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Detection and confirmation of diagnostic microsatellite loci in *Populus nigra* and *Populus deltoides* to identify their hybrids (*P.* × *canadensis*)

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Abstract: Poplar cultivation has economic, social, ecological, and environmental importance in Turkey. Even though, *Populus* × *canadensis* hybrids play an important role for the commercial poplar cultivation with good growth performance and adaptability, hybrid clones introduced the different region of Turkey have started to replace the native stands of *Populus nigra* gradually. To continue sustainable cultivation of poplar and to conserve the native stands of *P. nigra* at the same time, genetic identification, and registration of the *Populus* cultivars from different origins are essential in breeding programs. Genetic identification and characterization of a natural population of *P. nigra* growing along the Melendiz River, *Populus deltoides*, and hybrid *P.* × *canadensis* trees were performed by 12 microsatellites loci. In addition, 18 *P. nigra* trees from EUFORGEN core collection were screened as reference samples. *P. deltoides* specific alleles were detected at four microsatellite loci previously reported as having diagnostic alleles. Several *P. nigra* trees from natural population and EUFORGEN core collection also included species-specific alleles of *P. deltoides*. It is concluded that there is an introgression event between the natural population and cultivated hybrids or *P. deltoides* clones in the surrounding area. Also, some members of the EUFORGEN core collection could have been mixed up or misidentified as *P. nigra*. The obtained results indicated that the application of the rigorous standards for reliable identification and registration with large sample size is a necessity for future breeding and conservation programs. Also, conservation of the native *Populus nigra* trees is an important requirement to prevent the genetic pollution of the species' gene pool and reduction of effective population size.

Key words: *Populus* × *canadensis*, loci, diagnostic, breeding, conservation

1. Introduction

There are 260,681 ha of natural poplar stands composed of *Populus nigra, P. tremula, P. alba, P. canescens,* and *P. euphratica* species in different regions of Turkey (Velioğlu, 2020). Hybrid poplar (*P.* × *canadensis)* and American black poplar (*P. deltoides*) are also cultivated extensively in the temperate regions of our country. To ascend demand for energy sources for the developing and growing world population, there is an increasing interest in the cultivation of poplar as an energy crop for biomass production in the world (Volk et al., 2018; Stolarski et al., 2020). Parallel to studies in the world, breeding and conservation programs of poplar species have been undertaken under the framework of the European Forest Genetic Resources Program (EUFORGEN) since 1965 in Turkey. The best growing poplar individuals from their natural populations were selected throughout the country and registered for cultivation. Also, hybridization studies between *P. deltoides* and native *P. nigra* have been performed to improve the wood quantity and quality (Toplu, 2005). The obtained hybrid *P.* × *canadensis* with its good growth performance, adaptability, and strong hybrid viability indicated outstanding success for the extensive cultivation in different regions of Turkey (Tunçtaner, 1991; Smulders et al., 2008b; Velioğlu et al., 2020).

Although poplar plantations meet the needs of the economy, many studies indicated that the growth of large numbers of some *P. x canadensis* clones suppress the development of native stands of *P. nigra.* Hybrid clones in plantations effectively spread to river ecosystems and intercross with wild *P. nigra* trees (Fossati et al., 2003; Khasa et al., 2005; Jelic et al., 2015; Çiftçi and Kaya 2019). In addition to the loss of its natural habitats due to anthropogenic effects, competition and introgression with exotic poplar trees have caused to reduced genetic diversity and effective population size of the *P. nigra* in Turkey and Europe (Arnold et al., 2001; Meyer et al., 2018; Vanden Broeck et al., 2021).

Since poplar cultivation has economic, social, ecological, and environmental importance in Turkey, it must be sustainable depending on the long-term survival of the native populations in changing environmental and

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climatic conditions. To ensure that, remaining *P. nigra* stands acting as source populations for recolonisation should be conserved to continue sufficient genetic diversity (Booy et al., 2000). Effective gene conservation requires reliable genetic markers for accurate identification and genetic characterization of clones for all the phases of poplar breeding and conservation studies (Heinze, 2008). Microsatellites which contain species-specific alleles are excellent markers because of their transferability on different *Populus* species and usefulness in introgression and hybridization processes (Fossati et al., 2003; Lexer et al., 2007; Smulders et al., 2008a, 2008b). The purpose of this study was to detect diagnostic alleles at several microsatellite loci to quantify the extent of introgression occurred in poplars sampled throughout the species' natural range. Also, accurate identification of the *Populus* cultivar was made by discriminating among pure *P. deltoides*, *P. nigra,* and *P.* × *canadensis* trees for the national breeding and conservation programs in Turkey.

2. Materials and methods

2.1. Plant material

To characterize and identify the natural population of *P. nigra,* 24 trees were sampled along the Melendiz River and its tributaries. Eighteen *P. nigra* trees from the European Forest Genetic Resources Programme (EUFORGEN) Core Collection, provided by the Poplar and Fast-Growing Tree Institute, Kocaeli, Turkey, were used as reference samples. Leaves of ten different commercial clones of *P. deltoides* were also kindly provided by the Poplar and Fast-Growing Tree Institute. Ten hybrid trees as positive controls for verifying species-specifity of the microsatellite markers were sampled from Ceyhan River during a field trip survey. Mature and young hybrid trees were selected throughout the river. The related information about the trees are given in Table 1.

2.2. DNA extraction and PCR reactions

The nuclear DNA was extracted from leaf samples stored in silica gel by using Doyle and Doyle (1990) CTAB extraction protocol with some minor modifications. The DNA concentration was measured by BioDrop Spectrophotometer with the determination of 260:280 OD ratios and by checking the suitability of the DNA as a template in the PCR procedures. To fingerprint trees of different *Populus* species and to determine the diagnostic alleles for the accurate identification of poplar clones, 12 highly polymorphic microsatellite loci were selected based on published results of previous studies (Table 2).

The selected loci PMGC14, PMGC2163, WPMS05, WPMS09, WPMS14, WPMS15, WPMS18, and WPMS20 produce species-diagnostic alleles which are useful to identify offspring of the frequently planted cultivars of *P.* × *canadensis* (Table 2). The polymerase chain reaction was implemented in a 25-µL total volume with 10 ng template DNA in an Eppendorf thermocycler. Thermocycling was performed following the protocol of Van Der Schoot et al. (2000) and Smulders et al. (2001) with different annealing temperatures between 52 °C and 60 °C. PCR products for microsatellite genotyping were detected by Capillary Electrophoresis using ABI PRISM 310 Genetic Analyzer (Applied Biosystems) in BM Laboratory Systems Facilities in Ankara. Peak Scanner v2.0 (Applied Biosystems) was implemented to determine the allele sizes of the studied microsatellite loci with the GeneScan 400 size standard.

2.3. Genetic analysis

MICRO-CHECKER 2.2.3 program (Van Oosterhout et al., 2004) was used to find null allele, check scoring errors, and allele dropouts. To detect the discriminatory power of the multilocus allelic information and the patterns of inter and intra-specific polymorphism of *Populus* species, the number of alleles, observed (Ho) and expected heterozygosity (He), and Shannon Index value for each locus and combined loci were calculated by GenAlEx Software (Peakall and Smouse, 2006). Allelic richness was calculated based on the rarefaction approach in FSTAT v2.9.3.2 (Gaudet, 1995).

To visualize the distance among *P. nigra*, *P. deltoides* and their hybrid, MINIMUM SPANNING NETWORK (MSN) analysis was carried out by using genetic distance matrix based on calculated number of allelic differences among samples in *poppr* and *magrittr* packages of R (Kamvar et al., 2014). STRUCTURE v2.3.4 (Pritchard et al., 2000) Software based on Bayesian iterative algorithm was used to assign individuals of different poplar species to clusters with a burn-in of 50,000 and 250,000 Markov chain Monte Carlo iterations. The web-based tool STRUCTURE HARVESTER (Earl Dent and Vonholdt Bridgett, 2012) determined the most likely value of K from 10 STRUCTURE runs. Multiple runs for the true K value were analyzed with the CLUMPP (Jakobsson and Rosenberg, 2007). POPHELPER Software (Francis, 2016) was implemented to identify the best alignment to the replicate results of the cluster analysis and to visualize the output generated from the admixture analysis. Principal Coordinate Analysis (PCoA) based on the pairwise F_{ST} values was used to show the genetic distance between two *Populus* species and their hybrid.

3. Results

Sixty-two poplar trees were screened with 12 microsatellite markers and their genotypes were determined from multilocus allelic combination. All the loci yielded consistent amplification products were polymorphic for the studied samples except for *P. deltoides*. Two trees in the natural population of Melendiz had identical multilocus genotype.

Table 1. Information on the studied *Populus nigra, P. deltoides*, and *P.* × *canadensis* trees.

Tree code	Origin	Location	Species
N1–N24 (24 trees)	Natural population in Turkey	Melendiz River	*P. nigra*
LVUBAK A.5	Slovakia	EUFORGEN Core Collection provided by Poplar and Fast-Growing Tree Research Institute	*P. nigra*
ICAS5.5	Romania		
EFN.2.1	Portugal		
ICAS 4.5	Romania		
IZT.NS.00.1.1	Yugoslavia		
LVUBAC.5	Slovakia		
FBS 215/63.1	Germany		
EFN.1.1	Portugal		
FRA71017.401.1	France		
FBS.87/65	Germany		
FARCFLHH2 35.1	Austuria		
URFFMH.5	Ukrain		
ICAS.3.5	Romania		
ERTİ.33.3.2.1	Hungary		
FCRA HUNT.5	England		
IZT.NS.00.2.5	Yugoslavia		
URIFFMK.ELI.1	Ukrain		
ALTERRA.1	Holland		
ONDA	Cultivated	Poplar and Fast-Growing Tree Research Institute	*P. deltoides*
LUX			
89 M-060			
89M-061			
İZMİT			
89 M-063			
SAMSUN			
89 M-004			
89M-047			
89 M-040			
H1–H10 (10 trees)	Naturally grown	Ceyhan River	*P.* × *canadensis*

Observed allele number was changed from 6 (WPMS05, WPMS12) to 17 (PMGC2163) with an average of 11.92 (Table 3). Alleles were normalized to the minimum sample size in FSTAT. The observed alleles per locus was found to be lower in *P. deltoides* than in *P. nigra.* WPMS03, WPMS14, PMGC14, and PMGC2163 were found to be the most informative loci with the higher Shannon Index values changed from 1.24 to 1.48. Allelic richness did not vary significantly for the studied loci. Generally, all studied loci showed high observed and expected heterozygosity with 0.63 and 0.56 average value, respectively. The highest expected and observed heterozygosity values were found at WPMS14 locus (He = 0.74, Ho = 0.91) (Table 4).

By considering the results of previous studies reported that six loci PMGC14, PMGC2163, WPMS09, WPMS15, WPMS18, and WPMS20 include diagnostic alleles for *P. deltoides*, I tried to detect/confirm possible diagnostic alleles for those loci by determining allele frequencies for *P. nigra*, *P. deltoides*, and *P.* × *canadensis* (Table 5, Table 6). It was found that there were one, two or three bp differences in allele size of the *P.deltoides* specific alleles detected in different laboratory settings and the current study (Table 5). Species affiliation according to *P. nigra*, and *P. deltoides* was made by the help of species-specific allele information previously reported (Fossati et al., 2003; Khasa et al., 2005; Smulders et al., 2008a; Liesebach et al.,

Table 2. Information on the studied 12 microsatellite markers. IPGC (2016). International *Populus* Genome Consortium (Online). Website: http://www.ornl.gov/sci/ipgc/ssr_resources. htm [Accessed February 2020]

Microsatellite locus	Primer sequences (Forward, Reverse,5'-3')	Repeat motif	References	Studies detected diagnostic allele in *P. deltoides*
WPMS03	TTTACATAGCATTTAGCCTTTAGA TTATGATTTGGGGGTGTTATGGTA	$(GT)_{26-1}$	Van Der Schoot et al. (2000)	
WPMS04	TACACGGGTCTTTTATTCTCT TGCCGACATCCTGCGTTCC	$(GT)_{25}$	Van Der Schoot et al. (2000)	
WPMS05	TTCTTTTTCAACTGCCTAACTT TGATCCAATAACAGACAGAACA	$(GT)_{27}$	Van Der Schoot et al. (2000)	Liesebach et al. (2010)
WPMS09	CTGCTTGCTACCGTGGAACA AAGCAATTTGGGTCTGAGTATCTG	$(GT)_{21}(GA)_{24}$	Van Der Schoot et al. (2000)	Fossati et al. (2003); Smulders et al. (2008a); Rathmacher et al. (2010); Vanden Broeck et al. (2012, 2021)
WPMS10	GATGAGAAACAGTGAATAGTAAGA GATTCCCAACAAGCCAAGATAAAA	$(GT)_{23}$	Van Der Schoot et al. (2000)	
WPMS12	TTTTTCGTATTCTTATCTATCC CACTACTCTGACAAAACCATC	$(GT)_{19}$	Van Der Schoot et al. (2000)	
WPMS14	CAGCCGCAGCCACTGAGAAATC GCCTGCTGAGAAGACTGCCTTGAC	$(CGT)_{28-3}$	Smulders et al. (2001)	Liesebach et al. (2010)
WPMS15	CAACAAACCATCAATGAAGAAGAC AGAGGGTGTTGGGGGTGACTA	$(CCT)_{14-3}$	Smulders et al. (2001)	Liesebach et al. (2010)
WPMS18	CTTCACATAGGACATAGCAGCATC CACCAGAGTCATCACCAGTTATTG	$(GTG)_{13}$	Smulders et al. (2001)	Fossati et al. (2003); Smulders et al. (2008a); Rathmacher et al. (2010); Vanden Broeck et al. (2012)
WPMS20	GTGCGCACATCTATGACTATCG ATCTTGTAATTCTCCGGGCATCT	$(TTCTGG)_{8}$	Smulders et al. (2001)	Fossati et al. (2003); Vanden Broeck et al. (2021)
PMGC14	TTCAGAATGTGCATGATGG GTGATGATCTCACCGTTTG	(CTT)	IPGC SSR Resource	Fossati et al. (2003); Smulders et al. (2008a); Liesebach et al. (2010); Rathmacher et al. (2010); Vanden Broeck et al. (2012, 2021)
PMGC2163	CAATCGAAGGTAAGGTTAGTG CGTTGGACATAGATCACACG	(GA)	IPGC SSR Resource	Khasa et al. (2005); Rathmacher et al. (2010); Liesebach et al. (2010)

2010; Rathmacher et al., 2010; Vanden Broeck et al., 2012, 2021).

The microsatellite data detected and confirmed the presence of species-diagnostic alleles for loci PMGC2163, PMGC14, WPMS15 and WPMS18 but not for WPMS09 and WPMS20. For the PMGC2163, the allele 186 was found as characteristic for all reference *P. deltoides* clones and all hybrids with *P. nigra* with highest allele frequency (1, 0.5, respectively) (Table 6, Figure 1). Two *P. nigra* clones from Melendiz natural population also included the possible diagnostic allele 186.

At locus PMGC14, the allele 190 was seen with the higher frequency for *P. deltoides*, and *P.* × *canadensis*. Two trees from EUFORGEN core collection (ICAS 3.5 and ICAS 4.5 from Romania) and one tree Melendiz population included the possible diagnostic allele 190 (Table 7). Even though, alleles 192 or 193 and 198 or 199 were accepted typical for *P. deltoides* and for *P.* × *canadensis* in the previous studies (Fossati et al., 2003; Liesebach et al., 2010; Rathmacher et al., 2010; Vanden Broeck et al., 2012, 2021), *P. nigra* samples had these alleles as 193 and 199 with high frequencies in the current study. The alleles 193 and 196 were observed in *P. nigra* and *P. deltoides*, while the allele 199 was observed only in *P. nigra* clones (Table 6, Figure 1).

P. deltoides exhibited the diagnostic allele 220 with the highest allele frequency (0.95) at locus WPMS18. Hybrid trees did not have this allele because hybrids may not always have inherited diagnostic alleles. One *P. nigra* tree from EUFORGEN core collection (URIFFMKELI. 1

Table 3. Base pairs of observed alleles for the studied microsatellite loci in *Populus* species. (*P. deltoides* specific alleles are bold and italics.)

Loci	*P. nigra* (Melendiz River)	*P. nigra* EUFORGEN Core collection	*P. deltoides*	*P* × *canadensis*
WPMS03	264,270,272,280	222,228,234,240,246,260,264,266,270,272,276	242,246,248,256	200,264,266,270,280
WPMS04	248,260,274	236,248,250,252,258,274,276		246,256,260,274
WPMS05	278,280,284,286,288	268,276,278,280,286,288	262,268,274,278,280,286,294	230,234,278,284,286,290
WPMS09	236,246,252,260,274	246,250,256,258,260	218,220,230,232,236,246	210,234,250
WPMS10	220,236,246,248	236,240,244,246,248,250,252	were not amplified	240,246,248
WPMS12	164,166,174	160,164,166,170,172,174	were not amplified	164,166,174
WPMS14	210,231,243,252	231,234,243,252,255,258,267,270,279	252,255,258,261,264,270,273	210,231,234,242,251
WPMS15	183,204,210	204,210,213	192,***195***	183,***195***,204,210
WPMS18	226,232,247,250,262	205, 223, 235, 241, 247	***214***,***220***	198,217,***229***,253,256,259
WPMS20	220,226,234	212,214,226,228,232	220,228,234	220,224,228
PMGC14	193,196,199,205,208,217	193,196,199,202,205,208,211,217,223,226	***190***,193,196	***190***,193,199,211
PMGC2163	192,198,210,218,224,228,242,268	220,222,224,230,232,242,244,248,254,258	***186***	***186***,242,256

Table 4. Genetic diversity values for 12 microsatellite loci.

Loci	**N**	**Na**	**Ne**	**Ar**	**I**	**Ho**	**He**	**F**
WPMS03	60	16	4.26	3.16	1.41	0.83	0.73	-0.25
WPMS04	51	10	1.71	–	0.63	0.1	0.3	0.53
WPMS05	61	11	2.55	3.12	1.19	0.64	0.62	-0.1
WPMS09	61	13	2.46	3.78	1.05	0.48	0.55	0.15
WPMS10	51	10	2.03	–	0.84	0.35	0.44	0.08
WPMS12	50	6	1.78	–	0.71	0.45	0.38	–0.07
WPMS14	59	14	4.07	3.13	1.48	0.91	0.74	–0.33
WPMS15	62	6	2.3	3.31	0.92	0.71	0.56	–0.22
WPMS18	62	19	2.36	3.38	0.97	0.63	0.48	–0.25
WPMS20	62	8	2.64	2.5	1.05	0.91	0.64	–0.51
PMGC14	62	13	3.68	4.18	1.38	0.83	0.66	–0.28
PMGC2163	59	17	3.94	5.66	1.24	0.68	0.58	–0.23
Mean	58	11.92	2.82	3.58	1.07	0.63	0.56	–0.14

N = Mean number of individuals with amplification, Na = mean number of different alleles, Ne = mean number of effective alleles
I = Shannon Information Index, Ho = observed heterozygosity, He = expected Heterozygosity, F = inbreeding coefficient Allelic richness
was not calculated for the WPMS04, WPMS10, and WPMS12 loci because those loci were not amplified for *P. deltoides* samples.

from Ukrain) has the allele 220. The allele 229 is present in hybrid trees with highest allele frequency (0.75) and in one tree from natural population and three trees from EUFORGEN core collection (ICAS3.5, ICAS 4.5 from Romania and FBS.87/65 from Germany) (Table 7).

The allele 195 at WPMS15 locus was detected in hybrid and *P. deltoides* clones with the higher allele frequencies (0.81, 0.45, respectively). Three trees from natural population and one tree from EUFORGEN core collection (ICAS.4.5 from Romania) included the allele 195 (Table 7). At loci WPMS04 and WPMS09, the alleles 256 and 250 are present in all hybrids with 0.90 frequency. Even though, Rathmacher et al. (2010) detected the allele 237 (236 in the current study) as diagnostic, the allele was present mainly

Table 5. Comparison of the diagnostic alleles among current and previous studies for *P. deltoides* (Differences of allele sizes in different laboratory settings generally range between 1, 2, and 3 bp).

	Khasa et al. (2005)	Fossati et al. (2003)	Lucas et al. (2008)	Liesebach et al. (2010)	Rathmatcher et al. (2010)	Smulders et al. (2008a)	Vanden Broeck et al. (2012)	Vanden Broeck et al. (2021)	Current study
WPMS09		234			237	234	234	232	
WPMS15				197					195
WPMS18		220			218 220 225	220	220		214 220 229
WPMS20		224					218 224		
PMGC14		193 199		192 195	194 197	268 270	193 99	192 198	190
PMGC2163	181 195			188	190			185	186

Table 6. The frequencies of possible diagnostic alleles detected for *P. nigra* and *P. deltoides* and *P.* × *canadensis* trees.

Loci	**Allele (bp)**	**The frequencies of possible diagnostic alleles**			
		***P nigra* from natural pop.**	***P. nigra* from EUFORGEN collection**	***P. deltoides***	***P.* × *canadensis***
PMGC21A	186	0.04	0.0	1.000	0.50
PMGC14A	190 193 196 199	0.02 0.38 0.06 0.44	0.06 0.08 0.03 0.25	0.25 0.70 0.05 0.00	0.45 0.00 0.00 0.00
WPMS18	220 229	0.00 0.02	0.03 0.08	0.95 0.00	0.00 0.75
WPMS15	195	0.06	0.03	0.81	0.45
WPMS04	256	0	0	0	0.90
WPMS09	250	0	0	0	0.90

in *P. nigra* individuals in the current study. Also, the allele 232 was seen only in one *P. deltoides* tree. Previous studies reported significantly different allele frequencies between *P. nigra* and P. *deltoides* for locus WPMS20. However, any diagnostic alleles were not found in the current study (Table 5). Loci WPMS05, WPMS14, and WPMS20 have many common alleles in both *Populus* species.

Five out of ten *P.* × *canadensis* clones sampled from Ceyhan River were heterozygous at locus PMGC14, WPMS15, WPMS18, and PMGC2163. The diagnostic loci WPMS18 and PMGC2163 were heterozygous in the ten hybrid and homozygous in the ten reference *P. deltoides* clones. WPMS15 locus included *P. deltoides* specific allele of 195 at homozygous status and hybrid trees exhibited 183/195 allele combination.

The separation of *P. deltoides* and *P.* × *canadensis* trees from *P. nigra* trees was indicated by MSN analysis. Individuals of the *P. nigra* were closely grouped together and clearly separated from individuals of *P. deltoides*. The members of hybrid were placed a middle point between *P. nigra* and *P. deltoides* (Figure 2). The members of each species were mainly found to be genetically close to each other in their groups.

The results of STRUCTURE and PCoA analysis showed that a remarkable subdivision of *P. nigra* and *P. deltoides* was detected with the applied set of marker loci. *P. deltoides* and *P.* × *canadensis* trees were placed into the Cluster 3, while *P. nigra* trees were placed into the cluster 1 and 2. *P. nigra* trees with the diagnostic alleles of *P. deltoides* are clearly recognized in the Figure 3a based on individual membership values. One hybrid tree, which was possibly misidentified, with mainly alleles of *P. nigra* was belonged to cluster 3. *P. deltoides* and *P* × *canadensis* trees were clearly separated from *P. nigra* trees by the PCoA, while they were

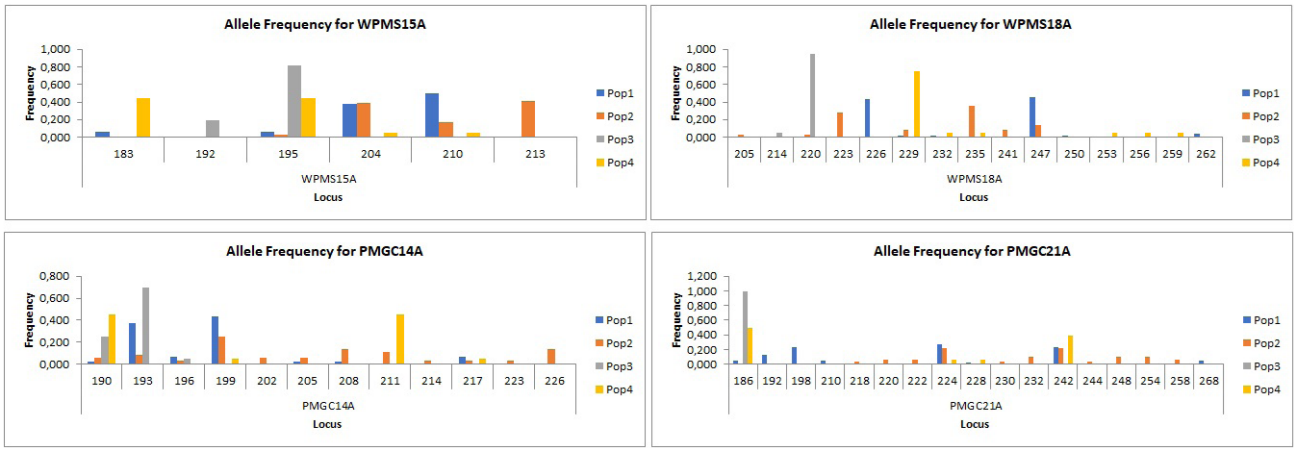

Figure 1. Allele frequency diagram for WPMS15, WPMS18, PMGC14, and PMGC2163 diagnostic loci. Pop 1: *P. nigra* population from Melendiz River, Pop 2: *P. nigra* population from EUFORGEN core collection, Pop 3: Reference *P. deltoides* samples, Pop 4: *P* × *canadensis* samples from Ceyhan River.

Table 7. *P. nigra* trees with *P. deltoides* specific alleles observed at four diagnostic loci. (*P. deltoides* specific alleles are bold and italics.)

Tree code	Origin	Species	WPMS15A	WPMS15B	WPMS18A	WPMS18B	PMGC14A	PMGC14B	PMGC21A	PMGC21B
N2	Melendiz River	*P. nigra*	***195***	210	226	247	193	199	198	210
N4	Melendiz River	*P. nigra*	***195***	210	226	247	193	199	224	242
N20	Melendiz River	*P. nigra*	204	210	226	247	193	199	***186***	242
N21	Melendiz River	*P. nigra*	183	210	***229***	250	***190***	208	***186***	198
N24	Melendiz River	*P. nigra*	***195***	210	226	247	193	199	224	242
ICAS 4.5	Romania	*P. nigra*	***195***	213	223	***229***	***190***	208	218	242
FBS.87/65	Germany	*P. nigra*	204	213	***229***	241	193	196	248	258
ICAS.3.5	Romania	*P. nigra*	204	213	223	***229***	***190***	208	224	242
URIFFMK.ELI.1	Ukrain	*P. nigra*	204	204	***220***	235	199	226	232	254

to be in the same genetic group in the STRUCTURE analysis due to shared diagnostic alleles with higher frequencies.

4. Discussion

In this study, 12 nuclear microsatellite markers transferred across the *P. nigra, P. deltoides* and *P* × *canadensis* species clearly proved their effectiveness to characterize and distinguish species and hybrids. Although only ten reference *P. deltoides* clones were used in this study, a high degree of homology was observed between the genomes of *P. deltoides* and *P. nigra*. The observed alleles for *P. deltoides* clones were significantly lower than natural population and EUFORGEN core collection of *P. nigra* (Table 3). This is an expected result that the studied SSR loci developed for *P. nigra* have a low degree of polymorphism for *P. deltoides* as well as a lower sample size for the *P. deltoides*.

Observed and expected heterozygosities were found generally to be high. Similarly, Ratcmatcher et al. (2010) studied seven common loci same as the current study, also reported high observed heterozygosity values (0.67–0.96). A high level of heterozygosity could be caused by hybrid effects of an elevated level of heterozygosity and open pollination/outcrossing characteristics of poplar trees (Stark et al., 2006). WPMS04, WPMS10, and WMPS12 loci did not amplify in *P. deltoides*. Lack of amplification in *P. deltoides* and the heterozygote deficiency at those loci could be resulted from the occurrence of null alleles which are possibly caused by geographically restricted mutations in primer binding sites which cause to the prevention of effective amplification of the loci (Çiftçi et al., 2017).

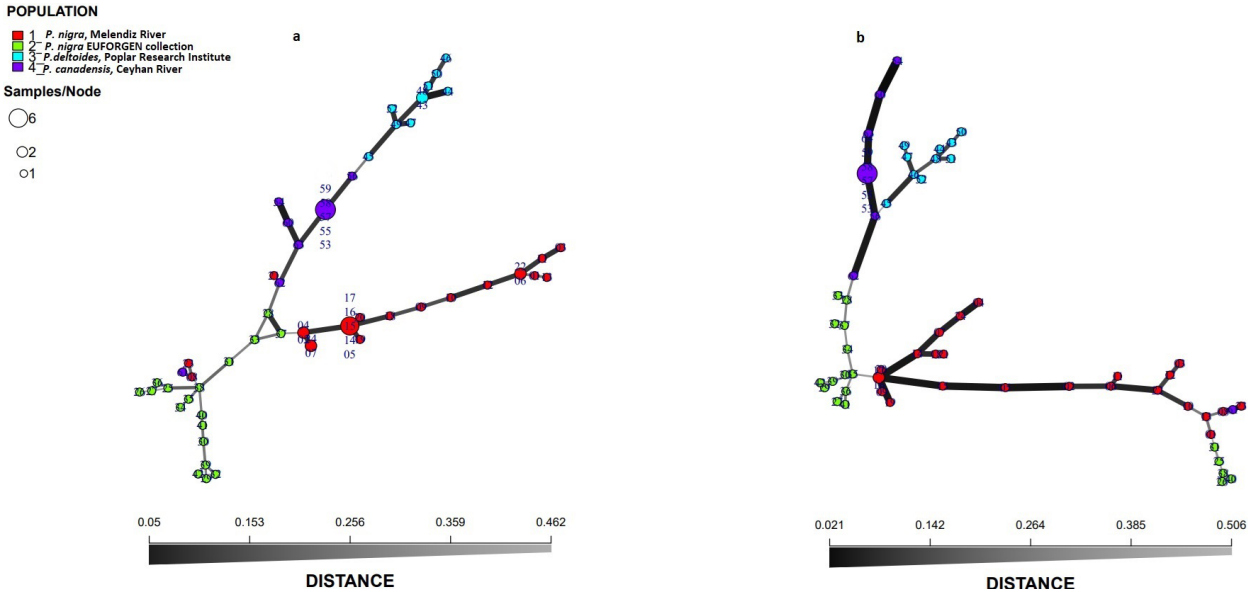

Figure 2. MSN analysis for *P. nigra, P. deltoides* and *P.* × *canadensis* **a.** Genetic distance among trees for four diagnostic microsatellite loci, **b.** Genetic distance among trees for 12 microsatellite loci.

Species-specific alleles are considered as diagnostic to detect hybrids in natural population of *P. nigra*. For *P. deltoides*, diagnostic alleles at loci WPMS18 (220 bp), PMGC2163 (186 bp), and WPMS15 (195 bp) described by previous studies were verified by this study with one or two base differences (Table 5). Another specific allele for *P. deltoides* at locus WPMS18 (previously described by Khasa et al., 2005) was observed for one *P. deltoides* individual (214 bp). The alleles 195, 229, and 190 were detected as possibly new species-specific alleles for *P. deltoides* at loci WPMS15, WPMS18, and PMGC14, respectively.

For the WPMS09 locus, Fossati et al. (2003), Smulders et al. (2008a) and Vanden Broeck et al. (2012) reported 234 bp as a diagnostic allele for *P. deltoides*, while Rathmacher et al. (2010) and Vanden Broeck et al. (2021) reported diagnostic allele as 237 and 232 bp, respectively. In this study, 232 and 236 bp alleles were in *P. deltoides*. This corroborates the results of Rathmacher et al. (2010) and Vanden Broeck et al. (2021). The allele 236 also found in *P. nigra* which shows that this allele is probably not diagnostic for *P. deltoides*. The alleles 256 and 250 with the highest frequency for *P.* × *canadensis* and *P. deltoides* at loci WPMS04, and WPMS09 could be classified as informative, as they contain information on the likelihood of the sample belonging to a certain species or hybrid.

Four trees in EUFORGEN core collection included diagnostic alleles for *P. deltoides* could have been mixed up or misidentified as *P. nigra*. Those trees should be revised with new reference *P. nigra* trees which are accurately identified by markers.

The presence of diagnostic alleles for *P. deltoides* in *P. nigra* trees from Melendiz natural population indicated that morphologically sampled trees may be F2 hybrids or backcrosses to *P. nigra* because of an introgression event. Arens et al. (1998) and Winfield et al. (1998) reported cases of introgression of foreign germplasm together with clonal duplication of black poplar in the Netherlands and the UK. Smulders et al. (2008a) similarly reported that half of the 44 sampled poplar trees were not pure *P. nigra* but progeny of natural hybridization along the Rhine River. It is a common event that the black poplar germplasm can be introgressed by commercial hybrids and consequent backcrosses.

Five *P.* × *canadensis* clones sampled from Ceyhan River were likely F1 hybrids because PMGC14, WPMS15, WPMS18, and PMGC2163 loci showed one allele diagnostic for *P. deltoides* and another specific for *P. nigra*. The other four trees with two *P. deltoides* or *P. nigra* specific alleles at those loci were in a different hybrid class (F2, BC to *P. nigra*, and BC to *P. deltoides*). These ten trees represented nine different hybrid genets. The occurrence of different hybrid classes could be explained by natural or human assisted hybrid tree propagation. Along the Ceyhan River, there are many hybrid trees despite of a smaller number of *P. nigra*. In addition to mature hybrid trees, high level of hybrid seedlings was observed along the river due to hybrid plantations growing near the Ceyhan River. It is known from the former studies that cultivated hybrid poplar can produce viable seeds and seedlings (Smulders et al., 2008a; Heinze, 2008). Due to the proximity of a hybrid plantation to the river; the transport of seeds, viable branches,

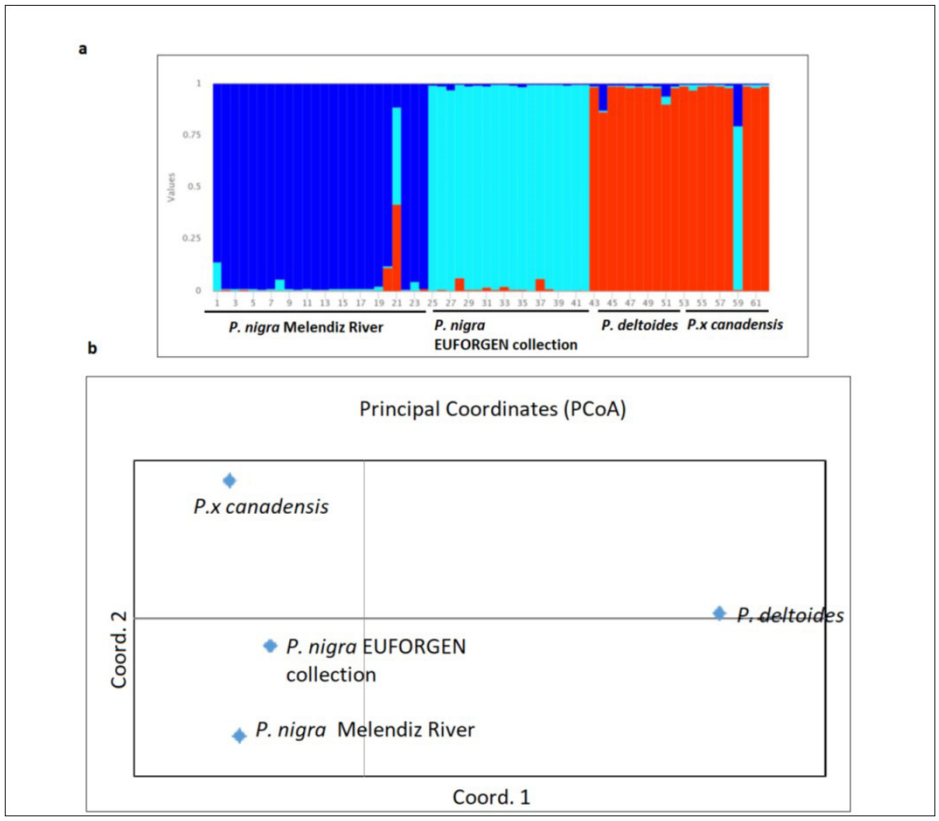

Figure 3 a. STRUCTURE grouping for *P. nigra, P. deltoides*, and *P.* × *canadensis* (Cluster 1: Blue, Cluster 2: Turquoise, Cluster 3: Red) **b.** PCoA result for *P. nigra, P. deltoides and P.* × *canadensis*.

trunks, or cuttings from hybrid clones could be relatively frequent. Hybrid trees along the Ceyhan River could also be introduced by local farmers, however, there is evidence that some of these trees were completely naturally grown by hybrid trees' seed or vegetative material via wind and water from the plantation site. Some of trees were inside of the river where cannot be planted by humans. For both conditions, growing hybrid seedlings could possibly start to naturalize along the river. Similarly, Fossati et al. (2003) identified some old trees in their Italian population as hybrids, but they concluded that hybrids were grown by human-mediated planting of cuttings. Çiftçi and Kaya (2019) reported that hybrid poplar trees identified along the Göksu River are derived from direct mating between *P.* × *canadensis* clones cultivated on commercial plantations that provide raw materials for the Paper Mill in Taşucu town in Göksu Delta. Vanden Broeck et al. (2012) found evidence for gene flow between cultivated hybrid poplars and native black poplars in West Flanders. Hybrid trees on commercial plantations naturally produce seeds and hybridize naturally with *P. nigra* (Cagelli and Lefevre, 1995; Vanden Broeck, 2004; Smulders et al., 2008a). This is a significant threat to the conservation of *P. nigra* in its natural habitat.

The result of MSN analysis indicated that *P. nigra* and *P. deltoides* were connected genetically by their intersectional hybrids. Even though, trees from Melendiz natural population were genetically close to each other, *P. nigra* trees from EUFORGEN core collection were found to be genetically more distant to each other and to trees from Melendiz natural population due to their different origin. Detection of the three genetic clusters, which two of them included *P. nigra* trees and other included *P. deltoides* and *P.* × *canadensis*, is an expected condition that *P. deltoides* and *P.* × *canadensis* clones with the diagnostic alleles share more similar genetic structure compared to *P. nigra*. Hybrid trees which morphologically sampled as *P. nigra* represent genetic structure of the determined three clusters (Figure 3).

With the help of the determined diagnostic alleles, I was able to detect hybrid trees within the natural population and EUFORGEN core collection and diagnose hybrid status of hybrid clones from Ceyhan River concerning *P. nigra* and *P. deltoides*. In the future studies, more detailed sampling is needed for more reliable detection of diagnostic alleles. The diagnostic

loci standardized by allelic ladders with the same size scoring and the same set of EUFORGEN genotypes should be used in all countries to review certification protocols and to perform correct identification of members of the poplar, which is essential in selection and breeding programs and conservation and management of *Populus* genetic resources.

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