Evaluation of the standard DNA barcodes in *Adenophora* (Campanulaceae) and its phylogenetic analysis based on ITS sequences

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**Abstract:** *Adenophora* (Campanulaceae) is a perennial herbs and diverse genus, which occurs mainly in eastern Asia, particularly in China. Due to the complex morphological variation and overlapping geographic distribution, the delimitation and systematic arrangement of the species within this genus are still on debate. In this study, we assessed the species discriminating power of four standard DNA barcodes (*rbcl*, *matK* and *trnH-psbA* from chloroplast genome and ITS from nuclear genome) on the basis of 30 accessions of nine *Adenophora* species obtained mainly from China. The results showed that all four barcodes can be easily amplified and sequenced with the currently established primers, but we did not find any distinct barcoding gaps in the distributions of any marker, and none of the single or combined markers achieved high species discrimination (11.1% – 44.4%), indicating the effectiveness of the standard DNA barcodes for species identification in *Adenophora* was very limited. Meanwhile, a phylogenetic analysis was performed for about 70% of the representatives of *Adenophora* based on ITS sequences. None of the two sections of *Adenophora* were monophyletic, and the topologies obtained do not suggest a new division for the genus.


**Key words:** DNA barcoding; *Adenophora*; Molecular phylogeny; ITS; Species identification

**1. Introduction**

*Adenophora* Fischer (1823: 165), a genus of perennial herbs, is mainly distributed in East Asia, south to India and Vietnam, with one species extending into Europe and another endemic to Crimea (Fedorov, 1957; Baranov, 1963; Hong, 1983; Lee and Lee, 1994; Tu et al., 1998, Wu et al., 2003, Hong et al., 2011). Almost all species occur on sheltered screen in the mountain zone, and usually grow at edges of thickets and forests, mainly in conifer forest, grasslands, grassy slopes, and scrub among debris (Hong, 1983; Fu and Liu, 1986; Wu et al., 2003, Hong et al., 2011). With more than one-half of the total recognized species and 23 endemics, China was considered as the differentiation center of *Adenophora* (Hong et al., 2011). In *Flora Reipublicae Popularis Sinicae* (Hong, 1983), *Adenophora* was subdivided into two sections, i.e., sect. *Microdiscus* Fedorov (1957: 348) including species with corolla usually funnelform and style shorter than corolla or a little elongation, while sect. *Adenophora* comprising species with corolla tubular and style obvious elongation. However, the delimitation and systematic arrangement of the species within *Adenophora* are still debate mainly due to high variation in morphology, habitats, phenotypic plasticity, and potential hybridization (Baranov, 1963; Qiu and Hong, 1993; Ge and Hong, 1995; Hong and Ge, 2010). A large number of specific and infraspecific taxa were described mainly based on a single morphological character (Hong, 1983; Fu and Liu, 1986; Qiu and Hong, 1993; Ge and Hong, 1995; Qian, 1998; Tu et al., 1998; Zhao, 2002; 2004). Many species and varietal names published after 1983 have been reduced to synonymy (e.g., Le and Le, 1994; Tu et al., 1998; Hong and Ge, 2010; Wang et al., 2012).

The complexity of the taxonomy and the high morphological variation of this genus make species identification difficult, especially for those widely distributed species, such as *A. stricta* Miquel (1866: 192), *A. polydentata* Nakai (1909: 188), and *A. capillaries* Hemsley in Forbes and Hemsley (1889: 10).

DNA barcoding aims to provide a rapid, accurate and automated method to identify all recognized species, and to help flag possible new species by using one or a combination of several DNA regions (Hebert et al., 2003; Savolainen et al., 2005; Hollingsworth, 2011). A two-marker combination of *rbcl* + *matK* was recommended as core barcode for land plants by the Consortium for the DNA Barcode of Life (CBOL) Plant Working Group (2009). Subsequently, the nuclear ribosomal internal transcribed spacer (ITS) and plastid *trnH-psbA* region were proposed to incorporate into core barcode for seed plants.
(Hollingsworth et al., 2011; China Plant BOL Group, 2011). In the present study, we try to test the effectiveness of the four standard barcodes for Adenophora species identification. And then, we reconstruct phylogenetic relationships of the genus based on ITS sequences to test the taxonomic system based on morphological characters (Hong, 1983).

2. Material and Methods

2.1 Plant materials

For evaluating the effectiveness of the four standard barcodes (rbcL, matK, psba-trnH and ITS) in Adenophora, a total of 30 samples representing nine species were collected in this study. At least two individuals were sampled from different populations for each species, and more individuals were collected for those widespread species in order to cover their geographic variation and genetic diversity (Table 1). In phylogenetic reconstruction, 30 Adenophora taxa representing about 70% of the total species in Adenophora were included in an ITS dataset (Table 2). Most taxa represented by only one ITS sequence except A. lilifoliioides Pax and K. Hoffmann in Pax (1922: 499). The sister relationship between Adenophora and Campanula Linnaeus (1753: 163) was supported in most previous studies, such as morphological (e.g. Fedorov, 1957; Baranov, 1963; Hong, 1983; Yoo and Lee, 1996), and molecular analyses (Ge et al., 1997; Kim et al., 1999; Eddie et al., 2003). Thus, C. repunculoides Linnaeus (1753: 165) was selected as the outgroup species. All corresponding voucher specimens were deposited in the Herbarium of the Kunming Institute of Botany, Chinese Academy of Sciences (KUN). Nomenclature followed Hong (1983) and Hong et al. (2011).

2.2 DNA extraction, PCR amplification and sequencing

Total DNA was extracted from silica-gel dried leaf materials using the CTAB procedure (Doyle and Doyle, 1987). Polymerase chain reaction (PCR) amplifications were performed in a 20 μL reaction mixture containing 1 × Taq buffer [50 mM (NH4) 2SO4; 75 mM Tris-HCl (pH 8.3); 50 mM KCl; 0.001% gelatin]; 2.5 mM MgCl2, 0.4 mM of dNTPs, 0.5 μM of each primer, 1.0 U of Taq DNA Polymerase (TaKaRa Biotechnology Co. Ltd., Dalian, China), and 1 μL of genomic DNA (25-30 ng). The primer information and thermocycling conditions for the four markers used in this study are listed in Table 3. Purified PCR products were sequenced in both directions with the PCR primers on an ABI 3730 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). The newly acquired DNA sequences have been deposited in GenBank and their accession numbers were provided in Table 1.

2.3 Data analysis

Sequences of each region were aligned with Clustal X v.2.0. (Larkin et al., 2007) and then manually adjusted in BioEdit Sequence Alignment Editor v.7.0.0 (Hall, 1999). The genetic pairwise distance for each marker was calculated using MEGA 4 (Tamura et al., 2007) with the Kimura 2-parameter (K2P) distance model. Additionally, inter- and intra-specific genetic divergences of the four candidate DNA regions were analyzed by Wilcoxon signed-rank tests (Meyer and Paulay, 2005). To evaluate whether individual sample of a species clustered into species-specific monophyletic clade, neighbour-joining (NJ) trees were constructed based on single marker and all possible combinations of the four candidate markers in MEGA 4, with pairwise deletion and K2P distance model. Bootstrap values (BP) were calculated over 5,000 replications.

2.4 Phylogenetic analyses

Phylogenetic analyses were carried out by using the maximum parsimony (MP) and Bayesian inference (BI) methods based on an ITS dataset with 16 newly obtained sequences and 15 downloaded from the GenBank. MP analysis was performed using PAUP v.4.0 b10 (Swofford, 2002) with all characters unordered and equally weighted. Heuristic searches were implemented with 100 random addition sequence replicates, tree bisection reconnection (TBR) branch swapping and MulTrees in effect, and steepest descent off. Bootstrap support values (BS) were estimated using a heuristic search strategy with 500 bootstrap replicates and 1000 random sequences additions.

BI analysis was executed using MrBayes version 3.2.2 (Ronquist et al., 2012). The best substitution types (Nst) and rate distribution models (rates) was determined by the Akaike information criterion (AIC) using Model Test v.3.7 (Posada and Crandall, 1998) with the hierarchical likelihood ratio tests. Four chains (one cold, three heated) of the Markov chain Monte Carlo (MCMC) were run, sampling one tree every 100 generation from the cold chain. We stopped the MCMC after 1,000,000 generations because the value of average standard deviation was below 0.01, suggesting that the tree samples from the two simultaneous runs became increasingly similar. For the calculation of the Bayesian posterior probabilities (PP), the burn-in period was the first 25% of the sampled generations as determined by the program Tracer v.1.6 (Rambaut et al., 2014). The 50% majority-rule consensus tree for the PP was generated by PAUP* v.4.0b10 (Swofford, 2002).

3. Results

3.1 Variation among sequences

All Adenophora samples were successfully amplified and sequenced using universal primer pair
for the four DNA regions, respectively (Table 3). A total number of 120 sequences were obtained from the nine sampled *Adenophora* species. The variability of the four DNA markers for all examined samples was summarized in Table 4.

**Table 1.** Samples of *Parnassia* and outgroup (*Campanula*) included in the present barcoding study, with voucher information and GenBank accession numbers

<table>
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<th>Taxon</th>
<th>Origin</th>
<th>Voucher</th>
<th>rbcL</th>
<th>matK</th>
<th>trnH-psbA</th>
<th>ITS</th>
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Table 2. Voucher information and GenBank accession numbers of species used in phylogenetic analyses based on nrDNA ITS dataset

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Table 3. Primers and reaction condition used in this study

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ITS showed the highest interspecific sequence distance (4.94%), followed by trnH-psbA (2.89%) and matK (1.55%). rbcL had the lowest interspecific (0.75%) and intraspecific (0.05%) distance divergence. ITS has the highest intraspecific sequence distance (0.28%), followed by trnH-psbA (0.23%). In addition, ITS and trnH-psbA showed much more intensive and dense variable sites than rbcL and matK. The rbcL region was highly conserved with dispersive and sparse variable sites without indel in Adenophora (Table 4).

3.2 Assessment of barcoding gap

We estimated interspecific and intraspecific genetic divergence of the four DNA regions based on Wilcoxon signed-rank test. ITS exhibited the highest divergence and rbcL showed the lowest divergence at the interspecific level (Table 5). At the intraspecific level, the lowest divergence was provided by rbcL, while there were no significant differences in intraspecific sequence divergence between rbcL and matK (Table 5). We did not find any distinct barcoding gap in the distributions of any marker, especially in the core barcode rbcL and matK (Figure 1). The results demonstrated that there was a larger range distribution of inter- and intra-specific distance of ITS and trnH-psbA than that of rbcL or matK (Table 5), indicating the higher sequence variation among individuals / species for ITS with higher species resolution.

3.3 Applicability for species discrimination

In the tree-based analysis, ITS provided the highest species discrimination (33.3%), followed by trnH-psbA (22.2%), matK (22.2%), and rbcL (11.1%). ITS combined with rbcL, matK, and rbcL+matK provided the same ability for species discrimination as ITS alone. A combination of ITS+trnH-psbA provided the highest species identification (44.4%) among all combinations (Figure 2). Individuals for four of the nine sampled Adenophora species formed monophyletic clade in the NJ tree, and most of the monophyletic species had high BP of over 85%. The samples of A. liliifolioides grouped into two different clades.

3.4 Molecular Phylogenetics of Adenophora

A phylogenetic analysis is provided for about 70% of the representatives of Adenophora based on ITS sequences. The MP tree revealed tree length with 183 steps, a consistency index (CI) of 0.902 excluding uninformative characters, and a retention index (RI) of 0.869. BI analysis yielded similar tree topologies with MP analysis (Figure 3 and Figure 4). None of the two sections of Adenophora (Hong, 1983) were monophyletic, and the topologies obtained do not suggest a new division of the genus.

<table>
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<th>Table 4. The comparisons of variability of the four DNA markers</th>
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<tr>
<td><strong>DNA region</strong></td>
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<td>Universality to primer</td>
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<td>Percentage PCR success</td>
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<td>Percentage sequencing success</td>
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<td>Aligned sequence length (bp)</td>
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<td>Indels (length, bp)</td>
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<td>No. information sites / variable sites</td>
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<td>Distribution of variable sites</td>
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<td>No. sampled species (individuals)</td>
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<td>Mean Interspecific distance (%)</td>
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<td>Mean Intraspesific distance (%)</td>
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Di, dispersive; S, sparse; I, intensive; D, dense.

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<th>Table 5. Wilcoxon signed rank tests of inter- and intra-specific divergence among four single loci</th>
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<td><strong>Markers</strong></td>
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<td>matK</td>
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<td>trnH-psbA</td>
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</table>
Figure 1. Relative distribution of interspecific and intraspecific distance for the four DNA markers of *Adenophora*. X-axes relate to K2P distances arranged in intervals, and the y-axes correspond to the percentage of occurrences.
Figure 2. Neighbour-joining tree based on the combination of the ITS+trnH-psbA sequences. Bootstrap values (>50%) are shown above the relevant branches.
Figure 3. The single most parsimonious tree of *Adenophora* based on ITS sequences. Bootstrap values (>50%) are shown above the relevant branches.

Figure 4. Majority rule consensus Bayesian phylogenetic tree of *Adenophora* based on ITS sequences. Bootstrap values (>50%) are shown above the relevant branches.
4. Discussion
4.1 Applicability for species discrimination

Species discriminatory power is an important criterion for a DNA barcode (Hebert et al., 2003; Kress et al., 2005; CBOL Plant Working Group, 2009). An ideal DNA barcode should provide high ability of species identification (Kress et al., 2005; Lahaye et al., 2008; China Plant BOL Group, 2011), and exhibit a ‘barcode gap’ between intraspecific divergence and interspecific divergence (Meyer and Paulay, 2005). ITS showed relatively well separated between intraspecific and interspecific divergence in *Adenophora* among the four DNA markers (Figure 1). There is no any distinct barcoding gap found in *rbcL*, however, based on the distribution of intra- versus inter-specific sequence divergence.

Using DNA sequences for species discrimination rests in part on the assumption that species are monophyletic with respect to barcode haplotypes (Hebert et al., 2003). Tree-based method (NJ trees) were used to perform the species identification of *Adenophora*. The results showed that the discriminating power of the four standard markers at the species level was very low. For the single DNA barcode level, ITS region provided the highest species resolution (33.3%). High level of species discrimination of ITS was also reported in other groups (e.g. Li et al., 2011; Liu et al., 2012; Yang et al., 2012; Xu et al., 2015).

Combinations of different markers provided higher resolution than that of the single marker which showed a little contribution to increase the discrimination. Majority preference was to recommend a core-barcode of two coding genes, *rbcL* + *matK* (CBOL Plant Working Group, 2009). Species discrimination power for combinations of two-marker ranged from 22.2% to 44.4% with ITS + *trnH-psbA* providing the highest species discrimination power of 44.4%(Figure 2). Although combining the DNA barcodes may give much higher resolution in terms of species discrimination, the differentiation of closely related herbal materials using the standard DNA barcodes may be difficult (Li et al., 2011).

Though only 20% of the species within this genus was analyzed, the results suggested that species discrimination using the standard DNA barcodes was very difficult in *Adenophora*. Based on the present result, we concluded that using of these three chloroplast makers, i.e., *matK*, *rbcL*, and *trnH-psbA* solely, are not suitable for the candidate barcoding of this genus. In recent years, considerable efforts have been made for searching of suitable DNA barcodes for specific herbs. A possible way to solve the problem is to supplement DNA barcoding with the isolation of polymorphic DNA obtained from whole-genome fingerprint and to use this as sequence characterized amplified region DNA marker (e.g. Kuang et al., 2011; Ma et al., 2011; Nock et al., 2011; Wolf et al., 2011).

4.2 Phylogenetic relationships within *Adenophora*

Although classification and evolution of *Adenophora* has been suggested by different authors (e.g. Fedorov, 1957; Baranov, 1963; Hong, 1983; Tu et al., 1998; Wu et al., 2003), the conclusions of previous studies have lacked robust support due to limited sampling or a paucity of phylogenetic characters. Hong (1983) divided the genus *Adenophora* into two sections: *Microdiscus* and *Adenophora*, which was accepted by other authors (Fu and Liu, 1986; Lee et al., 1997; Wu et al., 2003; Hong and Ge, 2010). In our study, *Adenophora* was splits into five clades based on Bayesian and parsimony analysis, however, neither sect. *Microdiscus* nor sect. *Adenophora* were monophyletic (Figure 4). The topologies obtained inside *Adenophora* were poorly resolved and were inconsistent between sections, making it impossible to further analyze the internal relationships among species. So perhaps the inclusion of more markers will shed light on the relationships among species of *Adenophora*.

Tu et al., (1998) considered that the indumentum and leaf shape appear to be a continuous character in ontogenetic and historical view. Therefore, they divided *Adenophora* into four groups based on external morphology and pollen morphology. The evolutionary trends of the genus are suggested: the leaf has evolved from petiolate to sessile; the flower from large and campanulate to small and cylindric; the ornamentation of the pollen from reticulate to striate. However, all morphological treatments use the shape of the corolla and basal leaves as the fundamental characters, which was not supported by molecular studies (Kim et al., 1999). The ITS sequence phylogeny suggested that some morphological characters, such as the relative length of the style in comparison with the length of corolla, were homoplastic in the genus *Adenophora*. The diagnostic characters of sections (i.e. the form and the position of calyx lobes, the size and the form of the disc, and cauline leaves alternate or verticillate) were optimized in one of the trees from the nuclear analysis.

Acknowledgements

This study was funded by The National Natural Science Foundation of China (no. 31360046), the Large-scale Scientific Facilities of the Chinese Academy of Sciences (grant number: 2009-LSF-GBOWS-01) and the Science and Technology Project of the Education Bureau of Jiangxi (GJJ13785). The authors thank Drs. Zhang Shu-Dong, Ren Zong-Xin and Mr. Cai Jie for their help of field and lab work, and/or providing plant materials. We are grateful to Profs. Gao Lian-Ming, Jin Qiao-Jun and
Zhang Zhi-Yong for their helpful discussion and critical reading of the manuscript.

References

8/24/2016