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Efficient development of dinucleotide microsatellite markers in Norway spruce (*Picea abies* Karst.) through dot-blot selection

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Abstract The development of microsatellite markers can be a time-consuming process, especially in species such as conifers where many microsatellites have been shown to be associated with the repetitive fraction of the genome and to produce complex banding patterns following electrophoresis. Therefore, procedures to eliminate this fraction from further processing are sought. In this paper, we report on the development of 53 dinucleotide SSR markers in Norway spruce, 35 of which (66%) produce simple, polymorphic patterns. This high efficiency is obtained by introducing a dot-blot selection against high copy number sequences, performed on the microsatellite-containing clones. The resulting markers turned out to be polymorphic and useful for population genetic studies and for linkage mapping. Seven additional markers that were not subject to the dot-blot selection are also presented.

Keywords SSR · *Picea abies* · Dot-blot · Molecular markers · Enrichment

Introduction

Microsatellites (or SSRs, simple sequence repeats) have in recent years become the marker system of choice in population genetics and linkage analysis, due to their codominant nature and their polymorphism. Nevertheless, the procedures to develop markers are complex and time-consuming, and this somehow prevents researchers from setting out for the isolation of such loci. Large numbers of SSRs, comparable to those usually seen for anonymous markers, such as AFLPs (amplified fragment length polymorphisms; Vos et al. 1995), are so far available only in a few species, in which a large-scale effort towards the construction of an SSR database has been made (Humans, mouse, rat). In plants, specific projects have aimed at searching for large numbers of microsatellite markers in important crops, mostly cereals and soybean (e.g. for maize: Maize database, http://www.agron.missouri.edu/ssr.html; for rice: RiceGenes, http://ars-genome.cornell.edu/rice; for soybean: SoyBase, http://129.186.26.94/SSR.html, and Cregan et al. 1999; for wheat: Roder et al. 1998; for barley: Ramsay et al. 2000). Beside these relatively few extensive searches, SSR markers for plant species are usually produced in relatively small numbers. A typical example is reported by Teulat et al. (2000), who developed 37 SSRs for coconut palm. Such cases indirectly show that isolating useful microsatellite sequences can be a high-throughput process only if systematic investment is made on this task. Identifying and sequencing microsatellite-containing clones is only the first step towards the successful development of microsatellite markers, which depends upon the capacity to amplify efficiently a single locus based on the target sequence. While some papers report on the efficiency in the marker development process (e.g. Pfeiffer et al. 1997; Huang et al. 1998), which will be variable from species to species, others simply report the sequences (e.g. Elsik et al. 2000) without mentioning whether they can provide useful markers or not.

In conifers, the problems encountered during the development of markers are even bigger. Despite the general observation that microsatellites in plants are preferentially associated with the low-copy DNA fraction of the genome (Morgante et al. 2002) and that this can be true for some microsatellite sequence motifs even in pines (Elsik and Williams 2001), it has been shown that a high proportion of the primer pairs derived from ge-

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nomic clones produces multiple-band patterns, and must be discarded in conifers due to their occurrence within repetitive DNA sequences. This is most-likely due to the size of the genome, which on average is ten-times larger than in maize, and displays a high proportion of repeated sequences (Elsik et al. 2000), as well as to the ancient origin of the repetitive DNA amplification (Stuart-Rogers and Flavell 2001). Multiple-band patterns can be used, but only a segregation test allows one to properly assign bands to loci, and therefore these markers are of limited use except in mapping. The numbers of good microsatellite markers reported in conifers are therefore even lower than for other species: Hicks et al. (1998) reported the development of five markers in lodgepole pine, Echt (1996) developed 19 SSRs for Eastern white pine, Scotti et al. (2000) isolated three markers for Norway spruce, Khasa et al. (2000) report 14 in larch, while the most extensive study for this species is described in Pfeiffer et al. (1997). Here just 7 out of 36 primer pairs produced the desired single-locus pattern.

Pfeiffer et al. (1997) showed that it is possible to identify low- or single-copy clones among those containing microsatellites from a Norway spruce genomic library through dot-blot selection after hybridisation to labelled total genomic DNA. We report here on the development of 50 microsatellites using a library enriched for AC/GT microsatellites and selection for low-copy number clones. We also describe ten additional markers that were not selected against the presence of repetitive DNA. AC/GT repeats were chosen, although Pfeiffer et al. (1997) report that AG/CT repeats are more abundant, because the latter tend to be over-represented in a highly repetitive DNA family (Zuccolo et al., in preparation).

Materials and methods

DNA of a single tree [V23, from the population of Val Meledrio (TN), Italy] was used for the construction of a genomic library. The markers were tested on a panel of six individual trees from different natural populations of Norway spruce belonging to the Italian alpine range of the species, including the tree used for library construction [V23 and V34, Val Meledrio (TN); VdF37, Val di Fiemme (TN); T37, Tarvisio (UD); F8, Fusine (UD); I1121, Passo Giau (BL)]. The progeny of a controlled cross between clones N2022 and E2006 (Skogforsk, Sweden) was used for testing the segregation of the markers. DNA was extracted from leaf tissue using the Qiagen DNeasy kit.

The enrichment for microsatellites was performed as described in Tenzer et al. (1999).

The selection of low-copy number clones was performed as described in Pfeiffer et al. (1997), using Norway spruce total genomic DNA as a probe. Plasmids were purified (Wizard Minipreps; Promega Corp.) and sequenced on an ALF automated sequencer (Pharmacia Biotech., Inc.) using the ThermoSequenase fluorescent-labelled primer cycle sequencing kit (Amersham Pharmacia Biotech).

Oligonucleotide primers flanking the microsatellite regions were designed using the computer program PRIMER (version 0.5; Whitehead Institute for Biomedical Research, Cambrige, Mass.). The theoretical annealing temperature of the primers was chosen to be 58 °C for all markers, and tested in the range 53–63 °C.

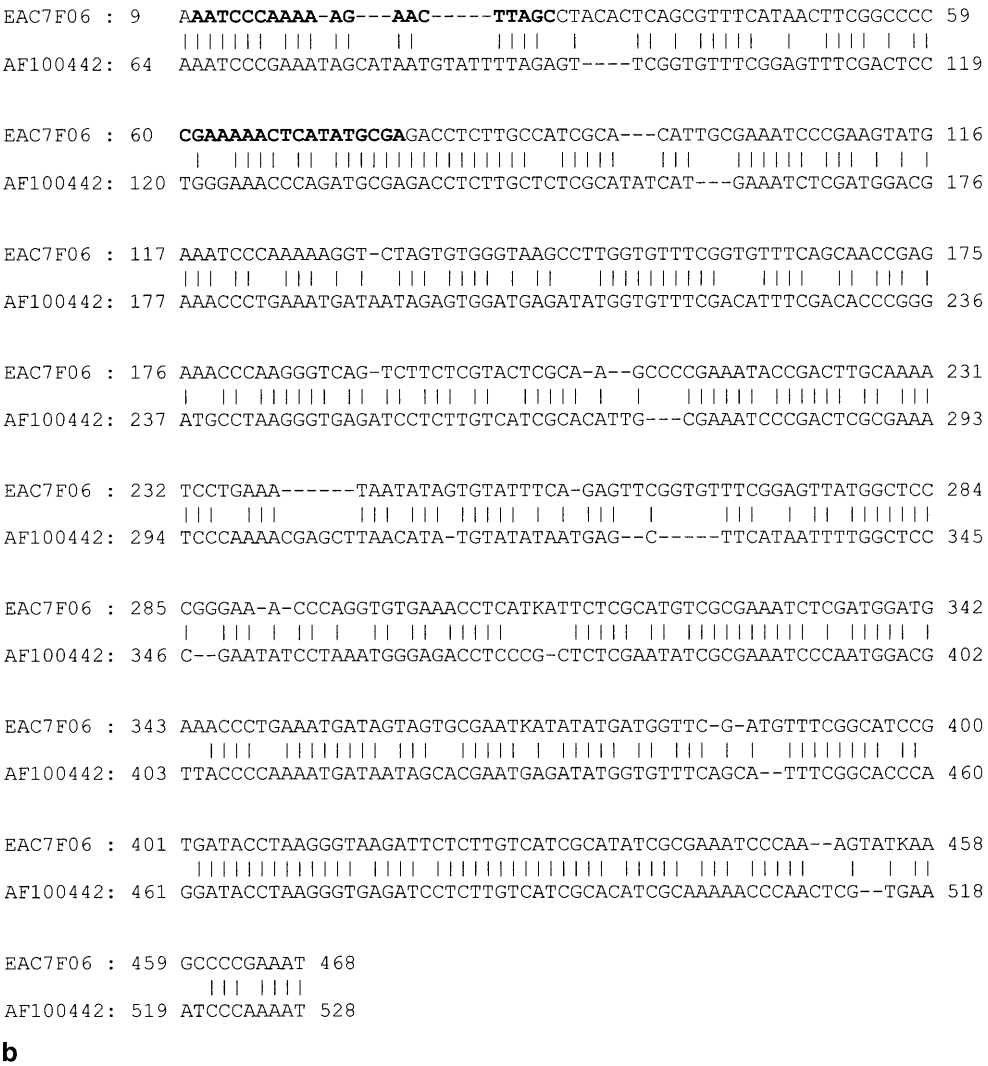
For each primer pair, the products were resolved on agarose gels or on polyacrylamide gels, depending on their pattern. PCR amplifications were performed in a final volume of 10 µl containing 1.5 ng of template (genomic DNA of *Picea abies*), 200 µM of each dNTP, 50 mM of KCl, 10 mM of Tris-HCl pH 8.3, 2.5 mM of $MgCl_2$, 0.25 µM of each forward and reverse primer, and 0.4 U of Ampli*Taq* Gold *Taq* polymerase (Perkin Elmer, Inc., Foster City, Calif.). When amplification products were separated on acrylamide gels, one of the two primers was end-labelled in the following way: 2.5 µM of the primer were combined with 1 µCi of [γ^{33}P]ATP and 0.5 U of T4 polynucleotide kinase (New England BioLabs, Inc., Missisauga, Canada) in 1 × kinase buffer and incubated for 35 min at 37 °C; the enzyme was de-activated by heating for 10 min at 70 °C. The labelling reaction was then combined with the PCR mix. Amplifications were performed in 9600 or 9700 Gene Amp PCR systems (Perkin Elmer, Inc., Foster City, Calif.) with the following profile: one cycle of hot start (95 °C for 10 min); seven cycles of touch down [95 °C for 30 s, (Ta+7 °C) for 30 s $\Delta\downarrow$1.0 °C, 72 °C for 30 s]; 28 cycles of amplification (95 °C for 45 s, Ta for 45 s, 72 °C for 45 s); one cycle of final extension (72 °C for 10 min). Non-radioactive amplification products were separated on 3.5% Metaphor agarose gels (FMC BioProducts, Inc.) in 1 × TBE, stained with 1 × ethidium bromide; gels were visualised on a UV lamp and photographed on Polaroid film. Radioactively labelled amplification products were run on acrylamide gels and visualised as described in Paglia et al. (1998).

All sequences of microsatellite-containing clones were compared to a set of highly repetitive Norway spruce sequences that we isolated (EMBL accession numbers: AF100427-AF100453; AF101009-AF101028; AF104476-AF104495; AF105147-AF105148; AF105962-AF105966; AF107119-AF107153; AF144643-AF144644; AF152541-AF152546; AF180427-AF180429; AF180922-AF180937; AF187273-AF187300; AF305095-AF305192) using BlastN (gapped Blast, score for matches: 4; score for mismatches: –5), as well as to each other using the FastA sofware.

Results

The 150 clones showing the weakest hybridisation signal in the dot-blot assay were selected for further processing, out of 600 putative AC-positive clones screened. Based on sequencing, 108 clones (72%) contained the expected repeat. When compared to each other using FastA, all of these clones were unique, confirming the efficiency of the enrichment procedure and of the selection for single-copy regions. A comparison of these sequences with our database, containing more than 100 kb of Norway spruce repetitive sequences showed that six clones (EAC1E03, EAC6C10, EAC6D11, EAC6H01, EAC7E06 and EAC7F06) share homology with different repetitive sequences (score greater than 100, E value less than 1e-20). Therefore, these six clones must in principle be assigned to the repeated fraction of the genome; that is, at least six repetitive sequences, out of 108 clones (5.6%), are still present after the dot-blot selection step. If we take the figure given in Elsik et al. (2000), with approximately 85% of the genome of Pinaceae composed of repetitive sequences as the baseline, and if we assume that dinucleotide microsatellites are randomly distributed across the genome, the upper-limit estimate of efficiency of enrichment in low-copy number sequences is 15-fold (if all the SSR clones belonging to the repetitive fraction of the genome have been correctly identified). Fifty three clones were selected for primer design (see Table 1), and their performances and features are described below; the last seven markers in Table 1 were developed as described in

a



b

Fig. 1a,b Homology of microsatellite clones with repetitive elements. **a** Homology of clone EAC7E06 with clone EMBL AF107133 (*P. abies* repetitive DNA clone PAB13D6). **b** Homology of clone EAC7F06 with clone EMBL AF100442 (*P. abies* repetitive DNA clone PAB7F12R). Primer sequences are displayed in *bold*. The microsatellite stretches have been deleted for the FastA search, and are found at position 56 in clone EAC7E06, and at position 33 of clone EAC7F06

Table 1 List of the markers. EMBL = EMBL accession numbers. Map = maps in which the markers are included (PA = Paglia et al. 1998; SC = Scotti et al. in preparation). Ta = annealing temperature; Exp. size = size of the amplified fragment, as deduced from the sequence. Na = number of alleles. The "*" in the Na column indicates a di-locus pattern. C = complex pattern. N = not amplifying

SSR name	EMBL	Map	Forward primer	Reverse primer	REPEAT	Ta	Exp. size	Na
EAC1A07	AJ292690		CTGTGACACTCAAGTGCCTCT	CTTTCTAACCACGCACACATT	(AC)52	60	157	6
EAC1C02	AJ292691		ACTATCCCTAGCCACCCAAC	ACTATAGGTCACTCCAGTCAGAGG	(AC)24	60	230	C
EAC1C08	AJ292692	SC	AGCCAAGGCTCTACAAGTAGC	TCCCAAATACTGTGCAAATGT	(AC)36	61	273	5
EAC1D10	AJ292693	PA	ACGATGTGGTATGTACATGGA	CAATGCACACTGAACATCAAA	(AC)31	60	150	2
EAC1E03	AJ292694		CAGTCCATAAAATCCGGAGAT	TTAACAGGTGACTTTTGTTCCC	(AC)11	58	166	4
EAC1F04	AJ292695	PA,SC	TGTAAGTCTGCTTGAAGGTGG	CAGATGGGGGTTGGGTAT	(AC)34	58	233	6
EAC1G03	AJ292696	PA	GAACACGGAAGGAACTACACAT	ATGTAGTTGGCAAGTGGCAT	(AC)23	60	117	C
EAC1G04	AJ292697		CAAGGCACCCCTAGTTCTTAG	GGTCGAAGAAGGATGTTCATT	(AC)23(AT)4	53	190	N
EAC1G05	AJ292698	PA,SC	TGAATAGTTGGTTATTTTTTCC	CATTGTGAATCATTCTGTCTC	(TC)4GT(AC)33	53	208	9
EAC1H01 Rev1	AJ292699		ATGGAAATAAATACACAAAATACA	TTTCATGGAGGAGGTTAGTAT	(AC)48	60	172	C
EAC2C08	AJ292700		TGATTATGTCTATTTAAAGTTTG	ATACAGATCTATAGCACACCC	(AC)25	50	139	4
EAC2E03	AJ292701		TTGAATGAAACGTTGGAACAT	ACATAATCGATGAGCATGGAA	(TTG)6TTA(TTC)-3(TTA)6(AC)23	57	244	C
EAC2H07	AJ292702		TCCAATAACACCTTACAATCTGG	TCCCCAAAAATGTCCTTAAAC	(AC)28	58	220	6
EAC2H08	AJ292703		TCATTGTAACACGAAGGGGTA	AAACAAGTCCTTCTTTGCTATCC	(CT)7(CA)20	58	217	6
EAC6A01	AJ292704		CACCTCGGTGTGATCAATTC	TTTAAAGAAATACCATGATCGTAGC	(AC)13	60	189	1
EAC6A02	AJ292705		TTTCACATCTTCGTGTTCCAT	ATGTCTTTTGATTTATGTCATTGTG	(CA)23	61	117	6
EAC6A06	AJ292706	PA,SC	AATTAAGGGGTAATGTGCCAC	AATGATGTTAAAGCAATATGTCTTG	(AC)20	57	117	6
EAC6A07	AJ292707		CATCGTAGGCCCTGAAGAC	GTGATTCTTTCAAAATGTAATCCA	(AC)17(AT)7	53	125	N
EAC6A08	AJ292708		CGTAACACATGCTTTTGTAGA	CAAAAGGGTGTTATTCAAAAC	(AC)20	53	118	N
EAC6A10	AJ292709		TCTGCCCTTGAACCTCTAAAC	TGAGTGATAAGCCAAACATTTC	(AC)20	60	199	8
EAC6B01	AJ292710	SC	ACCCACCAACACCATAACC	TTGAAGTGCACATTGAACTGA	(AC)28	60	128	5
EAC6B02	AJ292711	PA,SC	ACTCTGTGTTGTTTGATTACAACTC	ACCGCGGAACATACATATACA	(AC)27	58	149	5
EAC6B03	AJ292712		GAAGGTTATAATATTCAGTGAAGG	TAATGCTTATCAATGAGGTTG	(AC)25	57	125	4
EAC6B11	AJ292713		TGAACATTTTCTTTTGTTTTTTAGA	TCATAAGACATGACTACTTTGTCAA	(AC)23	60	181	8
EAC6C02	AJ292714	SC	TACTACTAGGTCGACCCTATTTCA	ACCCAAGGGGGGAATATATAG	(CA)26(TA)3	60	119	8
EAC6C10	AJ292715	PA	CCACCATAGAAGGCCTACCT	TACTTGCACAACACGCATAGA	(AC)9AA(AC)5	58	110	C
EAC6D01	AJ292716		ATTGGAAAGACCATGTATGGC	TGAGAGCAGACTTGAGAATCTG	(TA)6CT(CA)62	60	198	7
EAC6D03	AJ292717		CAATGTTCATATTCCTTTATTAA	TCCTATTATTTTCTTTAGAATTGC	(AC)24	53	196	1
EAC6D11	AJ292718	PA,SC	GGGACAACTCAAGTGGTT	TAATGTTTCTTTCAAGTTTATAATG	(TC)20(AC)27	57	149	C
EAC6E02	AJ292719	PA,SC	CGCACAGCCAAACAAACT	TGTGATGAACTGTTAGTATGCATG	(AC)13AG(AC)-38(AT)5	58	188	C
EAC6E07	AJ292720	SC	GAGACCTGACAAATAGGTATTCTTG	TTCCTGGAACAACAACCATC	(CA)40	58	200	1
EAC6E09	AJ292721	PA,SC	CCCCTCCAATATTTATGC	CCTTTTGTTATAGAAGATAATGTG	(AC)20	53	102	5
EAC6F04	AJ292722	PA,SC	CTTAGACGGTGCTTGCATG	TCGCATGTGTGCATAATAAGA	(CA)20(TA)3	58	129	C
EAC6F05	AJ292723	SC	ATCCTATTGACAATGGCTACT	AATATAGATCCTACACAACATCG	(AT)4(AC)28	53	257	1
EAC6G03	AJ292724	PA,SC	TCGCATGTGTGCATAATAAGA	GTGTCTGCACGATGACTTTTT	(CA)51	62	185	2
EAC6G07	AJ292725	PA	TAAGTCGGGGGATGTTAC	TGTAAATACTTTGACGTTTATTG	(CA)40	53	144	C
EAC6G08	AJ292726	PA,SC	AATTTAGATGATATTGAATACATACA	CATACATTCATCAACAGAAAA	(ACAT)7(AC)20(AT)7	57	159	C
EAC6H01	AJ292727		CTTTGTTTTCTCAAACCTCTT	AGCATAAATAATCTAAAATTCACA	(CA)36	57	194	1
EAC7A07	AJ292728	PA,SC	ATCATTTTTCATGTTTTGGGT	TGCTCTTTGTCTCTCCCTCTC	(AC)49	58	225	C
EAC7B09	AJ292729	PA,SC	TGTGTTGTGTTTGTTGTCGAG	GACGCATAACCAGTGCAATAT	(AC)28	58	136	12*
EAC7C11	AJ292730		AACTCTATAAAATAACGCACCTCG	CCAAAACAAAGGAAGGATGTT	(AC)19	60	126	4
EAC7D10	AJ292731	PA,SC	AAGGGTGTTATTCAAAACATGA	AGCCTTCGTATGTTCAAATAAA	(CA)13CG(CA)9	56	149	2
EAC7E06	AJ292732		GGTCCTAAGACCATGTCTCCT	GTTAATCCCTAGGCAAAGCC	(CA)47	57	199	3
EAC7E10	AJ292733		AATTGGAATGAACCCATCATT	TTATGGAAAATCTTTGTCAGGG	(AC)28	60	102	1
EAC7F06	AJ292734	PA,SC	AATCCCAAAAAGAACTTAGC	TCGCATATGAGTTTTTCG	(CA)32(TA)6	53	140	6*

Table 1 (continued)

SSR name	EMBL	Map	Forward primer	Reverse primer	REPEAT	Ta	Exp. size	Na
EAC7F08	AJ292735	PA,SC	AATTCATTAGAAATACATGTTGCG	AATTTGTAACCTCCCCAACC	(AC)15AT(AC)43	58	190	3
EAC7F10	AJ292736	PA,SC	TCCACAAATAATATAACACTATACA	TTCCATTTGTCTTTGTTTGTA	(AC)42	53	197	8
EAC7G07	AJ292737	PA,SC	TTCCTTTTTCCAGATAACCCA	AATGCTTGCTCTGGTGATATG	(AC)28(AT)3(GTAT)4	58	141	C
EAC7G08	AJ292738		AATTCAATATGCATATATATGTGTG	CATGTATGGCTTACGTTTATG	(AC)28(AT)4	53	108	7
EAC7H07	AJ292739	PA,SC	GGTTCAAACCTCCCACCTAC	ACCAACTAAGCCACAAGTGC	(CA)23	58	136	8
NACA08	AJ297481		AAGTTATTGAAATCAGAAGTTGGG	TTTTTCAAAATGTAATCCAGGA	(CA)19(TA)7	57	228	C
NACB06	AJ292741	PA,SC	CTCGTGCCCAATATACAAGTG	GTAGTCTCCAGAGCATCAAAGC	(AC)13(AT)6	58	229	C
NACG07	AJ292742	PA	GTTGGGCAAAAGAGAATAACC	TGCGATTTTTGTCGTCTTTAG	(CA)26(TA)11	58	231	C
Markers from non-enriched libraries (see text)								
SpL2AGH1	AJ292740	PA	GACACTCCACCCGTCATTAG	TTTTCAACAACCCCTCTACCT	(AG)18	58	147	C
SPL3AG1H4_A	G31835		TTAGGAGAGGAAAGGAGGAGG	GATCCCTGTGAGTTGACTAGATC	(AG)19	58	203	C
SPL3AGB4	AJ297477		AACATAGTGGACAACAACCCA	ACAAAAAACGAGGCAGAATTT	(GA)19	61	226	C
SPL3AGE3	AJ297478		TGTCCCTAGAATTCAAGAATGG	ACTCTCTTTGTCATTTTGGTTAGG	(AG)29	57	258	C
SPL3AGG2	AJ297479		ATAACCACCCAGAATAATAAGTCG	AATTCCTCTCCCAAGAATGC	(AG)24	61	221	C
SPL3B3A1	AJ297480		AATTGTATTTTCCAAGTCCAACC	GAGGTAAAGAGAGATATGGGGG	(CT)5TTTCTCCT-(TC)7	58	178	C
SpAGF1Rev2	G31832		CTATGGAGAGATTTAAGTTTTGGG	CCTATTACAATTGTGGATCTCATG	(CT)17AA(TA)21	57	186	C

Pfeiffer et al. (1997) and used by Paglia et al. (1998), but they have not been published yet. Therefore they are reported here, but will not be further discussed.

After optimisation of the reaction conditions, 33 primer pairs out of 53 (62%) produced a simple pattern, with no more than two bands per sample, and can therefore be considered as single-locus markers; two pairs (4%) produced two to four bands and could correspond to a two-locus system; 12 gave a complex pattern (28%); three gave no amplification at any annealing temperature in the range 53–63 °C. All the markers are reported in Table 1, including the multiple-band and unsuccessful ones, with the EMBL accession number for the corresponding sequence, since it is possible that the re-design of the primers gives a positive amplification, or a simplified pattern. Three of the primer pairs from clones showing similarity to repetitive sequences gave single-locus markers, although two of them display homology with repetitive sequences in the region of primer annealing (Fig. 1); one gave a two-locus marker; the remaining two gave complex patterns.

The number of alleles per locus ranged from one (six markers) to nine, with an average of 4.6, as estimated from the analysis of the sample of six Italian trees. These estimates do not include the markers showing a two-locus segregation, where up to 12 bands were visible on the same set of six trees. For 16 of the 33 single-locus markers we have evidence of Mendelian segregation in either or both of two linkage mapping experiments (Paglia et al. 1998; Scotti et al., in preparation). Two markers that were monomorphic in our panel of six trees were polymorphic in one mapping population (Table 1). The attribution of bands to loci can only be obtained, for markers showing a complex banding pattern, through segregation analysis. In the two linkage mapping experiments we additionally observed Mendelian segregation of at least one locus for 11 primer pairs showing complex patterns. When more than one band was segregating in these primer pairs, several dominant markers (presence/absence of band) assorted independently.

Discussion

The process of marker development needs to be made reliable and economic if a large set of microsatellite loci is desired. In order to obtain this, strategies must be adopted to improve the efficiency of recovery of the most useful microsatellite markers, i.e. those amplifying a single multiallelic locus. In plant species with a large genome, microsatellites can be found embedded within repetitive DNA sequences, and produce complex or non-reproducible banding patterns on gels (Roder et al. 1998; Ramsay et al. 1999).

One option is to select for microsatellites that intrinsically belong to the low-copy fraction of the genome, such as tri- and tetra-nucleotide repeats, as shown by Elsik et al. (2000); another possibility is to select against

those microsatellites that are embedded or flanked by repetitive fragments.

This work shows that several clones belonging to the repeated fraction of the genome can be excluded from further processing through dot-blot analysis. We obtained 35 primers with a simple pattern out of 53 (66%), which is a rather high rate compared to what is usually found in conifers: Pfeiffer et al. (1997) reported an efficiency of 19% (seven successful primer pairs out of 36) in the process of marker development without using such a selection procedure. Due to the dot-blot selection, the percentage of single-locus markers is thus more than tripled. It is worth noting that in species with a smaller genome, such as rye, it has been reported that only 47% of the primer pairs amplify simple single-locus patterns (Saal and Wricke 1999), and therefore this method could be helpful also with less problematic species. The dot-blot selection, in combination with down-scaling of the reaction and with the amplification of very small amounts of genomic DNA, allowed us to obtain cleaner PCR profiles.

Only two of the six markers developed on sequences showing similarity to our set of spruce repetitive DNA sequences (clones EAC6C10, EAC6D11) gave complex patterns while the other ones identified one (EAC1E03, EAC6H01, EAC7E06) or two (EAC7F06) loci. The lack of a strong signal in the dot-blot for these clones may indicate that they belong to middle or lowly repetitive families, or that they are quite divergent from the consensus sequence for the family. The success in amplifying some but not others may simply depend on where the primers are designed from and on the degree of conservation of that specific sequence between the different members of the repetitive sequence family. If this is the case, then the dot-blot selection worked also for these clones, and with a very high accuracy. On the other hand, a subset of primer pairs (13 out of 53, or 24%), that did not appear to be repetitive on the basis of either the dot-blot hybridisation experiment or the sequence similarity searches, still produce complex patterns. Dinucleotide microsatellites derived from expressed sequences (Scotti et al. 2000), which by definition should belong to the low-copy fraction of the genome, are affected, to an even larger extent, by the same problem. The presence of multiple bands cannot therefore be taken as a proof that these clones are actually repeated. While the dot-blot procedure like other reassociation-kinetic based procedures can effectively discriminate between high and low copy number sequences, the separation between low and single-copy sequences may be impossible to achieve. The fact that these primer pairs giving complex patterns are not derived from highly repetitive sequences is confirmed by the observation that in many cases they still give discrete banding patterns with bands that are inherited in a simple Mendelian fashion and thus correspond to single loci. Re-designing of primers may improve these markers.

The variability of these microsatellites, as tested on a panel of six trees from natural provenances sampled across the alpine range, is rather high, as is expected for this class of markers. Also for the primer pairs producing complex patterns it was possible to identify variable markers on acrylamide gels, as can be noted from Table 1, where the inclusion of the marker on at least one map implies that at least one Mendelian marker appeared.

The inclusion of these markers in genetic linkage maps makes them an even more powerful tool for different purposes (such as population genetics and provenance identification), allowing one to select for markers that provide an even coverage of the genome, or to identify markers at the desired map distance (e.g. unlinked markers for diversity studies, or linked markers for linkage disequilibrium analyses).

By allowing us to rescue 35 “good” markers out of only 53 primer pairs designed, the introduction of a simple procedure, such as the dot-blot selection, has made the otherwise cumbersome task of isolating tens of SSRs in a conifer a reasonable job. This increase in efficiency in the future will allow one to obtain large numbers of SSR markers, a valuable and flexible tool for all genetic studies.

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