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Research Article
Genetic Diversity and Population Structure of 29 Species of Genus *Populus* Assessed by AFLP Markers

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Abstract

Background and Objective: *Populus* species are one of the world's most important groups of forest trees, but distinguishing between cultivars and hybrids is a little difficult due to widespread interspecific hybridization and introgression and the high level intraspecific morphological variation. The objective of this study was to understand the genetic variation and heterozygosity of 29 *Populus* species from the Jianghan Plain of China and provided scientific guidance for the introduction of superior poplar varieties in Hubei province of China. **Materials and Methods:** The DNA was isolated from leaves of 29 poplar clones, digested by *Mse*I and *Eco*RI endonucleases and subsequently used Amplified Fragment Length Polymorphisms (AFLP) analysis. The AFLP data was analyzed by GeneMapper software to display the fragment sizes as electropherograms and binary data. Fingerprint was constructed according to the AFLP analysis. Genetic similarity coefficients were calculated using Numeric Taxonomic System (NTSYS) PC version software 2.11 and the phylogenetic tree was constructed by cluster analysis using the UPGMA. **Results:** A total of 959 fragments were amplified, 806 of which were polymorphic with an average polymorphism percentage of 84.67%. Genetic similarity coefficients of the 29 *Populus* species ranged from 0.6111-0.8478 with an average of 0.7217. The highest average genetic similarity coefficient (0.7549) was found between "Danhongyang" with other *Populus* species and the lowest (0.6532) was found between "6204" with other *Populus* species. According to the genetic similarity coefficient of 0.7117, the 29 *Populus* species were divided into four categories by UPGMA cluster analysis. Finally, we built DNA fingerprint of the 29 *Populus* genotypes. **Conclusion:** It was concluded that 29 *Populus* species shared high genetic similarity and low genetic diversity. "107", "6204" and "6102" were three unique species and worth further utilizing in hybridization.

Key words: *Populus*, AFLP, genetic similarity, genetic relationship, molecular markers

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Poplar is one of the most important economical and ecological trees in the world, populating the most widely cultivated areas with the highest timber yield[1-7]. It is mainly distributed in the temperate regions between 19-70°N, whereas, several types are found in Africa[8]. According to the characteristics of organ composition, pollen, leaf, bud, branch and trunk, researchers divided the genus *Populus* into five major groups: *Leuce*, *Aigeiros*, *Tacamahaca*, *Turanga* and *Leucoides* [9,10]. Extensive genetic variations exist among the *Populus* groups and their inter-species. Therefore, identification of poplar species and determination of genetic correlation bear importance for cultivation of new varieties and collection and preservation of germplasm resources[11,12].

In the past few decades, a variety of molecular markers have emerged, enabling the exploration of genetic variation between *Populus* groups, related inter-species and different clones at the genome level[13,14]. At present, the molecular markers that are widely used in identification of poplar varieties include the following: Restriction Fragment Length Polymorphism (RFLP)[15-17], Amplified Fragment Length Polymorphism (AFLP)[18,19], Random Amplified Polymorphism DNA (RAPD)[20-22] and Simple Sequence Repeat (SSR)[18,22-24]. Among these markers, AFLP is considered as an ideal and effective molecular marker technique. This technology combines the advantages of RFLP and RAPD, that is, the reliability of RFLP and convenience of RAPD. The AFLP features high polymorphism, co-dominant expression and absence of multiple allelic effects. The AFLP is present in small dosage and shows high sensitivity and can provide significant information in a short time[25-30]. Given these advantages, AFLP can provide accurate and reliable genetic markers for poplar production.

With the rapid development of plant molecular biology, AFLP molecular markers have been widely applied in some fields, such as poplar genetic diversity studies[31,32], determining the genetic structure[23,33], screen interspecific hybrids[34],genetic map construction[35,36] and variety identification[18,37], providing a more clear genetic background for poplar. At present, although there are many poplar varieties, there are still many problems. Firstly, numerous poplar varieties are mixed and severely commercialized. Secondly, many areas breed poplar blindly regardless of the regional features. Consequently, they fail to plant the optimum varieties. Hubei province is located in the central part of China and features a relatively wide geographical area. Except that, climate, altitude and soil span across the province are widely different. Therefore, an simple and accurate germplasm identification approach is required urgently to rationally protect and utilize different germplasm resources for cultivation of suitable new varieties. Undoubtedly, identification of germplasm resources at the molecular level is the most fundamental and accurate approach. In this study, 29 clones from different *Populus* groups were used and their genetic diversities were analyzed by AFLP molecular marker technology. The present study aimed to determine genetic diversity and genetic differentiation among *Populus* clones, inter-species, inter-groups and parents and progenies at the DNA level to provide scientific guidance for the introduction of superior poplar varieties in Hubei province.

MATERIALS AND METHODS

Plant materials and DNA extraction: Leaves of 29 poplar clones were provided by Qianjiang Research Institute of Forestry (Hubei, China, Table 1), which basically covered the main poplar cultivars in the Jianghan Plain and even the entire Yangtze River basin. Collected samples were dried with silica gel and stored at room temperature until DNA extraction. All of the samples were collected in early May, 2017 and this study was ended until November, 2017. The DNA isolation was carried out according to a modified protocol suggested by Murray and Thompson[38]. The DNA concentration and purity of each sample was determined by a spectrophotometer which equipped with the Thermo Scientific Nano Drop 2000 system (Wilmington, USA). The absorbance ratio at 260 and 280 nm (A260/280) was used to evaluate the purity of the DNA. The value of A260/280 in 1.8-2.0 represents that DNA meet the experimental requirements. All samples DNA were diluted to 200 ng L^{-1} and stored at -20°C. Primers used in this study were synthesized by The Beijing Genomics Institute (Table 2), in China.

AFLP analysis: The AFLP analysis was performed according to Vos *et al*.[25] with a slight modification. Frequent cutter *Mse*I and rare cutter *Eco*RI endonucleases purchased from New England Biolabs (NEB) were used for restriction digestion of genomic DNA. Restriction and ligation were then carried out together in which restriction ligation master mix. Pre-amplification mixture in a total volume of 20 μL containing 10 μL of 2×PCR Mix, 1 μL of each pre-PCR primers, 4 μL of restriction and ligation and 4 μL DNA template. The DNA amplification was performed using the following program: 94°C for 3 min,

Table 1: *Populus* species used in this study

Number	Name of the clone	Parents or source	Species
1	Chulin-2	I-69 (American black poplar) and I-45/51 (Canadian poplar)	*P. deltoides* (S. America)×*P. canadensis*
2	Zhonglin-2025	I-69 (American black poplar) and American black poplar (North)	*P. deltoides* (S. America)×*P. deltoides* (N. America)
3	DD86-24	Seeding center of Hubei province introduced this American black poplar from Japan in 1998	*P. deltoides*, Seeding center of Hubei province introduced this species from Japan in 1998
4	107	American black poplar	*P. deltoides*×*P. canadensis*
5	Lushanyang	I-69 (American black poplar)×Shanhaiguanyang (LeuceDuby)	*P. deltoides*×*P. tomentosa*
6	222	50 yang (American black poplar)× 36 yang (American black poplar)	*P. deltoides*
7	I-69	American black poplar (South)	*P. deltoides* (S. America)
8	95-4	Seeding center of Hubei province introduced this American black poplar from Japan in 1998	*P. deltoides*
9	Hanyang-8	I-69 (American black poplar)×I-72 (Canadian poplar)	*P. deltoides*
10	Hanyang-2	I-69 (American black poplar)×I-72 (Canadian poplar)	*P. deltoides*
11	DD375	Seeding center of Hubei province introduced this American black poplar from Japan in 1998	*P. deltoides*
12	6204	Xiaoyeyang (TacamahacaSpach)×I-72 (Canadian poplar)	*P. simonii*
13	Zhongqian-3	I-69 (American black poplar)×I-63 (American black poplar)	*P. deltoides*
14	Xianglin-75	American black poplar	*P. deltoides*
15	6102	I-69 (American black poplar)×Xiaoyeyang (TacamahacaSpach)	*P. simonii*
16	Xianglin-90	American black poplar	*P. deltoides*
17	Danhongyang	50 yang (American black poplar)×36 yang (American black poplar)	*P. deltoides*
18	DD66-0	Seeding center of Hubei province introduced this American black poplar from Japan in 1998	*P. deltoides*
19	DD66-1	Seeding center of Hubei province introduced this American black poplar from Japan in 1998	*P. deltoides*
20	89-104	Seeding center of Hubei province introduced this American black poplar from Japan in 1998	*P. deltoides*
21	Nanlin-95	I-69 (American black poplar)×I-45/51 (Canadian poplar)	*P. deltoides* (S. America)×*P. canadensis*
22	Zhongjia-8	I-69 (American black poplar)×I-63 (American black poplar)	*P. deltoides*
23	I-63	American black poplar (South)	*P. deltoides* (S. America)
24	I-72	Canadian poplar	*P. canadensis*
25	Zhonghuahongye	bud mutation of I-69 (American black poplar)	*P. euramericana*
26	261	50 yang (American black poplar)×36 yang (American black poplar)	*P. deltoides*
27	Nanlin-895	I-69 (American black poplar)×I-45/51 (Canadian poplar)	*P. deltoides* (S. America)×*P. canadensis*
28	Zhongshi-8	I-69 (American black poplar)×I-63 (American black poplar)	*P. deltoides*
29	DD102-4	Seeding center of Hubei province introduced this American black poplar from Japan in 1998	*P. deltoides*

followed by 30 cycles of 94°C for 30 sec, 50°C for 30 sec and 72°C for 1 min and a finally 72°C for 5 min. Pre-PCR products were diluted 20-fold and used as DNA template for selective amplification. Selective amplification was carried out using the following touchdown program: 95°C for 5 min, 95°C for 35 sec, 60°C for 35 sec and 72°C for 1 min, followed by 12 cycles of each with 0.7°C lowering of annealing temperature and finally 23 cycles of 94°C for 30 sec, 56°C for 30 sec and 72°C for 1 min with a final hold of 72°C for 5 min. The AFLP products were denatured by heating at 95°C for 10 min and ran on a 6% polyacrylamide sequencing gel. The gels were viewed on a trans-illuminator and photographed. Following successful amplification viewed on the gel, the products were separated using automated capillary array system (ABI prism 3730 DNA analyser).

Data analysis: Data was analyzed using GeneMapper software to display the fragment sizes as electropherograms and binary data. The presence of an unambiguous band was scored as 1 and its absence as 0. All AFLP markers, both mono- and polymorphic, were scored in the data matrix. Only reproducible bands across two PCR amplification replicates were used in the subsequent analysis. The genetic relationship between sample populations was shown based on Nei's genetic distances[39] produced by TFPGA 1.3 software[40]. The NTSYS-PC software package version 2.10 was used for the cluster analysis[41]. Based on these genetic distances, cluster analysis was performed using the un-weighed pair group method with arithmetic mean (UPGMA) and a dendrogram was constructed[42]. Bootstrap analysis, with 1000 re-samples, was computed using Win boot to determine the confidence limits of the UPGMA dendrogram[35].

Table 2: Sequences of oligonucleotide adapters and primer combinations used in AFLP

No.	Primer combinations and adapters	Sequences (5'-3')	Primer combinations and adapters	Sequences (5'-3')
	*Eco*R I adapter 1	CTCGTAGACTGCGTACC	*Eco*R I adapter 2	AATTGGTACGCAGTCTAC
	Mse I adapter 1	GACGATGAGTCCTGAG	*Mse* I adapter 2	TACTCAGGACTCAT
	Pre-amplification	GACGATGAGTCCTGAG	Pre-amplification	GACTGCGTACCAATTCGTG
	*Eco*R I primer		*Mse* I primer	
P1	E35	GACTGCGTACCAATTCACA	M65	GATGAGTCCTGAGTAAGAG
P2	E50	GACTGCGTACCAATTCCAT	M48	GATGAGTCCTGAGTAACAC
P3	E39	GACTGCGTACCAATTCAGA	M50	GATGAGTCCTGAGTAACAT
P4	E75	GACTGCGTACCAATTCGTA	M61	GATGAGTCCTGAGTAACTG
P5	E85	GACTGCGTACCAATTCTCG	M60	GATGAGTCCTGAGTAACTC
P6	E83	GACTGCGTACCAATTCTCA	M49	GATGAGTCCTGAGTAACAG
P7	E86	GACTGCGTACCAATTCTCT	M65	GATGAGTCCTGAGTAAGAG
P8	E44	GACTGCGTACCAATTCATC	M48	GATGAGTCCTGAGTAACAC
P9	E76	GACTGCGTACCAATTCGTC	M50	GATGAGTCCTGAGTAACAT
P10	E84	GACTGCGTACCAATTCTCC	M60	GATGAGTCCTGAGTAACTC
P11	E40	GACTGCGTACCAATTCAGC	M60	GATGAGTCCTGAGTAACTC
P12	E77	GACTGCGTACCAATTCATC	M84	GATGAGTCCTGAGTAATCC
P13	E12	GACTGCGTACCAATTCAC	M50	GATGAGTCCTGAGTAACAT
P14	E13	GACTGCGTACCAATTCAG	M61	GATGAGTCCTGAGTAACTG
P15	E14	GACTGCGTACCAATTCAT	M84	GATGAGTCCTGAGTAATCC
P16	E15	GACTGCGTACCAATTCCA	M65	GATGAGTCCTGAGTAAGAG
P17	E16	GACTGCGTACCAATTCCC	M48	GATGAGTCCTGAGTAACAC
P18	E20	GACTGCGTACCAATTCGC	M22	GATGAGTCCTGAGTAAGT
P19	E21	GACTGCGTACCAATTCGT	M23	GATGAGTCCTGAGTAATA
P20	E23	GACTGCGTACCAATTCTA	M25	GATGAGTCCTGAGTAATG
P21	E12	GACTGCGTACCAATTCAC	M22	GATGAGTCCTGAGTAAGT
P22	E13	GACTGCGTACCAATTCAG	M17	GATGAGTCCTGAGTAACG
P23	E14	GACTGCGTACCAATTCAT	M25	GATGAGTCCTGAGTAATG
P24	E16	GACTGCGTACCAATTCCC	M12	GATGAGTCCTGAGTAAAC

RESULTS

AFLP polymorphism: In 29 *Populus* species from Jianghan Plain, a total of 24 combinations were screened and ten combinations successfully produced clear high-intensity, reproducible and relatively high polymorphism bands (Fig. 1). The total number of 959 common AFLP fragments were obtained (Table 3), of which 806 were polymorphic bands with an average polymorphism percentage of 84.67%. The number of polymorphism bands ranged from 56 by P2 (E50M48) to 95 by P12 (E77M84), while percentage of polymorphism bands varied from 74.74% for P10 (E84M60) to 96.55% for P2 (E50M48). Thus, each primer set generated an average of 95.5 fragments, 80.6 being polymorphic. Among them, the P2 (E50M48) presented the lowest number of amplification bands but the highest proportion of polymorphism. Therefore, specific levels of genetic differences exist among the 29 poplar samples.

Genetic similarity analysis: To compare the genetic differences among 29 poplar varieties, genetic similarity coefficients among the tested *Populus* species were calculated by using NTSYS PC version software 2.11 according to the AFLP data. Similarity coefficients among the 29 poplar clones measured were between 0.6111 and 0.8478, with the average value of 0.7217. Among them, the highest average similarity coefficient (0.7549) was measured between "Danhongyang" with other poplar varieties and the lowest average similarity coefficient (0.6532) was observed between "6204" and other poplar clones.

Genetic relationships: According to the genetic similarity, the phylogenetic tree was constructed by the UPGMA cluster to estimate the genetic relationship among 29 clones. As shown in Fig. 2, at a genetic similarity coefficient of 0.7117, the 29 poplar species were divided into four categories (A, B, C and D): category A includes two germplasm resources, "Chulin-2" and "DD66-1" (accounting for 6.90%). Category B contains 24 germplasm resources, including "Zhonglin-2025", "DD86-24", "Lushanyang", "222", "I-69", "95-4", "Hanyang-8", "Hanyang-2", "DD375", "Zhongqian-3", "Xianglin-75", "Xianglin-90", "Danhongyang", "DD66-0", "89-104", "Nanlin-95", "Zhongjia-8", "I-63", "I-72", "Zhonghuahongye", "261", "Nanlin-895", "Zhongshi-8" and "DD102-4" (accounting for 82.76%). Category C only comprises "107" (accounting for 3.45%). Category D contains two germplasm resources, "6204" and "6102" (accounting for 6.89%).

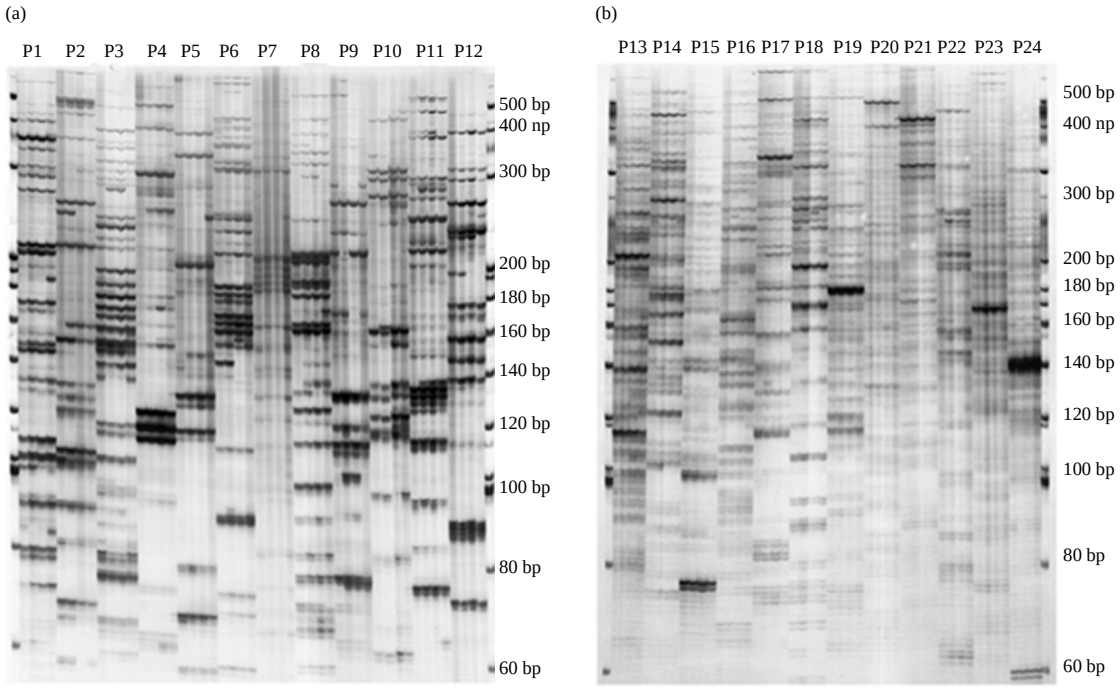

Fig. 1(a-b): Amplified patterns by 24 pair of primer combinations. P1, P2, P3.....P24 represent the number of primer combinations, respectively. P1 (E35M65), P2 (E50M48), P3 (E39M50), P4 (E75M61), P5 (E85M60), P6 (E83M49), P7 (E86M65), P8 (E44M48), P9 (E76M50), P10 (E84M60), P11 (E40M60), P12 (E77M84), P13 (E12M50), P14 (E13M61), P15 (E14M84), P16 (E15M65), P17 (E16M48), P18 (E20M22), P19 (E21M23), P20 (E23M25), P21 (E12M22), P22 (E13M17), P23 (E14M25), P24 (E16M12)

Table 3: Result of AFLP analysis of polymorphic bands of 29 poplar species

No.	Primer combinations	Total bands	Polymorphic bands	Percentage of polymorphic bands (%)
P1	E35M65	101.0	92.0	91.09
P2	E50M48	58.0	56.0	96.55
P3	E39M50	87.0	67.0	77.01
P4	E75M61	96.0	84.0	87.50
P6	E83M49	89.0	79.0	88.76
P8	E44M48	104.0	91.0	87.50
P9	E76M50	91.0	79.0	86.81
P10	E84M60	95.0	71.0	74.74
P11	E40M60	115.0	92.0	80.00
P12	E77M84	123.0	95.0	77.24
Total		959.0	806.0	-84.67
Mean		95.9	80.6	

Fingerprint: Based on the 806 polymorphic bands from AFLP analysis, the fingerprints of 29 clones belonging to three major *Populus* groups (*Aigeiros*, *Tacamahaca* and *Leuce*) were constructed. In the fingerprints, each poplar manifested a unique band type, which distinguished it from the others and the fingerprints showed high polymorphism.

DISCUSSION

In this study, intersectional, interspecific and intraspecific genetic and phylogenetic relationships among 29 *Populus* species belonging to the three sections (*Aigeiros*, *Tacamahaca* and *Leuce*) from Jianghan Plain, have been determined using AFLP markers for the first time. A total number of 959 common AFLP fragments were obtained, of which 806 were polymorphic bands with an average polymorphism percentage of 84.67%. The genetic similarity coefficient among the 29 poplar clones ranged from 0.6111-0.8478 and the average value was 0.7217. The cluster analysis of 29 poplar germplasms showed that all poplar germplasms can be classified into four categories at the genetic similarity coefficient of 0.7111.

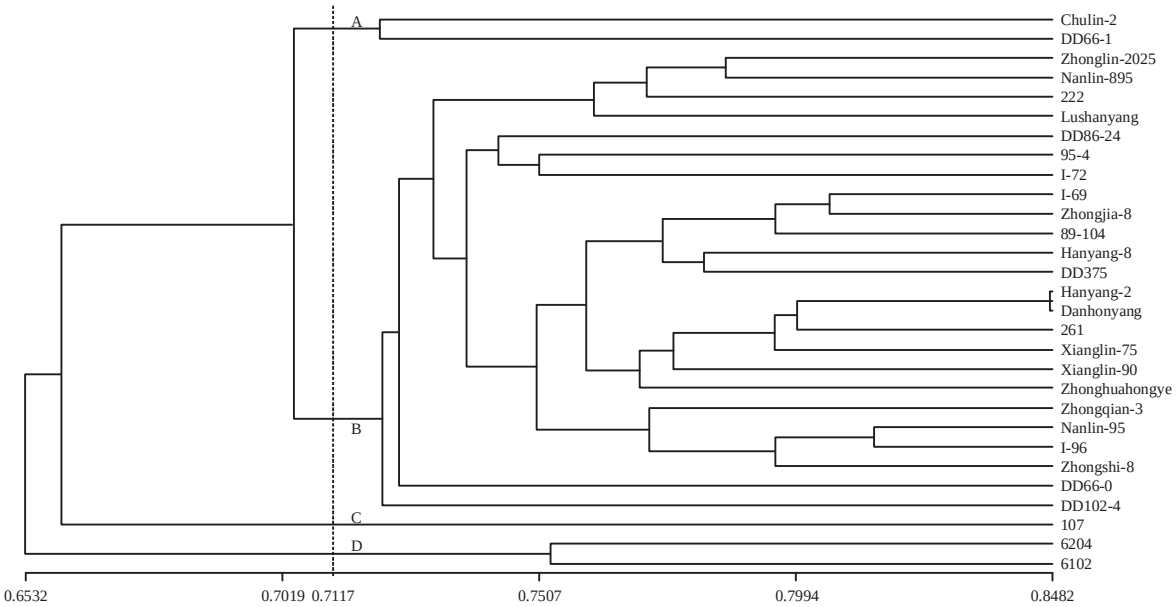

Fig. 2: Dendrogram of 29 *Populus* genotype from cluster analysis based on AFLP data

As we know, AFLP is one of the most reliable and repeatable molecular marker for evaluating the genetic diversity of plant and animal populations[43-51]. Its ability of screening many polymorphic fragments throughout the genome of this species made it possible to show the genetic diversity within and among populations. The AFLP polymorphism analysis showed there are many clear bands with high polymorphism and signal strength and easy to distinguish (Fig. 1), which clearly demonstrated that AFLP markers can be used for the genetic diversity analysis of *Populus* species. In the past decades, AFLP was first used in trees to genetically map a disease resistance in *Populus* species[52]. In addition, AFLP technology also applied in the evaluation of *Populus* genetic diversity and obtained a lot of achievements[18,53,54]. Results showed that *Populus* possesses high genetic diversities, except that, different individuals in groups, inter-species within a group and intra-species also possess abundant genetic variations[55-58].

Genetic diversity is the core of biological diversity, which reflects the capability of a given species to adapt the environment and the potential for long-term survival and evolution under continuous environmental changes[59-61]. UPGMA cluster analysis divided 29 *Populus* into four groups (Fig. 2). Among these groups, category B contained 24 germplasm resources, which basically involved the main poplar cultivars in the whole Yangtze River basin. This result indicated that these cultivars have relatively narrow genetic relationship. According to sources and parents of these germplasm resources, the vast majority of poplar germplasm resources are derived from hybrid progenies of a few poplar clones, such as "69", "63" and "72", which were introduced from Italy in 1970s (Table 1) and small genetic variations exist between these germplasm resources. In addition, each category features distinct primary and secondary compositions. These findings imply germplasms of the same species feature relatively high similarity. Poplar "107" formed a single category in cluster analysis, indicating that it possesses wide genetic variation. The large genetic differences in forest intra-species benefit hybrid breeding.

Taken together, combined with AFLP polymorphism, genetic similarity, fingerprints and cluster analysis, we understand if we want to cultivate superior new varieties that are suitable for cultivation in Jianghan Plain, we can start from two aspects. First, we should expand the introduction range, such as Poplar "107" which possesses wide genetic variation, and will benefit hybrid breeding. Secondly, we can hybridize black poplar with local poplar resources in Hubei province and introduce the genes of simon poplar (*Tacamahaca*), such as "6204" and "6102". These two clones were obtained by hybridizing "Lushanyang" and "Shengshanyang", respectively, with "Yichangxiaoyeyang" in our research group. These varieties grow well in low mountains and hills and not only have the fast-growing property of black poplar, but also the drought and barren resistance of simon poplar. On the one hand, the genetic basis of cultivated poplar species can be effectively expanded through these two ways. On the other hand, findings also indicated that clones, such as "107", "6204" and "6102", have further research value and can be used as parent resource for breeding superior new varieties.

CONCLUSION

It was concluded that 29 *Populus* species from Jianghan Plain share high genetic similarity and low genetic diversity. Among them, "107", "6204" and "6102" were three unique species and worth to further utilizing in hybridization and they provided a scientific guidance for the introduction of superior poplar varieties in Hubei province. In the future, the theory and method of molecular marker technology will also be developed and improved in genetic breeding of poplar.

SIGNIFICANCE STATEMENTS

This study assessed the genetic diversity and phylogenetic relationships of the 29 *Populus* species using the powerful AFLP technique. The genetic similarity analysis showed the existence of some genetic similarities among poplar varieties and abundant genetic variation among these varieties. The knowledge of genetic variation and the genetic relationship between genotypes can be an important consideration for efficient rationalization and utilization of poplar germplasm resources. This is the first report on molecular analysis of genetic diversity and population structure of *Populus* from Jianghan Plain. Moreover, this study addressed important phylogenetic questions within 29 *Populus* species and recommended three varieties of poplars for use in future breeding programs, genomic studies and varieties improvement.

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