



Research paper

Morphological and genomic evidence for a new species of *Corallorhiza* (Orchidaceae: Epidendroideae) from SW ChinaJia-Xin Yang^{a, b, c, 1}, Shuai Peng^{a, b, c, 1}, Jun-Jie Wang^{a, b, c}, Shi-Xiong Ding^{a, b, d}, Yan Wang^{a, b, c}, Jing Tian^{a, b}, Han Yang^e, Guang-Wan Hu^{a, b, *}, Qing-Feng Wang^{a, b}^a CAS Key Laboratory of Plant Germplasm Enhancement and Specialty Agriculture, Wuhan Botanical Garden, Chinese Academy of Sciences, Wuhan, 430074, China^b Sino-Africa Joint Research Center, Chinese Academy of Sciences, Wuhan, 430074, China^c University of Chinese Academy of Sciences, Beijing, 100049, China^d Jiangxi Provincial Key Laboratory for Bamboo Germplasm Resources and Utilization, Forestry College, Jiangxi Agricultural University, Nanchang, 330045, China^e Administration of Siguniang Mountains National Nature Reserve, Xiaojin, 624206, China

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ABSTRACT

Corallorhiza sinensis, a new species of mycoheterotrophic orchid from western Sichuan, China, is described and illustrated based on molecular and morphological evidence. It is morphologically similar to *Corallorhiza trifida*, but can be distinguished by bigger flowers, both sepals and petals with 3 veins, and longer lateral lobes of lip. To distinguish the new *Corallorhiza* species and explore its phylogenetic position within subtribe Calypsoinae, this study employed sequences of the nuclear ribosomal DNA (nrDNA) and whole plastome assembled from the genome skimming approach. The plastome is 148,124 bp in length, including a pair of inverted repeats (IRs) of 26,165 bp, a large single-copy region (LSC) of 82,207 bp, and a small single-copy region (SSC) of 13,587 bp. Further, phylogenetic analyses were performed using nrDNA sequence and 79 coding sequences (CDSs) from 26 complete plastomes of subtribe Calypsoinae. The phylogenetic tree based on nrDNA sequence suggested that *Corallorhiza* is a monophyletic group, and strongly support *C. sinensis* as sister to the rest species of *Corallorhiza*. The plastid tree showed that 10 *Corallorhiza* species grouped into two clades and *C. sinensis* is most closely related to the North American *C. striata* and *C. bentleyi* instead of *Oreorchis foliosa* and *O. angustata* in the same clade. The plastid tree and nrDNA tree indicate *Oreorchis* is a paraphyletic. Although the topological conflicts are displayed between plastome and nrDNA phylogenies of *C. sinensis*, it is still the most closely related to *Corallorhiza*. Comparative analysis showed that *C. sinensis* populations are characteristic of the intermediate morphological traits between *Corallorhiza* and *Oreorchis*. The finding of this new species from China shed new light on the phylogeny of *Oreorchis* and *Corallorhiza*.

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1. Introduction

Orchidaceae is one of the largest families of flowering plants, comprising about 736 known genera and 28,000 species

worldwide (Chase et al., 2015; Christenhusz and Byng, 2016). During an investigation on wild orchid resources in western Sichuan, China in 2020, a distinctive orchid species was discovered in the Siguniang Mountains National Nature Reserve under shady *Populus* forests. Some morphological characters such as lack of leaf and coralloid rhizome immediately caught our attention, and it is morphologically similar to the species in the genus *Corallorhiza*. *Corallorhiza* Gagnebin is the largest temperate genus of leafless putative heterotrophs in Orchidaceae, comprising 11 species that are restricted to the northern hemisphere of the New World, except for a single circumboreal species, *C. trifida* Chatelain (Chase et al.,

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2015; Freudenstein, 1997, 1999; Freudenstein and Senyo, 2008). According to the *Flora of China*, only *C. trifida* has been recorded in China, distributed in Gansu, Guizhou, Hebei, Jilin, Nei Mongol, Qinghai, Sichuan, Xinjiang (Chen and Stephan, 2009). We compared the new collection with *C. trifida*, and discovered that it can be distinguished from the latter by bigger flowers, both sepals and petals with 3 veins, and longer lip lateral lobes. We further compared it with other congeneric species. It is also similar to *C. striata* Lindley and *C. bentleyi* Freudenstein in sharing a distinctive morphological character: lack a mentum (i.e. a vestigial nectar spur at the base of the perianth), but can be easily distinguished from these two species by its lip broad oblong and obviously 3-lobed the toward base. After carefully comparing the plant with all previously known congeneric taxa, it could not be matched with any known species. Hence, our collection might be a new species of *Corallorhiza*.

Chase et al. (2015) presented a revised classification of Orchidaceae and divided the family into five subfamilies, namely Apostasioideae, Vanilloideae, Cyripedioideae, Orchidoideae, and Epidendroideae. The genus *Corallorhiza* was placed in the subtribe Calypsoinae (Epidendreae, Epidendroideae) which contains 13 genera, namely *Corallorhiza*, *Aplectrum* Nutt., *Calypso* Salisb., *Changnienia* S.S. Chien, *Coelia* Lindl., *Cremastra* Lindl., *Dactylostalix* Rchb. f., *Danxiaorchis* J.W. Zhai, F.W. Xing & Z.J. Liu, *Ephippianthus* Rchb. f., *Govenia* Lindl., *Oreorchis* Lindl., *Tipularia* Nutt., and *Yuania* Maxim. Freudenstein et al. (2017) reconstructed phylogenetic relationships among subtribe Calypsoinae. Based on *matK* marker, results showed *Corallorhiza* was paraphyletic, but the support for the node was relatively weak. In the results of ITS sequence and combined analyses (*matK* + ITS sequences), *Corallorhiza* was shown to be monophyletic with strong support. An earlier study based upon combined ITS, plastid datasets, and morphological character matrix strongly support *Oreorchis* and *Corallorhiza* are both monophyletic groups, but the *Corallorhiza* is far from *Oreorchis* (Zhai et al., 2013). In recent molecular phylogenetic studies based on the plastomes, however, *Corallorhiza* is still non-monophyletic, and the *Oreorchis* and *Corallorhiza* were nested together and formed a single clade (Kim et al., 2020; Li et al., 2020). In the taxonomic revision of *Oreorchis* (Pearce and Cribb, 1997), except for *Kitigorchis itoana*, the remaining species of *Kitigorchis* were transferred into *Oreorchis*. Moreover, Pearce and Cribb (1997) remarked that *Kitigorchis* be a monotypic genus intermediate between the *Oreorchis* and *Corallorhiza*, and suggested that *K. itoana* has coral-like rhizomes quite different from the subterranean organs of *Oreorchis*. Interestingly, the final study revealed that *K. itoana* and *O. indica* were conspecific (Yukawa et al., 2003). In fact, *O. indica* often possesses coralloid rhizomes (Suetsugu et al., 2020). An earlier study has shown that coralloid rhizomes occur in the *Aplectrum hyemale* (Muhl. ex Willd.) Nutt. (Macdougall and Dufrenoy, 1944). Thus the habit has happened many times in Calypsoinae. Similarly, in the Calypsoinae, a previous study also demonstrated that leafless habit has happened many times (Freudenstein et al., 2017). Suetsugu et al. (2020) suggested that *O. indica* is a partially mycoheterotrophic orchid, and the full mycoheterotrophy evolved after the establishment of partial mycoheterotrophy, rather than through direct shifts from autotrophy in the *Oreorchis*-*Corallorhiza* clade. In the genus *Corallorhiza*, it is well known that *C. trifida* is partial mycoheterotrophy (Zimmer et al., 2008). Due to the phylogenetic relationship of *Oreorchis* and *Corallorhiza* remaining uncertain, we hypothesize that our collection might be a new species of *Corallorhiza*.

Here we describe and illustrate the new species *Corallorhiza sinensis*. To distinguish the new *Corallorhiza* species, we sequenced its complete plastome. Its plastome was analyzed in comparison with other members of subtribe Calypsoinae. Finally, we

reconstructed its phylogenetic relationship within subtribe Calypsoinae based upon the nrDNA sequence and 79 coding sequences (CDSs).

2. Materials and methods

2.1. Morphological analyses

Morphological characters of the new species were based on the study of living plants and herbarium specimens. Measurements of flowers and rhizomes were taken from living plants. Photographs of *Corallorhiza sinensis* and *C. trifida* were taken in the field and are included in Figs. 1 and 2, respectively. A total of 11 diagnostic characteristics of these two species are compared in Table 1. Measurements of *C. trifida* were based on the combination of *Flora of China* (Chen and Stephan, 2009) and observation on living plants. Given that the phylogenetic relationship of *Oreorchis* and *Corallorhiza* remains uncertain, we compared the new collection with the members of *Oreorchis* as well and found the new species resembles *O. patens* and *O. foliosa* var. *indica*. To better distinguish the new species, we also presented its supplementary photograph (Fig. S1). Furthermore, the photographs of *O. patens* and *O. foliosa* var. *indica* were combined in the Fig. S2, which will provide important morphological evidence for this study. The voucher specimens of the new species were deposited in the Herbarium of Wuhan Botanical Garden, Chinese Academy of Sciences (HIB).

2.2. Specimens examined of *Oreorchis*

Oreorchis patens Lindl., China: Hunan, Sangzhi county, July–4–1998, Beijingdui, 2362 (PE, 01340734).

Oreorchis foliosa var. *indica* (Lindl.) N. Pearce & P.J. Cribb, China: Xizang, June–2–1972, Xizang Herbal Medicine Investigation Team 89 (PE, 00850746).

2.3. DNA extraction and sequencing

Purified total DNA of *Corallorhiza sinensis* was fragmented to construct short-insert libraries for sequencing (Illumina HiSeq, 2000 platform from the Biomarker Technologies, Inc.), and 6 GB of reads was obtained.

2.4. Plastome and nrDNA assembly, annotation

The complete plastome of *Corallorhiza sinensis* was assembled using GetOrganelle v.1.7.1 with appropriate parameters: K-mer is “21,45,65,85,105”, the Word size is 0.6 (Jin et al., 2020). Five key steps were included in the workflow: 1. Mapping reads to seed and assembling seed-mapped reads for parameter estimation; 2. Recruiting more target-associated reads through extending iterations; 3. Conducting de novo assembly; 4. Roughly filtering for target-like contigs; 5. Identifying target contigs and exporting all configurations (Bankevich et al., 2012; Camacho et al., 2009; Jin et al., 2020; Langmead and Salzberg, 2012). Final assembly graphs were visualized by Bandage to assess the completeness of the final graph (Wick et al., 2015). The nrDNA sequence (18S-ITS1-5.8S-ITS2-26S) of *C. sinensis* was assembled using GetOrganelle v.1.7.1 with default parameters.

Initially, PGA (Qu et al., 2019) was utilized to annotate the complete plastome of *Corallorhiza sinensis*, with the *C. trifida* (GenBank: MN990435) selected as the reference, and then manually adjusted protein-coding genes and exons boundaries using software Geneious 11.0.4 (Kearse et al., 2012). The final plastome and nrDNA sequence of *C. sinensis* have been submitted to GenBank under accession numbers MW191791 and MW413395, respectively.

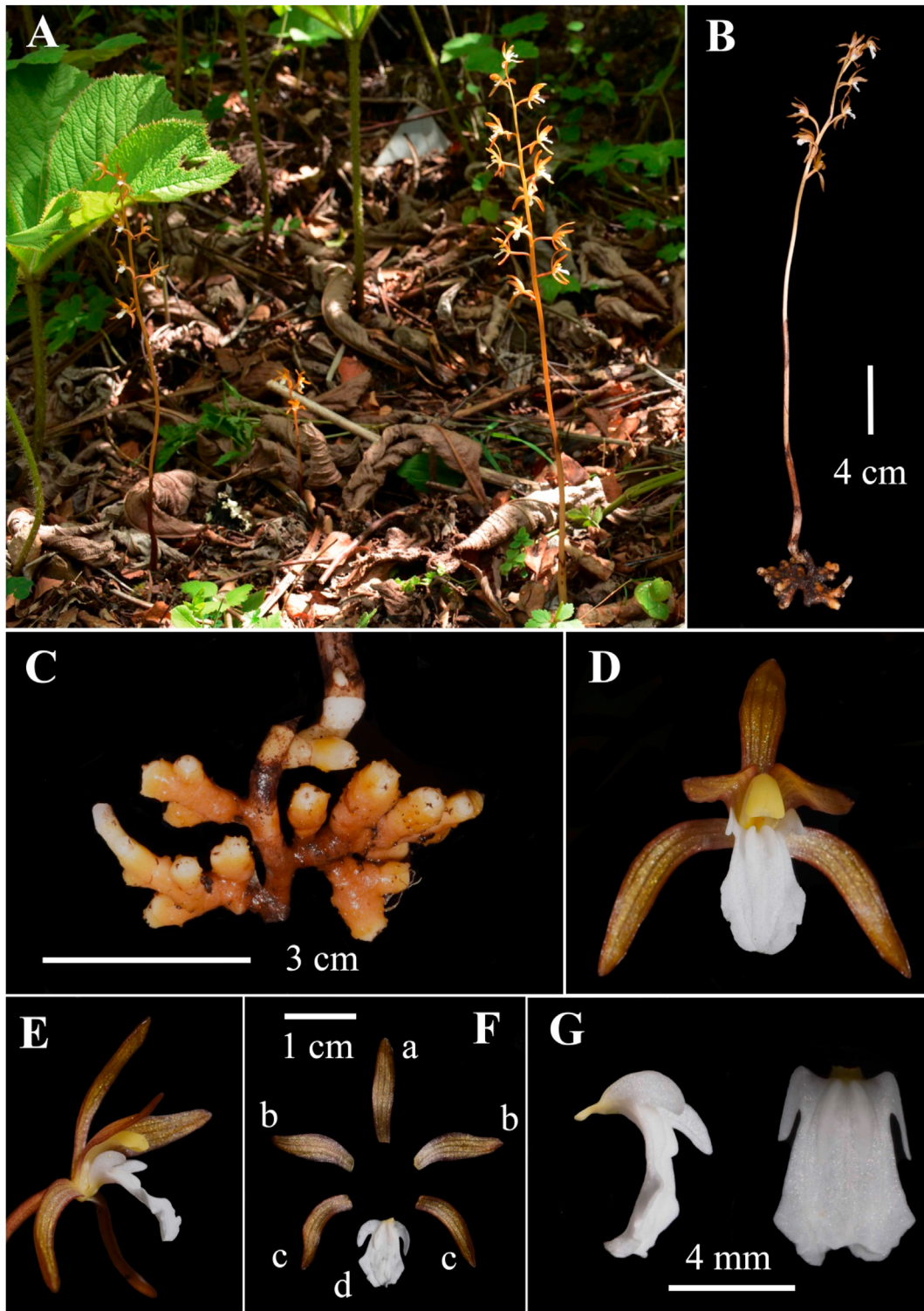


Fig. 1. Photographs of *Corallorhiza sinensis*. (A) habit; (B) individual; (C) root; (D) front view of flowers; (E) lateral view of flowers; (F) anatomy of flowers (a = dorsal sepal, b = petal, c = lateral sepal, d = lip); (G) lateral and front view of lips.

Gene map of the plastome of *C. sinensis* was drawn online using OGDRAW (Lohse et al., 2007).

2.5. Genome features and comparative plastid genomic analysis

Length of the whole plastome of *Corallorhiza sinensis*, numbers of genes, and categories of Genes were analyzed in Geneious 11.0.4

(Kearse et al., 2012). The software MEGA (Kumar et al., 2016) was used for calculating the guanine-cytosine (GC) content. In order to further identify potential variations between *C. sinensis* and other *Corallorhiza* species, the genome rearrangement analyses of ten *Corallorhiza* sequences were performed using Mauve (Darling et al., 2004) plugin in Geneious 11.0.4 (Kearse et al., 2012). Geneious 11.0.4 (Kearse et al., 2012) was used to detect the contraction/

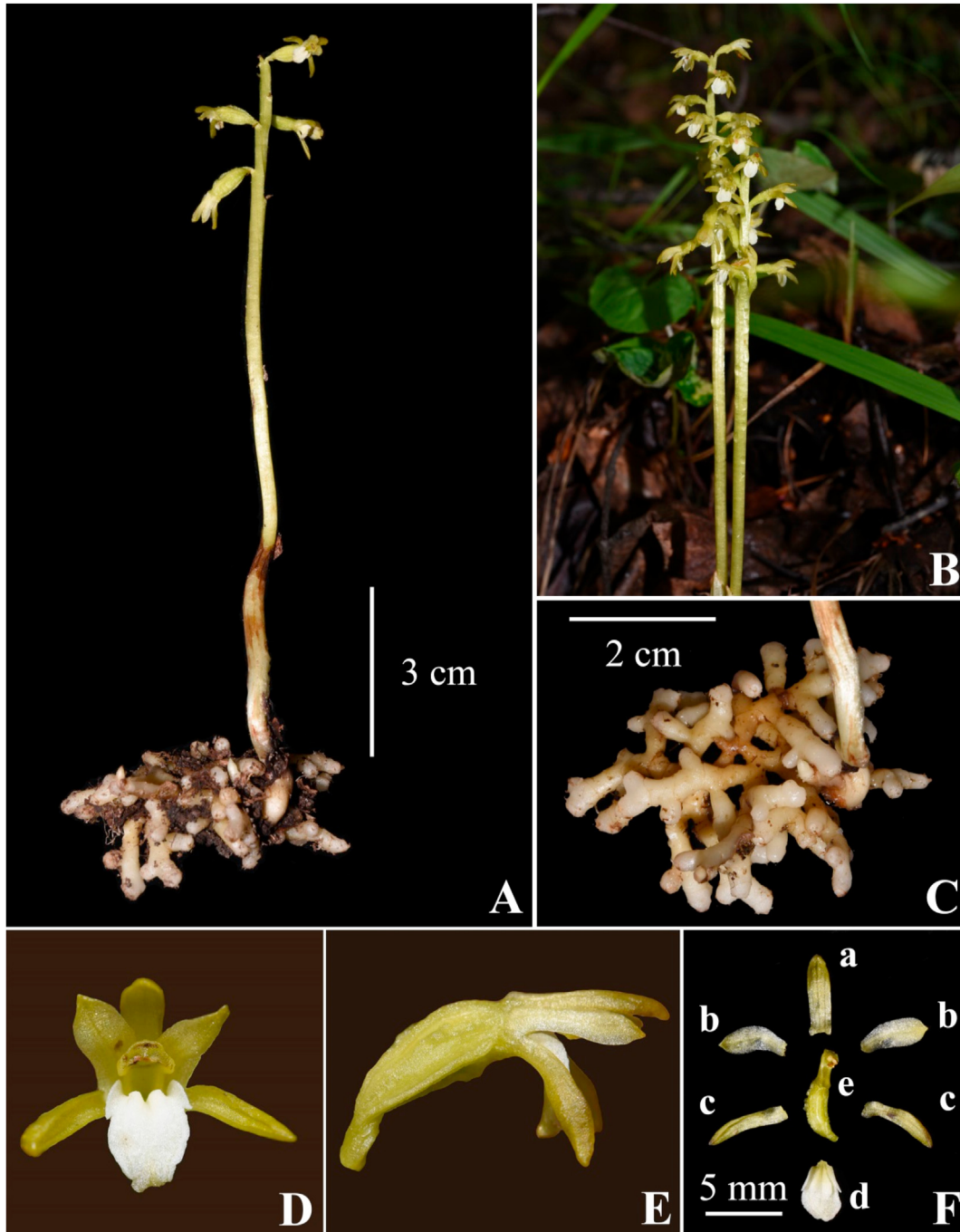


Fig. 2. Photographs of *Corallorhiza trifida*. (A) individual; (B) habit; (C) root; (D) front view of flowers; (E) lateral view of flowers; (F) anatomy of flowers (a = dorsal sepal, b = petal, c = lateral sepal, d = lip); (G) lateral and front view of lips.

expansion of the inverted repeat regions (IRs), and the graph of expansions/contractions was visualized using Adobe Illustrator.

2.6. Phylogenetic analysis

To estimate the evolutionary history of *Corallorhiza sinensis* within Calypsoinae, phylogenies were reconstructed by Maximum likelihood (ML) and Bayesian Inference (BI) analyses using the nrDNA sequence and coding sequences (CDSs), the total of 79 CDSs from 27 plastomes were used for constructing the phylogenetic relationship of the members within Calypsoinae (Table S1). Lost

genes and pseudogenes were treated as missing data. All nrDNA sequences and plastomes were downloaded from the NCBI database except *C. sinensis*. In the plastid tree and nrDNA tree, *Cattleya crispata* and *Chysis bractescens* are selected as outgroup, respectively. We extracted 79 CDSs using Geneious 11.0.4 (Kearse et al., 2012). Under the codon model, each single CDS matrix was aligned and edited by MAFFT (Katoh and Standley, 2013) and Gblocks (Talavera and Castresana, 2007) plugin in PhyloSuite (Zhang et al., 2020), respectively, and each matrix is eventually concatenated into one supermatrix by PhyloSuite (Zhang et al., 2020). ModelFinder was utilized to select the best-fit model for

Table 1
Comparison of the diagnostic characteristics of *Corallorhiza sinensis* and *C. trifida*.

Characters	<i>Corallorhiza sinensis</i>	<i>C. trifida</i>
Plant height	10–46 cm	10–28 cm
Inflorescence	5–13-flowered	3–7-flowered
Flower colour	light reddish brown	pale yellowish green to white
Pedicel and ovary	9–12 mm	3.5–5 mm
Lateral sepals	ca. 10 × 2 mm, 3-veined	4–6 × 1–1.5 mm, 1-veined
Dorsal sepal	ca. 12 × 2 mm, 3-veined	4–6 × 1–1.5 mm, 1-veined
Petal	long ovate-lanceolate, 11 × 4 mm, 3-veined	lanceolate to oblanceolate, 3–5 × ca. 1.5–2 mm, 1-veined
Lip	ca. 8 × 6 mm, obviously 3-lobed toward base	2.5–4 × ca. 1.5–3 mm, obscurely 3-lobed toward base
Midlobe of lip	oblong to broadly dilated toward apex, ca. 7 × 5 mm	elliptic to oblong, 1–1.5 × ca. 0.8 mm
Lateral lobe of lip	ca. 3 mm long	less than 1 mm long
Column	yellowish, ca. 8 mm long, arcuate	greenish, 2.5–3 mm long, erect

accurate phylogenetic estimates (Kalyaanamoorthy et al., 2017). ML analysis was performed using IQ-TREE with 1000 bootstrap replicates (Nguyen et al., 2015), BI analysis was performed in MrBayes 3.2.2 (Ronquist et al., 2012) using the Markov chain Monte Carlo (MCMC) method, with two independent runs for 10 million generations (Number of Chains is four). Phylogenetic trees were sampled every one thousand generations, the first 25% of trees generated were discarded as burn-in and the remaining trees were used to construct majority-rule consensus tree (Ronquist et al., 2012). Finally, the phylogenetic trees were edited using FigTree 1.4 (<https://github.com/rambaut/figtree>).

3. Results

3.1. Taxonomic treatment

3.1.1. *Corallorhiza sinensis* G.W. Hu & Q.F. Wang (Figs. 1 and 3)

Type: China. Sichuan: Xiaojin County, Siguniang Mountains National Nature Reserve, 102°44′31.5″E, 30°58′26.5″N, under *Populus* forests ca. 2984 m in elevation, 9 Jun 2020, S. Peng, J. X. Yang et J. J. Wang YJX-0060 (holotype: HIB, isotype: HIB).

Diagnosis: *Corallorhiza sinensis* resembles *C. trifida*, but prominently differs from the latter in having bigger flowers, both petals and sepals with 3 veins, and longer lateral lobes of lip. It is also similar to *C. striata* and *C. bentleyi* by sharing a distinctive morphological character: mentum absent, but can be easily distinguished by its lip broadly oblong and obviously 3-lobed toward base. Comparative analysis of morphology with the *Oreorchis* species showed that *C. sinensis* resembles *O. patens* and *O. foliosa* var. *indica*. The labellum of *C. sinensis* with two lamellae looks almost exactly like that of *O. patens* (Fig. S2, D–F), but *O. patens* has smaller flowers than *C. sinensis* and the subterranean system of *O. patens* indicated it is similar to normal (Fig. S2, D–F). The *O. foliosa* var. *indica* possess coralloid rhizomes and normal roots, which present obvious dual subterranean system, while the pseudobulb is still an important morphological character. Moreover, *O. foliosa* var. *indica* has a broadly 3-lobed to unlobed lip (Fig. S2, A–C). By observation of different populations, the *C. sinensis* has no leaves, not even visible green tissue in all individuals. Hence, our collection most closely resembles the *Corallorhiza*.

3.1.2. Description

Perennial mycoheterotrophic herbs, 10–46 cm tall. Rhizome fleshy, extensively branching, coralloid, nodiferous, internodes 2–4 mm long, often brown at the base of branch and beige at the apex of branch, diameter ca. 5 mm. Stems erect, cylindrical. Inflorescence light reddish brown, glabrous, leafless, covered with 3 or 4 membranous tubular sheaths from base to middle; sheaths amplexicaul, reddish brown, 2–12 cm long; rachis 5–18 cm, laxly 5–13-flowered; floral bracts small, lanceolate, apex acuminate,

1–3.5 mm long. Flowers light reddish brown, pedicel and ovary 9–12 mm long, torsion at base. Dorsal sepal arching forward, narrowly oblong-lanceolate, ca. 12 × 2 mm, 3-veined, apex acuminate; lateral sepals similar to dorsal sepal, slightly oblique, 3-veined, ca. 10 × 2 mm, connate at base, apex obtuse or acute. Petals long ovate-lanceolate, ca. 11 × 4 mm, 3-veined, apex obtuse or acute; lip broadly oblong, pure white with a yellowish claw, ca. 8 × 6 mm, obviously 3-lobed toward base; lateral lobes 2, erect, lanceolate but slightly curved, ca. 3 × 1 mm; middle lobe oblong to broadly dilated toward apex, ca. 7 × 5 mm, margin entire and slightly wavy at apex, with 2 distinct basal lamellae. Column yellowish, ca. 8 mm long, arcuate, basally with ridges or poorly developed auricles; ovary ca. 8.5 mm; mentum absent. Fruit not seen.

3.1.3. Etymology

The specific epithet “*sinensis*” refers to China, because the new species is endemic to China.

3.1.4. Distribution, habitat and conservation status

Currently, *Corallorhiza sinensis* is known only from Siguniang Mountains National Nature Reserve in Xiaojin County, Sichuan, China. The plants grow in shady *Populus* forests, flowering from May to June. Only two small populations of the new species were discovered, each population comprises approximately 12 mature individuals. Based on a previous study, *Corallorhiza* cannot be cultivated reliably because of the require of fungal associations for growth (Freudenstein, 1997). A preliminary assessment of the risk of extinction was conducted according to the International Union for Conservation of Nature (IUCN) Red List Categories and Criteria (<https://www.iucn.org/>), we thereby consider that *C. sinensis* should be classified as critically endangered (CR).

3.1.5. Additional specimens examined (paratypes)

China. Sichuan: Xiaojin County, Siguniang Mountains National Nature Reserve, grows *Populus* forests, elev. 2984 m, 102°48′21″E, 30°59′15.6″N, 8 Jun 2020, S. Peng, J. X. Yang et J. J. Wang PS-00166 (HIB).

3.2. Characteristics of the *Corallorhiza sinensis* plastome

The complete plastome of *Corallorhiza sinensis* is 148,124 bp in length, and exhibits a typical quadripartite structure, consisting of a large single copy (LSC) region of 82,207 bp and a small single copy (SSC) region of 82,207 bp, which were separated by a pair of 26,165 bp inverted repeat regions (IRs). The gene map of *C. sinensis* plastome is presented in Fig. 4. The gene composition in plastome of *C. sinensis* could be divided into four categories: genes related to photosynthesis, genes related to self-replication, protein-coding genes with unknown functions, and other genes. A total of 109

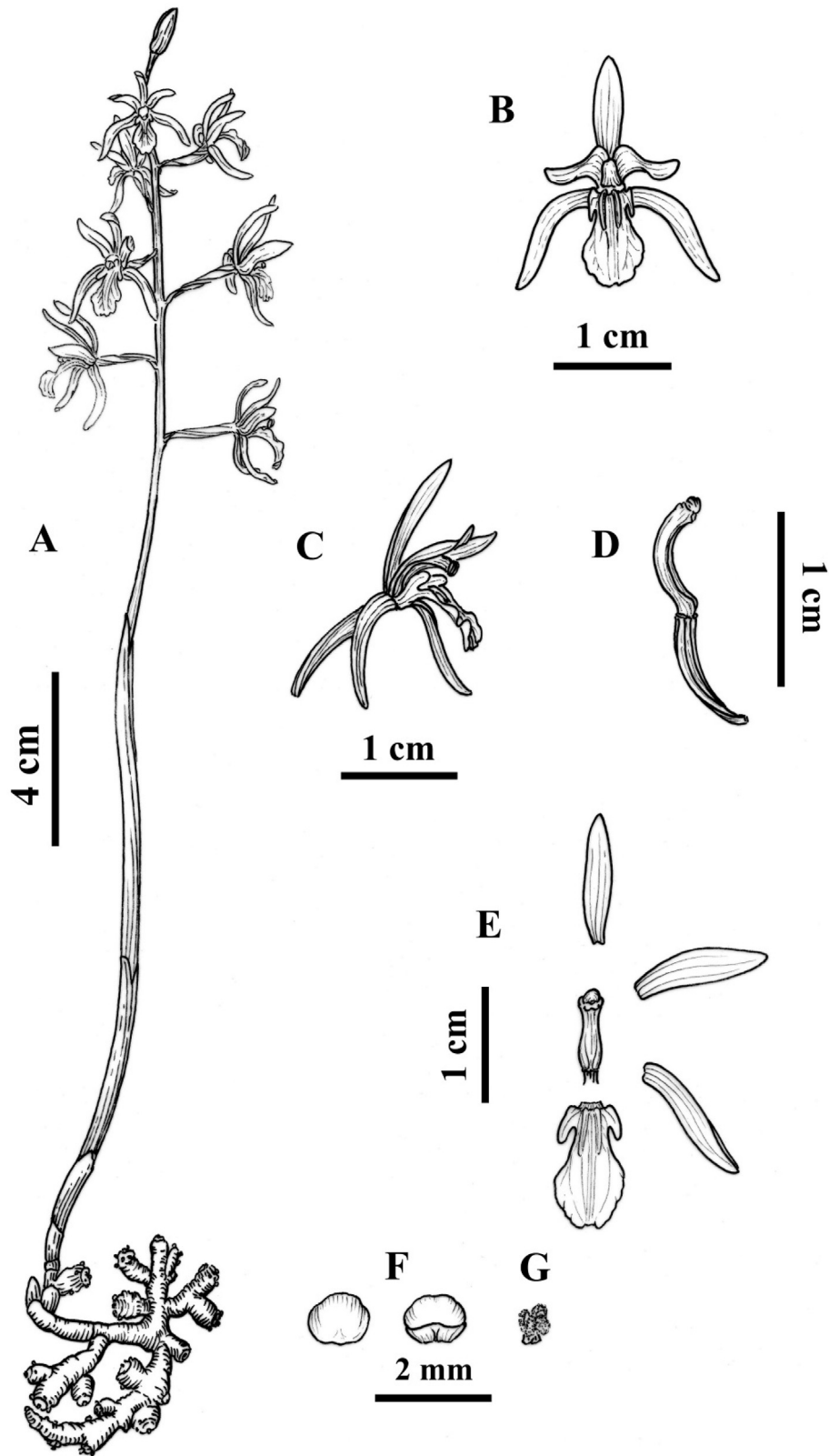


Fig. 3. Illustrations of *Corallorhiza sinensis*. (A) individual; (B) front view of flowers; (C) lateral view of flowers; (D) column and ovary; (E) anatomy of flowers; (F) anther cap; (G) pollinia. Drawn by Jing Tian.

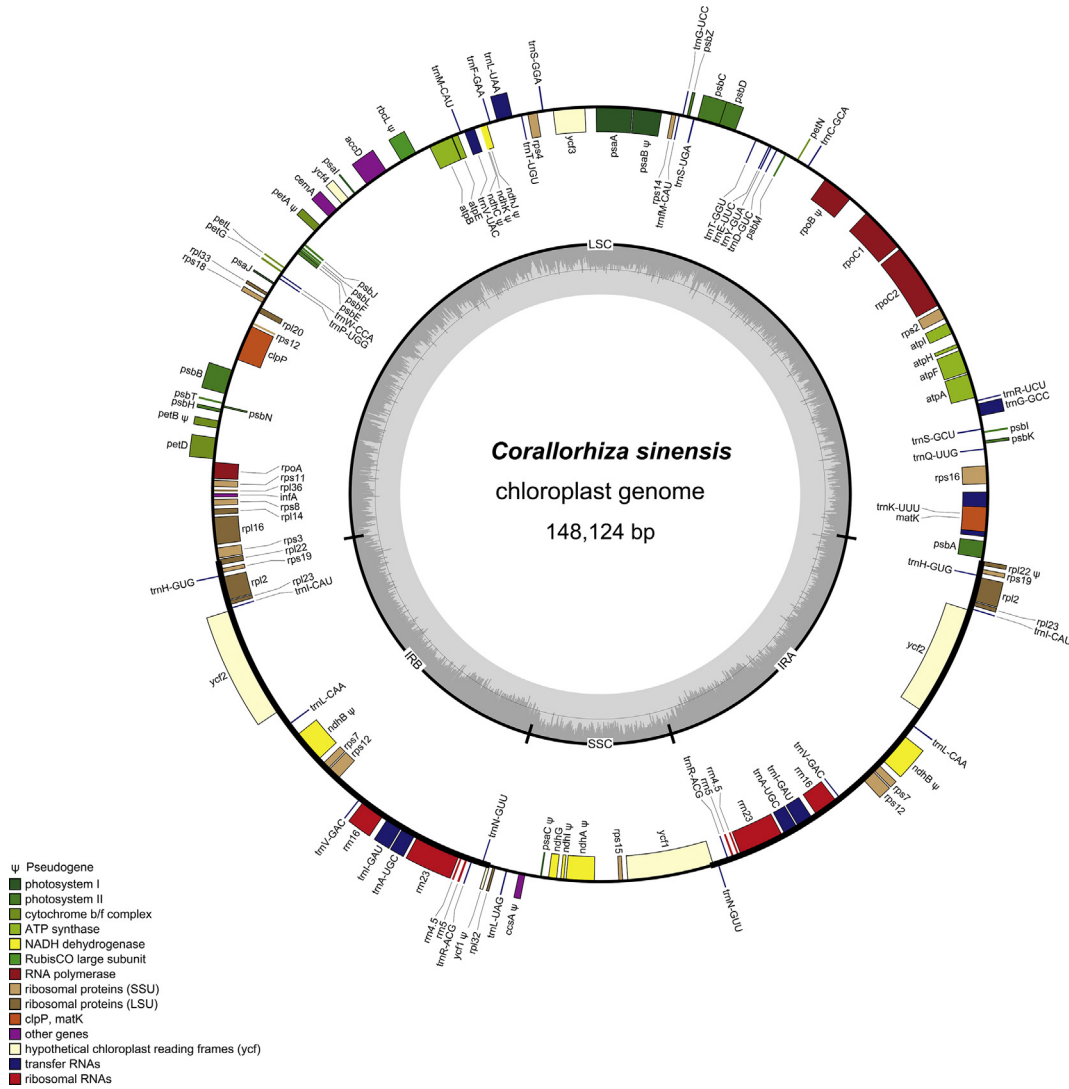


Fig. 4. Circular gene map of the plastid genome of *Corallorhiza sinensis*. Genes drawn within the circle are transcribed clockwise, while those drawn outside are transcribed counterclockwise. Genes are color-coded according to their functional groups.

unique genes were identified in the plastome, it contains 68 protein-coding genes, 30 tRNAs, and four rRNAs (Table 2). Among these genes, 15 genes are annotated as pseudogenes (*ccsA*, *ndhA*, *ndhB*, *ndhC*, *ndhI*, *ndhJ*, *ndhK*, *petA*, *petB*, *psaB*, *psaC*, *rbcl*, *rpoB*, *ycf1*, *rpl22*). A total of 21 genes were duplicated in the IR regions, including eight tRNA genes (*trnA-UGC*, *trnH-GUG*, *trnI-CAU*, *trnI-GAU*, *trnL-CAA*, *trnN-GUU*, *trnR-ACG*, *trnV-GAC*), four rRNA genes (*rrn4.5*, *rrn5*, *rrn16*, *rrn23*), and nine PCGs (*ndhB*, *ndhG*, *rpl2*, *rpl22*, *rpl23*, *rps7*, *rps19*, *rps12*, *ycf1*). A total of seven protein-coding genes and six tRNA genes contain a single intron, only *clpP* and *ycf3* contain two introns.

3.3. Comparative analysis of the plastomes

A comparison of the *Corallorhiza* and *Oreorchis* plastomes was conducted in the Table 3. The full-length plastome of the 10 *Corallorhiza* species ranged from 124,482 bp (*C. bentleyi*) to 151,031 bp (*C. macrantha*), and the *C. bentleyi* has the shortest plastome. The range of length variation among members of the

Corallorhiza for the LSC and SSC region is obvious. Further, we also compared them with published *Oreorchis* plastomes (Table 3). The length of the plastome ranges from 158,256 bp in *O. patens* to 158,654 bp in *O. angustata*, obviously larger than *Corallorhiza*. In addition, the results indicated that *Oreorchis* plastome have longer LSC region than *Corallorhiza*. Comparative analysis of gene content showed that the number of PCGs in *Oreorchis* is very similar to most angiosperms, but it is very low in some *Corallorhiza* members. As our results displayed, GC content and the number of tRNA and rRNA genes are highly conserved in all these plastomes.

Expansion and contraction of the IRs were compared among Calypsoinae (Fig. S3). The LSC/IRb boundary information of *Corallorhiza sinensis* is similar to that of ten plastomes, including *Changnienia amoena*, *Calypso bulbosa*, *Cremastra appendiculata*, two *Corallorhiza* species (*C. bentleyi* and *C. striata* var. *striata*), three *Oreorchis* species (*O. angustata*, *O. foliosa*, and *O. foliosa* var. *indica*), and two *Tipularia* species (*T. josephi* and *T. szechuanica*). The gene *rpl22* spanned the IRb/LSC region and the length of

Table 2
Gene contents in the plastid genome of *Corallorhiza sinensis*.

Category, Group of Genes	Gene Names
Photosynthesis:	
Subunits of ATP synthase	<i>atpA, atpB, atpE, atpF*, atpH, atpI</i>
Subunits of NADH dehydrogenase	<i>ndhA^a, ndhB^a(x2), ndhC^a, ndhG(x2), ndhI^a, ndhJ^a, ndhK^a</i>
Cytochrome b/f complex	<i>petA^a, petB^a, petD*, petG, petL, petN</i>
Subunits of photosystem I	<i>psaA, psaB^a, psaC^a, psal, psaj</i>
Subunits of photosystem II	<i>psbA, psbB, psbC, psbD, psbE, psbF, psbH, psbl, psbj, psbK, psbl, psbM, psbN, psbT, psbZ</i>
Large subunit of rubisco	<i>rbcl^a</i>
Other genes	
Subunit of Acetyl-CoA-carboxylase	<i>accD</i>
c-type cytochrome synthesis gene	<i>ccsA^a</i>
Envelope membrane protein	<i>cemA</i>
Protease	<i>clpP**</i>
Translational initiation	<i>infA</i>
Maturase	<i>matK</i>
Self-replication	
Large subunit of ribosome	<i>rpl14, rpl16*, rpl2*(x2), rpl20, rpl22^b(x2), rpl23(x2), rpl32, rpl33, rpl36</i>
DNA dependent RNA polymerase	<i>rpoA, rpoB^b, rpoC1*, rpoC2</i>
Small subunit of ribosome	<i>rps2, rps3, rps4, rps7(x2), rps8, rps11, rps12*(x2), rps14, rps15, rps16*, rps18, rps19(x2)</i>
rRNA Genes	<i>rrn4.5(x2), rrn5(x2), rrn16(x2), rrn23(x2)</i>
tRNA Genes	<i>trnA-UGC*(x2), trnC-GCA, trnD-GUC, trnE-UUC, trnF-GAA, trnJ-M-CAU, trnG-GCC*, trnG-UCC, trnH-GUG(x2), trnI-CAU(x2), trnI-GAU*(x2), trnK-UUU*, trnL-CAA(x2), trnL-UAA*, trnL-UAG, trnM-CAU, trnN-GUU(x2), trnP-UGG, trnQ-UUG, trnR-ACG(x2), trnR-UCU, trnS-GCU, trnS-GGA, trnS-UGA, trnT-GGU, trnT-UGU, trnV-GAC(x2), trnV-UAC*, trnW-CCA, trnY-GUA</i>
Unknown function	
Conserved open reading frames	<i>ycf1^b(x2), ycf2, ycf3**, ycf4</i>

Note: ^a indicates the pseudogene; ^c indicates trans-spliced gene; ^b indicates one of them is a pseudogene; * genes containing a single intron; ** genes containing two introns; (x2) genes present as two copies in the IR regions.

Table 3
Statistics on the basic features of the plastid genomes of species from *Corallorhiza* and *Oreorchis*.

Species	Accession no.	Length (bp)	LSC (bp)	SSC (bp)	IR (bp)	GC content (%)	No. of PCGs	No. of tRNA	No. of rRNA
<i>Corallorhiza sinensis</i>	MW191791	148,124	82,207	13,587	26,165	36.6	68	38	8
<i>C. bulbosa</i>	KM390013	148,643	82,851	12,368	27,712	37.1	74	38	8
<i>C. mertensiana</i>	KM390018	147,941	81,109	13,774	26,529	36.8	60	38	8
<i>C. wisteriana</i>	KM390020	146,437	82,350	11,743	26,172	37.1	73	38	8
<i>C. odororhiza</i>	KM390021	147,317	82,259	13,508	25,775	37.0	73	38	8
<i>C. trifida</i>	MN990435	149,408	83,171	14,397	25,920	37.2	74	38	8
<i>C. macrantha</i>	NC_025660	151,031	84,263	12,544	27,112	37.2	72	38	8
<i>C. striata</i> var. <i>striata</i>	NC_040978	141,202	75,881	12,999	26,161	36.3	50	37	8
<i>C. bentleyi</i>	NC_040979	124,482	63,581	7,781	26,560	36.6	45	39	8
<i>C. maculata</i>	NC_046814	146,198	79,612	13,018	26,784	36.8	55	38	8
<i>Oreorchis angustata</i>	MN990443	158,654	86,294	18,312	27,024	37.0	86	38	8
<i>O. patens</i>	MN990436	158,256	86,253	18,341	26,831	37.0	86	38	8
<i>O. foliosa</i> var. <i>indica</i>	MN990440	158,599	86,109	17,616	27,437	36.9	82	38	8
<i>O. foliosa</i>	MN990441	158,496	86,061	18,361	27,037	36.9	82	38	8

rpl22 ranged from 94 bp to 364 bp in LSC region. For the most species, the *rpl22* locates in the LSC region near the LSC/IRb border, and it is separated from the LSC/IRb border by a spacer varying from 17 to 68 bp. Notably, the LSC/IRb border of *D. singchiana* is *rps3* gene. The SSC/IRa border is crossed by the gene *ycf1* in all plastomes, and the fragment located in IRa region ranged from 61 to 1565 bp. Correspondingly, the gene *ycf1* in the junction region between SSC and IRb is treated as a pseudogene because of the incomplete duplication of the normal copy. However, for SSC/IRb border, gene *ndhF* located in SSC has a 71 bp extension to the IRb of four plastomes (*O. angustata*, *O. patens*, *Ch. amoena*, *Cr. appendiculata*). Similarly, the *rpl22* in the junction region between LSC and IRb is annotated as a pseudogene because of the incomplete duplication. The *psbA* gene locates in the LSC region, 70–147 bp from the LSC/IRa border. These results showed the obvious expansion of the IR region in *C. sinensis* plastome.

The result of the plastome structure comparison showed that there is one small inversion in *Corallorhiza sinensis* but none in other members of *Corallorhiza* and *Oreorchis* (Fig. S4). The inversion occurred in the LSC region between *trnE-UUC* and *psbD* genes, and was approximately 700 bp in length. Gene *trnT-GGU* was contained in the inversion region. We also detected an obvious inversion in plastome of *C. maculata* var. *maculata* and *C. maculata*. Furthermore, two inversions were detected in *D. singchiana*.

3.4. Phylogenetic analysis

Phylogenetic result of CDSs dataset showed *Corallorhiza sinensis* is sister to the clade comprised of *C. striata* var. *vreelandii* + *C. striata* var. *striata* + *C. cf. striata* + *C. bentleyi* + *C. striata* var. *involuta* with strong support in both Maximum likelihood and Bayesian Inference (ML: BS = 100; BI: PP = 1.00) (Fig. 5). In the plastid tree, *Corallorhiza* did not form

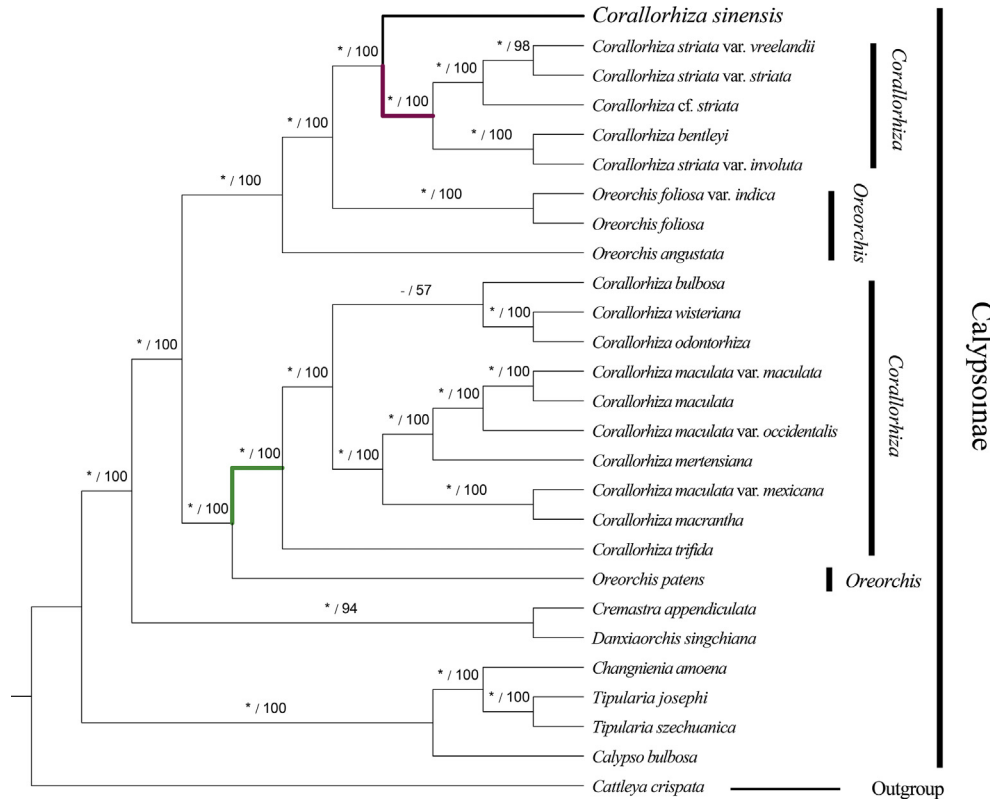


Fig. 5. Phylogenetic tree reconstruction of Calypsoinae using the maximum likelihood (ML) method based on 79 coding sequences. Only the ML tree is shown, because its topology is nearly identical to that of the obtained BI tree. Clade support values in the tree are posterior probabilities/bootstraps in percent from Bayesian Inference/Maximum likelihood, “**” indicates that the node is 1.00 supported. “-” indicates that the node is incongruent between the topology of the ML tree and the Bayesian tree. Red and green clade indicates mentum absent and present, respectively.

a monophyletic clade within Calypsoinae. Both ML and BI phylogenetic trees strongly indicated that the *Oreorchis* is nested within *Corallorhiza* (ML: BS = 100; BI: PP = 1.00). However, phylogenetic analysis based on nrDNA sequence suggest that *Corallorhiza* is a monophyletic group, and strongly support *C. sinensis* as sister to the rest species of *Corallorhiza* (ML: BS = 98; BI: PP = 1.00) (Fig. 6).

4. Discussion

The study is the first time to report new species of myco-heterotrophic orchid from western Sichuan: *Corallorhiza sinensis*. Previous studies indicated the members of *Oreorchis* possess coralloid rhizomes (Pearce and Cribb, 1997; Suetsugu et al., 2020), while the new species exhibits more abundant coralloid rhizomes than *Oreorchis*. *C. sinensis* present many synapomorphies that involved in *Corallorhiza*, such as leafless, no visible green tissue in all individuals. Despite the populations are characteristic of the intermediate morphological traits between *Corallorhiza* and *Oreorchis*, it most closely resembles the *Corallorhiza*. The complete plastome of *C. sinensis* was sequenced, assembled, and analyzed in comparison with these members of subtribe Calypsoinae. The plastome of *C. sinensis* shows a typical quadripartite structure, as reported for most angiosperms (Khayati et al., 2020; Wu et al., 2020; Zhu et al., 2020). A total of 109 unique genes were identified in the plastome, which contains 68 protein-coding genes, 30 tRNAs, and four rRNAs. *C. sinensis* further shows evidence of some photosynthesis-related genes either lost or pseudogenized, which is consistent with some previous studies of *Corallorhiza*

(Barrett and Davis, 2012; Kim et al., 2020; Li et al., 2020). In the current study, a common feature of the plastome of *Corallorhiza* and *Oreorchis* species was that IR regions were more conserved than the LSC and SSC regions. *C. sinensis* is closely related to *Corallorhiza* in terms of the length of whole plastome. Comparative analysis of plastomes showed that *C. sinensis* has one small inversion occurred in the LSC region. However, the larger inversions appear in *Danxiaorchis singchiana* (Lee et al., 2020), *C. maculata* var. *maculata* (Barrett et al., 2014) and *C. maculata* (Kim et al., 2020), which had been reported. IR boundaries of *C. sinensis* present obvious expansion in these members of Calypsoinae.

Phylogenetic analysis based on coding sequences (CDS) from 26 Calypsoinae members strongly supports *Corallorhiza sinensis* to be sister to the clade consisting of *C. striata* var. *vreelandii* + *C. striata* var. *striata* + *C. cf. striata* + *C. bentleyi* + *C. striata* var. *involuta*. All members of the sister clade of *C. sinensis* are distributed in North America (Freudenstein, 1997, 1999). *C. sinensis* is extremely similar to these members in sepals and petals morphology, but can be easily distinguished by its lip broad oblong and obviously 3-lobed toward base. In the clade consisting of *C. sinensis*, *C. striata* complex, and *C. bentleyi*, their mentum is absent. According to a monograph of *Corallorhiza* (Freudenstein, 1997), *Corallorhiza* was divided into two clades by the mentum absent or present. In current phylogenetic trees, red and green clade indicates mentum absent and present, respectively. The monograph indicated that the mentum is one of the characters to distinguish *C. striata* and other congeneric species (Freudenstein, 1997), thus the character presents a possible synapomorphy for the clade. Our phylogenetic

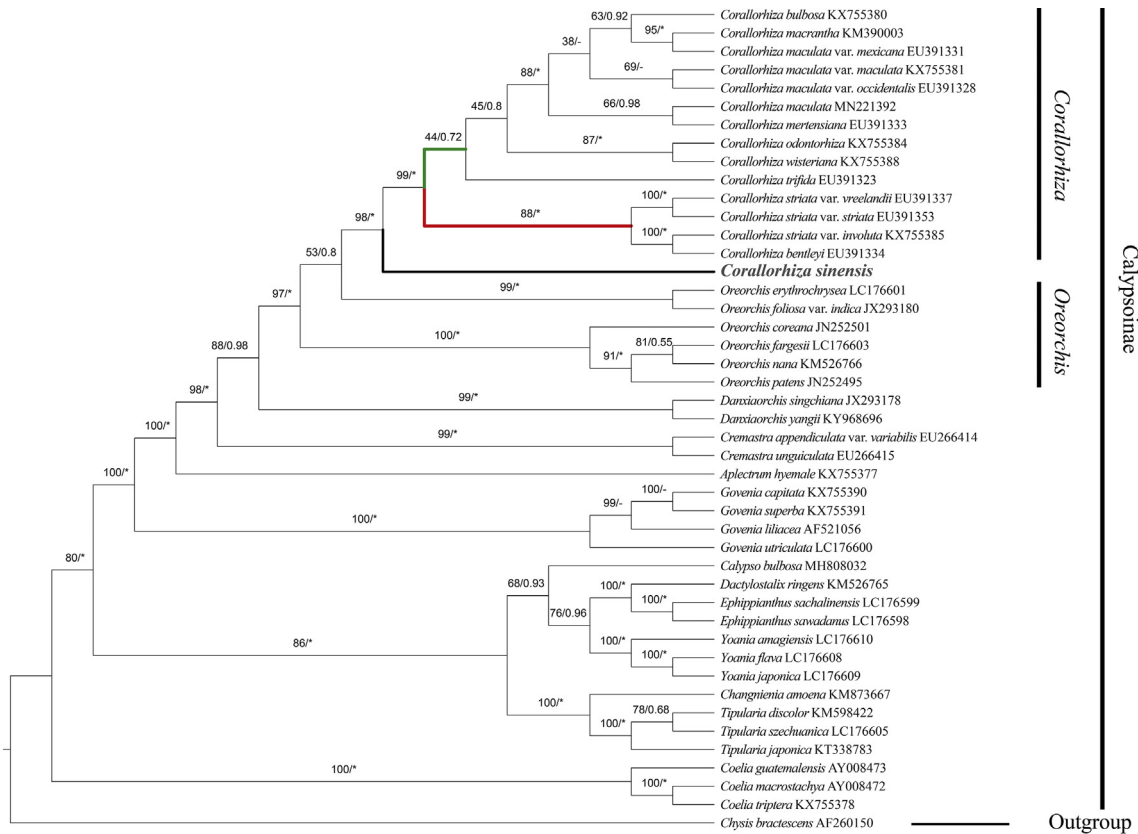


Fig. 6. Phylogenetic tree reconstruction of Calypsoinae using the maximum likelihood (ML) method based on nrDNA sequences. Only the ML tree is shown, because its topology is nearly identical to that of the obtained BI tree. Numbers associated with the branches are ML bootstrap value (BS) and BI posterior probabilities (PP). “*” indicates that the node is 1.00 supported. “-” indicates that the node is incongruent between the topology of the ML tree and the Bayesian tree. Red and green clade indicates mentum absence and presence, respectively. The species name is followed by accession number of the nrDNA sequences.

result is consistent with the previous studies (Kim et al., 2020; Li et al., 2020), that *Corallorhiza* is not a monophyletic group, because the genus *Oreorchis* is nested within the clade of *Corallorhiza* with strong bootstrap support (BS = 100) and posterior probability (PP = 1.00). In the Calypsoinae, however, phylogenetic analysis based on the nrDNA sequence suggests that *Corallorhiza* is a monophyletic group and strongly supports *C. sinensis* as sister to *Corallorhiza* (ML: BS = 98; BI: PP = 1.00). Although the topological conflicts are presented between plastome and nrDNA phylogenies of *C. sinensis*, it is still the most closely related to *Corallorhiza*. Both the plastid tree and nrDNA tree indicate *Oreorchis* is paraphyletic. The finding of this new species from China shed new light on the phylogenetic relationship of *Oreorchis* and *Corallorhiza*. Finally, we treat it here as a new species of *Corallorhiza* based upon morphological and molecular evidence.

Author Contributions

JXY, SP, JJW and HY collected these materials; GWH and QFW identified species; SX D, YW, JT, and HY designed and performed the experiments; JXY, SP, and JJW performed the analyses; JT drew the illustrations of the species; JY and SP wrote the manuscript; GWH and QFW revised the paper. All authors read and approved the final manuscript.

Declaration of competing interest

The author declares no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.pld.2021.01.002>.

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