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RESEARCH ARTICLE

Application of *pet*G-*trn*P sequence to systematic study of Chinese *Cupressus* species

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Abstract Chinese *Cupressus* L. includes five species. The molecular phylogenetic relationships of the *Cupressus* species and *Chamaecyparis* L. were determined by comparing 417—479 bp of chloroplast *pet*G-*trn*P intergenic spacer sequence. In PAUP* analysis, *Platycladus orientalis* was used as the functional out group. By using the maximum likelihood method 1 077 trees were examined and the result showed that one tree had a best score of –Ln=2 232.47. The phylogenetic tree clearly showed that *Chamaecyparis nootkatensis* was diverged from other *Chamaecyparis* species. Based on the results, together with evidences from other aspects, we consider that *Cupressus funebris* and *Chamaecyparis nootkatensis* should be placed in the genus *Cupressus.* The use of cpDNA intergenic spacer *pet*G-*trn*P in *Cupressus* was also discussed.

Keywords *Cupressus*, *pet*G-*trn*P sequence, molecular phylogeny

1 Introduction

Cupressus L. (Cupressaceae), including about 17 species, is an old, distinctive genus in the family Cupressaceae. It originated from the area of the Mediterranean Sea, and now the family is found in North Africa, Asia, South Europe, and south-western North America. There are nine species (four endemic, four introduced, and one variety) in China (Fu et al., 2000). *Cupressus funebris* (Endlicher) Franco is widely distributed and is also widely cultivated in South China, and grows at an elevation below 2 000 m. *Cupressus ducloxiana* Hickel disperses on the dry slopes at an elevation of 1 400 to 3 300 m in Guizhou, South-Western Sichuan Province, Central and North-Western Yunnan Province, South-Eastern Tibet, China. *Cupressus chengiana* S. Y. Hu appears at an elevation of 800 to 2 900 m in South Gansu Province, Central and Northern and Western Sichuan Province, China. While *Cupressus gigantea* W. C. Cheng and L. K.Fu is at an elevation of 3 000 to 3 400 m in South-Eastern Tibet, China, which often form a pure forest in slopes along the rivers. *Cupressus torulosa* D. Don is distributed at an elevation of 1 800 to 2 800 m in Eastern and Southern Tibet, China, and also in Bhutan, Northern India, Kashmir, Nepal, Sikkim, and Vietnam. Four species, except *C. funebris*, growing along the edge of the Qinghai-Tibet Plateau, in turn form a narrow distributing region of *Cupressus* from west to east, with other two species, *C. cashmeriana* Royle ex Carrière and *C. sempervirens* Linnaeus which discontinuously distributes from the Mediterranean Sea to West Asia (Guan, 1981). Therefore, the systematic study on Chinese *Cupressus* species is very important in illuminating the phylogenetic relationships of the genus *Cupressus*.

Past studies on the Chinese *Cupressus* species just focused on the morphology, cytogeography, and isozyme analysis (Jiang and Wang, 1985; Li and Fu, 1996; Fu et al., 2000), but there still exists a dispute about the taxonomic position of some species. For instance, *C. funebris* was placed in the genus *Cupressus* by Dallimore and Jackson (1966) and Zheng and Fu (1978). While Bailey and Bailey (1976) placed it in the genus *Chamaecuparis*. On the other hand, *Chamaecyparis nootkatensis* (D. Don)Spach whose morphological characteristics are similar with those of *C. funebris* was placed in *Cupressus* by Welch (1991) and Frankis (1993). But Farjon et al. (2002) and Damon et al. (2004) classified it as a new genus *Xanthocyparis*. Because of its confused taxonomic situation, it is necessary to re-analyze the phylogeny of *Cupressus* at a molecular level for the evolutionary pathway of these species.

Recently, chloroplast DNA has been used in the phylogenetic study of plants because of its low evolutionary rate at high taxonomic levels. And amplification of non-coding cpDNA sequences using several universal

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primers have reconstructed the phylogeny at lower taxonomic levels (Taberlet et al., 1991; Demesure et al., 1995), even at both interspecific (Gielly and Taberlet, 1994; Bruneau, 1996; Asmusen and Liston, 1998) and intraspecific taxon (Dumolin et al., 1997; Petit et al., 1997). With regard to the cypress, Wang et al. (2003) has used the *pet*G-*trn*P sequence on the phylogenetic reconstruction in *Chamaecyparis*.

In the present study, the *pet*G-*trn*P intergenic spacer sequences of five *Cupressus* species and four *Chamaecyparis* were amplified, and molecular phylogeny of these species were examined for the objectives:

(1) To explore the phylogenetic relationships between the two genus;

(2) To provide a molecular evidences for the taxonomic position of some cryptic species.

2 Materials and methods

2.1 Plant materials

We examined DNA sequences from one individual for *Platycladus orientalis* (L.) Franco, and five Chinese *Cupressus* species, and other DNA sequences of the *Chamaecyparis* species were obtained from GeneBank (Table 1). Young leaf tissue of *C. chengiana* was collected from natural forest at Lixian, Sichuan Province, China. *C. gigantean* was collected at Linzhi, Tibet, China. *C. torulosa*, *C. ducloxiana,* and *C. funebris* were collected from the cultivated trees at Kunming Arboretum, Kunming, Yunnan Province, China.

Table 1 The origin of materials used in the research

Taxon	Voucher	Locality or reference
C. chengiana	SZ00016188	Lixian, Sichuan Province, China
C. gigantean	TE1255896	Linzhi, Tibet, China
C. torulosa	TE1605744	Kunming Yunnan Province, China
C. ducloxiana	SZ00016199	Kunming, Yunnan Province, China
C. funebris	SZ00016267	Kunming, Yunnan Province, China
Ch. nootkatensis	AF435875	Wang et al. (2003)
Ch. pisifera	AF435879	Wang et al. (2003)
Ch. formosensis	AF435873	Wang et al. (2003)
P. orientalis	SZ00016768	Chengdu, Sichuan Province, China

Note: *C* is abbreviation of *Cupressus; Ch* is abbreviation of *Chamaecyparis; P* is abbreviation of *Platycladus*

2.2 Molecular techniques and sequencing strategy

Leaves (0.1 g) were ground with liquid nitrogen. Ground leaf powder was placed in 1 mL extraction buffer for genomic DNA extraction based on a modified CTAB procedure (Doyle and Doyle, 1987).

Double-stranded templates for direct sequencing were amplified by the polymerase chain reaction (PCR). PCR amplification was achieved using the primers for *pet*G-*trn*P (5′-GGTCTAATTCCTATAACTTTGGC-3′ and 5′-GGG-ATGTGGCGCAGCTTGG-3′) (Hwang et al., 2000). Reactions of PCR amplification were conducted on a Biorad MyCycler™ PCR instrument. The cycle of the PCR reaction consisted of 3 min at 95°C for denaturation, followed by 40 cycles of 45 sec at 94°C for denaturation, 1 min at 55°C for annealing, and 1 min at 72°C for polymerization. The final PCR reaction was 7 min at 72°C for polymerization. PCR products were purified using Omega purification kit and then sequenced using Model ABI377 automated sequencer (Applied Biosystems). For sequencing, we used the same primers as those used for amplification.

2.3 Sequence alignment and phylogeny assessment

Multiple alignments of the sequences were obtained using CLUSTAL X (Julie et al., 1997) with manual correction. Then statistical analysis on the sequences data were computed by MEGA 2.0 (Kumar et al., 2000). A maximum likelihood analysis was conducted with PAUP*4.0 (beta version 4.0b10) (Kumar, 2002) after random addition of sequences. Heuristic searches with 100 random entries were performed using HKY (Hasegawa et al., 1985) options of PAUP*. Gaps were treated as missing data and all characteristics were accorded with equal weight. The amount of support for monophyletic groups was evaluated by 1000 bootstrap replicates (Felsenstein, 1985).

3 Results

3.1 Nucleotide sequences and variation

Double-stranded DNA amplifications and sequences were obtained for all studied taxa. The non-coding sequences of *pet*G-*trn*P intergenic spacer comprised 31.6% A and 31.2% T (Table 2), which agreed with high A+T rich data from most non-coding spacers (Li, 1997). The chloroplast *pet*G-*trn*P intergenic spacer sequences generated for the five species of *Cupressus* varied from 417 to 478 bp in length before alignment, and it was closed to the *Chamaecyparis* species. The aligned *pet*G-*trn*P intergenic spacer sequences in *Cupressus*, *Chamaecyparis,* and out group contained a total length of 517 bp. Absolute distances and mean distances were calculated by MEGA (Table 2). Among the 10 sequences of *Cupressus*, *Chamaecyparis,* and out groups, 328 of the 539 aligned characteristics were variable and 289 (55.9%) were phylogenetically informative (Table 3).

Sequence data of chloroplast *pet*G-*trn*P intergenic spacer were used to deduce preliminary phylogenetic analysis with *Platycladus orientalis* as an out group species. Using the maximum likelihood method (HKY model), we examined 1 077 trees and found one tree with a best score of –Ln= 2 232.47, and several features were indicated (Fig. 1). All Chinese *Cupressus* formed a strong supported monophyletic clade (bootstrap= 91%), inside which *Ch. nootkatensis* was nested deeply. *C. chengiana* and *C. gigantean* shared a most

recent common ancestor and was a sister group compared to other *Cupressus*. Meanwhile, *Chamaecyparis* group containing *Ch. obtuse*, *Ch. taiwanensis* and *Ch. Thyoides*, was also monophyletic with high bootstrap support (97%).

Table 2 Size and nucleic acid content of *pet*G-*trn*P sequence of nine species in *Cupressus* and *Chamaecyparis*

	T /%	C /%	A /%	G /%	Size /bp
C. chengiana	30.5	18.5	32.4	18.7	417
C. gigantean	30.6	18.2	32.4	18.9	435
C. torulosa	30.7	17.4	33.4	18.5	437
Ch. nootkatensis	31.7	17.9	31.3	19	457
C. ducloxiana	31.6	19	31.6	17.8	478
C. funebris	32	18.1	32	17.9	469
Ch. thyoides	30.5	20.3	30.9	18.4	479
Ch. taiwanensis	31.1	18.9	30.7	19.3	476
Ch. obtuse	31.1	19.1	30.5	19.3	476
Mean	31.2	18.5	31.6	18.8	450.6

Note: *C* is abbreviation of *Cupressus; Ch* is abbreviation of *Chamaecyparis; P* is abbreviation of *Platycladus*

Table 3 Pairwise distances between taxa used in the present study

	1	2	3	4	5	6	7	8	9
1 *C. chengiana*		0.004	0.026	0.022	1.059	1.036	0.150	0.140	0.140
2 *C. gigantean*	1		0.022	0.018	1.041	1.019	0.145	0.136	0.136
3 *C. torulosa*	7	6		0.007	1.078	1.073	0.154	0.154	0.154
4 *Ch. nootkatensis*	6	5	2		1.064	1.059	0.149	0.149	0.149
5 *C. ducloxiana*	153	152	154	153		0.011	1.123	1.046	1.046
6 *C. funebris*	152	151	154	153	3		1.16	1.079	1.079
7 *Ch. thyoides*	37	36	38	37	157	59		0.038	0.038
8 *Ch. taiwanensis*	35	34	38	37	153	155	10		0
9 *Ch. obtuse*	35	34	38	37	153	155	10	0	

Below diagonal: Absolute distances; Above diagonal: Mean distances

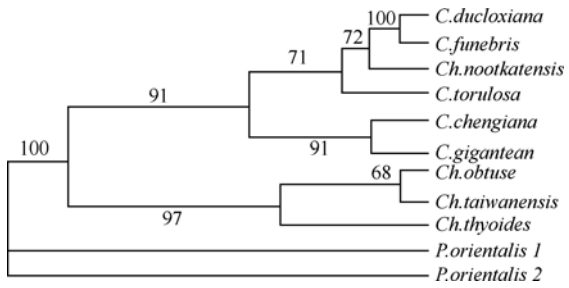

Fig. 1 Maximum likelihood tree generated from cpDNA *pet*G-*trn*P sequences using *Platycladus orientalis* as an out group

4 Discussion

4.1 Sequence variations and phylogenetic utility

The phylogenetic utility of the chloroplast *pet*G-*trn*P intergenic spacer in the study on *Chamaecyparis* and *Cunninghamia* has been successful (Hwang et al., 2003; Wang et al., 2003). In the genus *Chamaecyparis*, the aligned length is 517 characters with 113 (21.9%) variable and 41 (7.9%) informative (excluding the out groups). Comparatively, there are 505 characters with 281 (55.6%) variable and 253 (50.1%) informative in the genus *Cupressus*. The sequences of *pet*G-*trn*P offered enough information and clearly illustrated the interspecies relationship in the genus *Cupressus*. In addition, *pet*G-*trn*P intergenic spacer could be useful in the phylogeographic study on *Cupressus* in the future. In our study, we have confirmed that chloroplast non-coding sequences could be utilized as genetic markers in the study on interspecific variation in the genus *Cupressus*.

4.2 Phylogenetic relationships of *Cupressus*

Rushforth et al. (2003) have studied the variation among *Cupressus* species from the eastern hemisphere based on RAPD (Random Amplified Polymorphic DNAs), and found that the clade of *C. ducloxiana* and *C. gigantean* diverged from the clade of *C. chengiana*, *C. torulosa,* and *C. funebris*. Nevertheless, the studies on Chinese *Cupressus* species with plant chemotaxonomy by Laurence et al. (1998) found that *C. chengiana*, *C. torulosa*, *C. ducloxiana,* and *C. funebris* constitute a group and *C. gigantean* forms another group solely. In the present research, the result that Chinese *Cupressus* species shared a most recent common ancestor and formed a monophyletic group (91% bootstrap support), was similar with that of RAPDs (Rushforth et al., 2003) and chemotaxonomy data (Laurence et al., 1998). But phylogenetic analysis with non-coding sequence *pet*G-*trn*P intergenic spacer showed that Chinese *Cupressus* species consisted of two groups: one clade comprising *C. chengiana* and *C. gigantean* (91% bootstrap support) and another clustered by *C. torulosa*, *C. ducloxiana*, *C. funebris*, and *Ch. nootkatensis* (71% bootstrap support), which showed some differences from previous studies. In addition, for the similarities of morphologic characters to *C. torulosa*, Fu et al. (2000) suggested *C. gigantean* was better treated as a variety of *C. gigantean*. However, *C. torulosa* and *C. gigantean* were separated into two clades respectively in the present studies, suggesting that the two species have no close relationship at the molecular level.

4.3 The phylogenetic relationship of *C. funebris*

C. funebris was often classified into *Chamaecyparis* based on its flattened foliage sprays and relatively few seeds in small cones. However, its developmental characteristics

(cones maturing in second year) was similar to that of *Cupressus* species (Fu et al., 2000). In the present study, a clade consisting of five *Cupressus* species (100% bootstrap support) strongly supported the results provided by Dallimore et al. (1966) and Zheng et al. (1978) that *C. funebris* should be placed in genus *Cupressus*. Furthermore, the opinions of Li and Fu (1996) on cytotaxonomy and Laurence et al. (1998) on chemotaxonomy also supported this affiliation. On the other hand, the relationship of *C. funebris* and *C. ducloxiana* was closely related based on the molecular evidences despite of their differences in morphology (Fu et al., 2000).

4.4 The phylogenetic relationship of *Ch. nootkatensis*

Ch. nootkatensis was placed in *Cupressus* by Lambert (1824), Welch (1991), and Frankis (1993). While Spach (1842) treated it as *Chamaecyparis*. However, Farjon et al. (2002) transfered it to a new genus *Xanthocyparis,* which had been supported by Damon et al. (2004). Based on the analysis of DNA sequence, *Ch. nootkatensis* clusters with all *Cupressus* species. Therefore, it supported the opinion that *Ch. nootkatensis* was distant from *Chamaecyparis*, and should be in the genus *Cupressus*.

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