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RESEARCH ARTICLE

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Phylogenetic relationship of genus *Lespedeza* by ITS sequence data

Abstract ITS sequences of ten kinds of plants of *Lespedeza* and an out group were obtained by primer design PCR, sequencing and cluster analysis. The results show that ITS1 section length was 228–243 bp, 5.8S sequence length was 165 bp which was very conservative; ITS2 section lengths varied from 215 to 220 bp and the conservative sites occupied 88.1%. Phylogenetic analysis of ITS sequences using PAUP 4.0 software and their genetic relationship were discussed.

Keywords *Lespedeza* Michx, internal transcribed spacer (ITS), phylogeny

1 Introduction

Most plants which belong to Leguminosae *Lespedeza* distribute widely, with the excellent characteristics of tolerance to drought, deficiency resistance, cold tolerance, heat resistance, acid resistance, as well as tolerance to frequent cutting. These plants are pioneer species for afforestation on barren hills and wastelands and they can serve as feed, soil and water conservation, soil improvement, fuel wood, honey plant and medical use. Due to variable morphology and interspecific similarity, morphology classification of Leguminosae plants is relatively difficult. Therefore, there are various estimations on the number of genus *Lespedeza* from different documents, from 40 to 100. At present, the classical classification method is mainly used in the study of genus *Lespedeza* plants, on the basis of morphological characteristics and ecological distribution. There are some reports in the aspects of cytogenetics (Xia, 1989; Zhang, 1990), palynology (Huang, 1987; Zhang, 1994; Sun, 2001) and isozyme (Zhang et al., 2006), however, there is little connection with phylogeny. As a result, the genetic relationship and phylogeny among taxa are hardly reflected.

Internal transcribed spacer (ITS) is between the rRNA of 18 and 26S, which is divided into two segments by 5.8S, namely ITS1 and ITS2. ITS is of high repetition in nuclear genome, and moreover, there is concerted evolution of intra-site and inter-site among the repetition units by unequal crossing over and gene conversion. That is to say, the sequences among different ITS copies tend to be similar or totally concerted. With high-speed evolution, ITS can provide more variable sites and informative sites, which has been confirmed as an important molecular marker in the study of systematics and evolution concerning angiosperm populations. In recent years, ITS is widely used in plants systematic evolution and genetic relationship, since it can better demonstrate the intra-family, inter-genus and inter-species genetic relationship (Gao et al., 2002; Janet and Ivana, 2003; Zhao et al., 2004).

In this study, the direct sequencing of sequence products was adopted to make an analysis of partial genus *Lespedeza* plants in China. The genetic relationship of inter-species was discussed at DNA molecular level, in order to provide basis for study on ways of systematic evolution regarding genus *Lespedeza* plants, molecular evaluation of germplasm resources, identification, as well as classification. In addition, the study provided theoretical basis for rational utilization and genetic improvement of germplasm resources.

2 Materials and methods

2.1 Material resources

Materials for experiments and their resources are seen in Fig. 1, including three kinds of *Lespedeza* (large) plants

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and seven kinds of *Lespedeza* plants, *Campylotropis macrocarpa* as an out group plant as well.

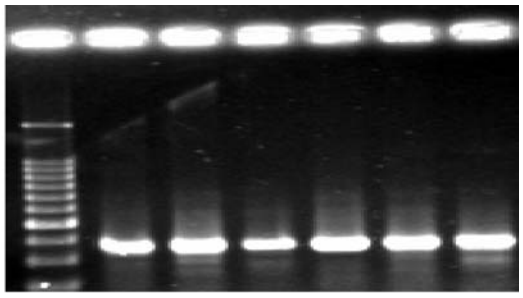

Fig. 1 Profile of PCR products of ITS region

2.2 Extraction of gross DNA of leaves

CTAB method was adopted to extract gross DNA of the plant, agarose gel with the concentration of 1.0% was used to extract quality of DNA by electrophoresis detection, and concentration of DNA was measured by spectrophotometer. The solution was diluted to the same concentration of 1.0% for reserve by aseptic water.

2.3 Amplification, purification and sequencing of ITS sequences

PCR amplification reaction was done on PCR amplifier of 9700 type of PE Company, with reaction system of 50 μL. The reaction system included double-stranded DNA template (about 25 ng), dNTP (200 μmol/L), positive primers and reverse primers (2 μmol/L respectively), *Taq* DNA polymerase (1.5 U), 10×buffer (200 mmol/L Tris-HCl, 100 mmol/L $(NH_4)_2SO_4$, 200 mmol/L KCl, 15 mmol/L $MgCl_2$). The process of amplification reaction is as follows: 10 min pre-degeneration at the temperature of 95°C, 1 min degeneration under the same condition, 1 min annealing at 55°C, and 90s extension at 72°C. The above process should be recycled for 35 times, and then extension should be done for 7 min at 72°C. The primer sequence was 5′-GAAGTAAAAGTCGTAACAAGG-3′, 5′-CCTCCTCCGCTTATTGATATGC-3′. The amplification segments included total ITS sequence, partial sequences of 18S and 16S. Purification and sequencing of amplification products were completed by Qinke Biotechnology Company of Beijing.

2.4 ITS sequences analysis

The sequence ranges of ITS1 and ITS2 were determined by primers and relative sequences (Z97662 and Z97687) of Melilotus officinalis of Leguminosae. The acquired sequences were sequenced by Clustal X version 1.83

3 Results and analysis

3.1 Length and G + C content of ITS sequences regarding genus *Lespedeza* plants

The direct sequencing of PCR products was adopted to measure the ITS sequences of 10 kinds of *Lespedeza* plants, with the length about 760 bp, which are shown in Fig. 1, and G + C content can be seen in Table 2. The length of ITS1 ranged from 228 to 243 bp, with the maximum difference of 15 bp and the average of 236 bp, which shows not much difference with other Leguminosae plants (Janetl and Ivanas, 2003). There was consistency in 5.8S of *Lespedeza*, with 165 bp respectively, which shows that this segment tends to be conservative; the length of ITS2 ranged from 215 to 220 bp, with the maximum difference of 5 bp and the average of 216 bp; the gross lengths of ITS sequences concerning 10 kinds of *Lespedeza* plants was all from 612 to 622 bp, with the average G + C content of 51%. In addition, the length of ITS sequences of *Lespedeza* plants shows consistency with the length of angiosperms which have been reported.

Table 1 The sources of materials

number	species	sources	note
1	*Lespedeza bicolor*	Pingshan, Heibei Province, China	*Lespedeza* (large)
2	*Lespedeza formosa*	Lushan, Jiangxi Province, China	*Lespedeza* (large)
3	*Lespedeza cyrtobotrya*	Haicheng, Liaoning Province, China	*Lespedeza* (large)
4	*Lespedeza mucronata*	Yantai, Shandong Province, China	*Lespedeza* (small)
5	*Lespedeza tomentosa*	Miyun, Beijing Province, China	*Lespedeza* (small)
6	*Lespedeza floribunda*	Yantai, Shandong Province, China	*Lespedeza* (small)
7	*Lespedeza daurica*	Chifeng, Inner Mongolia, China	*Lespedeza* (small)
8	*Lespedeza potaninii*	Yanchi, Ningxia Province, China	*Lespedeza* (small)
9	*Lespedeza inschanica*	Pingliang, Gansu Province, China	*Lespedeza* (small)
10	*Lespedeza cuneata*	Tianshui, Gansu Province, China	*Lespedeza* (small)
11	*Campylotropis macrocarpa*	Xinyang, Henan Province, China	out group

Table 2 Lengths (bp) and G + C contents of ITS sequences of 10 species of *Lespedeza*

species	ITS1		ITS2		5.8S	ITS (including 5.8S)	
	length/bp	G + C/%	length/bp	G + C/%	length/bp	length/bp	G + C/%
L. bicolor	241	49.7	215	51.8	165	620	50.0
L. formosa	241	47.7	216	51.1	165	621	49.8
L. cyrtobotrya	243	47.0	215	52.3	165	622	49.8
L. mucronata	228	49.9	220	53.2	165	612	51.3
L. tomentosa	228	49.9	220	53.3	165	612	51.3
L. floribunda	230	49.5	218	52.5	165	613	50.8
L. daurica	239	50.5	216	54.3	165	619	52.0
L. potaninii	239	50.5	215	54.0	165	618	52.0
L. inschanica	239	50.9	215	54.0	165	618	52.2
L. cuneata	228	50.4	216	53.8	165	618	52.1
mean	235.6	49.6	216.6	53.03	165	617.3	51.13

3.2 Clustering analysis of ITS sequences of *Lespedeza* plants

As the default, gap was used to analyze 637 sites by the software PAUP 4.0 after sequencing, and there were 507 constant characters among them, 76 variable sites (including 54 informative sites, taking up 8.48%), occupying 11.93%. The genetic distances among 10 samples were calculated and are showed in Table 3. And what's more, UPGMA method was used to make an analysis of the phylogenetic relationship of 10 kinds of *Lespedeza* plants, and phylogenetic relationship tree was set up, which is shown in Fig. 2. The length of the simplest tree is 158 steps, with consistency index (CI) of 0.9051 and retention index (RI) of 0.8074. CI, which is larger than 0.9, demonstrates that the phylogenetic relationship tree has rather high credibility. Bootstrap was used in the largest simple tree to analyze the bootstrap value of each branch, taking *Campylotropis macrocarpa* as an out group. The results show that three kinds of *Lespedeza* plants of the group of *Lespedeza* (large) clustered as one group, with 100% bootstrap value. In the *Lespedeza* (small) group, *L. inschanica* and *L. potaninii* first clustered together, and then they clustered with *L. daurica* and *L. cuneata* in order, with 99% bootstrap value. The two large branches connected with another branch, which was composed of *L. floribunda*, *L. mucronata* and *L. tomentosa*, and they got the bootstrap value of 76%.

4 Conclusions and discussion

Plants of *Lespedeza* distribute widely, which form the interval distribution from East Asia to North America across the Pacific Ocean, reaching subfrigid zone northward and Australia southward. Apart from Xinjiang Uygur Autonomous Region, plants of *Lespedeza* distribute all the other provinces of China. There are huge differences among geographical and ecological factors in the distribution area, which leads to the huge morphological variation

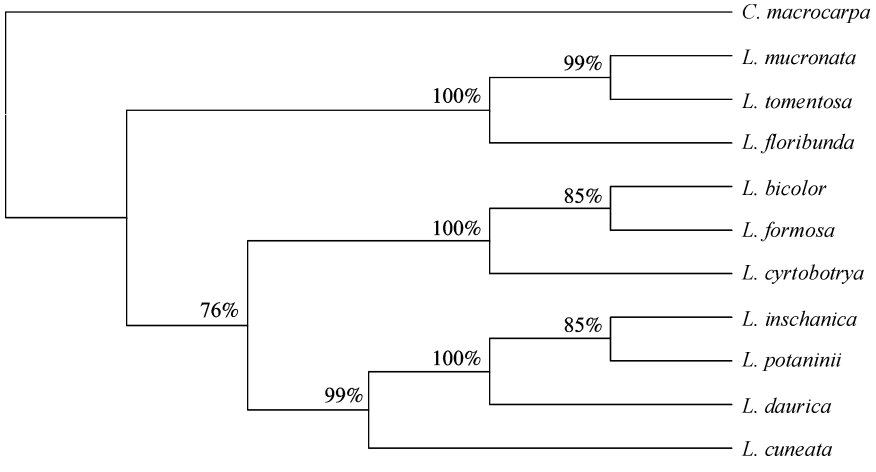

Fig. 2 Phylogenetic tree reconstructed using UPGMA with maximum likelihood distance based on ITS nucleotide sequence

Table 3 Pairwise genetic distances of nrITS and 5.8S rDNA sequences of 10 species of *Lespedeza* and *C. macrocarpa*

species	1	2	3	4	5	6	7	8	9	10	11
L. bicolor	0										
L. formosa	0.0081	0									
L. cyrtobotrya	0.0306	0.0372	0								
L. mucronata	0.0597	0.0682	0.0879	0							
L. tomentosa	0.0547	0.0632	0.0828	0.0049	0						
L. floribunda	0.0582	0.0634	0.0881	0.0197	0.0148	0					
L. daurica	0.0642	0.0727	0.0884	0.0662	0.0629	0.0684	0				
L. potaninii	0.0623	0.0708	0.0865	0.0661	0.0612	0.0666	0.0016	0			
L. inschanica	0.0625	0.0711	0.0852	0.0663	0.0614	0.0666	0.0032	0.0010	0		
L. cuneata	0.0573	0.0658	0.0833	0.0679	0.0629	0.0699	0.0309	0.0292	0.0293	0	
C. macrocarpa	0.1150	0.1220	0.1364	0.0102	0.0999	0.1054	0.1109	0.1107	0.1107	0.1144	0

of *Lespedeza* plants. Different experts have various estimations on the species number of *Lespedeza* plants, ranging from 40 to 100, with large difference. According to *China Flora*, there are 26 kinds of *Lespedeza* plants that distribute in China, which can be divided into 2 groups based on chasmogamous and cleistogamous flower: the group with chasmogamous flower is *Lespedeza* (large), which has 12 kinds of plants; the group with cleistogamous flower is *Lespedeza*, with 14 kinds of plants. As can be seen in Fig. 2, classification of the ten kinds of *Lespedeza* plants does not accord with the traditional morphological classification. Instead, the classification is like this: in the group of *Lespedeza* (large), *L. bicolor* and *L. formosa* first cluster together, and then they cluster with *L. cyrtobotrya*; in the group of *Lespedeza* (small), *L. inschanica* and *L. potaninii* first cluster together, and cluster with *L. daurica* and *L. cuneata*, while the group member *L. floribunda*, *L. mucronata* and *L. tomentosa* do not cluster with them directly. Instead, they separate and show far genetic relationship with other four kinds of *Lespedeza* plants in the group of *Lespedeza* (small). These three kinds of separate species are the middle types of evolution developing from a lower grade to a higher grade. This finding is similar to the result by ISSR molecular marker (Zhao et al., 2006).

Hong (2003) took the cladistic analysis to discuss the relationship of origin and evolution of 12 groups of *Lespedeza* plants populations distributed in Inner Mongolia by their 28 stable morphological shape. She pointed out that the phylogenetic order of *Lespedeza* plants distributed in Inner Mongolia was as follows: *L. cyrtobotrya*→ *L. bicolor*→ *L. floribunda*→ *L. inschanica*→ *L. juncea*→ *L. caraganae*→ *L. tomentosa*→ *L. daurica*→ *L. potaninii.* There was close genetic relationship among *L. floribunda*, *L. bicolor* and *L. cyrtobotrya.* However, in this study, the genetic relationship among them was not very close.

At present, the study on genetic diversity and genetic relationship of plants *Lespedeza* just begins, and because of the differences of adopted materials and methods by various researchers, the acquired results of phylogenetic order and inter-species genetic relationship are not the same. Meanwhile, the species for study were few with a single method, which cannot give a comprehensive evaluation on genetic diversity and genetic relationship regarding plants of *Lespedeza*. Therefore, it is necessary that new methods should be adopted to select more species to do further research on genetic diversity and genetic relationship, which will provide theoretical basis for classification and phylogeny concerning plants of *Lespedeza*.

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References

Gao L M, Zhu L D, Zhang C Q (2002). Infrageneric and sectional relationships in the genus *Rhododendron* (Ericaceae) inferred from ITS sequence data. J Plants, 44(11): 1351–1356

Hong X (2003). Research on classification of *Lespedeza* Michx plants of Inner Mongolia. Huhhot: Inner Mongol Normal Univ (in Chinese)

Huang P H (1987). Research on pollen morphology and classification of *Lespedeza* and its related genus in northeastern China. J Syst Evol, 25 (5): 366–369 (in Chinese)

Janet L, Ivana S (2003). Christian brochmann ribosomal ITS sequences and plant phylogenetic inference. Mol Phylogenet Evol, 29 (3): 417–434

Sun J T (2001). Pollen morphology and its taxonomic signification of *Lespedeza* Michx. from Shandong. J Shandong Normal Univ (Nat Sci), (2): 190–193 (in Chinese)

Xia Y J (1989). Research on karyotype of ten types of *Lespedeza* plants. Chin J Grassland, 3: 17–19 (in Chinese)

Zhang J Y, Yuan Q H, Wang Y R, Zhang W S (2006). RAPD analysis on genetic diversity of wild population of *Lespedeza* Michx. Acta Agrest Sin, 14(3): 214–218 (in Chinese)

Zhang Y L (1994). Research on pollen morphology of some honey plants in China. Wuhan Plants Study, 9(4): 317–320 (in Chinese)

Zhang Y X (1990). Research on karyotype of *Lespedeza* plants. J Shanxi Agric Univ (Nat Sci Ed), 13(1): 87–89 (in Chinese)

Zhao W G, Pan Y L, Zhang Z F (2004). Phylogenetic relationship of genus *Morus* by ITS sequence data. Acta Sericol Sin, 30(1): 11–14 (in Chinese)

Zhao Y, Chen X Y, Wang X R, Sun W S (2006). Inter-simple sequence repeat (ISSR) analysis on genetic relationship in *Lespedeza* spp.. Jilin For Sci Technol, 35(3): 1–4 (in Chinese)