appropriate weight to each trait according to its relative economic importance, its heritability, and genetic and phenotypic correlations between traits. Such index selection for WSG will provide the flexibility to breeders during culling of families (provenances) in later ages when late wood production is pronounced and provenance differences for WSG may be expressed better.

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Molecular Differentiation of Pine Species using a Primer Pair

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(Received 5th May 2003)

Summary

The identification of informative PCR-based markers is difficult in species with large, complex genomes such as conifers. We have isolated and characterized few microsatellite DNA markers from *Pinus resinosa.* The designed pairs resolved DNA variants showing consistent but complex multilocus patterns. A primer pair that allows the unambiguous differentiation of pine species was identified. This primer pair generated nine alleles which were highly informative. In fact, eight of the nine loci identified were polymorphic in trees from different populations. Each of the seven pine species examined had unique one or two locus genotypes, but each also had at least one common allele with *P. resinosa* from which the microsatellite clones were derived. The data set generated with a marker such as this can be of use in phylogenetic studies and the identification of interspecies hybrids.

Key words: Pinus resinosa, Simple sequence repeat, allelic frequency, DNA fingerprinting.

Introduction

The genus *Pinus* includes more than 100 species, more than any other gymnosperm. Rational management and usage of forests should be combined with measures aimed to preserve the biological diversity. This would be facilitated by a good understanding of taxonomy and phylogenetic relationships of pine species. The systematic of the genus Pinus has been extensively studied and has been repeatedly reconstructed. The first classifications were based on morphology of cones, position of resin ducts in the needle, shape of seed wings, intercrossing abilities of the species, pollen characteristics, anatomy of the seeds and needles, and resin compound. A classification which encompasses all well argumented views from other classifications was given by LITTLE and CRISTCHFIELD (1969). This has been for more than 25 years the most widely accepted classification of the genus *Pinus.* The authors summarized the worldwide distribution of the pines (CRISTFIELD and LITTLE, 1966) and presented a detailed intrageneric classification of the genus (LITTLE and CRISTCHFIELD, 1969). With the event of molecular techniques, this classification has been refined.

Comparative studies of chloroplast (cp) DNA variation have shed new light on the phylogenetic structure within the genus *Pinus.* There has been a surge of studies carried out on chloroplast, nuclear, and mitochondrial DNA in recent years, aimed at inferring phylogenetic relationships at intrageneric or higher taxonomic levels (BREMER, 1991; STRAUSS and DOERKSEN, 1990; WANG and SZMIDT, 1993). Phylogenetic analysis of plastid DNA restriction site and rearragement mutations by KRUPLIN et al. (1996) suggested a number of major revisions to tax-

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onomy and phylogenetic concepts in the hard pines. These authors also reported a number of strong conflicts with the classification scheme of LITTLE and CRISTCHFIELD (1969) and other scientists. Sequence analysis of species-specific RAPD markers revealed some new insights information in relationships among pine species (NKONGOLO et al., 2002). In the present study, we have developed a pair of primers that reveals taxonomically interesting differences among pine species.

Material and Methods

Genetic materials

Seeds of 21 populations of different pine species including *Pinus sylvestris, P. strobus, P. rigida, P. resinosa, P. monticola, P. contorta* and *P. banksiana* from different provenances (*Table 1*) were provided by the Canadian Forest Service (Fredericton). The seed samples were collected from experimental plots and the identity of each species was verified by the suppliers. Seeds were placed in clear polycarbonate "Petawawa germination boxes" containing wet "Kimpak" cellulose paper and kept in a germinator at 25 °C. Ten-day-old seedlings were collected and roots and seed debris were discarded. Seedlings were weighed, frozen in liquid nitrogen and stored at –70 °C until use for DNA extraction.

Table 1. – Plant materials used in this study⁺.

Species	Lot no.	Provenance
Pinus sylvestris	8880700	Hallestad District, Sweden
	8181130	Tatarskaya, Soviet Union.
	7383480	Balnagowan Wood, Scotland
Pinus strobus	9230121	Petawawa, Ontario, Canada
	8930585	Mackey, Ontario, Canada
	8930576	Petawawa, Ontario, Canada
Pinus rigida	8630177	Hill Island, Ontario, Canada
	8630122	Hill Island, Ontario, Canada
	8620282	St-Antoine-Abbe, Québec, Canada
Pinus resinosa	9230450	Cedar Lake, Ontario, Canada
	5780350	Delta County, Michigan, USA
	7023040	Norway-Bay, Québec, Canada
Pinus monticola	9170096	Pete Lake, British Columbia, Canada
	7679320	West Adams, British Columbia, Canada
	7071810	Adams Lake, British Columbia, Canada
Pinus contorta	7877170	Carmacks, Yukon, Canada
	6881120	Prairie City, Oregon, USA
	7779550	Cassiar, British Columbia, Canada
Pinus banksiana	7333410	Audrey Lake, Ontario, Canada
	7020470	Riviere Manicouagan, Québec, Canada
	7010380	Grand Lake, Ontario, Canada.

DNA extraction

Total DNA was isolated from individual seedlings from *Pinus resinosa* following a method described in NKONGOLO et al. (2002) for both construction of genomic libraries and microsatellite DNA genotyping.

Library construction and screening for SSR sequences

The partial genomic library enriched for (AG/TC)n containing microsatellites was constructed by following a protocol modified from KIJAS et al. (1994). The genomic DNA was digested with Sau3AI, and size fractionated on a 1% agarose gel. DNA fragments were extracted and purified using the Qiaquick gel extraction kit (Qiagen, Santa Clarita, Calif.). Purified DNA fragments were ligated to the pGEM-T-Easy vector (Promega). The ligated DNA mix was enriched for (AG/TC)n microsatellites by following the protocol of KIJAS et al. (1994).

The library enriched for the (AG/TC)n microsatellites were screened for clones containing microsatellites, as described by DAYANDAN et al. (1998).

Sequencing of positive clones, design of primers and resolution of SSR polymorphisms

Positive plaques were picked with a sterile pipette tip and placed in 15-ml culture tubes containing 2 mL of LB tetracycline (30 µg /ml) and 100 ul of overnight culture of XL1BlueMRF bacterial cells. Tubes were incubated at 37 °C with shaking for 6 h. A portion of each culture was transferred to 2-ml microfuge tubes and centrifuged at maximum speed (14,000 rpm) for 5 min. Single-stranded DNA was isolated from the supernatant using the Wizard M13 DNA purification system (Promega). Purified DNA from isolated clones with putative microsatellites was sequenced using the ABI Prism 377 automated DNA sequencer (Applied Biosystems, Foster City, Calif.). Oligonucleotide primers complementary to the regions flanking the identified repeats were synthesized and used for DNA amplification by PCR.

PCR amplification, primer optimization, and resolution of microsatellites

DNA was individually primed with pairs of designed oligonucleotide primers synthesized by CORTEC (Queen's University). In a 25-uL volume, 50 ng plant DNA, 0.25 uM primer, and 200 uM each of dATP, dCTP, dGTP, dTTP were mixed with 10 X reaction buffer II (Perkin Elmer), 0.02% BSA, 1.5 mM MgCl2 and 1 unit of Taq DNA polymerase (Perkin Elmer). Samples were amplified on a DNA thermal cycler (Perkin-Elmer). The PCR protocol of microsatellite analysis was optimized on a Gene Amp thermal cycler (Perkin Elmer Cetus, NorWalk, Conn.) With a touchdown program as follows: 3 min pre-incubation at 94 °C followed by 1 cycle of 1 minute each at 94 °C (denaturation), 64 °C (annealing), and 72 °C (extension) with stepwise lowering of the annealing temperature from 64 °C to 55 °C and 30 cycles of 1 minute each at 94 °C, 55 °C, and 72 °C prior to a final extension of 7 min at 72 °C, and subsequent cooling to 4°C. For analysis on 1% Gibco agarose gels in TAE buffer, 7 uL of the PCR reaction was mixed with 5 uL 1 X gel loading buffer (MANIATIS et al., 1989) to check for positive amplification. The rest of the amplified product was diluted with an equal volume of denaturing loading buffer consisting of 10 mM sodium hydroxide, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol. Six uL of this mix was electrophoresed on a 6% denaturing polyacrylamide gel containing 8 M urea and 1 x TBE buffer on a SEQUI-GEN sequencing electrophoresis apparatus at 45 W for 2 h. Following electrophoresis, the gel was silver-stained using a silver sequencing kit (Promega), and photographed. The number of alleles observed at each locus in each of the seven pine species and the numbers shared with or different from *P. resinosa* were determined.

Results and Discussion

Molecular identification of pine species has been subject of many studies. Highly informative genetic markers are still needed to facilitate pine genetic improvement, breeding, conservation, and sustainable management. In the present study, we have isolated and characterized few microsatellite DNA markers from *Pinus resinosa.* Several clones were developed but only three clones were sequenced and their nucleotide

sequences appear in the EMBL, GenBank, and DDBJ nucleotide sequence database under the accession numbers BH 792923, BH 792924, and BH792925 for micro-17, micro-3, and micro-5, respectively. As expected, for the clones analyzed, AG/CT repeats had the highest frequency. This supports previous reports, which indicated that (AG)n is the most frequent microsatellite motif in conifer (ECHT and MAY-MARQUARDT, 1997; ELSIK and WILLIAMS, 2001; SMITH and DEVEY, 1994 and SCHMIDT et al., 2000). Most of the repeat motifs identified were complex. In fact for the three sequences analyzed in detail, micro-5 contains (AG)6… (AG)12… (AC)3….. (AC)2…. (AC)7….. (AG)2; micro 17 contains (AG)7….. (CT)7…. and (CT)2… and micro 3 contains (AG)8….. (CT)2….(AG)3. A high frequency of (AC)n repeat motif was observed in micro 5. According to ELSIK and WILLIAMS (2001), this dinucleotide repeat motif appears to occur more frequently in the low-copy DNA than (AG)n. In pines, the frequency of (AG)n and (AC)n motifs vary from species to species. In fact, ECHT and MAY-MARQUARDT (1997) reported that Eastern pine (*Pinus strobus*) had more than twice as many (AC)n than (AG)n loci, in contrast with loblolly pine and most other plant species in which (AG)n is more abundant.

The primer pairs designed flanking the three sequences were 5'-AGA GAG AGA GAG AGA GCG-3' (forward) and 5'-GCA GTT TGA CCA TAG GGA-3' (reverse) for micro-3; 5'- AGA GAG AGA GAG CGA GAG-3' (forward) and 5'-GAA GTT GTT GAT CTC TAT GT-3' (reverse) for micro-5; and 5'-AGA ATA ACC GGA CAA GCA TC-3' (forward) and 5'-GAG GTT GGC TTT GGG CAT-3' (reverse) for micro-17. These primer pairs were tested for amplification of the expected fragment and for polymorphism on a panel of 88 seedlings representing different populations. In two of the sequences, the microsatellite region was too close to one end of the inserts. Therefore, the primers designed in these cases did not include a large portion of the microsatellite regions. A SSR marker amplified with a primer pair would be suitable when a single locus amplified efficiently at the expected size and with a minimum of stutter bands when assayed on silver-stained denaturing polyacrylamide gels (ECHT et al., 1996).

Overall, the three primers pairs tested yielded amplification products. None of the primer pairs for the different loci was classified as monomorphic. The SSR primer pairs micro-3 and micro-17 resolved DNA variants showing consistent but complex, multilocus patterns. The various bands that were obtained using a defined primer pair corresponded to different loci than the microsatellite locus. The primer pairs designed from micro-3 generated nine alleles in the pine samples examined (*Table 2*). These alleles were highly informative for allelic diversity. With the exception of allele seven which was monomorphic, all the alleles were polymorphic (*Fig. 1*). Each of the seven pine species had unique one or two-locus genotypes, but each also had at least one common allele with *P. resinosa* from which the microsatellite clones were derived (*Table 3*). The presence of a high level of unique and multi locus genotypes makes these designed primer pairs an excellent molecular tool for the various genetic studies, conservation and tree forensic fingerprinting and the identification of *Pinus* Species. If these findings remain consistent for a larger sample size, this primer pair could be used to differentiate unambiguously between closely related pines species. However, this marker cannot be used as codominant for diversity analysis which requires that a single locus be amplified in order to unambiguously assign individual genotypes based on the molecular phenotypes.

Clones giving multiple bands are usually associated with repetitive DNA sequences. Amplification of multiple bands may be due to large or small scale duplication within the pine genome. The identification of informative PCR-based markers is difficult in species with large, complex genomes such as pines because of the relative scarcity of unique, nonrepeated, DNA sequences (ECHT and MAY-MARQUARDT, 1997; ECHT et al., 1999). Consequently, only a small fraction of SSR clones selected from genomic libraries can be converted to informative markers. In the present study, primer pairs designed from *P.*

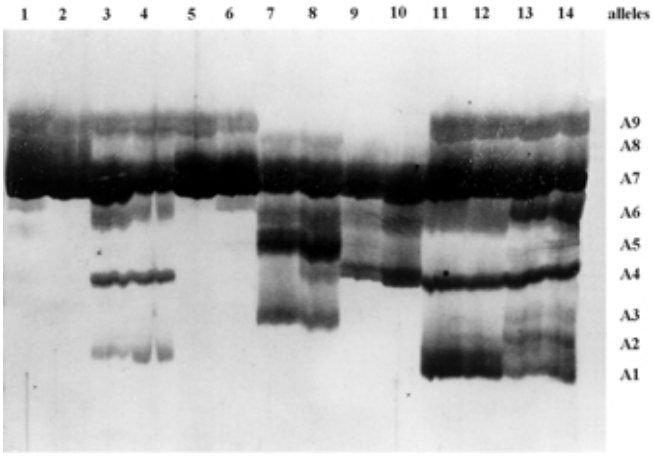

Figure 1. – Acrylamide gel showing PCR amplification products obtained by using a primer pair micro-3 (5' AGA GAG AGA GAG AGA GCG - 3' (forward) and GCA GTT TGA CCA TAG GGA -3' (reverse) for seven pine species. Lanes 1 and 2 were *Pinus resinosa,* lanes 3-4 were *P. rigida,* lanes 5-6 were *P. sylvestris,* lanes 7-8 were *P. monticola,* lanes 9-10 were *P. strobus,* lanes 11-12 were *P. contorta,* and lanes 13-14 were *P. banksiana.*

Table 3. – Number of alleles generated with the primer pair micro-3 in seven pines species.

Species	# alleles	Alleles shared with *P. resinosa*
P. resinosa	2	2
P. rigida	4	2
P. sylvestris	2	2
P. monticola	4	1
P. strobus	3	1
P. contorta	4	2
P. banksiana	6	2

Table 2. – Distribution of alleles generated with primer pair micro 3 derived from *P. resinosa* in seven pine species.

	Alleles								
Species	1	2	3	4	5	6	7	8	9
P. resinosa	-	-	-	-	-	-	+	-	+
P. rigida	+	-	-	+	-	-	+	-	+
P. sylvestris	-	-	-	-	-	-	+	-	+
P. monticola	-	-	+	-	+	-	+	+	-
P. strobus	-	-	-	+	-	-	+	-	-
P. contorta	+	+	-	+	-	-	+	-	+
P. banksiana	+	-	-	+	-	+	+	-	+

resinosa did amplify homologous sites in distantly related species, suggesting that the targeted region was highly conserved in a broader range of species within the genus *Pinus*.

Although the simultaneous amplification of multi-copy loci can sometimes be difficult to analyse when the bands overlap, there are advantages when their inheritance patterns can be discerned. Firstly, many alleles can be scored per individuals from one set of specific primers. A second advantage occurs when the multiple copies are at the same locus. This increases the chance that the locus will show polymorphism and can be used for genetic analysis. Lastly, unlinked multiple loci can be assessed simultaneously, providing a natural multiplex for mapping, parentage testing, and other analyses (FISHER et al., 1998).

Microsatellite markers are abundant in the genomes of nearly all eukaryotes. But the development of SSR markers is technically demanding, expensive and time consuming compared to other genetic markers. The use of DNA sequence similarities between taxa should contribute to leverage SSR and related markers development efforts. In the present study, the informative primer pair was derived from *P. resinosa* sequence around the SSR region. This pair was useful for genotyping in other species analyzed. If suitable numbers of informative SSR and related marker primer pairs developed from one conifer species could be used for genotyping in related species, then overall SSR marker development costs would be available, and SSR marker analysis could be more widely incorporated in many conifers genetics programs (ECHT et al., 1999). The polymorphic loci observed among species could be useful as species-specific markers if additional testing on a broader sampling of individuals in each species confirm these putative specific markers. The data set generated can be of use in phylogenetic studies and the identification of interspecies hybrids.

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Variation in Chilling Requirements for Completing Bud Rest Between Provenances of Norway Spruce

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(Received 5th May 2003)

Abstract

Winter dormancy in forest trees starts with a rest stage, during which buds remain inactive even if they are exposed to growth-promoting conditions, and is followed by quiescence. The transition from rest to quiescence requires chilling. The amount of chilling needed to complete the rest phase was tested in different provenances of Norway spruce (*Picea abies* (L.) Karst.). One-year-old seedlings from eight provenances were grown in a climate-chamber experiment, and from ten provenances (with one Sitka spruce provenance, for comparison) in a nursery experiment. The rest status of these seedlings after exposure to chilling for various durations was tested by measuring the time they took to break bud after transfer to growth-promoting conditions. In the nursery experiment, the chilling requirement was high in provenances from Denmark, Germany and Belorussia, and low in provenances from northern Sweden and Russia. Provenances from southern Sweden and Norway had intermediate chilling requirements. The Sitka spruce provenance had a very high chilling requirement. A high chilling requirement suggests better adaptation to maritime conditions and warm winters. However, absolute differences between provenances were small. The chilling requirement was

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