Theor Appl Genet (1995) 91:142–149

M. Perron · A. G. Gordon · J. Bousquet

Species-specific RAPD fingerprints for the closely related *Picea mariana* and *P. rubens*

Received: 15 November 1994 / Accepted: 15 December 1994

Abstract Species-specific molecular markers were designed to assist in the identification of closely related black spruce (*Picea mariana* [B.S.P.] Mill.) and red spruce (*P. rubens* Sarg.) in northeastern North America. Trees from six provenances of black spruce and three provenances of red spruce were sampled from outside the sympatric zone. They were first classified using a composite index of five qualitative morphological traits. The species-specific genetic markers were developed using random amplified polymorphic DNAs (RAPD) and a combination of bulk sample and individual tree analyses. Each species bulk sample was constructed from DNAs obtained from 12 trees that were from outside the sympatric zone and showed a morphological composite index specific of each species. A total of 161 primers were screened with the bulk samples. From these, 52 primers showing segregating fingerprints were further screened with the individual trees. Most of the markers observed were shared by the two species, and there was less diversity in *P. rubens*. A small number of markers were found to be monomorphic or nearly monomorphic and specific to either *P. mariana* or *P. rubens*. These markers remained species-specific when F_1 progenies derived from independent intraspecific crosses were screened, and they were subsequently found to co-segregate in hybrids derived from independent interspecific crosses here used as controls.

Key words DNA fingerprint · Molecular markers · *Picea mariana* · *Picea rubens* · RAPD

Communicated by P. M. A. Tigerstedt

M. Perron · J. Bousquet (✉)
Centre de recherche en biologie forestière, Faculté de foresterie et de géomatique, Université Laval, Sainte-Foy, Québec, Canada G1K 7P4

A. G. Gordon
Ontario Forest Research Institute, Ministry of Natural Resources, Sault Ste. Marie, Ontario, Canada P6A 5N5

Introduction

Black spruce (*Picea mariana* [B.S.P.] Mill.) and red spruce (*P. rubens* Sarg.) are closely related species (Manley 1972; Gordon 1976; Eckert 1989; Bobola et al. 1992; Sigurgeirsson and Szmidt 1993). They share an extensive sympatric zone in northeastern North America, where they have been reported to hybridize naturally (Manley 1972). However, the extent of this phenomenon appears to be limited (Gordon 1976; Manley and Ledig 1979). While morphological characters can be used to discriminate between these species (Manley 1971; Gordon 1976), interpretation of the quantitative variation in morphology must be conducted with caution.

In a study of the black spruce – red spruce complex with isozymes Eckert (1989) was unable to determine species-specific alleles out of 13 polymorphic loci surveyed; however, species could be distinguished on the basis of multivariate analysis of allele frequencies, as in isozyme studies in the Sitka spruce – white spruce complex (*P. sitchensis* [Bong.] Carr. – *P. glauca* [Moench] Voss) (Yeh and Arnott 1986). Restriction fragment length polymorphisms (RFLPs) have enabled the identification of species-specific markers in the interior spruce complex (white and engelmann spruce [*P. engelmanii* Parry]) and Sitka spruce complex (Szmidt et al. 1988; Sutton et al. 1991, 1994) in western North America. But the study of the black spruce-red spruce complex with polymorphisms detected at five restriction sites in the IGS and ITS regions of the nuclear rDNA repeat unit did not allow for species-specific markers, although species could be distinguished on the basis of multivariate analysis of restriction site frequencies (Bobola et al. 1992).

One limitation in previous isozyme or restriction site studies in the black spruce-red spruce species complex has been the limited number of loci surveyed and the small portion of the genomes sampled. Indeed, if black spruce and red spruce are truly differentiated species but

are still closely related, it is likely that a large number of loci would need to be screened before species-specific alleles could be identified. Therefore, a working hypothesis could be that the two species, even if closely related, could still be unambiguously distinguished at the genetic level if their genomes are sufficiently sampled and if natural hybridization is localized and not frequent. If any, these species-specific markers could then be used to monitor the extent of natural hybridization between the two species.

The development and identification of species-specific markers for the closely related black spruce and red spruce species require a correct a priori identification of individuals typical of each of the two species. This is crucial, and if it is not done properly, no discriminating alleles will be found because of the bias in the sample of trees. Consequently, in the study described here, trees were chosen so as to be from outside the sympatric zone where the occurrence of natural hybridization is less likely. Second, the selected trees were required to show a morphological composite index specific of each species (Manley 1971; Gordon 1976) to increase the probability that no hybrid or introgressed trees were included in the survey. Third, an independent set of trees used in a control crossing scheme involving intraspecific and interspecific crosses were sought to verify the specificity of the markers and their inheritance in artificial hybrids. Fourth, due to the lack of species-specific alleles at isozyme or rDNA restriction site loci, a large portion of the genome was sampled in order to identify such alleles with random amplified polymorphic DNA markers (RAPD) (Welsh and McClelland 1990; Williams et al. 1990). These markers have been shown to be dominant with Mendelian inheritance in black spruce (Isabel et al. 1993, 1995). They have also been used previously for the study of natural hybridization in plants (Arnold 1991; Orozco-Castillo et al. 1994; Fritz et al. 1994).

Materials and methods

Plant material

To avoid environmentally induced variation in morphology, on which our a priori classification of the trees used for the identification of species-specific molecular markers was based, trees (here called pro- venance trees) were selected from genecological tests at the same test site (Valcartier at 40 km north of Québec City, Canada, Lat. = 46.9° N, Long. = 71.5° W). The sample was composed of at least 5 trees from each of six provenances of black spruce and three provenances of red spruce. The nine provenances were selected to be from outside the sympatric zone between the two species. All trees had a dominant or co-dominant crown position in the plantations. For each tree, two twigs were sampled from the same radial area in the top portion of the crown exposed to the sun to further avoid environmentally induced variation in morphology.

For the validation of species-specific molecular markers, twigs were also collected from an independent sample of 30 trees (here called F_1 progeny trees) derived from different controlled crosses conducted in 1971 and 1975 by one of us (Gordon 1975, 1978). Parent trees involved in the controlled crosses were from natural stands or arboretum in Ontario and Nova Scotia and not readily accessible for analysis, but they were typical of either species based on morphology (Gordon 1976). All available F_1 progeny trees were sampled: 10 progeny trees from two crosses between *P. mariana*♀ × *P. mariana*♂, 9 progeny trees from two crosses between *P. rubens*♀ × *P. rubens*♂, 9 progeny trees from two crosses between *P. mariana*♀ × *P. rubens*♂, and 2 progeny trees from two crosses between *P. rubens*♀ × *P. mariana*♂.

Morphological classification

The provenance and progeny trees were classified using a composite index of five qualitative morphological traits similar to those used previously by Manley (1971) and Gordon (1976). The qualitative morphological traits used were analyzed on the 2-years portion of the twig and were needle color, needle configuration, twig ridges, twig color, and the color of the lignified terminal vegetative bud. Each morphological trait was scored independently three times, with the trees being scored in a random order. The scores from each repetition were compared for the same trees, and those trees with heterogenous scores were checked randomly during a fourth repetition where a final decision was taken. A composite index scaled from 0 for pure black spruce to 1 for pure red spruce was constructed. We gave each character an equal weight except bud color and twig color, which were each given half of the weight assigned to the other characters because of an obvious character correlation between them. From the index scores, the 12 provenance trees with the most typical morphology for each species were retained for the identification phase of species-specific molecular markers. The black spruce individuals retained were from six provenances with 2 trees from each, and the red spruce were from three provenances with 4 trees from each.

Total DNA isolation

DNA was extracted from each of the 24 provenance trees selected on the basis of morphology and from the 30 progeny trees derived from controlled crosses according to the method of Bousquet et al. (1990) with some minor modifications. If necessary, the DNA concentration of each sample was adjusted by dilution so as to be similar among samples.

Construction of bulk samples

Two bulk samples, one for each species, were constructed with DNA samples obtained from the 12 provenance trees selected a priori on the basis of morphology for each species. The bulk samples were used to facilitate the survey of primers that would show segregating RAPD markers between species (see below). An equal aliquot of each DNA sample was used to construct the bulk sample for each species.

DNA amplification (RAPD)

The DNA amplifications were performed following the method of Isabel et al. (1993) with some modifications. Reaction mixtures (13 µl) contained 1 × Boehringer Mannheim reaction buffer, 200 µ*M* of each dNTP (Pharmacia), 50 pmol of primer, and 0.5 unit *Taq* DNA polymerase (Boehringer Mannheim). For the DNA amplification, a Perkin Elmer Gene Amp PCR System 9600 was programmed for 45 cycles, each consisting of a denaturation step of 15 s at 94 °C, followed by an annealing step of 15 s at 35 °C, and an extension step of 1 min 30 s at 72 °C. The last 25 extension steps were progressively extended by 5 s per cycle, and the last cycle was followed by 10 min at 72 °C. After amplification was completed, 5 µl of the sample was loaded and electrophoresed on 0.5% synergel (Diversified Biotech) - 1% agarose gel and visualized by ethidium bromide staining. The molecular weight marker used was ϕX 174-RF DNA digested with *Hae*III (Pharmacia). Ten-mer primers were from Operon Technology and 11-mer primers were those previously described by Roy et al. (1992) and Isabel et al. (1993).

Identification of species-specific genetic markers

Following the screening of 161 primers on the species bulk samples, we retained 52 primers that showed apparent segregating RAPD fragments between the two species bulk samples for further analyses on individual provenance trees that composed each bulk sample. DNA amplification and visualization of fragments were as above, except that they were repeated twice for every sample. Only major and reproducible fragments were retained for analysis. Primers for which RAPD segregating fragments were observed between the two groups of 12 provenance trees per species were further checked in duplicate with DNA samples from the 30 progeny trees derived from independent controlled crosses, following procedures described above.

Results

Morphological analysis

The average morphological indices obtained for the black spruce trees sampled (from six provenances and from intraspecific controlled crosses) were close to the theoretical value of 0 expected for pure black spruce. For the 31 black spruce provenance trees from the genecological test, the average was 0.06 ± 0.07 (Table 1). The average of the 12 black spruce provenance trees selected to construct the species bulk sample was 0.03 ± 0.04 (data not shown). For the 10 black spruce progeny trees derived from intraspecific crosses, the average was 0.03 ± 0.05 (Table 1). The average morphological indices obtained for the red spruce trees sampled (from three provenances and from intraspecific controlled crosses) were comparatively more distant from the expected value for pure red spruce (1). For the 23 red spruce provenance trees from genecological test, the average was 0.67 ± 0.21 (Table 1). The average of the 12 red spruce provenance trees selected to construct the species bulk sample was 0.74 ± 0.08 (data not shown). For the 9 red spruce progeny trees derived from intraspecific crosses the average was 0.84 ± 0.16 with only 1 tree (value of 0.52) departing from typical red spruce values (Table 1). The average morphological index of artificial hybrids obtained from the controlled interspecific crosses was 0.37 ± 0.16 (Table 1), close to the expected mid-point value (0.40) between the morphological indices of black or red spruce progeny trees derived from intraspecific crosses. However, some of the trees were morphologically close to either red spruce or black spruce.

Identification of species-specific molecular markers in provenance trees

The analysis of species bulk samples conducted with 161 primers generated 665 scorable fragments, for an average of 4 fragments per primer tested. The analysis of bulk samples showed that most RAPD fragments were shared by black and red spruces, with about 14.0% of fragments being specific to black spruce and 13.6% to red spruce. This number of species-specific fragments was much further reduced by the individual tree analysis conducted with the 52 primers showing putative segregating fingerprints between the two species bulk samples. These 52 primers generated a total of 158 scorable fragments monomorphic in at least one species, of which 109 were monomorphic for both species and putatively homologous between the two species. Of the remaining 49 fragments, 34 were monomorphic in red spruce while polymorphic in black spruce, and 8 were monomorphic in black spruce while polymorphic in red spruce. The remaining 7 fragments had a high diagnostic

Table 1 Results from morphological analysis of provenance and progeny trees

	Number of trees	Needle color	Needle configuration	Twig ridges	Twig color	Bud color	Index[a]
Theoretical values							
Black-red spruce		0–2	0–3	0–2	0–2	0–2	0–1
Observed mean values							
Black spruce provenances	31	0.10	0.24	0.03	0	0.15	0.06
		(0.31)[e]	(0.37)	(0.18)		(0.23)	(0.07)
Red spruce provenances	23	1.10	1.32	2.00	0.82	0.68	0.67
		(0.31)	(0.48)	(0)	(0.25)	(0.25)	(0.21)
Black spruce progeny F_1^b	10	0	0.13	0	0	0.10	0.03
			(0.28)			(0.21)	(0.05)
Red spruce progeny F_1^c	9	1.44	1.69	1.89	0.89	0.83	0.84
		(0.53)	(0.58)	(0.33)	(0.22)	(0.35)	(0.16)
Hybrid progeny F_1^d	11	0.55	0.72	0.82	0.36	0.50	0.37
		(0.52)	(0.46)	(0.75)	(0.23)	(0)	(0.16)

[a] Composite index, obtained by the addition of five qualitative character scores with an equal weight except twig color and bud color, which were given half of the weight each. The needle configuration scores were corrected by a factor of 0.67 to have the same weight as the other characters. The index was scaled to be between 0 and 1

[b] Obtained from *P. mariana* × *P. mariana* crosses

[c] Obtained from *P. rubens* × *P. rubens* crosses

[d] Obtained from *P. mariana* × *P. rubens* and *P. rubens* × *P. mariana* crosses

[e] In parentheses: standard deviations

value since they were monomorphic or nearly monomorphic in one species (3 fragments in *P. mariana* and 4 fragments in *P. rubens*) while absent in the other with no exception. For red spruce, the four species-specific markers were present in all of the selected provenance trees (Tables 2 and 3; Figs. 1–4.) For black spruce, the three species-specific markers were present in most of the provenance trees selected with some exceptions (Tables 2 and 3; Figs. 1–4).

The Opd14-590 species-specific marker for *P. mariana* was absent in 3 of the 4 individuals from Western Canada but present in all other individuals. Therefore, it has a good diagnostic value because it is monomorphic in black spruce trees sampled in longitudes near the sympatric zone. To obtain more precise information on the frequency of this dominant allele in Western Canada, we extracted DNA and obtained fingerprints a posteriori for 30 zygotic embryos derived from seeds representing three black spruce provenances, one from British Columbia (Lat. = 59.1° N, Long. = 123.2° W) and two from Saskatchewan (Lat. = 52.3° N, Long. = 101.43° W and Lat. = 58° N, Long. = 103.5° W). Embryos instead of haploid megagametophytes were used in order to sample two haploid genomes per DNA sample for detecting the presence of this apparently rare dominant marker in Western Canada. We found that the dominant marker was not rare. It was present in 63% of the embryos (that is, the frequency s_k of genotypes AA + Aa), indicating that the frequency of the dominant allele A (p_k) would be 0.39 if one is willing to assume a Hardy-Weinberg equilibrium (that is, $p_k = 1 - (1 - s_k)^{1/2}$.

The Opa19-1250 species-specific marker for *P. mariana* was not present in 1 provenance individual (Table 2), suggesting that when the dominant marker is present, trees might be heterozygote for this marker. To obtain information on the frequency of this nearly monomorphic marker in black spruce, we have tested a posteriori the presence of this marker in DNA from 96 haploid megagametophytes (see Isabel et al. 1993 for methods) derived from seeds representing two black spruce provenances in Québec (Lat. = 50.2° N, Long. = 74.2° W and Lat. = 50.4° N, Long. = 68.4° W). Haploid megagametophytes were used here in order to avoid dominance problems in detecting the presence of the rare recessive allele (fragment absence). We found that the marker was present in 85% of the megagametophytes (p_k value), confirming that in diploid individuals, it is likely to be found either as homozygote (AA) or heterozygote (Aa), at frequency $s_k = p^2 + 2pq = 0.98$, if one is willing to assume a Hardy-Weinberg equilibrium. Therefore, the marker would be occasionally absent at a frequency of 2% for recessive homozygotes (aa).

The Ope06-900 species-specific marker for *P. mariana* was not present in 1 provenance tree (Table 2). To obtain a posteriori information on the frequency of

Table 2 Fingerprints of provenance and progeny trees morphologically typical of *P. mariana*

P. mariana	Latitude (°N)	Longitude (°W)	Morphological index[a]	RAPD fragments						
				Opa19 1250 bp	Opd14 590 bp	Ope06 900 bp	Opa06 900 bp	Ope09 603 bp	Opf17 800 bp	Opg12 1000 bp
Provenance trees										
77	48.3° N	81.2° W	0	1	1	1	0	0	0	0
80	48.3° N	81.2° W	0	1	1	1	0	0	0	0
83	49.0° N	90.3° W	0.06	1	1	1	0	0	0	0
86	49.0° N	90.3° W	0	1	1	1	0	0	0	0
S58	57.0° N	97.5° W	0.13	1	1	1	0	0	0	0
S60	57.0° N	97.5° W	0.13	1	0[c]	1	0	0	0	0
S70	56.4° N	121.3° W	0	1	0[c]	0	0	0	0	0
S71	56.4° N	121.3° W	0	1	0[c]	1	0	0	0	0
S75	50.4° N	68.5° W	0.06	1	1	1	0	0	0	0
S76	50.4° N	68.5° W	0.06	1	1	1	0	0	0	0
S78	50.3° N	73.4° W	0.06	0	1	1	0	0	0	0
S80	50.3° N	73.4° W	0	1	1	1	0	0	0	0
Progeny trees[b]										
314–71–1	–	–	0.06	1	1	1	0	0	0	0
314–71–2	–	–	0.15	1	1	1	0	0	0	0
314–71–3	–	–	0	1	1	1	0	0	0	0
314–71–4	–	–	0	0	1	0	0	0	0	0
314–71–5	–	–	0	1	1	1	0	0	0	0
315–71–1	–	–	0	1	1	1	0	0	0	0
315–71–2	–	–	0	1	1	1	0	0	0	0
315–71–3	–	–	0	1	1	0	0	0	0	0
315–71–4	–	–	0	1	1	0	0	0	0	0
315–71–5	–	–	0.08	1	1	1	0	0	0	0

[a] See Table 1 for definition

[b] F_1 (23 years old) from two *P. mariana* × *P. mariana* crosses (314 and 315)

[c] Trees from provenances in Manitoba and northern British Columbia

Table 3 Fingerprints of provenance and progeny trees morphologically typical of *P. rubens*

P. rubens	Latitude (°N)	Longitude (°W)	Morphological index[a]	RAPD fragments						
				Opa19 1250 bp	Opd14 590 bp	Ope06 900 bp	Opa06 900 bp	Ope09 603 bp	Opf17 800 bp	Opg12 1000 bp
Provenance trees										
S20	38.6° N	79.8° W	0.70	0	0	0	1	1	1	1
S22	38.6° N	79.8° W	0.73	0	0	0	1	1	1	1
S23	38.6° N	79.8° W	0.73	0	0	0	1	1	1	1
S24	38.6° N	79.8° W	0.67	0	0	0	1	1	1	1
S25	35.6° N	83.5° W	0.81	0	0	0	1	1	1	1
S26	35.6° N	83.5° W	0.65	0	0	0	1	1	1	1
S27	35.6° N	83.5° W	0.67	0	0	0	1	1	1	1
S29	35.6° N	83.5° W	0.73	0	0	0	1	1	1	1
S44	42.4° N	73.2° W	0.83	0	0	0	1	1	1	1
S46	42.4° N	73.2° W	0.88	0	0	0	1	1	1	1
S48	42.4° N	73.2° W	0.65	0	0	0	1	1	1	1
S49	42.4° N	73.2° W	0.81	0	0	0	1	1	1	1
Progeny trees[b]										
323-71-1	–	–	0.52	0	0	0	1	1	1	1
323-71-2	–	–	0.81	0	0	0	1	1	1	1
323-71-3	–	–	0.75	0	0	0	1	1	1	1
323-71-4	–	–	0.94	0	0	0	1	1	1	1
323-71-5	–	–	1	0	0	0	1	1	1	1
324-71-1	–	–	0.71	0	0	0	1	1	1	1
324-71-3	–	–	0.88	0	0	0	1	1	1	1
324-71-4	–	–	1	0	0	0	1	1	1	1
324-71-5	–	–	1	0	0	0	1	1	1	1

[a] See Table 1 for definition
[b] F_1 (23 years old) from two *P. rubens* × *P. rubens* crosses (323 and 324)

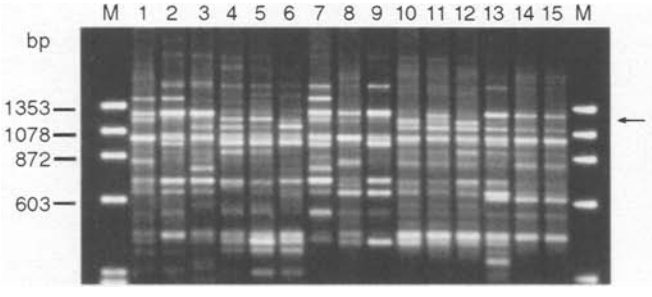

Fig. 1 Gel electrophoresis of RAPD fragments obtained with primer Opa19. This primer produces a 1250-bp fragment nearly monomorphic and specific to *Picea mariana* (indicated by *arrow*). *Lanes M* are the size marker ϕX 174-RF DNA digested with *Hae*-III, *lanes 1–3 P. mariana* provenance trees, *lanes 4–6 P. rubens* provenance trees, *lanes 7–9 P. mariana* F_1 progeny trees from controlled crosses, *lanes 10–12 P. rubens* progeny trees from artificial crosses, lanes *13–15* are hybrids F_1 trees from interspecific crosses.

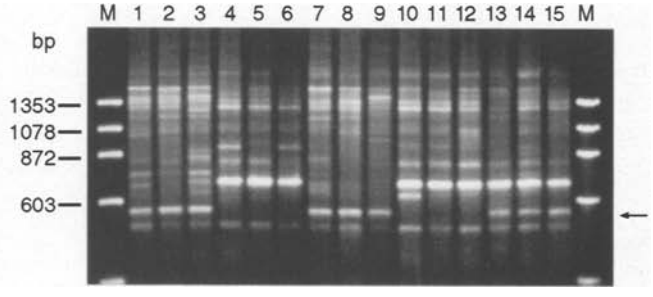

Fig. 2 Gel electrophoresis of RAPD fragments obtained with primer Opd14. This primer produces a 590-bp fragment nearly monomorphic and specific to *Picea mariana* (indicated by *arrow*). *Lanes M* and *1–15* are the same as those described in Fig. 1

this marker in black spruce, we tested the same megagametophytes used for Opa19-1250 (see above). We found that the marker was present in 67% of the megagametophytes (p_k value), confirming that in diploid individuals it is likely to be found either as homozygote (AA) or heterozygote (Aa) at frequency $s_k = p^2 + 2pq = 0.89$, if one is willing to assume a Hardy-Weinberg equilibrium. Therefore, the marker would be absent at a frequency of 11% for recessive homozygotes (aa).

Validation of species-specific molecular markers in progeny trees

The seven species-specific markers were validated with F_1 progeny trees derived from independent intraspecific and interspecific controlled crosses. In progeny trees derived from intraspecific crosses (19 trees), there was generally a good agreement between expected and observed fingerprints (Tables 2 and 3), in spite of the fact that there was no a priori selection of the parents involved in the crosses based on molecular fingerprints. The Opa19-1250 species-specific marker for *P. mariana*,

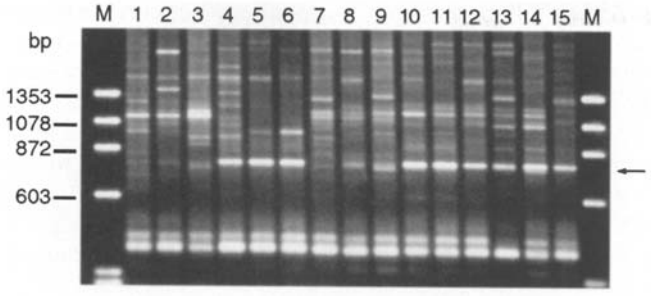

Fig. 3 Gel electrophoresis of RAPD fragments obtained with primer Opf17. This primer produces a 800-bp fragment monomorphic and specific to *Picea rubens* (indicated by *arrow*). *Lanes M* and *1–15* are the same as those described in Fig. 1

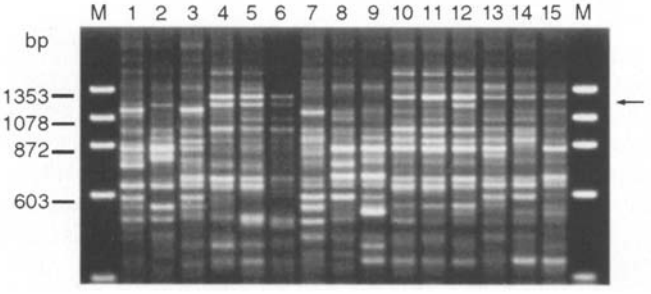

Fig. 4 Gel electrophoresis of RAPD fragments obtained with primer Opg12. This primer produces a 1000-bp fragment monomorphic and specific to *Picea rubens* (indicated by *arrow*). *Lanes M* and *1–15* are the same as those described in Fig. 1

shown to be absent from 1 of the 12 provenance trees used to identify black spruce specific markers, was also absent in 1 of the 10 black spruce progeny trees (Table 2). The Ope06-900 species-specific marker for *P. mariana*, shown to be absent from 1 of the 12 provenance trees used to identify black spruce specific markers, was absent in 3 of the 10 black spruce progeny trees (Table 2). In all crosses, markers found to be specific for one species were absent in the F_1 progeny trees of the other species with no exception.

In all artificial hybrids derived from interspecific crosses (11 trees), species-specific markers were found to co-segregate, as expected, in spite of the fact that there was no a priori selection of the progeny or parents involved in the crosses as determined by molecular fingerprints (Table 4, Figs. 1–4). In some progeny trees, all of the markers co-segregated; for all of the progeny trees, at least 2 markers, each specific to a different species, co-segregated. This was never observed in F_1 progeny trees derived from intraspecific crosses (Tables 2 and 3).

Discussion

In this survey for species-specific markers in the black spruce - red spruce complex, it was important to select a priori, for each species, trees from outside the sympatric zone where hybridization between these species is less likely to occur in order to minimize the possibility of including true hybrids or introgressants, which would have biased the analysis. The selection of individuals typical of each species from outside the sympatric zone was based on a morphological index obtained from five qualitative characters. The morphological indices obtained for the black spruce trees were significantly different than those obtained for the red spruce trees; the former were close to the value expected for pure black spruce, while the values for the red spruce trees were more distant from the expected value for pure red spruce. Furthermore, the mean of the red spruce values had a larger variance than the mean of black spruce values, indicating that the characters included in the morphological index showed more variation within red spruce than within black spruce. This is in contrast with

Table 4 Fingerprints obtained for hybrids from interspecific crosses

Hybrids	Morphological index[a]	RAPD fragments						
		Opa19 1250 bp	Opd14 590 bp	Ope06 900 bp	Opa06 900 bp	Ope09 603 bp	Opf17 800 bp	Opg12 1000 bp
P. mariana♀ × *P. rubens*♂								
318-71-1[b]	0.42	1	1	0	1	1	1	1
318-71-2[b]	0.58	1	1	0	1	1	1	1
318-71-3[b]	0.46	1	1	1	1	1	1	1
318-71-4[b]	0.42	1	1	1	1	1	1	1
318-71-5[b]	0.46	1	1	1	1	1	1	1
349-75-1[c]	0.27	1	1	0	0	1	1	0
349-75-2[c]	0.33	1	1	1	1	0	1	1
349-75-3[c]	0.06	1	0	1	1	1	1	1
349-75-4[c]	0.13	0	1	0	1	1	1	0
P. rubens♀ × *P. mariana*♂								
321-71-2[d]	0.40	0	1	0	1	1	1	1
322-71-4[d]	0.48	1	1	0	1	1	1	0

[a] See Table 1 for definition
[b] 23 years-old F_1 derived from one cross (318)
[c] 19 years-old F_1 derived from one cross (349)
[d] 23 years-old F_1 derived from two crosses (321 and 322)

the intraspecific diversity observed in RAPDs from individual tree analysis: more fragments were found to be monomorphic or nearly monomorphic for *P. rubens* (34) than for *P. mariana* (8), suggesting that *P. rubens* might be genetically depauperated. These results are in agreement with those obtained with isozyme markers (Eckert 1989; Hawley and Dettayes 1994). However, the RAPD fingerprints obtained with the individual tree samples showed that most of the markers (109) were monomorphic and shared by the two species. This result confirms that black and red spruce are genetically closely related (Manley 1972; Gordon 1976; Eckert 1989; Bobola et al. 1992; Sigurgeirsson and Szmidt 1993).

The analysis based on individual trees has enabled us to reduce the number of species-specific fragments previously determined with the bulk analysis. As previously reported (Michelmore et al. 1991), bulk analysis is subject to potential competition effects for amplification among alleles of low proportion in the mixture and among templates that have variable levels of mismatch with the primer during the initial stages of polymerase chain reaction (PCR) amplification. However, the preliminary screening procedure of 161 primers by bulk analysis was useful for reducing the number of candidate primers to be screened on individual trees.

The 4 species-specific markers found for *P. rubens* were monomorphic and present in all provenance and progeny trees tested. They were never observed in any *P. mariana* individuals. The 3 species-specific markers found for *P. mariana* were nearly monomorphic in all of the provenance and progeny trees tested. They were never observed in any *P. rubens* individuals. The Opd14-590 species-specific marker for *P. mariana* was not observed in 3 of the 4 provenance trees from Western Canada. An a posteriori survey of 30 zygotic embryos from Western Canada, however, showed this marker to be quite frequent. It remains valuable for surveying natural hybridization in the black spruce–red spruce complex because it was observed to be always present in the provenance and progeny trees of eastern black spruce and was never observed in any red spruce tree. The Opa19-1250 species-specific marker for *P. mariana* was nearly monomorphic in provenance trees, and the analysis of haploid megagametophytes revealed a low expected frequency of fragment absence in the diploid population. The presence of this marker was never observed in any red spruce tree, making it a valuable discriminating factor between the two species. The analysis of megagametophytes indicated that the Ope06-900 species-specific marker for *P. mariana* can be expected to be absent from approximately 10% of the individuals. Indeed, it was absent from only 1 provenance tree (out of 12) and from 3 progeny trees (out of 10), but it was still never observed in any red spruce tree. Among the markers retained, it is therefore the less reliable.

The F_1 progeny trees used to validate the species-specific markers were morphologically typical of either species, and the F_1 hybrids were morphologically intermediate. There was good agreement between expected and observed fingerprints. This is even more significant when one considers that the F_1 progeny trees available and used for the validation of markers were not selected a priori based on morphology. Furthermore, the morphological classification of parent trees used in the controlled crosses was conducted more than 20 years ago by one of us (A.G. Gordon) with a slightly different set of characters, while the morphological characterization of provenance trees used to screen fingerprints and the morphological characterization of F_1 progeny trees was done by a different person (M. Perron). Therefore, these two samples of trees (provenance-progeny) have been derived independently.

Because RAPDs are mostly dominant markers (Williams et al. 1990), the absence of some fragments typical to one or the other species in some of the F_1 progeny trees is likely to be due to some parent trees being heterozygote (Aa) for the marker, as shown indirectly for the marker Opa19-1250. Megagametophytes from the parents used in controlled crosses and the parents themselves were not readily accessible for genotyping. However, it can be seen from the crosses involving *P. mariana* as male parent and *P. rubens* as female parent (Table 4) that the *P. mariana* parent for cross 318 was likely heterozygote for the marker Ope06-900, that the *P. mariana* parent for the cross 349 was likely heterozygote for all markers specific to black spruce, and that the *P. rubens* parent for the same cross was heterozygote for 3 of the 4 markers found specific to red spruce. In spite of the apparent dominance problems associated to RAPDs, black spruce species-specific fragments were never observed in any red spruce tree analyzed. These species-specific fragments were found to co-segregate in all artificial hybrids in spite of some hybrids departing from intermediate morphology. In this regard, the markers used were more reliable than the set of morphological criteria employed here.

In conclusion, RAPD markers appear to be better suited than isozymes (Eckert 1989) or a limited set of RFLPs (Bobola et al. 1992) for identifying species-specific markers in this closely related species complex because large numbers of loci can be surveyed, that are likely more randomly distributed across the genome (Williams et al. 1990). The use of unbiased samples of trees representative of each species also appears to be essential in the identification of such diagnostic markers. These markers could be used in the survey of natural hybridization between the two species as well as for certification operations in tree improvement programs. In both cases, marker-aided species identification of young seedlings or seedlots would be helpful because of the limited number of discriminating morphological characters at such early developmental stages.

Acknowledgements We thank M. Villeneuve, G. Gagnon, and G. Numainville (Québec Ministry of Natural Resources) for their help with field sampling, A. Roy and N. Isabel (CRBF, Université Laval) for comments on previous drafts of this manuscript and providing

megagametophyte DNAs, and J. Beaulieu (Canadian Forest Service, Québec) and H. Markussen (Petawawa National Forestry Institute, Ontario) for providing some seed collections. This research was supported by grants provided by the Québec Ministry of Natural Resources, NSERC of Canada, and FCAR of Québec to J.B.

References

Arnold ML, Buckner CM, Robinson JJ (1991) Pollen-mediated introgression and hybrid speciation in Louisiana irises. Proc Natl Acad Sci USA 88:1398–1402

Bobola MS, Eckert RT, Klein AS (1992) Restriction fragment variation in nuclear ribosomal DNA repeat unit within and between *Picea rubens* and *Picea mariana*. Can J For Res 22:255–263

Bousquet J, Simon L, Lalonde M (1990) DNA amplification from vegetative and sexual tissues of trees using polymerase chain reaction. Can J For Res 20:254–257

Eckert RT (1989) Genetic variation in red spruce and its relation to forest decline in northeastern United States. In: Bucher JB and Bucher-Wallin I (eds) Air pollution and forest decline. (Proc. 14th Int Meet Specialists Air Pollution Effects For Ecosystems, IUFRO P2.05). IUFRO, Birmensodorf, pp 319–324

Fritz RS, Nichols-Orians CM, Brunseld SJ (1994) Interspecific hybridization of plants and resistance to herbivores: hypotheses, genetics, and variable responses in a diverse herbivore community. Oecologia 97:106–117

Gordon AG (1975) The contribution of genetic variation to productivity systems in spruce forest ecosystems. In: Illingworth K and Yeatman CW (eds) Proc 14th Meet Can Tree Improv Assoc, Part 1. Can For Serv, Ottawa, Canada, pp 73–74

Gordon AG (1976) The taxonomy and genetics of *Picea rubens* and its relationship to *Picea mariana*. Can J Bot 54:781–813

Gordon AG (1978) Genecology and the contribution of genetic variation to productivity systems in spruce forest ecosystems. In: Yeatman CW (ed) Proc 16th Meet Can Tree Improv Assoc Part 1. Can For Serv, Ottawa, Canada, pp 89–91

Hawley GJ, Dettays DH (1994) Genetic diversity and population structure of red spruce (*Picea rubens*). Can J Bot 72:1778–1786

Isabel N, Tremblay L, Michaud M, Tremblay FM, Bousquet J (1993) RAPDs as an aid to evaluate the genetic integrity of somatic embryogenesis derived populations of *Picea mariana* (Mill.) B.S.P. Theor Appl Genet 86:81–87

Isabel N, Beavlieu J, Bousquet J (1995) Complete congruence between gene diveristy estimates derived from genotypic data at enzyme and RAPD loci in black spruce. Proc Natl Acad Sci USA (in press)

Manley SAM (1971) Identification of Red, Black and Hybrid Spruce. Can Dep Environ For Can For Serv Ottawa, Canada, Publ. 1301

Manley SAM (1972) The occurrence of hybrid swarms of red spruce and black spruce in central New Brunswick. Can J For Res 2:381–391

Manley SAM, Ledig F (1979) Photosynthesis in black and red spruce and their hybrid derivatives: ecological isolation and hybrid adaptive inferiority. Can J Bot 57:305–314

Michelmore RW, Paran I, Kesseli RV (1991) Identification of markers linked to disease-resistance gene by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. Proc Natl Acad Sci USA 88:9828–9832

Orozco-Castillo C, Chalmers KJ, Waugh R, Powell W (1994) Detection of genetic diversity and selective gene introgression in coffee using RAPD markers. Theor Appl Genet 87:934–940

Roy A, Frascaria N, Mackay J, Bousquet J (1992) Segregating random amplified polymorphic DNAs (RAPDs) in *Betula alleghaniensis*. Theor Appl Genet 85:173–180

Sigurgeirsson A, Szmidt AE (1993) Phylogenetic and biogeographic implications of chloroplast DNA variation in *Picea*. Nord J Bot 13:233–246

Sutton BCS, Flanagan DJ, Gawley JR, Newton CH, Lester DT, El-Kassaby YA (1991) Inheritance of chloroplast and mitochondrial DNA in *Picea* and composition of hybrids from introgression zones. Theor Appl Genet 82:242–248

Sutton BCS, Pritchard SC, Gawley JR, Newton CH (1994) Analysis of Sitka spruce-interior spruce introgression in British Columbia using cytoplasmic and nuclear DNA probes. Can J For Res 24:278–285

Szmidt AE, EL-Kassaby YA, Sigurgeirsson A, Aldèn T, Lindgren D, Hällgren J-E (1988) Classifying seedlots of *Picea sitchensis* and *P. glauca* in zones of introgression using restriction analysis of chloroplast DNA. Theor Appl Genet 76:841–845

Welsh J, McClelland M (1990) Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res 18:7213–7218

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nuclei Acids Res 22:6531–6535

Yeh FC, Arnott JT (1986) Electrophoretic and morphological differentiation of *Picea sitchensis*, *Picea glauca*, and their hybrids. Can J For Res 16:791–798