Characterization of the complete chloroplast genomes of five *Populus* species from the western Sichuan plateau, southwest China: comparative and phylogenetic analyses

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Species of the genus *Populus*, which is widely distributed in the northern hemisphere from subtropical to boreal forests, are among the most commercially exploited groups of forest trees. In this study, the complete chloroplast genomes of five *Populus* species (*Populus cathayana, P. kangdingensis, P. pseudoglauca, P. schneideri* and *P. xiangchengensis*) were compared. The chloroplast genomes of the five *Populus* species are very similar. The total chloroplast genome sequence lengths for the five plastomes were 156,789 bp, 156,523 bp, 156,512 bp, 156,513 bp and 156,465 bp, respectively. A total of 130 genes were identified in each genome, including 85 protein-coding genes, 37 tRNA genes and eight rRNA genes. Seven genes were duplicated in the protein-coding genes, whereas 11 genes were duplicated in the RNA genes. The GC content was 36.7% for all plastomes. We analyzed nucleotide substitutions, small inversions, SSRs and long repeats in the chloroplast genomes and found nine divergence hotspots (*ccsA*+*ccsA-ndhD, ndhC-trnV, psbZ-trnfM, *trnG-atpA, trnL-ndhJ, trnR-trnN, ycf4-cemA, ycf1*, and *trnR-trnN*), which could be useful molecular genetic markers for future population genetic and phylogenetic studies. We also observed that two genes (*rpoC2 and rbcL*) were subject to positive selection. Phylogenetic analysis based on whole cp genomes showed that *P. schneideri* had a close relationship with *P. kangdingensis* and *P. pseudoglauca*, while *P. xiangchengensis* was a sister to *P. cathayana*. aœ) ^= ;| M0Y
Characterization of the complete chloroplast genomes of five *Populus* species from the western Sichuan plateau, southwest China: comparative and phylogenetic analyses

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Abstract

Species of the genus Populus, which is widely distributed in the northern hemisphere from subtropical to boreal forests, are among the most commercially exploited groups of forest trees. In this study, the complete chloroplast genomes of five Populus species (Populus cathayana, P. kangdingensis, P. pseudoglaucu, P. schneideri and P. xiangchengensis) were compared. The chloroplast genomes of the five Populus species are very similar. The total chloroplast genome sequence lengths for the five plastomes were 156,789 bp, 156,523 bp, 156,512 bp, 156,513 bp and 156,465 bp, respectively. A total of 130 genes were identified in each genome, including 85 protein-coding genes, 37 tRNA genes and eight rRNA genes. Seven genes were duplicated in the protein-coding genes, whereas 11 genes were duplicated in the RNA genes. The GC content was 36.7% for all plastomes. We analyzed nucleotide substitutions, small inversions, SSRs and long repeats in the chloroplast genomes and found nine divergence hotspots (ccsA+ccsA-ndhD, ndhC-trnV, psbZ-trnfM, trnG-atpA, trnL-ndhJ, trnR-trnN, ycf4-cemA, ycf1, and trnR-trnN), which could be useful molecular genetic markers for future population genetic and phylogenetic studies. We also observed that two genes (rpoC2 and rbcL) were subject to positive selection. Phylogenetic analysis based on whole cp genomes showed that P. schneideri had a close
relationship with *P. kangdingensis* and *P. pseudoglauc*a, while *P. xiangchengensis* was a sister to *P. cathayana*.

**Introduction**

The species of the genus *Populus*, collectively known as poplar, are widely distributed in the northern hemisphere from subtropical to boreal forests and one of the most commercially exploited groups of forest trees (*Hamzeh & Dayanandan, 2004*). Because of their small genome size, fast growth rates, profuse vegetative propagation, adaptability to a variety of ecological sites, and their wood’s numerous uses, *Populus* species have become one of the most economically important groups of forest trees and a model organism for the study of tree biology (*Braatne, et al., 1992; Stettler, et al., 1996*). According to a recent classification, the genus *Populus* is classified into six sections (*Fang, et al., 1999; Zsuffa, 1975; Eckenwalder, 1996*). To date, more than 100 *Populus* species or varieties have been reported worldwide, of which approximately 53 are endemic to China (*Wan & Zhang, 2013*).

As a concentrated area of *Populus* resources in southwest China, the western Sichuan Plateau is dominated by mountainous and plateau geomorphology, and the mountains play a critical role in isolating plant distribution (*He, et al., 2015*). Meanwhile, the complex and unique natural and geographical conditions of this area provide not only diversified refuges where plants retreat in response to climatic changes but also great opportunities to develop new hybrid species (*Lu, et al., 2014*). However, the extensive interspecific hybridization and the high levels of morphological variation in *Populus* have posed great difficulties in species delimitation for systematic and comparative evolutionary studies (*Hamzeh & Dayanandan, 2004; Eckenwalder, 1996; Cronk, 2005*).

*Populus kangdingensis, P. pseudoglauc*a, *P. schneideri* and *P. xiangchengensis* are native to the western Sichuan Plateau, and they are distributed at altitudes above 3000 m and even above 4000 m, whereas *P. cathayana* widely occurs in China at altitudes ranging from 800 m to 3000 m. All five species overlap in the western Sichuan Plateau. Previous research has focused on their phylogenetic relationships. Liu & Fu (2004) considered *P. xiangchengensis* a hybridization of *P. schneideri* and *P. pseudoglauc*a based on morphological characteristics, while another study suggested that *P. xiangchengensis* was a likely hybrid species of *P. kangdingensis* and *P. pseudoglauc*a based on morphological characteristics (*Wan, et al., 2009*).

*P. schneideri* was classified into section *Tacamahaca*. Meanwhile, it was also considered a natural hybrid formed by *P. kangdingensis* and *P. cathayana* based on ISSR (inter-simple sequence repeat) and ITS (internal transcribed spacer) molecular markers (*Chen et al, 2007; Wang, 2012*). *P. pseudoglauc*a was originally classified in section *Leucoides*, although it was suggested to be assigned to section *Tacamahaca* (*Zhao, 1994*), and this assignment was supported by ISSR and nuclear ITS sequence markers (*Wang, 2012*). The relationship between the five *Populus* species is shown in Figure 1. All these findings suggested that the phylogenetic relationship of the five *Populus* species is rather complex and unclear.

Organellar DNA, which has its own genome and is inherited from one parent, is well conserved and allows for the development of informative universal markers (*Howe, et al., 2003;
The chloroplast (cp) genome, because of its relatively conserved size, gene content, structure and slow rate of nucleotide substitution within protein-coding genes, has been an ideal source of data on the phylogenetic relationships of plant taxa and their evolution and has been used to make significant contributions concerning evolutionary mechanisms for species and phylogenetic reconstruction (Khan et al., 2012; Asheesh & Vinav, 2012; Liu et al., 2017).

With the development of sequencing technology in recent years, in addition to nuclear genome sequences, cp genes, gene spacer regions, and cp genome sequences have been widely used to study plant molecular systematics. Whole cp genomes of several species from the genus *Populus* have been sequenced and deposited in GenBank. Here, we compare the complete cp genomes of *P. cathayana* (GenBank accession number: MK267318), *P. kangdingensis* (GenBank accession number: MK267308), *P. schneideri* (GenBank accession number: MK267305), *P. pseudoglauca* (GenBank accession number: MK267308), and *P. xiangchengensis* (GenBank accession number: MH910611). The codon usage bias, sequence divergences, mutation events, single nucleotide polymorphism (SNP) patterns and SSR distributions are compared, and a phylogenetic tree is reconstructed based on 27 complete cp genome sequences from Salicaceae. Our study provides cp genomic information for further phylogenetic reconstruction, molecular evolution research, and resources for breeding the genus *Populus*.

**Materials & Methods**

**Plant materials and DNA extraction**

The fresh leaves of *P. cathayana* were collected in Kangding (101°56′26″E, 29°59′36″N, Sichuan, China; altitude: 3109 m), while the samples of *P. kangdingensis*, *P. pseudoglauca*, *P. schneideri* and *P. xiangchengensis* were collected in Kangding (101°36′43″E, 30°05′20″N, Sichuan, China; altitude: 3554 m), Yajiang (100°54′06″E, 29°59′14″N, Sichuan, China; altitude: 3598 m), Litang (101°36′43″E, 30°05′20″N, Sichuan, China; altitude: 4018 m) and Xiangcheng (99°40′33″E, 28°55′47″N, Sichuan, China; altitude: 3530 m), respectively. The voucher specimens of the five species were deposited at the herbarium of Southwest Forestry University, Kunming, China. Total genomic DNA was extracted with the Ezup plant genomic DNA prep kit (Sangon Biotech, Shanghai, China), and DNA samples were stored at -80°C at the Key Laboratory of State Forestry Administration on Biodiversity Conservation in Southwest China, Southwest Forestry University, Kunming, China.

**Genome sequencing, assembly and annotation**

Total DNA was used to generate libraries with an average insert size of 400 bp, which were sequenced using the Illumina HiSeq X platform. Approximately 15.0 GB of raw data were generated from each genome with 150 bp paired-end read lengths. Then, the raw data were used to assemble the complete cp genome using GetOrganelle software (Jin et al., 2018) with *P. trichocarpa* as the reference. Genome annotation was performed with the program Geneious R8 (Biomatters Ltd, Auckland, New Zealand) by comparing the sequences with the cp genome of *P.
trichocarpa. The tRNA genes were further confirmed through online tRNAscan-SE web servers (Schattner et al., 2005). A gene map of the annotated Populus cp genome was drawn by OGdraw online (Lohse, et al., 2013).

Indices of codon usage

As an important indicator of codon usage bias, the relative synonymous codon usage (RSCU) value is the frequency observed for a codon divided by its expected frequency (Sharp, et al., 1986; Sharp & Li, 1987). The amino acid compositions and RSCU values of the five Populus cp genomes were calculated using the CodonW program (Peden, 1999). Because short CDSs generally result in large estimation errors for codon usage, CDSs shorter than 300 bp in length were excluded in codon usage calculations to avoid sampling bias (Rosenberg et al., 2003). Finally, 58 CDSs from the five cp genomes were analyzed in this study.

Genome comparison

To investigate divergence in cp genomes, identity across the whole cp genomes was visualized using the mVISTA viewer in Shuffle-LAGAN mode for the five species, with the P. xiangchengensis genome as the reference. MAFFT version 7 software (Katoh et al., 2005) was used to align the five plastome sequences, followed by adjustment with BioEdit. To elucidate the level of sequence variation, we then performed sliding window analysis to assess the pairwise variability (Pi) over the plastomes in DnaSP version 5 software (Librado & Rozas, 2009). The window length was set to 600 bp, and the step size was set to 200 bp. The SNP variation was detected using the “find variation” function in Geneious R8.

Identification of simple sequence repeats (SSRs) and long sequence repeats

SSRs in five Populus cp genomes were detected using MISA (Thiel et al., 2003) with the minimal repeat number set to 12, 6, 5, 5 and 5 for mono-, di-, tri-, tetra-, penta-, and hexa nucleotide sequences, respectively. We used the online REPutter software to identify and locate forward (F), reverse (R), complemented (C) and palindromic (P) repeats. The following settings for repeat identification were used: (1) Hamming distance equal to 3; (2) minimal repeat size was set to 30 bp; (3) maximum computed repeats was set to 90 bp (Kurtz et al., 2001).

Gene selective pressure analysis of five Populus plastomes

To examine variation in the evolutionary rates of cp genes, we calculated the nonsynonymous substitution rates (Ka), synonymous rates (Ks), and their ratios (Ka/Ks) using model averaging in the Ka_Ks Calculator program according to the LWL85 method (Yang & Bielawski, 2000; Zhang et al., 2006).

Phylogenetic analysis

To explore the genetic relationships among the five species of the Populus genus, a total of 17 complete cp genomes of Populus and five plastomes of Salix were obtained from GenBank,
and Itoa orientalis and Idesia polycarpa were used as the outgroups (Table S1). To examine the phylogenetic utility of different regions, phylogenetic analyses were performed based on the following data: (1) the complete cp DNA sequences, (2) the large single copy (LSC) region, (3) the small single copy region (SSC), (4) one inverted repeat region (IR), (5) the LSC+SSC region, (6) the LSC+SSC+IR region, and (7) a set of 85 common protein coding genes. All of the datasets were aligned using MAFFT under default settings. jModelTest 2.0 (Darriba et al., 2012) was used to determine the best-fitting model for each dataset based on the Akaike information criterion (AIC). A maximum likelihood method for phylogenetic analysis was performed based on the GTR+I+G model in RAxML version 8 (Stamatakis, 2014).

Results

Features of the five Populus plastomes

The complete cp genomes of the five Populus species ranged from 156,465 bp (P. xiangchengensis) to 156,789 bp (P. cathayana) in length. The plastome size of P. schneideri was only 1 bp larger than that of P. pseudoglauca. The plastome size of P. kangdingensis was 11 bp larger than that of P. pseudoglauca and 166 bp smaller than that of P. cathayana (Table 1 and Fig. 2). The five cp genomes included a pair of inverted repeats (IRs) of 27,620 bp in the three species P. kangdingensis, P. pseudoglauca and P. schneideri and an IR pair of 27,672 bp in P. cathayana and 27,570 bp in P. xiangchengensis. The GC contents were consistent in P. kangdingensis, P. pseudoglauca and P. schneideri, with 34.5%, 30.5% and 42.0% in the large single copy (LSC), small single copy (SSC) and IR regions, respectively (Tables 1 and 2).

Each of the P. cathayana, P. kangdingensis, P. pseudoglauca, P. schneideri and P. xiangchengensis cp genomes encoded 130 functional genes; 112 of these were unique genes, including 78 protein-coding genes, 30 tRNA genes and 4 rRNA genes (Table S2). Most of these genes occurred as a single copy, while 18 genes were double copies: seven protein-coding genes, seven tRNA genes and four rRNA genes. The LSC region contained 59 protein-coding genes and 22 tRNA genes, whereas the SSC region contained 10 protein-coding genes and one tRNA gene.

Codon usage

Most protein-coding genes had the standard AUG sequence as the start codon, but ndhD started with GUG, and rpl16 started with ATC. ATC as an initiation codon has been reported in other cp genomes (Raubeson et al., 2007; Wu et al., 2017). GUG start codons have been reported in tobacco, but they are very rare in eukaryotic genomes (Kuroda et al., 2007). When GUG was the start codon of a protein, it was still translated as Met because of the separate tRNA used for initiation. Furthermore, the codon usage patterns of the 58 distinct protein-coding genes in the five plastomes were examined, and the plastomes of P. kangdingensis, P. pseudoglauca, and P. schneideri were consistent, with a length of 75,990 bp and encoding 25,330 codons, while those of P. cathayana and P. xiangchengensis were 75,864 bp and 75,840 bp in size and encoded 25,288 and 25,280 codons, respectively, as presented in Table S3. Coding ending with A and T/U had RSCU values >1 for the five Populus cp genomes, indicating that they were used more
frequently than synonymous codons and may play major roles in the A+T bias of entire cp genomes. There was a general excess of A- and U-ending codons. All three stop codons were present, with UAA being the most frequently used among the five plastomes (Table S3). In addition, leucine (Leu, 10.67%, 10.65%, 10.65%, 10.65% and 10.65%) and cysteine (Cys, 1.14%) were the most and least commonly coded amino acids, respectively, among the five plastomes (Table S3 and Fig. 3).

Comparative analysis of the five Populus plastomes

In this study, the cp genomes of the five Populus species were well conserved, and no gene organization rearrangement occurred when P. xiangchengensis was used as a reference (Fig. 4 and Fig. 5). LSC, SSC and IR sections of the three Populus species of P. kangdingensis, P. pseudoglaucu, and P. schneideri were highly conserved and smaller than those of P. cathayana, while the IR regions were larger than those of P. xiangchengensis. Detailed comparisons of the IR-SSC and IR-LSC boundaries among the cp genomes of the five species are presented in Figure 6. Two complete or fragmented copies of rpl22 and ycf1 were located at the boundaries between the LSC or SSC regions and IR regions among the five Populus plastomes. The rpl22 gene crossed the IR-LSC with only 1 bp variation in sequence length among the five plastomes.

Parts of the ycf1 gene (15 (P. cathayana) -158 bp (P. kangdingensis, P. pseudoglaucu, and P. schneideri) were found in the SSC region at the IRb-SSC junction, whereas a portion of the ycf1 gene (1689 bp (P. xiangchengensis) to 1707 bp (P. cathayana)) was present in both IRs. A 61 bp overlap between ycf1 and ndhF was found in P. kangdingensis, P. pseudoglaucu, and P. schneideri. The Pi values within the slide window of 600 bp in the five plastomes varied from 0.00001 to 0.00335 (Table 3), with a mean of 0.00210. However, nine highly variable loci (Pi>0.01), including trnG-atpA, psbZ-trnfM, trnL-ndhJ, ndhC-trnV, ycf4-cemA, trnN-trnR, ycf1, ccsA+ccsA-ndhD and trnR-trnN, were located in the five Populus plastomes (Fig. 7). Among these regions, trnG-atpA, psbZ-trnfM, trnL-ndhJ, ndhC-trnV and ycf4-cemA were located in the LSC region, ycf1 and ccsA+ccsA-ndhD were in the SSC region, and trnN-trnR and trnR-trnN were in the IR regions.

Number and forms of mutations

We investigated SNPs, the most abundant type of mutation, in the five plastomes, with P. xiangchengensis as the reference. In gene-coding regions, we detected 70 SNPs in the comparative combination of P. cathayana-P. xiangchengensis, including 33 transition (Ts) and 37 transversion (Tv) SNPs, as well as 160 (97 Ts and 63 Tv), 166 (101 Ts and 65 Tv) and 164 (99 Ts and 65 Tv) SNPs in the plastomes of P. kangdingensis-P. xiangchengensis, P. pseudoglaucu-P. xiangchengensis and P. schneideri-P. xiangchengensis, respectively (Table 4). Furthermore, 106 (38 Ts and 68 Tv), 323 (130 Ts and 193 Tv), 316 (130 Ts and 186 Tv) and 314 (131 Ts and 183 Tv) SNPs were detected in noncoding regions among the four comparative combinations, respectively (Table S4).
In our study, a total of six small inversions (petA-psbJ, ndhC-trnV, trnN-trnR, ccsA-ndhD, ndhD-psaC and ndhF-trnL) were identified based on the sequence alignment of the five complete cp genomes (Fig. 8). The small inversions from ndhC-trnV and ndhD-psaC occurred in only P. xiangchengensis, those from ndhF-trnL occurred in P. pseudoglauca and P. schneideri, those from trnN-trnR occurred in P. kangdingensis, P. pseudoglauca and P. schneideri, and those from ccsA-ndhD occurred in the four species other than P. cathayana, while the inversion from petA-psbJ occurred in the four species other than P. xiangchengensis (Fig. 8).

Synonymous (Ks) and nonsynonymous (Ka) substitution rate analysis
In this study, ratios of nonsynonymous (Ka) versus synonymous (Ks) substitutions were calculated for 78 shared unique protein coding genes in P. cathayana, P. kangdingensis, P. pseudoglauca and P. schneideri, with P. xiangchengensis as the reference. Among these genes, only 19 protein-coding genes had Ka/Ks values (Fig. 9 and Table S5). The Ka/Ks values of the remaining protein-coding genes could not be calculated because Ka or Ks was equal to 0, indicating that these sequences were conserved without nonsynonymous or synonymous nucleotide substitution. The Ka/Ks ratios of all genes except rpoC2 in P. pseudoglauca (1.00903) and the rbcL gene in P. kangdingensis (2.26407), P. pseudoglauca (2.26407) and P. schneideri (2.26407) were less than 1 (Fig. 9).

SSR and long repeat analysis
With MISA, a total of 170 SSR loci were detected, of which mononucleotide repeats (P1) comprised 148 (87.06%) of all SSRs and all of the mononucleotides composed of poly A (polycytosine) and poly T (polythymine) repeats (Table 5). Within the five plastomes, SSR loci were primarily located in the LSC region, followed by the SSC region. A total of 15 SSR loci were detected in the protein-coding genes rpoB, rpoC2 and rps8, with all others situated in intergenic spacers and introns (Table S6). A total of 28, 39, 39 and 25 SSR loci were detected in the P. cathayana, P. kangdingensis, P. pseudoglauca, P. schneideri and P. xiangchengensis cp genomes, respectively (Table 5). The corresponding numbers of these repeats in P. kangdingensis, P. pseudoglauca and P. schneideri matched each other and consisted of 33 P1, two dinucleotide (P2) and four compound (C) repeats. Comparison among the five plastomes revealed that four P1 loci were found in only P. xiangchengensis, one C locus was found only in P. cathayana, and 15 SSR loci (11 P1, 1 P2 and 3C repeats) were detected in the plastomes of P. kangdingensis, P. pseudoglauca and P. schneideri (Table S6).

In the plastomes of the five Populus species, we found 58 repeats in P. cathayana, which was a higher number than those found in the other four species (49, 48, 48 and 48 repeats, respectively) (Fig. 10A). P. pseudoglauca and P. schneideri shared the same number and types of repeats (17 forward repeats (F), 23 palindromic repeats (P), 5 reverse repeats (R) and 3 complement repeats (C)) (Fig. 10A). The majority of repeats (84.86%) varied from 30 to 39 bp in length (Fig. 10B). Variation in the number of repeat sequences situated in the four parts of the plastome was observed between species (Table S7).
Phylogenetic analysis based on the cp genome

Seven data partitions (complete cp genomes, LSC, SSC, IR, LSC+SSC, LSC+SSC+IR region and protein coding regions) from 22 Salicaceae cp genomes were used to construct phylogenetic trees (Figures S1-S6 and Fig. 11). However, the best resolution in phylogenetic relationships was achieved using full cp genome sequences; thus, we discuss phylogenetic relationships mainly based on Figure 11. All of Populus was divided into four main highly supported clades (Fig. 11). Three species of the section Turanga were clade I members. Clade II included seven species (P. adenopoda, P. alba, P. davidiana, P. qiongdaoensis, P. rotundifolia, P. tremula, and P. tremula × alba) in section Populus and one species in section Aigeiros (P. nigra). Clade III consisted of three species in section Tacamahaca (P. kangdingensis, P. schneideri and P. yunnanensis) and two species in section Leucoides (P. lasiocarpa and P. pseudoglauc). Clade IV included the four species in section Tacamahaca (P. balsamifera, P. cathayana, P. trichocarpa and P. xiangchengensis), one species in section Aigeiros (P. fremontii) and one species in section Leucoides (P. wilsonii). Our results showed that P. kangdingensis, P. pseudoglauca, and P. schneideri were in clade III, while P. xiangchengensis formed a sister relationship with 100% bootstrap support to P. cathayana in clade IV.

Discussion

In the present study, we compared five Populus plastomes, all of which assembled into single, circular, double-stranded DNA sequences presenting a typical quadripartite structure with a length of 156,465 bp to 156,789 bp, similar to most Populus cp genomes (Wang et al, 2016; Zhang & Gao, 2016; Zheng et al, 2016; Han et al., 2017). The GC contents in the LSC, SSC and IR regions were consistent among the three species P. kangdingensis, P. pseudoglauc and P. schneideri, and the high GC content in the IR regions was possibly due to the presence of four ribosomal RNA sequences in these regions (Yang et al., 2016). In addition, LSC, SSC and IR sections of the three Populus species were highly conserved and smaller than those of P. cathayana, while the IR regions were larger than those of P. xiangchengensis. IRs are the most conserved regions of the cp genome, and the construction and expansion of IR borders are common evolutionary events and the major reasons for size differences between cp genomes (Shen et al., 2017; Wu et al., 2018).

The whole aligned sequences revealed surprisingly low divergence; however, nine regions (trnG-atpA, psbZ-trnM, trnL-ndhJ, ndhC-trnV, ycf4-cemA, trnN-trnR, ycf1, ccsA+ccsA-ndhD and trnR-trnN) displayed high variation. Further work is still necessary to determine whether these nine variable loci could be used in phylogenetic analyses of related Populus species or as potential molecular markers for population genetics and phylogenetics.

Understanding nucleotide substitution rates is of fundamental importance in molecular evolution (Muse & Gaut, 1994). During the process of searching for SNPs, we found that the cp genome sequences of P. kangdingensis, P. schneideri and P. pseudoglauc had similar numbers of mutations, while P. cathayana had a smaller number of mutations relative to P.
xiangchengensis. Therefore, the phylogenetic relationships of these five species may be affected by different mutation modes (Yang et al., 2016). Furthermore, we found that the numbers and types of SSRs and long repeats of the three species P. kangdingensis, P. schneideri and P. pseudoglauca were basically identical. These SSR repeats and long repeats could be used to examine genetic structure, diversity, differentiation and relative species in future studies.

The Ka and Ks nucleotide substitution patterns are very important markers in gene evolution studies (Kimura, 1979). The Ka/Ks ratio is indicative of changes in selective pressures. Ka/Ks values >1, =1, and <1 indicate positive selection, natural evolution and purifying selection affecting the coding portions, respectively (Sharp & Li, 1987; Yang & Bielawski, 2000; Lawrie et al., 2013). However, the Ka/Ks ratio is less than one in most protein-coding regions (Makalowski & Boguski, 1998). When we compared the 78 common unique protein coding genes, the Ka/Ks ratios were less than one, except the ratio for the gene rpoC2 in one pairwise comparison of P. pseudoglauca-P. xiangchengensis and the ratio for the gene rbcL in the pairwise comparisons of P. kangdingensis-P. xiangchengensis, P. pseudoglauca-P. pseudoglauca and P. schneideri-P. xiangchengensis, indicating that these two genes are undergoing positive selection and that at least some of the mutations concerned must be advantageous.

Small inversions in the cp genome of angiosperms are ubiquitous and commonly associated with a hairpin secondary structure (Kim & Lee, 2005; Catalano et al., 2009). A distinctive feature of these inversions is that they are flanked by inverted repeats that range from 8 to 50 bp, such that the inverted repeats form the stem and the segment between them forms the loop (Catalano et al., 2009). These small inversions are generally recognized by pairwise comparisons between sequences. In our study, six small inversions were discovered based on the sequence alignment of the five complete cp genomes. Two small inversions from ndhC-trnV and ndhD-psaC were only present in P. xiangchengensis, while three from ccsA-ndhD, ndhF-trnL and trnN-trnR were shared by P. pseudoglauca and P. schneideri. These small inversion regions will provide abundant information for marker development in phylogenetic analyses of related Populus species. Small inversions in the ccsA-ndhD and petA-psbJ intergenic regions have been reported in other studies (Song et al., 2015; 2016; Dong et al., 2017). However, small inversions of noncoding sequences may influence sequence alignment and character interpretation in phylogeny reconstructions, so caution is necessary when using cp noncoding sequences for phylogenetic analysis.

The cp genome is widely employed to study evolution through phylogenetics, and it has been suggested to be useful for phylogenetic reconstruction at low taxonomic levels (Zhang et al., 2011; Ma et al., 2014; Yang et al., 2014; Zhang et al., 2016). It has also been postulated to be a potential ultrabarc ode or organelle-scale barcode for taxonomically complex groups (Kane et al., 2012). The key interest of the current study is to resolve previous phylogenetic controversies in Populus (Zhao, 1994; Liu & Fu, 2004; Chen et al., 2007; Wan et al., 2009; Wang, 2012) by using complete cp genome sequences. The results revealed that P. nigra (section Aigeiros) was nested among members of the section Populus, which is supported by previous studies (Rajora et al., 2018).
Both previous studies found that *P. nigra* showed higher similarities to *P. alba* than to other species.

The position of *P. pseudoglauc*a confirms the previously published phylogeny described by Chao & Liu (1991), in which *P. pseudoglauc*a was classified into section *Tacamahaca* according to fossil evidence, paleogeography, paleoclimate, and modern distribution. The species *P. schneideri*, which is distributed in the western Sichuan Plateau at altitudes of 3000 to 4000 m, has remained a topic of debate among scientists. According to its morphology, it is similar to *P. cathayana* (Fang et al., 1999). Wan et al. (2013) suggested that *P. schneideri* is generally closer to *P. cathayana* than *P. kangdingensis*, and it is a natural hybrid between the ancestors of *P. cathayana* and *P. kangdingensis* based on cpDNA and nuclear DNA sequence data as well as amplified restriction fragment polymorphism (AFLP) analyses. Other studies considered *P. schneideri* to be a variety of *P. kangdingensis* based on morphological traits (Chao & Liu, 1991; Yu et al., 2003; Liu & Fu, 2004). Chen et al. (2007) suggest that *P. schneideri* is generally more highly related to *P. kangdingensis* than to *P. cathayana* based on cpSSR analysis. Our data revealed that *P. schneideri* had a close relationship with *P. pseudoglauc*a and *P. kangdingensis*. *P. schneideri* and *P. kangdingensis* are both unique to the western Sichuan Plateau, and they share similar altitude and habitat requirements (Yu et al., 2003). In addition, the plastome characteristics, SNP variations, SSR and long repeats and small inversions all showed that *P. kangdingensis*, *P. pseudoglauc*a and *P. schneideri* had similar structure and variation models. Therefore, we speculated that these three species may be the same species; however, more population data are needed for further analyses. *P. xiangchengensis* was a sister to *P. cathayana*, as revealed by cp genome sequence analysis, which did not support the viewpoint that *P. xiangchengensis* is a natural hybrid species of either *P. schneideri* and *P. pseudoglauc*a or *P. kangdingensis* and *P. pseudoglauc*a. It is our hope that the five plastomes will provide useful resources for better understanding the phylogeny and relationships of the genus *Populus*.

**Conclusions**

This study reports the comparative analysis of five *Populus* cp genome sequences with detailed gene annotation. Comparing the five plastomes showed that the plastomes were similar in structure and had a high degree of synteny. Nine divergent regions (*trnG-atpA, psbZ-trnfM, trnL-ndhJ, ndhC-trnV, ycf4-cemA, trnN-trnR, ycf1, ccsA+ccsA-ndhD* and *trnR-trnP*) were identified and may be utilized as potential molecular markers for population genetic and phylogenetic studies in *Populus*. Furthermore, among the five cp genomes, *P. kangdingensis*, *P. pseudoglauc*a and *P. schneideri* showed little difference in their SNP loci and SSRs. The results of the phylogenetic analyses showed that *P. schneideri* had the closest affinity to *P. kangdingensis* and was a sister to *P. pseudoglauc*a, while *P. cathayana* had a close relationship with *P. xiangchengensis*. The characterization of these five plastomes will provide useful resources for better understanding the phylogeny and relationships of the genus *Populus*. 

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Figure captions

Figure 1 The hybridization schematic diagram of five *Populus* species. (A) *P. kangdingensis*; (B) *P. cathayana*; (C) *P. schneideri*; (D) *P. pseudoglauca*; (E) *P. xiangchengensis*. *P. schneideri* = *P. kangdingensis* × *P. cathayana*, *P. xiangchengensis* = *P. kangdingensis* × *P. pseudoglauca*.

Figure 2 Gene map of the five *Populus* species cp genomes. The genes that are drawn outside of the circle are transcribed clockwise, whereas those that are drawn inside the circle are transcribed counterclockwise. Large single copy (LSC), small single copy (SSC) and inverted repeats (IRa and IRb) are indicated.

Figure 3 Amino acid frequencies of the protein-coding sequences of the five plastomes.

Figure 4 Whole chloroplast genome alignments for five *Populus* species using the mVISTA program, with *P. xiangchengensis* as the reference. The Y-scale indicates identity from 50% to 100%, Gray arrows indicate the position and direction of each gene. Red indicates non-coding sequences (CNS), blue indicates the exons of protein-coding genes (exon) and line green indicates the tRNA or rRNA genes.

Figure 5 Mauve alignment of the cp genomes of five *Populus* species. (A) Cp genome of *P. xiangchengensis*; (B) Cp genome of *P. cathayana*; (C) Cp genome of *P. kangdingensis*; (D) Cp genome of *P. pseudoglauca*; (E) Cp genome of *P. schneideri*.

Figure 6 Comparison of LSC, SSC and IR region borders among cp genomes of five *Populus* species. (A) Cp genome of *P. xiangchengensis*; (B) Cp genome of *P. cathayana*; (C) Cp genome of *P. kangdingensis*; (D) Cp genome of *P. pseudoglauca*; (E) Cp genome of *P. schneideri*.

Figure 7 Sliding window analyses of the whole plastomes of five *Populus* species. The genetic divergence among the plastomes of *P. cathayana*, *P. kangdingensis*, *P. pseudoglauca*, *P. schneideri* and *P. xiangchengensis* was calculated with DnaSP 5.0 software (window length: 600 bp, step size 200 bp). X-axis: position of the midpoint of a window, Y-axis: nucleotide diversity of each window.

Figure 8 Predicted hairpin loops of inversions in the five plastomes of *Populus*. The structures of hairpin loops in the of the (A) *ccsA-ndhD*, (B) *ndhC-trnV*, (C) *ndhD-psaC*, (D) *ndhF-trnL*, (E) *petA-psbJ* and (F) *trnN-trnR* regions were drawn with RNAstructure. The arrows in the figure indicate the break points in inversion events.

Figure 9 Ka/Ks values of 19 protein-coding genes of the four comparative combinations. Ka: nonsynonymous, Ks: synonymous.

Figure 10 Comparison of long repeats among five *Populus* plastomes. (A) Number of each repeat type; F: forward repeats, P: palindromic repeats, R: reverse repeats and C: complement repeats (B) Frequency of each repeat type by length.

Figure 11 Molecular phylogenetic tree of 27 species in the family *Salicaceae* inferred from ML analyses based on the complete plastome sequence. The numbers associated with each node are bootstrap support values.
Supporting Information

Supplementary file1

The raw data from the four *Populus* species *P. cathayana*, *P. kangdingensis*, *P. pseudoglauc* and *P. schneideri*.

Table S1 GenBank accession numbers of *Populus*, *Salix* and outgroups with cp genome sequences used for phylogenetic analyses

Table S2 List of genes in the cp genomes

Table S3 Codon usage in the five *Populus* plastomes

Table S4 Transitions (Ts) and transversions (Tv) in the four plastomes relative to the plastome of *P. xiangchengensis*

Table S5 Synonymous (Ks) and nonsynonymous (Ka) analysis of the five species based on 78 unique CDS genes, with *P. xiangchengensis* as the reference

Table S6 SSRs in the five cp genomes

Table S7 Repeat sequences in the five cp genomes

Figure S1 Molecular phylogenetics tree of 27 species in the family *Salicaceae* inferred from ML analyses based on LSC regions. The numbers associated with each node are bootstrap support values.

Figure S2 Molecular phylogenetics tree of 27 species in the family *Salicaceae* inferred from ML analyses based on SSC regions. The numbers associated with each node are bootstrap support values.

Figure S3 Molecular phylogenetics tree of 27 species in the family *Salicaceae* based on IR regions. The numbers associated with each node are bootstrap support values.

Figure S4 Molecular phylogenetics tree of 27 species in the family *Salicaceae* based on LSC+SSC regions. The numbers associated with each node are bootstrap support values.

Figure S5 Molecular phylogenetics tree of 27 species in the family *Salicaceae* based on LSC+SSC+IR regions. The numbers associated with each node are bootstrap support values.

Figure S6 Molecular phylogenetics tree of 27 species in the family *Salicaceae* based on protein-coding regions. The numbers associated with each node are bootstrap support values.
Figure 1

The hybridization schematic diagram of five *Populus* species.

(A) *P. kangdingensis*; (B) *P. cathayana*; (C) *P. schneideri*; (D) *P. pseudoglauc*a; (E) *P. xiangchengensis*. *P. schneideri* = *P. kangdingensis* × *P. cathayana*, *P. xiangchengensis* = *P. kangdingensis* × *P. pseudoglauc*a, *P. xiangchengensis* = *P. schneideri* × *P. pseudoglauc*a.
Figure 2

Gene map of the five *Populus* species cp genomes

The genes that are drawn outside of the circle are transcribed clockwise, whereas those that are drawn inside the circle are transcribed counterclockwise. Large single copy (LSC), small single copy (SSC) and inverted repeats (IRa and IRb) are indicated.
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Amino acid frequencies of the protein-coding sequences of the five plastomes
Whole chloroplast genome alignments for five *Populus* species using the mVISTA program, with *P. xiangchengensis* as the reference.

The Y-scale indicates identity from 50% to 100%, Gray arrows indicate the position and direction of each gene. Red indicates non-coding sequences (CNS), blue indicates the exons of protein-coding genes (exon) and line green indicates the tRNA or rRNA genes.
Figure 5

Mauve alignment of the cp genomes of five *Populus* species

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Comparison of LSC, SSC and IR region borders among cp genomes of five *Populus* species

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Figure 7

Sliding window analyses of the whole plastomes of five *Populus* species

The genetic divergence among the plastomes of *P. cathayana*, *P. kangdingensis*, *P. pseudoglauc*, *P. schneideri* and *P. xiangchengensis* was calculated with DnaSP 5.0 software (window length: 600 bp, step size 200 bp). X-axis: position of the midpoint of a window, Y-axis: nucleotide diversity of each window.
Figure 8

Predicted hairpin loops of inversions in the five plastomes of *Populus*.

The structures of hairpin loops in the of the (A) ccsA-ndhD, (B) ndhC-trnV, (C) ndhD-psaC, (D) ndhF-trnL, (E) petA-psbJ and (F) trnN-trnR regions were drawn with RNAstructure. The arrows in the figure indicate the break points in inversion events.
Figure 9

Ka/Ks values of 19 protein-coding genes of the four comparative combinations.

Ka: nonsynonymous, Ks: synonymous.
Figure 10

Comparison of long repeats among five *Populus* plastomes.

(A) Number of each repeat type; F: forward repeats, P: palindromic repeats, R: reverse repeats and C: complement repeats (B) frequency of each repeat type by length.
Figure 11

Molecular phylogenetic tree of 27 species in the family Salicaceae inferred from ML analyses based on the complete plastome sequence.

The numbers associated with each node are bootstrap support values.
Table 1 (on next page)

The features of five *Populus* plastomes
Table 1. The features of five *Populus* plastomes

| Species             | Size (bp) | LSC (bp) | SSC(bp) | IR(bp) | Number of protein coding genes | Number of tRNA genes | Number of rRNA genes | GC content (%) |
|---------------------|-----------|----------|---------|--------|--------------------------------|----------------------|---------------------|-----------------|
| *P. cathayana*      | 156,789   | 84,851   | 16,594  | 27,672 | 85(7)                           | 37(7)                | 8(4)                | 36.7            |
| *P. kangdingensis*  | 156,523   | 84,788   | 16,495  | 27,620 | 85(7)                           | 37(7)                | 8(4)                | 36.7            |
| *P. pseudoglauca*   | 156,512   | 84,777   | 16,495  | 27,620 | 85(7)                           | 37(7)                | 8(4)                | 36.7            |
| *P. schneideri*     | 156,513   | 84,778   | 16,495  | 27,620 | 85(7)                           | 37(7)                | 8(4)                | 36.7            |
| *P. xiangchengensis*| 156,465   | 84,812   | 16,513  | 27,570 | 85(7)                           | 37(7)                | 8(4)                | 36.7            |
**Table 2** (on next page)

Base composition of the five *Populus* plastomes
Table 2. Base composition of the five *Populus* plastomes

| Region   | P. cathayana | P. kangdingensis | P. pseudoglauc | P. schneideri | P. xiangchengensis |
|----------|--------------|------------------|----------------|---------------|--------------------|
| LSC(%)   |              |                  |                |               |                    |
| A        | 32.0         | 32.1             | 32.1           | 32.1          | 32.1               |
| T        | 33.4         | 33.4             | 33.4           | 33.4          | 33.4               |
| C        | 17.7         | 17.7             | 17.7           | 17.7          | 17.7               |
| G        | 16.8         | 16.8             | 16.8           | 16.8          | 16.8               |
| GC       | 34.6         | 34.5             | 34.5           | 34.5          | 34.5               |
| SSC(%)   |              |                  |                |               |                    |
| A        | 34.9         | 34.9             | 34.9           | 34.9          | 34.9               |
| T        | 34.5         | 34.6             | 34.6           | 34.6          | 34.3               |
| C        | 16.1         | 16.1             | 16.1           | 16.1          | 16.1               |
| G        | 14.6         | 14.4             | 14.4           | 14.4          | 14.6               |
| GC       | 30.6         | 30.5             | 30.5           | 30.5          | 30.7               |
| IR(%)    |              |                  |                |               |                    |
| A        | 28.9         | 29.0             | 29.0           | 29.0          | 29.0               |
| T        | 29.1         | 29.0             | 29.0           | 29.0          | 29.1               |
| C        | 21.8         | 21.8             | 21.8           | 21.8          | 21.8               |
| G        | 21.8         | 20.1             | 20.1           | 20.1          | 20.2               |
| GC       | 41.9         | 42.0             | 42.0           | 42.0          | 42.0               |
| Overall length (%) |       |                  |                |               |                    |
| C        | 18.7         | 18.7             | 18.7           | 18.7          | 18.7               |
| G        | 18.0         | 18.0             | 18.0           | 18.0          | 18.1               |
| GC       | 36.7         | 36.7             | 36.7           | 36.7          | 36.7               |
Table 3 (on next page)

Pairwise nucleotide divergences of the five *Populus* plastomes
Table 3. Pairwise nucleotide divergences of the five *Populus* plastomes

| Species          | P. cathayana | P. kangdingensis | P. pseudoglauc | P. schneideri | P. xiangchengensis |
|------------------|--------------|------------------|----------------|---------------|-------------------|
| P. cathayana     | -            | -                | -              | -             | -                 |
| P. kangdingensis | 0.00124      | -                | -              | -             | -                 |
| P. pseudoglauc   | 0.00333      | 0.00326          | -              | -             | -                 |
| P. schneideri    | 0.00335      | 0.00327          | 0.00003        | -             | -                 |
| P. xiangchengenis| 0.00333      | 0.00325          | 0.00001        | 0.00002       | -                 |
**Table 4** (on next page)

Transitions (Ts) and transversions (Tv) in the protein-coding regions of the four compare combinations with the plastome of *P. xiangchengensis*
Table 4. Transitions (Ts) and transversions (Tv) in the protein-coding regions of the four compare combinations with the plastome of *P. xiangchengensis*

|                  | Ts |       |       |       |       |       |       |       |
|------------------|----|-------|-------|-------|-------|-------|-------|-------|
|                  | A-G | C-T   | A-T   | A-C   | T-G   | G-C   | Total |       |
| *P. cathayana*   | 14  | 19    | 13    | 7     | 9     | 8     | 70    |       |
| *P. kangdingensis* | 42  | 55    | 12    | 14    | 24    | 13    | 160   |       |
| *P. pseudoglauca* | 44  | 57    | 12    | 15    | 24    | 14    | 166   |       |
| *P. schneideri*  | 43  | 56    | 12    | 15    | 24    | 14    | 164   |       |
Table 5 (on next page)

Statistics of chloroplast SSRs detected in five *Populus* plastomes

**Notes:** P1: mononucleotide repeats; P2: dinucleotide repeats; C: compound repeats
### Table 5. Statistics of chloroplast SSRs detected in five *Populus* plastomes

| SSR type | P. cathayana | P. kangdingensis | P. pseudoglauc | P. schneideri | P. xiangchengensis |
|----------|--------------|------------------|----------------|---------------|-------------------|
| (A)12    | 3            | 9                | 9              | 9             | 3                 |
| (A)13    | 5            | 5                | 5              | 5             | 4                 |
| (A)14    | 2            | 2                | 2              | 2             | 3                 |
| (A)15    | 2            | 0                | 0              | 0             | 1                 |
| (A)16    | 1            | 2                | 2              | 2             | 0                 |
| (A)17    | 0            | 1                | 1              | 1             | 0                 |
| P1       |              |                  |                |               |                   |
| (T)12    | 4            | 5                | 5              | 5             | 3                 |
| (T)13    | 3            | 3                | 3              | 3             | 3                 |
| (T)14    | 3            | 1                | 1              | 1             | 1                 |
| (T)15    | 1            | 1                | 1              | 1             | 3                 |
| (T)16    | 2            | 4                | 4              | 4             | 1                 |
| (T)17    | 0            | 0                | 0              | 0             | 1                 |
| ALL      | 26           | 33               | 33             | 33            | 23                |
| P2       |              |                  |                |               |                   |
| TA/AT    | 1            | 2                | 2              | 2             | 1                 |
| C        | 1            | 4                | 4              | 4             | 1                 |
| Total    | 28           | 39               | 39             | 39            | 25                |

**Notes:**
- P1: mononucleotide repeats; P2: dinucleotide repeats; C: compound repeats.