis*, *G. leucocarpa*, *G. pyrolifolia*, *G. straminea*, *G. suborbicularis*, *G. dumicola*, *G. notabilis G. borneensis*, *G. cardiosepala*, *G. longibracteolata*
rbcL + *matK* + *trnH-psbA*	*G. codonantha*, *G. dolichopoda*, *G. jingdongensis*, *G. leucocarpa*, *G. pyrolifolia*	*G. codonantha*, *G. dolichopoda*, *G. jingdongensis*, *G. leucocarpa*, *G. pyrolifolia*, *G. dumicola*, *G. notabilis*, *G. straminea*, *G. suborbicularis*	*G. codonantha*, *G. dolichopoda*, *G. jingdongensis*, *G. leucocarpa*, *G. pyrolifolia*, *G. dumicola*, *G. notabilis*, *G. straminea*, *G. suborbicularis*, *G. cardiosepala*, *G. cuneata*, *G. longibracteolata*
rbcL + *matK* + ITS	*G. codonantha*, *G. dolichopoda*, *G. jingdongensis*, *G. leucocarpa*, *G. longibracteolata*, *G. pseudonotabilis*, *G. pyrolifolia*, *G. straminea*, *G. suborbicularis*, *G. trigonoclada*, *G. wardii*	*G. codonantha*, *G. dolichopoda*, *G. jingdongensis*, *G. leucocarpa*, *G. longibracteolata*, *G. pseudonotabilis*, *G. pyrolifolia*, *G. straminea*, *G. suborbicularis*, *G. trigonoclada*, *G. wardii*, *G. nummularioides*	*G. codonantha*, *G. dolichopoda*, *G. jingdongensis*, *G. leucocarpa*, *G. longibracteolata*, *G. pseudonotabilis*, *G. pyrolifolia*, *G. straminea*, *G. suborbicularis*, *G. trigonoclada*, *G. wardii*, *G. nummularioides*, *G. borneensis*, *G. cardiosepala*
rbcL + *matK* + *trnH-psbA* + ITS	*G. codonantha*, *G. dolichopoda*, *G. jingdongensis*, *G. leucocarpa*, *G. longibracteolata*, *G. pyrolifolia*, *G. trigonoclada*, *G. wardii*	*G. codonantha*, *G. dolichopoda*, *G. jingdongensis*, *G. leucocarpa*, *G. longibracteolata*, *G. pyrolifolia*, *G. trigonoclada*, *G. wardii*, *G. dumicola*, *G. nummularioides*, *G. pseudonotabilis*, *G. straminea*, *G. suborbicularis*	*G. codonantha*, *G. dolichopoda*, *G. jingdongensis*, *G. leucocarpa*, *G. longibracteolata*, *G. pyrolifolia*, *G. trigonoclada*, *G. wardii*, *G. dumicola*, *G. nummularioides*, *G. pseudonotabilis*, *G. straminea*, *G. suborbicularis*, *G. borneensis*, *G. cardiosepala*
matK + ITS	*G. cardiosepala*, *G. codonantha*, *G. dolichopoda*, *G. jingdongensis*, *G. leucocarpa*, *G. longibracteolata*, *G. pseudonotabilis*, *G. pyrolifolia*, *G. straminea*, *G. suborbicularis*, *G. trigonoclada*	*G. cardiosepala*, *G. codonantha*, *G. dolichopoda*, *G. jingdongensis*, *G. leucocarpa*, *G. longibracteolata*, *G. pseudonotabilis*, *G. pyrolifolia*, *G. straminea*, *G. suborbicularis*, *G. trigonoclada*, *G. dumicola*, *G. nummularioides*	*G. cardiosepala*, *G. codonantha*, *G. dolichopoda*, *G. jingdongensis*, *G. leucocarpa*, *G. longibracteolata*, *G. pseudonotabilis*, *G. pyrolifolia*, *G. straminea*, *G. suborbicularis*, *G. trigonoclada*, *G. dumicola*, *G. nummularioides*, *G. borneensis*, *G. cuneata*

Gaultheria leucocarpa includes two varieties, *G. leucocarpa* var. *yunnanensis* and *G. leucocarpa* var. *crenulata*. ITS, internal transcribed spacer.

resolving species as distinct lineages and separated the greatest number of species with parsimony bootstrap support >50%, whereas *rbcL* and *trnH-psbA* showed low clade support in NJ trees (only two species can be discriminated). For different combinations of the four loci, *rbcL* + *matK* separated only seven species, which has less capacity of species discrimination than that of *matK* alone. In most cases, multi-loci analyses resolved more species with parsimony bootstrap support >50% and some specific lineages in NJ trees. Although the bootstrap support increased when more regions were combined, the combination of all four loci only identified eight species in the NJ tree. Three loci combinations, namely, *rbcL* + *matK* + *trnH-psbA*, merely divided five out of 30 species. Nevertheless, the *rbcL* + *matK* + ITS and *matK* + ITS combinations showed highest species discrimination and each separated 11 species. Whether species were shown as monophyletic for each barcode was evaluated by ML analysis. The *matK* + ITS, *rbcL* + *matK* + ITS, and *rbcL* + *matK* + *trnH-psbA* + ITS barcodes recovered the highest value of species monophyly (11 species, bootstrap value >50%), whereas *rbcL* recovered the lowest value (two species).

3 Discussion

3.1 DNA barcode evaluation

We evaluate genetic loci for DNA barcoding mainly based on the criteria of Kress et al. (2005), that is, to detect significant species-level genetic variability and divergence. Polymerase chain reaction and sequencing success is an important standard for DNA barcoding regions. All four loci tested in this study showed 100% PCR and sequencing success. Nevertheless, the discrimination levels of these loci from the *P*-distance analyses are low. The *rbcL* and *trnH-psbA* regions have the lowest level, by which only two out of 30 species were determined, whereas *matK* showed the highest level, with eight out of 30 species separated. Combining barcoding markers has benefits for species discrimination (Fazekas et al., 2008; Ford et al., 2009). Although the CBOL Plant Working Group (2009) recommended a two-locus combination of *rbcL* + *matK* as the universal barcode for flowering plants, it resolves only six out of 30 species of Chinese *Gaultheria*. The NJ tree of the combination of *rbcL* + *matK* + *trnH-psbA* has relatively low support value for its specific lineages (seven species resolved). Nevertheless, the combinations of *rbcL* + *matK* + ITS and *matK* + ITS achieve maximum taxon discrimination in Chinese *Gaultheria*, which is better than the combination of all four loci (*rbcL* + *matK* + *trnH-psbA* + ITS). Based on the results of Wilcoxon signed rank tests, ITS and *matK* + ITS are the most appropriate barcodes for determining interspecific level variation among the loci. Maximum species (11 spp.) can be recovered as monophyletic from the ML analyses of *matK* +ITS, *rbcL* + ITS +*matK*, and *rbcL* + *matK* + *trnH-psbA* + ITS. Therefore, based on these statistical results, we suggest that a combination of *matK* and ITS represents the most efficient DNA regions to discriminate the Chinese species of *Gaultheria* in our analyses, notwithstanding that only 11 of 30 species can be separated by it (Fig. 1).

3.2 Taxonomic significance of DNA barcoding in Chinese species of *Gaultheria*

The phylogenetic study of Lu et al. (2010) revealed that reticulate evolution, cryptic species, character convergence, and rapid radiation characterized the Chinese species of *Gaultheria*. Although complicated evolutionary processes occur in this group, species reidentification of some taxa such as *Gaultheria griffithiana* var. *insignis* R. C. Fang (as a new species) and of ser. *Trichophyllae* is well resolved by molecular data. In ser. *Trichophyllae*, such non-monophyly was largely attributed to extreme morphological reduction. In our analysis, some species are delimitated in varying degrees by different DNA loci or combinations. *Gaultheria leucocarpa* var. *yunnanensis* and *G. suborbicularis* W. W. Sm., which occur in China but are not related to the species from the core East Asian clade, can be discriminated with 100% resolvability in almost all NJ analyses (Table 5) and ML analyses (except for *rbcL*, and *trnH-psbA* and its related combinations *rbcL* and *trnH-psbA* failing for *G. leucocarpa* var. *yunnanensis*, *rbcL* + *matK* + *trnH-psbA* and *rbcL* + *matK* + *trnH-psbA* + ITS failing for *G. suborbicularis*). For the core East Asian clade, the four loci and their various combinations better resolved the species discrimination of ser. *Leucothoides* s.l. than that of ser. *Trichophyllae*. Species from ser. *Leucothoides* s.l. such as *G. codonantha* Airy Shaw, *G. longibracteolata* R. C. Fang, *G. pseudonotabilis* H. Li ex R. C. Fang, *G. pyrolifolia* Hook. f. ex C. B. Clarke, *G. straminea* R. C. Fang, and *G. trigonoclada*, and species from ser. *Trichophyllae* such as *G. cardiosepala* Hand.-Mazz., *G. dolichopoda*, and *G. jingdongensis*, can be discriminated with 100% resolvability only by one of the four loci, however, these species can also be well discriminated by morphology. *Gaultheria leucocarpa* var. *yunnanensis*, *G. nummularioides*, and *G. fragrantissima* are the most widespread species among the Chinese *Gaultheria.* The loci or combinations in the study more or less discriminate the former two species, whereas none of them can resolve that of

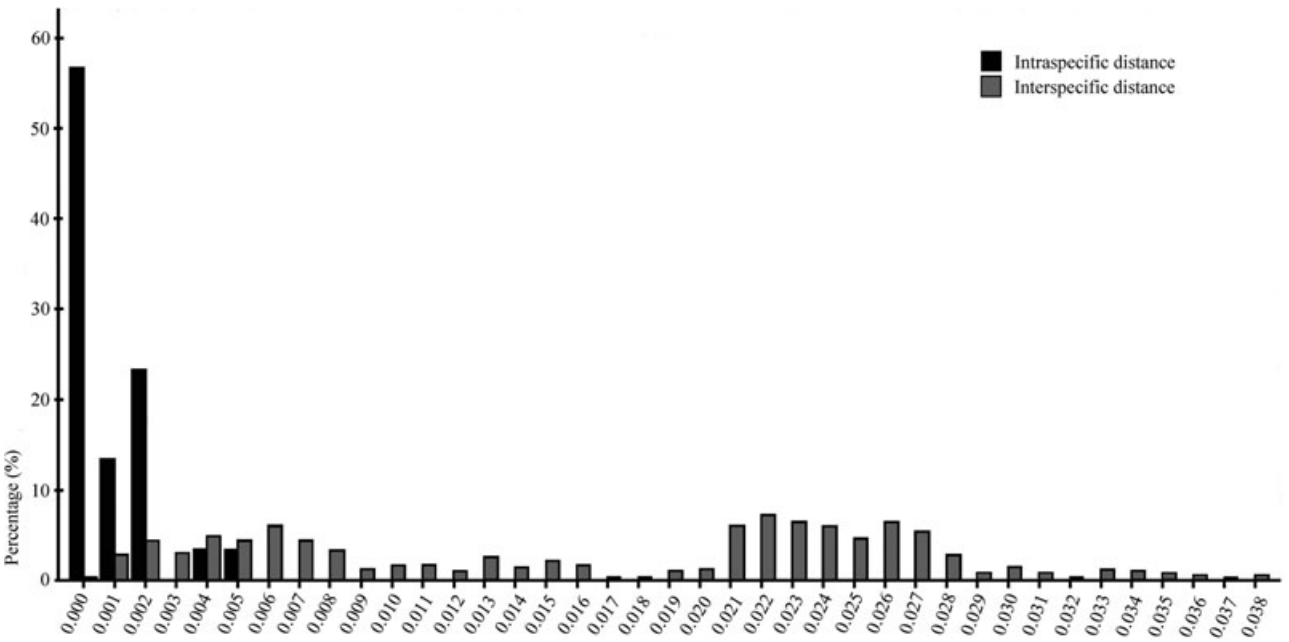

Fig. 1. Intraspecific versus interspecific distances from the combination of *matK* + internal transcribed spacer (ITS).

the latter, probably due to reticulate evolution (see Lu et al., 2010).

Chen et al. (2010) proposed that ITS2 can serve as a novel universal barcode for the identification of a broader range of plant taxa. Kress et al. (2005) proposed the *trnH-psbA* spacer, although short (ca. 450 bp), was the most variable plastid region in angiosperms and was easily amplified across a broad range of land plants. This region has been tested having a high discrimination level in *Pedicularis* L. (Orobanchaceae) (Yu et al., 2011, unpublished data, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, China.). Nevertheless, both ITS2 and *trnH-psbA* loci have very poor capacity of species separation for the Chinese *Gaultheria* in our analyses. Instead, the *cp*DNA regions, e.g., *trnS-trnG*, complete *matK* (ca. 1500 bp), *rpl16*, and *trnL-trnF* (see Lu et al., 2010), possess more valuable characters for species discrimination on *Gaultheria* than that of the four loci in the study. Therefore, DNA barcoding still faces many issues of species separation to some genera like *Gaultheria*, with a complicated evolutionary history or through rapid radiation. More efforts are needed for searching for appropriate DNA loci for the barcoding of such plants.

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Appendix I Species, their collection information, and GenBank accession numbers (ITS)

Taxon	Locality	Voucher	*rbcL*	*matK*	*trnH-psbA*	ITS
Gaultheria borneensis Stapf	RBGE, UK	19411001A, RBGE (E)	JF941568	JF953750	JN044551	JF976336
	RSF, USA	Van der Kloet, 2101092 (RSF)	JF941567	AF366629	JN044550	AF358881
G. brevistipes (C. Y. Wu & T. Z. Hsu) R. C. Fang	Medog, Tibet, China	L. Lu et al. 07300-1 (KUN)	JF941569	HM597340	JN044552	HM597253
		L. Lu et al. 07300-3 (KUN)	JF941572	JF953753	JN044555	JF976339
		L. Lu et al. 07300-4 (KUN)	JF941571	JF953752	JN044554	JF976338
		L. Lu et al. 07300-5 (KUN)	JF941570	JF953751	JN044553	JF976337
G. cardiosepala Hand.-Mazz.	Dali, Yunnan, China	L. Lu et al. 0516-1 (KUN)	JF941574	HM597395	JN044557	HM597308
		L. Lu et al. 0516-2 (KUN)	JF941577	JF953756	JN044560	JF976342
	Pianma Yunnan, China	L. Lu et al. 060022-1 (KUN)	JF941573	HM597394	JN044556	HM597307
		L. Lu et al. 060022-3 (KUN)	JF941576	JF953755	JN044559	JF976341
	Yongde, Yunnan, China	Y. X. Zhang, 001 (KUN)	JF941575	JF953754	JN044558	JF976340
G. codonantha Airy Shaw	Chayu, Tibet, China	L. Lu et al. 07303-1 (KUN)	JF941578	HM597343	JN044561	HM597256
		L. Lu et al. 07303-3 (KUN)	JF941581	JF953759	JN044564	JF976345
		L. Lu et al. 07303-4 (KUN)	JF941580	JF953758	JN044563	JF976344
		L. Lu et al. 07303-5 (KUN)	JF941579	JF953757	JN044562	JF976343
G. cuneata (Rehder & E. H. Wilson) Bean	Qiaojia, Yunnan, China	S. D. Zhang & L. Lu, 031543 (KUN)	JF941583	HM597337	JN044566	HM597250
	RBGE, UK	19091023B, RBGE (E)	JF941582	HM597338	JN044565	HM597251
G. discolor Nutt. ex Hook. f.	Gongshan, Yunnan, China	GLGS32542 (CAS)	JN098404	HM597366	JN098398	HM597279
G. dolichopoda Airy Shaw	Gongshan, Yunnan, China	L. Lu et al. 060005-1 (KUN)	JF941584	HM597405	JN044567	HM597318
		L. Lu et al. 060005-2 (KUN)	JF941587	JF953762	JN044570	JF976348
		L. Lu et al. 060005-3 (KUN)	JF941586	JF953761	JN044569	JF976347
		L. Lu et al. 060005-4 (KUN)	JF941585	JF953760	JN044568	JF976346
G. dumicola W. W. Sm.	Pianma, Yunnan, China	L. Lu et al. 06101-1 (KUN)	JF941591	HM597345	JN044574	HM597258
		L. Lu et al. 06101-3 (KUN)	JF941594	JF953765	JN044577	JF976351
	Tengchong, Yunnan, China	L. Lu et al. 07009-1 (KUN)	JF941589	HM597347	JN044572	HM597260
		L. Lu et al. 07009-3 (KUN)	JF941592	JF953763	JN044575	JF976349
G. dumicola var. *aspera* Airy Shaw	Gongshan, Yunnan, China	L. Lu et al. 0666-1 (KUN)	JF941590	HM597348	JN044573	HM597261
		L. Lu et al. 0666-3 (KUN)	JF941593	JF953764	JN044576	JF976350
	Gongshan, Yunnan, China	GLGS 20245 (CAS)	JF941588	HM597344	JN044571	HM597257
G. eciliata (Rae & D. G. Long) P. W. Fritsch & L. H. Zhou	Gongshan, Yunnan, China	GLGS16874 (CAS)	JF941596	HM597419	JN044579	HM597328
	Medog, Tibet, China	L. Lu et al. 07149 (KUN)	JF941595	HM597420	JN044578	HM597329
G. fragrantissima Wall.	Yuanjiang, Yunnan, China	L. Lu et al. 06002-1 (KUN)	JF941613	JF953777	JN044596	JF976363
		L. Lu et al. 06002-2 (KUN)	JF941612	JF953776	JN044595	JF976362
	Dali, Yunnan, China	L. Lu et al. 060027-1 (KUN)	JF941601	HM597350	JN044584	HM597263
		L. Lu et al. 060027-2 (KUN)	JF941611	JF953775	JN044594	JF976361
	Baoshan, Yunnan, China	L. Lu et al. 07007-1 (KUN)	JF941600	HM597349	JN044583	HM597262
		L. Lu et al. 07007-3 (KUN)	JF941610	JF953774	JN044593	JF976360
	Tengchong, Yunnan, China	L. Lu et al. 07008-1 (KUN)	JF941609	JF953773	JN044592	JF976359
		L. Lu et al. 07008-2 (KUN)	JF941608	JF953772	JN044591	JF976358
	Gongshan, Yunnan, China	GLGS16548 (CAS)	JF941599	HM597353	JN044582	HM597266
	Lijiang, Yunnan, China	L. Lu et al. JMC-1 (KUN)	JF941607	JF953771	JN044590	JF976357
		L. Lu et al. JMC-2 (KUN)	JF941606	JF953770	JN044589	JF976356
	Linzhi, Tibet, China	L. Lu et al. 07305-2 (KUN)	JF941598	HM597352	JN044581	HM597265
		L. Lu et al. 07305-3 (KUN)	JF941605	JF953769	JN044588	JF976355
	Jingdong, Yunnan, China	L. Lu et al. 0607-1 (KUN)	JF941597	HM597351	JN044580	HM597264
		L. Lu et al. 0607-2 (KUN)	JF941604	JF953768	JN044587	JF976354
	Pingbian, Yunnan, China	L. Lu et al. LU001-1 (KUN)	JF941603	JF953767	JN044586	JF976353
		L. Lu et al. LU001-2 (KUN)	JF941602	JF953766	JN044585	JF976352
G. griffithiana Wight	Dali, Yunnan, China	L. Lu et al. 060026-1 (KUN)	JF941616	HM597355	JN044599	HM597268
		L. Lu et al. 060026-2 (KUN)	JF941625	JF953786	JN044608	JF976372
	Jingdong, Yunnan, China	L. Lu et al. 06008-1 (KUN)	JF941624	JF953785	JN044607	JF976371
		L. Lu et al. 06008-2 (KUN)	JF941623	JF953784	JN044606	JF976370
	Caojian, Yunnan, China	L. Lu et al. 06100-1 (KUN)	JF941622	JF953783	JN044605	JF976369
		L. Lu et al. 06100-2 (KUN)	JF941621	JF953782	JN044604	JF976368
	Weixi, Yunnan, China	J. Liu, 09511 (KUN)	JF941620	JF953781	JN044603	JF976367
	Gongshan, Yunnan, China	L. Lu et al. 06DSF-1 (KUN)	JF941619	JF953780	JN044602	JF976366
		L. Lu et al. 06DSF-2 (KUN)	JF941618	JF953779	JN044601	JF976365
	Medog, Tibet, China	L. Lu et al. 07169-1 (KUN)	JF941615	HM597357	JN044598	HM597270
		L. Lu et al. 07169-3 (KUN)	JF941617	JF953778	JN044600	JF976364
	RBGE, UK	19911115B, RBGE (E)	JF941614	HM597356	JN044597	HM597269

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Taxon	Locality	Voucher	*rbcL*	*matK*	*trnH-psbA*	ITS
G. heteromera R. C. Fang	Medog, Tibet, China	L. Lu et al. 07316A-1 (KUN)	JF941626	HM597358	JN044609	HM597271
		L. Lu et al. 07316A-2 (KUN)	JF941629	JF953789	JN044612	JF976375
		L. Lu et al. 07316A-3 (KUN)	JF941628	JF953788	JN044611	JF976374
		L. Lu et al. 07316A-4 (KUN)	JF941627	JF953787	JN044610	JF976373
G. hookeri C. B. Clarke	Qiaojia, Yunnan, China	S. D. Zhang & L. Lu 031500-1 (KUN)	JF941632	HM597360	JN044615	HM597273
		S. D. Zhang & L. Lu 031500-2 (KUN)	JF941636	JF953793	JN044619	JF976379
	Gongshan, Yunnan, China	L. Lu et al. 06DYK-1 (KUN)	JF941635	JF953792	JN044618	JF976378
		L. Lu et al. 06DYK-2 (KUN)	JF941634	JF953791	JN044617	JF976377
	Medog, Tibet, China	L. Lu et al. 07089-2 (KUN)	JF941631	HM597362	JN044614	HM597275
		L. Lu et al. 07089-3 (KUN)	JF941633	JF953790	JN044616	JF976376
	Gonggashan, Sichuan, China	S. D. Zhang & W. B. Yü 009 (KUN)	JF941630	HM597364	JN044613	HM597277
G. hypochlora Airy Shaw	Gongshan, Yunnan, China	GLGS16817 (CAS)	JF941640	HM597412	JN044623	HM597336
	Fugong, Yunnan, China	GLGS28628 (CAS)	JF941639	HM597409	JN044622	HM597330
	Gongshan, Yunnan, China	L. Lu et al. 060012-1 (KUN)	JF941638	HM597408	JN044621	HM597321
		L. Lu et al. 060012-3 (KUN)	JF941642	JF953795	JN044625	JF976381
	Medog, Tibet, China	L. Lu et al. 07135-1 (KUN)	JF941637	HM597423	JN044620	HM597333
		L. Lu et al. 07135-3 (KUN)	JF941641	JF953794	JN044624	JF976380
G. jingdongensis R. C. Fang	Jingdong, Yunnan, China	L. Lu et al. 0619A-1 (KUN)	JF941643	HM597407	JN044626	HM597320
		L. Lu et al. 0619A-2 (KUN)	JF941646	JN098400	JN044629	JF976384
		L. Lu et al. 0619A-3 (KUN)	JF941645	JF953797	JN044628	JF976383
		L. Lu et al. 0619A-4 (KUN)	JF941644	JF953796	JN044627	JF976382
G. leucocarpa var. *yunnanensis* (Franchet) T. Z. Hsu & R. C. Fang	Qiaojia, Yunnan, China	S. D. Zhang & L. Lu, 031607-1 (KUN)	JF941666	JF953815	JN044649	JF976404
		S. D. Zhang & L. Lu, 031607-2 (KUN)	JF941665	JF953814	JN044648	JF976403
	Tengchong, Yunnan, China	L. Lu et al. 07011-1 (KUN)	JF941664	JF953813	JN044647	JF976402
		L. Lu et al. 07011-2 (KUN)	JF941663	JF953812	JN044646	JF976401
	Baoshan, Yunnan, China	J. Liu, Liu1001-1 (KUN)	JF941660	JF953810	JN044643	JF976398
		J. Liu, Liu1001-2 (KUN)	JF941659	JF953809	JN044642	JF976397
	Jingdong, Yunnan, China	L. Lu et al. 0609-1 (KUN)	JF941658	JF953808	JN044641	JF976396
		L. Lu et al. 0609-2 (KUN)	JF941657	JF953807	JN044640	JF976395
	Yuanjiang, Yunnan, China	L. Lu et al. 0610-1 (KUN)	JF941656	JF953806	JN044639	JF976394
		L. Lu et al. 0610-2 (KUN)	JF941655	JF953805	JN044638	JF976393
	Pingtang, Guizhou, China	T. X. Liu, LTX001-1 (KUN)	JF941654	JN098401	JN044637	JF976392
		T. X. Liu, LTX001-2 (KUN)	JF941653	JF953804	JN044636	JF976391
	Pingbian, Yunnan, China	R. F. Lu, LU002-1 (KUN)	JF941652	JF953803	JN044635	JF976390
		R. F. Lu, LU002-2 (KUN)	JF941651	JF953802	JN044634	JF976389
	Anning, Yunnan, China	R. F. Lu, LU1001-1 (KUN)	JF941650	JF953801	JN044633	JF976388
		R. F. Lu, LU1001-2 (KUN)	JF941649	JF953800	JN044632	JF976387
	Kunming, Yunnan, China	R. F. Lu, LU2001-1 (KUN)	JF941648	JF953799	JN044631	JF976386
		R. F. Lu, LU2001-2 (KUN)	JF941647	JF953798	JN044630	JF976385
G. leucocarpa var. *crenulata* (Kurz) T. Z. Hsu	Wuding, Yunnan, China	L. Lu et al. HE001-1 (KUN)	JF941662	JN098402	JN044645	JF976400
		L. Lu et al. HE001-2 (KUN)	JF941661	JF953811	JN044644	JF976399
G. longibracteolata R. C. Fang	Yuanjing, Yunnan, China	L. Lu et al. 0601-1 (KUN)	JF941667	HM597365	JN044650	HM597278
		L. Lu et al. 0601-2 (KUN)	JF941669	JF953817	JN044652	JF976406
		L. Lu et al. 0601-3 (KUN)	JF941668	JF953816	JN044651	JF976405
G. notabilis J. Anthony	Tengchong, Yunnan, China	L. Lu et al. 07005-1 (KUN)	JF941670	HM597370	JN044653	HM597282
		L. Lu et al. 07005-2 (KUN)	JF941673	JF953820	JN044656	JF976409
		L. Lu et al. 07005-3 (KUN)	JF941672	JF953819	JN044655	JF976408
		L. Lu et al. 07005-4 (KUN)	JF941671	JF953818	JN044654	JF976407
G. nummularioides D. Don	Tengchong, Yunnan, China	L. Lu et al. 07010-1 (KUN)	JF941679	HM597374	JN044662	HM597286
		L. Lu et al. 07010-3 (KUN)	JF941682	JF953823	JN044665	JF976412
	Fugong, Yunnan, China	GLGS20182 (CAS)	JF941681	JF953822	JN044664	JF976411
	Gongshan, Yunnan, China	GLGS22006 (CAS)	JF941678	HM597372	JN044661	HM597284
	Jingdong, Yunnan, China	L. Lu et al. 0618A (KUN)	JF941677	HM597375	JN044660	HM597287
	Medog, Tibet, China	L. Lu et al. 07304-1 (KUN)	JF941676	HM597376	JN044659	HM597288
		L. Lu et al. 07304-3 (KUN)	JF941680	JF953821	JN044663	JF976410
	RBGE, UK	19891041B, RBGE (E)	JF941675	HM597371	JN044658	HM597283
	Gonggashan, Sichuan, China	S.D. Zhang & W.B. Yü 010 (KUN)	JF941674	HM597373	JN044657	HM597285

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Taxon	Locality	Voucher	*rbcL*	*matK*	*trnH-psbA*	ITS
G. praticola C. Y. Wu & T. Z. Hsu	Gongshan, Yunnan, China	L. Lu et al. 060056-1 (KUN)	JF941690	JF953831	JN044673	JF976420
		L. Lu et al. 060056-3 (KUN)	JF941689	JF953830	JN044672	JF976419
		L. Lu et al. 060056-4 (KUN)	JF941688	JF953829	JN044671	JF976418
		L. Lu et al. 060056-5 (KUN)	JF941687	JF953828	JN044670	JF976417
	Medog, Tibet, China	L. Lu et al. 07140-1 (KUN)	JF941686	JF953827	JN044669	JF976416
		L. Lu et al. 07140-3 (KUN)	JF941685	JF953826	JN044668	JF976415
		L. Lu et al. 07140-4 (KUN)	JF941684	JF953825	JN044667	JF976414
		L. Lu et al. 07140-5 (KUN)	JF941683	JF953824	JN044666	JF976413
G. prostrata W. W. Sm.	Gonggashan, Sichuan, China	S. D. Zhang & W. B. Yü 011 (KUN)	JN098405	JN098403	JN098399	JN098397
G. pseudonotabilis H. Li ex R. C. Fang	Gongshan, Yunnan, China	L. Lu et al. 060045-1 (KUN)	JF941694	JF953835	JN044677	JF976424
		L. Lu et al. 060045-2 (KUN)	JF941693	JF953834	JN044676	JF976423
		L. Lu et al. 060045-4 (KUN)	JF941692	JF953833	JN044675	JF976422
		L. Lu et al. 060045-5 (KUN)	JF941691	JF953832	JN044674	JF976421
G. pyrolifolia Hook. f. ex C. B. Clarke	Medog, Tibet, China	L. Lu et al. 07117-1 (KUN)	JF941695	HM597381	JN044678	HM597292
		L. Lu et al. 07117-3 (KUN)	JF941698	JF953838	JN044681	JF976427
		L. Lu et al. 07117-4 (KUN)	JF941697	JF953837	JN044680	JF976426
		L. Lu et al. 07117-5 (KUN)	JF941696	JF953836	JN044679	JF976425
G. semi-infera (C. B. Clarke) Airy Shaw	Jingdong, Yunnan, China	L. Lu et al. 0617-1 (KUN)	JF941701	HM597385	JN044684	HM597298
		L. Lu et al. 0617-2 (KUN)	JF941706	JF953843	JN044689	JF976432
	Pianma, Yunnan, China	L. Lu et al. 06103-1 (KUN)	JF941700	HM597386	JN044683	HM597299
		L. Lu et al. 06103-2 (KUN)	JF941705	JF953842	JN044688	JF976431
	Gongshan, Yunnan, China	L. Lu et al. 06QQ-1 (KUN)	JF941704	JF953841	JN044687	JF976430
		L. Lu et al. 06QQ-2 (KUN)	JF941703	JF953840	JN044686	JF976429
	Medog, Tibet, China	L. Lu et al. 07312-1 (KUN)	JF941699	HM597388	JN044682	HM597301
		L. Lu et al. 07312-2 (KUN)	JF941702	JF953839	JN044685	JF976428
	Gongshan, Yunnan, China	L. Lu et al. 060070-1 (KUN)	JF941723	HM597389	JN044706	HM597303
		L. Lu et al. 060070-2 (KUN)	JF941726	JF953857	JN044709	JF976446
		L. Lu et al. 060070-3 (KUN)	JF941725	JF953856	JN044708	JF976445
	Weixi, Yunnan, China	J. Liu, 09490 (KUN)	JF941724	JF953855	JN044707	JF976444
G. sinensis J. Anthony	Pianma, Yunnan, China	L. Lu et al. 060021-1 (KUN)	JF941710	HM597402	JN044693	HM597317
		L. Lu et al. 060021-3 (KUN)	JF941714	JF953847	JN044697	JF976436
	Gongshan, Yunnan, China	L. Lu et al. 060040-1 (KUN)	JF941709	HM597404	JN044692	HM597315
		L. Lu et al. 060040-2 (KUN)	JF941713	JF953846	JN044696	JF976435
	Medog, Tibet, China	L. Lu et al. 07133-1 (KUN)	JF941708	HM597399	JN044691	HM597313
		L. Lu et al. 07133-3 (KUN)	JF941712	JF953845	JN044695	JF976434
	Dali, Yunnan, China	L. Lu et al. 0615-1 (KUN)	JF941707	HM597397	JN044690	HM597310
		L. Lu et al. 0615-2 (KUN)	JF941711	JF953844	JN044694	JF976433
G. straminea R. C. Fang	Medog, Tibet, China	L. Lu et al. 07306-1 (KUN)	JF941715	HM597390	JN044698	HM597302
		L. Lu et al. 07306-3 (KUN)	JF941718	JF953850	JN044701	JF976439
		L. Lu et al. 07306-4 (KUN)	JF941717	JF953849	JN044700	JF976438
		L. Lu et al. 07306-5 (KUN)	JF941716	JF953848	JN044699	JF976437
G. suborbicularis W. W. Sm.	Deqin, Yunnan, China	L. Lu et al. 07307-1 (KUN)	JF941722	JF953854	JN044705	JF976443
		L. Lu et al. 07307-2 (KUN)	JF941721	JF953853	JN044704	JF976442
		L. Lu et al. 07307-3 (KUN)	JF941720	JF953852	JN044703	JF976441
		L. Lu et al. 07307-4 (KUN)	JF941719	JF953851	JN044702	JF976440
G. trichophylla Royle	Gongshan, Yunnan, China	L. Lu et al. 060007 (KUN)	JF941732	HM597414	JN044715	HM597323
	Dali, Yunnan, China	L. Lu et al. 060019-1 (KUN)	JF941731	HM597413	JN044714	HM597322
		L. Lu et al. 060019-2 (KUN)	JF941734	JF953859	JN044717	JF976448
	Medog, Tibet, China	L. Lu et al. 07155-1 (KUN)	JF941730	HM597416	JN044713	HM597325
		L. Lu et al. 07155-3 (KUN)	JF941733	JF953858	JN044716	JF976447
	Linzhi, Tibet, China	L. Lu et al. 07308-1 (KUN)	JF941729	HM597418	JN044712	HM597327
	Deqin, Yunnan, China	L. Lu et al. 07400-1 (KUN)	JF941728	HM597415	JN044711	HM597324
	Gonggashan, Sichuan, China	S. D. Zhang & W. B. Yü 013 (KUN)	JF941727	HM597417	JN044710	HM597326
G. trigonoclada R. C. Fang	Medog, Tibet, China	L. Lu et al. 07216-1 (KUN)	JF941735	HM597391	JN044718	HM597304
		L. Lu et al. 07216-3 (KUN)	JF941738	JF953862	JN044721	JF976451
		L. Lu et al. 07216-4 (KUN)	JF941737	JF953861	JN044720	JF976450
		L. Lu et al. 07216-5 (KUN)	JF941736	JF953860	JN044719	JF976449
G. wardii var. *elongata* R. C. Fang	Gongshan, Yunnan, China	L. Lu et al. 060067-1 (KUN)	JF941740	HM597392	JN044723	HM597305
		L. Lu et al. 060067-3 (KUN)	JF941741	JF953863	JN044724	JF976452
		L. Lu et al. 060067-4 (KUN)	JF941750	JF953872	JN044733	JF976461
		L. Lu et al. 060067-5 (KUN)	JF941749	JF953871	JN044732	JF976460

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Appendix I Continued

Taxon	Locality	Voucher	*rbcL*	*matK*	*trnH-psbA*	ITS
G. wardii C. Marquand & Airy Shaw	Medog, Tibet, China	L. Lu et al. 07301-1 (KUN)	JF941739	HM597393	JN044722	HM597306
		L. Lu et al. 07301-3 (KUN)	JF941748	JF953870	JN044731	JF976459
		L. Lu et al. 07301-4 (KUN)	JF941747	JF953869	JN044730	JF976458
		L. Lu et al. 07301-5 (KUN)	JF941746	JF953868	JN044729	JF976457
	Linzhi, Tibet, China	L. Lu et al. 07ZQ-1 (KUN)	JF941745	JF953867	JN044728	JF976456
		L. Lu et al. 07ZQ-2 (KUN)	JF941744	JF953866	JN044727	JF976455
		L. Lu et al. 07ZQ-3 (KUN)	JF941743	JF953865	JN044726	JF976454
		L. Lu et al. 07ZQ-4 (KUN)	JF941742	JF953864	JN044725	JF976453

CAS, California Academy of Sciences Herbarium; E, Royal Botanic Garden Edinburgh Herbarium; ITS, internal transcribed spacer; KUN, Kunming Institute of Botany Herbarium; RSF, Rhododendron Species Foundation, USA.