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DNA barcoding and phylogenetic relationships in *Omphalogramma* (Primulaceae) from the Hengduan Mountain region of China

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Abstract

Omphalogramma is a small genus with about 13 species and rather isolated distribution on high mountain range of the Himalayas. As the large degrees of similarity in morphological characters between species, it is difficult to distinguish closely related species in the genus. In this study, we used one nuclear ITS and other three chloroplast DNA markers (*rbc*L, *mat*K, and *trn*H-*psb*A) to evaluate 39 accessions of *Omphalogramma* (Primulaceae) from the Hengduan Mountains, representing seven species. Our results showed that the discrimination power of the markers at the species level was 14.3% (*rbc*L), 28.4% (*trn*H-*psb*A), 42.9% (*mat*K), and 100% (ITS). The mean value of genetic distance between species for ITS was more than 3-fold that of intraspecific distance; all species were distinguished. Species were differentiated by unique characters, and phylogenetic analysis showed that the *Omphalogramma* species formed two clear monophyletic clades comprising *O. vinciflorum* (bootstrapping: 96.8%) and remaining *Omphalogramma* species (bootstrapping: 97.5%). This study indicated that DNA barcoding is a useful technique in the phylogenetic and taxonomic description of *Omphalogramma* species.

Keywords: *Omphalogramma*, DNA barcoding, ITS, phylogenetic relationships

Introduction

The *Omphalogramma* Franchet (1898: 178) is a small genus in the Primulaceae (Fletcher, 1949; Pax and Knuth, 1905) comprising 13 species that are restricted to north-east Burma (Hu and Kelso, 1996; Pax and Knuth, 1905; Richards, 1993, 2003), south-east Tibet, and north-west Yunnan in China (Fletcher, 1949); the genus contrasts with the extraordinarily species-rich *Primula* Linnaeus (1753: 142) (de Vos *et al.*, 2014; Hu and Kelso, 1996). Although solitary ebracteate flowers, flat-winged seeds, and involuted leaves of *Omphalogramma* are distinct from those of *Primula* spp., the *Omphalogramma* was originally classified as a subgenus, and then a section of *Primula* by Franchet, who described the first species *O. delavayi* Franchet (1898: 179), before it was recognized as a distinct genus in 1898 (Fletcher, 1949). *Omphalogramma* continued to be accepted as a genus in the Primulaceae until 1928, when there was a conference on *Primula* (The Fouth Primula Conference, 1929, The Royal Horticultural Society).

Omphalogramma is an entirely Asiatic group, with its center of diversity in the Hengduan Mountain region of China (Fletcher, 1949), containing nine species have been recorded from China and mainly grow in alpine meadows and forest margins at altitudes of 2200–4700 m, of which seven species are endemic (Hu and Kelso, 1996). The classification of *Omphalogramma* has historically focused on morphological characters, of which the key diagnostic features are thick woody rhizomes, leaves that are round or cordate at the base, campanulate or infundibuliform corolla, corolla tube length, and glabrous or glandular pilose style and filaments. However, large degrees of overlapping and similarity in these characters among species lead to taxonomic confusion and uncertainty of taxonomic status of several species, such as the complex groups of *O. elegans* Forrest (1923: 55), *O. forrestii* Balfour (1920: 13), *O. minus* Handel-Mazzetti (1922: 248), *O. souliei* Franchet (1898: 180) and *O. tibeticum* Fletcher (1949: 153). However, due to the limited geographic distribution and small populations, the field surveys of collections of these species are rather difficult, which results in that a comprehensive collection of *Omphalogramma* plant material to validate the species

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identity of the small genus has been almost impossible (Huang *et al.*, 2006; Huang *et al.*, 2009). Furthermore, there is a lack of integrative phylogenetic analyses to provide the relationships among the species in *Omphalogramma*, because of limited plant materials, even in herbarium specimens (Anderberg *et al.*, 1998; Conti *et al.*, 2000; Martins *et al.*, 2003; Mast *et al.*, 2006; Mast *et al.*, 2001; Richards, 1993, 2003; Zhang and Kadereit, 2002). Moreover, for the specimens without flowers, the species identification is extremely difficult. Considering its rarity and small population size, therefore complementary methods of identification and classification are urgently required for this genus to provide elementary species diversity information for conservation practice.

DNA barcoding is a technique used for taxonomic identification that relies on sufficient variation in sequences to recognize species and correctly identify individuals (Hollingsworth *et al.*, 2009; Hollingsworth, 2011; Kress and Erickson, 2007; Kress *et al.*, 2005). In plants, the nuclear ribosomal internal transcribed spacer region (nrITS) and several plastid regions, including *rbc*L, *mat*K, and *trn*H-*psb*A, have been proposed as potential candidates for DNA barcoding (Li *et al.*, 2011). Here, we used standard molecular barcodes, based on the three main criteria (universality, sequence quality, and discriminatory power) of three cpDNA regions (*rbc*L, *mat*K, *trn*H-*psb*A) and nuclear marker ITS to evaluate barcode universality and discriminatory power in *Omphalogramma* species and reconstruct phylogenetic relationships within the genus.

Materials and Methods

Plant material

The 39 plant materials of genus *Omphalogramma* representing seven species were collected from 19 sites, primarily located in the east Himalaya-Hengduan Mountains (HDM) in China (Table 1). We used *Primula agleniana* Balfour & Forrest (1920: 3)*, P. beesiana* Forrest (1911: 242) and *P. blinii* Léveillé(1915: 2) as outgroups. All samples of DNA and voucher specimens have been deposited at the Germplasm Bank of Wild Species and the herbarium of the Kunming Institute of Botany (KUN), respectively.

TABLE 1. *Omphalogramma* taxon, voucher, collection information, and Genbank accession numbers.

Species	Voucher ID	Location	Co-ordinates		Altitude	Genbank accession number			
						*trn*H-*psb*A	*rbc*L	*mat*K	ITS
O. delavayi	HY200501D1	Yunnan, Dali, Changshan	N25º40’37.48”	E100º05’33.8”	3750m	JN045603	JF942663	JF954757	JF977177
	HY200501D2					JN045602	JF942662	JF954756	JF977176
	HY200501D3					JN045601	JF942661	JF954755	JF977175
	HY200501D4					JN045600	JF942660	JF954754	JF977174
	HY200501D5					JN045599	JF942659	JF954753	JF977173
O. vinciflorum	HY20030610V1	Yunnan, Shangri-la, Daxue Mountain	N28º37’19.1”	E99º49’29.8”	3790m	JF942690	JF954784	JN045630	JF977204
	HY20030605V2	Yunnan, Shangri-la, Qianhu Mountain	N27º24’33.8”	E99º47’37.7”	3800m	JF942694	JF954788	JN045634	JF977208
	HY20030607V3	Yunnan, Shangri-la, Tianbao Mountain	N27º36’29.1”	E99º52’48.8”	3830m	JF942692	JF954786	JN045632	JF977206
	HY004V4	Yunnan, Shangri-la, Hong Mountain	N26º53’26”	E99º36’36”	3877m	JF942697	JF954791	JN045637	JF977211
	Hao560V5	Sichuan, Muli,916 forest	N27º56’32.41”	E101º11’17.32”	3000m	JF942689	JF954783	JN045629	JF977203
	HY20030601V6	Yunnan, Lijiang, Leidazhan	N27º14’02.3”	E99º24’34.3”	3200m	JF942695	JF954789	JN045635	JF977209

...Continued on next page

TABLE 1. (Continued)

Species	Voucher ID	Location	Co-ordinates		Altitude	Genbank accession number			
						*trn*H-*psb*A	*rbc*L	*mat*K	ITS
O. vinciflorum	HY20030606V7	Yunnan, Shangli-la, Bitahai	N27º46'51.0"	E99º55'00.3"	3480m	JF942693	JF954787	JN045633	JF977207
	Hao561V8	Sichuan, Muli,912 forest	N27º56'32.41"	E101º11'17.32"	3200m	JF942688	JF954782	JN045628	JF977202
	HY20030609V9	Yunnan, Shangli-la, Bilacuo	N28º32'19.7"	E99º56'32.3"	4370m	JF942691	JF954785	JN045631	JF977205
	HY005V10	Yunnan, Lijiang, Yulong Snow Mountain	N26º54'10.51"	E99º37'07.62"	3500m	JF942696	JF954790	JN045636	JF977210
O. souliei	HY20030602S1	Yunnan, Lijiang, Fenshuiling	N27º13'25.9"	E99º24'36.5"	3450m	JF942683	JF954777	JN045623	JF977197
	HY20030602S2					JF942682	JF954776	JN045622	JF977196
	HY20030612S3	Yunnan, Lijiang, Mandaying	N27º14'02.3"	E99º24'34.3"	3480m	JF942680	JF954774	JN045620	JF977194
	HY20030612S4					JF942679	JF954773	JN045619	JF977193
	HY20030603S5	Yunnan, Lijiang, Fenshuiling	N27º13'46.9"	E99º24'10.0"	3370m	JF942681	JF954775	JN045621	JF977195
O. forrestii	HY20030608F1	Yunnan, Shangri-la, Tianchi	N27º37'14.3"	E99º38'10.5"	3900m	JF942673	JF954767	JN045613	JF977187
	HY20030608F2					JF942672	JF954766	JN045612	JF977186
	HY20030608F3					JF942671	JF954765	JN045611	JF977185
	HY20030611F4	Yunnan, Shangri-la, Qianhu Mountain	N27º23'51.4"	E99º46'40.3"	3940m	JF942670	JF954764	JN045610	JF977184
	HY20030611F5					JF942669	JF954763	JN045609	JF977183
O. elegance	Huang101E1	Yunnan, Gaoligongshan, Dulongjiangyakou	N27º47.061'	E98º27.636'	3400m	JF942668	JF954762	JN045608	JF977182
	Huang101E2					JF942667	JF954761	JN045607	JF977181
	Huang101E3	Yunnan, Gaoligongshan, Dulongjiang	N27º47.061'	E98º27.636'	3480m	JF942666	JF954760	JN045606	JF977180
	Huang103E4					JF942665	JF954759	JN045605	JF977179
	Huang103E5					JF942664	JF954758	JN045604	JF977178
O. minus	Huang001M1	Yunnan, Weixi, Biluo Snow Mountain	N27º15'14"	E99º27'12"	3620m	JF942678	JF954772	JN045618	JF977192
	Huang001M2					JF942677	JF954771	JN045617	JF977191
	Huang001M3					JF942676	JF954770	JN045616	JF977190
	Huang001M4					JF942675	JF954769	JN045615	JF977189
	Huang001M5					JF942674	JF954768	JN045614	JF977188
O. tibeticum	Huang006T1	Tibet, Motuo	N29°45'42"	E95°41'24"	3565m	JF942687	JF954781	JN045627	JF977201
	Huang006T2					JF942686	JF954780	JN045626	JF977200
	Huang006T3					JF942685	JF954779	JN045625	JF977199
	Huang006T4					JF942684	JF954778	JN045624	JF977198

DNA extraction, amplification and sequencing

Genomic DNA was isolated from silica gel-dried leaves using a modified cetyltrimethylammonium bromide (CTAB) protocol (Doyle, 1987). PCR amplifications of two coding plastid genes (*rbc*L, *mat*K), one intergenic plastid spacer (*trn*H-*psb*A), and the nuclear ribosomal internal transcribed spacer (ITS) were sequenced directly using BigDye Terminator Cycle Sequencing Ready Reaction Kit and an ABI 3730 DNA Sequencer (Applied Biosystems). The primer pair sequences were summarized in Appendix S1.

Data analysis

The raw sequences were edited using Geneious 9.0 and aligned using Clustal X software (Thompson *et al.*, 1997), and pair distances of all four DNA regions were calculated using MEGA7 and the Kimura two-parameter (K2P) distance model (Kumar *et al.*, 2016). Barcoding gaps between intraspecific and interspecific distances were calculated as the frequency distributions of K2P distances (0.5% categories), based on a histogram, using TaxonDNA version 1.7 (Meier *et al.*, 2006), and the distance method was used to assess barcode performance. TaxonDNA was used to identify species based on best match, best close match, and all species barcode, using K2P model. If both sequences were from the same species, the identification was deemed correct, whereas mismatched names were classed as incorrect; several, equally good best matches from different species were classified as ambiguous.

Maximum likelihood trees was estimated for concatenated alignments of plastid *rbcL, trn*H-*psb*A*, matK,* and nuclear ITS concatenated sequence data and ITS sequence data separately, using IQTREE 1.6.7 (Trifinopoulos *et al.*, 2016), where we used the best substitution model of HKY+F+R3 for concatenated sequence data and TIM2e+G4 for ITS sequence data respectively, based on a comparison of Bayesian information criterion (BIC) values calculated by IQTREE (Nguyen *et al.*, 2015). Support for the estimated topology was inferred with rapid bootstrapping and the Shimodaira-Hasegawa (SH)-approximate likelihood ratio test (aLRT) for 10,000 replicates (Hoang *et al.*, 2018). Clades with support values of Sh-aLRT ≥80% and ultrafast bootstrap ≥95% were considered as credible.

Results

Sequence characteristics and genetic divergence

All plastid and nuclear markers (rbcL, *trn*H-*psb*A, *mat*K, and ITS) were successfully amplified using single primer pairs across all individuals, where universality of primer and success of sequence amplification at each of the four region was 100%. In general, consensus sequences were directly assembled from reads of each DNA strand, the aligned length of the four DNA markers ranged from 246bp (*trn*H-*psb*A) to 701bp (ITS), and, with the exception of *rbc*L, markers possessed indels ranging from 1 to 9 bps (Table 2). The frequency of variable sites was lower in coding regions *rbc*L and *mat*K than in non-coding regions ITS and *trn*H-*psb*A; marker *rbc*L displayed the lowest interspecific divergence (0.2%), while *mat*K exhibited the lowest intraspecific divergence (0.1%). Greatest interspecific distance was in ITS (3.8%), followed by *trn*H-*psb*A (3.6%).

We found evidence of a barcoding gap, where intra-specific distances were greater and did not fully overlap with inter-specific distance. There were relatively clear barcoding gaps in two barcodes (ITS and *mat*K) and three combinations (ITS+*mat*K, ITS+*rbc*L, and *mat*K+*rbc*L), whereas overlaps between intra- and inter-specific distances for all other markers and their combinations indicated absence of clear barcoding gaps (Fig. 1).

Discrimination ability of candidate barcodes

The distance threshold of each barcode varied from 0.44% to 6.57% (*rbc*L and *trn*H-*psb*A, respectively) (Fig. 2), and discrimination success for candidate barcodes varied from 14.3% to 100% (*rbc*L and ITS, respectively) using best match. ITS showed the best species discrimination (100%) among all the candidates based on best match and best close match (Fig. 3), and discrimination power at the species level for *mat*K, *trn*H-*psb*A, and rbcL was 42.9%, 28.6%, and 14.3%, respectively.

Relationships within Omphalogramma

With the exception of *O. souliei*, which shared a robust clade with *O. minus*, *Omphalogramma* species formed robust monophyletic clades, where there were two main clades that comprised *O. vinciflorum* (Franchet 1887: 574) Franchet (1898: 180) and the remaining species (*O. delavayi, O. souliei, O. minus, O. forrestii, O. tibeticum,* and *O. elegans*) (Fig. 3). *O. delavayi* was shown to be a sister to the other five species, and *O. elegans* and *O. tibeticum* formed monophyletic clades as sisters to a clade comprising *O. forrestii*, and *O. souliei* and *O. minus* (Fig. 4).

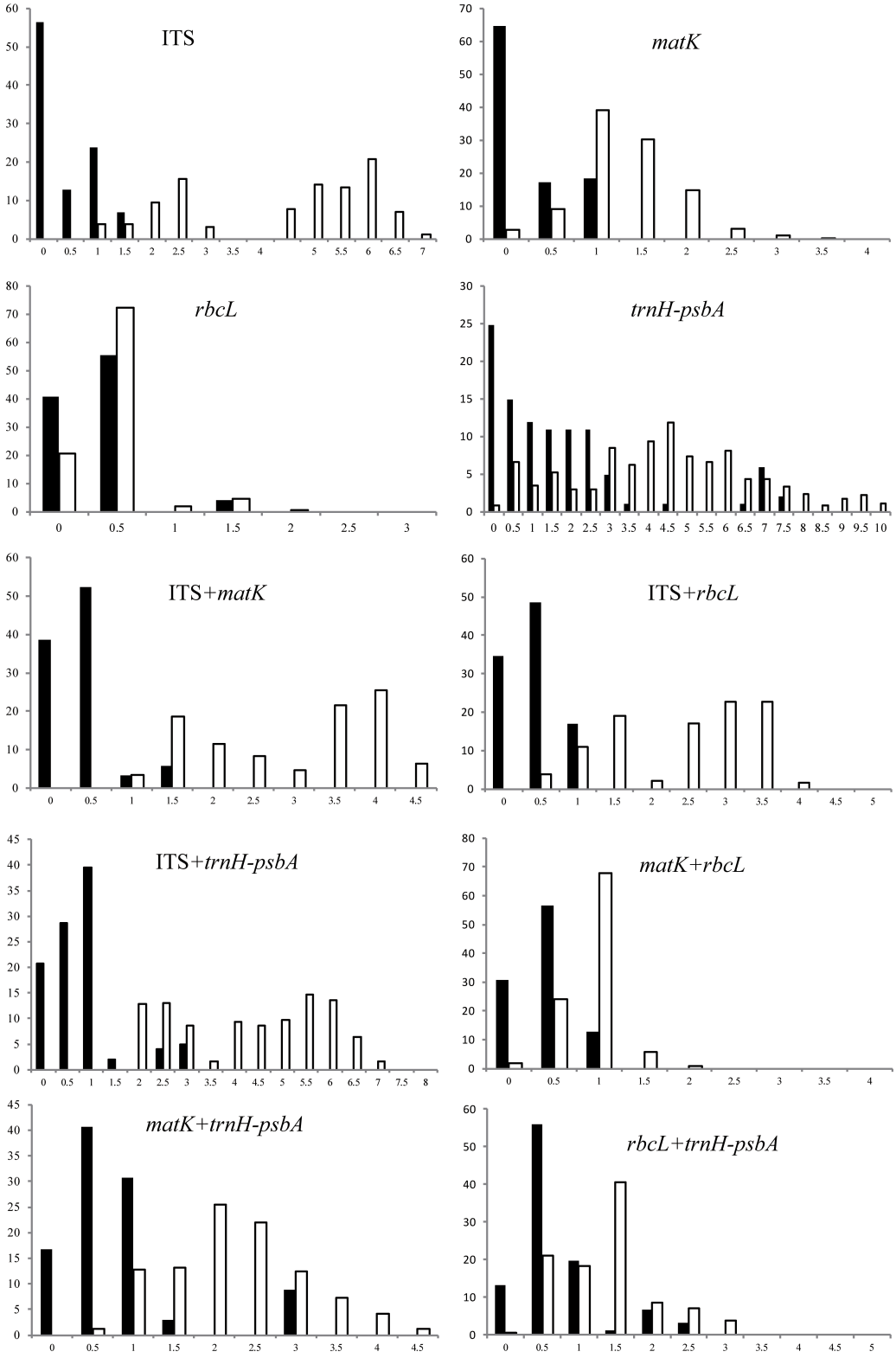

FIGURE 1. Frequency distribution of intra- (Black) and inter-specific (white) Kimura 2-parameter (K2P) distances among *Omphalogramma* samples for individual and combined barcode loci.

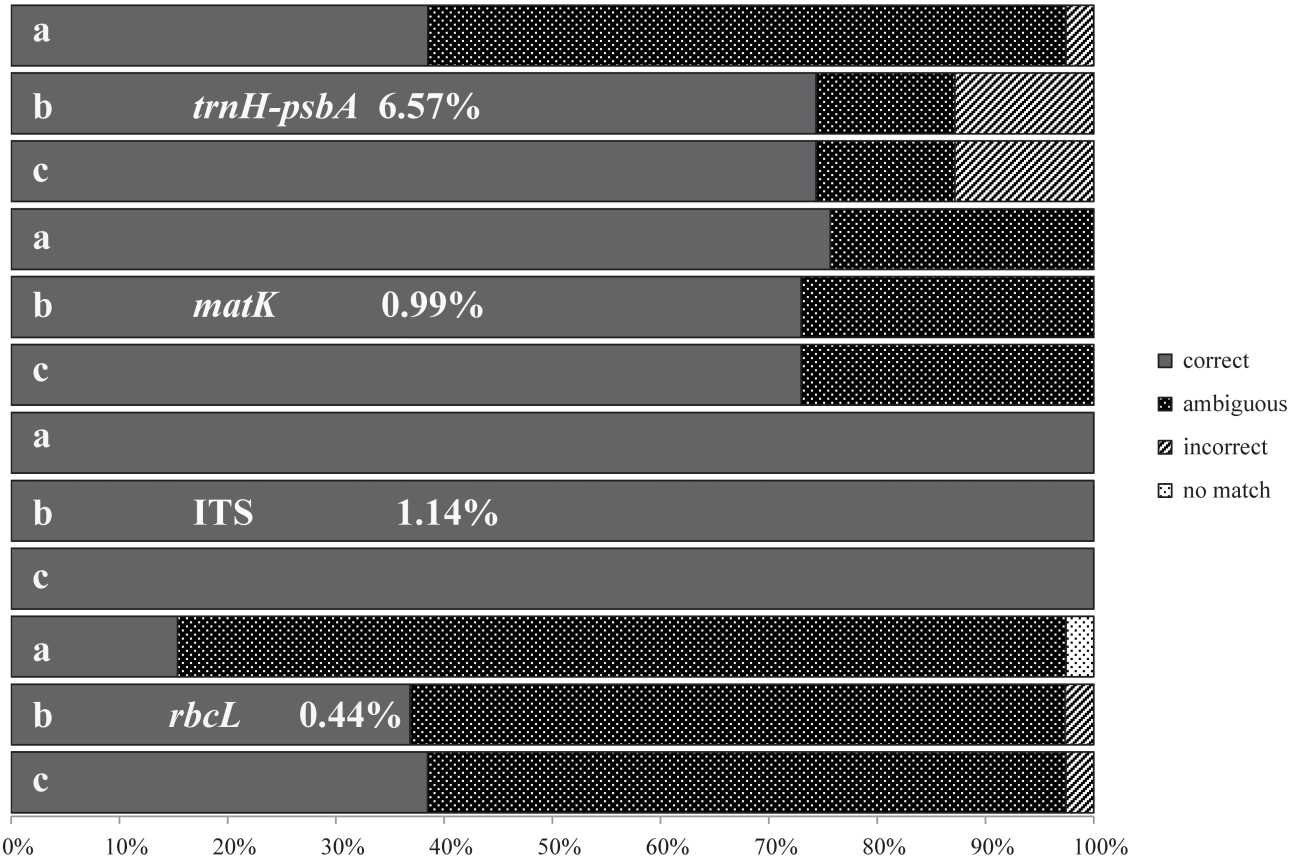

FIGURE 2. Performance of barcode candidates based on best match (a), best close match (b), and all species barcodes (c).

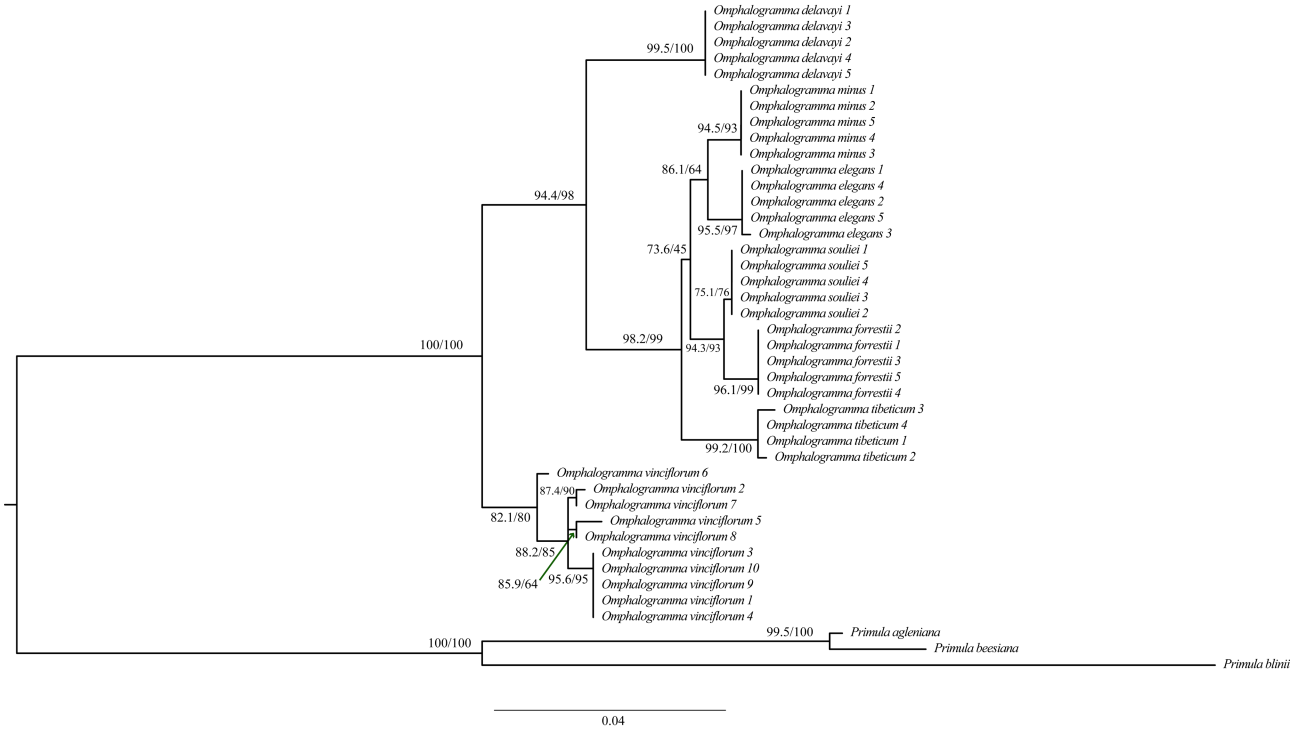

FIGURE 3. Species resolution success at the species level for ITS. Maximum likelihood phylogenetic tree based on nuclear ITS sequence data. Support values (SH-aLRT ≥80%; ultrafast bootstrap ≥95%) are SH-aLRT support (%) / ultrafast bootstrap support (%).

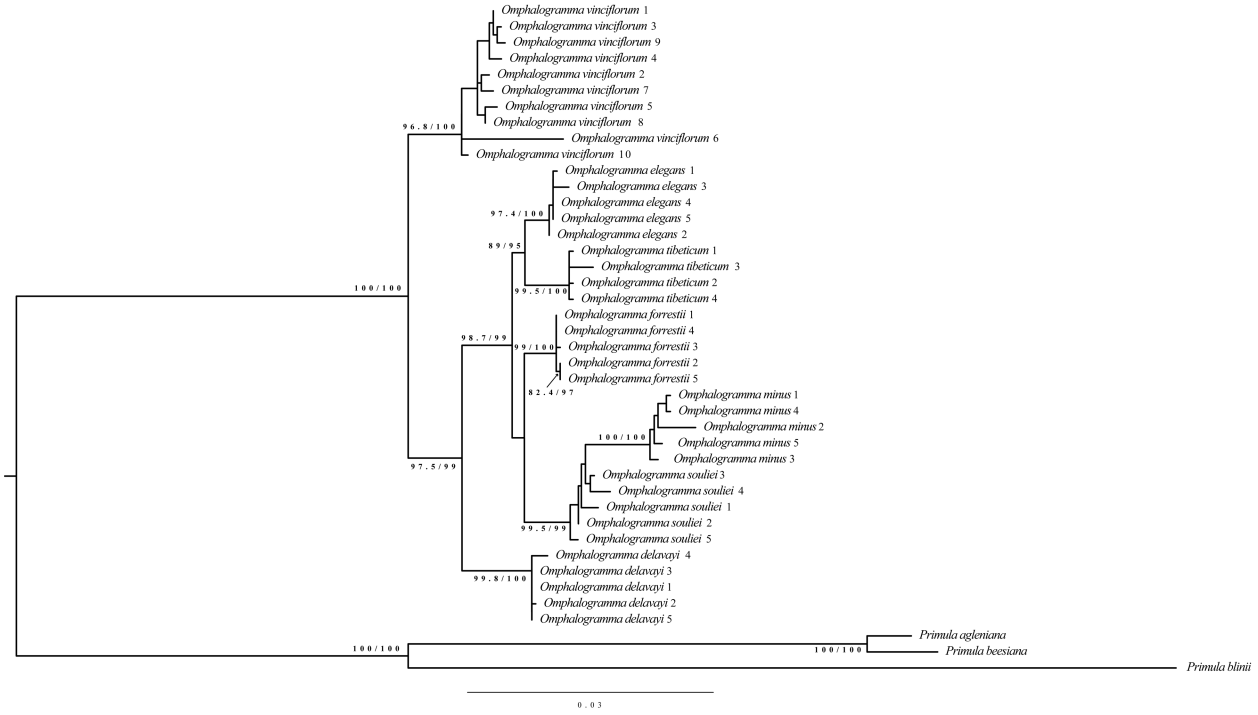

FIGURE 4. Maximum likelihood phylogenetic tree based on plastid *rbc*L, *trn*H-*psb*A, *mat*K, and nuclear ITS concatenated sequence data. Support values (SH-aLRT ≥80%; ultrafast bootstrap ≥95%) are SH-aLRT support (%) / ultrafast bootstrap support (%).

TABLE 2. Evaluation of the four DNA markers.

DNA region	rbcL	*mat*K	ITS	*trn*H-*psb*A
Universal ability to primer	100	100	100	100
Percent PCR success	100	100	100	100
Percent sequencing success	100	100	100	100
Aligned sequence length (bp)	667	521	701	246
Indel length (bp)		1(9)	5(1–3)	4(2–9)
No. of variable / parsimony informative sites	14/5	21/13	38/27	75/68
Ratios of variable/parsimony informative site (%)	2.1/0.7	4.0/2.5	15.4/11.0	10.7/9.7
No. of species (individuals)	7(39)	7(39)	7(39)	7(39)
Interspecific distance mean (range), (%)	0.2(0–0.6)	1.0(0.2–1.4)	3.8(0.5–7.7)	3.6(0.7–6.2)
Intraspecific distance mean (range), (%)	1.16(0–2.49)	0.1(0–0.31)	1.15(0–2.17)	0.12(0–0.57)
Ability to discriminate (%)	14.3 (1/7)	42.9 (3/7)	100 (7/7)	28.6 (2/7)

Discussion

Evaluation of DNA barcodes

Primer universality is the most important criterion for a meaningful DNA barcode (Kress and Erickson, 2007). In this study, all the four barcodes for *Omphalogramma* were successfully amplified and sequenced from 100% of individuals, indicating that these four regions are probably suitable as universal barcodes. However, we found that the *rbc*L+*mat*K combination provided lowest discrimination, whereas ITS performed best and showed the highest level of species discrimination, consistent with previous studies (Chen *et al.*, 2010; Liu *et al.*, 2017; Yan *et al.*, 2011a; Yan *et al.*, 2015; Yu *et al.*, 2011). Although ITS has occasionally been criticized as an unsuitable marker, because of its incomplete concerted evolution, our results indicated this may not be problematic in the *Omphalogramma*. Generally, species discrimination on the basis of pairwise distance is likely to be successful, if the inter-specific distances are

greater than intra-specific distances [8]; here, we found the greatest intra- and inter-specific sequence divergences based on distance analysis were for ITS. Assignment to best match, best close match, and all species barcodes showed highest species discrimination rate for ITS, followed by *mat*K. In general, combined DNA barcodes improve species identification. For example, combinations of ITS and plastids loci are most appropriate for some plant genera (Liu *et al.*, 2017; Yan *et al.*, 2011b; Yan *et al.*, 2015; Yu *et al.*, 2011). Our results demonstrated that highest discrimination power achieved when combining ITS and *mat*K among the four barcode loci, which is also consistent to the previous studies in other genera in the Primulaceae (Yan *et al.*, 2011b; Yan *et al.*, 2015). Thus, we suggested that single barcode ITS or combined barcode markers ITS+*mat*K are likely to be most suitable and efficient DNA barcoding markers for species identification in the *Omphalogramma*.

Implications of phylogenetic relationships

Our phylogenetic analysis revealed that *Omphalogramma* is a robust monophyletic clade, and supported that the terminal and solitary flowers is a distinguishing synapomorphism of this genus (Fig. 4). The relationship of *O. vinciflorum* as a sister to the rest species is consistent with its morphological traits: *O. vinciflorum* is unique in this genus with its lack of a thick woody rhizome that is typical of the other species. Throughout its range in Yunnan and Sichuan (China), *O. vinciflorum* is subject to much variation, chiefly in the size and shape of the leaves and corolla-lobes. The remaining species were divided into two clades: all accessions of *O. delavayi* were clustered to a single clade, characterized by a round or cordate leaf base and glandular-hairy style, while *O. souliei* was clustered with the *O. minus* clade, indicating that these two species are closely related. The close relationship between *O. souliei and O. minus* is consistent with their similar morphological features. However, the larger flowers and wider distribution area of *O. souliei* than that of *O. minus* in HDM indicates that *O. minus* probably diverged from *O. souliei*. The similarity between the flowers of *O. souliei* and *O. forrestii* (stigma longer than anthers, possibly extending beyond the corolla aperture) led to the classification of *O. forrestii* as a subspecies of *O. souliei*. However, the glabrous filaments of the stamens in *O. forrestii* are distinct from the glandular filaments in *O. souliei*, which suggested that it is a distinct species and the name should be retained. However, other morphoanatomical evidences should be supplemented in the future to clarify the taxonomy status on *O. forrestii.*

Accessions of *O. tibeticum* and *O. elegans* clustered to a monophyletic clade. *O. tibeticum* is believed to be endemic to south-eastern Tibet, while the distribution of *O. elegans* is much wider and overlaps with that of *O. tibeticum*. These two species are closely related and are distinguished only by small differences in leaf and petal shape.

In conclusion, our study robustly resolved the species phylogenetic relationship of the genus *Omphalogramma*, and inferred ITS or ITS-*mat*K combination can achieved highest species discrimination ability, which are suitable DNA barcoding markers for resolving the species phylogeny and efficiently aid the species identification.

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APPENDIX S1. Table. PCR primers used in the present study.

Locus	Forward primer	Reverse primer
*rbc*L	ATGTCACCACAAACAGAGACTAAAGC	TCGCATGTACCTGCAGTAGC
*mat*K	CGTACAGTACTTTTGTGTTTACGA G	ACCCAGTCCATCTGGAAATCTTGGTTC
ITS	GGAAGTAAAAGTCGTAACAAGG	TCCTCCGCTTATTGATATGC
*trn*H–*psb*A	CGCGCATGGTGGATTCACAATCC	GTTATGCATGAACGTAATGCTC