African Journal of Biotechnology Vol. 8 (20), pp. 5215-5224, 19 October, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Molecular phylogeny of Ranunculaceae based on internal transcribed spacer sequences

Ying-fan Cai[1#*], Sheng-wei Li[1,2#], Yi Liu[1], Sun Quan[1], Min Chen[2], Yong-fang Xie[1], Huai-zhong Jiang[1], En-zhao Wei[1], Neng-wen Yin[1], Ling Wang[1], Rui Zhang[1], Cheng-lin Huang[1], Xiao-Hong He[1] and Ming-feng Jiang[2]

[1]Chongqing University of Posts and Telecommunications, Chongqing 400065, China.
[2]Southwest University for Nationalities, Chengdu 610041, China.

Accepted 10 September, 2009

The botanical family Ranunculaceae contains important medicinal plants. To obtain new evolutionary evidence regarding the systematic classification of Ranunculaceae plants, we used molecular phylogenies to test relationships based on the internal transcribed spacer region. The results of phylogenetic analysis of 92 species of Ranunculaceae, Paeoniaceae and Berberidaceae not only supported the monophyly of each of these genera but also suggested a number of additional points: (1) All of the inferred genera were clearly rooted together and most supported previous classifications; (2) *Helleborus*, which has sometimes been placed in *Helleboroideae*, should be retained within *Ranunculoideae*; (3) *Adonis*, which has sometimes been placed in *Ranunculoideae*, should be in *Helleboroideae*; (4) *Callianthemum*, which currently belongs to *Ranunculoideae*, is separated by a large genetic distance from other genera in this family, suggesting that it should be removed from this family and (5) the current generic classification of Ranunculaceae should be revised.

Key words: Ranunculaceae, genetic relationship, ITS sequences, genealogy.

INTRODUCTION

Ranunculaceae is a family of flowering plants known as the “buttercup family,” and is composed of 59 genera and *ca*. 2,500 species (Tamura, 1993). Although Ranunculaceae species are distributed worldwide (Wang et al., 1979), the plants are most common in the temperate and cold areas of the northern hemisphere. In China Ranunculaceae plants mainly concentrated in southwest (Delecctis Florae Reipublicae Popularis Sinicae Agendae Academiae Sinicae Edita 1979). Numerically, the most important genera in this family are *Ranunculus* (600 species), *Delphinium* (365 species), *Thalictrum* (330 species), *Clematis* (325 species) and *Aconitum* (300 species). The family contains many ornamental flowering plants common to the Himalayas, many taxa within the family are pharmaceutically important and some have confirmed medicinal value. Ranunculaceae plants are used to cure jaundice and are used as herbal medicines in many countries because of their antibiotic and anti-inflammatory, heat-clearing and detoxification, anticancer, antiarrhythmia, immunomodulatory, depressurisation, analgesic and diuretic effects (Serkedjieva and Velcheva, 2003; Chen et al., 2002; Li et al., 2002; Wang et al., 2004). Plants belonging to the Ranunculaceae have complex chemical compositions, many of which represent important taxonomic characteristics and the same chemical constituents are shared among different genera (Peng et al., 2006a). This is a phylogenetically important family (Ro et al., 1997).

Researchers have used various taxonomic characters to determine the importance of the relationships between phylogeny, chemical composition and even pharmacological effects. However, circumscription of the Ranunculaceae, especially the membership of several genera, is controversial (Ro et al., 1997). Based on chromosomal

*Corresponding author. E-mail: caiyf3000@yahoo.com.cn. Tel: +92-023-62461884.

Abbreviations: ITS, Internal transcribed spacer; **IPTG,** isopropyl β-D-1-Thiogalactopyranoside; **X-Gal**, 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside.

#These authors contributed equally to this paper

and floral characteristics, Tamura recognised five subfamilies: Hydrastidoideae, Thalictroideae, Isopyroideae, Ranunculoideae and Helleboroideae (Tamura, 1993). The results of pharmaphylogenic research (Peng et al., 2006a) were in accordance with the phylogenetic system presented by Tamura and supported the establishment of Cimicifugoideae; chemical characteristics supported the separation of Isopyrum from Thalictroideae, constituting an independent subfamily namely Isopyroideae (Peng et al., 2006b). Yang (2001) and Lehnebach et al. (2007) performed cytological investigations, Jensen (1966, 1968) used serological approaches, Loconte and Estes (1989) and Hoot (1991) performed cladistic analyses of the Ranunculaceae and Ro and Bruce (1997) and Hörandl et al. (2005) performed molecular analyses of the *Aquilegia* and *Ranunculus* groups. Some of these results were roughly compatible with the current classification of the Ranunculaceae, but they were scattered and even inconsistent. Therefore, it is necessary to carefully reevaluate relations in light of independent phylogenetic estimates.

In recent years, a number of groups have used molecular systematics methods to reconstruct plant systematics and resolve systematic problems that are difficult to resolve by way of classical taxonomy. Ribosomal internal transcribed spacer (ITS) sequences (including ITS1 and ITS2) and 5.8S rRNA sequences have conserved lengths and a high degree of variability and are well suited for classification studies. Nuclear rDNA ITS sequences have been used in the analysis of angiosperms (Baldwin et al., 1992; Stanford et al., 2000) and in the analysis of the evolutionary rate for lower level phylogeny (genus level and below) as well as in advanced phylogenetic studies (Fior et al., 2006; Huang et al., 2005; Ding et al., 1996; Tian and Li, 2002).

Here we report the sequencing and analysis of ITS sequences of Ranunculaceae and related Paeoniaceae and Berberidaceae plants and discuss the significance of distinguishing plants at the molecular level. The results provide new information regarding monophyly and molecular phylogenetic relationships of Ranunculaceae and related Paeoniaceae and Berberidaceae plants.

MATERIALS AND METHODS

Plant materials and sequences

Plant materials were collected mainly from southwest China, where most Chinese Ranunculaceae species occur (Wu et al., 2003). Plant materials included *Thalictrum simplex* from Sichuan Jianyang of China and cultivated in tissue culture at Chongqing University of Posts and Telecommunications; *Coptis chinensis* from Shizhu, Chongqing, provided by Chen Daxia; *Ranunculus japonicus*, *Paeonia suffruticosa*, *Paeonia lactiflora*, *Mahonia fortunei* and *Nandina domestica* collected from the botanical gardens of the Chongqing Academy of Chinese Materia Medica and other species collected from the Jinfo Mountains in Chongqing. All 16 voucher specimens (Table 1) have been deposited at the Chongqing Academy of Chinese Materia Medica, China. All plant materials were re-identified by Dr. Liu Yi. In addition, ITS sequences of 76 species downloaded from GenBank were included in the final analyses (Table 1).

Strains and agents

Escherichia coli DH5α was preserved in the laboratory and the pMD18-T vector was purchased from TaKaRa (Kyoto, Japan). Genomic DNA extraction reagent kits, Taq DNA polymerase and dNTPs were purchased from Huashun Biotechnology (Shanghai, China). DNA Ladder was purchased from Tiangen Biotechnology (Beijing, China).

Genomic DNA extraction and PCR amplification

Genomic DNA was extracted from freshly frozen leaf material using a Genomic DNA extraction reagent kit. The ITS sequence primers were designed according to Wang and Zhou (1997): primer ITS-F, 5'-GGA AGT AAA AGT CGT AAC AAG G-3'; primer ITS-R, 5'-TCC TCC TCC GCT TAT TGA TAT GC-3'. The ITS sequencing reactions were carried out in a final volume of 20 µL, with 2 µL DNA, 5 pmol of each primer and 0.5 U Taq DNA polymerase in 1.8 mM $MgCl_2$, 100 mM dNTP and 10× reaction buffer. The cycling parameters were 94℃ for 5 min followed by 35 cycles of 94℃ 1 min, 55℃ 1 min and 72℃ 1 min, with a final extension for 5 min at 72℃.

T/A clone and sequencing

Gene fragments were amplified by PCR, separated by 1.0% agarose gel electrophoresis and purified using an Agarose Gel DNA Purification Kit (TaKaRa). The purified fragments were inserted into the pMD18-T cloning vector and transformed into competent *E. coli* DH5, which was incubated in growth medium and finally spread on agar LB medium plates containing ampicillin, IPTG and X-gal and incubated at 37℃ for 12-16 h. White colonies were picked and incubated in LB liquid culture medium. The plasmids were then extracted, digested with restriction enzymes and subjected to sequence analysis by Beijing Sunbiotech Co., Ltd. using an ABI3730XL DNA Sequencer.

DNA sequence analysis and phylogenetic tree construction

Online blastn at the NCBI website was used for analysis of the target gene and sites with missing data or gaps were excluded from all analyses. Neighbour-joining (NJ) and Maximum-parsimony analysis (Saitou and Nei, 1987) were performed using MEGA (Kumar et al., 1993). The Jukes-Cantor model of nucleotide substitution was selected for analyses based on the guidelines of Nei (1991) for choosing the most appropriate distance measure. For Neighbor-joining (NJ) and Maximum-parsimony analysis, MEGA was used to implement the heuristic search procedure with 2000 replicates of random addition of taxa to minimize possible bias from input order. The reliability of clustering patterns in trees was tested by bootstrapping in the case of the NJ tree and by the standard error test for the internal branches of the NJ tree (Rzhetsky and Nei, 1991).

RESULTS

PCR amplification and sequencing

The gene fragments were amplified by PCR. The ampli-

Table 1. The ITS Sequences of Ranunculaceae and related plants.

Species	Author	GenBank Accession no.	Voucher	Specimen no.
Aconitum racemulosum	Cai, Y. F	FJ424224	T. Y. Zhang	SM1043
Aquilegia vulgaris	Cai, Y. F	FJ424225	T. Y. Zhang	SM793
Clematis gratopsis	Cai, Y. F	FJ424226	W. T. Wang	SM0574
Clematis montona	Cai, Y. F	FJ424227	C. Ho	SM96482
Coptis Chinensis	Cai, Y. F	EU370101	W. T. Wang	SM1137
Epimedium brevicornum	Cai, Y. F	FJ424228	S. G. Tang	SM0106
Mahonia Bealei	Cai, Y. F	FJ424229	Z. O. Gu	SM543
Mahonia fortunei	Cai, Y. F	EU926492	Z. O. Gu	SM589
Nandina domestica	Cai, Y. F	EU926493	T. Y. Zhang	SM0348
Paeonia lactifloza	Cai, Y. F	EU926491	D. Y. Hong	SM1481
Paeonia suffruticos	Cai, Y. F	EU369007	D. Y. Hong	SM0068
Ranunculus cantoniensis	Cai, Y. F	FJ424230	H. X. Luo	SM0027
Ranunculus japonicus	Cai, Y. F	EU370102	J. L. Li	SM26297
Ranunculus sieboldii	Cai, Y. F	FJ424231	C. Ho	SM1751
Thalictrum simplex	Cai, Y. F	EU370100	S. Y. Chen	SM6572
Thalictrum thunbergii	Cai, Y. F	FJ467345	W.T. Wang	SM0401
Aconitum carmichaeli	Luo, Y	AY571352		
Aconitum chrysotrichum	Luo, Y	AY164642		
Aconitum crassiflorum	Luo, Y	AY150230		
Aconitum finetianum	Luo, Y	AY164643		
Aconitum gigas	Kita, Y	AB004963		
Aconitum scaposum	Luo, Y	AY150231		
Actaea asiatica	Compton, J. A.	Z98282		
Actaea pachypoda	Compton, J. A.	Z98277		
Actaea rubra	Compton, J. A.	Z98278		
Actaea spicata	Compton, J. A.	Z98279		
Adonis amurensis	Kaneko, S	AB361620		
Adonis multiflora	Kaneko, S	AB361622		
Adonis ramosa	Kaneko, S	AB361618		
Adonis shikokuensis	Kaneko, S	AB361623		
Anemone cernua	Pfosser, M	AM267279		
Anemone hupehensis	Schuettpelz, E	AY055397		
Anemone montana	Pfosser, M	AM267281		
Anemone patens	Pfosser, M	AM267280		
Anemone virginiana	Kress, W. J.	DQ006033		
Anemonopsis macrophylla	Compton, J.A.	Z98275		
Aquilegia canadensis	Ro, K.-E	ACU75656		
Aquilegia ecalcarata	Ro, K.-E	AEU75657		
Aquilegia fragrans	Ro, K.-E	AFU75658		
Beesia calthifolia	Compton, J.A.	AJ496613		
Callianthemum anemonoides	Schuettpelz, E.	AY365390		
Callianthemum coriandrifolium	Schuettpelz, E.	AY365393		
Caltha natans	Schuettpelz, E	AY365398		
Caltha palustris	Lee, W. K.	AY515398		
Caltha sagittata	Schuettpelz, E	AY365399		
Caltha scaposa	Schuettpelz, E	AY365396		
Cimicifuga dahurica	Yamaji, H	AB194179		
Cimicifuga heracleifolia	Yamaji, H	AB194180		
Cimicifuga simplex	Yamaji, H	AB194149		

Table 1. contd.

Clematis pierotii	Miikeda, O	AB120191		
Clematis potaninii	Miikeda, O	AB120198		
Clematis serratifolia	Miikeda, O	AB120205		
Clematis vitalba	Miikeda, O	AB120207		
Clematis vitalba	Miikeda, O	AB120207		
Clematis williamsii	Miikeda, O	AB120181		
Consolida ajacis	Luo, Y	AY150244		
Consolida ambigua	Koontz, J. A.	AF259282		
Coptis quinquefolia	Lin, T. C	EF206702		
Delphinium andersonii	Koontz, J. A.	AF258773		
Delphinium distichum	Koontz, J. A.	AF258774		
Delphinium hesperium	Koontz, J. A.	AF258772		
Delphinium recurvatum	Koontz, J. A.	AF258782		
Eranthis pinnatifida	Compton, J.A.	AJ496615		
Helleborus multifidus	Lee, W.K.	AY515403		
Hepatica acutiloba	Pfosser, M	AM267282		
Hepatica asiatica	Pfosser, M	AM267289		
Hepatica henryi	Pfosser, M	AM267290		
Hepatica insularis	Pfosser, M	AM267288		
Hepatica nobilis	Pfosser, M	AM267292		
Hepatica transsilvanica	Pfosser, M	AM267283		
Isopyrum fumarioides	Hang, S	AJ347912		
Megaleranthis saniculifolia	Lee,W.K.	AY515402		
Myosurus minimus	Hang, S	AJ347913		
Paeonia delavayi	Sang, T	PDU28279		
Paeonia japonica	Sang, T	PJU28281		
Paeonia lutea	Sang, T	PLU28283		
Paeonia mairei	Sang, T	PMU28282		
Paeonia mlokosewitschii	Sang, T	PMU28287		
Ranunculus amerophyllus	Hoerandl, E	AY680146		
Ranunculus eichleranus	Hoerandl, E	AY680138		
Ranunculus trigonus	Ling, X	DQ410724		
Semiaquilegia adoxoides	Ling, X	DQ410731		
Souliea vaginata	Compton, J.A.	Z98274		
Thafictrum dasycarpum	Ro, K.-E	U75665		
Thalictrum cooleyi	Ro, K.-E	U75666		
Thalictrum coriaceum	Ro, K.-E	U75664		
Thalictrum revolutum	Ro, K.-E	U75662		
Thalictrum thalictroides	Ro, K.-E	U75663		
Trautvetteria grandis	Hoerandl, E.	AY680202		
Trollius chinensis	Despres, L.	AY148269		
Trollius pumilus	Despres, L.	AY148282		
Trollius ranunculinus	Despres, L.	AY148277		
Trollius riederianus	Despres, L.	AY148279		

fied ITS sequences of all 16 species were determined with reference to the sequences in GenBank (Table 1). The lengths of the ITS sequences included in the final data matrix ranged from 494 to 654 bp. ITS1 and ITS2 were 180 - 271 bp and 207-249 bp in length, respectively. The sequence of 5.8 S was relatively well conserved, whereas those of ITS1 and ITS2 differed among species. For the 92 species studied, we obtained 692 bp of aligned sequence. Among these 692 sites, 505 were variable and 448 were informative for parsimony analysis. The average A: T: C: G ratio was 23.5:20.1:28.5:27.9, with a narrow standard error around the means. Thus, the ITS had high

G+C content consistent with earlier observations in other plant taxa (Baldwin, 1992). The average transition/ transversion (ts/tv) ratio was 1.1, which was higher than the expected ratio of 0.5 when all types of substitutions were assumed to be equally likely.

Phylogenetic analyses

Clustal X software was used to align the ITS sequences and then ITS sequence phylogenetic trees were constructed using MEGA (Figures 1 and 2). Ranunculaceae group relationships were inferred from the NJ tree and Maximum-parsimony trees using Jukes-Cantor distances based on all pairwise comparison of ITS1, 5.8S and ITS2 sequences from 82 species of Ranunculaceae and 10 outgroup taxa in Paeoniaceae and Berberidaceae. We used 10 outgroup sequences in the phylogenetic analysis, although large portions of the ITS regions were too variable to be reasonably aligned with the Ranunculaceae group taxa. The alignable portions between the ingroup and outgroup, which included the 5.8S sequences, did not provide any information to resolve ingroup phylogeny. As can be seen in Figures 1 and 2, the outgroup taxa (Out groups 1 and 2; Table 2) were located independently outside the phylogenetic tree and were separated by a large genetic distance from the Ranunculaceae groups (Groups 1-26).

In the Ranunculaceae groups, nearly all species were concentrated together in genera. Figure 2 shows seven major clusters in addition to the outgroup clade. The results indicated that *Coptis* (Group 26) was a sister group to a monophyletic clade consisting of the remaining Ranunculaceae (bootstrap value [BV] = 89%). The first clade included *Clematis* (Group 1), *Anemone* (Group 2), *Hepatica* (Group 3) and *Helleborus* (Group 4), with strong support for *Helleborus* as a sister group to the other three genera (BV = 90%).

The second clade was composed of *Trautvetteria* (Group 5), *Myosurus* (Group 6), and *Ranunculus* (Group 7; BV = 95%). *Trautvetteria* was a sister group to the *Myosurus–Ranunculus* clade (BV = 93%), whereas *Myosurus* and *Ranunculus* formed a group (BV = 93%) closely allied to the rest of this second clade.

The third clade was composed of *Actaea* (Group 8), *Eranthis* (Group 9), *Souliea* (Group 10), *Cimicifuga* (Group 11), *Anemonopsis* (Group 12), *Beesia* (Group 13), *Caltha* (Group 14), *Adonis* (Group 15), *Trollius* (Group 16) and *Megaleranthis* (Group 17; BV = 75%). *Caltha* was a sister group to the *Adonis–Trollius–Megaleranthis* clade (BV = 60%), whereas *Adonis*, *Trollius* and *Megaleranthis* formed a group (BV = 99%) closely allied to the rest of this clade. The *Actaea–Eranthis–Cimicifuga–Souliea–Anemonopsis–Beesia* lineage (BV = 96%) further supported several structural rearrangements (Ro et al., 1997).

Surprisingly, *Callianthemum* (Group 21) is a monophyletic group in the fourth clade.

The fifth clade consisted of *Delphinium* (Group 18), *Consolida* (Group 19), and *Aconitum* (Group 20; BV = 88%). The *Delphinium–Consolida* clade (BV = 57%) was a sister group and *Aconitum* was positioned outside these two genera.

The sixth major clade was composed of *Isopyrum* (Group 22), *Aquilegia* (Group 23), *Semiaquilegia* (Group 24) and *Thalictrum* (Group 25; BV = 99%). The two clades *Aquilegia-Semiaquilegia* (BV = 90%) and *Thalictrum* (BV = 52%) were positioned together and as a sister group with Isopyrum. The outgroup lineage consisted of *Mahonia*, *Epimedium*, *Nandina* (Berberidaceae, Outgroup 1) and *Paeonia* (Paeoniaceae, Outgroup 2; BV = 94%) and the two weakly supported clades *Mahonia–Epimedium* (BV = 74%) and *Nandina-Paeonia* (BV = 50%) were positioned outside the phylogenetic tree.

Taken together, the results were in accordance with those of studies in morphology and other disciplines, but there were some discrepancies that require further investigation. Groups 1-7 were evolutionarily more closely related and there were close relationships between groups 8-17. Moreover, groups 18 -20 and groups 22-25 were also closely related. Group 26, which was separated from other ingroup taxa, was separated by a large genetic distance from Ranunculaceae groups. In addition, the results indicated that Outgroup 1 (Berberidaceae) and Outgroup 2 (Paeoniaceae) were closely related.

DISCUSSION

Plant systematics and phylogenetic studies are based mainly on morphological and molecular characters. In this study, we sequenced and analysed ITS sequences of 92 species of Ranunculaceae and related plants that have not been reported before. The results provide an initial understanding of the evolutionary relationship and process in Ranunculaceae and other related plants.

Consistent with the classification and nomenclature of Ranunculaceae presented by Tamura (1993) (Table 2), most clades found in NJ and MP analyses can be characterized by morphological or karyological features reported in the literature and may represent a basis for a revised classification (Figures 1 and 2). Many species of the Ranunculaceae groups have a very interesting morphology and chemical structure (Ro et al., 1997) that could be mapped on an inferred topology derived from ITS sequences. Analysis of ITS sequences among Ranunculaceae ingroups and outgroups can provided useful molecular data for the classification of the genera in Ranunculaceae. Moreover, it provided useful evidence with chemical composition (Xiao, 1980), especially in terms of ranunculin, thalictrine, and magnoflorine, which play important roles in the genealogy of both ingroups and outgroups.

Our analyses included 82 species of Ranunculaceae

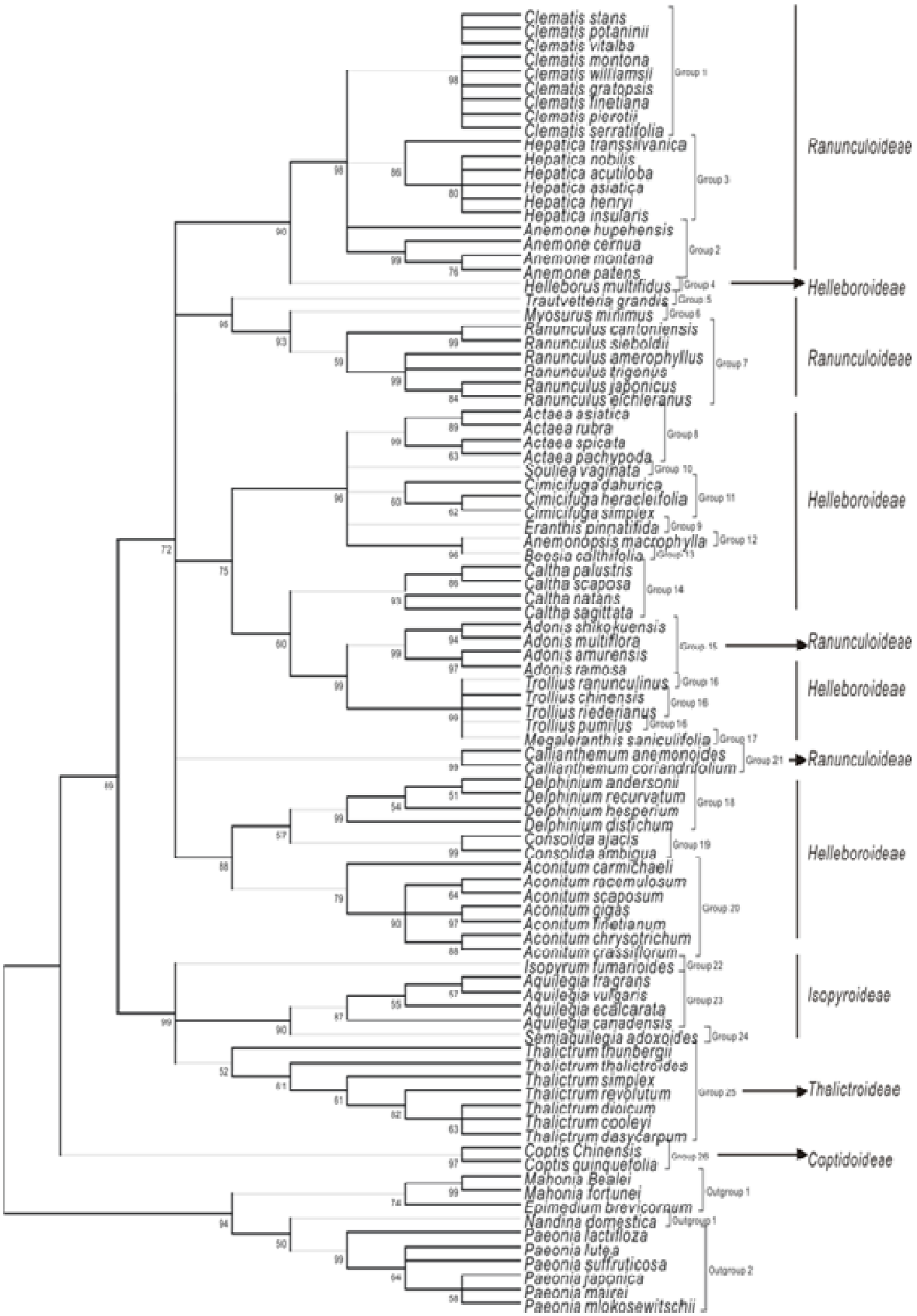

Figure 1. Ranunculaceae group relationships inferred from Neighbor-Joining tree using Jukes-Cantor distances based on all pairwise comparison of ITS 1, 5.8S and ITS 2 sequences (692bp).

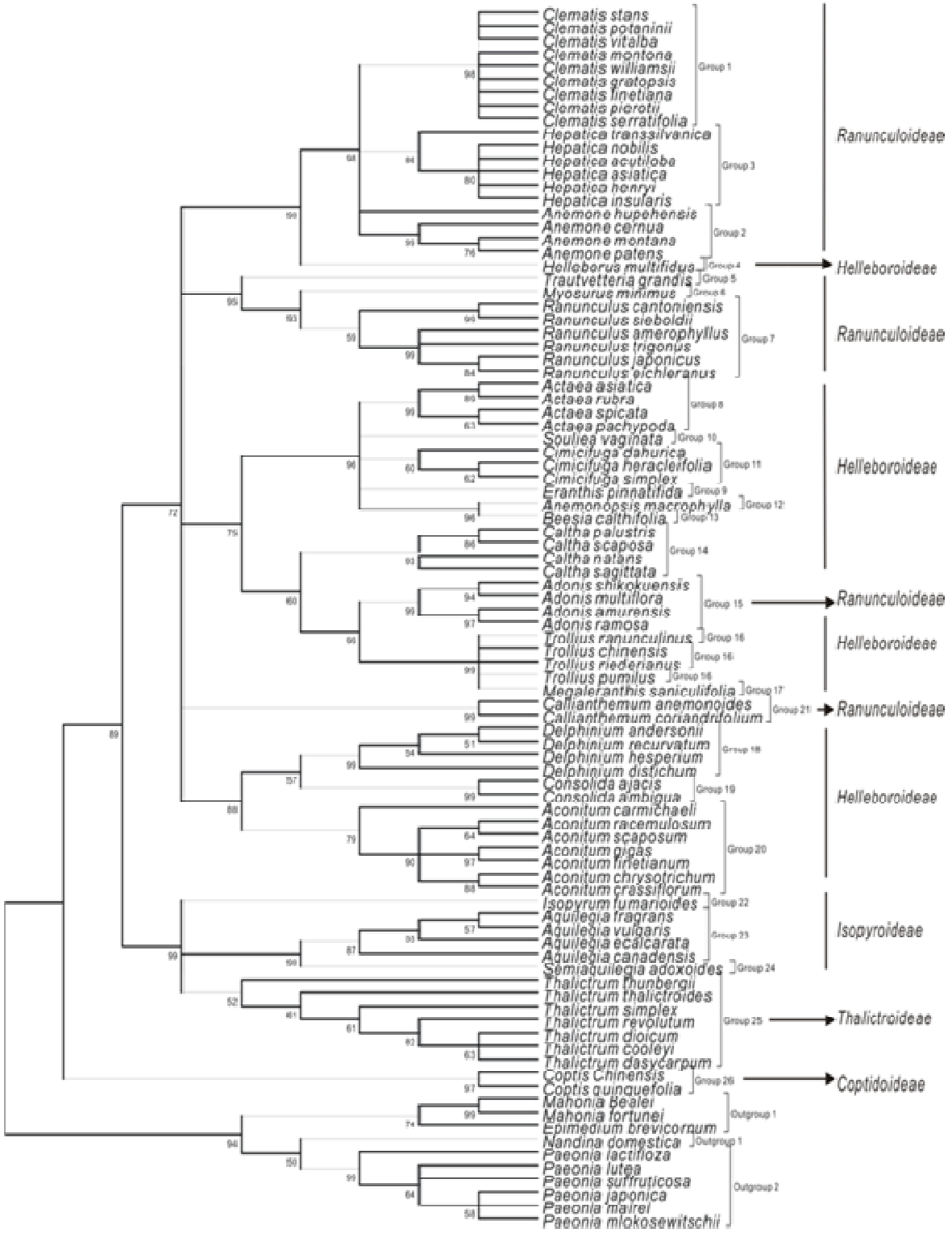

Figure 2. Ranunculaceae group relationships inferred from Maximum-parsimony tree treating all characters as equally weighted. Numbers indicate Pc value from the standard error test (higher than 50%).

and 10 species of outgroup genera from Paeoniaceae and Berberidaceae. The Paeoniaceae represented a new branch that separated from Ranunculaceae relatively recently. We chose Berberidaceae and Paeoniaceae as outgroups because they both possess several types of protoberberine alkaloids (Peng et al., 2006a) and some

Table 2. The classification by Tamura (1993) for Ranunculaceae.

(Ranunculaceae) subfamily	Tribe	Genus	Group no.
Helleboroideae	**Trollieae**	***Caltha***	**Group 14**
		Trollius	Group 16
		Megaleranthis	Group 17
		Beesia	Group 13
	Cimicifugeae	*Actaea*	Group 8
		Cimicifugea	Group 11
		Anemonopsis	Group 12
		Souliea	Group 10
	Helleboreae	*Eranthis*	Group 9
		Helleborus	Group 4
	Delphineae	*Aconitum*	Group 20
		Delphinium	Group 18
		Consolida	Group 19
Ranunculoideae	**Anemoneae**	***Anemone***	**Group 2**
		Hepatica	Group 3
		Clematis	Group 1
	Ranunculeae	*Adonis*	Group 15
		Ranunculus	Group 7
		Callianthemum	Group 21
		Myosurus	Group 6
		Trautvetteria	Group 5
Thalictroideae	**Thalictreae**	***Thalictrum***	**Group 25**
Isopyroideae	**Isopyreae**	***Isopyrum***	**Group 22**
		Semiaquilegia	Group 24
		Aquilegia	Group 23
		Coptis	Group 26
Hydrastidoideae			

older classifications included *Paeonia* (peony) in Ranunculaceae (Prantl, 1891).

This study included 26 genera of Ranunculaceae and the monophyly of each of these genera was supported by our inferred phylogeny as well as by that of Ro et al. (1997), who drew the same conclusions based on nuclear 26S-rDNA sequences. Because of the use of multiple outgroups, the inferred phylogeny of each genus was clearly rooted, showing a contrasting pattern of evolution. A close relationship among *Clematis*, *Anemone*, *Hepatica*, *Helleborus*, *Trautvetteria*, *Myosurus* and *Ranunculus* was supported by our data (Figure 1); with the exception of *Helleborus*, which has been placed in *Helleboroideae*, should be retained within *Ranunculoideae*. These results differed from the classification reported by Tamura (1993); hence, further investigations are required. All of the genera in Ranunculoideae have similar chemical compositions; they possess ranunculin but no magnoflorine or other benzylisoquinolines (Peng et al., 2006a), suggesting that they may have the same origin. Tamura (1993) placed these members clade in the subfamily Ranunculoideae based on their follicular fruits, a position that was supported by our data (Peng et al., 2006b).

A monophyletic group including *Actaea*, *Eranthis*, *Cimicifuga*, *Souliea*, *Anemonopsis*, *Beesia*, *Caltha*, *Adonis*, *Trollius* and *Megaleranthis* was revealed by our data and those of Ro et al. (1997). Taxology and phytochemistry showed that the first five genera have a close relationship with one another, and do not possess magnoflorine or ranunculin (Peng et al., 2006a; Xiao, 1980). The final four genera all have a close relationship with one another and they all contain magnoflorine but no ranunculin (Peng et al., 2006a; Xiao, 1980). However, there is strong disagreement over the tribal and subfamilial circumscriptions within the family. Based on the classification of Tamura et al. (1993), all 10 genera belong to the Helleboroideae except for *Adonis*, which belongs to the Ranunculaceae tribe in Ranunculoideae (Table 2) and so is inconsistent with our analyses (Ro et al., 1997; Xiao, 1980). We contend that it is more reasonable for *Adonis* to belong to the Helleboroideae.

Our molecular data also indicated affinity among

Delphinium, *Consolida* and *Aconitum*, with high statistical support. Morphologically speaking, all of the genera have racemiform inflorescences, zygomorphic flowers and free follicles. With regard to phytochemistry, they all contain distinctive diterpene alkaloids (Xiao, 1980) that are not present in any other genera of Ranunculaceae. Tamura (1993) placed this clade in the tribe Delphineae, a position that is supported by our data (Jensen, 1992), but further evidence is required to determine whether this clade can be placed into the subfamily Helleboroideae. The genus *Callianthemum*, which belongs to the Ranunculoideae, is genetically distant from the genera of Ranunculoideae, suggesting that Callianthemum should be separated from Ranunculoideae (Figures 1 and 2).

There was a closely genetic relationship between *Aquilegia*, *Semiaquilegia*, *Isopyrum* and *Thalictrum*. It is strongly consistent with Ro's cladogram (Ro et al. 1997) and the Florae's for the Thalictroideae Classification (Delecctis Florae Reipublicae Popularis Sinicae Agendae Academiae Sinicae Edita 1979). The first three genera belong to the subfamily Isopyreae, whereas the last belongs to the Thalictroideae. All of these genera have similar chemical compositions and contain magnoflorine, saponin and flavone, but no ranunculin (Peng et al., 2006a), suggesting that they may have the same origin. In addition, our results indicated that the two subfamilies had a close relationship and that they may be put in the same subfamily. With regard to phytochemistry, the chemical composition of *Coptis* showed characteristics similar to those of *Thalictrum* (Peng et al., 2006a), but *Coptis* had a larger genetic distance than all other genera of Ranunculaceae based on our data, which supports Tamura's classification for the subfamily Coptidoideae (Tamura, 1993).

This study has introduced new information about the phylogenetic relationships among Ranunculaceae plants. Molecular genetic methods have demonstrated several advantages over classical morphological and chemical analyses. Genetic methods are based on genotype rather than phenotype and therefore are not dependent on the environment. Moreover, the molecular genetic approach provides a rapid and reliable method for the identification and botanical classification of medicinal plants both within and between genera (Ma et al., 2001). These results will be helpful in identifying new components of medical plants in Ranunculaceae and related plants. The research will be beneficial to the rational utilization and protection of precious Ranunculaceae and related plant resources.

ACKNOWLEDGMENTS

This work was supported by grants from the National Nature Science Foundation of China (Nos. 30771311), Natural Sciences Foundation of Chongqing, China (No. cstc2007 BB1328) and project of Chongqing Education Committee, China (KJ050510, KJ080504).

REFERENCE

Baldwin BG (1992). Phylogenetic utility of the internal transcribed spacers of nuclear ribosomal DNA in plants: an example from the Compositae. Mol. Phylogen Evol. 1: 3-16.

Baldwin BG, Sanderson MJ, Porter JM, Wojceichowski MF, Campbell CS, Donoghue MJ (1992). The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. Ann. Missouri Bot. Gard. 82: 247-277.

Chen Q, Peng WL, Qi SJ, Xu AL (2002). Apoptosis of human highly metastatic lung cancer cell line 92-D induced by acutiaporberine, a novel bisalkaloid derived from *Thalictrum* acutifolium. Planta Medica, 68: 550-553.

Delecctis Florae Reipublicae Popularis Sinicae Agendae Academiae Sinicae Edita (1979) Flora Reipublicae Popularis Sinicae, Tomus 27, Science Press, Beijing, p. 502

Ding SY, Zhang CL, Gu HY, Chen ZL (1996). Progresses of the studies on plant systematics at DNA level. Acta Botanica Boreali-Occidentalia Sinica, 16: 446-456.

Fior S, Karis PO, Casazza G, Minuto L, Sala F (2006). Molecular phylogeny of the Caryophyllaceae (CaryophyHales) inferred from chloroplast matK and nuclear rDNA ITS sequences. Am. J. Bot. 93: 399-411.

Hoot SB (1991). Phylogeny of the Ranunculaceae based on epidermal microcharacters and macromorphology. Syst. Bot. 16: 741-755.

Hörandl E, Paun O, Johansson JT, Lehnebach C, Armstrong T, Chen L, Lockhart P (2005). Phylogenetic relationships and evolutionary traits in *Ranunculus s.l.* (Ranunculaceae) inferred from ITS sequence analysis. Mol. Phylogenet. Evol. 36: 305-327.

Huang WZ, Dong TX, Qi HY, Lu ZQ, Zhan HQ (2005). Identification of Stellaria media by PCR. J. Chin. Pharmaceut. Sci. 14: 144-148.

Jensen U (1992). Secondary compounds of the Ranunculiflorae. Plant Syst. Evol. (Suppl.), 9: 82-97.

Jensen U (1966). Die Verwandtschaftsverhaltnisse innerhalb der Ranunculaceae aus serologischer Sicht. Ber. Deutsch. Bot. Ges. 79: 407-412.

Jensen U (1968). Serologische Beitrage zur Systematik der Ranunculaceae. Bot. Jahrb. 88: 269-310.

Jensen U (1995). Secondary compounds of the Ranunculaceae. Plant Syst. Evol. (Suppl.), 9: 85-97.

Ro KE, Bruce A (1997). McPheron Molecular Phylogeny of theAquilegia Group (Ranunculaceae) Based on Internal Transcribed Spacers and 5.8S Nuclear Ribosomal DNA. Biochem. Syst. Ecol. 25 445-461.

Ro KE, Keener CS, McPheron BA (1997) Molecular Phylogenetic Study of the Ranunculaceae: Utility of the Nuclear 26S Ribosomal DNA in Inferring Intrafamilial Relationships.Mol. Phylogenet. Evol. 8: 117-127.

Kumar S, Tamura K, Nei M (1993). MEGA: Molecular Evolutionary Genetics Analysis, Version 1.01. The Pennsylvania State Univ. University Park, PA.

Lehnebach CA, Cano A, Monsalve C, McLenachan P, Horandl E, Lockhart P (2007). Phylogenetic relationships of the monotypic Peruvian genus Laccopetalum (Ranunculaceae). Plant Syst. Evol. 264: 109-116.

Li YL, Ma SC., Yang YT (2002) Antiviral activities of flavonoids and organic acid from *Trollius chinenssi* Bunge. J Ethnophamukeol. 79:365-368.

Loconte H, Estes JR (1989). Phylogenetic systematics of Berberidaceae and *Ranunculales (Magnoliidae).* Syst. Bot. 14: 565-579.

Ma XQ, Zhu DY, Li SP, Dong TT, Tsim KW (2001). Authentic identification of *Stigma Croci* (stigma of *Crocus sativus*) from its adulterants by molecular genetic analysis. Planta Medica, 67: 183-186.

Nei M (1991). Relative efficiencies of different tree-making methods for molecular data. In Phylogenetic Analysis of DNA Sequences (Miyamoto MM and Cracraft J Eds.). Oxford Univ. Press, New York, pp. 90-128

Peng Y, Chen SB, Chen SL, Xiao PG (2006a). Preliminary pharmaphylogenetic study on Ranunculaceae China. J. Chin. Materia Medica, 31: 1124-1128.

Peng Y, Chen SB, Liu Y, Wang LW, Xiao PG (2006b). Preliminary pharmaphylogenetic study on Isopyroideae (Ranunculaceae) China. J. Chin. Materia Medica, 31: 1210-1214.

Prantl K (1891). Ranunculaceae. In Enoler A, Prantl K (Eds): Die natfirlichen Pflanzenfamilien. 3: 43-66. Leipzig: Engelmann.
Rzhetsky A, Nei M (1992). A simple method for estimating and testing minimum-evolution trees. Mol. Biol. Evol. 9: 945-967.
Saitou N, Nei M (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406-425.
Serkedjieva J, Velcheva M (2003). In vitro anti-influenza virus activity of the pavine alkaloid (-)-thalimonine isolated from *Thalictrum* simplex L. Antiviral Chem. Chemother. 14: 75-80.
Stanford AM, Harden R, Parks CR (2000). Phylogeny and biogeography of Jugland (Juglandaceae) based on matK and ITS sequence data. Am. J. Bot. 87: 872-882.
Tamura M (1993). Ranunculaceae. In: Kubitzki K, Rohwer JG and Bittrich V (eds.). The Families and Genera of Vascular Plants. Berlin: Springer-Verlag. 2: 563-583.
Tian X, Li DZ (2002). Application of DNA Sequences in Plant Phylogenetic Study. Acta Botanica Yunnanica, 24: 170-184.
Wang RF, Yang XW, Ma CM, Cai SQ, Li JN, Shoyama Y (2004). A bioactive alkaloid from the flowers of Trollius chinensis. Heterocycles, 63: 1443-1448.
Wang WC (1979). Ranunculaceae. Flora of China (Vol. 27, 28). Beijing: SciencePress (in Chinese).
Wang YQ, Zhou KY (1997). A preliminary study on the identification of crude snake drug by Molecular Genetic Markers. Acta Pharm. Sinica, 32: 384-387.
Wu ZY, Lu AM, Tang YC (2003) The families and genera of angiosperms in China. Science press, Beijing, pp. 378-411.
Xiao PG (1980). A Priliminary Study of the Correlation Between Phylogeny, Chemical Constituents and Pharmaceutical Aspects in the taxa of Chinese Ranunculaceae. Acta Phytotaxonomica Sinica, 18: 143-153.
Yang QE (2001). Cytology of 12 species in *Aconitum* L. and of 18 species in *Delphinium* L. of the tribe *Delphineae* (Ranunculaceae) from China. Acta Phytotaxonornica Sinica, 39: 502-514.