# 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.

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**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 only 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. liliifolioides* 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. rapunculoides* 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 $MgCl_2$, 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

| Taxon | Origin | Voucher | *rbcL* | *matK* | *trnH -psbA* | ITS |
|---|---|---|---|---|---|---|
| *A. capillaris* | Shennongjia District, Hubei | LiJP0199 | KF175333 | KF175273 | KF175374 | KF175324 |
| | Qiaojia County, Yunnan | 08CS327 | KF175334 | KF175274 | KF175375 | KF175325 |
| *A. coelestis* | Shennongjia District, Hubei | LiJP0231 | KF175360 | KF175300 | KF175367 | KF175321 |
| | Shangri-La County, Yunnan | YangQ1997 | KF175361 | KF175301 | KF175368 | KF175322 |
| | Shangri-La County, Yunnan | YangQ2844 | KF175362 | KF175302 | KF175369 | KF175323 |
| *A. himalayana* | Daufu County, Sichuan | SCU10203 | KF175335 | KF175275 | KF175376 | KF175326 |
| | Dege County, Sichuan | 08CS799 | KF175336 | KF175276 | KF175377 | KF175327 |
| | Yushu County, Qinghai | XianH0326 | KF175337 | KF175277 | KF175378 | KF175328 |
| *A. khasiana* | Shangri-La County, Yunnan | LiJ622 | KF175349 | KF175289 | KF175383 | KF175306 |
| | Wuhua District, Yunnan | 08CS385 | KF175350 | KF175290 | KF175384 | KF175307 |
| | Shangri-La County, Yunnan | LiJ683 | KF175351 | KF175291 | KF175385 | KF175308 |
| *A. liliifolioides* | Jiangda County, Tibet | 08CS775 | KF175338 | KF175278 | KF175379 | KF175329 |
| | Ganzi County, Sichuan | 08CS825 | KF175339 | KF175279 | KF175380 | KF175330 |
| | Xiahe County, Gansu | LJQ-08GN346 | KF175340 | KF175280 | KF175381 | KF175331 |
| | Nang County, Tibet | L105 | KF175341 | KF175281 | KF175382 | KF175332 |
| | Changdu County, Tibet | YangYP-Q3207 | KF175342 | KF175282 | KF175392 | KF175303 |
| | Dingqen County, Tibet | YangYP-Q3115 | KF175343 | KF175283 | KF175390 | KF175304 |
| | Ganzi County, Sichuan | YangYP-Q3047 | KF175344 | KF175284 | KF175391 | KF175305 |
| *A. polyantha* | Xinpu,District, Jiangsu | HangYY8512 | KF175356 | KF175296 | KF175363 | KF175317 |
| | Taishan District, Shandong | ZhaoZT0203 | KF175357 | KF175297 | KF175364 | KF175318 |
| | Laoshang District, Shandong | LuoY427 | KF175358 | KF175298 | KF175365 | KF175319 |
| | Feidong County, Anhui | XuZD028 | KF175359 | KF175299 | KF175366 | KF175320 |
| *A. stenanthina* | Thungren County, Qinghai | ChenSL0903 | KF175345 | KF175285 | KF175386 | KF175309 |
| | Lintan County, Gansu | LJQ-08GN345 | KF175346 | KF175286 | KF175387 | KF175310 |
| | Chonghe County, Qinghai | ChenSL0489 | KF175347 | KF175287 | KF175388 | KF175311 |
| | Dare County, Qinghai | ChenSL0278 | KF175348 | KF175288 | KF175389 | KF175312 |
| *A. tetraphylla* | Laoshang District, Shandong | LuoY148 | KF175354 | KF175294 | KF175370 | KF175313 |
| | Helong District, Jilin | YangLM0212 | KF175355 | KF175295 | KF175372 | KF175314 |
| *A. trachelioides* | Laoshang District, Shandong | LuoY386 | KF175352 | KF175292 | KF175371 | KF175315 |
| | Zhifu District, Shandong | BianFH0122 | KF175353 | KF175293 | KF175373 | KF175316 |
| *C. rapunculoides* | Ontario, Canada | AP217 | HQ589981 | HQ593198 | HQ596619 | HQ823434 |

**Table 2.** Voucher information and GenBank accession numbers of species used in phylogenetic analyses based on nrDNA ITS dataset

| Taxon | Origin | Voucher | ITS |
|---|---|---|---|
| *A. capillaris* | Qiaojia County, Yunan, China | 08CS327 | KF175325 |
| *A. coelestis* | Shangri-La County, Yunnan, China | YangQ2844 | KF175323 |
| *A. coronpifolia* | Mt. Halla, South Korea | Yoo18845 | HQ704519 |
| *A. divaricata* | Mt. Daedeok, South Korea | Yoo73736 | HQ704520 |
| *A. erect* | Ullengdo, South Korea | Kim2005-3230 | HQ704554 |
| *A. grandiflora* | Mt. Daedeok, South Korea | Yoo54257 | HQ704522 |
| *A. himalayana* | Dege County, Sichuan, China | 08CS799 | KF175327 |
| *A. kayasanensis* | Mt. Gaya, South Korea | Yoo75385 | HQ704523 |
| *A. khasiana* | Wuhua District, Yunnan, China | 08CS385 | KF175307 |
| *A. lamarckii* | Mt. Samyeong, South Korea | Yoo53180 | HQ704553 |
| *A. liliifolia* | Mt. Seorak, South Korea | Yoo143 | HQ704551 |
| *A. liliifolioides* 1 | Jiangda County, Tibet, China | 08CS775 | KF175329 |
| *A. liliifolioides* 2 | Changdu County, Sichuan, China | YangYP-Q3115 | KF175304 |
| *A. lobophylla* | Jinchuan County, Sichuan, China | GS-301 | KF279691 |
| *A. morrisonensis* | Hualien Hsien, Taiwan, China | Hsiulan-Ho1214 | KF279686 |
| *A. paniculata* | Mt. Baihua, Beijing, China | GS-01 | KF279687 |
| *A. petiolata* | Longxian County, Shaanxi, China | GS-G9514 | KF279688 |
| *A. polyantha* | Laoshang District, Shandong, China | LuoY427 | KF175319 |
| *A. potaninii* | Danfeng county, Shaanxi, China | GS-G9518 | KF279690 |
| *A. racemosa* | Seonjaryeong, South Korea | Yoo75332 | HQ704545 |
| *A. remotiflora* | Mt. Botheon, South Korea | Yoo75355 | HQ704526 |
| *A. remotiflora* var. *hirticalyx* | Mt. Jiri, South Korea | Yoo19500 | HQ704528 |
| *A. stenanthina* | Dare County, Qinghai, China | ChenSL0278 | KF175312 |
| *A. stricta* | Podaebong, South Korea | Yoo61921 | HQ704529 |
| *A. taquetii* | Mt. Halla, South Korea | Yoo75384 | HQ704555 |
| *A. tetraphylla* | Laoshang District, Shandong, China | LuoY148 | KF175313 |
| *A. trachelioides* | Laoshang District, Shandong, China | LuoY386 | KF175315 |
| *A. triphylla* | Mt. Samak, South Korea | Yoo63275 | HQ704530 |
| *A. verticillata* var. *hirsuta* | Ullengdo, South Korea | KHB090825-262 | HQ704532 |
| *A. verticillata* | Seonjaryyeong, South Korea | Yoo75356 | HQ704531 |
| *A. wawreana* | Mt. Xiangshan, Beijing, China | GS-G01 | KF279689 |
| *C. rapunculoides* | Ontario, Canada | AP217 | HQ823434 |

**Table 3.** Primers and reaction condition used in this study

| DNA region | Primer pairs | Primer sequences (5'-3') | Thermocycling conditions |
|---|---|---|---|
| *rbcL* | *rbcL*a_f | ATGTCACCACAAACAGAGACTAAAGC | 95℃ 4min; [35cycles: 94℃ 50sec; 52℃ 1min; 72℃ 80sec]; 72℃ 10min |
| | 724R | TCGCATGTACCTGCAGTAGC | |
| *matK* | Xf | TAATTTACGATCAATTCATTC | 95℃ 4min; [35cycles: 94℃ 50sec; 52℃ 1min; 72℃ 80sec]; 72℃ 10min |
| | 5r | GTTCTAGCACAAGAAAGTCG | |
| *trnH-psbA* | *trnH* | ACTGCCTTGATCCACTTGGC | 95℃ 4min; [35cycles: 94℃ 30sec; 55℃ 45sec; 72℃ 1min]; 72℃ 10min |
| | *psbA* | CGAAGCTCCATCTACAAATGG | |
| ITS | ITS5 | GGAAGTAAAAGTCGTAACAAGG | 95℃ 4min; [35cycles: 94℃ 50sec; 55℃ 1min; 72℃ 80sec]; 72℃ 10min |
| | ITS4 | TCCTCCGCTTATTGATATGC | |

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 4.** The comparisons of variability of the four DNA markers

| **DNA region** | ***rbcL*** | ***matK*** | ***trnH-psbA*** | **ITS** |
|---|---|---|---|---|
| Universality to primer | Yes | Yes | Yes | Yes |
| Percentage PCR success | 100% | 100% | 100% | 100% |
| Percentage sequencing success | 100% | 100% | 95% | 100% |
| Aligned sequence length (bp) | 724 | 830 | 405 | 716 |
| Indels ( length, bp) | 0 | 0 | 9(1-41) | 18(1-2) |
| No. information sites/ variable sites | 12/22 | 27/74 | 21/46 | 38/89 |
| Distribution of variable sites | Di and S | Di and S | I and D | I and S |
| No. sampled species (individuals) | 9(30) | 9(30) | 9(30) | 9(30) |
| Mean Interspecific distance (%) | 0.75 | 1.55 | 2.89 | 4.94 |
| Mean intraspecific distance (%) | 0.05 | 0.14 | 0.23 | 0.28 |

Di, dispersive; S, sparse; I, intensive; D, dense.

**Table 5.** Wilcoxon signed rank tests of inter- and intra-specific divergence among four single loci

| **Markers** | | **inter-specific divergence** | | **intra-specific divergence** | |
|---|---|---|---|---|---|
| | | **P value** | **Result** | **P value** | **Result** |
| *rbcL* | *matK* | P ≤ 8.143×10-9 | *rbcL*< *matK* | P ≤ 0.138 | *rbcL* < *matK* |
| *rbcL* | *trnH-psbA* | P ≤ 5.179×10-9 | *rbcL* < *trnH-psbA* | P ≤ 0.068 | *rbcL* < *trnH-psbA* |
| *rbcL* | ITS | P ≤ 5.179×10-9 | *rbcL* < ITS | P ≤ 0.043 | *rbcL* < ITS |
| *matK* | *trnH-psbA* | P ≤ 7.760×10-9 | *matK* < *trnH-psbA* | P ≤ 0.345 | *matK* < *trnH-psbA* |
| *matK* | ITS | P ≤ 7.760×10-9 | *matK* < ITS | P ≤ 0.176 | *matK* < ITS |
| *trnH-psbA* | ITS | P ≤ 5.891×10-7 | *trnH-psbA* < ITS | P ≤ 0.612 | *trnH-psbA* = ITS |

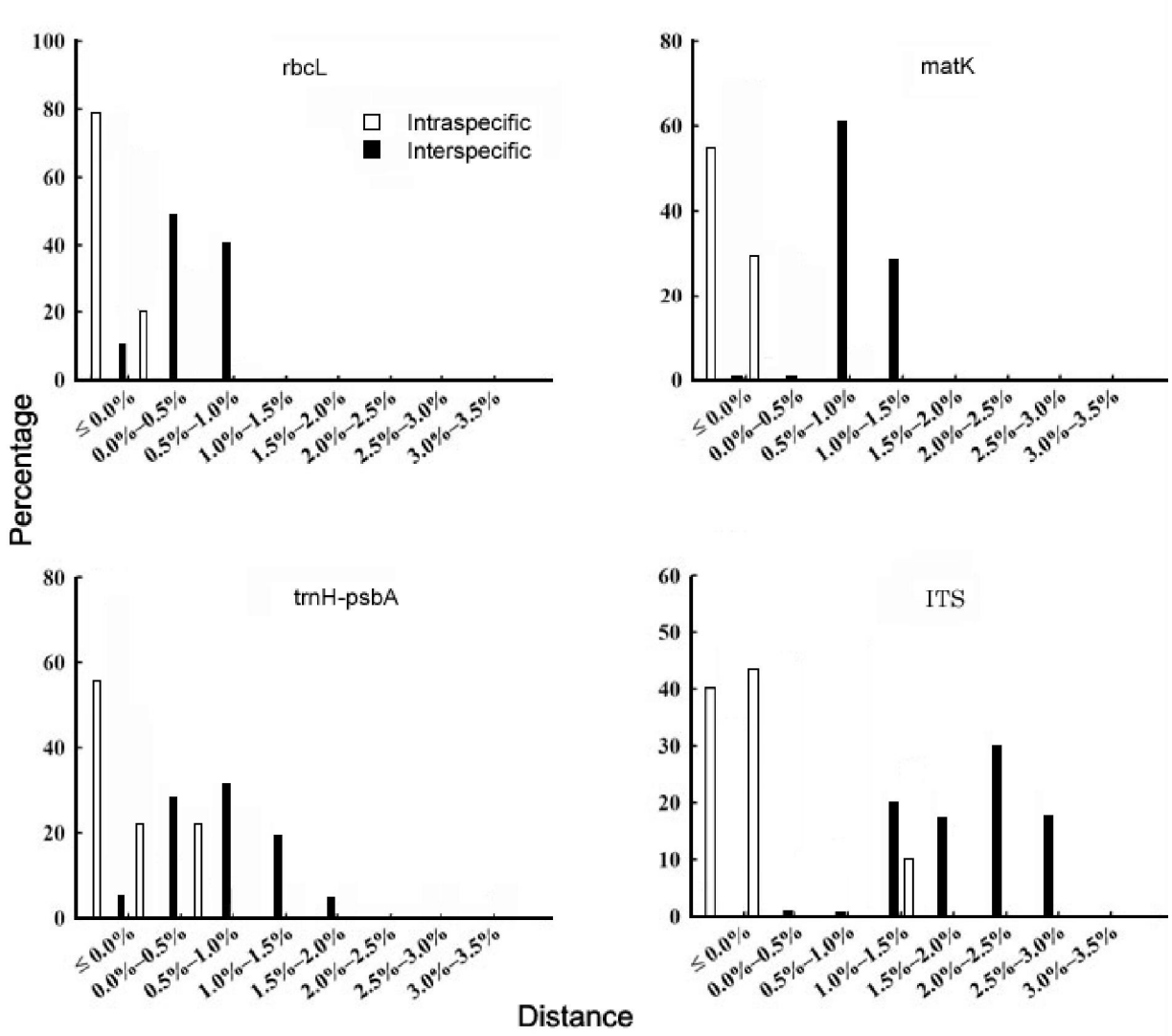


**Figure 1.** Relative distribution of interspecific and intraspecific distance for the four DNA markers of *Adenophora.* x-axes relate to K2P distances arranged in intervals, and the y-axes correspond to the percentage of occurrences

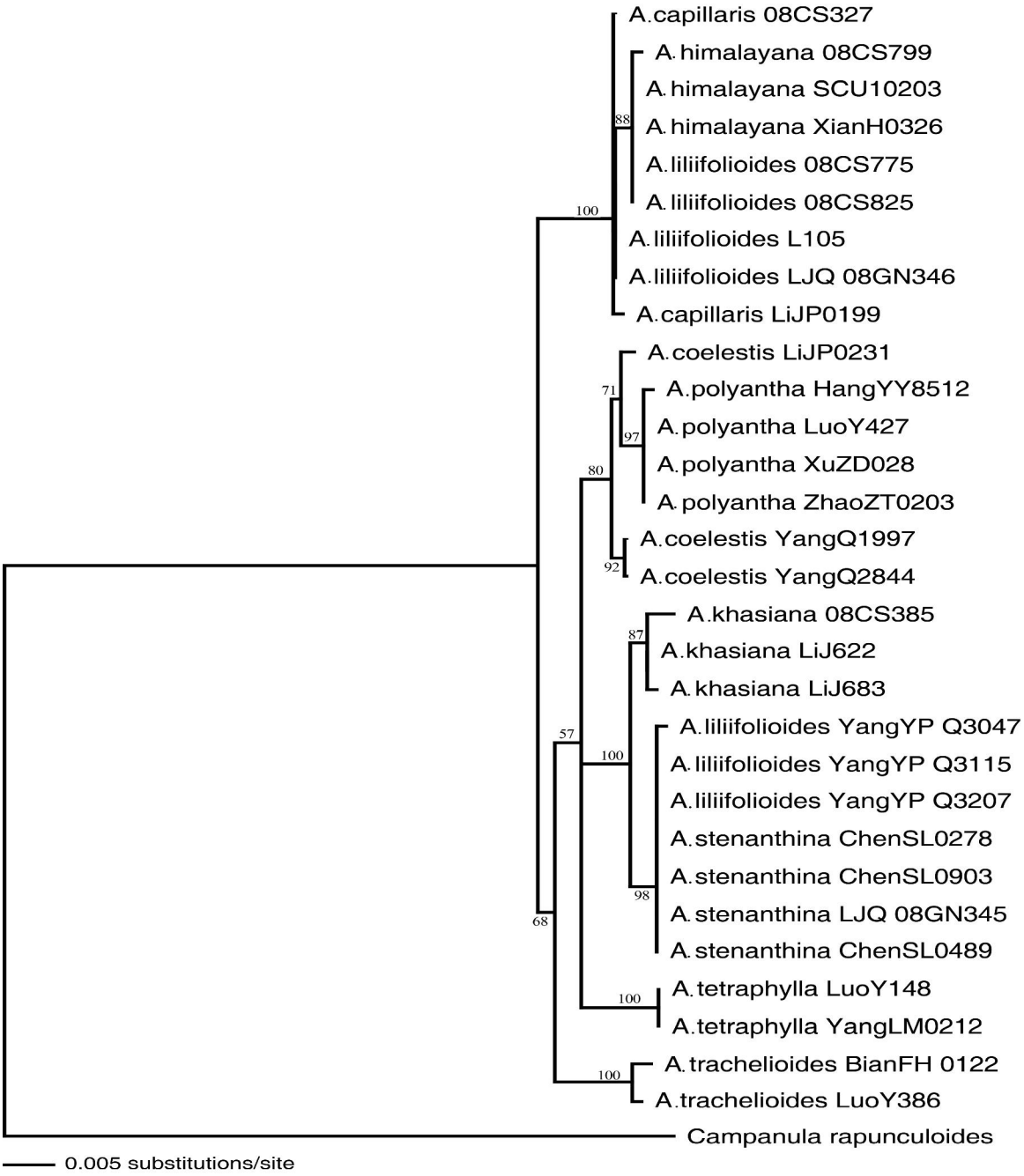


**Figure 2.** Neighbour-joining tree based on the combination of the ITS+*trnH-psbA* sequences. Bootstrap values (>50%) are shown above the relevant branches

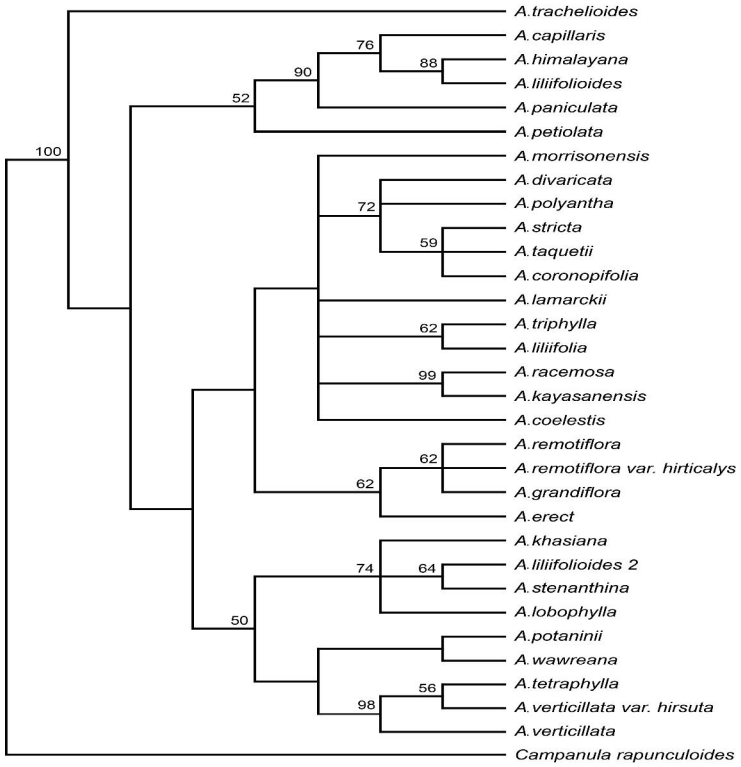


**Figure 3.** The single most parsimonious tree of *Adenophora* based on ITS sequences. Bootstrap values (>50%) are shown above the relevant branches

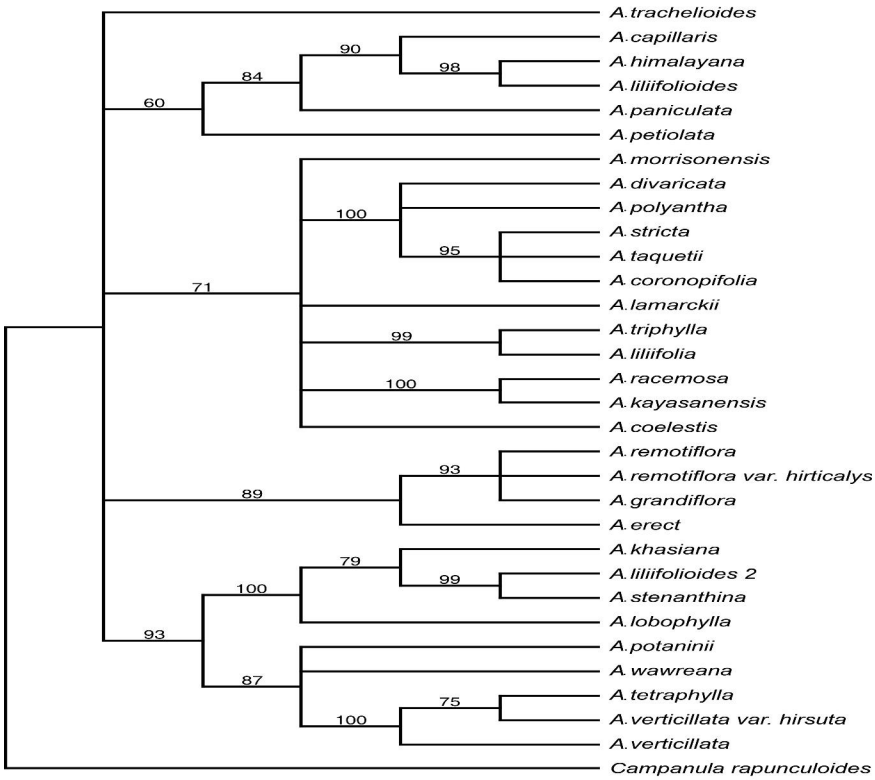


**Figure 4.** Majority rule consensus Bayesian phylogenetic tree of *Adenophora* based on ITS sequences. Bootstrap values (>50%) are shown above the relevant branches

## 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.

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**References**


1. Barranov AI, 1963. Materials to the monograph of the species of *Adenophora* of N. E. China. *Quarterly Journal of the Taiwan Museum* 16: 143-179.
2. CBOL Plant Working Group, 2009. A DNA barcode for land plants. *Proceedings of the National Academy of Sciences of the United States of America* 106: 12794-12797.
3. China Plant BOL Group, 2011. Comparative analysis of a large dataset indicates that ITS should be incorporated into the core barcode for seed plants. *Proceedings of the National Academy of Sciences of the United States of America* 108: 19641-19646.
4. Doyle JJ, Doyle JL, 1987. A rapid DNA isolation procedure for small quantities of fresh leaf material. *Phytochemical Bulletin* 19: 11-15.
5. Eddie WMM, Shulkina T, Gaskin J, Haberle RC, Jansen RK, 2003. Phylogeny of Campanulaceae s. str. inferred from ITS sequences of nuclear ribosomal DNA. *Annals of the Missouri Botanical Garden* 90: 554-575.
6. Fedorov AA, 1957. *Adenophora. In*: Shishkin, B.K. & Bobrov, E.G. eds *Flora of the U. R. S. S.,* Vol. 24. Israel Program for Scientific Translations, Jerusalem, pp. 343-371.
7. Fischer FEL, 1823. Adumbratio generis *Adenophorae*. *Mémoires de la Société Impériale des Naturalistes de Moscou* 6: 165-169.
8. Forbes FB, Hemsley WB, 1889. Enumeration of all the plants known from China Proper, Formosa, Hainan, the Corea, the Luchu Archipelago, and the island of Hongkong: together with their distribution and synonymy continued Vol. 23. *Botanical Journal of the Linnean Society* 26: 1-592.
9. Fu CX, Liu HQ, 1986. A classification study on the genus *Adenophora* from Heilongjiang, China. *Journal of Harbin Normal University* 2: 41-52.
10. Ge S, Hong DY, 1995. Biosystematic studies on *Adenophora potaninii* Korsh. complex Campanulaceae, III. genetic variation and taxonomic value of morphological characters. *Acta Phytotaxonomica Sinica* 33: 433-443.
11. Ge S, Schaal BA, Hong DY, 1997. A reevaluation of the status of *Adenophora lobophylla* based on ITS sequence, with reference to the utility of its sequence in *Adenophora*. *Acta Phytotaxonomica Sinica* 35: 385-395.
12. Hall TA, 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows95 / 98 / NT. *Nucleic Acids Symposium Series* 41: 95-98.
13. Hebert PDN, Cywinska A, Ball SL, de Waard JR, 2003. Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London Series B: Biological Sciences* 270: 313-321.
14. Hollingsworth PM, 2011. Refining the DNA barcode for land plants. *Proceedings of the National Academy of Sciences of the United States of America* 108: 19451-19452.
15. Hollingsworth PM, Graham SW, Little DP, 2011. Choosing and using a plant DNA barcode. *PLoS ONE* 6: e19254.
16. Hong DY, 1983. *Adenophora*. *In:* Hong, D.Y. ed., *Flora Reipublicate Popularis Sinicae*, Vol. 732. Science Press, Beijing, pp. 92-139.
17. Hong DY, Ge S, 2010. Taxonomic notes on the genus *Adenophora* Campanulaceae in China. *Novon* 20: 426-428.
18. Hong DY, Ge S, Thomas GL, Laura LK, 2011. *Adenophora. In:* Wu, C.Y., Raven, P.H. & Hong, D.Y. eds. *Flora of China*, Vol. 19. Science Press, Beijing, pp. 536-551.
19. Kim YD, Lee J, Suh Y, Lee S, Kim SH, Jansen RK, 1999. Molecular evidence for the phylogenetic position of *Hanabusaya asiatica* Nakai Campanulaceae, an endemic species in Korea. *Journal of Plant Biology* 42: 168-173.
20. Kress WJ, Wurdack KJ, Zimmer EA, Weigt LA, Janzen DH, 2005. Use of DNA barcodes to identify flowering plants. *Proceedings of the National Academy of Sciences of the United States of America* 102: 8369-8374.
21. Kuang DY, Wu H, Wang YL, Gao LM, Zhang SZ, Lu L, 2011. Complete chloroplast genome sequence of *Magnolia kwangsiensis* Magnoliaceae: implication for DNA barcoding and population genetics. *Genome* 54: 663-673.
22. Lahaye R, van der Bank M, Bogarin D, Warner J, Pupulin F, Gigot G, Maurin O, Duthoit S, Barraclough TG, Savolainen V, 2008. DNA barcoding: the floras of biodiversity hotspots. *Proceeding of the National Academy of Science of the United States of America* 105: 2923-2928.
23. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG, 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* 23: 2947-2948.
24. Lee J, Lee S, 1994. A taxonomic study of the genus *Adenophora* in Korea. *Jounal National Science Sung Kyun Kwan University* 45: 15-34.
25. Li DZ, Liu JQ, Chen, ZD, Wang H, Ge XJ, Zhou SL, Gao LM, Fu CX, Chen SL, 2011. Plant DNA barcoding in China. *Journal of Systematics and*

*Evolution* 49: 165-168.
26. Liu J, Provan J, Gao LM, Li DZ, 2012. Sampling strategy and potential utility of indels for DNA barcoding of closely related plant species: a case study in *Taxus*. *International Journal of Molecular Sciences* 13: 8740-8751.
27. Ma PF, Guo ZH, Li DZ, 2011. Rapid sequencing of the bamboo mitochondrial genome using Illumina technology and parallel episodic evolution of organelle genomes in grasses. *PLoS ONE* 7: e30297.
28. Meyer CP, Paulay G, 2005. DNA barcoding: error rates based on comprehensive sampling. *PLoS Biology* 3: e422.
29. Miquel FAW, 1866. *Annales Musei Botanici Lugduno-Batavi,* Vol. 2. C.G. van der Post, Amsterdam, 313 pp.
30. Nakai T, 1909. Plantae novae Asiaticae. *Botanical Magazine Tokyo* 23: 185-192.
31. Nock CJ, Waters DLE, Edwards MA, Bowen SG, Rice N, Cordeiro GM, Henry RJ, 2011. Chloroplast genome sequences from total DNA for plant identification. *Plant Biotechnol Journal* 9: 328-333.
32. Posada D, Crandall KA, 1998. MODELTEST: Testing the model of DNA substitution. *Bioinformatics* 14: 817-818.
33. Qian YY, 1998. A new species of *Adenophora* from Yunnan. *Guihaia* 18: 9-10.
34. Qiu JZ, Hong DY, 1993. A biosystematic study on *Adenophora gmelinii* complex Campanulaceae. *Acta Phytotaxonomica Sinica* 31: 17-41.
35. Rambaut A, Suchard MA, Xie D, Drummond AJ, 2014. Tracer v1.6. Available from http://beast.bio.ed.ac.uk/Tracer.
36. Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP, 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic biology* 61: 539-542.
37. Savolainen V, Cowan RS, Vogler AP, Roderick GK, Lane R, 2005. Towards writing the encyclopaedia of life: an introduction to DNA barcoding. *Philosophical Transactions of the Royal Society of London, Series B: Biological Sciences* 360: 1805-1811.
38. Swofford DL, 2002. PAUP*: Phylogenetic analysis using parsimony * and Other Methods, version 4.0 b10. Sinauer Associates, Sunderland, MA.
39. Tamura K, Dudley J, Nei M, Kumar S, 2007. MEGA 4: Molecular Evolutionary Genetics Analysis MEGA software version 4.0. *Molecular Biology and Evolution* 24: 1596-1599.
40. Tu PF, Chen HB, Xu GJ, Xu LS, 1998. Classification and evolution of the genus *Adenophora* Fisch. in China. *Acta Botany Boreal* 18: 613-621.
41. Wang YP, Cai J, Zhang ZR, Wu D, 2012. Natural resources and a new synonym of the genus *Adenophora* Campanulaceae from Yunnan, China. *Acta Agirculturae Universitatis Jiangxiensis* 34: 200-202.
42. Wolf PG, Der JP, Duffy AM, Davidson JB, Grusz AL, Pryer KM, 2011. The evolution of chloroplast genes and genomes in ferns. *Plant Molecular Biology* 76: 251-261.
43. Wu ZY, Lu AM, Tang YC, Chen ZD, Li DZ, 2003 *The families and genera of angiosperms in China, a comprehensive analysis.* Science Press, Beijing, 1209 pp.
44. Xu SZ, Li DZ, Li JW, Xiang XG, Jin WT, Huang WC, Jin XH, Huang LQ, 2014. Evaluation of the DNA barcodes in *Dendrobium* Orchidaceae from mainland Asia. *PloS ONE* 10: e0115168.
45. Yang JP, Wang YP, Möller M, Gao LM, Wu D, 2012. Applying plant barcodes to identify species of *Parnassia* Parnassiaceae. *Molecular Ecology Resources* 12: 267-275.
46. Yoo KO, Lee WT, 1996. External morphology of Korea Campanulaceae. *Korean Journal of Plant Taxonomy* 26: 77-104.
47. Zhao YT, 2002. *Adenophora urceolata* Campanulaceae, a new species from Inner Mongolia, China. *Annales Botanici Fennici* 39: 335-336.
48. Zhao YT, 2004. *Adenophora biloba* Campanulaceae, a new species from Inner Mongolia, China. *Annales Botanici Fennici* 41: 381-382.

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