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Development and characterization of microsatellite markers in black poplar (*Populus nigra* L.)

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Abstract Using an enrichment procedure, we have cloned and sequenced microsatellite loci from black poplar *(Populus nigra* L.) and developed primers for sequence-tagged microsatellite (STMS) analysis. Twelve primer pairs for dinucleotide repeats produced fragments of sufficient quality which were polymorphic in *P. nigra*. Some of them also showed amplification in other *Populus* species (*P. deltoides*, *P. tricocarpa*, *P. tremula*, *P. tremuloides*, *P. candicans*, and/or *P. lasiocarpa*). The best nine and (GT) (GA) microsatellite markers were tested on a set of 23 *P. nigra* genotypes from all over Europe. The microsatellites were highly polymorphic, with 10–19 different alleles per microsatellite locus among these 23 genotypes. WPMS08 sometimes amplified three fragments. Using the other eight marker loci, the level of heterozygosity among the plants was on average 0.71 (range 0.25–1.00). The microsatellite markers developed will be useful for screening the genetic diversity in natural populations and in gene bank collections.

Key words SSR · Genetic diversity · Dinucleotide repeat

Introduction

Populus nigra L. is a tree of social and economic importance. It is a unique pioneer species of riparian ecosystems, which contribute to the natural control of flooding and water quality. Such ecosystems are characterized by a high level of diversity of the fauna and flora. Unfortunately, large areas of this natural habitat have been lost due to drainage of rivers, management of riverbanks, intensive grazing and wood cutting. Currently, there is great interest in Europe to restore the natural borders of rivers, not only for the control of flooding but also because the river borders will serve as corridors to connect natural areas now separated from each other. Because existing black poplar stands may act as source population for the re-colonization of floodplains, it is important to know if the genetic variation of black poplar stands has been reduced, and how much genetic variation in black poplar is present along different rivers (Cottrell et al. 1997; Arens et al. 1998, Winfield et al. 1998).

To establish the extent of genetic variation, levels of heterozygosity, and dispersal in black poplar, it is necessary to have codominant genetic markers that are able to distinguish individuals and establish parent/offspring relations. Microsatellites or simple sequence repeats (SSRs) are short (1–5 bp long) tandemly repeated DNA sequences. These sequences have been shown to be highly polymorphic with regard to the number of repeats (Schlötterer and Tautz 1992). Since the flanking sequences of each SSR locus are usually unique, primers to flanking regions can be designed to produce a sequence-tagged microsatellite (STMS) marker (Beckmann and Soller 1990; Morgante and Olivieri 1993). This marker can be amplified by PCR and separated on sequencing gels to detect the polymorphism in repeat length.

For such an STMS approach, information about the flanking sequences has to be available or be obtained. The primers developed by Dayanadan et al. (1998) in trembling aspen were not useful in black poplar. Only one set gave some amplification product. A number of microsatellites developed for *Populus trichocarpa* are listed in the PMGC database (http://poplar2.cfr.washington.edu/pmgc). Almost all of these microsatellites contain (GA) repeats. Only recently, a new set of primer pairs was added, of which some produce polymorphic fragments in black poplar. Therefore we cloned and sequenced microsatellite loci from black poplar using an enrichment procedure (van de Wiel et al. 1999; Smulders

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et al., in preparation), designed primers and tested the STMS markers on 23 genotypes from all over Europe.

The microsatellites markers developed in this study will be useful for studies on genetic diversity and population dynamics as well as the characterisation of genebank collections of black poplar.

Materials and methods

Plant material and DNA extraction

Twenty three *P. nigra* trees were used from the EUFORGEN Core Collection (Turok et al. 1998). This collection consists of *P. nigra* trees from different countries and is designed to incorporate as diverse material as possible. Young leaves were collected from individual Core Collection trees grown in the nursery of Alterra, Wageningen, The Netherlands (see Table 1). Plant material of *Populus deltoides* was also obtained from Alterra. All materials were immediately frozen in liquid nitrogen and stored at –70°C until freeze-drying. Nuclear DNA was extracted from single freeze-dried leaves using a DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. At the end of the procedure the column was eluted with two-times 100 µl of AE buffer. The average yield was 10 µg of DNA. DNA from other *Populus* species (see Table 3) was a gift of V. Storme (Gent, Belgium).

Enriched library construction

Microsatellite-enriched libraries were produced by a selective hybridisation procedure (Karagyozov et al. 1993), using the method described by Van de Wiel et al. (1999), with minor modifications. In short, genomic DNA from *P. nigra* (clone IBN1749) was digested with *Alu*I. The fragments were separated on a 2% agarose gel and those in the size range of 200–700 bp were cut out and ligated to an adapter, consisting of a 21-mer (5′ CTCTTGCTTAGATCTGGACTA-3′) and a 25-mer (5′-ACACGAGAACGAATCTAGACCTGAT-3′), containing a *Bgl*II site. The fragments were amplified and microsatellite-containing fragments were selected by hybridization to nylon membranes with a combination of different synthetic oligonucleotides attached to them. Three combinations of oligonucleotides were used (see Table 2). After hybridization, membranes were washed two-times in 1.5×SSC, 0.1% SDS at 62°C, for 20 min each. Elution of the fragments was performed in 0.5 ml of 0.5×SSC, 0.1% SDS for 30 min at 62°C.

After precipitation and dissolving in 25 µl of distilled water, 3 µl of this solution was amplified, by 30 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C. For cloning, the fragments were digested with *Bgl*II and ligated into the *Bam*HI site of pBluescript SK+ (Stratagene). Before transformation to *Escherichia coli* DH5α (Life Technologies) the ligation mixes were digested with *Bam*Hl. Fragment-containing clones were transferred onto Hybond N+ membranes and screened by hybridization to a mixture of the appropriate oligonucleotides (at 62°C).

Primer design and selection of microsatellite loci

Clones giving a positive hybridization signal were sequenced with the PE Biosystems *Taq* DyeDeoxy Terminator Cycle Sequencing Kit, using the M13 forward and reverse primers. Sequences were run on an ABI 377 Automated DNA Sequencer. For microsatellite-containing sequences, primers were designed complementary to flanking regions of the repeats using Lasergene (DNAstar). Primers were synthesized by Isogen (Maarssen, The Netherlands).

Two different PCR protocols were used on the TouchDown thermal cycling system (Hybaid). One program (NP) has short amplification cycles: one cycle for 3 min at 94°C, 30 cycles (5 s at 94°C, 15 s at the annealing temperature, 60 s at 72°C), followed by 10 at min 72°C. The other program (LP) has longer steps: one cycle for 3 min at 94°C, 30 cycles (45 s at 94°C, 45 s at the annealing temperature, 105 s at 72°C), followed by 10 min at 72°C. Amplification was performed in a 20-µl solution containing 75 mM Tris-HCl pH 9.0,1.5 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$, 0.01% (w/v) Tween 20, 100 µM of each dNTP, 200 nM of each primer, 8 µl of a 2 ng/µl dilution of template DNA in sterile distilled water and a 0.1 unit of Goldstar DNA polymerase (Eurogentec). Amplification products were mixed with an equal volume of 8 M urea containing 10 mM NaOH and 0.05% bromophenolblue, denatured for 5 min at 80°C and separated on 6% denaturation polyacrylamide gels (National Diagnostics). The products were visualized by silver staining according to the Promega Silver Sequence DNA Sequencing System. We optimized the quality of the allelic patterns by testing different annealing temperatures (55, 60 or 65°C) and/or the two different PCR programs.

The primer pairs that gave a good amplification product at the given annealing temperature, assessed on a 2% agarose gel, were subsequently separated on a 6% sequencing gel. After visualization by silver staining, the patterns were analyzed for the presence of polymorphisms and the quality of the banding pattern.

The best nine primer pairs were tested on a larger set of 23 genotypes (Table 1). The final quality of the patterns was assessed according to Smulders et al. (1997); quality 1, weak stutter bands, well scorable; quality 2, "stutter" bands present, but product still scorable; quality 3, ladders of bands of equal intensity, making scoring difficult.

Table 1 *P. nigra* genotypes from the euforgen core collection used in this study

Clone	Country of origin	H_o[a]
IBW-N004	BEL	1.00
IBW-N009	BEL	1.00
SEEFAR-SVICHTOV-N2	BGR	0.75
FBS-215/63-JUG-1	DEU	0.88
FBS-87/65-OFFENBERG-1	DEU	0.75
SIA-PA1	ESP	0.75
SIA-LUC2	ESP	0.88
FCR-HUNTINGTON	GBR	0.88
FCRA-HOBSONS-CONDUIT	GBR	0.88
FF-V336	HRV	0.63
FF-V408	HRV	0.63
ERTI-33-3-1	HUN	0.75
ISP-N068	ITA	0.63
IBN-1238	NLD	0.75
IBN-1792	NLD	0.63
LVU-BAKA	SKV	0.88
LVU-IVACHNOVA	SKV	0.38
KAE-N.90.013	TUR	0.50
IZT-NS002	YUG	0.50
IZT-NS001	YUG	0.25
VULHM-88044	CZE	0.75
ICAS-4	ROM	0.50
Cultivar Italica		0.88

[a] Determined using eight loci: WPMS03-07, WPMS09-10, and WPMS12

Results

Enrichment and characterization

A microsatellite-enriched library was constructed by hybridization to different pools of synthetic repeats. In total, three separate enrichments were done, using different pools. Pool 1 was a mixture of a (GA) and a (GT) dinu-

Table 2 Results of microsatellite enrichment using three mixtures of synthetic oligonucleotides

Pool of synthetic oligonucleotides	Number of white clones screened	Number of clones with a positive hybridisation signal	Number of clones containing a microsatellite	Number of unique microsatellite inserts[a]	Microsatellites found	
					Repeat motif	Number of clones
Pool 1: mixture of $(GA)_{12}$ and $(GT)_{12}$	356	61	57	47	GA	2
					GT	43
					GT/GA[b]	1
					TTCTGG	1
Pool 2: mixture of $(TGT)_9$, $(GTG)_8$, $(GAG)_8$, $(GCT)_8$, $(TGTT)_8$, and $(GATA)_8$	345	27	27	18	GCT	8
					GTG	3
					CTG	2
					GAG	1
					TGT	1
					GTC/GCT[b]	1
					TGC/TGA/TGC[c]	1
					TGC/TGA/TGC[c]	1
Pool 3: mixture of $(TCT)_{10}$, $(CGT)_8$, $(AGT)_{10}$, $(TGA)_{10}$, and $(GTAT)_8$	181	12	3	3	GCT	1
					TGA	1
					GTC/ATC/CTC[d]	1

[a] Duplicates have been excluded
[b] Two repeat stretches adjacent to each other
[c] One repeat flanked by two stretches of another repeat motif
[d] Stretches of three different repeats adjacent to each other

cleotide repeat. Pool 2 and pool 3 were mixtures of six different tri- and tetra-nucleotide repeats (Table 2). For the dinucleotide repeats a separate pool was formed because they are very abundant in plants. In total 882 white colonies were screened. To select for the presence of a microsatellite, they were hybridized to a mixture of the corresponding oligonucleotide probes. The efficiency of enrichment was variable (Table 2). In the pools with trinucleotide repeats the efficiency was between 1 and 5% only, but the dinucleotide repeat produced 13% (47 out of 356) different microsatellite-containing clones.

Most of the microsatellite sequences were perfect repeats. We found five compound repeats (Table 2). In pool 1 (GA/GT) we found 43 (GT) repeats and, remarkably, only two (GA) repeats. In pools 1 and 2 six clones were picked up more than once.

Marker development

Of the clones obtained, 13% were not useful because sufficient flanking sequence was lacking, either on one side or on both sides. We started with the development of markers for the GA/GT repeats found, because dinucleotide repeats are considered to be among the most polymorphic motifs. Most primer pairs produced polymorphic fragments in a test set of four genotypes, even though the scorability was poor in a number of cases. We optimised the quality of the allelic patterns by varying the cycling conditions of the PCR reactions.

Nevertheless, still many had quality 4 (additional fragments of unexpected sizes) or quality 5 (very weak bands or no amplification at all) patterns. In summary 12 out of 43 primer pairs for GA/GT repeats gave quality 1–3 patterns (Table 3).

Marker evaluation

The nine primer pairs with quality 1 or 2 were tested on a set of 23 *P. nigra* genotypes (see Table 1), part of the Core Collection of the EUFORGEN network, aimed to represent the diversity across Europe. In these trees, the nine microsatellites amplified between 10 and 19 alleles each. Due to the large number of alleles, loci were present in a heterozygous form in 57% (WPMS06) to 96% (WPMS05) of all trees analysed. Obviously, it was no problem to identify each individual based on its own unique pattern. Almost all alleles found differ in size by two base pairs or multiples of two base pairs, and form allelic ladders for each microsatellite locus. The allelic ladders span between 13 (WPMS12) and 41 (WPMS04) repeat units (average of 21 repeat units). In the series of WPMS04 and WPMS09 there is one case where three successive alleles differ by only one base pair. WPMS08 amplified three fragments in some genotypes. We did not determine the identity of these fragments.

Genetic diversity of the trees

Using the eight loci that produced one or two fragments in each genotype, the heterozygosity of the genotypes was calculated. The heterozygosity was on average 0.71, ranging from 0.50 to 1.00 with two exceptions: IZT-NS001 (from Yugoslavia) had a heterozygosity of only 0.25 and LVU-IVACHNOVA (from Slovakia) had a heterozygosity of 0.38. The most genetically similar trees were the two from Great Britain, with a proportion of shared alleles of 56% (data not shown). This is in agreement with the low level of natural genetic diversity in black poplar found in the Upper Severn area of the UK (Winfield et al. 1998).

Table 3 Characterization of microsatellites markers and their products in 23 *P. nigra* plants from the EUFORGEN core collection

Micro-satellite locus	Repeat[a]	Expected Product length	Primer sequences (forward, reverse)	Amplification conditions[b]	Quality of patterns in *P. nigra*[c]	Number of alleles among 23 *P. nigra* plants	Allelic range	Amplification in other species[d]
WPMS01	$(GA)_{20}$	141	5′-AACCACTATGCCACCTTCTT-3′ 5′-AACTAACTCCATTCATTGCTAAA-3′	50NP	3		137 – 169	Not tested
WPMS02	$(GA)_{23}$	253	5′-AGAAATACCCCTGCTAATC-3′ 5′-AATGTTTTTGGTCCGTGAAT-3′	55LP	3		196–244	Not tested
WPMS03	$(GT)_{26–1}$	297	5′-TTTACATAGCATTTAGCCTTTAGA-3′ 5′-TTATGATTTGGGGGTGTTATGGTA-3′	50LP	1	15	262–302	D, T, C, L
WPMS04	$(GT)_{25}$	213	5′-TACACGGGTCTTTTATTCTCT-3′ 5′-TGCCGACATCCTGCGTTCC-3′	55NP	2	19	245–318	None
WPMS05	$(GT)_{27}$	300	5′-TTCTTTTTCAACTGCCTAACTT-3′ 5′-TGATCCAATAACAGACAGAACA-3′	50LP	1	13	270–308	D, T, Ta, To, C
WPMS06	$(GT)_{24}$	205	5′-GTATAACGATGACCCCACGAAGAC-3′ 5′-TATAAATAAAGGCATGACCAGACA-3′	60LP	2	12	182–226	D, C, L
WPMS07	$(GT)_{24}$	258	5′-ACTAAGGAGAATTGTTGACTAC-3′ 5′-TATCTGGTTTCCTCTTATGTG-3′	55LP	1	12	218–270	D, T, Ta, To, C, L
WPMS08	$(GT)_{25}$	244	5′-TAACATGTCCCAGCGTATTG-3′ 5′-TTTTTAGAGTGTGCATTTAGGAA-3′	55LP	1/2	12	218–250	T, Ta, To, C, L
WPMS09	$(GT)_{21}(GA)_{24}$	295	5′-CTGCTTGCTACCGTGGAACA-3′ 5′-AAGCAATTTGGGTCTGAGTATCTG-3′	60LP	1	11	246–298	T, C, L
WPMS10	$(GT)_{23}$	258	5′-GATGAGAAACAGTGAATAGTAAGA-3′ 5′-GATTCCCAACAAGCCAAGATAAAA-3′	50LP	2	13	234–262	T, Ta, To, C, L
WPMS11	$(GT)_{26}$	217	5′-TAAAGATGATGGACTGAAAAGGTA-3′ 5′-TAAAGGAGAATATAAGTGACAGTT-3′	50NP	3		221–253	Not tested
WPMS12	$(GT)_{19}$	178	5′-TTTTTCGTATTCTTATCTATCC-3′ 5′-CACTACTCTGACAAAACCATC-3′	50NP	1	10	157–183	D

[a] Stretches shorter than five repeats are not taken into account

[b] 50, 55, 60: T_m; NP: 1 cycle 3 min 94°C, 30 cycles (5 s 94°C, 15 s at the annealing temp., 60 s 72°C) 10 min 72°C
LP: 1 cycle 3 min 94°C, 30 cycles (45 s 94°C, 45 s at the annealing temp., 105 s 72°C) 10 min 72°C

[c] Quality 1, weak stutter bands, well scorable; quality 2, "stutter" bands present, but product still scorable; quality 3, ladders of bands of equal intensity, making scoring difficult. Primer pairs producing products of quality 4, bands of unexpected sizes, and quality 5, very weak bands or no amplification at all, have not been tested on the set of 23 genotypes

[d] D=*P. deltoides*, T=*P. trichocarpa*, Ta=*Populus tremula*, To=*Populus tremuloides*, C=*Populus candicans*, and L=*Populus lasiocarpa*

Transferability

The nine loci were also tested on six different *Populus* species. Apart from WPMS04, which did not amplify in any of the other species, all microsatellite markers amplified fragments in a number of the other species (Table 3). We did not determine whether the markers were polymorphic in the other species, nor if they really amplified a microsatellite. However, the fragments vary only moderately in size between the different poplar species, the fragment sizes were in the same range as those of the *P. nigra* genotypes, and the shape of the amplification products was comparable to *P.nigra*.

Discussion

Microsatellites have been shown to differentiate between closely related genotypes (Smulders et al. 1997; van de Wiel et al. 1999) and can also be used to investigate genetic diversity in natural populations (Chase et al. 1996). In this study we employed a microsatellite-enrichment protocol (Van de Wiel et al. 1999) to efficiently clone microsatellite-containing DNA fragments. Based on the (GT) repeats, which were the most abundant class, we developed 12 primer pairs that showed a considerable level of polymorphism in black poplar. The number of alleles was between 10 and 19 in 23 genotypes tested. Most primer pairs also gave an amplification product in one or more other poplar species (Table 3).

The levels of heterozygosity were very high, in line with what is generally found for cross-hybridizing species, although some trees were less than 50% heterozygous.

After our enrichment procedure using a mix of GA and GT synthetic oligonucleotides, we isolated 20-times as many GT repeats as GA repeats from the black poplar genome. This is different from most other species, GA usually being more abundant (Lagercrantz et al. 1993; Morgante and Olivieri 1993; Taramino et al. 1997). Relative and absolute frequencies of types of repeats may vary among species but, in the case of poplar, at the same time a large number of GA repeats has been developed for *Populus trichocarpa* and published on the PMGC web site. Therefore, it is not very likely that GA repeats are rare in *P. nigra*. Our isolation method involves several PCR steps and a selective hybridisation step. In this process, the use of a mixture of different repeat motifs can affect the frequency of repeats cloned (Van de Wiel et al. 1999). Most probably, the use of separate GA and GT filters would allow us to pick up a fair amount of repeats of both classes.

Taramino and Tingey (1996) found in maize that GT/AC repeats were less polymorphic and difficult to score. The GT repeats in poplar that we have cloned, are mostly perfect repeats, and from our amplification results (Table 2) it is clear that they are highly polymorphic loci, and have a high quality. It is possible that the tri- and tetra-nucleotide repeats also cloned from *P. nigra* may be easier to score (but perhaps less polymorphic). We will continue to develop markers using the trinucleotide repeats cloned.

The microsatellites developed here may also be useful for clonal identification. With a lower number of loci compared to, e.g., AFLPs, but with a higher information content (more than two alleles) per locus, the resolving power of the 12 microsatellite markers taken together is large. In addition, the codominant nature of microsatellites makes them ideally suited for population genetic studies, as it allows assessing loss of heterozygosity, population subdivision and inbreeding.

The 23 trees from the Core Collection were selected to represent as much diversity as possible based on morphological criteria (Frison et al. 1995; Turok et al. 1998). This does not necessarily coincide with genetic diversity. However, as is clear from our analysis with only eight microsatellite markers, the total genetic diversity present in the species may actually be quite large. To assess the diversity in more detail, populations across Europe should be sampled, preferably along different rivers. To assess diversity at the population level, it is necessary to sample populations in such a way that genets can be identified. This is currently being carried out, employing the markers developed here.

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