Full Length Research Paper

Internal transcribed spacers (ITS) identification of *Angelica anomala* Lallem Chuanbaizhi (in Chinese) cultivars collected in Sichuan and their molecular phylogenetic analysis with other *Angelica* L. species

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Nuclear ribosomal DNA (nrDNA) targets, especially internal transcribed spacers (ITS) ITS1 and ITS2, have been widely used for molecular identification of some plants. We therefore, conducted an investigation in the identification of the *Angelica anomala* cultivars collected in Sichuan using the ITS universal primers. About 700 bp fragments were initially obtained from the famous-region drugs sample of Zhongji village, Chuanshan County (ZJCS), depending on the ITS sequence of ZJCS, the identification results suggested that 7 samples are famous-region drugs, but the others cannot count as famous-region drugs. Meanwhile, a comparative analysis was conducted to evaluate their molecular phylogenetic analysis with other 17 *Angelica* L. species. Our analysis revealed that *A. anomala* and *Angelica dahurica* are closely related and nested in this phylogeny tree. *A. dahurica* was shown the high similarity with *A. anomala* than any other species.

Key words: *Angelica anomala* Chuanbaizi, internal transcribed spacers (ITS) identification, phylogenetic analysis.

INTRODUCTION

Angelica anomala Lallem (Chuanbaizhi in Chinese) is a well-known Traditional Chinese Medicine. Its original herbs belong to *Angelica* L., which is one of the largest genera of the family Umbelliferae, and *Angelica dahurica* is the original plant of *A. anomala* (Delectis Florae Reipublicae Popularis Sinicae Agendae Academiae Sinicae Edita, 1992). *A. anomala* of famous-region drug is distributed in Sichuan province, China. Its main product areas have been distributed in Suining, Nanchong and Ziyang in Sichuan (Yang et al., 2002). Because the root of *A. anomala* has analgesic, antibacterial, antidotal, carminative, depurative, diaphoretic and poultice medicinal properties, and it has also been widely used to treat women's complaints. The drug (an extract of the root) lowers arterial pressure, increases diuresis and stimulates contraction of the smooth muscles, especially the uterus, but without causing abortion (Chinese Pharmacopoeia Commission, 2010).

To identify crude drugs precisely is a pivotal point for chemical and pharmacological research of Traditional Chinese Medicine and for their clinical applications. Traditional means of *A. anomala* identification rely on the inspection of morphological markers such as shape, colour, texture and odour. However, these methods are not always sufficient for the identification of herbs that morphologically resemble each other, particularly when the herbs are in a dried and/or sliced state, the common forms of herbs when they are traded (Lee et al., 2008). Its accuracy depends heavily on the examiner's experience. Therefore, more objective and canonical methods are

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Table 1. List of plant samples used in this study, nrDNA ITS sequence size and its G+C content of different places of Chuanbaizhi

No.	Place of collection	ITS region (ITS1+5.8S+ITS2)	G+C content (ITS1+5.8S+ITS2) (%)
1	Buying village, Pengxi County (BYPX)	599 (216+162+221)	53.76% (53.70+54.94+52.94%)
2	Yongyi village, Pengxi County (YYPX)	599 (216+162+221)	54.09% (53.70+54.32+54.30%)
3	Sangshulin village, Shehong County (SSLSH)	599 (216+162+221)	53.76% (52.31+54.94+54.30%)
4	Weijiaying village, Shehong County (WJYSH)	599 (216+162+221)	54.26% (53.70+54.94+54.30%)
5	Baishi village, Shehong County (BSSH)	599 (216+162+221)	54.26% (53.70+54.94+54.30%)
6	Qikou town, Daying County (QKDY)	599 (216+162+221)	54.26% (53.70+54.94+54.30%)
7	Zhongji village, Chuanshan County (ZJCS)	599 (216+162+221)	54.26% (53.70+54.94+54.30%)
8	Shuizhaimen village, Chuanshan County SZMCS)	599 (216+162+221)	54.26% (53.70+54.94+54.30%)
9	Zhonglinsi village, Chuanshan County (ZLSCS)	599 (216+162+221)	54.26% (53.70+54.94+54.30%)
10	Shuyuan village, Chuanshan County (SYCS)	599 (216+162+221)	54.26% (53.70+54.94+54.30%)
11	Longpinghu village, Chuanshan County (LPHCS)	599 (216+162+221)	54.26% (53.70+54.94+54.30%)
12	Xiaoba village, Chuanshan County (XBCS)	599 (216+162+221)	54.09% (53.24+54.94+54.30%)
13	Lijia village, Anyue County (LJAY)	599(216+162+221)	54.26% (53.70+54.94+54.30%)
14	Xinglong town, Anyue County (XLAY)	599 (216+162+221)	54.26% (53.70+54.94+54.30%)
15	Qinglin village, Gaoping County (QLGP)	599 (216+162+221)	54.09% (53.24+54.94+54.30%)

necessary. To overcome difficulties of these methods, kaleidoscopic molecular biology techniques utilizing different genetic information of organism, are used for the discrimination of plant species (McClell and Welsh, 1994).

Eukaryotic nrDNA is tandemly organized with high copy numbers and each repeat unit consists of genes coding for the nuclear ribosomal small subunit (SSU), large subunit (LSU) and 5.8S nrDNA. These coding regions are separated from each other by spacers. ITS1 and ITS2 spacers, in addition to the 5.8S nrDNAcoding DNA is referred as nrDNA ITS region. 5.8S nrDNAis embedded in between two internal transcribed spacers (ITS1 and ITS2) (Baldwin, 1992; Baldwin et al., 1995; Li, 1997). Herein, the ITS region of nrDNA was employed to assist identification of the traditional Chinese medicinal plant (Shiba et al., 2006; Sahin et al., 2007; Yang et al., 2007; Xie et al., 2009; Lee et al., 2010). Moreover, the numerous ITS sequences available in the GenBank enable immediate comparative analyses. Up to date, more publications have revealed that ITS2 can serve as a novel universal barcode for the identification of a broader range of medicinal plant species and genera (Chen et al., 2010; Yao et al., 2010), but no method was reported to accurately identify medicinal plant of a specie.

Characterization of the genetic diversity and examination of the genetic relationship among *Angelica* L. species are important for the sustainable conservation and can enhance use ratio of plant genetic resources. Recent studies have focused almost exclusively on the phylogenetic analyses of DNA sequences, particularly that of the ITS region of nrDNA (Spalik et al., 2004; Xue et al., 2007). Molecular systematic studies revealed that the *Angelica* genus is monophyletic upon the transfer of several of its species to other genera (Feng et al., 2009). The phylogenetic relationships among several taxonomically complex species, such as *A. anomala* and *A. decursiva*, have yet to be resolved unequivocally.

In this study, we developed reliable DNA markers based on the ITS sequence to discriminate the famous-region drug of *A. anomala* and consummated the phylogenetic analysis of *Angelica* genus. Through cloning the ITS sequences and analyzing the information given by ITS sequences, we first identify the famous-region drug of *A.anomala* in Sichuan, China used by ITS sequences and pave way for further phylogenetic and/or evolutionary studies on *A. anomala* and other *Angelica* L. species.

MATERIALS AND METHODS

Plant materials and genomic DNA extraction

Fresh leaves of samples were kept in silica gel upon collection; the voucher specimens were deposited in the College of Ethnomedicines, Chengdu University of Chinese Traditional Medicine, Chengdu, China. Totally 15 populations covering all populations currently used in Sichuan, China, were examined for sequence variations in the ITS sequence region of nrDNA (Table 1). Details of locations of herbarium vouchers were provided in Table 1.

The materials were frozen with liquid nitrogen and milled into a fine powder. Total genomic DNA was extracted from the ground powder by using a modified cetyltrimethylammonium bromide method from Doyle and Doyle (1987). The concentration of genomic DNA was measured by Eppendorf Biophotometer.

PCR amplification

The complete ITS region (including ITS1, 5.8S nrDNAand ITS2) was amplified using the primers ITS1 (5'-GAAGTCGTAACAAGGTTTCCGTAGG-3') and ITS4 (5'-TCCTCCGCT TATTGATATGC-3') (Figure 1) (Takaiwa et al., 1985; White et al., 1990). PCR amplification was carried out under the following conditions: initial denaturation at 94ºC (4 min); followed
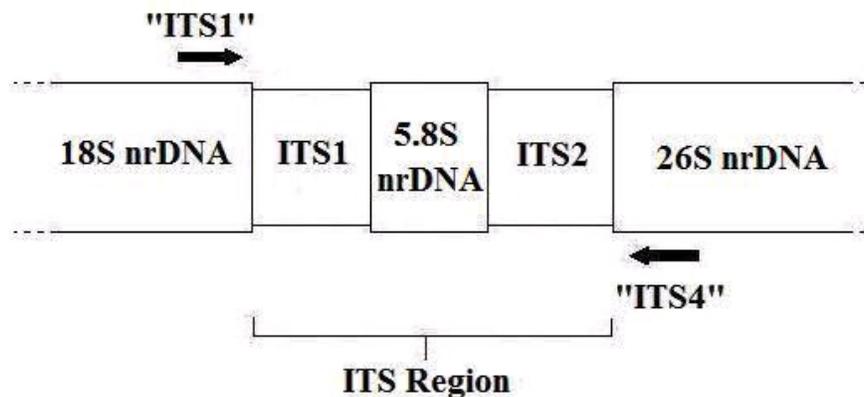
Figure 1. Organization of the internal transcribed space (ITS) region of 18-26S rDNA. Approximate positions of primers used for DNA amplification and sequencing are indicated by arrows.

by 30 cycles of 94ºC denaturation (30 s), 57ºC annealing (30 s), and 72ºC extension (45 s); a final extension at 72ºC for 10 min.

The PCR amplification was carried out in a GeneAmp® PCR System 9700 (PE Applied Biosystems Inc.) with a 25 µL reaction mixture containing 5 µL 5 x PCR buffer (including 2.5 mM MgCl$_2$), 2 µL dNTP Mix (2.5 mM each), 1 µL 10 µM each primer, 2-5 µg template DNA and 0.25 µL Pfu DNA polymerase. Successful PCR amplification produced a single DNA band of approximately 700 bp in length on a 1% agarose gel and was visualized by ethidium bromide staining under UV.

PCR products were subsequently purified using TIANgel mini purification Kit (Tiangen Biotech (Beijing) Co. Ltd.). Then the PCR products were ligated with the pEAZY-Blunt vector (Transgen Biotechnology Co. Ltd.). Competent T cells were transformed into the ligation products, and the correct colonies were identified by LB solid medium (IPTG, X-gal, Amp$^+$) and bacteria PCR. The sequencing products were analyzed by Invitrogen Biotechnology (Shanghai) Co. Ltd. All products were sequenced by forward and reverse reactions for sequence confirmation.

Sequence analysis

The sequencing chromatograms were assembled using SeqMan of DNAStar software package (Burland, 2000), and the sequences were aligned using the Clustal X (Thompson et al., 1997) and adjusted manually as necessary. The complete sequence of the ITS region for each sample was then stored as a separated text file, and later deposited in GenBank (Table 1). Sequence boundaries of ITS1, 5.8S and ITS2 were determined by utilizing the following submitted sequence data from GenBank.

Searching for ITS sequences in other *Angelica* L. species

For a meaningful comparison, the ITS sequences of other *Angelica* L. species (Table 2) were downloaded from NCBI Database (http://www.ncbi.nlm.nih.gov). Most analyses were based on deriving common ITS sequence of *Angelica* L. species. The algorithm was as described in the website, and a BLAST program reported the similarity in *Angelica* L. species.

Phylogenetic analysis

In order to compare the evolutionary relationship of ITS sequences in different species of *Angelica* L., we compared the nucleotide similarity of ITS sequences, and constructed phylogenetic trees using molecular evolutionary genetics analysis (MEGA) software version 4.0 (Tamura et al., 2007). The scoring matrix was substituted from sequence similarity to ITS sequence similarity between each two species. Phylogenetic trees were reconstructed by MEGA 4.0 with maximum parsimony (MP) method and neighbor-joining (NJ) method respectively.

RESULTS AND DISCUSSION

Determination and analysis of ITS sequences

ITS sequence of each sample including 5.8S nrDNA coding region was amplified using primers ITS1 and ITS4 (Figure 2) (Takaiwa et al., 1085; White et al., 1990). The approximately 700 bp PCR products were amplified in samples of *A. anomala* (Figure 2). To precisely reveal ITS variations in samples of *A. anomala*, we defined the ITS1, 5.8S nrDNA and ITS2 regions, and analyzed the nucleotide sequences of each region separately after the determination of their PCR products. On the basis of the nucleotide sequence of ITS deposited in NCBI GenBank, we defined a 216 bp ITS1 in *A. anomala* (Table 1). In addition, we defined 162 bp 5.8S nrDNA and 221 bp ITS2 in both *A. anomala* (Table 1). ITS1 was slightly shorter than ITS2, but it provided parsimony informative sites almost as much as ITS2. The means of guanine-cytosine (GC) content were approximate in ITS1 and ITS2 sequences (Table 1).

The GC balance between ITS1 and ITS2 is a common feature and obviously presented in all eukaryotic taxa (Feng et al., 2010), which phenomenon called molecular coevolution and might linked to the functional structures. The ITS1 and ITS2 sequences appeared to function in the maturation of nuclear ribosomal RNAs, that is, the ITS1 has probably been a spacer, and the ITS2 was considered to be a former expansion segment (Liang and Fournier, 1997). The balanced G+C content and conserved motif of

Table 2. List of ITS sequences in other *Angelica* L. species.

No.	Species	Genus	GenBank Acc. No.
1	*Angelica acutiloba*	*Angelica* L.	DQ278165.1
2	*Angelica morii*	*Angelica* L.	DQ263578.1
3	*Angelica amurensis*	*Angelica* L.	DQ263581.1
4	*Angelica valida*	*Angelica* L.	DQ263569.1
5	*Angelica cartilaginomarginata*	*Angelica* L.	DQ263589.1
6	*Angelica dahurica*	*Angelica* L.	EU418374.1
7	*Angelica decursiva*	*Angelica* L.	EU418375.1
8	*Angelica laxifoliata*	*Angelica* L.	EU647210.1
9	*Angelica gigas Nakai*	*Angelica* L.	DQ263575.1
10	*Angelica likiangensis*	*Angelica* L.	DQ263587.1
11	*Angelica maowenensis*	*Angelica* L.	EU236157.1
12	*Angelica nitida*	*Angelica* L.	EU418378.1
13	*Angelica omeiensis*	*Angelica* L.	DQ263571.1
14	*Angelica oncosepala*	*Angelica* L.	EU418382.1
15	*Angelica polymorpha maxim*	*Angelica* L.	DQ263590.1
16	*Angelica sinensis*	*Angelica* L.	DQ263570.1
17	*Angelica tsinlingensis*	*Angelica* L.	DQ263577.1
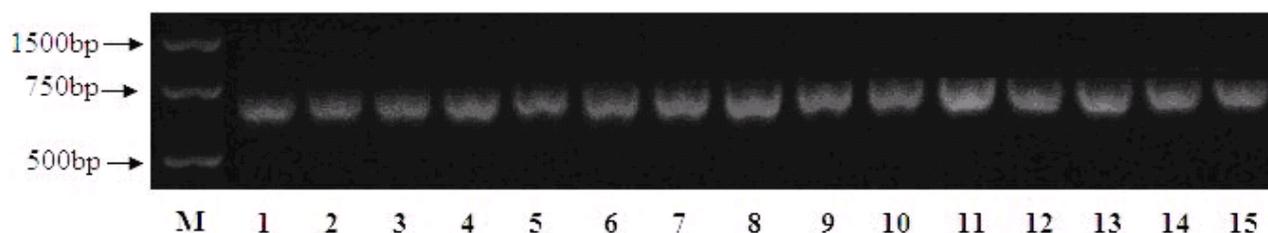
Figure 2. PCR products of ITS sequence of samples listed in Table 1 amplified by specific primers. Lane M: DNA marker; Lane 1~15: BYPX, YYPX, SSLSH, WJYSH, BSSH, QKDY, ZJCS, SZMCS, ZLSCS, SYCS, LPHCS, XBCS, LJAY, XLAY, QLGP.

ITS indicated the significant functional role of ITS for the rRNA primary transcript processing (Torres et al., 1990).

ITS identification of *A. anomala*

Traditionally, the methods for the *A. anomala* identification are mainly based on the morphological characters observed from their original plants. The analysis of compounds relies on the amounts of samples and the stability of compounds. In general, the identification based on morphological characters and analysis of compounds is subtle and ambiguous (Yang et al., 2007). ITS sequences were proposed to be a molecule marker to identify *A. anomala*. Chen et al. (2010) concluded ITS2 can accurately identify medicinal plant of species and genera, but no other methods could identify medicinal plant in species, because of cytochrome coxidase 1 sequence, which has been found to be widely applicable in animal DNA barcoding, and it is not appropriate for most species of plants because of a much slower rate of cytochrome coxidase 1 gene evolution in higher plants than in animals (Kress et al., 2005). In our study, the sample of ZJCS sourced from the GAP nursery of Zhongji village, Chuanshan County, which is the first-rank product area of *A. anomala*. According to the identification by Prof. Zhang Yi (College of Ethnomedicines, Chengdu University of Chinese Traditional Medicine), we decided to take the sample of ZJCS (GenBank number: HQ699461) as the famous-region drug, the other samples identified with ZJCS to judge whether they were famous-region drugs. Alignment of all sequences between the sample of ZJCS and other samples, 7 samples were identical with ZJCS, but the other samples had tiny differences. The environmental factor, developmental stage and the intraspecific variation of the plants possibly produce gene mutation in conservation region (for example: ITS region). The reports on mutational analysis revealed that transitions are more probable than transversions (Li and Graur, 1991). Therefore, transversions are considered as
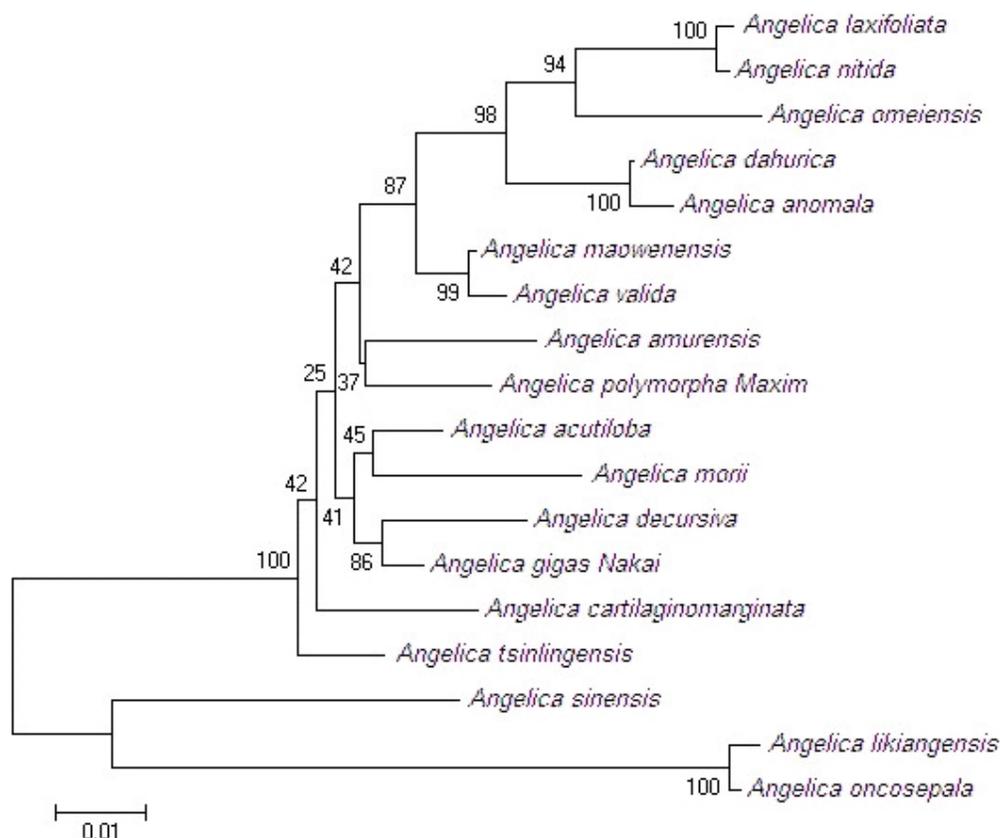
Figure 3. Neighbor-joining tree constructed from the ITS sequences based on Kimura 2-parameter distance. A scale bar showing the distance of 0.01 is at the bottom left.

more reliable mutations in samples (Quicke, 1993). According to the ITS sequence of ZJCS, we identified 14 *A. anomala* collected in Sichuan samples, 7 samples are famous-region drugs, but the others cannot count as famous-region drugs. ITS sequences may serve as a useful tool for identifying herbs of *A. anomala* cultivars collected in Sichuan.

Phylogenetic analysis with other *Angelica* L. species

The internal transcribed spacer is the most commonly sequenced locus used in plant phylogenetic analysis at the species level, and it shows high levels of interspecific divergence (Kress et al., 2005). The maximum parsimony and the neighbor joining trees confirmed that *A. dahurica* had the high similarity with *A. anomala* than any other species. Both trees (Figures 3 and 4) resulted in two major clades, but minor differences exist in these clades between the NJ tree and the MP tree.

The NJ tree (Figure 3) was constructed based on the Kimura 2-parameter distance. Based on this NJ tree (Figure 3), two putative types of ITS might be recognized. The obtained bootstrapping values were strongly supported by the NJ tree (Figure 3), which is indicated by

the high bootstrap support (1,000 replicates). The major clade was formed with *A. anomala*, *A. dahurica, A. tsinlingensis, A. acutiloba, A. laxifoliata, A. nitida, A. omeiensis, A. maowenensis, A. valida, A. amurensis, A. polymorpha Maxim, A. decursiva, A. gigas Nakai* and *A. morii*. The minor clade was formed with *A.sinensis, A. likiangensis* and *A. oncosepala*. It is shown that *A. anomala* and *A. dahurica* are closely related and nested in this phylogeny tree. *A. dahurica* had the high similarity with *A. anomala* than any other species, and it was clustered with *A. anomala* clade and supported by high bootstrap percentages value (100). This might be a result of the lower degree of concerted evolution across ITS multiple copies (Sun et al., 2007). Moreover, more reported data showed that *A. dahurica* was the original plant (Delectis Florae Reipublicae Popularis Sinicae Agendae Academiae Sinicae Edita, 1992). In the minor clade of the NJ tree, *A. likoangensis* and *A. oncosepala* are clustered with least distance, and followed by *A. sinensis*.

The maximal parsimony tree (Figure 4) was constructed by MEGA 4.0. Compared with the NJ tree, the MP tree showed the similar results. The clades of MP tree were similar to those of NJ tree. *A. dahurica* had the high similarity with *A. anomala* than any other species, and   it
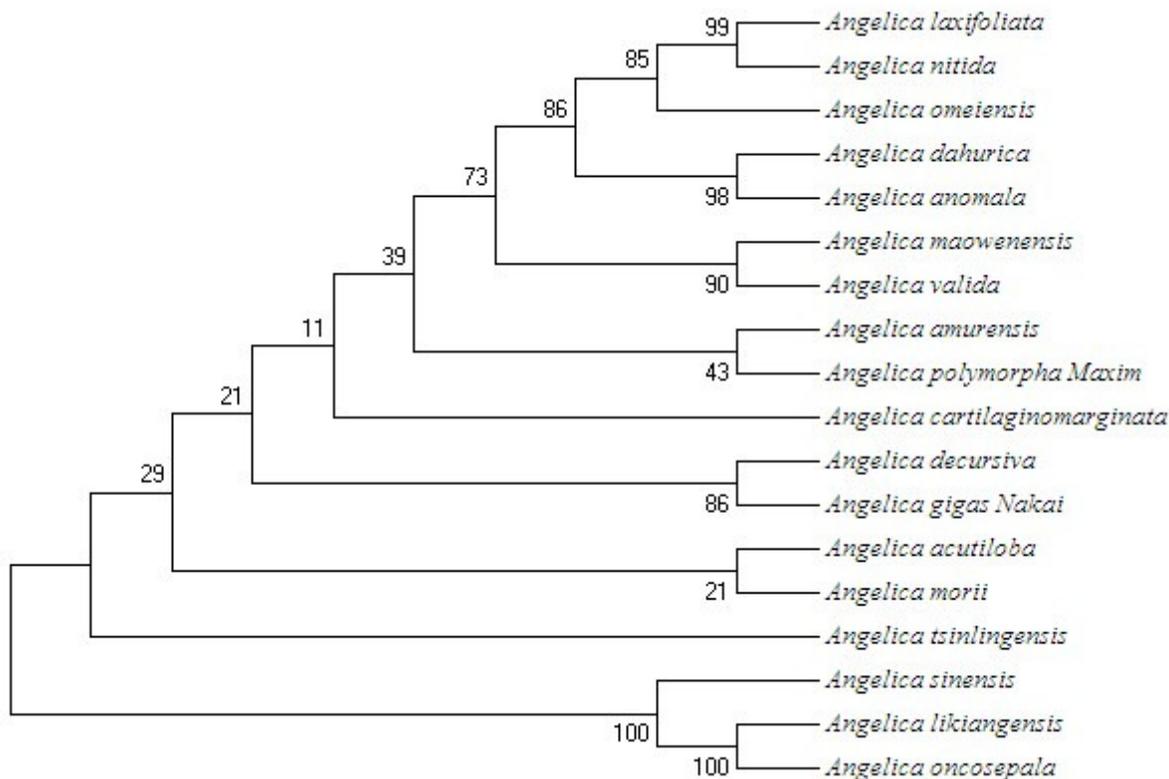
Figure 4. Phylogenetic tree of *Angelica* L. generated using ITS sequence according to Maximum-Parsimonious method supported with bootstrapping analysis.

was clustered with *A. anomala* clade and supported by the high bootstrap percentages value (98). The MP tree further confirmed that *A. anomala* and *A. dahurica* are closely related and nested in this phylogeny tree. In addition, *A. dahurica* was shown the high similarity with *A. anomala* than any other species.

The present study proved the validity of the nrDNA ITS sequence in phylogenetic analysis of *Angelica* genus and paved way for further phylogenetic and/or evolutionary studies of *A. anomala*. The sequence data generated will fine-tune our study of intraspecies population, phylogentic origins, biogeography and molecular evolution.

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