



## *Liparis wenshanensis*, a new orchid species from China: Evidence from morphological and molecular analyses

YONG-YU SU<sup>1,2</sup>, YI-LANG HUANG<sup>1,2</sup>, LI-JUN CHEN<sup>2</sup>, PEI-WEN ZHANG<sup>2</sup>, ZHONG-JIAN LIU<sup>1,2,3</sup> & GUO-QIANG ZHANG<sup>2,3</sup>

<sup>1</sup>College of Forestry, South China Agricultural University, Guangzhou, 510640, China.

<sup>2</sup>Shenzhen Key Laboratory for Orchid Conservation and Utilization, The National Orchid Conservation Center of China and The Orchid Conservation and Research Center of Shenzhen, Shenzhen, 518114, China; E-mail: zhang2384539@163.com.

<sup>3</sup> College of Forestry, Fujian Agriculture and Forestry University, Fuzhou 350002, China.

### Abstract

A new orchid species, *Liparis wenshanensis*, discovered in Yunnan, China is described and illustrated in this study based on morphological and molecular analyses. A detailed comparison between the newly discovered orchid and other members of the genus, *Liparis*, was conducted. The new plant is characterized by the combination of the following features: a long rachis with 45 to 55 flowers; white sepals, petals and column; a greenish lip with a purplish center; strongly recurved and revolute dorsal sepals and petals; strongly recurved, oblong lateral sepals; a cordate lip that is strongly deflexed below the middle, with a two-lobed apex and a two-lobed callus at the base; an arcuate column with a lamella extending along the center almost to the stigma, and with a pair of broad wings toward the apex. These features distinguish the new orchid from all other known species of *Liparis*. We proceeded to a phylogenetic analysis to ascertain the systematic position of this enigmatic species. Molecular analyses based on nuclear ribosomal ITS and plastid *matK* DNA sequence data supports the recognition of *L. wenshanensis* as a distinct species.

**Keywords:** *Liparis wenshanensis*, orchid, Malaxideae

### Introduction

The genus *Liparis* Richard (1817: 39)(Orchidaceae, Malaxideae) consists of approximately 320 species. Plants in the *Liparis* genus are widely distributed throughout tropical Asia, New Guinea and the southwestern Pacific islands and also extend into subtropical and tropical Americas. Sixty-five species are found in China, of which 22 are endemic (Chen *et al.* 2009), including two new species that have been recently described and named *Liparis pingxiangensis* L. Li & H. F. Yan (2013:e78112 ) and *Liparis funingensis* Y. Y. Su, Y. Meng & Z. J. Liu (2014: 85). *Liparis* plants are terrestrial, lithophytic, or epiphytic; rhizomatous; and, rarely, mycotrophic, and their leaves are reduced to scales (Chen *et al.* 2009). These plants' flowers usually have narrow linear petals; a larger and unlobed lip that is incurved and divided to the hypochile and the epichile; an incurved-arcuate, winged column; and four pollinia in two pairs, with each pair having a small viscidium (Su *et al.* 2014). As a new species, *Liparis funingensis* was described based on it having four pollinia with long caudicles but without viscidium and a rostellum with a mucilaginous sac and a three-lobed lip with a notably short and deep two-lobed mid-lobe (Su *et al.* 2014). During a recent field trip, six terrestrial populations of *Liparis* species were observed in Yunnan, China. The plant is similar in appearance to *Bletilla sinensis* Schlechter (1911: 256) and grows in population with *B. sinensis*, but the floral structure is similar to *Liparis* with a lip incurved to the hypochile and the epichile and four pollinia in two pairs. The newly identified species behaves similar to *Liparis odorata* Lindley (1830: 26) and *Liparis habenarina* Benth. (1873: 335) but differs greatly by having an inflorescence with 45 to 55 flowers, and flowers with white sepals, petals and column, and a greenish lip; a dorsal sepal and cylindrical petals, lateral sepals that are oblong and strongly curved; lip cordate, apical two-lobed; a column with a lamellae extending from its base to near the stigma along the centre way, a base of lamellae fused to the base of

the lip, and with widened wings on both sides of the stigma. The molecular phylogenetic analysis also indicated that it represents an unknown species, which is described and illustrated in this report as a new species.

## Materials and Methods

### Morphological observations

Gross morphological data were obtained during fieldwork, and specimens were deposited in the National Orchid Conservation Center (NOCC) located in Shenzhen city, Guangdong province, China. The floral structure in the fully opened flowers of the new species were observed under a Leica M205A stereomicroscope with a digital camera. We compared the key characteristics, such as inflorescences, floral color, dorsal sepal, lateral sepals, petals, lip and column between the newly discovered orchid and other related members of *Liparis*, and a detailed comparison was generated (table 1).

**TABLE 1.** Comparison of diagnostic characteristics for *L. wenshanensis*, *L. odorata*, *L. habenarina* and *L. sootenzanensis*.

Characteristics	<i>L. wenshanensis</i> (Z.J.Liu8072)	<i>L. odorata</i> (Z.J.Liu5044)	<i>L. habenarina</i> (Bentham, 1873)	<i>L. sootenzanensis</i> (Z.J.Liu4879)
Inflorescences	With 45–55 flowers	Not over 20 flowers	Not over 20 flowers	With 5–15 flowers
Floral color	Sepals, petals and column white, lip greening with a purple center	Greenish yellow, lip brownish	Greenish brown	Greenish yellow
Dorsal sepal	Curved backward to cylindrical	Linear	Linear	Linear-oblong or narrowly elliptic
Lateral sepals	Oblong, strongly curved backward	Ovate-oblong, slightly oblique	Ovate-oblong, oblique	Linear-oblong or narrowly elliptic, slightly oblique
Petals	Deflexed to cylindrical	Linear	Linear	Filiform
Lip	Cordate, deflexed, apex two-lobed, with two-lobed callus at base	Obovate-oblong, apex subtruncate and emarginate, with two calli at base	Oblong apex subtruncate with a two-lobed callus at base	Obovate, base contracted, apical margin denticulate, disk with 2 carinae
Column	With a lamellae extending from its base to near stigma along centre way, base of lamellae fused to the base of lip, with widen wings on both side of the stigma	With wings on both sides, without lamellae on centre way	With wings on both sides, without lamellae on centre way	With wings on both sides, without lamellae on centre way

## Materials

A total of 75 species of seven genera were analyzed; five genera belonged to Malaxideae, and two genera, *Bulbophyllum* and *Dendrobium*, were selected as negative controls. Table 2 provides detailed information regarding the assessment.

### Amplification and sequencing

Total DNA was extracted from fresh material or silica gel-dried plant tissue using a Multisource Genomic DNA Miniprep Kit (Axygen Biosciences) following the manufacturer's instructions. The amplification and sequencing of the Internal Transcribed Spacer (ITS) of the nuclear ribosomal region was performed using the primer pairs ITS A and ITS B (Mile *et al.* 1999). For *matK* regions of the chloroplast, amplification was performed using the primer pair *matK*-19F and *trnK*-2R (Mile *et al.* 1999), and sequencing by *matK*-19F and *trnK*-2R and several primers developed by Liu *et al.* (Liu *et al.* 2011). Table 3 contains detailed information.

**TABLE 2.** Species and gene regions sequenced for analysis.

Species	ITS	<i>matK</i>
	Accession number	Accession number
Outgroup		
<i>Bulbophyllum odoratissimum</i>	HQ114230	FJ794042
<i>Dendrobium crumenatum</i>	HM590370	HM055235
Ingroup		
<i>Crepidium resupinatum</i>	JN114483	JN004407
<i>Dienia ophrydis</i>	AY907114	AY907181
<i>Liparis bracteata</i>	AY907076	AY907140
<i>L. brunnescens</i>	AY907098	AY907165
<i>L. cathcartii</i>	KJ021031	KJ021027
<i>L. clypeolum</i>	AY907079	AY907143
<i>L. condylobulbon</i>	AY907080	AY907144
<i>L. cordifolia</i>	AB289459	KJ459312
<i>L. disticha</i>	AY907081	AY907145
<i>L. formosana</i>	AY907082	AY907147
<i>L. fujisanensis</i>	EU024936	EU024937
<i>L. funingensis</i>	KJ021032	KJ021028
<i>L. gibbosa</i>	KJ459288	AY907149
<i>L. guangxiensis</i>	KF589875	KF589881
<i>L. habenarina</i>	AY240017	-
<i>L. japonica</i>	AB289462	EU017429
<i>L. kumokiri</i>	AY907087	AY907152
<i>L. layardii</i>	AY907089	AY907155
<i>L. liliifolia</i>	AF521067	AY907156
<i>L. loeselii</i>	AY907091	AY907157
<i>L. maingayi</i>	AB289476	-
<i>L. makinoana</i>	AB289474	EU017428
<i>L. nervosa</i>	AB289482	KJ459324
<i>L. nugentiae</i>	AY907093	AY907159
<i>L. odorata</i>	KJ021033	KJ021029
<i>L. pandurata</i>	AY907094	AY907160
<i>L. parviflora</i>	-	AY907162
<i>L. pauliana</i>	AY907096	AY907163
<i>L. pingxiangensis</i>	KF589872	KF589878
<i>L. purpureoviridis</i>	AB289478	-
<i>L. sootenzanensis</i>	KJ021034	KJ021030
<i>L. stricklandian</i>	KJ459298	KJ459328
<i>L. sula</i>	AY907104	AY907171
<i>L. terrestris</i>	AY907105	AY907172
<i>L. truncicola</i>	AY907106	AY907173
<i>L. viridiflora</i>	KJ459299	KJ459329
<i>L. wenshanensis</i>	KM391980	KM391981
<i>L. anopheles</i>	AY907075	AY907139
<i>Malaxis acuminata</i>	AB290884	AB290892
<i>M. boninensis</i>	AB290887	AB290897
<i>M. brachypoda</i>	AY907108	AY907175
<i>M. brevidentata</i>	AB290886	AB290894
<i>M. calophylla</i>	AY907109	-

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**TABLE 2.** (Continued)

Species	ITS	matK
	Accession number	Accession number
<i>M. corymbosa</i>	AY907110	AY907176
<i>M. hahajimensis</i>	AB290888	AB290896
<i>M. hastilabia</i>	-	EU214382
<i>M. latifolia</i>	AY907111	AY907177
<i>M. latisepala</i>	AY907112	AY907178
<i>M. mambulilingensis</i>	-	AY907179
<i>M. metallica</i>	AY907113	AY907180
<i>M. monophyllos</i>	-	EF065593
<i>M. oculata</i>	AB290890	AB290898
<i>M. perakensis</i>	AB290891	AB290899
<i>M. porphyrea</i>	AY907115	AY907182
<i>M. punctata</i>	AY907117	AY907183
<i>M. resupinata</i>	AY907118	AY907185
<i>M. soulei</i>	AY907119	AY907186
<i>M. spicata</i>	AF521068	AY368415
<i>M. steyermarkii</i>	-	EU214179
<i>M. taurina</i>	AY907127	AY907195
<i>Oberonia brunoniana</i>	JN114625	JN004518
<i>O. ensiformis</i>	KJ459300	KJ459331
<i>O. falconeri</i>	JN114629	JN004526
<i>O. heliophila</i>	AY907131	AY907199
<i>O. iridifolia</i>	AY907132	AY907200
<i>O. mucronata</i>	JN114641	JN004530
<i>O. neocaledonica</i>	AY907133	AY907201
<i>O. pachyrachis</i>	JN114644	JN004538
<i>O. recurva</i>	JN114647	JN004544
<i>O. setifera</i>	AY907136	AY907204
<i>O. wappeana</i>	AY907138	AY907206
<i>Oberonioides microtatantha</i>	KJ459302	KJ459333
<i>O. sp</i>	KJ459303	KJ459334

**TABLE 3.** Primers used in this study.

Primer	Sequence(5'→3')	Origin
ITS A	GGAAGGAGAAGTCGTAACAAGG	Mike <i>et al.</i> (1999)
ITS B	CTTTTCCTCCGCTTATTGATATG	Mike <i>et al.</i> (1999)
<i>trnK</i> -2R	AACTAGTCGGATGGAGTAG	Mike <i>et al.</i> (1999)
<i>matK</i> -19F	CGTTCTCATATGCACTATG	Mike <i>et al.</i> (1999)
<i>matK</i> -969R	CTTTTCCTTGATATCGAACAT	Liu <i>et al.</i> (2011)
<i>matK</i> -731F	AAGAAAAGATTCTTTTGGTTCC	Liu <i>et al.</i> (2011)

### Data analyses

The datasets included a nuclear ITS, *matK*, and their combination. Data analyses were conducted as per Liu *et al.* (Liu *et al.* 2011). The detailed information of each dataset is listed in Tables 4 and 5.

TABLE 4. Statistics from the analysis datasets.

Region Information	No. of taxa	Aligned length	No. variable characteristics	No. informative characteristics (%)	Tree length	Consistency index	Retention index
ITS	70	789	501	385(48.8%)	1720	0.4936	0.8271
<i>matK</i>	71	1391	387	233(16.8%)	654	0.7110	0.8854
Combined	75	2180	888	618(28.3%)	2386	0.5507	0.8397

## Results

### Morphological analysis

A detailed comparison between the newly discovered orchid and other members of *Liparis* was conducted. The new plant is characterized by having a long rachis with 45 to 55 flowers and with white sepals, petals and column, and a greenish lip; dorsal sepal and petals curved backward, lateral sepals that are oblong and strongly curved backward; a strongly deflexed, apical, two-lobed lip cordate; a column with a lamellae extending from it base to near the stigma along the center way with the base of the lamellae fused to the disc base of the lip, and with widened wings on both sides of the stigma. These features distinguish the new orchid from all other known species of *Liparis*.

TABLE 5. Best-fit model and parameter for the analysis datasets.

Region	AIC select model	Base frequencies				substitution model(rate matrix)						I	G
		A	C	G	T	A-C	A-G	A-T	C-G	C-T	G-T		
ITS	GTR+I+G	0.2268	0.2394	0.3117	0.2221	1.1052	2.9471	1.5672	0.5285	4.7013	1.0000	0.2049	1.7638
<i>matK</i>	GTR+ G	0.3220	0.1524	0.1342	0.3913	1.9017	1.8751	0.1842	0.3385	1.2491	1.0000	0.2488	1.0130
Combined	TVM+I+G	0.2729	0.2049	0.2119	0.3104	1.3706	2.6875	0.6928	0.5325	2.6875	1.0000	0.3402	0.7642

### Phylogenetic analysis

The length of the aligned matrix of nrITS was 789 base pairs (bp), of which 501 (63.5%) were variable and 374 (48.8%) were parsimony informative. The aligned *matK* matrix consisted of 1391 characteristics, of which 387 (27.8%) were variable, and 233 (16.8%) were parsimony informative. The combined matrix of nrITS and *matK* was 2180 bp, of which 888 (40.7%) were variable and 618 (28.3%) were parsimony informative. There were no significant differences in topologies between nrITS and *matK* (see Figure 1 and 2). For the combined data, the heuristic search found 2251 MPts with a length of 2386 steps. The consistency index (excluding uninformative characteristics) was 0.5507, and the retention index was 0.8397. The topologies from the MP and Bayesian analyses were congruent. The strict consensus tree based on combined data (with BS and BPP) is shown in Figure 3.

The phylogenetic trees generated based on the ITS sequence data analysis clearly show that there are three main clades of this tree (Fig. 1). The BI phylogram with most of the clades received strong support (PP>95%). Clade A comprised four subclades: *Liparis guangxiensis*, *Liparis truncicola*, *Liparis sula*, and *Oberonia*. Clade B included three subclades: *Oberonioides*, *Malaxis porphyrea*, and *Liparis*. Clade C was composed of three subclades: *Liparis sootenzanensis*, *Liparis layardii*, and *Dienia ophrydis*. The new species was sister to *Liparis habenarina* with a PP of 1.00 and nested into the *Liparis sootenzanensis* subclades.

Similarly, the phylogenetic trees generated based on the *matK* sequence data analysis was also divided into three main clades (Fig. 2). Most clade relationships agreed with ITS, but a number of subclades varied slightly. The new species was sister to *Liparis sootenzanensis*.

In this study, ITS and *matK* were combined into a single dataset. The strict consensus BI phylogram (Fig. 3) strongly supports the three main clades of the alliance. All of the main clades were identified with ITS and *matK* phylogram, but some subclades were formed, and most clades were strongly supported. Clade A comprised four subclades: *Liparis*

*sula*, comprising eight species; *L. guangxiensis*, comprising three species; *L. truncicola*, comprising four species; and *Oberonia*. Clade B included four subclades: *Oberonioides*, comprising four species; *Malaxis monophyllos* and *Malaxis*, comprising eight species; and *Liparis*, comprising nine species. Clade C comprised three subclades: *Liparis sootenzanensis*, comprising three species; *Liparis layardii*, comprising seven species; *Malaxis*, comprising *Malaxis* 15 species; and one *Crepidium* species. The new species was sister to *L. habenarina* and nested into the *Liparis sootenzanensis* subclade.

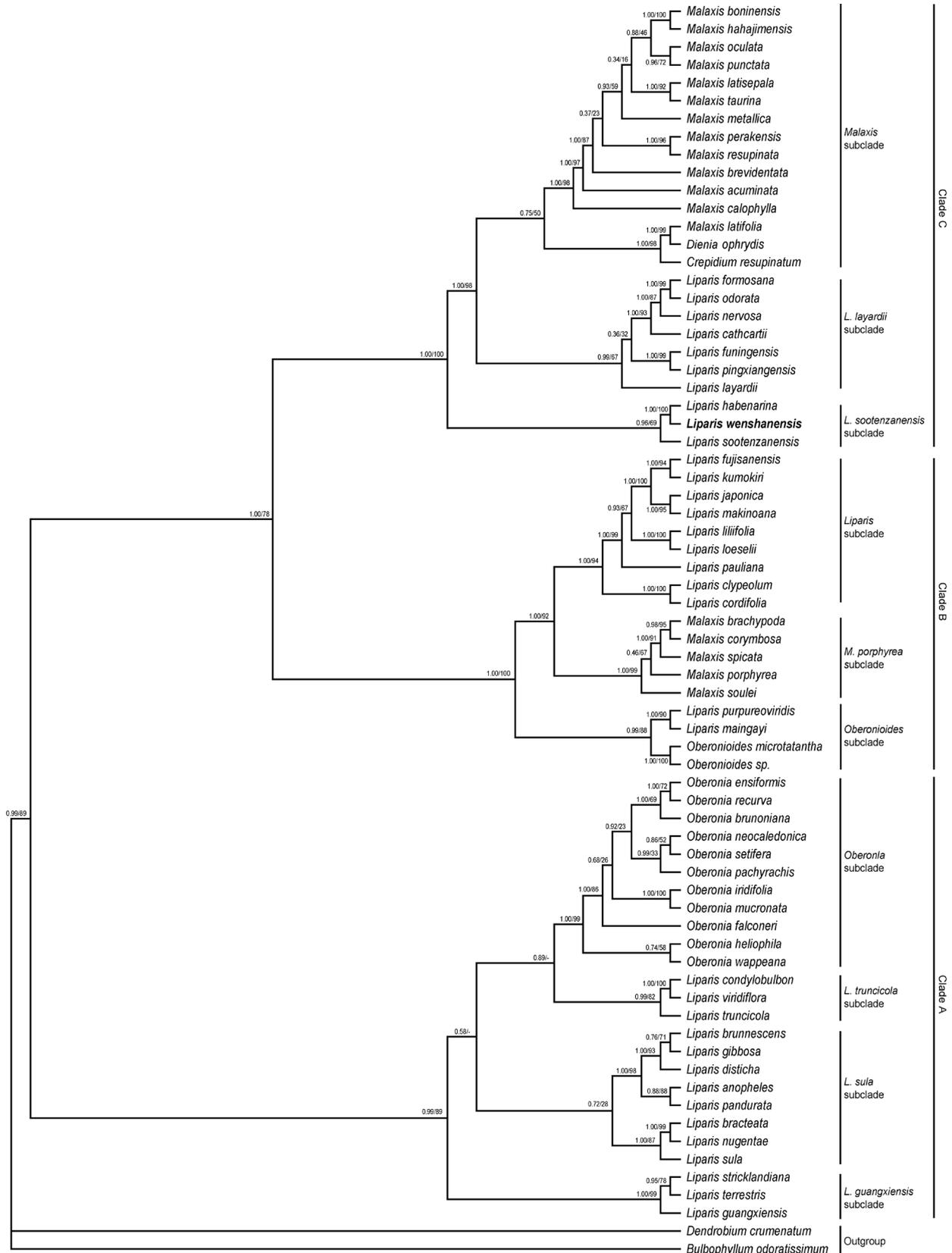


FIGURE 1. Consensus trees based on ITS data.

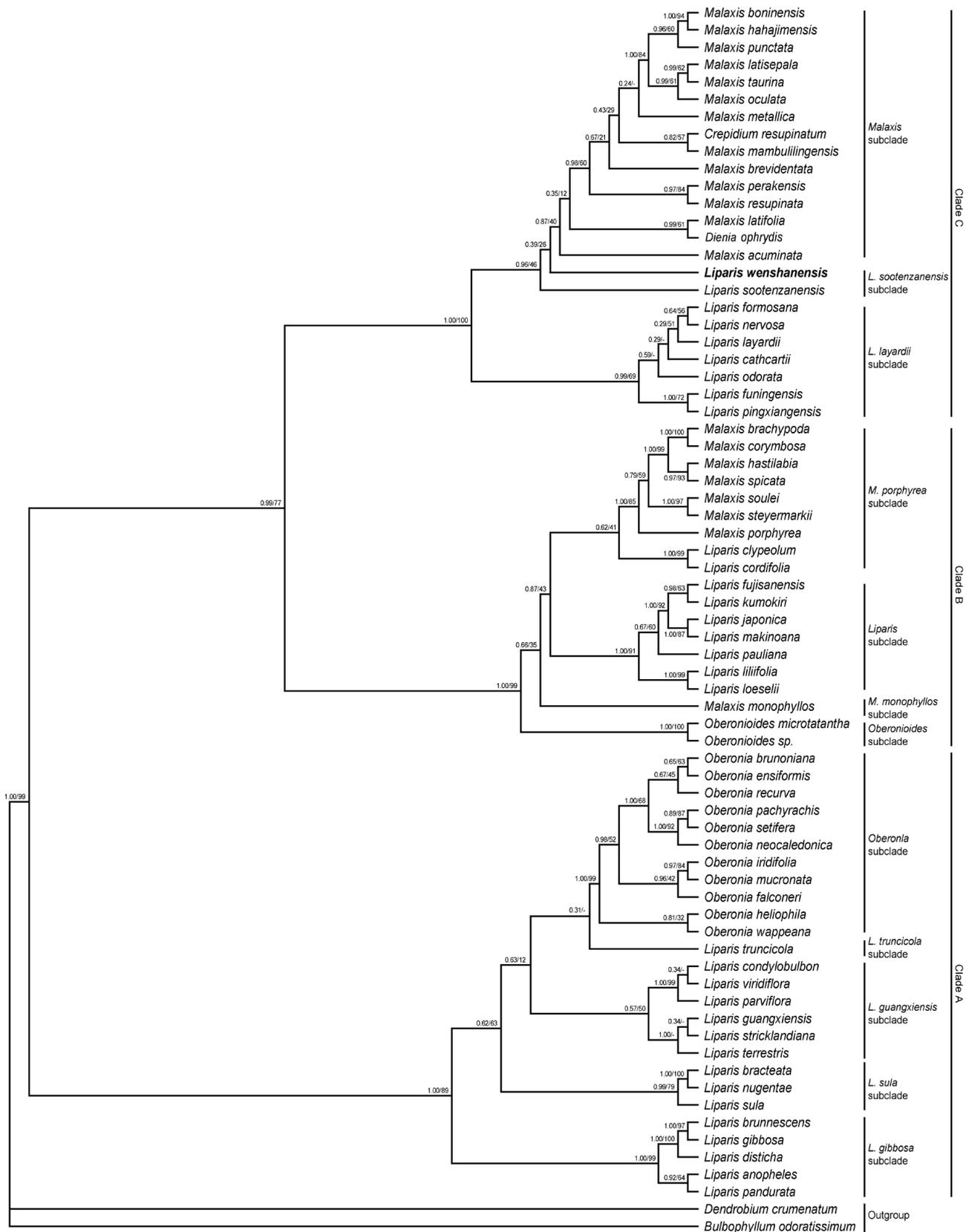


FIGURE 2. Consensus trees based on *matK* data.

## Discussion

*Liparis* has always been characterized by an unlobed lip with two parts forming the hypochile and the epichile and

four 'naked pollinia' in two pairs that do not have caudicles or stipes on their pollinia, although each pair has a tiny viscidium (Dressler, 1993; Chen *et al.* 2009). The new species described in this report has the characteristics of *Liparis*, indicating it is a member of this genus.

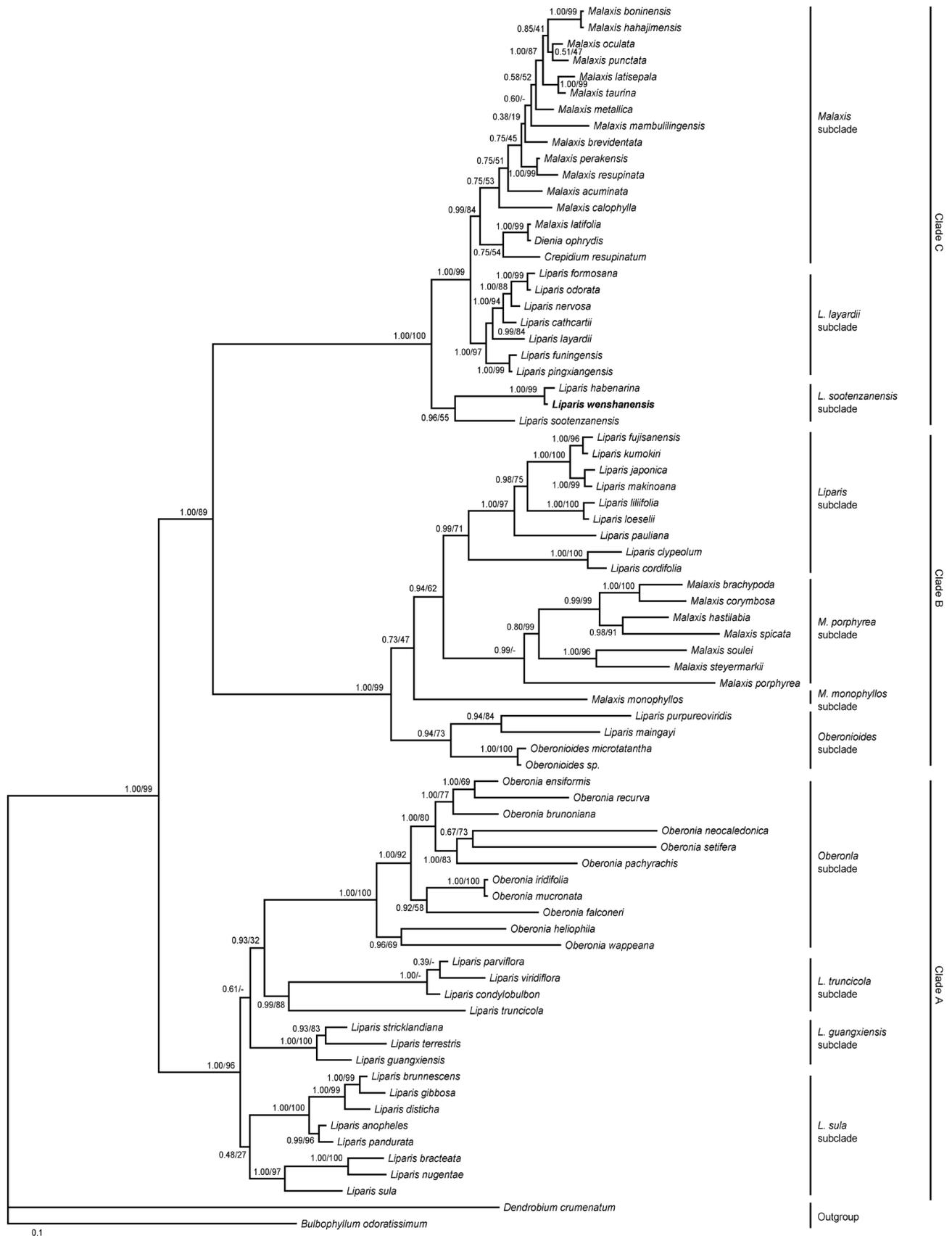
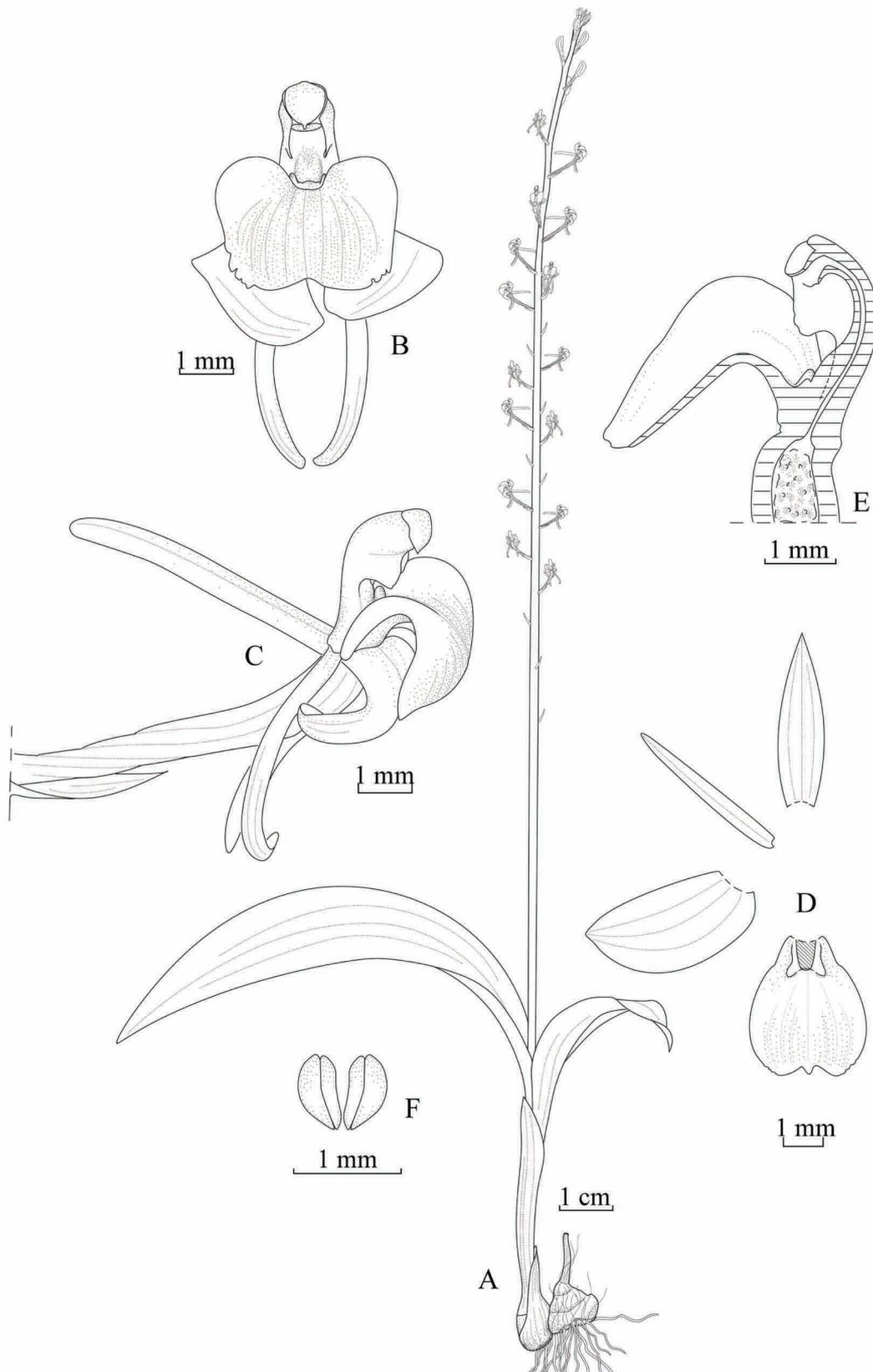


FIGURE 3. Consensus trees based on ITS and *matK* data.

The resulting cladograms reveal three clades, with *Liparis* found in all three. The results confirm the phylogenetic analyses of previous studies (Cameron, 2005; Li & Yan, 2013; Su *et al.* 2014) and suggest that the generic delimitations between *Liparis* and related genera need to be re-evaluated. The new species identified was nested into *Liparis sootenzanensis* subclade of clade C and sister to *L. habenarina* (PP1.00), supporting *L. habenarina* (F. Muell) Benth. as a member of *Liparis*.



**FIGURE 4.** *Liparis wenshanensis* Y. Y. Su, Y. L. Huang & G. Q. Zhang. A. Flowering plant. B. Flower, front view. C. Flower, side view. D. Dorsal sepal, petal, lateral sepal, and lip. E. Column and lip, longitudinal section. F. Pollinarium.

The subclade of clade C comprised three species, *L. sootenzanensis* Fukuyama (1933: 84), *L. habenarina*, and the new species, all of which have plicate leaves that widen and frontward lateral sepals that look much larger than the lip. *L. sootenzanensis* and *L. habenarina* have a larger plant with over four leaves, stem-like pseudobulbs, and shorter inflorescence with no more than 20 flowers, flowers with unlobed lips, and stigma with wings. However, the new species has a long rachis with 45 to 55 flowers, lip cordate, apical two-lobed; column with a lamellae extending from its base to near the stigma along the center way, the base of the lamellae fused to the base of the lip disc, and the base part without wings with widened wings on both sides of the stigma, indicating that the new species is a distinct species in *Liparis*.

## Taxonomic treatment

*Liparis wenshanensis* Y.Y. Su, Y. L. Huang & G. Q. Zhang, sp. nov. (Figs. 4, 5)

Type:—CHINA. Yunnan: Wenshan, terrestrial in evergreen broad-leaved forest on moist and shady slopes, alt. 1500 m, 10 July, 2014 Z. J. Liu 8072 (Holotype: NOCC).

Diagnosis: This new species is similar to *Liparis odorata* (Willdenow) Lindley (1830: 26) and *Liparis habenarina* (F. Muell) Benth., from which it differs by having a long rachis with 45–55 flowers and sepals, petals and column white, lip greenish with a purplish center; dorsal sepal and petals cylindrical; lateral sepals oblong, strongly curved; lip cordate, strongly curved, apical two-lobed, base with a two-lobed callus; column with a lamellae extending from its base to near stigma along centre way, base of lamellae fused to the base of lip, and with widened wings on both sides of the stigma.

Herbs, terrestrial. Pseudobulbs sub-ovoid, 1.7–2 × 0.8–1 cm, noded. Leaves 2, subsessile, base enclosed by two greenish brown cylindrical-sheaths, stem-like, 2.5–10 cm; blade ovate-elliptic, 8–13 × 4–5 cm, margin entire, apex acuminate. Inflorescence 45–55 cm long; rachis with 45–55-flowered; floral bracts lanceolate, 3–4 mm. Flowers full open. Sepals, petals and column white, lip greenish with purplish center; pedicel and ovary 1–1.2 cm. Dorsal sepal narrowly linear, margin revolute to cylindrical, 6–6.5 × 0.8–1 mm, apex obtuse; lateral sepals oblong, strongly curved backward, 4.5–5 × 2–2.2 mm, apex obtuse. Petals narrowly linear, margin revolute to cylindrical, 5–6 × 0.5–0.6 mm; lip cordate, strongly deflexed, 4–5 × 3–3.5 mm, apical two-lobed, with a two-lobed callus at base. Column upper part arcuate, 3–3.5 mm long, with a lamellae extending from its base to near stigma along centre way, base of lamellae fused to the base of the lip for 0.8–1 mm, and with widened wings on both sides of the stigma. Four pollinia in two pairs, waxy, elongate-obovoid, bilaterally flattened without a caudicle or viscidium. Capsule ellipsoid. Flowering period: July–August.

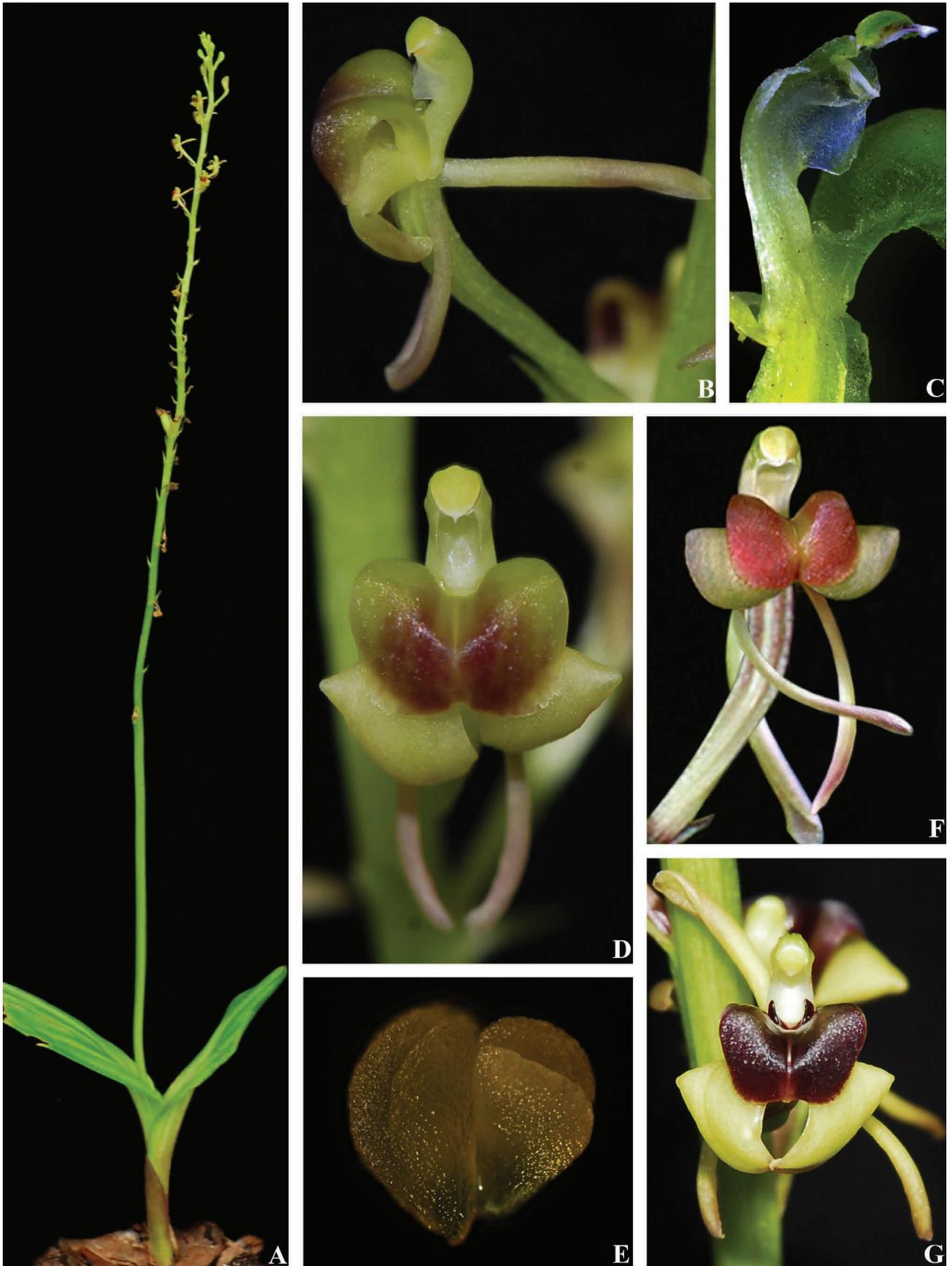
**Distribution and habitat:**—*Liparis wenshanensis* is terrestrial and grows in mixed populations with *Bletilla sinensis*, forming scattered colonies on shady and wet areas on the slopes at elevations of 1500 m in the evergreen broad-leaved forest of Wenshan, Yunnan Province, China.

**Etymology:**—The species epithet refers to the locality where this new species was first found.

**Taxonomic notes:**—*Liparis wenshanensis* is very distinctive among species in the genus. It is morphological similar to *L. odorata* and *L. habenarina*, but it can be easily distinguished by its having a long rachis with 45 to 55 flowers and with white sepals, petals and column, and greenish lip with a purple center; dorsal sepal and cylindrical petals, lateral sepals that are oblong and strongly curved backward; a lip cordate that is strongly deflexed, apical two-lobed; a column with a lamellae extending from its base to near the stigma along the center way, the base of the lamellae fused to the base of the lip, and with widened wings on both sides of the stigma (Table 1).

## Acknowledgments

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**FIGURE 5.** *Liparis wenshanensis* Y. Y. Su, Y. L. Huang & G. Q. Zhang. A. Flowering plant. B. Flower, side view. C. Column and lip, longitudinal section. D. Flower, front view. E. Pollinarium. F. A flower of *L. habenarina*. G. A flower of *L. odorata*.

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