Reproductive barriers and hybridity in two spruces, *Picea rubens* and *Picea mariana*, sympatric in eastern North America

John E. Major, Alex Mosseler, Kurt H. Johnsen, Om P. Rajora, Debby C. Barsi, K.-H. Kim, J.-M. Park, and Moira Campbell

Abstract: Hybridization between red spruce (*Picea rubens* Sarg.) and black spruce (*Picea mariana* (Mill.) BSP), late- and early-successional species, respectively, has resulted in identification and management problems. We investigated the nature and magnitude of reproductive and life-cycle success barriers in controlled intra- and inter-specific crosses of red and black spruce. We quantified a number of reproductive, germination, phenological, and performance traits, and examined traits by parental pedigree and hybrid index. Species' pollen had no effect on number of aborted or nonpollinated ovules. Controlled intraspecific crosses had, on average, 6.6 times more filled seeds than interspecific crosses. Cone and seed morphometric traits were species specific, with seed traits showing negative hybridization effects on both species. Germination, cotyledon number, and seedling height had significant species-specific traits, with hybrids showing an additive or slightly negative heterosis. Severe, negative heterosis appears to be of limited importance as an isolating barrier between red and black spruce. Reproductive phenology was remarkably similar among species and hybrid progenies when grown in common garden experiments. Crossability barriers are clearly paramount in maintaining the separation of the species. Ecological separation based on ecophysiological differences (e.g., shade tolerance) also represents an important prezygotic barrier for minimizing the negative effects of hybridization (e.g., postzygotic inviability) on reproductive fitness.

Key words: cone, seed and seedling traits, genetic variation, phenology, red and black spruce.

Résumé : L'hybridation entre l'épinette rouge (*Picea rubens* Sarg.) et l'épinette noire (*Picea mariana* (Mill.) BSP), des espèces de fin et de début de succession respectivement, soulève des problèmes d'identification et d'aménagement. Les auteurs ont étudié la nature et l'ampleur du succès des barrières et du cycle vital à contrôler les croisements intra- et inter-spécifiques, entre les épinettes rouges et noires. Les auteurs ont quantifié le nombre de caractères reproductifs, germinatifs, phénologiques et de performance, et ont examiné les caractères par pedigrees parentaux et index d'hybridation. Les pollens des espèces sont sans effet sur le nombre d'ovules non-fécondées ou avortées. En conditions contrôlées, les croisements intraspécifiques donnent en moyenne 6,6 fois plus de graines pleines que les croisements interspécifiques. Les caractères morphométriques des cônes et des graines sont spécifiques à l'espèce, les caractères des graines montrant des effets négatifs d'hybridation chez les deux espèces. La germination, le nombre de cotyles et la hauteur des plantules montrent des caractères significativement spécifiques à l'espèce, les hybrides montrant une hétérosis additive ou légèrement négative. L'hétérosis négative sévère semble être d'importance limitée comme facteur de barrière d'isolation entre les épinettes rouges et noires. La phénologie de la reproduction est remarquablement similaire entre les espèces et les progénitures hybrides, lorsqu'on les cultive dans des jardins expérimentaux communs. Les barrières aux croisements jouent clairement un rôle primordial dans le maintien de la séparation des espèces. La séparation écologique basée sur des différences écophysiologiques (p. ex., tolérance à l'ombre) représente également une importante barrière pré-zygotique dans la limitation des effets négatifs de l'hybridation (p. ex., mortalité postzygotique) sur l'adaptation reproductive.

Mots clés : cône, caractères des graines et des plantules, variation génétique, phénologie, épinettes rouges et noires.

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J.E. Major,[1] A. Mosseler, D.C. Barsi, and M. Campbell. Natural Resources Canada, Canadian Forest Service – Atlantic Forestry Centre, P.O. Box 4000, Fredericton, NB E3B 5P7, Canada.
K.H. Johnsen. USDA Forest Service, 3041 Cornwallis Road, Research Triangle Park, NC 27709, USA.
O.P. Rajora.[2] Department of Biology, Life Sciences Centre, Dalhousie University, Halifax, NS B3H 4J1, Canada.
K.-H. Kim and J.-M. Park. Faculty of Forest Science, College of Agriculture, Chonbuk University, Chonju, South Korea.

[1] Corresponding author (e-mail: jmajor@nrcan.gc.ca).

[2] Present address: Canada Research Chair in Forest and Conservation, Faculty of Forestry and Environmental Management, University of New Brunswick, Fredericton, NB E3B 6C2, Canada.

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Table 1. Selection number, geographic coordinates, and current locations of black spruce (B) and red spruce (R) parent trees.

Selection No.	Species and parent	Source	Coordinates	Current location[a]
1668	R1 ♀	Eighth Lake, Queens Co.	44°07′53″N, 65°12′43″W	CB36
1243	R2 ♀	Bear River, Digby Co.	44°31′N, 65°37′W	CB36
1400	R3 ♀	Merland, Antigonish Co.	45°34′40″N, 61°38′40″W	CB36
1494	B1 ♀	Lower Caledonia, Guysborough Co.	45°16′30″N, 62°18′30″W	SO14
1405	B2 ♀	Stanley, Hants Co.	45°10′30″N, 63°48′45″W	SO14
1409	B3 ♀	Upper Stewiacke, Colchester Co.	45°12′26″N, 63°02′30″W	SO14
1014	R4 ♂	Hodson, Pictou Co.	45°44′N, 62°57′W	CB36
1018	R5 ♂	Fox River, Cumberland Co.	45°24′N, 64°31′W	CB36
1248	R6 ♂	Big Lots, Lunenburg Co.	44°25′N, 64°25′W	CB36
1416	B4 ♂	Debert Sanctuary, Colchester Co.	45°24′N, 63°27′W	SO14
1427	B5 ♂	Masstown, Colchester Co.	45°24′N, 63°28′W	SO14
1435	B6 ♂	Shipley Road, Cumberland Co.	45°39′50″N, 64°23′W	SO14

[a] CB36, clone bank 36; SO14, seed orchard 14. Both are located near Debert, N.S.

Introduction

Red spruce (*Picea rubens* Sarg.) is a commercially important, characteristic component of late-successional mixed forests of eastern Canada and the northeastern United States (Rowe 1972). Black spruce (*Picea mariana* (Mill.) B.S.P.) is transcontinental, occurring in boreal and temperate zone forests (Viereck and Johnston 1990). Black spruce is an early successional species, and its forest stands are generally even aged. Except in the southern part of its geographic range along the Appalachian Mountains, red spruce is largely sympatric with black spruce (Blum 1990). Based on morphology (Manley 1971; Berlyn et al. 1990), and isozyme (Eckert 1989) and molecular marker profiles (Bobola et al. 1992; Perron et al. 1995), these two spruces appear closely related and are capable of natural hybridization (Manley and Ledig 1979; Gordon 1976).

Red spruce has declined substantially over most of its geographic range because of excessive harvesting (Korstian 1937; Seymour 1992) and adverse environmental and climatic changes (McLaughlin et al. 1987; Hamburg and Cogbill 1988). Interspecific hybridization with black spruce may also contribute to the decline of red spruce (Major et al. 2003*b*). Hybridization can result in a species hybrid complex that combines the traits of both species in a way that may undermine the specific ecophysiological adaptations of both species (Major et al. 2003*a*, 2003*c*), and can cause identification and forest management problems for these otherwise ecologically distinct species (Manley 1975; Beylor 1999).

Estimates of the extent of natural hybridization and introgression range from extensive (Morgenstern and Farrar 1964; Manley 1972; Bobola et al. 1996) to minor (Gordon 1976; Manley and Ledig 1979; Mosseler et al. 2000). Controlled crosses by Manley (1975) and Gordon (1976) indicated reproductive barriers to hybridization; however, there is no record in the literature of a rigorous quantification of crossability and reproductive phenological barriers between these closely related species. Manley and Ledig (1979) reported what could be termed severe negative performance heterosis, where hybrid seedlings perform worse than either parental species. They concluded that this hybrid inferiority was important in maintaining species identity. However, in a subsequent retrospective examination of some of the same seed used by Manley and Ledig (1979), Major et al. (2003*b*) reported that F_1 interspecific hybrids had a statistically significant negative heterosis, but it was not severe for mature tree height and was nonsignificant for seedling height. Furthermore, Major et al. (2003*b*) reported a positive heterosis for black spruce backcrosses for mature tree and seedling heights. Unlike Manley and Ledig's (1979) work with seedlings, Johnsen et al. (1998) found no negative heterosis in growth or photosynthesis using the same seedlots after 20 years in the field. Thus, the mechanisms that maintain the separation of these species in their sympatric range remain unclear.

We hypothesize that, as distinct sympatric species, red and black spruce possess reproductive, phenological, performance, and other barriers to interspecific hybridization. Our objectives were to determine reproductive success barriers from controlled intra- and inter-specific crosses of red and black spruce by (*i*) quantifying crossability levels of intra- and inter-specific crosses using a modified 6 × 6 diallel-mating design and (*ii*) examining phenological barriers to reproductive success using an established plantation experiment of pedigreed material with varying degrees of hybridization. We also investigated the nature of pollen, cone, seed, germination, and performance traits by parental analyses and hybrid index, and discuss these traits with respect to the ecological niches of the two species.

Materials and methods

Experiment 1

Mating design and protocols

The red spruce trees were selected from natural populations across Nova Scotia (NS) (Table 1), indexed (sensu Manley 1971) as pure red spruce and occurring as grafted clones located within a clone bank near Debert, NS (45°44′N, 62°57′W). The black spruce trees are located in a seed orchard near Debert, NS, and originated as open-pollinated seed from indexed selections from locations across NS (Table 1). Six red spruce and six black spruce trees were selected for controlled pollinations, which were performed in the spring of 1997, and are part of the NS tree improvement program. For each species, three of the six parents were used as females, and three were used as males in a 6 × 6 modified diallel-mating design (Table 2). In describing the four crosstypes (e.g., black × black, black × red, red × black, and red × red), the female parent always precedes the male parent. Nine full-sib families were produced for each crosstype.

Although pollen was only required from three parents from each species for the mating design, pollen was collected from all six sources of each species to measure pollen grain morphological characteristics. Pollen cone collection began at stage 4 of development (i.e., before anthesis; Copis 1990). Advanced pollen cones were placed in paper bags at 30 °C for accelerated drying.

Pollen morphometric traits

Pollen was stored in vials with loose cotton batting in a desiccator and placed in a refrigerator at 3 °C. Prior to morphological measurements, pollen was subjected to an acetolysis treatment using a Livingstone-modified Erdtman's solution (Kim 1982). Pollen samples were mounted in glycerine jelly on microscope slides and measured using a light microscope (Olympus BX 40) at × 400 magnification. Terminology according to Erdtman (1952) and Kim and Lee (1983) was used to describe pollen morphology. The following parameters were measured: pollen body length, total pollen length including air bladders, pollen body width, total pollen width including air bladders, air bladder length, air bladder width, and exine thickness. Surface patterns of the pollen body and air bladders from both polar and equatorial views were observed using a scanning electron microscope (JSM 5600/LV, JEOL) at magnifications ranging from × 1100 to × 15 000.

Seed extraction and processing

Cones were collected at their natural ripening time in September 1997. Five samples of three cones without external signs of physical defects or insect infestation were separated into individual paper bags in the field and stored under ambient conditions to allow for after-ripening, drying, and natural opening.

Seeds with developed, rotund (i.e., enlarged) seedcoats were processed and extracted using methods previously described (Mosseler et al. 2000). The number of flat, aborted, or unpollinated seeds (i.e., those without enlarged seedcoats) was calculated by subtracting the number of developed seeds (whether filled or empty) from twice the number of fertile cone scales representing reproductive potential (Bramlett et al. 1977). To assess reproductive success in terms of filled seed production, the proportions of aborted ovules, empty seeds, and filled seeds were calculated in relation to reproductive potential. The ratio of filled to full-size (rotund) seed was calculated. Following seed extraction, each cone sample was oven-dried at 65 °C for 48 h and weighed to the nearest 0.01 g to obtain the ovendry cone biomass. All seeds per sample were weighed and counted, and thousand-seed weight was calculated. A sample of 50 seeds was used to determine seed length, diameter, area, and volume, using WinSeedle™ Scanning software (Régent Instruments Inc., Québec City, Québec) together with a Hewlett Packard 6100C scanner and lighting system (Hewlett Packard, Palo Alto, California). Seed density was calculated as seed weight per unit volume (mg/mm^3).

Table 2. Mating design of black spruce (B) and red spruce (R) parent trees.

	Female parent					
Male parent	B1	B2	B3	R1	R2	R3
B4	BB1	BB2	BB3	RB1	RB2	RB3
B5	BB4	BB5	BB6	RB4	RB5	RB6
B6	BB7	BB8	BB9	RB7	RB8	RB9
R4	BR1	BR2	BR3	RR1	RR2	RR3
R5	BR4	BR5	BR6	RR4	RR5	RR6
R6	BR7	BR8	BR9	RR7	RR8	RR9

Growing conditions and assessment of seed germination and growth

Single seeds were sown in a hydrated 2:1 peat–vermiculite mixture in multipot No. 3 – 96 trays, which have an overall dimension of 60 cm × 35 cm × 12 cm (Ropak Corp., Springhill, NS) (single pot has 105 mL full volume) on 27 March 2002; they were placed randomly in a compartment at the Canadian Forest Service – Atlantic Forestry Centre (CFS-AFC) greenhouse in Fredericton, New Brunswick (NB), Canada (45°52′N, 66°31′W). Each seedlot was sown in three to five replicate trays, depending on seed availability (maximum number 480). Seedlings were watered and fertilized twice weekly from 15 to 25 April with 11:41:8 N–P–K plus micronutrients at 50 ppm N (Plant Products Co. Ltd., Brampton, Ontario); from 26 April to 15 July with 20:8:20 at 100 ppm N two or three times weekly; and from 5 August to 18 September with 8:20:30 at 35 ppm N twice weekly. Temperature and humidity were set for 22:18 °C and 50%:60% relative humidity (day:night) from 27 March to 14 May; 50%:40% relative humidity from 15 May to 3 June; and 20:16 °C from 15 May to 14 July with external vent set for 23:19 °C. Natural light was supplemented by sodium vapor lamps for 16 h. Seedlings were moved outside on 15 July.

Seed germination was assessed by counting the number of fully emerged germinants, where the seed coat was discarded and the cotyledons were fully displayed. Trays were assessed on 8, 10, 12, 14, 16, 18, and 24 April. Germination as a percentage was assessed from 12 to 22 d after sowing. As there was negligible germination after day 28 from sowing, overall percentage germination was calculated on that date. Sampling of 40 seedlings per seedlot, for 1-year seedling height and diameter, was random across all trays and was measured in the spring of 2003.

Experiment 2

Genetic material

Trees used for the phenological observations originated from a series of controlled pollinations made by Manley (1971, 1975). Using a hybrid index system based on 19 leaf, twig, cone, and crown characteristics, Manley (1971, 1975) selected parent trees that had hybrid indices of 0, 25, 50, 75, and 100. The hybrid index represents the percentage of the red spruce component estimated in the parent tree, with 0 denoting pure black spruce, and 100 denoting pure red spruce. At least three different sets of parent trees were used for controlled crosses performed over 3 years. These trees were located on or near the CFS-AFC's Acadia Research Forest, NB, Canada (46°N, 66°15′W).

In 1976, full-sib families from these crosses were established in a progeny test, at the Acadia Research Forest, as a randomized block design with three blocks and four-tree plots (2 × 2) planted at 1.8 m × 1.8 m spacing. In the early spring of 2000, all trees with hybrid indices 0 (0 × 0), 25 (0 × 50, and 50 × 0), 50 (0 × 100, 50 × 50, 100 × 0), 75 (50 × 100, 100 × 50), and 100 (100 × 100) were assessed for the presence of male and female buds. Ten to 12 trees for each hybrid index from an even mix of crosstypes that had both male and female buds were selected for phenological progression assessment of male, female, and vegetative buds. An adjacent experiment planted in 1973 with full-sib black, red, and hybrid spruces was used to supplement hybrid indices 0 and 100, as there were insufficient families available in the aforementioned experiment.

Phenology

Classification of reproductive phenology followed Copis (1990). The male cone developmental rating system is as follows: 0, small round buds; 1, bud scales loosening and purple color of cone barely showing; 2, bud scales flaking away exposing purple cone, clear liquid when cone is squeezed; 3, cone fully exposed, a yellow fluid is emitted when pollen cone is squeezed; 4, cone is swollen and limber, yellow paste when it is squeezed; and 5, cone ready to open or has opened. The female cone developmental rating system is, briefly, as follows: 0, bud larger than vegetative bud and pointed; 1, bud is enlarged and glossy; 2, bud scales loosening and purple color of cone barely showing; 3, bud scales flaking away exposing purple cone; 4, cone scales exposed and approaching but not perpendicular to cone axis; 5, receptive stage, cone scales perpendicular to cone axis; 6, cone scales beginning to close; 7, cone scales are closed; and 8, cone becomes pendant. Vegetative bud developmental rating system is as follows: 0, no bud swelling visible; 1, bud swelling visible; 2, bud scales loosening and green color of needles barely shows; and 3, bud scales open, green color of needles fully visible. Phenological assessments were made in 2000 on 16, 19, 22, 26, 29, and 31 May, and 2, 5, 8, 12, 14, 16, 19, 21, and 23 June.

On each date, each tree was evaluated according to the percentage of the developmental category for male, female, and vegetative buds. For example, on a certain date, a tree may have 60% and 40% of male buds as categories 2 and 3, respectively. An index was then calculated by summing the product of category number and proportion represented for that tree. Using the example above, the index would be $0.60 \times 2 + 0.40 \times 3 = 2.40$.

Growing degree-day values (GDD = ((maximum temperature + minimum temperature)/2) – 5) were calculated for each day summing from 1 March 2000 using data from Environment Canada for Fredericton, NB. If the result was negative for a day, no GDD were accumulated and a zero value was assigned to that day. Accumulated GDD were calculated for each day that a phenological assessment was made.

Statistical analyses

Experiment 1 reproductive analysis was established as a completely randomized design. The five sample bags per tree were averaged for each crosstype. Differences between species and crosstypes were determined by ANOVA, testing male effect, female effect, and male × female interaction using the residual error for all tests. The greenhouse or germination part of experiment 1 was established as a completely randomized experiment with three to five trays of seedlings for each family randomly located across greenhouse benches. Each tray was assessed for percentage germination, and each germinant was assessed for its cotyledon number. An arcsine transformation was performed for percentage or proportion data (Zar 1984). ANOVA, testing crosstype, was conducted for each date that germination was assessed. Residual error was used to test all sources of variation.

Experiment 2 was conducted as a completely randomized design. Hybrid index was considered a fixed effect. An arcsine transformation was performed for percentage or proportion data (Zar 1984). Tukey mean separation test ($p = 0.05$) was used to determine the statistical significance of differences among hybrid indices.

Results

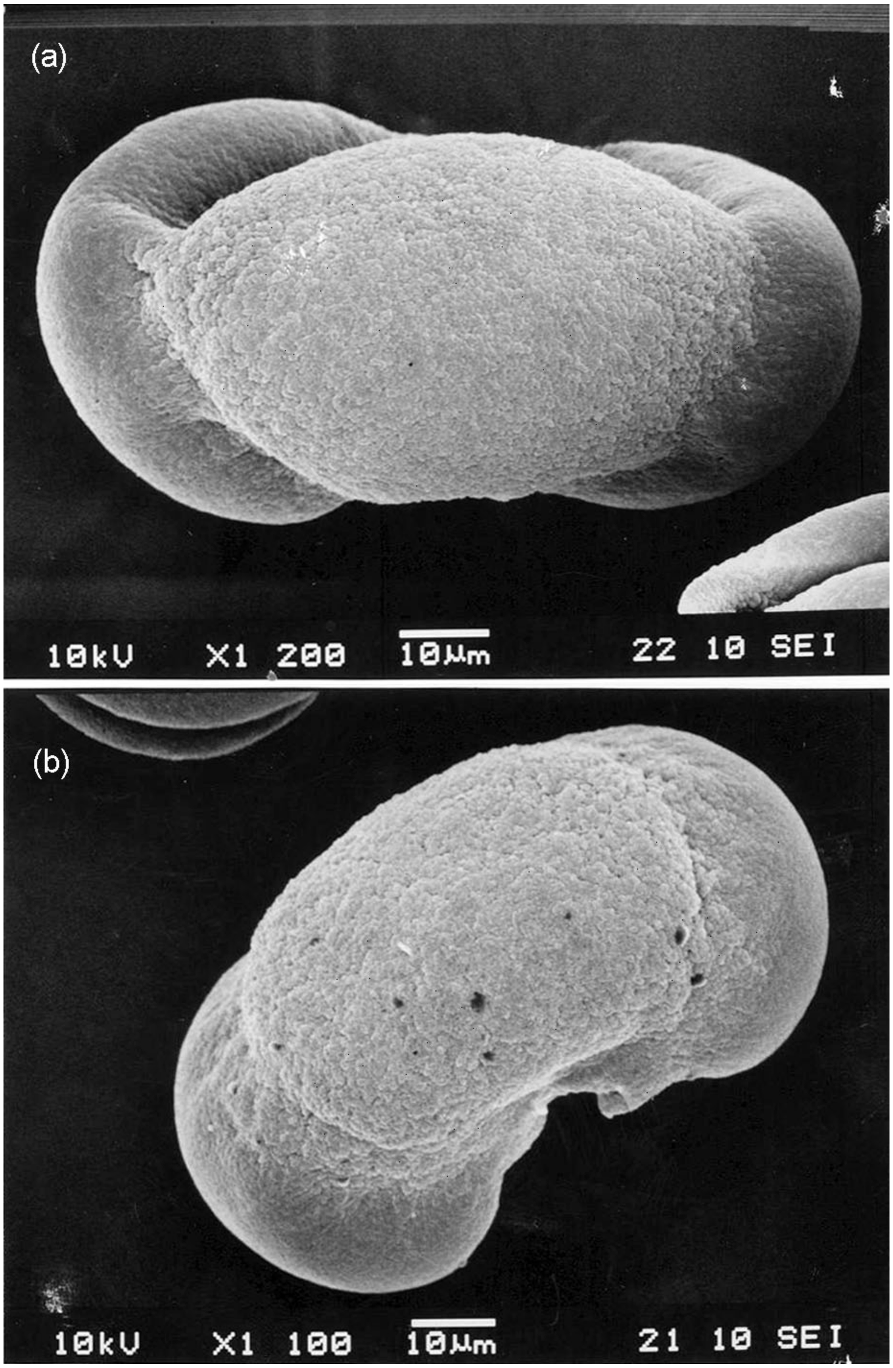
Pollen morphometric traits

The pollen grains of red and black spruce were monads with two air bladders (Fig. 1). There were no differences between the species in either pollen length parameters, PL1 and PL2 (Table 3). However, red spruce pollen body width (PW1) and overall pollen width (PW2) (Table 3) were significantly greater than those of black spruce. There were no significant differences in bladder dimensions. The foveolate surface patterns on the air bladders were indistinguishable between the two species. However, the surface pattern on the pollen body of black spruce was distinguished by a microverrucate sculpture pattern, whereas red spruce had a granulate pattern. The exine thickness of the pollen body was 13.7% greater for red spruce than for black spruce ($p = 0.025$).

Cone morphometric traits

There were no significant male effects for all cone traits examined (Table 4). Red spruce cones were significantly longer (40%) and heavier (31.3%) than black spruce (Table 4). Fully dried and flexed cone width was 18.6% greater for red spruce than for black spruce, with values of 24.2 and 20.4 mm, respectively, (not shown). Black spruce had less fertile cone scales (46) than red spruce (52). The above traits had no significant female × male interactions. The average number of seeds per cone did not have a significant female ($p = 0.446$) or male ($p = 0.591$) effect; however, there was a significant ($p < 0.001$) female × male interaction. Black × black and black × red crosstypes had, on average, 18.1 and 2.7 filled seeds per cone, respectively. Red × red and red × black crosstype had 14.6 and 2.1 filled seeds per cone, respectively.

Fig. 1. Scanning electron microscope pictures (polar view) of black spruce (*a*) and red spruce (*b*) pollen. Scale bars = 10 µm.



Seed morphometric traits

ANOVA of all seed traits showed significant female effects (Table 5). There were no significant male effects alone for all seed traits examined; however, there were numerous significant female × male interactions. Seeds produced from any cross in which red spruce was the female parent were 80% heavier than seeds from any cross with female black spruce, 2.36 and 1.31 g per 1000 seeds, respectively (Table 5). Significant negative interaction was a result of interspecific hybridization. Black × red crosstypes resulted in 8.3% lighter seeds than black × black crosstype. Red × black crosstypes resulted in 23.7% lighter seeds than red × red spruce crosstype.

Seed length, width, and silhouette (two-dimensional) area per seed were 10.9%, 14.4%, and 28% greater, respectively, for crosses with red spruce females than for crosses with black spruce females (not shown and Table 5). Female × male interactions were significant and negative. Black × red crosstypes were lower than or equal to black × black spruce crosstype, and similarly red × black crosstypes were smaller than red × red spruce crosstype. Red spruce female parent crosstypes had 65% greater volume than seeds with black

Table 3. Pollen morphometric traits (means ± SE) for black spruce and red spruce and ANOVA results (p and r^2 values).

Trait[a]	Black spruce	Red spruce	p	r^2
Pollen body length (μm)	48.5±1.4	49.9±1.0	0.420	0.073
Pollen length (μm)	76.2±1.5	76.9±1.3	0.722	0.015
Pollen body width (μm)	43.1±0.6	47.2±0.4	<0.001*	0.780
Pollen width (μm)	50.7±0.6	53.1±0.5	0.014*	0.510
Bladder length (μm)	36.7±0.2	40.1±0.7	0.105	0.264
Bladder width (μm)	31.4±0.7	31.6±0.8	0.869	0.003
Exine thickness (μm)	2.1±0.1	2.4±0.1	0.025*	0.444
Body sculpture pattern	Microverrucate	Granulate		
Bladder sculpture pattern	Foveolate	Foveolate		

Note: *, statistical significance at $p < 0.05$.

spruce female parent; nevertheless, the former had greater density than the latter.

Reproductive parameters

Approximately 25% of the potential fertile ovules were estimated to have been aborted in black spruce (Fig. 2*a*). This was significantly greater than the approximately 17.6% estimated for red spruce ($p < 0.001$). There was no pollen species effect on the number of aborted seed ($p = 0.229$). Black spruce females had significantly lower proportions of empty seed than red spruce females, with 63.1% and 74.7%, respectively (Fig. 2*b*). There were no pollen species effects on the number of empty seed ($p = 0.995$); however, there was a significant female × male interaction ($p < 0.001$). Red × black crosstype resulted in 11.6% more empty seed than the black × red spruce crosstype.

Approximately 19.7% of the potential fertile ovules for black × black spruce crosstype produced filled seed (Fig. 2*c*). On average, 13.4% of the potential ovules for red × red spruce crosstype contained filled seed. The black × red spruce crosstype produced 2.9% filled seed, on average, 38% higher than the red × black spruce crosstype which yielded 2.1% filled seed. Neither female nor male effects were significant, but the female × male interaction was significant ($p < 0.001$). Black × black spruce crosstype had the highest filled to full-sized seed ratio at 25.6%, followed by red × red spruce crosstype at 15.9% (Table 4). There were 56% more filled seed per full-sized seed for black × red than for red × black crosstype.

Germination and cotyledon traits

Full cotyledon emergence (germination) was first recorded on day 12 from sowing. ANOVA showed significant female and male effects and a significant female × male interaction (Fig. 3). At day 14, 33.0% of the black × black crosstype seeds had germinated; however, the red × red crosstype seeds had only 2.0% germination. Black × red crosstype had 18.5%, and red × black crosstype had 5.2% germination. Day 16 showed similar ANOVA ranking but higher cumulative germination. By day 18, cumulative germination from black spruce females had begun to plateau, with values of 86% and 71% for black × black and black × red crosstypes, respectively. By day 20, cumulative germination for the red × black crosstype began to plateau. By day 22, cumulative germination in the red × red crosstype had maximized. Final percentage germination on day 28, showed a significant female and male effect (Table 5). The values were 93.2%, 79.6%, 92.9%, and 92.7% for black × black, black × red, red × black, and red × red crosstypes, respectively.

Black × black and red × red crosstypes had, on average, 4.6 and 6.0 cotyledons per germinant, respectively. (Fig. 4*a*) The black × red and the red × black crosstypes had on average 5.2 and 5.6 cotyledons per seedling, respectively. There was a significant female, male, and female × male interaction. All crosstypes were significantly different from each other ($p = 0.05$).

Seedling height

There was a significant female, male, and female × male interaction for seedling height (Fig. 4*b*). Black × black and red × red crosstypes had, on average, 28.7, and 22.5 cm height growth in the first year, respectively. The black × red and red × black crosstypes had on average 23.4 and 23.6 cm height growth, respectively. Hybrids were not significantly different from each other but were intermediate and significantly different from the two parent species. Both F_1 hybrid crosstypes (0 × 100 and 100 × 0) showed a significant negative heterosis ($p < 0.001$) effect compared with expected hybrid index 50 (25.5 cm), with 8.6% lower than expected values.

Phenology

Male reproductive development showed a similar pattern across all hybrid indices, culminating at the mature stage (5) at 220 GDD, which began 2 June 2000 (Fig. 5*a*). Statistically, there was only one time where there were any differences among hybrid indices. At 199 GDD, red spruce was higher than hybrid 50, but the other indices were not different from any other, and there were no differences thereafter. Female reproductive development also showed a similar pattern across all hybrid indices up to and beyond stage 5 (Fig. 5*b*). The receptive stage lasted from approximately 220 to 245 GDD or from 2 to 5 June 2000. There were no statistical differences among hybrid indices at any time. Vegetative development did not reach an average stage 1 (bud swelling) development until approximately 300 to 320 GDD (Fig. 5*c*). This represents the end of the externally active reproductive phase and, up to this point, there were no differences between hybrid indices. Thereafter, vegetative

Table 4. Cone morphometric and reproductive fitness traits (means ± SE) for black spruce (B), red spruce (R), and hybrid crosstypes; results from ANOVA (p and r^2 values).

					Female × male interaction					
Trait	Species	Female	Male		Female B	Female R	Female	Male	F × M	r^2
Cone dry wt (g)	B	1.029±0.090	1.203±0.100	Male B	1.101±0.148	1.305±0.135	0.024*	0.858	0.166	0.166
	R	1.352±0.099	1.178±0.104	Male R	0.958±0.104	1.399±0.154				
Cone length (mm)	B	22.7±0.6	24.7±1.2	Male B	23.1±0.9	31.7±1.0	<0.001*	0.724	0.696	0.644
	R	31.8±1.0	27.0±1.5	Male R	22.2±0.9	31.9±1.8				
Avg. no. of fertile cone scales	B	46.3±1.1	48.9±1.2	Male B	45.8±1.2	52.0±1.5	0.002*	0.760	0.258	0.258
	R	52.0±1.2	49.4±1.5	Male R	46.8±2.0	52.0±2.0				
Avg. no. of seeds/cone	B	10.4±2.6	10.1±2.6	Male B	18.1±3.5	2.1±0.7	0.446	0.591	<0.001*	0.472
	R	8.3±2.4	8.6±2.4	Male R	2.7±0.9	14.6±3.8				
Filled to full-size seed (%)	B	14.7±1.6	14.5±1.7	Male B	25.6±2.2	2.5±0.4	<0.001*	0.008*	<0.001*	0.468
	R	9.3±1.2	9.7±1.2	Male R	3.9±0.6	15.9±1.9				

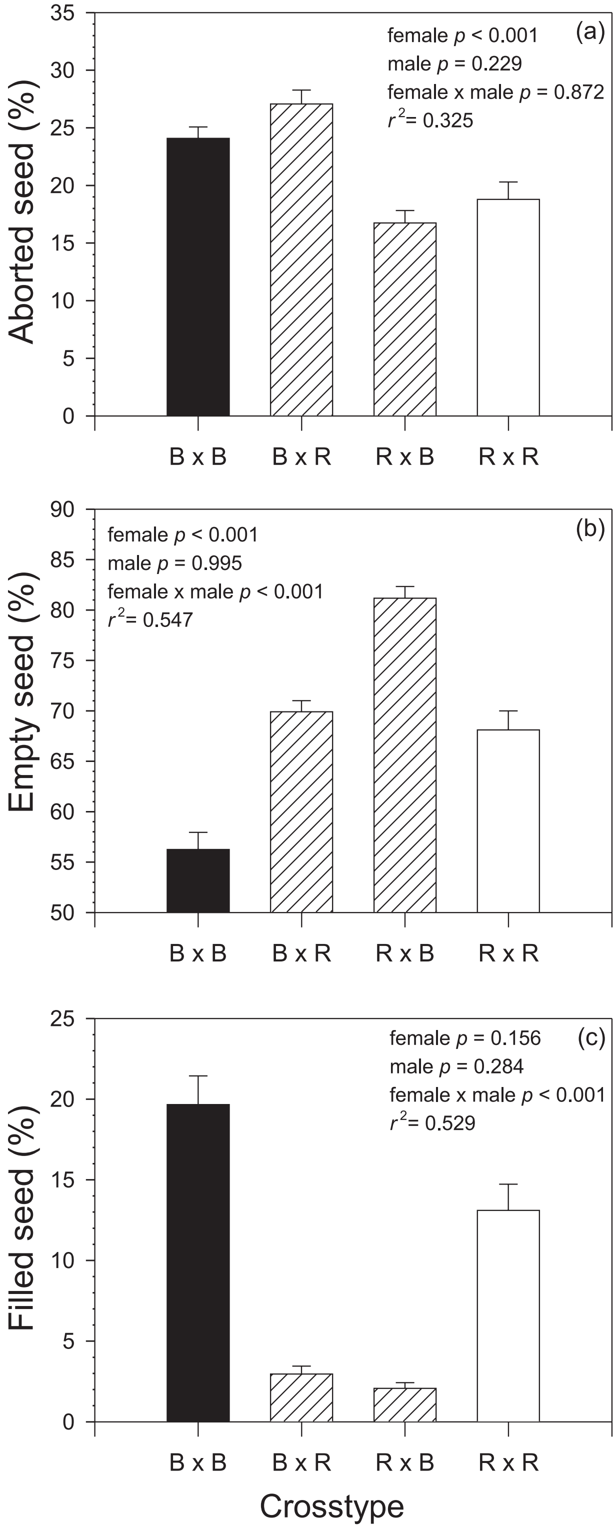
Note: df: female, 1; male, 1; female × male, 1; error, 32. *, statistical significance at $p < 0.05$.

Table 5. Seed and seedling morphometric traits (means ± SE) for black spruce (B), red spruce (R), and hybrid crosstypes; results from ANOVA (p and r^2 values).

					Female × male interaction					
Trait	Species	Female	Male		Female B	Female R	Female	Male	F × M	r^2
1000 seed wt (g)	B	1.312±0.039	1.677±0.114	Male B	1.361±0.045	2.082±0.149	<0.001*	0.151	0.031*	0.708
	R	2.361±0.141	1.956±0.196	Male R	1.256±0.062	2.577±0.200				
Silhouette area/seed (mm^2)	B	2.11±0.04	2.29±0.08	Male B	2.16±0.06	2.45±0.15	<0.001*	0.160	0.028*	0.523
	R	2.71±0.12	2.50±0.14	Male R	2.05±0.05	2.90±0.16				
Seed volume (mm^3)	B	1.23±0.04	1.50±0.10	Male B	1.29±0.05	1.77±0.16	<0.001*	0.198	0.024*	0.619
	R	2.03±0.14	1.73±0.17	Male R	1.15±0.05	2.24±0.18				
Seed density (mg/mm^3)	B	1.08±0.03	1.13±0.04	Male B	1.05±0.01	1.23±0.06	0.019*	0.911	0.124	0.226
	R	1.19±0.03	1.14±0.03	Male R	1.12±0.07	1.16±0.02				
Germination day 28 (%)	B	87.5±1.7	93.1±1.0	Male B	93.2±1.0	92.9±2.2	<0.001*	<0.001*	0.073	0.174
	R	92.7±1.1	87.3±1.7	Male R	79.6±3.3	92.7±1.3				

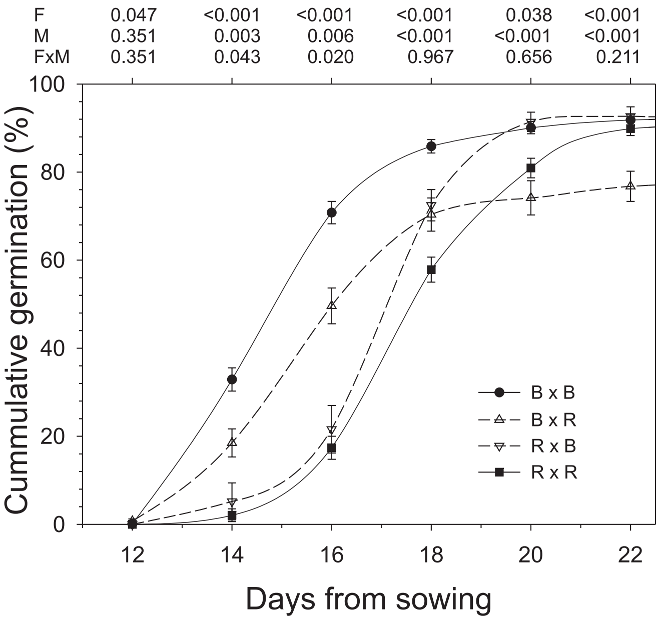
Note: df: female, 1; male, 1; female × male, 1; error = 32 (for germination day, error = 142).*, statistical significance at $p < 0.05$.

Fig. 2. Percent aborted (*a*), percent empty (*b*), and percent filled seed (*c*) for black spruce (B) and red spruce (R) intra- and inter-specific crosstypes. Values are means ± SE.



development accelerated, with red spruce advancing more rapidly than black spruce, with hybrid indices 25, 50, and 75 intermediate up to approximately 440 GDD.

Fig. 3. Cumulative germination by days from sowing for black spruce (B) and red spruce (R) intra- and inter-specific crosstypes. ANOVA results (*p*) for each sampling time are at the top of figure with female (F), male (M), and F × M interaction as sources of variation.



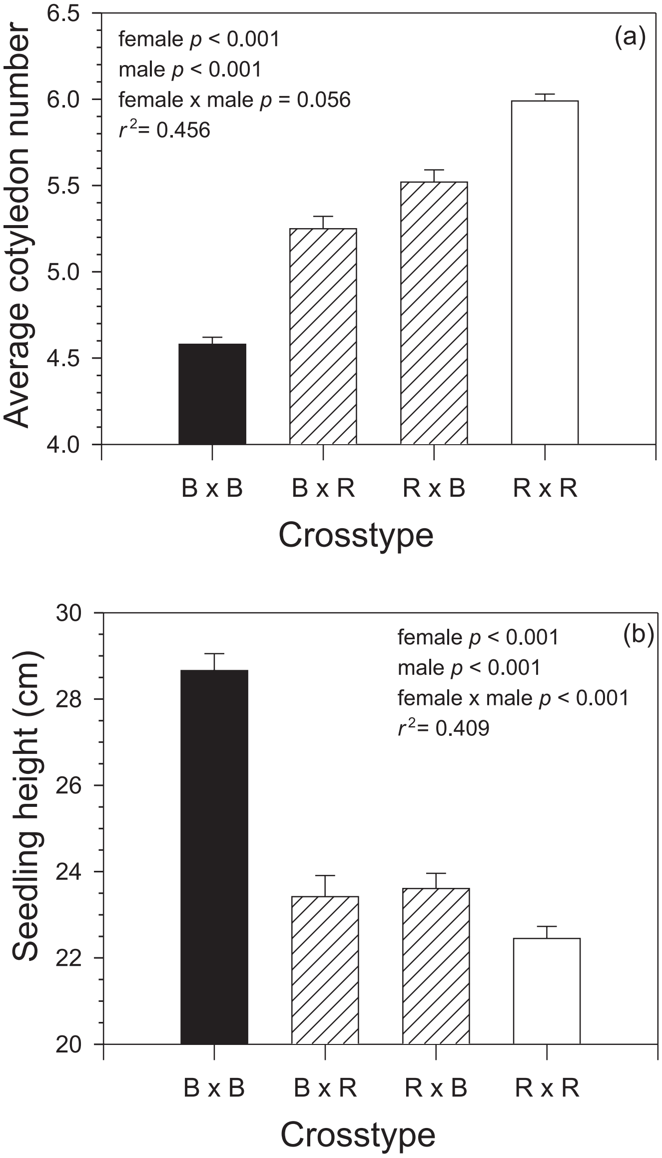
Discussion

Pollen, cone, and seed traits

Pollen dimensional differences between red and black spruce were due to pollen body size differences rather than to bladder dimensions (Table 3). The smaller black spruce pollen body in relation to bladder size probably makes the pollen lighter and increases its potential for dispersal, consistent with pioneer species ecology (Bazzaz 1979). However, pollen size traits are unlikely to be useful in characterizing natural hybrids or quantifying introgression, and are unlikely to find practical applications or be incorporated into hybrid indices based on morphological differences such as those described by Manley (1971). However, such differences are being used in paleobiological studies (Schauffler and Jacobson 2002). Previous studies have indicated the potential and consistent usefulness of cone and seed size traits (Gordon 1976; Mosseler et al. 2000; Major et al. 2003*a*), which are more readily observable and more likely to provide useful preliminary indicators of species, and levels of introgression, particularly for forest management decision-making purposes.

Seed size traits indicate that seed vigor may be adversely affected by hybridization, thereby reducing competitive ability in natural environments, particularly in hybrid seed from red spruce females. Important and highly consistent differences in cotyledon number between black and red spruce (Fig. 4*a*), and the intermediate cotyledon number in inter-specific hybrids present a useful indicator for assessing hybridization in seedling progeny, as does germination time, which was also seen in seeds from New Brunswick (Major et al. 2003*b*) produced by parents crossed by Manley (1975).

Fig. 4. Cotyledon number (*a*) and seedling height (*b*) for black spruce (B) and red spruce (R) intra- and inter-specific crosstypes. Values are means ± SE.



Phenological barriers

There were no reproductive phenology barriers for natural hybridization between black, red, or hybrid spruce, which all peaked between 220 and 240 GDD in a common garden experiment (Fig. 5). Phenological differences may still exist between some close natural stands of red and black spruce but are likely due to different local niche environments, such as cooler temperatures in black spruce bogs and warmer temperatures in upland hardwood red spruce mixedwood stands. It is interesting to compare the phenology of these spruces with white spruce (*Picea glauca* (Moench) Voss), the only other sympatric spruce, which is considered a mid- to late-successional species (Nienstaedt and Zasada 1990). White spruce has an earlier nonoverlapping reproductive phenology that peaks around 150 GDD compared with 220 for black spruce (P. Copis, unpublished data).

Crossability barriers

Crossability, defined as the percentage of filled to full-sized seeds, was 3.9% for black × red, and 2.5% for red × black (Table 4). Gordon (1976) reported an F_1 crossability between red spruce and black spruce of 0%–3%. Pollen species did not affect the number of aborted ovules, which represent undeveloped ovules without any pollen contact (Bramlett et al. 1977; Mosseler et al. 2000). Black spruce females had 8% more aborted ovules than red spruce, despite producing more filled seed. Both species had significantly more empty seeds as a result of being crossed with the pollen of the other species. The number of empty seeds was high, but this often occurs in controlled crosses when pollen from a single tree is applied (Gordon 1976).

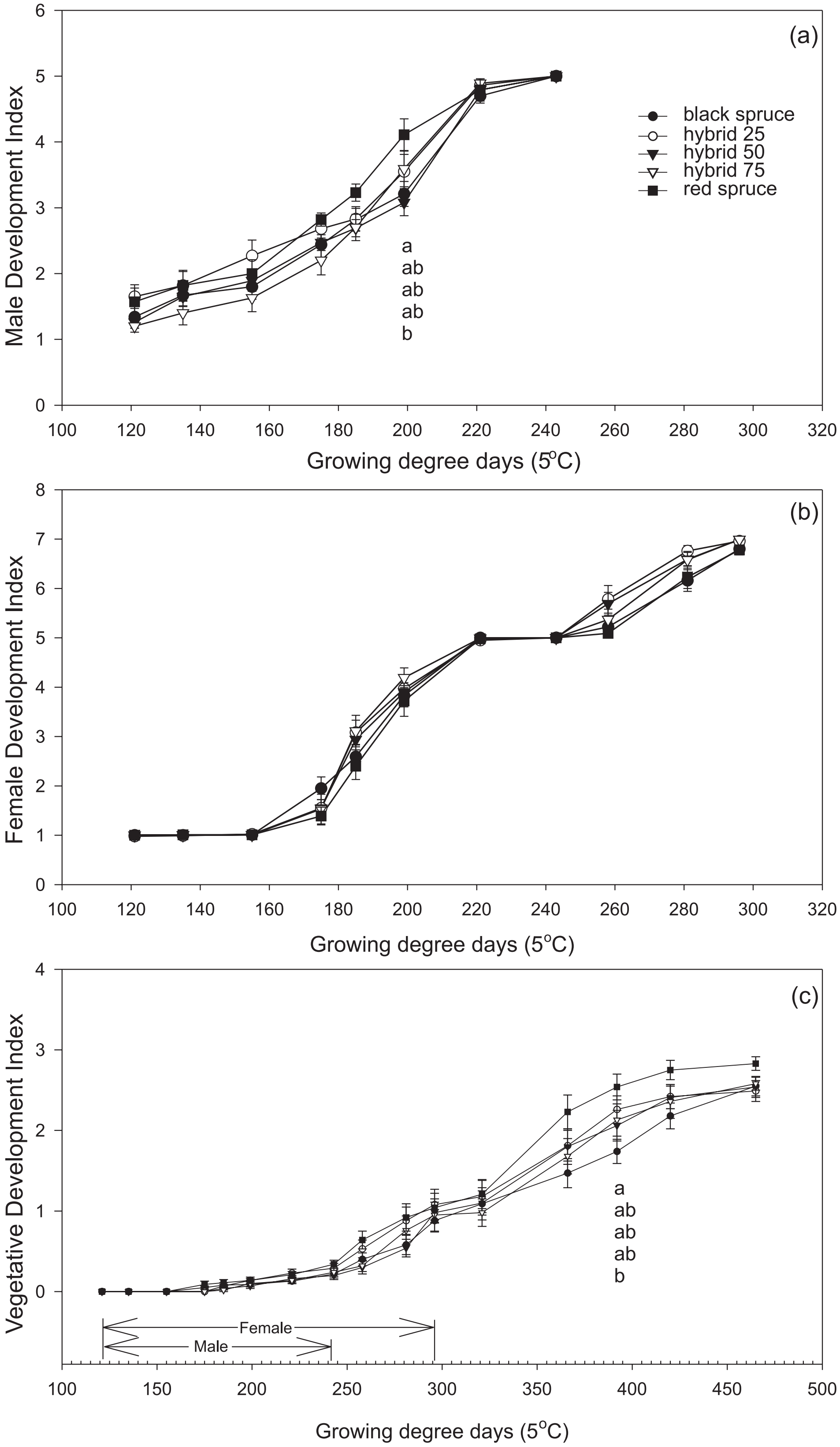
Filled seed in interspecific controlled crosses was 6.6 times lower than intraspecific crosses, and indicates a substantial crossability barrier to hybridization between red and black spruce. These low rates of viable seed production suggest that, under natural pollination conditions, very low levels of viable hybrid seed may be expected. Significant quantities of hybrid seed may only be produced under exceptional circumstances in which the supply of the same species pollen is overwhelmed by pollen of the other species (e.g., see Manley 1972).

Crossability of black and red spruce with white spruce was virtually zero as summarized by Gordon (1976). Both red and black spruce, but not white spruce, have high crossability with Serbian spruce (*Picea omorika* (Pancic) Purkyne), which is considered taxonomically similar to red spruce (Wright 1955), but allopatric to red and black spruce since the Cretaceous. However, using sequence-tagged site markers, Perron et al. (2000) suggested that black and red spruce are a progenitor–derivative species pair, with red spruce genetic diversity essentially a subset of that found in black spruce. The phenological and crossability barriers between white spruce and red and black spruce are very strong and distinct, and raise the question why stronger barriers did not develop between red and black spruce. This will be discussed further in the hybridization sections.

Germination barriers

Three of the four crosstypes showed little evidence of germination barriers, on average less than 10% (Table 5). Germination in one crosstype, black × red, was 12% lower than in the other crosstypes, because one cross had very low germination. Germination time was impacted by both species and interspecific crossing. Germination pattern follows both an additive and maternal effect, which reflects ecological adaptation differences between the species. For instance, early successional species, such as black spruce, germinate quickly and enter a logarithmic growth phase immediately upon germination, whereas red spruce seed germination is delayed over an extended period before accelerating. Species' germination times were similar to those reported for a range-wide experiment by Major et al. (2003*a*), in which black and red spruce had germination times of 17 and 24 d from sowing, respectively.

Fig. 5. Male (*a*), female (*b*), and vegetative bud (*c*) development indexes by growing degree days for hybrid indices 0, 25, 50, 75, and 100. Hybrid indices represent the percentage of red spruce, with the remaining proportion being black spruce. Lowercase letters indicate dates during which hybrid means were different at p = 0.05.



Performance barriers and heterosis

The F_1 interspecific hybrids produced a significant negative but not severe negative heterosis (performance worse than both parental species) in height consistent with a small but significant negative heterosis found in a mature tree study, but not with a 1-year-old seedling study using the same set of crosses (Major et al. 2003*b*). As discussed earlier, red and black spruces are so different in their adaptive and ecological profiles that negative heterosis might be expected in F_1 hybrids. Black spruce backcrosses produced a positive heterosis in seedling and mature tree height growth, as might be expected with more advanced crosses due to increased compatibility and effects on heterosis. In contrast with Manley and Ledig (1979), it appears that severe negative heterosis is of little importance as an isolating barrier between red and black spruce.

Genecological barriers

Ecological barriers would appear to be quite strong, given the very different ecological and adaptive traits (Major et al. 2003*a*, 2003*c*) and successional roles of red and black spruce (i.e., early- vs. late-successional forest types) (Blum 1990; Viereck and Johnston 1990; Seymour 1992). However, in areas of sympatry (most of the red spruce range), particularly those dominated by human disturbances such as clearcutting, increased opportunities for natural hybridization might be expected. Extensive harvesting of late-successional forest types, regeneration with early-successional species, and the increased juxtaposition of black and red spruce stands probably remove the natural ecological barriers to hybridization.

The formation of hybrids creates problems for forest managers in identification of species, determining the amount of hybridization and introgression, and thus in implementation of ecologically appropriate silvicultural practices (Manley 1971; Beylor 1999). As a long-lived, shade-adapted species, red spruce often responds poorly to clearcut harvesting (Major et al. 2003*b*, 2003*c*), whereas black spruce establishes and responds well. If the aim of forest management is to promote natural or artificial regeneration following forest harvesting, then it is important that species adaptation and hybridity be better understood to select the most appropriate silvicultural methods.

Hybridization

Petit et al. (1997) postulated that hybridization between sympatric early- and late-successional species of oak (*Quercus* spp.) was a step in the recolonization of the late-successional species in its migration north following glacial retreat. In the migration advance, the late-successional species would pollinate females of the early-successional species to establish hybrids. These hybrids would eventually be displaced by the late-successional species capitalizing on its adaptive traits (e.g., shade tolerance). It was suggested that interspecific hybridization in this direction would have higher success than hybridization in the opposite direction. A similar phenomenon could be postulated for the red, black, and hybrid spruce complex. Our data show a 56% higher seed set with significantly more filled seed for the black × red than red × black crosstypes (Table 4). A virtually identical reproductive phenology and differential crossability makes this late-successional migration theory plausible for these spruces. Nkongolo et al. (2003) recently claimed that the presence of putative species-specific random amplified polymorphic DNA (RAPD) markers suggests widespread introgression within natural black spruce and red spruce populations that would otherwise be identified as "pure" species based on their morphological traits or ecological contexts. They use this putative RAPD marker evidence to question the reliability and usefulness of conventional morphological and ecological evidence for distinguishing these species. We contend that their putative species-specific RAPD fragments could not distinguish among individuals that clearly fit into red or black spruce species based on morphological, ecological, reproductive, and ecophysiological adaptive characteristics, which are the traits that determine species delineation and identity in the first place. RAPD markers are dominant markers and have problems in reproducibility (Riedy et al. 1992; Ellsworth et al. 1993). In fact, none of the putative red spruce and black spruce species-specific RAPD markers identified so far has been found to be truly species specific in repeated analyses in our lab (unpublished data). Therefore, identification of such RAPD markers as species specific may be very imprecise. Many of the so-called species-specific RAPD markers for black and red spruce may indeed represent rare or low frequency intraspecific RAPD variants. More reliable, codominant, and reproducible makers, such as allozymes (e.g., Rajora 1990), microsatellites (e.g., Rajora and Rahman 2003), and actual DNA sequence data, would provide better tools for molecular identification of red spruce, black spruce, and their interspecific hybrids. Indeed, reliable molecular or genetic markers, in combination with and linked to morphological, ecological, reproductive, and ecophysiological characteristics, are the ones that we must focus on to study the extent of natural interspecific hybridization and to make sound management decisions with respect to silviculture, conservation, sustainable management, and restoration activities.

It is the ecological context that will determine the fate of interspecific hybrids and introgressants. Our results on reproductive barriers demonstrate that, under the absence of human disturbance, opportunities for interspecific hybridization are very low, and the suggestion that there are "likely no reproductive barriers, nor ecological isolation" (Nkongolo et al. 2003) between these species is untenable. The hybrid index approach proved useful for differentiating genotypes across a black spruce – red spruce continuum (Johnsen et al. 1998). In fact, the parents selected to produce the range of crosstypes used by Johnsen et al. (1998) were selected solely on needle, shoot, and cone morphological traits (Manley and Ledig 1979) and yet, not only was the hybrid index value highly heritable, but the progenies' hybrid index was strongly correlated with growth performance of the trees in the field, clearly showing the robustness and applicability of the approach.

Perron et al. (2000) examined 26 sequence-tagged site loci to explore the genetic relationship between black and red spruce. They found no unique alleles in red spruce and suggested red spruce may have evolved from black spruce via allopatric speciation because of glaciation-induced isolation. Their conclusions may be logical given the limited number of loci they studied; however, red spruce has genetic traits that clearly distinguish it from black spruce, including partial sexual incompatibility (this study), seedling growth rate (this study), growth rate in the field (Johnsen et al. 1998), cone, leaf, and stem morphology (Manley 1971; Johnsen et al. 1998), and adaptive traits (Major et al. 2003*a*, 2003*c*). Again, we emphasize caution must be applied to interpretations based solely on molecular markers as, so far, they provide a limited (albeit still important) view of the spruce genome and that available molecular data do not reflect adaptive differences.

The development and application of molecular genetic tools for differentiating red and black spruce and their hybrids would permit more extensive in situ sampling across the landscape relative to morphological and physiological screening in common garden experiments. However, the reliability of molecular markers in distinguishing between closely related species and their hybrids will only be confirmed when they are related to the morphological and ecophysiological traits that distinguish red and black spruce and their hybrids.

Conclusions

Crossability and ecological barriers appear most important in maintaining the separation of sympatric red and black spruce. Negative heterosis appears to be of lesser importance as an isolating barrier between the two species. Interspecific hybridization between red and black spruce results in a large decrease in reproductive fitness measured by seed set, and the few seeds produced appear to have decreased competitive ability. The lack of evidence for natural selection for phenological differences between red and black spruce suggests that physical ecological (e.g., niche or habitat) separation, based on the ecophysiological differences (e.g., shade tolerance, and habitat differences between early- and late-successional forest environments), represents an adequate and important prezygotic barrier for minimizing the negative fitness effects of hybridization. This has important implications for the proper silviculture to maintain these two spruces on the landscape. There are significant barriers to hybridization, except under exceptional circumstances created by disturbance of natural environments, such as clearcutting. In such cases, the relatively large amount of ubiquitous black spruce pollen may inundate the few remaining red spruce individuals, creating natural hybrids, despite the presence of substantial barriers. Thus, the elimination of late-successional forest types, and the increased juxtaposition of increasingly isolated individuals or small, remnant populations of red spruce within a forested landscape dominated by black spruce, may be exacerbating the impacts of hybridization on the red spruce gene pool. The development of a careful silvicultural strategy to maintain red spruce should be of high priority.

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