Microsatellite DNA markers in *Populus tremuloides*

Muhammad H. Rahman, S. Dayanandan, and Om P. Rajora

Abstract: Markers for eight new microsatellite DNA or simple sequence repeat (SSR) loci were developed and characterized in trembling aspen (*Populus tremuloides*) from a partial genomic library. Informativeness of these microsatellite DNA markers was examined by determining polymorphisms in 38 *P. tremuloides* individuals. Inheritance of selected markers was tested in progenies of controlled crosses. Six characterized SSR loci were of dinucleotide repeats (two perfect and four imperfect), and one each of trinucleotide and tetranucleotide repeats. The monomorphic SSR locus (*PTR15*) was of a compound imperfect dinucleotide repeat. The primers of one highly polymorphic SSR locus (*PTR7*) amplified two loci, and alleles could not be assigned to a specific locus. At the other six polymorphic loci, 25 alleles were detected in 38 *P. tremuloides* individuals; the number of alleles ranged from 2 to 7, with an average of 4.2 alleles per locus, and the observed heterozygosity ranged from 0.05 to 0.61, with an average of 0.36 per locus. The two perfect dinucleotide and one trinucleotide microsatellite DNA loci were the most informative. Microsatellite DNA variants of four SSR loci characterized previously followed a single-locus Mendelian inheritance pattern, whereas those of *PTR7* from the present study showed a two-locus Mendelian inheritance pattern in controlled crosses. The microsatellite DNA markers developed and reported here could be used for assisting various genetic, breeding, biotechnology, genome mapping, conservation, and sustainable forest management programs in poplars.

Key words: poplar, microsatellites, genetic mapping, simple sequence repeat (SSR) markers, DNA fingerprinting.

Résumé : Huit nouveaux marqueurs microsatellites ont été développés et caractérisés chez le peuplier faux-tremble (*Populus tremuloides*) à partir d'une banque partielle d'ADN génomique. L'informativité de ces marqueurs a été évaluée en déterminant le polymorphisme parmi 38 individus du *P. tremuloides*. L'hérédité des marqueurs sélectionnés a été vérifiée chez des progénitures issues de croisements contrôlés. Six microsatellites étaient dinucléotidiques (deux parfaits et quatre imparfaits) tandis que les deux autres microsatellites étaient respectivement trinucléotidiques et tétranucléotidiques. Le locus monomorphe (*PTR15*) possédait une répétition dinucléotidique composée imparfaite. Les amorces conçues pour l'amplification d'un locus très polymorphe (*PTR7*) amplifiaient deux loci et les allèles n'ont pu être assignés au locus correspondant. Pour les six autres microsatellites, un total de 25 allèles ont été détectés parmi les 38 individus du *P. tremuloides*. Le nombre d'allèles variait entre deux et sept pour une moyenne de 4,2 allèles par locus. L'hétérozyotie observée variait entre 0,05 et 0,61 pour une moyenne de 0,36 par locus. Les deux microsatellites dinucléotidiques parfaits et l'unique microsatellite trinucléotidique se sont avérés les plus informatifs. Les variants alléliques pour quatre microsatellites caractérisés précédemment ont montré une hérédité mendélienne monogénique tandis que ceux du locus *PTR7* (rapporté ici) ont montré une hérédité mendélienne digénique suite à des croisements contrôlés. Les microsatellites développés et rapportés ici pourront être utiles pour assister divers travaux en génétique, en amélioration, en biotechnologie, en cartographie, en conservation ainsi qu'en gestion forestière chez les peupliers.

Mots clés : peupliers, microsatellites, cartographie génétique, marqueurs SSR, empreintes génétiques.

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Introduction

Trembling aspen (*Populus tremuloides* Michx.) is a widespread tree species in North America, and commercially important for pulp, paper, and oriented strand board industries. There is an active tree improvement program for this species in Alberta. Highly informative genetic markers could facilitate this and other trembling aspen genetics and breeding programs.

Microsatellite DNA, or simple sequence repeats (SSRs), a relatively new class of DNA markers, are highly informative genetic markers. Microsatellites are abundant polymorphic elements in nuclear genomes and consist of tandemly reiter-

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Table 1. Repeat type, primer sequence (F, forward and R, reverse), number (Ao) and size range of alleles, and observed heterozygosity (Ho) at eight microsatellite DNA loci (*PTR 5*, *PTR6*, *PTR7, PTR 8*, *PTR11, PTR 12*, *PTR14*, and *PTR15*) in *Populus tremuloides*.

Locus	Repeat	Primer sequence (5′→3′)	Ao	Size (bp)	Ho
PTR5	$(TG)_7$	CTTCTCGAGTATAAATATAAAACACCA (F) TCACATCACCCTCTCAGTTTCGC (R)	3	250–256	0.61
PTR6	$(AT)_8$	AGAAAAGCAGATTGAGAAAAGAC (F) CTAGTATAGAGAAAGAAGAAGCAGAAA (R)	6	186–240	0.53
PTR7	$(CT)_5AT(CT)_6$	ATTTGATGCCTCTTCCTTCCAGT (F) TATTTTCATTTTCCCTTTGCTTT (R)	NA*	200–230	NA*
PTR8	$(A)_{11}(CT)_8$	TAGGCTAGCAGCTACTACAGTAACA (F) TTAAGTGCGCGTATCCCAAAGA (R)	4	132–140	0.37
PTR11	$(GT)_3G(GT)_2GGT$	ATGATTGAGCTCTCTCAAGGTTGCT (F) TTTGCAACCATGCTATCTACTTCAA (R)	2	138–142	0.05
PTR12	$(AAAG)_3A_6n_7(AAAG)_2$	AATAACCATCCCTCCAATAACCTAC (F) TATTTTGCACCTAAATGGCTGTTCT (R)	3	248–256	0.08
PTR14	$(TGG)_5$	TCCGTTTTTGCATCTCAAGAATCAC (F) ATACTCGCTTTATAACACCATTGTC (R)	7	130–204	0.50
PTR15	$(GA)_3AA(GA)_5n_{10}(GA)_5$	CGTGATTGAAGGCGCACTAACCAT (F) CTTTGTTCTCAGTGGCTGCCTATT (R)	1	220	0.00

Note: The DNA sequences for the full-length microsatellite DNA clones have been deposited to GenBank, accession numbers AF240755–AF240762.
*Primers for *PTR7* resolved two highly polymorphic microsatellite DNA loci. However, the amplification products were not clearly distinguishable between the loci, therefore alleles could not be assigned to a specific locus and observed heterozygosity could not be calculated.

ated, short DNA sequence motifs (Tauz 1989; Weber and May 1989; Wang et al. 1994). These regions are interspersed throughout eukaryotic genomes, generally embedded in unique DNA sequences, and polymorphism among individuals arises from changes in the number of repeats. This type of markers has also been referred to as simple sequence length polymorphism (SSLP, Tautz 1989), or sequence-tagged microsatellite sites (STMS, Beckman and Soller 1990). Due to their hypervariability, codominance, and high reproducibility, microsatellites are ideal markers for constructing high-resolution genetic maps and identifying loci controlling traits of interest (Hearne et al. 1992; Devey et al. 1996; Paglia et al. 1998), population and conservation genetics studies (Powell et al. 1995; Rajora et al. 2000), clonal identification (Dayanandan et al. 1998; Sanchez-Escribano et al. 1999), certification of controlled crosses, identification of species and hybrids, paternity determination (van de Den and McNicol 1996), marker assisted early selection (Weising et al. 1998), assessing the genetic effects of forest management practices, and developing strategies for conservation and sustainable management of forest genetic resources (Rajora et al. 2000).

The development of SSR primers is laborious in forest trees. Due to the limited amount of sequence data available in databases, it is often necessary to construct genomic libraries and screen for specific SSRs. Therefore, information on microsatellites in forest trees is quite rudimentary. A limited number of microsatellite DNA markers has been developed for trembling aspen (Dayanandan et al. 1998), and a few pine (*Pinus*) (Smith and Devey 1994; Kostia et al. 1995; Echt et al. 1996; Fisher et al. 1998), spruce (*Picea*) (van de Ven and McNicol 1996; Pfeiffer et al. 1997; Paglia et al. 1998; O.P. Rajora and associates, unpublished data), and eucalyptus (Marques et al. 1999) species.

We have recently developed microsatellite DNA markers and characterized four SSR loci for the first time in trembling aspen (Dayanandan et al. 1998). Here we report eight additional microsatellite DNA loci in trembling aspen. We also have examined the inheritance of one of these polymorphic and four microsatellite DNA markers earlier reported.

Materials and methods

Eight microsatellite DNA loci (Table 1) were identified and characterized following protocols described in Dayanandan et al. (1998).

DNA amplification and microsatellite polymorphism resolution

Total genomic DNA from leaf tissue from each individual was extracted following the protocol described in Rajora and Dancik (1995). DNA amplification was performed in a total volume of 10 µL, with 1 µL (20 ng) of extracted DNA, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 5 pmol of each primer, 2 µg of BSA (bovine serum albumin), 1× reaction buffer and 1 U of AmpliTaq DNA polymerase (Perkin–Elmer, Applied Biosystems, Foster City, Calif.), using GeneAmp 9600 thermal cycler (Perkin–Elmer, Applied Biosystems, Foster City, Calif.). The optimal temperature cycling parameters for amplification were: initial denaturation at 94°C for 3 min, followed by two cycles of 30 s each at 94°C, 60°C, and 72°C; 11 cycles of 15 s at 94°C, 60°C, and 72°C, with a stepwise lowering of the annealing temperature from 60°C to 54°C by the eleventh cycle; and 25 cycles with 15 s each at 94°C, 54°C, and 72°C followed by a final extension step at 72°C for 3 min. After thermal cycling, 5 µL of the amplified product was electrophoresed on a 1.5% horizontal agarose gel to check for positive amplification and to determine the approximate amount of the product. The rest of the amplified product (5 µL) was diluted with 5–20 µL of loading dye (formamide, xylene cyanol, and bromophenol blue) and about 3–4 µL of the mix was electrophoresed on 6% denaturing polyacrylamide gel with 6 M urea and 1× TBE buffer on a S2 electrophoretic apparatus (Owl Scientific, Woburn, Mass.) at 80 W for 3–4 h. Following electrophoresis, the gel was silver-stained using a silver sequencing kit (Promega Corporation, Madison, Wis.)

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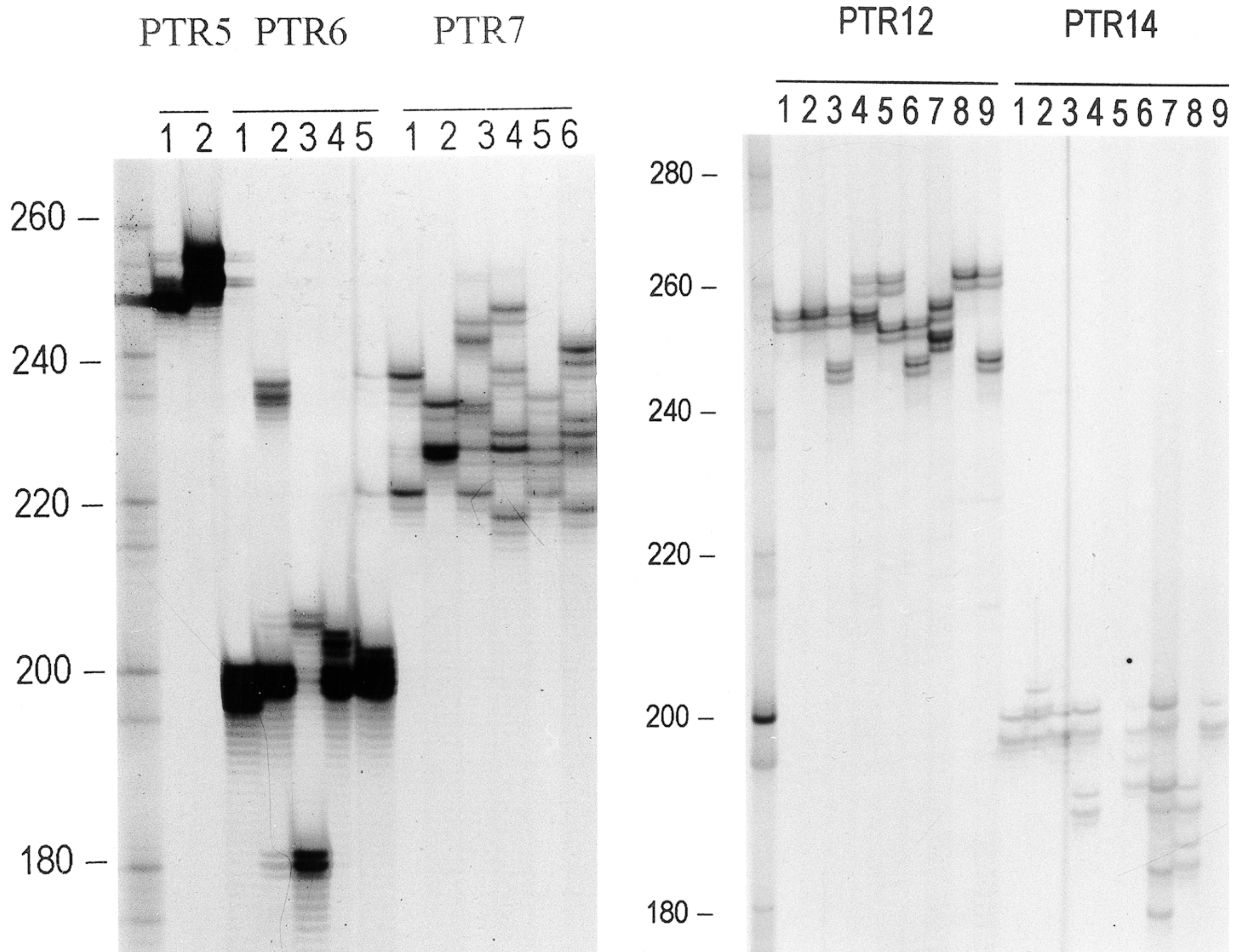
Fig. 1. Allelic variation at five microsatellite DNA loci (*PTR 5*, *PTR6*, *PTR7, PTR12,* and *PTR14*) in 2–9 individuals of *Populus tremuloides* visualized on silver-stained polyacrylamide gels. The numbers in left lane are DNA fragment size standard of a 20-bp ladder.



as described in Rajora et al. (2000) and photographed following the manufacturer's instructions.

Allelic variation, and inheritance

Allelic diversity and levels of observed heterozygosity were surveyed at each locus by genotyping 38 individuals of *P. tremuloides*, collected from various regions of the Canadian province of Alberta (see Dayanandan et al. 1998). Inheritance of microsatellite DNA markers was examined for the loci *PTR1*, *PTR2*, *PTR3*, and *PTR7* in progenies of a controlled cross between parents S2 and S4, and for markers of the locus *PTR4* in the progenies of a cross between S3 and T1–58. The SSR loci *PTR1–PTR4* were described previously (Dayanandan et al. 1998). These five SSR loci were selected on the basis of the availability of sufficient number of progenies from parents polymorphic for the given locus. Studying the inheritance of microsatellite DNA variants (alleles) for the remaining SSR loci was not feasible due to a lack of sufficient progenies from parents polymorphic for each locus. Mendelian inheritance of microsatellite variants was determined from the observed distribution of progeny genotypes compared with the expected distribution based on hypothesized genotypes of the parents by performing a χ^2 test and a *G* test with William's correction for small sample size (Sokal and Rohlf 1969).

Results and discussion

Primers for all eight new microsatellite DNA sequences produced consistent results. Six of these SSR loci were of dinucleotide repeats (two perfect and four imperfect), and one each of trinucleotide and tetranucleotide repeats (Table 1). Among 38 *P. tremuloides* individuals surveyed, seven SSR loci were found to be polymorphic, while one (*PTR15*) was monomorphic (Table 1).

The *PTR7* primer pair showed amplification products of three to four sizes (Fig. 1) in the majority of individuals tested, suggesting a possible occurrence of more than one copy of these microsatellite DNA sequences in *P. tremuloides*. Therefore, assignment of alleles to a specific locus in *PTR7* was not possible and allelic frequency and heterozygosity values could not be calculated. However, because of a high level of polymorphism, the microsatellite DNA

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Table 2. Size and frequency of alleles for six microsatellite DNA loci in 38 individuals of *Populus tremuloides*.

Locus	Allele size	Frequency
PTR5	250	0.224
	252	0.645
	256	0.131
PTR6	186	0.013
	202	0.053
	204	0.684
	206	0.079
	208	0.145
	240	0.026
PTR8	132	0.026
	136	0.092
	138	0.658
	140	0.224
PTR11	138	0.974
	142	0.026
PTR12	248	0.039
	252	0.948
	256	0.013
PTR14	131	0.039
	158	0.039
	161	0.093
	194	0.026
	197	0.724
	200	0.053
	203	0.026

variants of *PTR7* could still be used in many genetic studies, such as DNA fingerprinting and gene flow studies. The distribution of microsatellite DNA variants in parents and progeny of their controlled cross, resolved by *PTR7* primers, indicated that there are possibly two copies (loci) of *PTR7* microsatellite DNA sequences in the *P. tremuloides* genome. However, in other *Populus* species and interspecific hybrids, *PTR7* primers resolved microsatellite DNA variants typical of a single locus (not shown), suggesting possible duplication of this SSR locus in *P. tremuloides*. The other seven primer pairs amplified products as expected for the presence of a single copy, with one or two amplified product(s) in each individual (Fig. 1).

From 38 *P. tremuloides* individuals surveyed, 25 alleles in total were identified at the six polymorphic (excluding *PTR7*) SSR loci, with an average of 4.2 alleles per polymorphic locus. The highest allelic diversity of 7 alleles was observed for the trinucleotide SSR locus *PTR14* and the lowest allelic diversity for the imperfect dinucleotide repeat locus *PTR11* (Table1; Fig. 1). The frequency of a given allele varied from the lowest value of 0.013 at the *PTR6* and *PTR12* loci to the highest value of 0.974 at the diallelic locus *PTR11* (Table 2). The highest heterozygosity (0.61) was observed for the *PTR5* locus (perfect dinucleotide repeats) and the lowest for the *PTR11* (0.05) locus (imperfect dinucleotide repeats) (Table 1), with an average observed heterozygosity of 0.36 per locus. The observed heterozygosity was quite high (0.50) for the trinucleotide locus *PTR14*, which also showed the highest allelic diversity. This trinucleotide repeat sequence, $(TGG)_5$, was isolated by using the dinucleotide probe $(TG)_{15}$. A high degree of polymorphism observed at the trinucleotide locus *PTR14* is consistent with a considerable level of polymorphism observed earlier among the same 36 *P. tremuloides* individuals for the trinucleotide loci *PTR1* and *PTR2* (Dayanandan et al. 1998). However, our observations are contrary to the general view that trinucleotide repeats are much less polymorphic than dinucleotide repeats. Thus, our present and earlier (Dayanandan et al. 1998) results suggest that trinucleotide repeats are highly polymorphic in trembling aspen.

The observed distribution of the microsatellite DNA variants for each of *PTR1*, *PTR2*, *PTR3*, and *PTR4* loci (Dayanandan et al. 1998) in progeny of a controlled cross, was consistent and not significantly different from that expected for a single-locus Mendelian inheritance (data not shown). Thus our results suggest that microsatellite DNA variants of each of *PTR1*, *PTR2*, *PTR3*, and *PTR4* are under a single nuclear locus control.

The presence of a considerable level of polymorphism for five (*PTR5*, *PTR6*, *PTR7*, *PTR8*, and *PTR14*) of the eight microsatellite loci described makes them a valuable molecular tool for various genetics, breeding, genomics, and genetic resource management programs of trembling aspen, including fingerprinting and identification of clones. Two individuals of *P. tremuloides* from each of the two pairs could not be uniquely genotyped using *PTR1–PTR4* SSR loci (Dayanandan et al. 1998). These individuals in each pair could be separated by their genotypes at the *PTR8* locus. Thus, all 38 *P. tremuloides* individuals could be uniquely genotyped and distinguished based on five SSR (*PTR1–PTR4*, and *PTR8*) loci. These results further highlight the utility of the microsatellite DNA markers that we developed for clonal identification in *P. tremuloides*.

In conclusion, we have developed and characterized markers for eight additional microsatellite DNA loci in trembling aspen to follow up our first report of isolation and characterization of microsatellites in the genus *Populus* (Dayanandan et al. 1998). The microsatellite DNA markers developed could be used for various studies and programs on genetics, breeding, biosystematics, genome mapping, molecular breeding, and conservation and sustainable management of genetic resources in *Populus*.

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