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*Full Length Research Paper*

# Evaluation of interspecific DNA variability in poplars using AFLP and SSR markers

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**The objective of this paper was to examine interspecific DNA variation in poplars using AFLP and SSR markers. The AFLP and SSR markers polymorphism and its power of discrimination were determined within 13 genotypes of different genetic background (clones, cultivars, hybrids) of two sections (*Aigeiros* and *Tacamahaca*) of genus *Populus*. Twelve sets of PTR and four sets of WPMS microsatellite primers as well as 6 AFLP primer combinations were used in this study. SSR and AFLP markers revealed high power of discrimination, 0.81 and 0.92 respectively. Results obtained using SSR data showed a clear separation of three major clusters, while four major clusters were obtained using AFLP data. Both markers crearly separated two distinct clusters, one included *Populus nigra* and the other *Populus deltoides* genotypes. According to both markers, different genetic background were revealed between two clones of *Populus trichocarpa.* When evaluating polymorphisms within genotypes of interest, microsatellite and AFLP DNA markers proved to be a useful tool for distinguishing genetic background of tree clones, cultivars and hybrid genotypes thus grouping them according to their genetic dissimilarity. The results presented in this study could be of significant interest in poplar breeding programs and could also be used as a valuable annex in new bred clones registration process.**

**Key words:** Poplar, AFLP and SSR markers, genotyping, polymorphisms.

## INTRODUCTION

Genus *Populus* L. (Salicaceae) includes about 30 species classified in six sections (Dickman and Stuart, 1983), distributed widely, mainly in the forests of temperate and cold regions of the Northern Hemisphere. The economically most important species are in the section *Aigeiros* Duby, *Tacamahaca* Spach. and *Leuce* Duby. Although *Populus* has become the model genus for molecular genetics and genomics research on forest trees, genetic relationships within this genus have not yet been comprehensively studied at the molecular level (Cervera et al., 2005).

*Populus deltoides* Marsh, (cotton-wood) and *Populus nigra* L. (black poplar) of section Aigeiros, *Populus trichocarpa* Torr. Ex Gray (black cottonwood), *Populus balsamifera* L. (balsam poplar) and *Populus maximowiczii* Henry (Japanese poplar) of the section *Tacamahaca* are the most important species for interspecific poplar breeding programs worldwide (Zsuffa, 1975; FAO, 1979; Rahman and Rajora, 2002). These species and their interspecific hybrids are suitable for short-rotation intensive poplar culture and agro-forestry. Identification of *Populus* clones and cultivars and knowledge relating to their genetic interrelationship are essential for effective selection, breeding and genetic resource management programs. Furthermore, it is also essential for varietal control, protection and registration as well as for handling of planting and breeding stocks. A main concern confronting poplar breeders is to improve knowledge of genetic constitution, relationships and identification of clones, cultivars and hybrids on molecular basis.

The poplar plantations are very important in forest

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**Table 1.** Species of *Populus* used to test SSR and AFLP primer pair utility.

| Genotypes under observation: | Section |
|---|---|
| 1. B81 (*Populus deltoides*) | *Aigeiros* Duby |
| 2. B229 (*Populus deltoides*) | *Aigeiros* Duby |
| 3. Panonija - M1 (*P. x euramericana*) | *Aigeiros* Duby |
| 4. 182/81 (*Populus deltoides*) | *Aigeiros* Duby |
| 5. PD100 (*Populus deltoides*) | *Aigeiros* Duby |
| 6. NS001 (*Populus nigra L.*) | *Aigeiros* Duby |
| 7. NS002 (*Populus nigra L.*) | *Aigeiros* Duby |
| 8. I-214 (*P. x euramericana*) | *Aigeiros* Duby |
| 9. 102/81 (*P. nigra x P. maximowiczii*) | |
| 10. 9111/93 (*P. nigra x P. maximowiczii*) x *P. nigra var.* Italica | |
| 11. *P. nigra* var. Italica | *Aigeiros* Duby |
| 12. 1007(*P. trichocarpa*) | *Tacamahaca* Spach |
| 13. 1004 (*P. trichocarpa*) | *Tacamahaca* Spach |

sector in Serbia. In the past 50 years 16 new poplar cultivars created in Institute of Lowland Forestry and Environment (ILFE) were introduced into newly established plantations. Today, the share of poplar plantations is 1.3% of total forest area in Serbia, but the share of income is about 20%.

Serbian forest breeding programs in ILFE until now were based only on conventional clonal identification system, which was based on combination of morphological and phenological (Orloviæ et al., 1997) traits characterization. The DNA based background insights of the poplar genotypes in the ILFE nurseries were revealed in this paper for the first time. They were revealed and characterized using molecular markers systems like AFLPs and SSRs.

While AFLP markers have been utilized in evaluating hybrids and in parentage assessment in many other species (Van Toai et al., 1996; Krauss, 2000; Galoviæ et al., 2004; Galoviæ et al., 2006), the highly polymorphic, consistent and codominant markers, such as SSRs provided excellent markers for clone and cultivar identifycation and in poplars as well, according to Dayanandan et al. (1998), Rahman et al. (2000), Rajora and Rahman (2003), Van der Shoot et al. (2000), and Galoviæ and Orloviæ, (2007).

The objectives of this paper were to test the informational value of SSRs and AFLPs markers originally developed for other species and make differentiation of selected clones, cultivars and their hybrids from two sections of genus *Populus* by evaluating their DNA profiles and to determine their molecular and genetic interrelationships and get the information on interspecific DNA polymorphism in observed poplar individuals.

As preservance of genetic variability is the main task for maintenance of the adaptive potential in species, therefore indentification of poplar clones and cultivars and knowledge of their genetic interrelationship are essential for effective breeding and selection.

Characterization of diversity in forest species, until now, has been mainly based on revealing morphological and physiological traits in research programs. The contribution of this paper to future progress in the field of poplar breeding in Serbia is to acknowledge the genetic constitution of poplar clones by molecular techniques that from now on will be introduced into breeding programs of ILFE. This way could be possible to characterize Serbian genetic material and summarize its genetic potential.

## MATERIALS AND METHODS

### *Populus* species and their clones/cultivars/hybrids

Thirteen poplar individuals, with different genetic background (clones, cultivars/clones, hybrids/clones) from two sections: nine genotypes of *Aigeiros* Duby (4 clones of *P. deltoides* - B81, B229, 182/81; 2 clones of *P. nigra* - NS001, NS002 and 2 hybrids of *Pxeuramericana* - Pannonia, I-214; 1 cultivar - *P. nigra* var. Italica), two genotypes from *Tacamahaca* Spach (2 clones of *P. trichocarpa* -1004, 1007) and two hybrids (one clone of *P. nigra* x *P. maximowiczii* - 102/81; one clone of (*P. nigra* x *P. maximowiczii*) x *P. nigra* var. Italica) were sampled from the ILFE nursery (Table 1).

### Microsatellite (SSRs) and AFLPs markers protocols

DNA was extracted from freeze-dried leaf material using DNeasy Mini Kit (Qiagen, GmbH, www.qiagen.com) according to manufacturer's instructions. Twelve SSR marker loci (*PTR1, PTR2, PTR3 PTR4, PTR5, PTR6, PTR7, PTR8, PTR11, PTR12, PTR14, PTR15*), originally developed from *Populus tremuloides* (Dayanandan et al., 1998; Rahman et al., 2000); 4 *WPMS* SSR marker loci (*WPMS3, WPMS5, WPMS7 and WPMS12*) originally established from *P. nigra* (van der Schoot et al., 2000) and 6 AFLP primer combinations were used to determine the identity and genetic relationship of the genotypes.

PCR protocols followed for SSR analysis were: for WPMS markers according to van der Schoot et al. (2000) and for PTR markers according to Dayanandan et al. (1998). AFLP analysis was performed using the protocol of Vos et al. (1995). For digestion reactions *EcoRI* and *MseI* endonucleases were used. According

**Table 2.** Means for SSR DNA variability and informative parameters over the 13 SSR loci for differentiation of 13 poplar genotypes

| SSR loci | PTR1 | PTR2 | PTR3 | PTR4 | PTR5 | PTR6 | PTR11 | PTR12 | PTR14 | PTR15 | WPMS3 | WPMS7 | WPMS12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. of alleles | 6 | 3 | 6 | 1 | 6 | 6 | 2 | 1 | 6 | 3 | 6 | 6 | 4 |
| PD | 0.769 | 0.538 | 0.793 | 0 | 0.686 | 0.769 | **0.132** | 0 | 0.811 | 0.642 | **0.812** | 0.778 | 0.742 |

PD = Power of discrimination.
Characters in 'bold' signifies the lowest and the highest power of discrimination for locus under observation.

**Table 3.** Allelic constitution of *P. trichocarpa* clones.

| Clones no. | WPMS3 | WPMS7 | WPMS12 | PTR1 | PTR2 | PTR3 | PTR4 | PTR5 | PTR6 | PTR11 | PTR12 | PTR14 | PTR15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1007 | f | a | 0 | f | c* | f | 0 | a | d | a | a | e | 0 |
| 1004 | 0 | f | 0 | d | c | c | 0 | a | f | a | a | e | 0 |

*Similar allele signifies the same genetic fragments that two clones share.

to Cervera et al. (2005), pre-amplification reactions were carried out with *EcoRI*+A and *MseI*+AC primers. Due to high level of observed inter-specific polymorphisms, a combination of one *EcoRI* and one *MSeI,* with three and four selective nucleotides, was used to reduce the complexity of DNA finger-printing and facilitate scoring. The following six primer combinations were chosen for the final selective amplification: *Pes1/ Pms3, Pes1/Pms4, Pes1/Pms1, Pes1/Pms2, Pes2/Pms2 and Pes2/Pms3.* Only constant bands in range of 100 - 500 bp were screened. Sequencing was performed on 6% denaturing PAA gels at 75W constant current for 2 - 3 h. Visualization of PCR products for both systems, SSRs and AFLPs, was done using silver staining protocol. The gels were air-dried and contact prints were made on DIAS system (SERVA Electrophoresis, GmbH, www.serva.de) to obtain permanent images.

### Data analysis

For both markers, the locus and allele sizes were determined by including different DNA standard size markers: 100 bp Plus Ladder (GeneRuler$^{TM}$, Fermentas, life sciences, Lithuania, www.fermentas.com), 50 bp size ladder (O'RangeRulerTM, Fermentas) and a ultra low range size ladder (Gene Ruler$^{TM}$, Fermentas).

Microsatellite alleles at one locus were identified by their molecular sizes in base pairs (bp). Their single and multi-locus genotypes indicated in different allele paterns and ranges were constructed for genetic identification of each individual. Microsatellite DNA variability parameters were determined for each of SSR locus as well as over all 16 SSR loci for each *Populus* species: number of alleles, number of alleles/loci, number of observed SSR genotypes and power of discrimination (Table 2).

AFLP markers were scored in the range of 100-500 bp as a binary character for the presence (1), or absence (0) of fragments. Only intense, consistently amplified fragments, which were clearly separated from other fragments, were scored. Parameters of variation for AFLP loci for each *Populus* species were: number of overall scored bands in specific range, number of monomorphic and polymorphic bands, polymorphisms rate in percentage and power of discrimination, respectively (Table 3).

Statistical analysis for both SSRs and AFLPs were carried out with the software NTSYS-PC software package, version 2.1 (Rohlf, 2000). Genetic dissimilarity (GD) among accessions was estimated from the number of shared amplified fragments by using the dissimilarity coefficients Dice (Dice, 1945), GS(*ij*) = 2*a*/(a+b+c) where GS(*ij*) is the measure of GS between the individuals *i* and *j*, *a* is the number of polymorphic fragments that are shared by *i* and *j*, *b* is the number of fragments present in *i* and absent in *j* and *c* is the number of fragments present in *j* and absent in *i*. The agreement among the Dice's dissimilarity matrix was tested using Mantel's test (Mantel, 1967). The randomization procedure as implemented in NTSYS-pc version 2.1 (Rohlf, 2000) software package included $10^4$ random permutations.

The unweighted pair group method with arithmetic mean (UPGMA) analyses was performed based on the dissimilarity matrix using PHYLIP software v.3.63 (Felsenstein, University of Washington, Seattle, WA, USA). In addition, bootstrap analysis was perfor-med on 1000 bootstrap samples to test the reliability of branches (Felsenstein, 1985). UPGMA dendrograms were visualized with the Tree View program (Page, 1996). The power of discrimination (PD) for individual microsatellite and AFLP DNA loci was determined as follows (Klosterman et al., 1993): PD = $\sum G^2_i$ where *Gi* is the fre-quency of the $i^{th}$ genotype at a locus.

## RESULTS

### SSR markers analyses

13 out of 16 SSR loci were successfully amplified, while three loci (*PTR7, PTR8* and *WPMS5*) failed. All amplified loci showed single-locus pattern in all 13 *Populus* individuals examined. Three loci (*PTR4, PTR11* and *PTR12*) were monomorphic

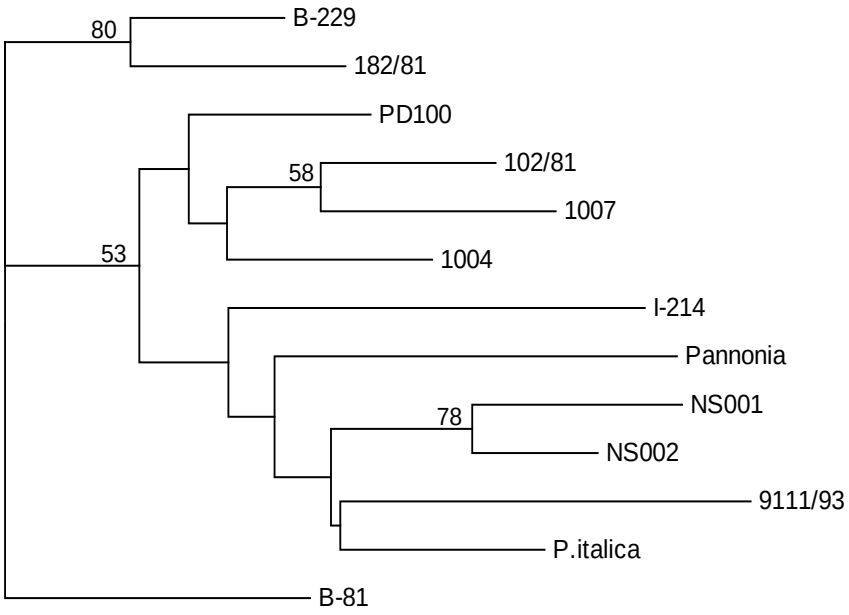


**Figure 1.** Unrooted NJ tree of *Populus* clones constructed from SSR fragment dissimilarities.

and 10 loci proved to be polymorphic. Among these loci, the individuals that failed to show any amplification product were designated as null genotypes. At the same time such individuals amplified well with the primers of the other DNA loci and they were not taken into account in the statistical analyses. Twenty-nine null alleles were found for all 13 loci.

In total, 56 alleles were scored out of 13 SSR loci. Loci *WPMS3* with 0,812 power of discrimination showed the highest polymorphisms among genotypes, while *PTR11* scored the lowest polymorphism with 0.132 power of discrimination (Table 2). Out of 13 loci and 56 alleles the average number of alleles per locus was 4.31.

Allelic frequency regarding genotypes under observation differed. Allele *a* had the highest frequency and appeared in all genotypes under observation with 100% of the cases. Allele *b* contributed with 28.6% and almost shared the rate with allele *d* that appeared with a slightly higher percentage (30.3%). Allele *c* was scored in 53.57% of the cases. Alleles *e* and *f* (with their 10.7% contribution) were observed mainly in the hybrid/clones I-214 (*P.* x *euramericana*), 102/81 (*P. nigra* x *P. maximowiczii*), 9111/93 (*P.nigra* x *P.maximowiczii*) x *P.nigra* var. Italica and in *P. trichocarpa* and once in the clone *P. nigra* var. Italica. Allele *e* was predominant in hybrid/clones genetic background while allele *f* was exclusively found in *P. trichocarpa* with the exception of *P. nigra* var. Italica where allele *f* was amplified using *PTR14* marker set. It is interesting to mention that both *P. trichocarpa* clones (1004 and 1007) had no identical genetic background but phenotipically were similar. Their allelic constitutions are shown in Table 3.

Cluster analyses performed according to SSR data showed a clear separation of three different *Populus* species and the hybrids (Figure 1). Three major clusters were obtained containing three *P. deltoides* genotypes B81, B229 and 182/81 as the cluster 1. Cluster 2 separated *P. deltoides* clone (PD100) and hybrid (*P.nigra x P.maximowiczii*) together and two genotypes (1007 and 1004) of *P. trichocarpa* with genetic dissimilarity ranged from 0.286 - 0.444. Clone PD 100 showed higher genetic dissimilarity to hybrid/clone 102/81 of 0.286 than to *P. trichocarpa* clones. Cluster 3 included two subclusters - genotypes Pannonia and I-214 and the main group consisted of *P. nigra* genotypes and one hybrid clone (*P. nigra* x *P. maximowiczii* x *P. nigra* var. Italica). In this cluster NS001 and NS002 were closely related to 0.273 genetic dissimilarity (supported by high bootstrap value of 78%).

## Analyses for AFLP markers

AFLP marker technique employed analysed 13 individuals by 6 selective primer combinations (Table 4). Sixty bands in total were scored in all primer combinations that gave in average 9.5 polymorphic bands per locus. All AFLP marker loci showed high polimorphic paterns, where *Pes1/Pms1* selective primer combination was the most polymorphic with 0.923 power of discrimination (PD). Then follows *Pes1/Pms4* primer combination with 0.883 PD that signified also high polymorphisms rate. The lowest PD of 0.207 was scored out of *Pes2/Pms3* primer combination.

Cluster analyses performed according to AFLP data showed a clear separation of three different *Populus*

**Table 4.** Means for AFLP DNA variability and informativeness parameter over the 6 AFLP loci for differentiation of 13 *Poplar* genotypes.

| AFLP loci | No. of bands | Polymorphic bands | Monomorphic bands | PD |
|---|---|---|---|---|
| Pes1/Pms1 | 18 | 17 | 1 | 0.923 |
| Pes1/Pms2 | 5 | 5 | 0 | 0.318 |
| Pes1/Pms3 | 12 | 11 | 1 | 0.828 |
| Pes1/Pms4 | 14 | 13 | 1 | 0.883 |
| Pes2/Pms2 | 5 | 5 | 0 | 0.817 |
| Pes2/Pms3 | 6 | 6 | 0 | 0.207 |
| Total | 60 | Polymorphic bands/locus 9.5 | | |

PD = Power of discrimination.

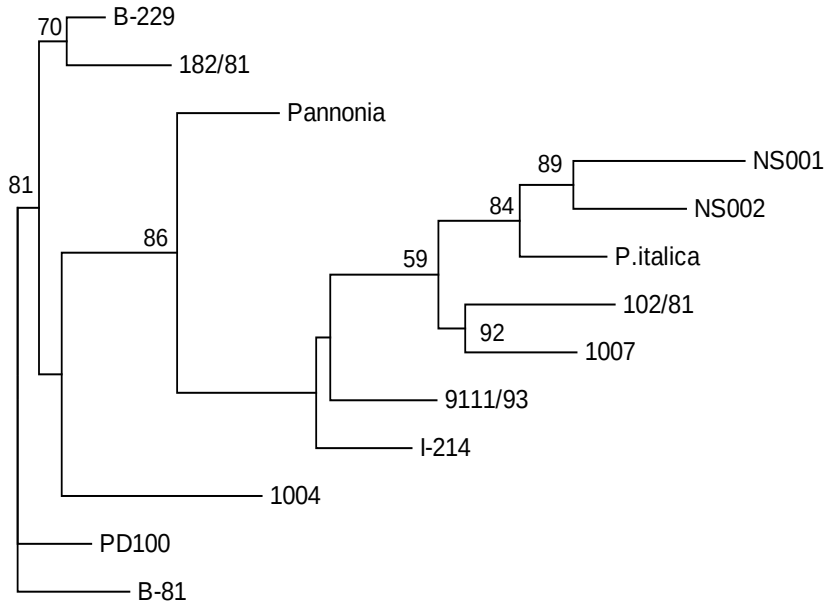


**Figure 2.** Unrooted NJ tree of *Populus* clones constructed from AFLP fragment dissimilarities.

species and hybrids (Figure 2). Four major clusters were obtained which contained the *P. deltoides* genotypes (cluster1), the *P. trichocarpa* genotype (cluster 2), hybrid genotypes (cluster 3) and the *P. nigra* genotypes (cluster 4) as four distinct groups. Four *P. deltoides* genotypes (B229, 182/81, PD100 and B81) were very close to each other and genetic dissimilarity ranged from 0.100 - 0.179. It is interesting to mention that *P. trichocarpa* genotype 1004 was distinguished as a separate group but more connected to the genotypes from cluster 3 at genetic dissimilarity of 0.333. Cluster 3 contained 3 hybrid genotypes: Pannonia and I-214 as *P x euramericana* and 9111/93 as (*P. nigra* x *P. maximowiczii* x *P. nigra* var. Italica) where dissimilarity among them ranged from 0.159-0.242. Cluster 4 had grouped closely related (0.200), mainly *P. nigra* genotypes (NS001, NS002, *P. nigra* var. Italica), but it had also grouped one clone - *P. Trichocarpa* as a subgroup, which showed a very close similarity (0,184) with the hybrid *P. nigra* x *P. maximowiczii* suggesting its greater similarity to the *P. nigra.*

Dendrogram created according to cluster analyses combining two markers AFLP/SSR data showed a clear separation of two different *Populus* species (*P. nigra* and *P. deltoides*) into two distinct clusters. The next two separated subgroups obtained hybrids such as *P. x euramericana* was connected with the second subgroup hybrid/clones *P. nigra x P. maximowiczii* and clone *P. trichocarpa* (Figure 3).

## DISCUSSION

Our study clearly demonstrates that the *PTR* SSR DNA markers developed from *P. tremuloides* (Dayanandan et al., 1998; Rahman et al., 2000) and *WPMS* SSR markers originally established from *P. nigra* (van der Schoot et al.,

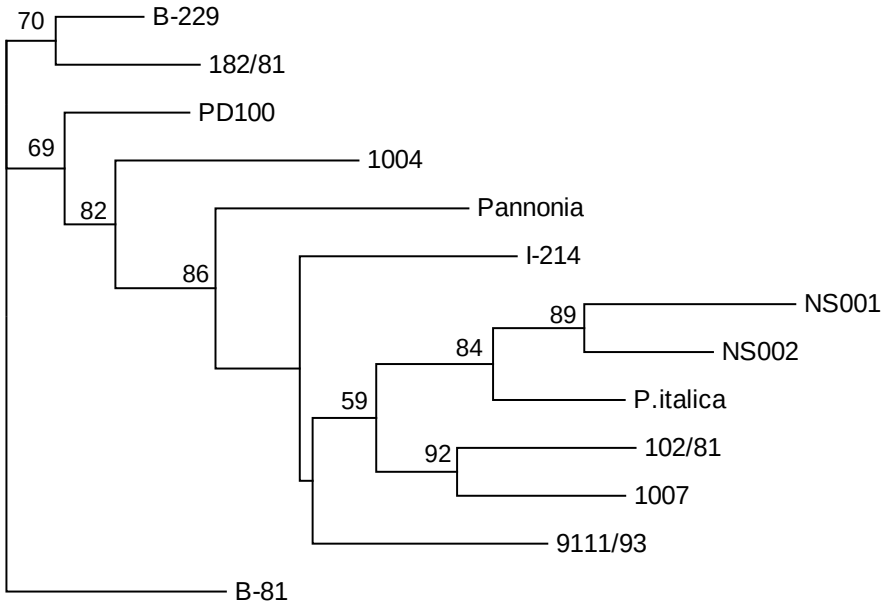


**Figure 3.** Unrooted NJ tree of *Populus* clones constructed from AFLP/SSR fragment dissimilarities.

2000) could be successfully used for differentiation of closely related genotypes e. g. for investigation of genetically specific and interspecific relationships of *Populus* species. Both markers could also be reliable for genetic fingerprinting of clones, cultivars and hybrids of *P. nigra, P. deltoides, P. maximowiczii and P. trichocarpa*. Our findings were in accordance with Rahman and Rajora (2002), Gomez et al. (2003) and Smulders et al. (1997).

All SSR primers resolved single-locus patterns and most of the SSR DNA loci showed high allelic and genotypic diversity in most of *Populus* species used for this study. High informativeness of the *P. tremuloides* and *P. nigra* SSR primers for clonal differentiation in *Populus* species from distant sections *Aigeiros* and *Tacamahaca* suggests that the genomic segments containing the studied SSR loci are homologous in the studied *Populus* species of the sections *Leuce*, *Aigeiros and Tacamahaca* (Rahman and Rajora, 2002). Also new phylogenetic analyses revealed by AFLP markers (Cervera et al., 2005) suggested close relationships between the *Leucoides, Tacamahaca* and *Aigeiros* sections thus proving this marker highly informative and reliable for interspecific genetic and phylogenetic relationships in the genus *Populus*.

Our study is in accordance with the previous findings and proved the high informativeness and discriminativeness of both markers applied. They showed very high polymorphisms and power of discrimination which for AFLP was scored 0.923 and for SSR 0.812.

In this study both markers, SSR and AFLP, clearly distinguished *Populus* species, cultivars and hybrids. The markers proved trueness to type and high genetic dissimilarity of the B81, B229, 182/81 genotypes thus grouped them to *P. deltoides* species. Also marked it clear that genotypes NS001, NS002 belongs to species *P. nigra*. And all other clones, cultivars and hybrid genotypes that have a certain amount of genomic segments of *P. nigra* (*P. nigra var*. Italica; 102/81- hybrid of *P. nigra x P. maximowiczii* and a clone 1007 - *P. trichocarpa*) both markers grouped together in subclusters. Both markers also grouped together the two hybrids of *P. x euramericana*.

However there is a slight discrepancy between the results obtained by SSR and AFLP markers, that is, AFLP analysis had grouped the PD100 clone into *P. deltoides* species, where as it was previously thought it belonged to. According to the SSR analysis this clone can not be classified into the *P. deltoides* species, but based on genetic traits it tended much more toward hybrid of *P. nigra x P. maximowiczii* and to *P. trichocarpa.*

This could be due to misleading or mislabeling events in the nurseries and this finding was also confirmed by Rajora and Zsuffa (1991) but more closely to the statement of Rahman and Rajora (2002) who found out that according to SSR analyses *P. deltoides* (section *Aigeiros*) group was closer to *P. trichocarpa* (section *Tacamahaca*) group than to the *P. nigra* (section *Aigeiros*) group.

Two clones of *P. trichocarpa*, 1004 and 1007 were supposed to be genetically identical but SSR analysis showed some allelic changes in the DNA pattern of those clones. They shared 53.85% dissimilarity including missing fragments. Both markers, however, suggested that clone 1007 revealed high genetic dissimilarity (SSR-

0,667; AFLP-0,816) to hybrid 102/81 with *P. nigra* x *P.maximowiczii* genetic background.

*P. nigra var*. Italica after AFLP analyses was closely related to *P. nigra* species with genetic dissimilarity of 0.159 and due to SSR analysis this cultivar was more toward 9111/93 hybrid (*P. nigra x P.maximowiczii) x P. nigra* var. Italica with genetic dissimilarity of 0.500. Those findings were both in accordance because regarding that this cultivar has germplasm of *P. nigra* species in 9111/93 hybrid due to a backcrossing event, there is 50% of *P. nigra* var. Italica germplasm.

## Conclusion

Our study clearly demonstrates that the *PTR* SSR DNA markers developed from *P. tremuloides* and *WPMS* SSR markers originally established from *P. nigra* could be successfully used for differentiation of closely related genotypes e. g. for investigation of genetically specific and interspecific relationships of *Populus* species. SSR data cluster analyses showed a clear separation of three major clusters, while four major clusters were obtained using AFLP data. According to both markers, two clones of *P. trichocarpa*, 1004 and 1007, were not genetically identical and SSR analysis showed some allelic changes in the DNA pattern of those clones. As far as *P. nigra* var. Italica is concerned, after AFLP analyses it seemed that it was closely related to *P. nigra* species with genetic dissimilarity of 0.159 but due to SSR analysis this cultivar was more toward 9111/93 hybrid (*P. nigra x P. maximowiczii) x P. nigra* var. Italica with genetic dissimilarity of 0.500.

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