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CHLOROPLAST MICROSATELLITE DIFFERENTIATION IN JACK PINE (*PINUS BANKSIANA*) POPULATIONS IN QUEBEC

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ABSTRACT. — In this study we investigated the genetic structure and diversity of jack pine populations in Quebec using chloroplast microsatellite markers. Six cpSSR loci were used to screen 15 *Pinus banksiana* populations from an economically important region of the boreal zone. The main objective was to quantify the variation within and among populations and to relate this to their geographic pattern. From 3 to 6 size variants were identified at each locus. A total of 29 size variants at the six loci were identified and 5 of the size variants occurred infrequently. The size variants were combined into 87 different haplotypes, of which 49 were unique. The AMOVA analysis revealed that 10.96% of the variation was distributed between populations and 89.04% within populations. Cluster analysis divided the studied populations into two large groups, one from north-western Quebec and the second from eastern Quebec. These results suggest that the genetic structure of the studied jack pine populations is the result of interactions between historical post-glacial migrations and climate-disturbance structuring factors.

KEY WORDS. – Pinaceae, *Pinus banksiana* Lamb., biogeography, chloroplast, microsatellites, population differentiation.

INTRODUCTION

Pinus banksiana Lamb. is a two-needled pine (*Diploxilon* section), well adapted to frequent forest fires, that colonizes recently burned and sandy sites. As a result, it has long been recognized as a fundamental species in forestry operations, and its biology, morphology and genetics have been studied. Family and provenance tests of *P. banksiana* have been established in all ecological zones across Quebec and Canada, as the economic importance of jack pine has resulted in the development of seed transfer zones and therefore better management of its genetic resources (MATYAS & YEATMAN 1992, PARKER & VAN NIEJENHUIS 1996).

The distribution of jack pine includes most of Canada and the north-eastern USA (CRITCHFIELD & LITTLE 1966, LITTLE 1971). The present distribution limit is between the 29°C mean annual maximum isotherm and the northern limit of continuous open boreal forest, with a few isolated populations in northern Ontario, northern Quebec and Labrador (RUDOLPH & LAIDLY 1990, ASSELIN *et al*. 2003). The differentiation in phenotype and genotype frequencies as a result of the ecological gradient created by this broad east-west distribution has not been well documented.

The structure of genetic diversity among forest tree species in natural ecosystems is a major topic of research in plant population genetics. This knowledge does not only further understanding of the selection and differentiation processes in forest tree populations, but can also be applied to the management of genetic resources. In *Pinus banksiana*, the discrimination between natural populations based on isozyme analysis is 1.8 to 7.0% (DANCIK & YEH 1983, DANZMANN & BUCHERT 1983, ROSS & HAWKINS 1986, WHEELER & GURIES 1989, GAUTHIER *et al.* 1992). However, new DNA analysis techniques often illustrate among population differentiation that was previously hidden, and the methods are open for development and the addition of new markers.

Different types of markers are used to investigate population genetic processes. Estimates of population subdivision based on paternally inherited chloroplast microsatellites are generally higher than those reported in isozyme studies (POWELL *et al.* 1995, VENDRAMIN *et al.* 1998, 1999, RIBEIRO *et al*. 2002). Population structuring can be expected to be more pronounced for chloroplastic genes than for nuclear genes because of their uniparental inheritance and the lower effective population size for the haploid chloroplast genome. Previous chloroplast SSR studies have provided information on the postglacial migration routes and the refugia locations for different *Pinus* species (VENDRAMIN *et al.* 2000, WALTER & EPPERSON 2001, MARSHALL *et al*. 2002). Recently, DYER & SORK (2001) used these markers to show a significant correlation between paired genetic and physical distances in shortleaf pine. Their findings strongly indicate that pollen movement within a continuous forest is restricted owing to both isolation by distance and vegetation structure.

The main objective of this paper is to quantify the variation within and among jack pine populations and their geographic pattern in Quebec by using cpSSR analysis.

MATERIAL AND METHODS

PLANT MATERIAL

The Ministry of Natural Resources of Quebec provided jack pine seeds that were collected following the IUFRO's and Canadian Forest Service's recommendations to insure that each seed lot is representative of each provenance. These seed lots were collected from sites with a minimum of 180 trees per site to insure a representative sample of the gene pool from each site. Fifteen provenances were chosen throughout the species range in Quebec (Table 1 and Fig. 1). Seeds were removed from their cones and kept in the dark at 4 ºC. Thirty-six seeds from each population were randomly chosen for DNA analysis. They were placed in Petri dishes at 26°C under a light regime of 14 hours of light per day for 2 days.

LABORATORY METHODS

Total DNA was extracted from 36 megagametophytes per population using the Sigma mini preparation kit (product Code : G2N350). Following extraction, chloroplast microsatellite (cpSSR) loci were surveyed using six primer pairs developed by VENDRAMIN *et al.* (1996) that showed good variability in preliminary tests (Pt-30204, Pt-36480, Pt-45002, Pt-71936, Pt-79951, and Pt-87268). The cpSSR primers were obtained from Sigma Genesys. Amplifications were realized in a 10 µl reaction volume on a Perkin-Elmer 9700 thermocycler with HotMaster Taq DNA Polymerase of Eppendorf AG (Hot start and Cool stop enzyme system). Multiplex PCR was done on two groups of primers (group A – Pt-30204, Pt-45002, Pt-71936 and group B – Pt-36480, Pt-79951, Pt-87268) as determined by the program OLIGO, ver. 9.9.8 (KALENDAR 1999-2002) and laboratory tests. After initial amplification the PCR products were diluted 5 to 8 times.

FRAGMENT ANALYSIS AND STATISTICAL ANALYSIS

Fluorescent dye labelled PCR products (0.65 µl), 12 µl Hi-Di Formamide (Applied Biosystems) and 0.36 µl internal standard Tamra-500 (Applied Biosystems) were denatured for 5 minutes at 95°C and separated by capillary electrophoresis on a PE Applied Biosystems 310 Genetic Analyser. The electropherograms were then analysed with GENESCAN™ software (PE Applied Biosystems). Basic steps such as extraction, PCR amplification and fragment analysis of each site were repeated two times. Haplotypes were deduced from individual size profiles from the cpSSR analysis. All of the statistic analyses were performed on the haplotype frequencies (chlorotype) as required for uniparentally inherited genomes (PRICE *et al.* 1998, VENDRAMIN *et al.* 1998, MITTON *et al.* 2000, SCHUSTER & MITTON 2000). Gene diversity statistics and *F*-statistics were calculated with a stepwise mutation model

TABLE 1

Studied jack pine (Pinus banksiana) *populations and estimates of haplotypic diversity*

Population*	Latitude N	Longitude W	Altitude (m)	SV/L	N_h	H_e
Pop-A	48°18'00	68°52'00	150	2.500	11	0.192
Pop-B	48°45'00	71°30'00	180	2.167	11	0.202
Pop-C	48°30'00	72°30'00	364	2.667	16	0.245
Pop-D	46°40'00	72°30'00	150	2.833	12	0.239
Pop-E	47°18'15	73°34'30	300	2.667	17	0.292
Pop-F	48°18'00	74°36'00	440	2.667	14	0.192
Pop-G	45°56'30	76°05'00	120	3.000	16	0.269
Pop-H	47°55'00	79°25'00	242	2.667	17	0.375
Pop-I	48°40'00	78°30'00	Unknown	2.667	16	0.250
Pop-J	48°42'00	69°15'11	100	2.167	12	0.206
Pop-K	48°00'00	66°25'00	Unknown	3.333	19	0.369
Pop-L	49°16'00	77°52'00	325	2.500	11	0.161
Pop-M	50°45'15	74°53'00	400	2.833	18	0.306
Pop-N	48°40'00	76°20'00	Unknown	3.000	20	0.314
Pop-O	49°16'00	76°52'00	Unknown	3.000	19	0.260
Average				2.711	15.3	0.258

*The number of analysed individuals per population was 36.

SV/L = average number of size variants per locus ; N_h = number of observed haplotypes ; H_e = unbiased observed haplotype diversity according to VENDRAMIN *et al.* (1998).

(SLATKIN & BARTON 1989, SLATKIN1995) using GENETIX 4.02 (BELKHIR 2002) and ARLEQUIN 2.001 (EXCOFFIER *et al*. 2002). An Analysis of Molecular Variance (AMOVA ; EXCOFFIER *et al.* 1992) was also calculated based on the number of different haplotypes. This is equivalent to a weighted F_{st} over all of the locus when estimating genetic structures (WEIR & COCKERHAM 1984, MICHALAKIS & EXCOFFIER 1996). The same analysis was also done based on the sum of the squared number of repeat difference between haplotypes, which is equivalent to the Slatkin R_{st} analogue of the stepwise mutation model (SLATKIN 1995). Gene flow (N_m) was estimated as the product of the proportion of migrants (m) from a population of size N. Estimation of N_m was based on F_{st} (SLATKIN 1987, SLATKIN & BARTON 1989). The association between the two squared distance matrices (Nei's 1978 genetic distances between pairs of stands and physical distances separating the stands) was then analysed with a Mantel test (MANTEL 1967). A dendogram was constructed using Nei's distance (NEI 1978) and the GENETIX hierarchic search clustering procedure.

RESULTS

SIZE VARIATION AND HAPLOTYPE FREQUENCIES

The six chloroplast primer pairs revealed fragments between 132 and 168 bp, which is within the range of very good detection by capillary electrophoresis. The difference between size variants at each locus was one bp. From 3 to 6 size variants were identified at each locus, for a total of 29. The mean number of size variants per locus varied among populations from 2.17 to 3.33 (Table 1). Of the 29 size variants observed, 9 occurred infrequently (< 1%). Out of these, five were unique (i.e., present in only one population). The most frequent size variants, present in all populations, were fragments 141 bp (97.0%) at locus Pt-36480, 160 bp (86.7%) at locus Pt-87268, 135 bp (83.7%) at locus Pt-30204, followed by 142 bp (80.9%) at locus Pt-71936, 138 bp (80.0%) at locus Pt-79951 and 165 bp (66.1%)

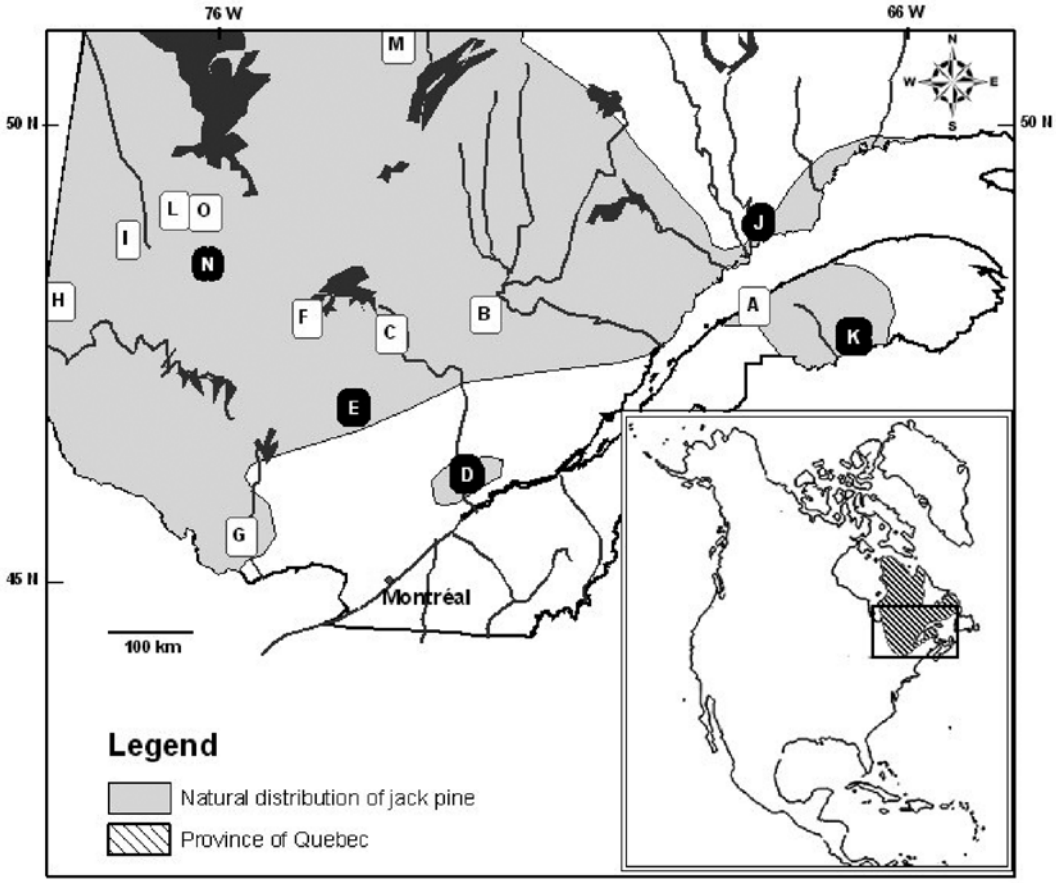

FIG. 1. — Jack pine (*Pinus banksiana*) range in Quebec (light grey area, modified from LITTLE 1971) showing the 15 populations studied. The northwestern group is represented by white rectangles, while the eastern group is represented by black rectangles.

at locus Pt-45002. The 29 size variants were combined in 87 different haplotypes. One haplotype was common to all of the studied populations, namely h-49. Two thirds (> 66%) of the total number of samples was represented by 8 haplotypes, each of which was present in at least 7 of the 15 studied populations (Table 2).

GENETIC STRUCTURE AND POPULATION DIFFERENTIATION

The unbiased population haplotype diversity (H_e) was on average 0.252 (Table 1), and varied from 0.161 to 0.375. Lower levels of diversity (H_e= 0.161 to 0.232) were observed in populations Pop-A, Pop-B, Pop-F, Pop-J and Pop-L and higher haplotypic diversity (H_e= 0.304 to 0.375) was found in populations that were located at the western (Pop-H), eastern (Pop-K) and northern (Pop-M and Pop-N) extremities of the sampled area. The observed number of haplotypes varied between 11 and 20. The AMOVA analysis revealed that 10.96% of variation was found between populations, with 89.04% of diversity distributed within populations (Table 3). The calculated G_{st} and F_{st}, used to estimate the degree of population differentiation, were 8.30% and 6.40% respectively, which confirmed the AMOVA results. Gene flow (N_m) was estimated to be slightly more than 2 migrants per generation.

Nei's genetic distance between populations varied between 0 and 0.098, with a mean of 0.0569. Cluster analysis divided the fifteen jack pine populations into two large groups. The north-

TABLE 2

*Frequency of the 8 most common haplotypes and diversity within the 15 jack pine (*Pinus banksiana *Lamb.) populations studied in Quebec (the predominant haplotype is indicated in bold type)*

Haplotype*	Label	POPULATION															Total
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	
135/141/165/141/138/160	h-44	-	2.78	11.11	5.56	5.56	-	2.78	8.33	2.78	5.56	-	-	13.89	8.33	-	4.44
135/141/165/142/138/160	**h-49**	**36.11**	**36.11**	**38.89**	**30.56**	**27.78**	**36.11**	**27.78**	**19.44**	**33.33**	**33.33**	**19.44**	**41.67**	**16.67**	**13.89**	**27.78**	**29.26**
135/141/165/142/138/161	h-50	8.33	2.78	-	2.78	5.56	2.78	5.56	5.56	-	11.11	5.56	5.56	-	-	2.78	3.89
135/141/165/142/139/160	h-51	2.78	25.00	5.56	2.78	-	19.44	5.56	-	8.33	5.56	5.56	2.78	5.56	-	2.78	6.12
135/141/165/143/138/160	h-53	11.11	-	-	8.33	2.78	-	-	13.89	2.78	-	2.78	2.78	11.11	-	5.56	4.07
135/141/166/142/138/160	h-61	19.44	11.11	5.56	19.44	13.89	-	19.44	-	8.33	25.00	2.78	22.22	13.89	11.11	11.11	12.22
135/141/166/142/139/160	h-64	2.78	2.78	-	-	-	5.56	5.56	8.33	2.78	2.78	8.33	-	-	5.56	-	2.96
136/141/165/142/138/160	h-78	-	-	-	8.33	-	-	5.56	-	11.11	-	8.33	2.78	5.56	5.56	-	3.15
	Total	80.55	80.56	61.12	77.78	55.57	63.89	72.24	55.55	69.44	83.34	52.78	77.79	66.68	44.45	50.01	66.11

*The first column shows the size variants at each of the six cpSSR loci studied (Pt-30204, Pt-36480, Pt-45002, Pt-71936, Pt-79951, and Pt-87268, respectively).

TABLE 3

Hierarchical analysis of molecular variance (AMOVA) based on the sum of squared size difference (R_{st})

Variance component	d.f.	Sum of squares	Variance components	Percent of variation*
Among populations	13	2310.019	0.13180	10.96
Within populations	9310	19933.607	1.07055	89.04
Total	18647	22243.626	1.20234	

*F_{st} = 6.4% and G_{st} = 8.3%

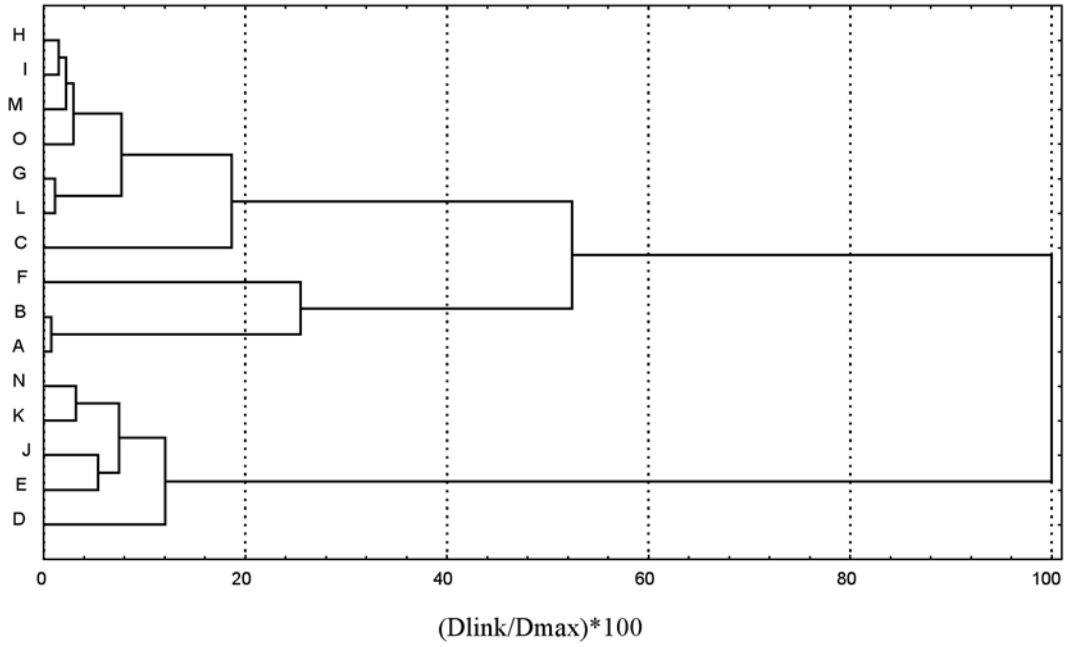

FIG. 2. — Dendrogram of the 15 jack pine (*Pinus banksiana*) populations derived from the genetic distances of NEI (1978). The north-western group is represented by populations H through A and the eastern group by populations N through D.

western Quebec group was composed of populations Pop-A, Pop-B, Pop-C, Pop-F, Pop-G, Pop-H, Pop-I, Pop-L, Pop-M and Pop-O (Fig. 2). Within this western group there was a divergence between Pop-A, Pop-B and Pop-F and the remaining six populations. The second group, in eastern Quebec, was composed of populations Pop-D, Pop-E, Pop-J, Pop-K and Pop-N (Fig. 2). The Mantel test revealed that when all the populations are included in the analysis there is no significant correlation between geographic and genetic distance (r = 0.343, P = 0.19). However, when we consider each group separately, the correlation between genetic and geographical distances is significant (r = 0.732, P = 0.05) in north-western group, while the eastern group approaches significance (r = 0.132, P = 0.06).

DISCUSSION

POPULATION STRUCTURE

The studied populations are representative of a significant part of the natural distribution of jack pine across Quebec (Fig. 1). Situated in the typical boreal zone, the jack pine stands are closely

interlinked and populations intensively exchange pollen and seeds. Despite this, the eastern and northwestern groups tend to differ, though not significantly (t-test), in the average number of size variants per locus (2.800 east vs. 2.667 west, P = 0.46), the number of observed haplotypes (16.0 vs. 14.9, P = 0.55) and the unbiased haplotype diversity (0.284 vs. 0.245, P = 0.28). In contrast, the predominant haplotypes are more frequent in the northwestern group (25.00% vs. 31.39%). These differences in the patterns of size variants and haplotypes within and among populations are a result of the influence of different factors in population dynamics, such as migration, isolation, genetic drift, fire and climatic history, and development. The results indicate that the populations in the northwestern group have more genetic diversity among them (F_{st} = 6.73%, G_{st} = 8.47%) than the populations in the eastern group (F_{st} = 5.94%, G_{st} = 6.96%). This may in part explain the relatively good differentiation between the two main population groups (F_{st} = 11.32% and G_{st} = 13.74%). The presence of geographically illogical sub-groups within the northwestern group may be due to the small number of populations analysed compared to the geographic distance covered.

The inter-population differentiation revealed by the analysis of chloroplast microsatellites is similar to the differentiation revealed by isozyme analysis of this species in this region in the past. While a small number of individuals per population may result in large inter-population differences, our study has sample sizes greater than 36, and the differences revealed are likely to reflect actual differentiation between populations.

Good inter-population differentiation (Table 3, Figs. 1 and 2) in jack pine is indicative of genetic drift and substantial isolation within populations. Our estimates showed that 10.96% of the total genetic variation was distributed between populations, which is a moderate level of differentiation for a coniferous species (see VIARD *et al.* 2001) and is indicative of genetic drift and significant isolation among populations. For example, in *Pinus contorta* Dougl. ex Loud., MARSHALL *et al.* (2002) reported a 15% level of differentiation between populations. In *Pinus pinaster* Ait. VENDRAMIN *et al.* (1998) found an average G_{st} of 0.233, thus 23% of the total gene diversity could be attributed to inter-population differentiation. Curiously, a very large level of differentiation (56%) among populations, obtained with the same groups of chloroplast primers, was published for *Pinus resinosa* Ait. by WALTER & EPPERSON (2001). Like us, these authors found higher levels of genetic differentiation than those revealed from using allozyme or other markers (BROWN & MORGAN 1981, BOSHERINI *et al.* 1994, KARHU *et al.* 1996, VENDRAMIN *et al.* 1998, ZHENG & ENNOS 1999, LEDIG 2000, GAMACHE *et al.* 2003). In contrast to other pine species, previous jack pine studies consistently reported a low level of population differentiation both at micro- and at macrogeographical scales (DANCIK & YEH 1983, ROSS & HAWKINGS 1986, XIU & KNOWLES 1991, GAUTHIER *et al.* 1992, SAETZ-ROMERO *et al.* 2001). The highest level detected in jack pine populations was sampled in the sympatric hybrid regions with 15% of the variability partitioned between populations (YE *et al.* 2002).

POST-GLACIAL MIGRATION HISTORY AND FIRE DEPENDENCE BIOLOGY OF JACK PINE

The natural dynamics of jack pine is largely controlled by fire regime, climatic and genetic factors expressed with different intensity in time and space (historical climate-disturbance-population structure interaction). Compared with other species from the boreal region, *Pinus banksiana*'s dependence on fire for regeneration and colonisation of new territories has generated a lag in its postglacial expansion (PAYETTE 1993). Fire characteristics, such as fire-free interval and fire size, play an important role in determining the success of reproduction, seed dispersion, seedling survival and development (CARROLL & BLISS 1982, CAMPBELL & FLANNIGAN 2000). As a result, forest tree species arrived late in Quebec, following the rim of newly exposed land revealed by the melting ice caps. In this period, during which Quebec was being colonized by jack pine, forest fires were rare, generating only a gradual opening of the landscape (LAMB 1980, RICHARD *et al.* 1982, GARRALLA & GAJEWSKI 1992, PIELOU 1992).

The separation of *P. banksiana* in Quebec into two groups by cpSSR analysis indicates the possibility of two principal migration and re-colonisation patterns following deglaciation. Factors such as differing migration speeds, and in some cases fragmentation and isolation, lead to the current differentiation observed in the genetic structure of the populations. The species survived the maximum extent of the Wisconsian ice age in two distinct refugia, west of the Appalachian Mountains south of the tundra zone bordering the glacial extent and at low elevations on the eastern side of the Appalachian Mountains (McLeod & MacDonald 1997). Migration north out of the western refugia was more rapid, as jack pine reached its present northern distribution maximum before the end of the warm Hypsithermal period, ca. 5000 years BP. In contrast, migration northwards was retarded in the east by the presence of large ice masses (Lauriol & Gray 1987) and the first eastern migrants arrived at the beginning of the cool Neoglacial period ca. 3000 years BP (Critchfield 1985, Jacobson *et al.* 1987, Desponts & Payette 1993, Gajewski *et al.* 1993).

Therefore, the separation into eastern and northwestern groups may reflect the different post-glacial origins of these populations. The eastern group may have migrated east from the Appalachians and the southern Great Lakes, while the western group may have migrated from northwestern Canada, where jack pine was already well established (Richard 1980, Davis 1981, Ritchie 1987, Cohmap 1988, Liu 1990, MacDonald 1992). This hypothesis is also supported by data from the North American Pollen Database (NAPD). An alternative hypothesis is that the observed groups are part of the continuous gradient of differentiation between the western and eastern populations of jack pine's natural distribution in Canada. Most likely, there was an interaction between post-glacial migration history and environmental gradients created by fire disturbance history.

CONCLUSION

This study showed good levels of genetic diversity in jack pine populations and supports previous reports that used other markers to show that this diversity is structured. As jack pine stands are often characterized by the absence of natural advance regeneration, in order to ensure regeneration the usual practice is to re-plant harvested areas. These planted trees are likely to come from a reduced number of individuals and/or populations. This will not jeopardize jack pine genetic diversity *per se* but will probably affect its natural structural pattern. Therefore, for the present range of jack pine in Quebec, it is important to conserve genetically unique populations and to continue selection, genetic, orchard and breeding projects. Identification of geneticalally unique populations is the first step to conservation of forest tree species, but this task is formidable. We must emphasize that the development of new genetic markers might reveal even higher levels of genetic differentiation. This could modify our understanding of how boreal forest tree genetic diversity is structured. This should be taken into account for the delineation of evolutionary significant units and integration in the sustainable forest management toolbox.

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