Communication

Development of *Populus alba* L. and *Populus tremula* L. species specific molecular markers based on the 5S rDNA non-transcribed spacer polymorphism

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Abstract: The *Populus* L. genus unites tree species, which are botanically grouped into several sections. The species successfully hybridize both in the same section and between some sections. The poplar hybridization widely occurs in nature and in variety breeding. Therefore, the development of poplar species specific molecular markers is very actual. The effective markers for trees of the *Aigeiros* Daby section have been recently developed using the polymorphism of the 5S rDNA non-transcribed spacers (NTSs). In this article, the 5S rDNA NTS based markers were designed for several species of the *Leuce* Daby section. The *alb9* marker amplifies one fragment with DNA matrix of *P. alba* and *P. × canescens* (natural hybrid *P. alba × P. tremula*). The *alb2* marker works the same way except the case with *P. bolleana*. In this case, the amplification of three fragments was observed. The *tremu1* marker amplification is detected with DNA matrix of *P. tremula* and *P. × canescens*. Thus, the developed markers may be applied as useful tool for the *P. alba*, *P. tremula*, *P. × canescens* and *P. bolleana* identification in such areas of plant science as botany, dendrology, genetics of populations, variety breeding etc.

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

1. Introduction

The *Populus* L. genus includes about several dozen of tree species which are mainly distributed in temperate and subtropical latitudes of the Northern Hemisphere. These species are grouped in the following botanical sections: *Turanga* Bge., *Leucoides* Spach., *Aigeiros* Daby, *Tacamahaca* Spach., and *Populus* (syn. *Leuce* Daby) [1]. Due to the rapid growth, suitable wood, ease of vegetative propagation and decorative qualities, various types of poplar are widely used for landscaping, protective afforestation, plantation forestry, construction and other industries [2]. The high economic importance makes for an enlargement of the scientific interest in poplars. In different countries, several poplar genomes have been recently sequenced [3–7].

The *Populus* species successfully hybridize both in the same section and between some sections [8]. The poplar hybridization is widespread in nature. Also, it is effectively used in poplar variety breeding [9–13]. Identification of hybrids and parents by analysis of morphological characters is often difficult due to different reasons. For example, these are the environmental influences on morphology or differences between juvenile (in hybrid seedlings) and mature (in parent trees) characters [14]. In this connection, the development of biochemical and DNA molecular markers were actively applied in the work with poplar species and their hybrids [15–16]. The different types of DNA markers were previously developed. The microsatellite and single nucleotide polymorpfism (SNP) markers are most commonly used [17–20]. Also, the 5S rDNA non-transcribed spacer (NTS) based markers (sequence-characterized amplified region (SCAR) markers) have been recently

designed for identification of some poplars (*P. nigra*, *P. deltoides* and *P. × canadensis*) in *Aigeiros* section [21].

The 5S rDNA arrays are organized as typical satellite repeats in high plants and animals. In this case, the repeated monomer consists of two parts. The first part is a 120 bp region that is coding 5S rRNA. It is high conservative. The second part is a NTS, which can be of different length and nucleotide sequence in different species [22–24]. In poplars, several NTSs were previously sequenced and studied [25–27]. The observed polymorphism in these NTSs has a powerful potential for the species specific marker development.

In this article, the 5S rDNA NTS based markers are presented for the identification of some poplar species in *Leuce* section. As a useful tool, these markers may be applied in botanical, dendrological, genetic and breeding works with *P. alba*, *P. tremula*, *P. × canescens*, *P. bolleana* and their hybrids.

2. Materials and Methods

2.1. Plant Material and DNA Isolation

Young poplar leaves were collected from 27 samples (Table 1) and used for DNA extraction according to Doyle and Doyle (1990) protocol [28], with some modifications [29]. After equalization of concentrations with the help of the NanoDrop® ND-1000 (Thermo Fisher Scientific, United States of America, USA), the quality of DNA samples was tested by PCR with a universal primer pair based on 5S rRNA gene (5S1: 5'-GGATGGGTGACCTCCCGGGAAGTCC-3'; 5S2: 5'-CGCTTAACTGCGGAGTTCTGATGGG -3') [30].

Table 1. Poplar tree samples and their co-ordinates.

Species	Parents of hybrid	Sample name	Co-ordinates
P. alba L.	-	tree#1	55°81'46.58" 37°55'98.41"
		tree#2	44°22'78.85" 38°89'55.87"
		tree#3	44°64'65.02" 39°14'36.91"
		tree#4	45°29'43.82" 36°42'96.96"
		tree#5	50°44'89.88" 39°63'36.73"
		tree#6	55°83'62.65" 37°56'70.35"
P. tremula L.	-	tree#7	55°83'50.41" 37°55'53.78"
		tree#8	56°23'92.56" 38°11'17.26"
		tree#9	56°06'58.33" 37°90'21.71"
		tree#10	55°90'05.39" 37°56'67.05"
		tree#11	55°41'81.88" 37°84'72.91"
		tree#12	67°08'07.96" 32°86'88.48"
P. × canescens (Aiton) Sm.	*P. alba* L. × *P. tremula* L.	tree#13	55°45'11.99" 36°94'16.11"

			tree#14	55°72′38.02″ 37°56′49.92″
			tree#15	55°85′23.54″ 37°54′33.94″
P. bolleana Lauche			tree#16	45°29′56.17″ 36°43′13.14″
			tree#17	45°30′05.94″ 36°42′79.16″
			tree#18	45°30′04.41″ 36°42′81.74″
P. nigra L.			tree#19	50°83′42.96″ 39°39′21.76″
P. deltoides Bartr. ex Marshall	-		tree#20	55°83′51.72″ 37°55′55.98″
P. × canadensis Moench.	*P. nigra* L. × *P. deltoides* Bartr. ex Marshall		tree#21	55°82′83.67″ 37°57′47.36″
P. trichocarpa Torr. et A. Gray	-		tree#22	55°83′48.51″ 37°55′55.97″
P. maximowiczii Henry	-		tree#23	55°83′52.52″ 37°55′53.57″
P. simonii Can.	-		tree#24	55°83′52.74″ 37°55′54.93″
P. candicans Ait.	-		tree#25	55°83′53.31″ 37°55′52.29″
P. × moskoviensis R. I. Schrod.	*P. suaveolens* Fish. × *P. laurifolia* Ldb.		tree#26	55°83′53.36″ 37°55′54.88″
P. × berolinensis K. Koch.	*P. laurifolia* Ldb. × *P. nigra* L.		tree#27	55°83′51.80″ 37°55′55,15″

2.2. Analysis of Sequences and Primer Design

Nine 5S rDNA sequences including NTSs of *P. alba* and *P. tremula* were taken from GenBank (*P. alba*: AJ843770, AJ843771, AJ843772, KU994877, KU994878; *P. tremula*: KU994872, KU994873, KU994874, AJ843814). The manipulation, alignment of sequences and identity calculation were carried out in GenDoc software [31]. The low identity NTSs were used for studying of their polymorphic regions. Processing of polymorphism analysis was described in detail by authors in Reference [21]. The *P. alba* NTS fragments with a high level of polymorphism were selected for the development of the *alb2* and *alb9* markers. The *tremu1* marker was created as follows. The *P. tremula* NTS fragment with high level of polymorphism was used for the *tremu1* forward primer design. The 120 bp sequence of 5S rRNA gene was used to design the *5Srev* revers primer. All designed primer pairs were checked by Multiple Primer Analyzer (see link in Figure S1). The self-dimers and cross-dimer free primers (Table 2) were synthesized by ZAO "Evrogen" (Moscow, Russia) and ZAO "Synthol" (Moscow, Russia).

Table 2. The primer sequences and the expected lengths of PCR products.

Primer Name	Sequence	PCR Product Length, bp
alb2-f	5′- TTTTGCCGTTTTCC -3′	110
alb2-r	5′- AATCGCCCGGGAAAGGAAA -3′	
alb9-f	5′- TCGGAGTAGCGATTCACAGC -3′	126
alb9-r	5′- GTTTGCGTCGGACCATAACA -3′	
tremu1	5′- AGCCTCCCGCTGGG -3′	113
5Srev	5′- CGCTTAACTGCGGAGT -3′	

2.3. PCR and Electrophoresis

The PCR conditions for the *alb2* and *alb9* markers were the following: 94 °C for 5 min; 35 cycles of 94 °C for 20 s, 60 °C for 20 s, and 72 °C for 20 s; 72 °C for 10 min. In the case with the *tremu1* marker, the number of cycles was 30 and the annealing temperature was 68 °C. Other parameters were the same. PCR products were separated by electrophoresis on 2.5% agarose gel at 6 V/cm in 0.5 M TBE buffer using a Sub-Cell Model 192 camera (Bio-Rad, United States of America, USA). In the electrophoresis, the marker of molecular lengths "100 bp DNA Ladder" (Jena Bioscience GmbH, Germany) was used. The results of the electrophoresis were visualized and photographed using the GelDoc XR Plus (Bio-Rad, USA) gel documentation system.

3. Results

The *P. alba* and *P. tremula* NTSs were isolated from the collected sequences *P. alba*: AJ843770, AJ843771, AJ843772, KU994877, KU994878 and *P. tremula*: KU994872, KU994873, KU994874, AJ843814 (see in Figure S2). All NTSs had a different length. In *P. alba*, it ranged from 153 to 323 bp. In *P. tremula*, it ranged from 132 to 477 bp. They were aligned (Figure S3) and compared in level of homology (Table S4). It was found that the *P. alba* NTSs had a 39–93% level of homology between themselves. In the case with the *P. tremula* NTSs, the value of this indicator was 15–76%. The NTS homology between the two studied species was at the 18–48% level. Some NTSs with the 18% level of homology in any case (in *P. alba* AJ843770 and AJ843772; in *P. tremula* KU994872) were selected for analysis of polymorphism in detail.

In the analysis of polymorphism between the *P. alba* AJ843770, AJ843772 NTSs and all *P. tremula* NTSs, two alignments were conducted. The first alignment (with *P. alba* AJ843770 NTS) consisted of 336 columns (Table S5). The analysis of polymorphism in ten-column fragments did not show regions with a high level of polymorphism (>90%). However, the region between 288 and 318 points of the alignment (it is indicated with orange letters in Table S5) was evaluated as promising for primer design, because it had four 1-5 bp deletions (it is indicated with yellow colored cells) in the AJ843770 NTS. As a result, this region was changed for PCR test (*alb2-r* primer, Table 2). The forward primer for *alb2-r* (*alb2-f* primer, Table 2) was designed in the beginning of the AJ843770 NTS (it is indicated with red letters in Table S5). The second alignment (with *P. alba* AJ843772) consisted of 471 columns (Table S6). In this case, the analysis of polymorphism in ten-column fragments detected four extended regions (between 1 and 40, 105 and 115, 127 and 136, 316 and 339 points of the alignment) with a high level of polymorphism (>90%). The fourth region (it is indicated with orange letters in Table S6) was changed as a basis for primer design (*alb9-r* primer, Table 2). The forward primer for *alb9-r* (*alb2-f* primer, Table 2) was designed in the region that is located in front of the 316-339 region (it is indicated with red letters in Table S6).

In the analysis of polymorphism between the *P. tremula* KU994872 NTS and all *P. alba* NTSs, the 509-column alignment (Table S7) showed two extended regions (between 234 and 334, 423 and 486 points of the alignment) with 56–76% level of polymorphism (regions with high level of polymorphism (>90%) were not detected). One of the second extended region parts (it is indicated with red letters in Table S7) was selected as a basis for primer design (*tremu1* primer, Table 2). The revers primer for *tremu1* (*5Srev* primer, Table 2) was designed in the region that is located in the middle of the 5S rRNA gene sequence.

Thus, three pairs of the designed primers were synthesized for PCR test. The test was conducted in two stages. In the first stage, the samples of the *Leuce* poplars were used only (Figure 1, a–c). In the second stage, the markers were checked with DNA matrices of poplars from other sections (Figure 1, d–f). PCR with the *alb2* marker (Figure 1, a) was resulted the amplification of one 110 bp fragment in the case with all *P. alba* and *P. × canescens* samples. This fragment was amplified in *P. bolleana* profile too. The profile also included two added fragments (approximately 85 and 135 bp). In the case with all *P. tremula* samples, there was no amplification. The results of the *alb9* marker work (Figure 1, b) were the following. The amplification of the expected 126 bp fragment was detected in the cases with all *P. alba*, *P. bolleana* and *P. × canescens* samples. In all *P. tremula* samples, the amplification was absent as in the case with the *alb2* marker. The work of the *tremu1* marker

consisted in the 113 bp fragment amplification in the cases with all *P. tremula* and *P. × canescens* samples. In the cases with all *P. alba* and *P. bolleana* samples, no fragment was amplified (Figure 1, c). Also, the second stage of the PCR test showed the absence of the amplification with all studied markers in the cases with poplars of other sections (Figure S8, a–c).
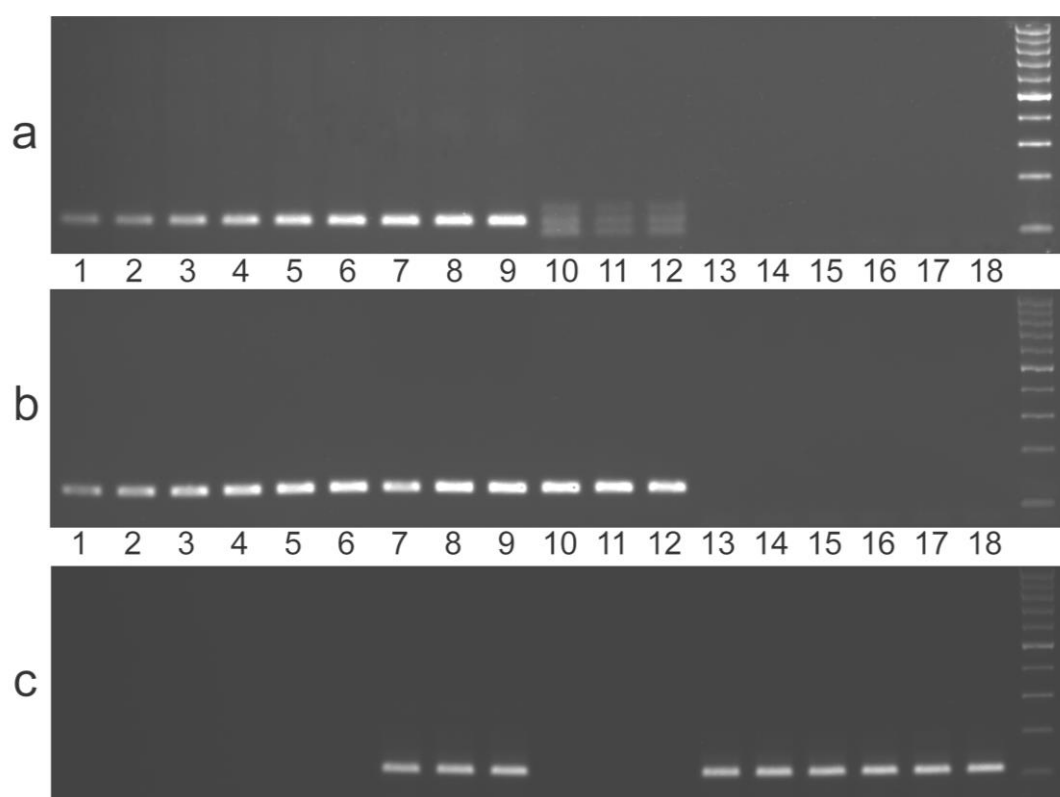

Figure 1. The results of the first stage of the PCR (polymerase chain reaction) test: (a) with the *alb2* marker; (b) with the *alb9* marker; (c) with the *tremu1* marker. The numbers in all panels correspond to the following samples: 1—*P. alba* L. tree#1; 2—*P. alba* L. tree#2; 3—*P. alba* L. tree#2; 4—*P. alba* L. tree#4; 5—*P. alba* L. tree#5; 6—*P. alba* L. tree#6; 7—*P. × canescens* (Aiton) Sm. tree#13; 8—*P. × canescens* (Aiton) Sm. tree#14; 9—*P. × canescens* (Aiton) Sm. tree#15; 10—*P. bolleana* Lauche tree#16; 11—*P. bolleana* Lauche tree#17; 12—*P. bolleana* Lauche tree#18; 13—*P. tremula* L. tree#7; 14—*P. tremula* L. tree#8; 15—*P. tremula* L. tree#9; 16—*P. tremula* L. tree#10; 17—*P. tremula* L. tree#11; 18—*P. tremula* L. tree#12.

4. Discussion

In this article, all included in GenBank sequences of the *P. alba* and *P. tremula* NTSs were analyzed as it was made in the previous study with *P. nigra* and *P. deltoides* [21]. It was previously described that NTSs of these black poplar species are separated into two classes [21,25]. In the case with the *P. alba* and *P. tremula* NTSs, such clear separation was not observed. All studied NTSs had a different length. The level of homology between NTSs of one species was different too. In general, the studied *P. tremula* NTS sequences were more homologous to each other than *P. alba* NTSs. The KU994872 and KU994873 NTSs were the most alike (with 93% level of homology). They were distinguished by 7 single nucleotide substitutions and one 14 bp deletion only (the mutations are indicated with a red shade in the Figure S9). Despite this example, the intraspecies level of NTS homology in *P. tremula* and *P. alba* was generally lower than in *P. nigra* and *P. deltoides* (as it was previously reported in Reference [21], this indicator was 71–98% in these species of black poplars). In these two compared cases, the interspecies level of NTS homology was also lower with the exception of several NTS pairs, which had a similar value of this indicator. Thus, the potential for the successful species-specific marker development was originally rated as quite high.

However it is worth noting, that in practice the development of the *alb2* and *tremu1* markers was connected with some difficulties. In the *alb2* development, the AJ843770 NTS had a small length

(in comparison with other analyzed NTSs) and the alignment included many gaps in the bottom line (the line with sequence, which is rated). Since the gap in this line corresponds to zero value of the polymorphism level in this point of the alignment, the polymorphism level of ten-column fragments with this point is decreased. As a result, the ten-column polymorphism was not high in the alignment with the AJ843770 NTS (a level of polymorphism was considered high, if it was >90% as in Reference [21]). This problem was not observed in the marker development in black poplar, when the length differences between the rated NTS and others were not large [21]. In sum, the problem with the AJ843770 NTS was solved by design a primer on the basis of the short region with a large number of deletions. This approach may be a useful solution, when the markers are developed in similar conditions. In the *tremu1* development, there were two problems. The first problem consisted of the presence of two short (in comparison with others) NTS sequences (AJ843770 and KU994878) in the alignment, which was compared with the rated sequence (KU994872). Since the gap in such lines (all lines except the bottom line) is considered as a polymorphic nucleotide, the polymorphism level of ten-column fragments with this point is increased. When other nucleotides in this column are polymorphic, there is no problem. However, if other nucleotides in this column coincides (or at least two of them: the first in the bottom line and the second in any other line), then the polymorphism level of ten-column fragments with this point is overpriced and the fragments may be not suitable for the marker development. Such situation was observed for the most part of the 234-334 and 423-486 extended regions in the *tremu1* alignment. As a solution of this problem, the short sequences may be excluded from the alignment. In the test with these *tremu1* extended regions, the exclusion of AJ843770 and KU994878 NTSs form the alignment decreases the polymorphism level of ten-column fragments (Table S10). The second problem in the *tremu1* development was in the following. The selected region for primer making stood out by the level of polymorphism in that alignment place. However there were regions in other alignment places, which had coincidences with the 5'-part of the selected region (the coincidences are indicated with a red shade in the Figure S11). Such regions are occasionally masked in the process of the alignment construction and may be detected with the help of Basic Local Alignment Search Tool (BLAST). As a solution of this problem in the *tremu1* development, the following approach was used. Since the coincidences were detected in 5'-part only, the primer was designed with a forward orientation and the annealing temperature was increased as much as possible.

In the cases with *P. alba*, *P. tremula* and *P. × canescens* samples, all markers amplified as it had been expected. The *alb2* and *alb9* amplification as well as no *tremu1* amplification were observed in *P. alba* samples. The *tremu1* amplification with no *alb2* and *alb9* amplification were fixed in *P. tremula* samples. The amplification of *alb2*, *alb9* and *tremu1* was detected in *P. × canescens* (natural hybrid *P. alba × P. tremula*) samples. Thus, the effective molecular marker system for the rapid identification of these two species and their hybrids were created. This result may have an important value in genetics of populations, as these plants are often used as model objects. Usually, the SSR marker sets are applied in such experiments [18,19]. The use of the presented marker system may decrease the volume of PCRs, increase the speed of the works and reduce their cost.

The work of the presented markers in other plants of the *Leuce* section has a significant interest for botanists. In this article, the *P. bolleana* (the white poplar with a pyramidal crown, which is considered by some scientists as a form or synonym of *P. alba* [32,33]) samples were tested with the developed markers. The results of the *alb9* and *tremu1* work with the *P. bolleana* samples were exactly the same as in the *P. alba* samples. However in the case with the *alb2* marker, the amplification of two added fragments was detected in the profile. The reason of this result may be revealed in the thorough study of the *P. bolleana* 5S rDNA NTSs. Nevertheless, the *alb2* marker application will be useful, for example, in checking of the *P. alba* and *P. bolleana* herbarium materials.

The PCR experiments with poplar trees of other sections showed no amplification for each of the presented markers. This fact testifies that they are *Leuce* section specific. Thus, the developed markers may be applied as a useful tool for the *P. alba*, *P. tremula*, *P. × canescens* and *P. bolleana* identification in such areas of plant science as botany, dendrology, genetics of populations, variety breeding etc.

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