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Development of 5S rDNA-Based Molecular Markers for the Identification of *Populus deltoides* Bartr. ex Marshall, *Populus nigra* L., and Their Hybrids

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Abstract: *Populus* L. is a tree genus that includes species with a high ability for interspecies hybridization. This process takes place in nature, and is used in poplar breeding. As a result, species identification in poplar populations and plantations is very difficult. In this study, a molecular marker system was developed for the identification of the most significant poplar species (*P. nigra* L. and *P. deltoids* Bartr. ex Marshall). The basis of the system is a polymorphism in non-transcribed spacers (NTSs) of 5S rDNA. The species-specific molecular markers were tested on a number of species and hybrids of poplars. It was shown that the marker system is a powerful tool for species identification, hybrid analysis, parent identification, and poplar breeding.

Keywords: poplars; molecular markers; non-transcribed spacers (NTSs); 5S rDNA; interspecies hybridization; species identification

1. Introduction

Poplars (*Populus* L. species) are widely-used forest trees that have strategic interest for many countries in the Northern Hemisphere. Their significant role in national economics was the reason for their study by chemists, agronomists, geneticists, breeders, and others [1–3]. From a botanical point of view, species of the *Populus* genus are often grouped into several sections: *Turanga* Bge., *Leucoides* Spach., *Aigeiros* Daby, *Tacamahaca* Spach., and *Populus* (syn. *Leuce* Daby) [4]. Hybridization successfully occurs among species in the same section and between some sections [5–7]. This feature of poplars has long been used in breeding programs. Interspecific hybrids of poplars are widespread in urban and industrial plantations. Species identification, the distinction of species and hybrids, and the determination of parents in hybrid poplars is almost impossible by morphological analysis alone. Therefore, it is very important to apply molecular methods.

Species identification with the help of molecular markers is a promising and rapidly developing area in molecular biology. The most popular method in this area is DNA barcoding based on fragments of chloroplast DNA and ITS1-5,8S-ITS2 in 45S rDNA [8,9]. However, there are cases where analysis by DNA barcoding is difficult. First of all, difficulties arise when studying young species, species with a low rate of evolution, species with a pseudogene of DNA barcode sequences and species involved in interspecific hybridization [10]. Attempts to use DNA barcoding for *Populus* species identification have been made worldwide, but a single polymorphic locus (single nucleotide polymorphism, SNP) for all poplars has not been determined. Therefore, systems of different markers for poplar barcoding (and PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) markers based on these SNPs) are repeatedly offered by many researchers [11–14].

In this study, we present another approach to identifying poplars. The approach involves the use of polymorphic fragments longer than SNPs to develop sequence-characterized amplified region (SCAR) markers. One of such loci is a non-transcribed spacer (NTS) of 5S rDNA. In seed plants and animals, the 5S rRNA genes (120 bp) form large arrays, in which they are separated by NTSs [15,16]. The length and nucleotide composition of NTSs are often detected as species-specific [17–19]. In contrast, the 120 bp coding sequences are conserved. The NTS amplification in different species is available with the help of the same primers which have been picked up on 5S rRNA gene borders [20].

In poplars, NTSs 5S rDNA has been studied for a long time. Two NTSs with different lengths were detected by authors in Reference [21]. The presence of two NTSs is typical for black poplars, and indirectly testifies to the hybridogenic origin of these species. The NTSs of *Aigeiros* Daby, *Tacamahaca* Spach., and *Populus* sections have been previously sequenced and aligned [21,22]. Long polymorphic sites were found in these NTSs that can be used for the design of species-specific primers. In this article, NTS polymorphism-based SCAR markers for the identification of two important *Populus* species were developed and tested in experiments with a wide range of poplars and hybrids.

2. Materials and Methods

2.1. Plant Material and DNA Isolation

Poplar cuttings and young leaves were collected from 22 trees (Table 1). DNA extraction was conducted according to the protocol of Doyle and Doyle (1990) [23], with some modifications [24]. The concentration of DNA in samples was equalized. DNA quality was tested by preliminary PCR with a universal primer pair based on 5S rRNA gene (5S1: 5′-GGATGGGTGACCTCCCGGGAAGTCC-3′; 5S2: 5′-GGATGGGTGACCTCCCGGGAAGTCC-3′) [20].

Table 1. Poplar tree samples and their characteristics.

Species	Parents of Hybrid	Sample Name	Co-Ordinates
P. nigra L.		tree#3	55°83′51.59″ 37°55′45.48″
		tree#10a	55°81′94.18″ 37°56′81.44″
		tree#15	55°76′77.41″ 37°56′47.46″
		tree#16	55°76′78.05″ 37°56′47.74″
		tree#10b	50°83′42.96″ 39°39′21.76″
P. deltoides Bartr. ex Marshall		tree#4	55°83′51.72″ 37°55′55.98″
		tree#1	55°81′89.74″ 37°56′83.83″
		tree#13	55°83′3.25″ 37°57′52.70″
P. × *canadensis* Moench.	*P. nigra* L. × *P. deltoides* Bartr. ex Marshall	tree#11	55°82′83.67″ 37°57′47.36″
		tree#14	55°83′5.53″ 37°57′47.93″
		tree#2	55°81′63.37″ 37°56′94.05″
		tree#17	55°77′0.49″ 37°56′56.19″
P. trichocarpa Torr. et A. Gray		tree#20	55°83′48.51″ 37°55′55.97″
P. maximowiczii Henry		tree#21	55°83′52.52″ 37°55′53.57″
P. simonii Can.		tree#22	55°83′52.74″ 37°55′54.93″

Table 1. *Cont.*

Species	Parents of Hybrid	Sample Name	Co-Ordinates
P. candicans Ait.		tree#23	55°83′53.31″ 37°55′52.29″
P. alba L.		tree#24	55°81′46.58″ 37°55′98.41″
P. tremula L.		tree#25	55°83′50.41″ 37°55′53.78″
P. × *moskoviensis* R. I. Schrod.	*P. suaveolens* Fish. × *P. laurifolia* Ldb.	tree#26	55°83′53.36″ 37°55′54.88″
P. × *sowietica pyramidalis* Jabl.	*P. alba* L. × *P. bolleana* Lauche	tree#27	55°83′67.63″ 37°55′52.73″
P. × *jablokovii* Jabl.	*P. tremula* L. × *P. bolleana* Lauche	tree#28	55°97′77.66″
			37°89′60.74″
P. × *berolinensis* K. Koch.	*P. laurifolia* Ldb. × *P. nigra* L.	tree#29	55°83′51.80″ 37°55′55.15″

2.2. Analysis of Sequences and Primer Design

Thirteen 5S rDNA fragments including NTSs of *P. nigra* and *P. deltoides* were collected from GenBank (*P. nigra*: AJ843800, AJ843801, AJ843802, KU994870, KU994871; *P. deltoides*: AJ292052, AJ292053, AJ292054, AJ292055, AJ292056, AJ292057, AJ843767, AJ843768). Sequence alignments were conducted with the help of GenDoc software [25]. NTS sequences with a low level of identity were selected for the analysis of polymorphic regions. The analysis was performed as follows. The NTS sequence of the studied species was aligned with all sequences of another species. Alignment was divided into ten-column fragments (the first column of each fragment was the second column of the previous fragment). The level of polymorphism in each fragment was calculated as the ratio of the number of polymorphic sites (nucleotides that differ from the nucleotides of the studied sequence or gaps) to the total number of sites in the fragment among all the sequences of another species. The polymorphism level in sites with gaps in the sequence of studied species was considered equal to zero. The neighboring fragments with a high level of polymorphism were used for the primer design. The design was conducted as follows. The previously designed 5S1 primer (see Section 2.1) was used as a basis to create a forward primer. The 5S1 primer is annealed in the center of the conserved 5S rRNA gene sequence, and can be used for a wide range of organisms.

This primer was checked with different variants of the reverse primer that is based on the above-mentioned polymorphic fragments. In order to check the primers, the Multiple Primer Analyzer and Basic Local Alignment Search Tool (BLAST) (see links in Figure S1) were used. When self-dimers and cross-primer dimers were identified, the length of the forward primer was modified and some other variant of the reverse primer was input.

The self-dimers and cross-dimer free primers are presented in Table 2. The primers were synthesized by ZAO "Synthol" (Moscow, Russia).

Table 2. The designed primers and the parameters of its use.

Primer Name	Sequence	Annealing, t °C [1]	PCR Product Length, bp
5S1mod1 NIG6-2r	5′-GGATGGGTGACCTCCCG-3′ 5′-TTCGAAGGAGCCGCGC-3′	62.8	485
5S1mod2 DEL121-4r	5′-GGATGGGTGACCTCCGG-3′ 5′-GGCGACCCTGGGAAGTGT-3′	66.0	433

[1] This is an "N" in Section 2.3.

2.3. PCR and Electrophoresis

The following PCR conditions were used: 94 °C for 5 min; 30 cycles of 94 °C for 20 s, N °C for 20 s, and 72 °C for 20 s; 72 °C for 10 min. For "N" values, see Table 2. PCR results were detected by electrophoresis on 2.5% agarose gel at 10 V/cm in 0.5 M TBE buffer using a Sub-Cell Model 192 camera

(Bio-Rad, Hercules, CA, USA) and photographed using the gel documentation system GelDoc XR Plus (Bio-Rad, Hercules, CA, USA).

3. Results

P. nigra and *P. deltoides* 5S rDNA sequences were found in GenBank and used for NTS isolation. The alignment of the NTSs showed that 11 sequences (AJ843800, AJ843801, AJ843802, KU994870 of *P. nigra* and AJ292052, AJ292053, AJ292054, AJ292055, AJ292056, AJ292057, AJ843767 of *P. deltoides*) had a 70%–99% level of homology between themselves (Figure S2, Table S1). *P. nigra* NTS KU994871 and *P. deltoides* AJ843768 NTS had a 39%–45% level of homology with others and 64% between themselves. These two NTSs were recognized as the most promising basis for the successful development of species-specific markers. They were selected for the detailed analysis of polymorphism.

The alignment of *P. nigra* NTS KU994871 with all NTSs of *P. deltoides* consisted of 517 columns (Table S2). The analysis of 508 ten-column fragments detected three fragments with a high (>90%) level of polymorphism (fragments 39, 40, and 469). The 14–20 bp regions containing the fragments were tested as primers. As a result, the region containing fragments 469–475 (NIG6-2r primer, Table 2) was changed for PCR assays. The alignment of *P. deltoides* NTS AJ843768 with all NTSs of *P. nigra* consisted of 512 columns (Table S3). The analysis of 503 ten-column fragments detected 12 fragments with a high (>90%) level of polymorphism (fragments 21, 22, 392–399, 414, and 415). The region containing fragments 386–395 (DEL121-4r primer, Table 1) was changed for PCR assays.

PCR tests of the designed primers were conducted with all collected DNA samples of *P. nigra*, *P. deltoides*, and *P.* × *canadensis* trees. Experiments with the 5S1mod1/NIG6-2r primer pair showed an amplification of one 485 bp fragment in cases with *P. nigra* and *P.* × *canadensis* samples (Figure 1). *P.* × *canadensis* fragments were dimly visualized by electrophoresis. PCR with the 5S1mod2/DEL121-4r primer pair showed an amplification 433 bp fragment in *P. deltoides* and *P.* × *canadensis* samples (Figure 1). Additionally, the electrophoresis showed that the signal brightness of the *P.* × *canadensis* fragments was lower.

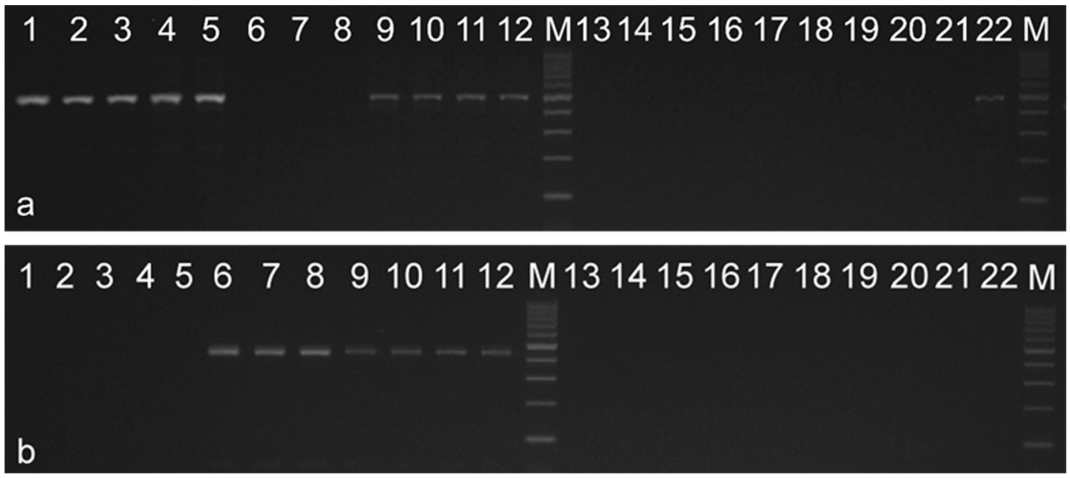

Figure 1. The electrophoresis picture with the results of the PCR (polymerase chain reaction) test: (**a**) with the 5S1mod1/NIG6-2r primer pair; (**b**) with the 5S1mod2/DEL121-4r primer pair. The numbers in both panels correspond to the following samples: 1—*P. nigra* L. tree#3; 2—*P. nigra* L. tree#10a; 3—*P. nigra* L. tree#15; 4—*P. nigra* L. tree#16; 5—*P. nigra* L. tree#10b; 6—*P. deltoids* Bartr. ex Marshall tree#4; 7—*P. deltoids* Bartr. ex Marshall tree#1; 8—*P. deltoids* Bartr. ex Marshall tree#13; 9—*P.* × *canadensis* Moench. tree#11; 10—*P.* × *canadensis* Moench. tree#14; 11—*P.* × *canadensis* Moench. tree#2; 12—*P.* × *canadensis* Moench. tree#17; 13—*P. trichocarpa* Torr. et A. Gray tree#20; 14—*P. maximowiczii* Henry tree#18; 15—*P. simonii* Can. tree#19; 16—*P. candicans* Ait. tree#23; 17—*P. alba* L. tree#24; 18—*P. tremula* L. tree#25; 19—*P.* × *moskoviensis* R. I. Schrod. tree#26; 20—*P.* × *sowietica pyramidalis* Jabl. tree#27; 21—*P. jablokovii* Jabl. tree#28; 22—*P.* × *berolinensis* K. Koch. tree#29.

PCR with DNA samples of other section poplars and their hybrids demonstrated no amplification in all cases. However, a weak amplification of the corresponding fragment with the 5S1mod1/NIG6-2r primer pair was observed in *P.* × *berolinensis*, which has a *P. nigra* in the parent generation (Figure 1).

In all described cases, the different level of the marker amplification in species and hybrids may be linked to the different number of marked 5S rDNA monomers in them.

4. Discussion

In this study, all described NTS classes of *P. nigra* and *P. deltoides* were analyzed. It was previously reported that all *P. deltoides* NTSs are separated into two classes [21,22]. Additionally, Wilson (2013) asserted that *P. nigra* has one NTS class. However, Alexandrov et al. (2014) reported a second NTS class in *P. nigra* [26]. This study confirmed the existence of two *P. nigra* NTS classes, because the second (described by Alexandrov et al., 2014) *P. nigra* NTS was used as the basis for the development of an efficient *P. nigra*-specific molecular marker.

Analysis of NTS alignments revealed that the majority of polymorphic fragments corresponded to inserts in the marked NTSs of *P. nigra* or *P. deltoides* (to deletion in other NTSs of the same and second species). Consequently, the annealing of NIG6-2r and DEL121-4r primers occurred in NTSs of one corresponding class only. Such a condition complex ensured reliable work for the developed species-specific markers in this case. However, a high level of NTS polymorphism is not observed in all closely related species. In cases where the polymorphic regions are short and cannot serve as a basis for primer development, molecular markers of other types can be created. For example, short polymorphic regions can be used to develop RLFP, SNP, or CAPS (cleaved amplified polymorphic sequences) markers [14,27]. Thus, the approaches for the development of species-specific markers presented in the article are not universal. However, they can be used in cases similar to this one. For example, they will be useful for marking some other poplar species.

Despite the importance of the presented approaches, the developed markers have a scientific value in themselves. They can be used as a tool for *P. nigra*, *P. deltoides*, and *P.* × *canadensis* identification in genetic or ecological studies, in dendrological and breeding practice (especially in the aphyllous period of the year), as well as in the analysis of other poplar hybrids. It is worth noting that the application of the markers requires conditions which exclude a PCR failure (because it may be taken as a false negative result). Most importantly, the quality of the used DNA must be high. The authors recommend testing DNA in a preliminary PCR experiment with universal 5S rRNA gene-based primers such as 5S1/5S2 primers [20].

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/9/10/604/s1, Figure S1: The Multiple Primer Analyzer link, Figure S2: The alignment of all described *P. nigra* and *P. deltoides* 5S rDNA NTSs, Table S1: The levels of homology between the described *P. nigra* and *P. deltoides* 5S rDNA NTSs, Table S2: The results of the polymorphism level analysis in ten-column fragments of KU994871 *P. nigra* 5S rDNA NTS, Table S3: The results of the polymorphism level analysis in ten-column fragments of AJ843768 *P. deltoides* 5S rDNA NTS.

Author Contributions: O.S.A. and G.I.K conceived and designed the experiments, and formulated the discussion; O.S.A. performed the experiments, analyzed the data, and wrote the paper.

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