

Full Length Research Paper

# Genetic relationships of poplar species in section *Tacamahaca* based on cpDNA and ISSR

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A molecular investigation on the species of *Populus* belonging to section *Tacamahaca* was undertaken with the aim of inferring genetic relationships within the section. The ISSR and cpDNA RFLP-based UPGMA analyses were performed for seven *Populus* species, with 50 specimens representing 10 accessions from southeastern Qinghai-Tibetan Plateau, China. The results provided molecular evidence suggesting that *Populus schneideri* is highly genetically similar to *Populus kangdingensis*, as also indicated by previous studies with respect to their morphological characters. Similarly, the combination of cpSSR and nuclear SSR analyses showed that *Populus trinervis* and *Populus simonii* have a close genetic affinity, indicating their closed sister relationships. Although *Populus yunnanensis* is distinct from the other species based on nuclear ISSR analysis; it formed a clade with *P. simonii* and *P. trinervis*. This reflects similar maternal phylogeny among the three species. In addition, the cluster analyses clearly showed a close relationship among accessions of the same species and suggested monophyly in *Populus przewalskii* and *Populus cathayana*. Our results demonstrated that the combination of cpDNA-RFLP and ISSR molecular markers enables a clear estimation of genetic relationships among *Populus* species and they provided information for preliminary conclusions that will be helpful in guiding future research.

**Key words:** *Populus*, section *Tacamahaca*, genetic relationships.

## INTRODUCTION

The genus *Populus* L. is a member of the Salicaceae family and is subdivided into six sections. They are widely distributed in the forests of temperate and cold regions of the Northern hemisphere (Brawdshaw et al., 2000; Cervera et al., 2005). *Populus* are major supplier of industrial wood and fiber in many countries and have become the model of choice for molecular genetics and genomics research on forest trees because of their fast growth rates, easy vegetative propagation, adaptability to a variety of ecological sites and amenability to genetic transformation (Fossati et al., 2005; Xu et al., 2009). The southeastern Qinghai-Tibetan Plateau is abundant in poplar gene resources including 17 species and 15 varieties identified. With 11 species, it alone comprise

46% of the 24 endemic poplar species of the section *Tacamahaca* occurring in China (Yu et al., 2003). In fact, southeastern Qinghai-Tibetan Plateau is regarded as the natural distribution and variation center for poplar species of the section *Tacamahaca* in China. The vertical distribution of these poplar trees ranges from 1,300 to 4,300 m above sea level (a.s.l.). Even under unfavourable climatic conditions at high altitudes and with a short growth season, some species (that is *Populus kangdingensis*) stand out due to their high vitality, remarkable growth potential and great adaptability. However, little is currently known about the majority of the poplar species occurring in this region. Although *Populus* is the model tree species for biological research, information on interspecific phylogenetic relationships in the genus is rather limited (Fussi et al., 2010). The placement of species within a section has traditionally been based on morphological and reproductive characters, as well as interspecific crossability. However,

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**Table 1.** The investigated *Populus* specimens, all of the section *Tacamahaca*, and their natural altitude distribution, codes and origin.

Species	Common name	Natural altitude distribution (m)	Codes	Origin
<i>P. kangdingensis</i> , C. Wang et Tung	Kangding poplar	2,100 ~ 4,300	KD	Kangding, Sichuan
<i>P. schneideri</i> , Rehd.	-	2,650 ~ 3,900	DC	Daocheng, Sichuan
<i>P. przewalskii</i> , Maxim	-	2,900 ~ 3,400	AB XN	Aba, Sichuan Xining, Qinghai
<i>P. cathayana</i> , Rehd	Green poplar	1,900 ~ 3,000	JZ PW HY	Jiuzhai, Sichuan Pingwu, Sichuan Hanyuan, Sichuan
<i>P. trinervis</i> , C. Wang et Tung	Threevein poplar	1,500 ~ 2,500	KDSM	Kangding, Sichuan
<i>P. simonii</i> , Carr.	Simon poplar	1,250 ~ 2,400	KDXY	Kangding, Sichuan
<i>P. yunnanensis</i> , Dode	Yunnan poplar	1,300 ~ 3,000	LD	Luding, Sichuan

classical taxonomic analysis based on morphological characteristics has proven to be very difficult because of wide intraspecific variability, high natural crossability among members of the genus, and the convergent morphology shown by hybrids and their parental species (Cervera et al., 2005).

Since the late 1980s, several molecular marker approaches have been successfully used in a number of poplar species for the fingerprinting and identification of clones and the determination of their interrelationships: isozymes, RAPD, ISSR, AFLP and SNP markers (Liu and Furnier 1993; Rahman and Rajora 2002; Gao et al., 2006; Olson et al., 2010), mitochondrial and chloroplast restriction site analyses (Barrett et al., 1993; Heinze 1998), and sequence analyses primarily on the nuclear ITS and on the noncoding chloroplast regions (Hamzeh and Dayanandan, 2004). In addition, the combined use of cpDNA (maternally inherited marker) and nuclear DNA (biparentally inherited marker) data is crucial when aiming to gain a more comprehensive understanding of the evolutionary history of plants (Hamzeh and Dayanandan, 2004). In the present study, cpDNA and nuclear ISSR markers were used to differentiate seven closely related *Populus* species belonging to the section *Tacamahaca* and to estimate their genetic relationships.

## MATERIALS AND METHODS

### Plant materials and DNA extraction

Seven species of the genus *Populus* in section *Tacamahaca* from southeastern Qinghai-Tibetan Plateau, China were analyzed in this study. Details of the collection sites, natural altitude distributions and accession codes are given in Table 1. DNA was extracted from 0.5 g fresh young leaf material from each individual following a

modified protocol of Castiglione et al. (1993). Concentrations and quality of DNA was checked by a DNA-Protein instrument (Bio-RAD). In our study, the DNA mixing strategy was used for the ISSR and cpDNA PCR-RFLP assay. For this template-mixing strategy, equal amounts of working solution DNAs from each five individuals of the same accession were pooled as template DNA prior to the PCR reaction.

### ISSR amplification

Initially, 29 ISSR markers which were used to construct a genetic map of the *Populus* genome (Yin et al., 2002) and 30 ISSR markers developed by the Biotechnology Laboratory, University of British Columbia (UBC, Vancouver, Canada) were tested. Based on the clarity and reproducibility of the band patterns, 11 primers were selected from the 59 primers tested for the further studies (Table 2). PCR amplification was performed in a 20- $\mu$ l reaction volume containing 2.0  $\mu$ l of the 10  $\times$  reaction buffer (TaKaRa, Dalian, Mg<sup>2+</sup> free), 1.8 mM MgCl<sub>2</sub> buffer, 150  $\mu$ M dNTP (Promega), 0.25  $\mu$ M primer, 1.0 U Taq polymerase (TaKaRa, Dalian) and 25 ng of genomic DNA. The mixture was overlaid with mineral oil and subjected to PCR on a GeneAmp® PCR System 9700 (Perkin Elmer Corp., Norwalk, CT, USA) programmed for an initial step of 5 min at 95°C, followed by 40 cycles of 1 min at 94°C, 1 min at annealing temperature (Table 2) and 2 min at 72°C, with a final extension step of 10 min at 72°C. PCR products were analyzed on 1.8% agarose gels and stained with ethidium bromide. The gel images were recorded and the band sizes were quantified using a Gel Doc 2000 system (Bio-RAD). Each PCR reaction was performed at least twice to ensure the reproducibility.

### PCR-amplification of cpDNA and mtDNA non-coding regions

Five sets of chloroplast primers and four sets of mitochondrial primers were used to amplify non-coding regions of cpDNA and mtDNA shown in Table 3 (Demesure et al., 1995). The amplification reactions were performed in a volume of 25  $\mu$ l containing reaction buffer, 200  $\mu$ M of dNTP (Promega), 0.2  $\mu$ M of each primer, 1.0 U of Taq polymerase (TaKaRa) and 20~50 ng of DNA. The PCR

**Table 2.** The nucleotide sequences of the eight selected ISSR primers and the number of bands scored.

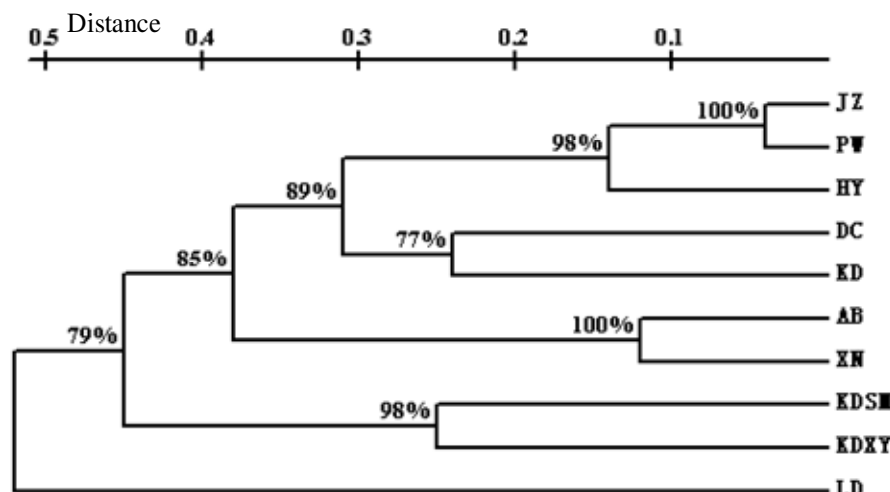
Primer	Primer sequences (5'-3')	Annealing temp. (°C)	No. of alleles	PIC value
IP-2	(AC) <sub>8</sub> C	54	12	0.454
IP-4	(GA) <sub>8</sub> CTT	54	8	0.455
24	(AC) <sub>8</sub> TC	55	16	0.486
33	(AG) <sub>8</sub> AT	53	17	0.398
177	(AC) <sub>8</sub> YG	52	9	0.432
807	(AG) <sub>8</sub> T	55	11	0.497
808	(AG) <sub>8</sub> C	56	13	0.401
809	(AG) <sub>8</sub> G	57	12	0.382
826	(AC) <sub>8</sub> C	53	11	0.392
834	(AG) <sub>8</sub> YT	53	7	0.334
848	(CA) <sub>8</sub> RG	55	14	0.422

**Table 3.** The cpDNA and mtDNA primer pair sequences and annealing temperatures.

Locus	Approximate size of fragment (bp)	Primers (5'-3')	Annealing temp. (°C)
ORF-M	1050	CTTGCTTTCCAATTGGCTGT CATAACCTTGAGGTCACGGG	55
CD	2300	TTCCCGGTAGCACATACACA ATTTGAACTGGTGACACGAG	58
cpDNA DT	800	TGTTCGATAGCAAGTTGATTG GAGTTAGTTGAACTTATCACTC	55
TF	1500	CATTACAAATGCGATGCTCT ATTTGAACTGGTGACACGAG	58
17+20	800	GAAGTAGTAGGATTGATTCTC CCCTACAACCTCATGAATTAAG	55
nad1 2-nad1 3r	1550	GCATTACGATCTGCAGCTCA GGAGCTCGATTAGTTTCTGC	55
nad4 1-nad4 2r	2000	CTGTGGGTTGGTCTGGTATG TCATATGGGCTACTGAGGAG	55
mtDNA nad4 2c-nad4 3r	2700	CTCCTCAGTAGCCCATATGA AACCAGTCCATGACTTAACA	55
nad5 1-nad5 2r	2200	TTTTTTCGGACGTTTTCTAG TTTGGCCAAGTATTCCTACAA	55
nad7 2-nad7 3r	1500	GCTTTACCTTATTCTGATCG TGTTCTTGGGCCATCATAGA	55

conditions for DT, CD and TF were as follows: 95°C for 15 min, followed by 40 cycles of 93°C for 45 s, 55°C or 58°C for 45 s and 72°C for 2 min followed by 72°C for 10 min. For ORF-M, there was an initial step of 95°C for 15 min followed by 30 cycles of 95°C for 50 s, 55°C for 50 s and 72°C for 1 min followed by 72°C for 10 min.

The PCR conditions for 17 + 20 were 95°C for 15 min followed by 20 cycles of 95°C for 50 s, 58°C for 50 s with a reduction of 0.5°C per cycle and 72°C for 1 min 45 s followed by further 20 cycles of 95°C for 50 s, 50°C for 50 s and 72°C for 1 min 45 s followed by 72°C for 10 min. For mtDNA primers: 5 min at 94°C; 35 cycles of 1



**Figure 1.** UPGMA dendrogram obtained for the *Populus* Section *Tacamahaca* by nuclear ISSR analysis using the Nei's index. Bootstrap values above 50% obtained from 1000 replicate analyses are shown.

min at 94°C, 1 min annealing at 55°C and 2 min extension at 72°C; followed by 72°C for 10 min. The success of each PCR reaction was verified by electrophoresing 2  $\mu$ l of the reaction products with 1% agarose gel. The PCR-amplified DNA fragments were digested using four restriction enzymes (HinfI, TaqI, EcoRI and BamHI; New England Biolabs, Ltd.) for the cpDNA regions and mtDNA region. The restriction digests were visualized on 2% agarose gel stained with ethidium bromide.

#### Data analysis

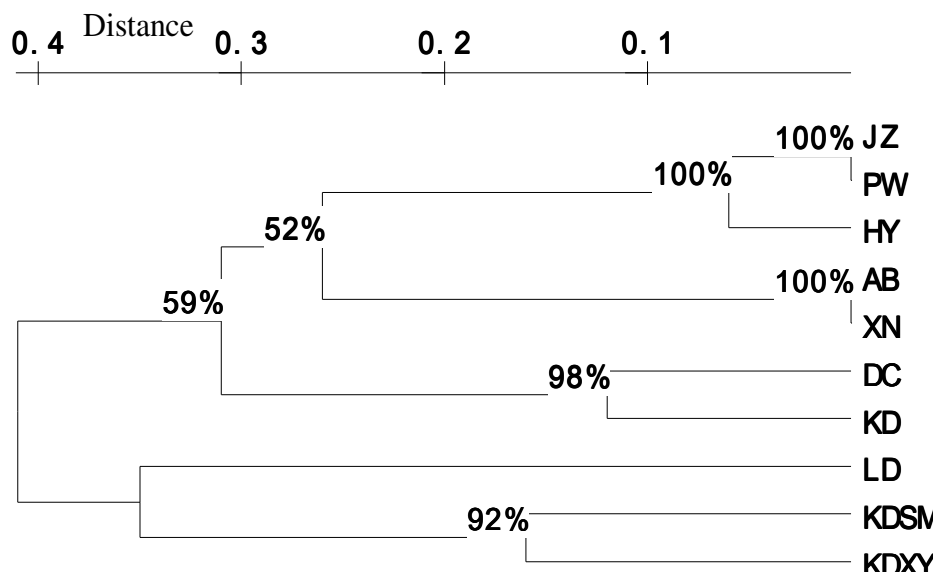
For the ISSR analysis, the fragment size scored ranged from 200 to 1500 bp. Weak bands were excluded from final data analysis. Since ISSRs were interpreted as dominant markers, the bands were scored as diallelic characters either as 1 (present) or 0 (absent). A pair-wise similarity matrix was calculated using the simple matching coefficient. This similarity matrix was employed to construct a dendrogram by UPGMA or neighbour joining (NJ) using the TREECON 1.3 b software. Branch support was estimated with 500 bootstrap replicates. A cophenetic matrix was derived from the similarity matrix to test the goodness of fit of the clusters by comparing the two matrices using the Mantel matrix correspondence test in the MXCOMP program of the NTSYS-pc package. In addition, the program DCENTER, EIGEN and MOD3D of NTSYS-pc 2.1p were used to perform a principle coordinates (PCO) analysis for the nuclear ISSR character matrix. For cpDNA and mtDNA PCR-RFLP data analysis, the digested DNA fragments were scored by presence (1) or absence (0) for each *Populus* accession. To facilitate comparison between different marker systems, the data matrices were analyzed by the same computer programs as described earlier for ISSR data analysis.

## RESULTS

In the current experiment, consistent results were obtained across at least two DNA mixture template replicates for the three marker systems with over 97% of scorable fragments reproducible for ISSR and 100% for cpDNA and mtDNA-RFLP. Very faint fragments were not

reproducible, thus such fragments were not scored in this study. ISSR amplifications using 11 primers generated a total of 130 reliable fragments from the template-mixing patterns of the ten *Populus* accessions. The size of these fragments ranged between 200 to 1500 bp (Table 2). The minimum and maximum number of fragments generated per primer in all the accessions studied were 7 (primer 834) and 17 (primer 33), respectively (Table 2), with an average of 11.8 fragments per primer. Of the total 130 scorable fragments, 119 (91.5%) bands were polymorphic among accessions and only 11 bands were simultaneously present in the 10 *Populus* species. 22 bands (approximately 16.9%) were found to be species-specific. The polymorphic information content (PIC) for each ISSR primers varied from 0.333 to 0.493 with a mean of 0.423 (Table 2). The primer 807 was found to be the most informative for species differentiation, whereas primer 834 was the least informative. The PCR-RFLP survey of the chloroplast DNA from the 10 *Populus* accessions investigated revealed 52 polymorphic fragments, which together allowed the identification of 9 haplotypes. Only the two accessions of *Populus przewaskii* shared the same haplotype. Four non-coding regions of mtDNA were also amplified with the universal primers. PCR amplification of these regions revealed no length polymorphism among accessions. These PCR products were further digested using four restriction enzymes, but neither site variation nor length polymorphism was revealed among the 10 accessions studied. Thus, no mtDNA-RFLP analysis was considered necessary for further investigation.

The genetic relationships among the 10 accessions of *Populus* were analyzed by cophenetic cluster methods (Figure 1). The ISSR phenogram had very high cophenetic correlation coefficient ( $r = 0.94$ ), indicating excellent fits of the similarity matrices to the resulting



**Figure 2.** UPGMA dendrogram obtained by cpDNA-RFLP analysis using the Nei and Li (1979) index for the *Populus* section *Tacamahaca*. Bootstrap values above 50% obtained from 1000 replicate analyses are shown.

phenogram while that with cpDNA-RFLP was lower ( $r = 0.84$ ). However, the topologies of the ISSR and cpDNA phenograms were similar to each other by inspection. Comparing the cpDNA-based cluster tree (Figure 2) with the ISSR-based tree (Figure 1), similar relationships were found. Firstly, the accessions from same *Populus* species clustered together with high bootstrap value support, that is accessions of *P. przewalskii* and *Populus cathayana*. Within the *P. cathayana* group, the accession HY was distinct from the accessions JZ and PW with high bootstrap value support. Moreover, the close relationships between KD and DC were confirmed, and the two same species were then grouped with *P. cathayana* and *P. przewalskii*. In addition, KDSM and KDXY formed one group with high bootstrap support in both cluster trees indicating their closed sister relationships. However, the major difference between the ISSR and cpDNA trees was the position of *Populus yunnanensis* which formed a clade with KDSM and KDXY based on a shared cpDNA fragment length polymorphism with low bootstrap value (below 50%). Additionally, the relationships among the *Populus* species were further illustrated by the results of a principal coordinate analysis based on the nuclear ISSR character matrix. The first three coordinates accounted for 28.1, 24.1 and 10.3% of the total variance, respectively and distinguished similar groupings of cpDNA-based tree, which support the placement of *P. yunnanensis* with KDSM and KDXY.

The cophenetic matrices computed from chloroplast and nuclear marker-based tree matrices and compared with the original similarity data showed significant correlations of 94.3 and 92.6%, respectively, revealing in both cases a good fit for the cluster analyses performed.

## DISCUSSION

The main objective of the present study was to assess the genetic relationships among the *Populus* species belonging to the section *Tacamahaca* on the basis of cpDNA-RFLP and ISSR markers. A striking feature of poplars is the occurrence of interspecific hybrids. In phylogenetic studies conducted on groups in which hybridization between lineages has played a substantial role in their evolution (Smith, 1988), the combined use of cpDNA and nuclear DNA markers is crucial when aiming to acquire a comprehensive understanding of the evolutionary history. ISSR and cpDNA-RFLP markers were proved to be the efficient and inexpensive way to provide molecular data. They have been used successfully in determining genetic relationships and for DNA fingerprinting (Huang, 2000). Traditionally, *Populus* species have been grouped within their respective sections based primarily on their interspecific crossability and morphological similarities. The most debates were mainly existed in the species identification in Section *Tacamahaca* and *Aigerios*. In present study, all the 7 species of *Populus* could be distinguished by ISSR and cpDNA-RFLP, respectively, which laid a solid foundation. Since *P. cathayana*, *Populus kangdingensis*, *P. schneideri*, and *P. przewalskii* clustered into one group or one clade especially in cpDNA analysis, one can conclude that these species are highly genetically related. Within this group, the highest genetic similarities were found between *P. kangdingensis* and *P. schneideri*. The species *P. schneideri*, which occurs in southeastern Qinghai-Tibetan Plateau at the altitudes of 2,500 ~ 4,000 m a.s.l., is morphologically similar to *P. cathayana*.

However, unlike *P. cathayana* which has glabrous branchlets and leaves, *P. schneideri* usually has pilose branchlets and leaves, which are common characteristics also in *P. kangdingensis*. In addition genetically closely alike *P. schneideri* and *P. kangdingensis* are both unique to western Sichuan and they share similar altitude and habitat requirements. Thus, the high interspecific ISSR genetic similarity values (data not shown) observed between the *P. kangdingensis* and *P. schneideri*, together with the ISSR and cpDNA analyses, partially corroborate previous suggestion (based on morphological classification) that *P. schneideri* was a variety of *P. kangdingensis*.

Additional molecular, morphological and other analyses with a larger sample size would be required. Another group of highly related species include *Populus trinervis* and *Populus simonii*. The high genetic similarities between the two species are consistent with their morphological characters and habitat requirements. Both the two species is the long ovoid capsule, while the other species of the section *Tacamahaca* usually have ovoid capsules. *P. trinervis* and *P. simonii* are adapted to dry and thermic valley habitats and both are distributed at low altitudes. Although *P. trinervis* and *P. simonii* showed high ISSR and cpDNA similarities, their accessions formed distinct separate groups. Thus, additional molecular, morphological and other analyses with larger sample sizes would be required to corroborate their relationships. The status of *P. yunnanensis* has been unclear. The cluster analysis based on ISSR markers clearly distinct *P. yunnanensis* from the other *Populus* species. However, these results are not consistent with those reported earlier that *P. yunnanensis* was grouped with *P. simonii* based on AFLP analysis (Cervera et al., 2005). *P. yunnanensis* may share the same chloroplast ancestor with *P. simonii* and *P. trinervis* as indicated by their identical cpDNA analysis (Figure 2). This may reflect their similar maternal phylogeny. Hence, the position of *P. yunnanensis* remains unclear and the analysis of a larger number of accessions is required to clarify its relationship with other *Tacamahaca* species, especially with *P. simonii*. Mitochondrial PCR-RFLPs showed no site or length polymorphism in the present study. This indicates that the slow evolutionary rate of the mitochondrial genome makes mtDNA PCR-RFLPs unsuitable for resolving phylogenetic relationships at the intrageneric level. In comparison with the high level of restriction site polymorphism detected in cpDNA for the same set of species, this study again provides evidence supporting that the nucleotide substitution rate of mtDNA in plants is much lower than that of cpDNA (Palmer, 1992). In conclusion, the present study showed the pattern of genetic interrelationships and intraspecific variability among the native poplar species in southeastern Qinghai-Tibetan Plateau. Consequently, the new knowledge supplies information for understanding the evolution of *Populus* from the section *Tacamahaca* and it

will help to guide future research.

Our results demonstrated that the combination of cpDNA and ISSR molecular markers can be practical when monitoring a defined but yet limited set of genetic changes that may have occurred during the evolution process among *Populus*. However, the range of *Populus* accessions and species included in this study is limited. Therefore, a more extensive survey of *Populus* specimens in the section *Tacamahaca*, based on a wide range of markers covering both non-coding and coding regions including data based on for example AFLPs, DNA sequencing and isozymes would enable us to obtain a more complete view of the genetic variation, interrelationships and evolution among *Populus* species.

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