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Rapid identification of white-Engelmann spruce species by RAPD markers

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Abstract Fragments of random amplified polymorphic DNA (RAPDs) were used as markers to distinguish *Picea glauca* (Moench) Voss (white spruce) and *Picea engelmannii* Parry (Engelmann spruce). These species and their putative hybrids are difficult to differentiate morphologically and are collectively known as interior spruce. Four oligodeoxynucleotide decamer primers showed species-specific amplification products between white spruce and Engelmann spruce. These fragments are highly conserved among seed lots and individual trees of each species from diverse geographic origins. The consistency and reproducibility of these species-specific amplification products were tested in more than two amplification reactions. Therefore, RAPD markers can provide genetic markers for easy and rapid identification of the specific genetic entry of these spruce species and their reported putative hybrids. According to the frequencies of the species-specific RAPD markers, it is possible to estimate the hybrid fraction, indicative of true introgression between the two species. These results are useful for quick identification of both species and their hybrid swarms at any stage in the sporophyte phase of the life cycle, for determining the occurrence and the magnitude of introgressive hybridization in an overlap zone between the two species, and for certification purposes in operational re-forestation and tree-improvement programs.

Key words Hybridization · PCR · *Picea* · RAPD fingerprints

Introduction

Picea glauca (Moench) Voss (white spruce), *Picea engelmannii* Parry (Engelmann spruce), and their natural hybrids, sometimes known collectively as interior spruce (Garman 1957; Roche 1969; Krajina et al. 1982; Coates et al. 1994), are important economically, ecologically, and aesthetically, in temperate and boreal ecosystems. White spruce is a subartic species whose geographic range in Canada and United States extends from the East to the West coasts and from near the Canada-U.S.A. border in the South to Alaska, Yukon and Northwest Territories in the North. Engelmann spruce is a sub-alpine species widely distributed in western Canada and United States, where it occurs from British Columbia and Alberta in the North to Arizona and New Mexico in the South (Fowler and Roche 1977). Although white spruce spans the range from lowlands to alpine timberline in Alaska, in the more southerly portions of its range, it becomes more and more restricted to lower altitudes (Daubenmire 1974). In contrast, Engelmann spruce is a tree mainly found in the sub-alpine forest belt throughout its range.

About 100 million spruce seedlings are planted every year in British Columbia, most of them from the white-Engelmann spruce complex (see Sutton et al. 1991a). White spruce is the most commonly planted species in Canada (Kuhnke 1989) and white and Engelmann spruces are wideley planted in parts of the USA. (see Fowler and Roche 1977). A substantial quantity of spruce seedlings produced annually are from seed lots collected from the zone of putative introgression (Sutton et al. 1991 a, b, 1994). Introgressive hybridization in the zone of range overlap between white spruce and Engelmann spruce may have played a significant role in the evolution of this species complex but it remains difficult to distinguish both species and their hybrids based on morphological characters (La Roi and Dugle 1968; Roche 1969; Daubenmire 1974; Krajina et al. 1982).

The question of species classification for seeds collected from spruce stands in which possible introgressive hybridization occurs is of obvious concern in spruce genetic resource management and in ensuring adequate cultural treatments. Thus, tools for rapid identification of seed lots for operational nursery practices and seed

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transfer guidelines must be developed. For the last few decades, the classification of white spruce-Engelmann spruce seed lots has used quantitative morphological characters and species crossability patterns (Wright 1955), a morphological index based on branchlet, cone, cone-scale, and cone-bract characters (Garman 1957), as well as hybrid index, discriminant analysis, and Wells' hybrid distance techniques based on morphological traits (Schaefer and Hanover 1989). Unfortunately, many of the morphological characters that distinguish white and Engelmann spruces and the varieties of white spruce, are either found only in mature, cone-bearing trees, or not found on herbarium specimens, or not annotated on more than a few herbarium sheets (La Roi and Dugle 1968). Therefore, markers which may apply at any stage in the sporophyte phase of the life cycle, available in most herbarium specimens, and which are little or not affected at all by ontogenetic and environmental factors, would better address the inter- and intra-specific chemotaxonomy of the species complex.

Since the late 1960s, biochemical markers have been developed in *Picea* including terpene compounds using gas-liquid chromatography (Hegnauer 1962; Rudloff 1964, 1967; Ogilvie and Rudloff 1968), flavonoid and phenolic compounds using one- and two-dimensional paper chromatography of needle extracts (La Roi and Dugle 1968), sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of total proteins (Flinn et al. 1991), and allozymes (Yeh and Arnott 1986; Ernest et al. 1990). More recently, molecular markers such as chloroplast (cp) and mitochondrial (mt) DNA restriction fragment length polymorphism (RFLP) markers (Szmidt et al. 1988; Sutton et al. 1991 a, b), RFLP of the nuclear ribosomal RNA genes (Sutton et al. 1994), and in situ hybridization probes (Brown et al. 1993), are being used to classify seed lots of spruce species and for physical mapping purposes. These methods, however, are time consuming, labour-intensive, and require quite large amounts of plant materials. Since the 1990s, a new class of DNA markers, called random amplified polymorphic DNAs (RAPDs), has been developed, in which DNA is amplified by the polymerase chain reaction (PCR) using arbitrary short (approximate 10-mer) primers (Welsh and McClelland 1990; Williams et al. 1990; Welsh et al. 1991). Applications of RAPD markers to differentiate closely related plant species and their hybrids have recently been reported (Arnold et al. 1991; Perron et al. 1995).

The objective of the present study was to develop species-diagnostic RAPD fingerprints for the white-Engelmann spruce species complex, which later can be used for certification purposes in tree-improvement programs and to assess the occurrence and extent of hybridization in reported introgression zones. We screened individuals of both species with 130 RAPD primers in order to identify DNA fragments that are conserved within a species but differ between the two species (monomorphic or nearly monomorphic fragments and specific to either white spruce or Engelmann spruce).

Materials and methods

Plant material and template DNA preparation

Fresh needles of 145 trees from seven allopatric (geographically separate) populations of both spruce species were collected in the summer of 1990 (see Table 1 for the population locations). Extracted DNA was stored in a freezer (−20 °C) until used in this study in 1994. The protocol of genomic DNA extraction was essentially the CTAB procedure (Murray and Thompson 1980) as modified by Dr. J. F. Sampson (unpublished data). Ten grams of needle were macerated in liquid nitrogen, 150 ml of CTAB extraction buffer was added, and the mixture homogenized with a polytron (< 10 s) and filtered through four layers of cheesecloth and one layer of miracloth. The filtered homogenate was centrifuged (5000 rpm, 15 min, 4 °C), and the resulting pellet was resuspended in 5 ml of wash buffer (0.35 M sorbitol, 0.05 M Tris-HCl, 2.2 nM EDTA, pH 8.0), then 1 ml of 10% N-laurylsarcosine was added and mixed by gentle inversion. The samples were incubated for 10 min at room temperature. Further, 1 ml of 5 mM NaCl and 1 ml of 10% CTAB were added, mixed gently, and then incubated at 60 °C for 10 min. Following the incubation, 10 ml of choroform: octanol (24:1) was added, mixed, and then the emulsion was centrifuged (10000 rpm, 15 min, 15 °C). The supernatant was collected gently with a wide-bore pipette, and 10 ml of ice-cold isopropanol was added to the aqueous phase to precipitate the nucleic acids. The nucleic acid pellet was hooked out and put into 5 ml of 3 M ammonium acetate buffer for 20 min. Ammonium acetate buffer was discarded and the nucleic acid pellet was dried and dissolved in 1 ml of TE buffer (Sambrook et al. 1989).

To eliminate RNA, 10 µl of RNase A from bovine pancreas (Boehringer Mannheim) at a concentration of 10 mg/ml (Sambrook et al. 1989) were added, and the samples were incubated for 60 min at 37 °C. The DNA was once again precipitated with ice-cold ethanol 95%, dried and dissolved in an appropriate volume of sterile TE. The concentration of DNA samples was estimated by gel electrophoresis against a DNA standard and by using a UV spectrophotometer at 260 nm. Finally, the DNA concentration was adjusted at the desired

Table 1 Locations of sampled populations of *P. glauca*, *P. engelmannii*, and the sample size for each population

Population surveyed	Latidue (N)	Longitude (W)	Sample size
P. engelmannii from outside the range of *P. glauca*			
1. Clarkia, Idaho	47°06′00″	116°14′00″	10
2. Garden Valley, Lowman, Idaho	44°11′36″	115°15′11″	10
3. Lolo Work Center, Montana	46°46′0″	114°15′11″	25
4. Lolo summit, Montana	46°46′31″	114°25′15″	25
P. glauca from outside the range of *P. engelmannii*			
5. Green Lake, Saskatchewan	54°10′25″	107°25′28″	25
6. Near Kluane Lake, Yukon	61°01′40″	138°24′32″	25
7. Near Stewart Crossing, Yukon	63°23′00″	136°40′00″	25

level for use in PCR amplication by dilution with sterilized double-distilled water.

RAPD protocol

The PCR protocol was modified from Williams et al. (1990). Reaction mixtures (20 µl) contained 50 mM Tris-HCl, pH 8.3, 500 µg/ml BSA, 0.5–1% Ficoll, 1 mM Tartrazine, 2 mM $MgCl_2$, 200 µM dNTP, 0.5 µM primer, approximately 20 ng of genomic DNA, 0.75 unit of Amplitaq DNA polymerase, overlaid with one drop of mineral oil to prevent evaporation. The 10 × PCR buffer Med Ficoll and dye, 10 × dNTP mix, and enzyme diluent were purchased from Idaho Technology (Idaho Falls, Idaho, U.S.A.) whereas Amplitaq DNA polymerase was obtained from Perkin-Elmer/Cetus (Perkin-Elmer/Cetus, Norwalk, Conn., U.S.A.). A DNA thermal cycler 480 (Perkin-Elmer/Cetus) was programmed for 4 min at 94 °C followed by 40 cycles of 1 min at 94 °C (denaturation), 1 min at 37 °C (annealing), and 2 min at 72 °C (extension) to carry out the amplification reactions. An incubation of 8 min at 72 °C was added at the final stage to ensure that the primer extension reactions proceeded to completion. The RAPD products plus 4 µl of 6 × blue-mix loading buffer (Sambrook et al. 1989) were resolved electrophoretically on 1% agarose gels in 1 × TBE (tris-borate-EDTA) buffer into which the ethidium bromide dye was incorporated [3 µl (10 mg/ml)/150 ml)]. To make clear comparisons of interespecific polymorphisms, amplified products of the two species were run side by side on gels for about 5 h at 80 V and photographed using a UV light source. One control (blank) containing all the components of a typical PCR reaction but lacking the template DNA was used to confirm that amplification products represent amplified genomic DNA and not an artifact of the primer (Ellsworth et al. 1993). Sizes of the amplified products were estimated by using a 1-kb DNA ladder (GIBCO BRL, Life Technologies, Gaithersburg, Md.).

The RAPD decamer oligodeoxynucleotide primers screened consisted of 60 (kits A, B and H with 20 primers in each kit) purchased from Operon Technologies (Alameda, Calif., U.S.A.) and of 70 (set #3, numbered from 201 to 300) purchased from the University of British Columbia, Oligonucleotide Synthesis Laboratory (Vancouver, BC, Canada) and selected in this study according to their constancy (Fritsch et al. 1993). Amplification of DNA was repeated at least twice, and only reproducible and unambiguous fragments were scored. Fragments were scored as present (1) or absent (0). A fragment was considered polymorphic if both the presence and absence of that fragment were observed in the same species and monomorphic if it was present among all individuals within a species. We also verified the inheritance of the species-specific fragments in another study involving interspecific crosses (Khasa et al., manuscript in preparation).

Results

Of the 130 RAPD primers screened, 29 resulted in good amplification patterns (Table 2). The primer amplification strength was similar between the two species. Re-

Table 2 Attributes of the selected RAPD primers showing reproducible banding patterns in white (WHI), and Engelmann (ENG) spruces

Primer	Nucleotide sequence (5′ to 3′)	% GC content	Amplification[a]	Number of fragments		Number of polymorphic fragments		Fixed differences between the two species	Size range of fragments
				WHI	ENG	WHI	ENG		
OPA-08	GTGACGTAGG	60	+ +	10	9	9	7	1	424–2036
OPA-09	GGGTAACGCC	70	+ +	13	13	12	12	0	259–1636
OPA-17	GACCGCTTGT	60	+ +	14	14	11	8	0	451–3054
OPB-06	TGCTCTGCCC	70	+ +	7	7	5	5	0	786–1836
OPB-08	GTCCACACGG	70	+ +	11	11	10	10	0	613–1903
OPB-11	GTAGACCCGT	60	+ +	9	9	5	5	0	507–2036
OPH-09	TGTAGCTGGG	60	+ +	6	6	4	4	0	839–3054
OPH-15	AATGGCGCAG	60	+ +	6	6	2	2	0	736–2545
OPH-18	GAATCGGCCA	60	+ +	13	13	10	10	0	507–3054
OPH-19	CTGACCAGCC	70	+ +	6	6	4	4	0	370–1327
OPH-20	GGGAGACATC	60	+ +	6	6	2	2	0	507–2036
UBC-203	CACGGCGAGT	70	+ +	11	11	10	10	0	469–1769
UBC-211	GAAGCGCGAT	60	+ +	11	12	9	9	1	370–2036
UBC-218	CTCAGCCCAG	70	+ +	8	9	3	3	1	341–1636
UBC-220	GTCGATGTCG	60	+ +	5	5	2	2	0	762–2290
UBC-234	TCCACGGACG	70	+ +	14	14	13	13	0	507–3562
UBC-237	CGACCAGAGC	70	+ +	11	11	11	11	0	608–3054
UBC-239	CTGAAGCGGA	60	+ +	8	8	5	5	0	528–2036
UBC-240	ATGTTCCAGG	50	+	10	10	10	10	0	660–2799
UBC-241	GCCCGACGCG	90	+ +	13	13	10	8	0	370–2036
UBC-243	GGGTGAACCG	70	+ +	11	11	9	9	0	396–1283
UBC-247	TACCGACGGGA	60	+ +	8	10	6	6	2	762–4072
UBC-248	GAGTAAGCGG	60	+ +	14	14	12	12	0	470–3054
UBC-273	AATGTCGCCA	50	+	5	5	4	4	0	933–1836
UBC-276	AGGATCAAGC	50	+	7	7	4	4	0	506–1283
UBC-277	AGGAAGGTGC	60	+ +	5	5	2	2	0	1018–1836
UBC-280	CTGGGAGTGG	70	+ +	7	7	4	4	0	839–3054
UBC-283	CGGCCACCGT	80	+ +	5	5	1	1	0	608–916
UBC-299	TGTCAGCGGT	60	+ +	10	10	6	6	0	451–2036
Total				264	267	195	188	5	
Average				9.1	9.2	6.7	6.5	0.2	

[a] +, good amplification (at least one moderately intense fragment was detected for most DNAs); + +, excellent amplification (at least one very intense fragment was detected for all DNAs). RAPD primers for which no, or poor, amplification was obtained are not reported here

producibility of the results was verified by repeating each amplification at least twice. The 29 primers produced a total of 264 and 267 fragments for white spruce and Engelmann spruce, respectively, with an average of nine per primer. The size of the amplified products ranged from 259 to 4 072 bp. The average proportion of polymorphic fragments was 74% for white spruce and 70% for Engelmann spruce. However, this percentage may be underestimated, due to the drawback of dealing with the dominance problem of the RAPD phenotypic information. Among the 29 primers, four were found to produce species-diagnostic fragments showing differences in frequency between the two species (Table 3). No amplification was found in the control samples in which no DNA was added to the reaction mixture, indicating that all fragments observed in the other samples are not artifactual but due to amplification of genomic DNA (Ellsworth et al. 1993).

The frequencies of the most discriminatory fragments between white spruce and Engelmann spruce using the five random primers OPA-08, UBC-211, UBC-218, UBC-241 and UBC-247 are given in Table 3. For instance, the combination of fragments OPA-08_{1520}, UBC-211_{1250}, UBC-218_{350}, UBC-247_{740} and UBC-247_{1080} can be considered as species-specific markers in identifying fixed differences between the two species and their hybrids. Representative amplified fragments are shown in Fig. 1 (A, B, C). According to the dominance-recessive fashion of RAPD inheritance, at least two markers showing fixed differences for alternate species are required to differentiate the two species and their putative hybrids. For instance, the fragment OPA-08_{1520} present in all individuals of white spruce, but absent in Engelmann spruce, will be present in all putative hybrids provided that the parental genotype of white spruce is homozygous. In fact, assuming the genotype of white spruce as *AA* and that of Engelmann spruce as *aa*, then the putative hybrids are *Aa*, showing the fragment OPA-08_{1520}. On the other hand, if the genotype of white spruce is *Aa* and that of Engelmann spruce as *aa*, then the fragment will segregate in the hybrids in a ratio of 1:1. For instance, all surveyed populations of white spruce had the fragment OPA-08_{1520} while populations of Engelmann spruce did not harbor this fragment. On the other hand, the fragment UBC-218_{350} was present in all individuals of Engelmann spruce, while absent in white spruce. Each of the Engelmann spruce individuals from Idaho (e.g., population 1) surveyed possessed UBC-211_{1250}, while it was nearly absent in the populations from Montana (e.g., population 3) and in all white spruce populations. In some cases, however, we observed one individual among several lacking the diagnostic fragment within a species. This deviation was not different from that due to chance alone assuming a binomial distribution at $\alpha = 0.05$.

The pattern of inheritance for the primers that demonstrated species-specific amplification products was examined using the experimental F_1 hybrids recovered from crosses of white spruce and Engelmann spruce. Each of the F_1 individuals demonstrated a combination of the white spruce and Engelmann spruce diagnostic markers (Khasa et al., manuscript in preparation). The distribution of the RAPD markers for white spruce and Engelmann spruce in the F_1 hybrids was consistent with a biparental mode of inheritance and showed segregation of the RAPD markers according to the dominance-recessive fashion (Khasa et al., manuscript in preparation).

Discussion

RAPDs have been shown to be very useful for the construction of genetic maps (Williams et al. 1990; Carlson et al. 1991; Tulsieram et al. 1992; Nelson et al. 1993; Chaparro et al. 1994), DNA fingerprinting (Welsh and McClelland 1990; Williams et al. 1990; Welsh et al. 1991), the detection of genetic polymorphism and varietal and clone identification (Welsh et al. 1991; Castiglione et al. 1993; Isabel et al. 1993; Lin et al. 1994), the identification of genetic markers linked to specific phenotypic traits of economic relevance (Kesseli et al. 1992; Grattapaglia et al. 1992), parentage determination (Welsh et al. 1991; Roy et al. 1992), population dynamics (Arnold et al. 1991; Fritsch and Rieseberg 1992), to help

Table 3 Frequencies of the most discriminatory fragments between *P. gluca* and *P. engelmannii* using the random primers OPA-08, UBC-211, UBC-218, UBC-241, and UBC-247, for each of the populations surveyed

Species/population surveyed	OPA-08	UBC-211	UBC-218	UBC-241		UBC-247		
	1 520 bp	1 250 bp	350 bp	450 bp	610 bp	740 bp	1 080 bp	1300 bp
P. engelmannii, pop. 1.	0.00	1.00	1.00	0.00	0.00	1.00	1.00	0.10
P. engelmannii, pop. 2.	0.00	1.00	1.00	0.00	0.10	1.00	1.00	0.10
P. engelmannii, pop. 3.	0.00	0.16	1.00	0.08	0.08	1.00	1.00	0.08
P. engelmannii, pop. 4.	0.00	0.40	1.00	0.12	0.00	1.00	1.00	0.24
P. glauca, pop. 5.	1.00	0.00	0.00	0.52	0.52	0.00	0.00	1.00
P. glauca, pop. 6.	1.00	0.00	0.00	0.64	0.64	0.00	0.00	1.00
P. glauca, pop. 7.	1.00	0.00	0.00	0.56	0.68	0.00	0.00	0.92

See locations of populations in Table 1

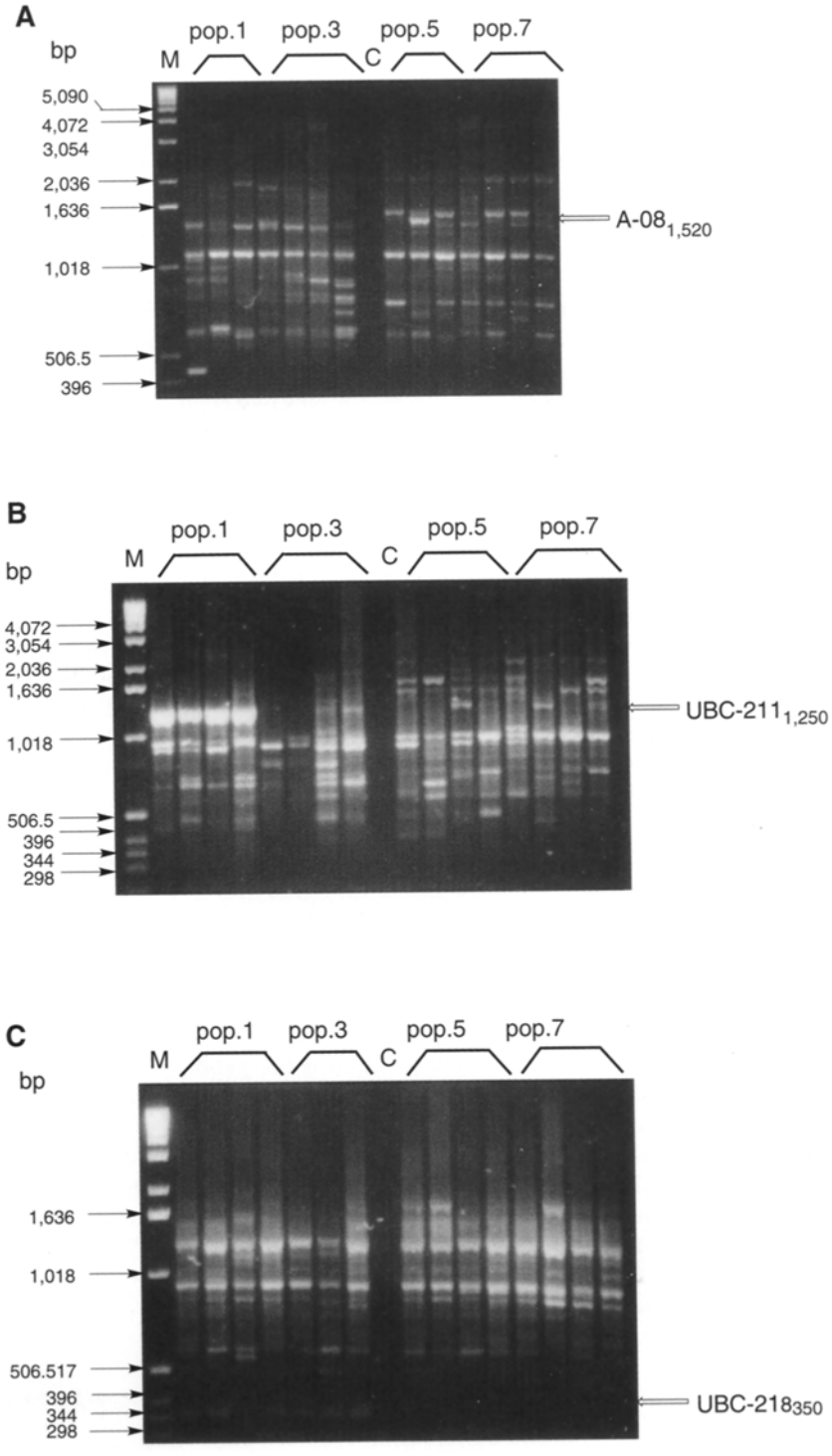

Fig. 1 **A** RAPD profiles of DNA samples of *P. engelmannii* (*populations 1 and 3*) and *P. glauca* (*populations 5 and 7*) using primer OPA-08. *M* a 1-kb marker ladder used to estimate sizes of the amplified fragments; the blank lane (*C*) in the middle is a control in which no DNA was added to the reaction mixture. See Table 1 for description of populations. *Empty arrows* indicate diagnostic fragments. **B** RAPD profiles of DNA samples of *P. engelmannii* (*populations 1 and 3*) and *P. glauca* (*populations 5 and 7*) using primer UBC-211. **C** RAPD profiles of DNA samples of *P. engelmannii* (*populations 1 and 3*) and *P. glauca* (*populations 5 and 7*) using primer UBC-218

document the occurrence of natural introgression (Arnold et al. 1991; Perron et al. 1995), and to estimate outcrossing rates in natural populations (Fritsch and Rieseberg 1992).

Recently, the RAPD technique has been applied to document the origin of the intergeneric hybrid × *Margyraceana skottsbergii* (Rosaceae) on the Juan Fernandez islands (Crawford et al. 1993). In this study, 18 species-specific fragments from *Acaena argentea* and 27 from *Margyricarpus digynus* were found in × *Margyraceana* using 13 primers. In our study, however, we did not find as many markers. This is not surprising given that white and Engelmann spruces are so closely related that they can be considered as subspecies of a single species (La Roi and Dugle 1968; Ogilvie and Rudloff 1968). Our results in the northwestern North

American species complex corroborated those of the northeastern species (red spruce-black spruce) complex, where only a small number of species-specific RAPD genetic fingerprints have been found (Perron et al. 1995). Using a minimum combination of two diagnostic markers, one for each species, it is possible to discriminate qualitatively between white-Engelmann spruces and their hybrids. Amplification of the internal transcribed spacers of the nuclear rDNA repeat unit can also be used to differentiate the two species (data not shown). Isozyme markers, however, proved inconclusive for addressing this question (Dr. Rajora, personal communication). The development and identification of species-diagnostic RAPD markers for the closely related white spruce and Engelmann spruce requires the use of the right individuals typical of each of the two species (say, individuals from far allopatric populations as sampled in this study) otherwise no discriminating fragments can be found.

One concern about the RAPD technique is its reproducibility and reliability. Therefore, for each new taxon, it is essential to conduct an extensive pilot survey with different short oligodeoxynucleotide primers under standardized optimal reaction conditions and internal controls (Williams et al. 1990) in order to select those that are consistent and can be subsequently used in population surveys. In our study, 29 primers showed good amplification with a total (G + C) content generally between 60 and 70%. With a (G + C) content of less than 50%, the primer amplification strength decreased drastically. Indeed, of the six characteristics of primer base sequences analysed in a study involving flowering-plant genera, the total (G + C) content was found most valuable in predicting primer amplification strength (Fritsch et al. 1993). Since there was a strong agreement between the two species as to the similarity of primer amplification strength, this suggests that the amplification-product yield of a particular primer is independent of the template-DNA source. Even if we did not test different amplification factors that can lead to artifactual variation, including magnesium concentration, primer and template-DNA concentration ratio, and annealing temperature (Ellsworth et al. 1993), we used the standardized optimal reaction conditions and internal controls as listed above (Williams et al. 1990; Tingey et al. 1992) to ensure reproducibility and consistency between separate amplification reactions.

Because RAPDs are usually inherited in a dominant-recessive mode (Williams et al. 1990; Carlson et al. 1991; Roy et al. 1992), the sole presence of a fragment could indicate heterozygous (*Aa*) or homozygous (*AA*) genotypes. Parental heterozygotes (*Aa*) which display a fragment may contribute the recessive allele (*a*) for the absence of a fragment to a given progeny. This was confirmed by the segregation of the species-diagnostic RAPD markers in F_1 individuals from controlled interspecific crosses between white spruce and Engelmann spruce (Khasa et al. 1995). Cloning of these species-specific RAPD markers is now underway in order to use them as probes in a Southern-blot hybridization analysis to determine the allelic nature of all variants.

With a minimum of two loci showing patterns of fixed differences between taxa and monomorphic within taxa, it is possible to define species boundaries (including hybridization) of the white spruce-Engelmann spruce complex (Baverstock and Moritz 1990). Recently, a combination of nuclear and organelle markers have been used to estimate the degree of introgression of white spruce-Engelmann spruce complex by employing a fractional index (Sutton et al. 1994). Using the RAPD technique, many loci can be surveyed in a short time, while requiring little plant material. Therefore, this technique appears to offer a quick and easy method for the identification of white-Engelmann spruces and their hybrids with clear advantages over many of the chemotaxonomic methods, including biochemical and RFLP markers, previously used. The hybrid nature can be estimated simply by the frequency of co-segregation of diagnostic markers each specific for a different species.

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