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Improvement of spring canola *Brassica napus* by use of winter canola

Habibur Rahman[1] and Berisso Kebede

Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Alberta, Canada T6G 2P5
[1]Corresponding author: Habibur.rahman@ualberta.ca

Abstract

Further improving of seed yield, other agronomic and seed quality traits in spring canola *Brassica napus* requires broadening of genetic diversity in this crop. The European winter oilseed *B. napus* is known to be genetically diverse from spring oilseed *B. napus*. We hypothesized that elite spring canola *B. napus* lines with greater seed yield and genetic diversity can be achieved through the use of European winter *B. napus*. For this, a winter x spring *B. napus* breeding program was undertaken at the University of Alberta, and pedigree and doubled haploid breeding techniques were applied. Spring canola lines with significantly increased seed yield were obtained in one cycle of breeding. Estimates of genetic diversity based on SSR markers revealed that some of the spring canola lines derived from winter x spring crosses possess greater diversity compared to the spring parents. The major constraint of this winter x spring breeding is that lateness of flowering and maturity are generally introduced into the spring type lines which would require repeated cycle of breeding for improvement.

Key words : *Canola, Brassica napus, genetic diversity, germplasm improvement*

Introduction

Genetic improvement in a crop through plant breeding essentially require existence of adequate genetic diversity within the gene pool. In a breeding program, development of best recombinant inbred lines through genetic recombination and selection may be exhausted when plant breeding is based on a restricted gene pool. A decline in allelic variation and genetic diversity over a period of breeding has been reported by Fu and Gugel (2010) in case of a Canadian spring *B. napus* breeding program. Similarly, loss of genetic diversity over several generation of breeding has been reported in case of Australian spring *B. napus* (Cowling, 2007). Therefore, broadening of genetic diversity is needed in spring *B. napus* for improvement in this crop from long-term perspectives. Furthermore, hybrid spring *B. napus* cultivars, despite of their higher seed price, is getting higher acceptance by the growers primarily due to high seed yield. The need of genetic diversity in the hybrid parental lines has been demonstrated by several researchers (Lefort-Buson, *et al.* 1987; Dies, *et al.* 1996; Ali *et al*. 1995; Riaz *et al.* 2001). The winter type *B. napus*, which is primarily grown in Europe, is known to be genetically diverse from spring type (Diers and Osborn, 1994; Becker *et al.* 1995; Hasan *et al.* 2006), hence can be used for the improvement of the spring type.

Several researchers have demonstrated the potential of using the winter *B. napus* germplasm for increasing seed yield in hybrid (Butruille *et al*., 1999; Quijada *et al*., 2004; Udall *et al*., 2004) or open-pollinated (Kebede *et al*. 2010) spring *B. napus* cultivars. A cultivar developed in a plant breeding program carry not only a single desirable trait, e.g. high seed yield or oil content; rather it is a package of different improved traits for the benefit of the growers and/or the end users. In case of hybrid cultivars, these traits are the result of genetic architecture of the male and female parents; and improvement for many of these traits is, therefore, often needed in both parental lines. Combining all desirable traits and making incremental progress in the cultivars and/or elite breeding lines is a major challenge to the breeders when using exotic gene pool which often carry many undesired traits. In case of a winter x spring breeding program, the breeding steps and selection process involved may differ greatly from that of spring x spring breeding program due to involvement of vernalization and other undesired genes from the winter type. Information on breeding for the development of elite spring canola lines/cultivars from a winter x spring program is not available in literature. In this paper we report a canola breeding program that has been undertaken at the University of Alberta for the development of RoundUp and Clearfield herbicide tolerant elite and genetically diverse spring canola *B. napus* lines/cultivars by use of the European winter canola *B. napus*

Materials and Methods

Parents, crosses and generation of F_2

A total of eight winter x spring canola (*B. napus*) crosses were made: six crosses, viz. Aviso x A03 3NR, Aviso x A01-20694NR, Tequila x A03-3NR, Pollen x A99-13NR, 21290 x A99-13NR and Smart x SP Banner, were aimed for the development of RoundUp herbicide tolerant spring canola, while two crosses, viz. Express-IMI x Cougar and Smart x A03-14NI, were aimed for the development of Clearfield herbicide tolerant spring canola. The parents Aviso, Tequila, Pollen and Smart are winter canola cultivars registered in Europe, 21290 is a winter canola breeding line, and all are non-herbicide tolerant type. Seeds of these genotypes were obtained from Dr. Werner Horn, SW Seeds, Germany. Express-IMI is a F_4 line developed from a cross between a non-herbicide tolerant winter canola cultivar Express and a Clearfield herbicide tolerant spring canola cultivar 45A71 with selection for winter growth habit (does not flower without vernalization) and tolerance to Clearfield herbicide. Seeds of Express and 45A71 were obtained from NPZ-Lembke, Germany, and Pioneer Hi-Bred through BASF, respectively. Cougar and A03-14NI are Clearfield herbicide tolerant canola, and A03-3NR, A99-13NR and A01-20694NR are RoundUp herbicide tolerant canola; and all these spring cultivars/lines were developed at the University of Alberta. SP Banner is a RoundUp herbicide tolerant spring canola cultivar developed by Saskatchewan Wheat Pool (currently, Viterra).

The winter canola lines/cultivars were seeded in a heated greenhouse ($20^0/16^0$C day/night, 16 hrs light); and the plants at the age of four weeks after seeding were transferred to a growth chamber set at 4^0C with photosynthetic flux density of 130 μE m^{-2} s^{-1} at plant level (9 hrs light, 15 hrs dark) for eight weeks for vernalization. After vernalization, plants were moved to the greenhouse, where the spring canola cultivar/lines were seeded about three weeks prior to this time, and crosses were made using winter canola as female. The F_1 plants were grown in a greenhouse, vernalized for six weeks as mentioned above, and were self-pollinated by bag isolation for F_2 seeds.

Study on segregation for flowering in F_2

QTL mapping of flowering time in *B. napus* disclosed that at least four genomic regions, with different degree of effect, are involved in vernalization responsive flowering (Osborn *et al.* 1997, Kole *et al.* 2002). However, information on F_2 segregation for vernalization-independent flowering is scarce in literature, and this information is needed for selection of spring growth habit type plants from winter x spring crosses. Therefore, the F_2 population of Aviso x A03-3NR together with its spring parent and F_1 were grown as a separate experiment to study segregation for this trait. Plants were grown in 32-cell tray (cell size, 6.5 cm x 6.5 cm x 8.5 cm, breadth x width x depth) in a greenhouse ($22^0/16^0$C day/night, 16 hrs light) in two times (H" replicates) with 19 days interval between first and second seeding (June 25th and July 14th). Flowering date of the individual plants was recorded at the time of first opened flower. The experiments were terminated at 130th day after seeding, and at this stage all non-flowering plants were considered to be winter growth habit type.

Generation of pedigree and doubled haploid (DH) lines

Six to nine hundred F_2 plants from each cross were grown in a heated greenhouse ($20^0/16^0$C day/night, 16 hrs light), and sprayed at 2-3 leaf stage with either RoundUp @ 1 ml RoundUp in 399 ml water or Clearfield herbicide @ 0.76g Odyssey plus 5 ml Merge as surfactant per litre water. Herbicide tolerance was evaluated one week after spray and 240 most tolerant plants from each cross were retained. The F_2 plants which flowered without the need of vernalization were self-pollinated for F_3 seeds. The F_2 plants of few crosses were subjected to microspore culture and DH lines were produced.

For production of DH lines, flower buds from 15-20 most early flowering F_2 plants were used and bulk culture of microspores was done. Isolated microspores were cultured in Nitsch and Nitsch medium (Lichter, 1985) without hormones but with 13% sucrose and 50 mg L^{1} colchicine (Möllers *et al.* 1994). Cotyledonary embryos were transferred to B5 medium containing gibberillic acid (0.1 mg L^{-1}) and solidified with 0.8% agar (Coventry *et al*., 1988). Germinated embryos, at an age of 4 to 6 weeks after transfer to B5 medium, were transplanted to a soil-free mix in the greenhouse. The pollen-producing plants were considered to be chromosome doubled, and were self-pollinated for harvest of seeds.

Field evaluation of the pedigree and DH lines

The F_3 families were evaluated in field nursery at the Edmonton Research Station of the University of Alberta in single replication nursery with checks in every 10 to 15 plots. Plot size was 3 m x 1 m with four rows. The early flowering F_3 plants were self-pollinated and F_4 families were generated. The F_4 families and the DH lines were evaluated in field nursery in 2006 at the same research farm in same size plots, as mentioned above. The selected F_4 families (evaluated as F_5 lines) and DH lines were evaluated in yield trials in 2007 in seven locations in Alberta and Manitoba, Canada, with three replications in each location. In field experimentations, the official check cultivars and/ or the available cultivar/elite breeding lines were used as checks. The following agronomic and seed quality data were recorded: herbicide tolerance, days to flowering, maturity, seed yield, and seed oil, protein, glucosinolate and saturated fatty acid contents. In case of the Clearfield program, herbicide tolerance was recorded in 0 to 9 scale, where 9 = no visible herbicide injury and 0 = plants died; and in case of the RoundUp program, the plants were scored either tolerant or susceptible (dead). Days to flower in greenhouse was recorded at first opened flower stage; while in field experimentation it was recorded when about 10-15% plants in the plots had at least one open flower. Days to maturity was recorded when silique of the spring canola checks started to turn to straw-brown colour, and was recorded in 1 to 9 scale, where 9 = most early (can be desiccated right away) and 1 = extreme late (cannot be matured in the growing season). Plots were harvested using plot combine, and seed yield was recorded as Hkg ha^{-1}.

The F_5 and DH lines were also evaluated for resistance to blackleg disease in Thornhill, Manitoba (Agriprogress Inc., Morden) in 2007 following the procedure recommended by the Western Canola/ Rapeseed Recommending Committee (WCC/RRC) for registration of canola cultivars in western Canada.

Seed quality analysis

Seed oil, protein and glucosinolate contents were estimated by near-infrared spectroscopy (NIRS, Model 6500, Foss North America, Eden Prairie, MN) following the protocol approved by the Canadian Grain Commission (Daun *et al*. 1994). Oil and protein contents were expressed as percent of whole seed dry weight basis, and glucosinolate content on whole-seed basis (8.5% moisture) and expressed as ìmol g^{-1} seed. Total saturated fatty acid content (sum of C12:0, C14:0, C16:0, C18:0, C20:0, C22:0 and C24:0) of oil was measured by gas chromatography. All these seed quality analyses were done in the Analytical Laboratory of the Canola Breeding program of the University of Alberta, which is accredited by the Canadian Grain Commission for these analyses.

Genotyping of the lines from winter x spring crosses

A total of 17 lines, 11 elite DH/F_5 lines from different winter x spring crosses and their 6 parents, were genotyped by use of simple sequence repeat (SSR) markers. For this, fresh leaf samples were used to extract DNA using a SIGMA DNA extraction Kit (Sigma-Aldrich, St Louis, USA) following the manufacturer's instructions. Publicly available SSR markers from Agriculture and Agri-Food Canada (http://www.brassica.agr.gc.ca/index_e.shtml) were used for genotyping the lines. Polymerase chain reactions (PCR) were carried out in a volume of 10 µl containing 15 ng of template DNA, 1 pmol of each forward and reverse primers, 0.2 mM dNTPs mix, 2.5 mM $MgCl_2$, 1 x PCR reaction buffer, and 0.25 unit of Taq DNA polymerase (ABI, California, USA). To reduce primer labelling cost, the PCR products were labelled following the M13-tailing technique as described by Schuelke (2000). The forward primer of each SSR was appended with the universal M13 primer sequence 5'-CACGACGTTGTAAAACGA C-3' fluorescently labelled with infra red (IRD), FAM, VIC, NED and PET dyes. The detection of the amplification products were performed on capillary ABI sequencer No. 3730 (ABI, California, USA).

Statistical Analysis

Basic descriptive statistics including mean, variance, standard error, correlation, etc. were calculated using EXCEL worksheet, and analysis of variance was calculated using PROC MIXED procedure of SAS (SAS Institute, 2003). In case of molecular marker analysis of the parents and F_5/DH lines, a genotypic data matrix was prepared from the results of SSR marker analysis. For this, the presence of a fragment (ABI peak) was scored as 1 and absence as 0. The percentage of winter alleles in each F_5/DH line was calculated based on the number of alleles unique to the winter parent divided by total number of alleles detected by all markers multiplied by one hundred. Pair-wise genetic similarities were used to calculate Dice's (Nei and Li, 1979) similarity coefficients followed by cluster analysis (unweighted pair-group method with arithmetic mean, UPGMA). The UPGMA coefficients were used to draw dendrogram using the computer software NTSYS PC 2.2 (Rohlf, 2000). Standardized SSR data matrix of the lines was used to generate eigenvalues for principal coordinate analysis using NTSYSpc (Rohlf, 2000). The principal coordinate were drawn using a two dimensional graph.

Results and Discussion

Segregation for days to flower in F_2

A total of 384 and 373 F_2 plants of Aviso x A03-

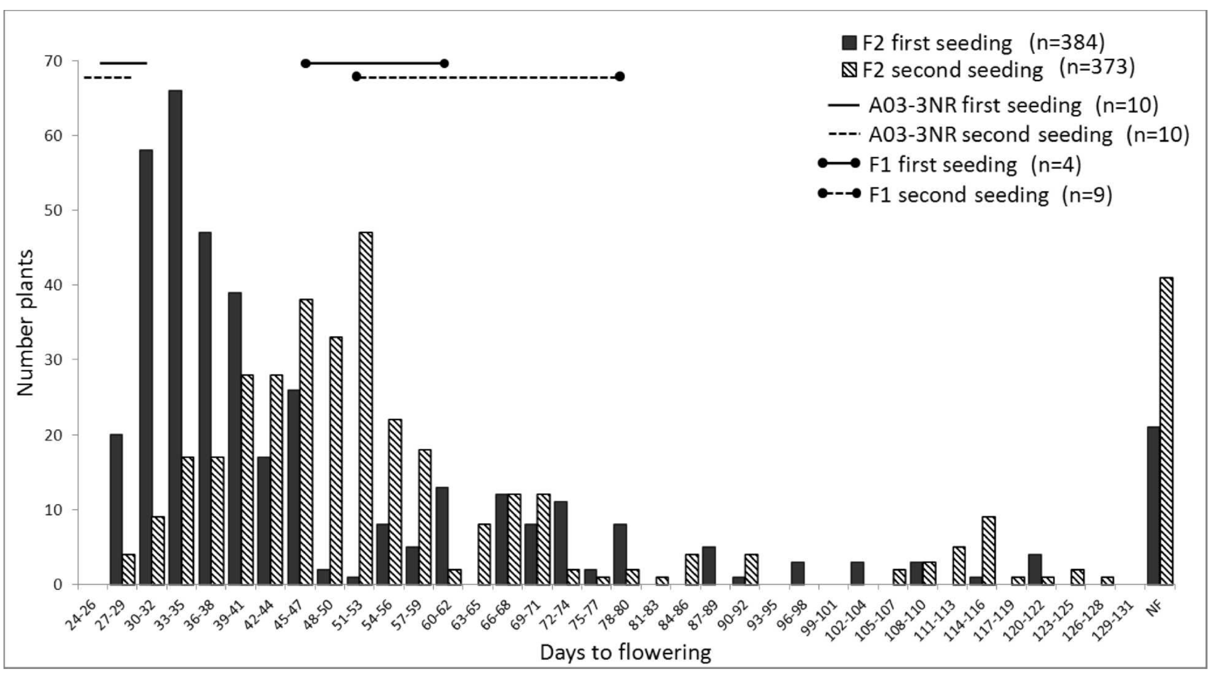

Fig. 1. Frequency distribution for days to flowering of F_2 populations of Aviso (winter) x A03-3NR (spring) cross of *Brassica napus* seeded in greenhouse at two different dates (June 25th and July 14th)

3NR were grown from first and second seeding. In both cases, the spring parent A03-3NR required almost similar number of days to flower and all plants flowered within five days. However, significant variation for flowering was found in F_1 despite this generation expected to be homogeneous. The distribution of the F_2 population differed significantly between the first and second seeding (Fig. 1). In case of first seeding, the distribution skewed towards earliness where 67.4% of the plants flowered within 46 days after seeding, i.e. before the F_1 started to flower. In case of the second seeding, the F_1 plants required six more days to flower, and the F_2 population followed almost a normal distribution until 80th days after seeding. In this case, 49.9% of the plants flowered by the time F_1 started to flower. In case of first seeding, 13.8% plants flowered within the flowering range of the spring parent A03-3NR while only 0.5% plants flowered within this range in case of the second seeding (Fig. 1). The effect of seeding time on flowering of the F_2 plants is also evident from the number of non-flowering plants. In case of first seeding, 5.5% F_2 plants failed to flower at 130th day after seeding, while almost double number of plants (11%) failed to flower at this stage in case of second seeding.

Table S1. The size of the winter x spring (*Brassica napus*) breeding program at the University of Alberta based on number of lines/families evaluated in field nursery in 2005 and 2006, and the selected lines evaluated in multi-location trials in 2007. Number crosses involved are given in brackets. Superscripted numbers indicate the number of F_3 families from where the F_4 families are derived.

Generation	**2005**	**2006**	**2007**
RoundUp herbicide tolerant			
F_3	148 (4)	-	-
F_4	-	166^{42} (2)	-
F_5	-	-	1 (1)
DH	-	459 (4)	6 (2)
Total	**148**	**625**	**7**
Clearfield herbicide tolerant			
F_3	86 (2)	-	-
F_4	-	167^{26} (1)	-
F_5	-	-	4 (1)
DH	-	22 (1)	-
Total	**86**	**189**	**4**

Agronomic and seed quality of the pedigree and DH lines

Of the six winter x spring crosses, where pedigree breeding was applied (Table S1), agronomic and seed quality data from two crosses, Aviso x A03-3NR and Express-IMI x Cougar, presented as example. Similarly, of the five crosses where DH breeding was applied, data only from Pollen x A99-13NR cross is presented. In general, the F_3 families flowered and matured much later compared to the spring check cultivars/lines as well as had lower oil content (Table 1 and 2). Most of the families showed wide variation for flowering between the individual

Table 1. Agronomic and seed quality data of the winter x spring F_3 and F_4 families of Aviso (winter) x A03-3NR (spring) cross for the development of RoundUp herbicide tolerant spring canola *Brassica napus.*

Year	Genotypes		Days to flower	Days to maturity	Seed yield (hkg ha^{-1})	Relative seed yield (%)	Seed oil (% DM)	Seed protein (% DM)	Glucosinolate (µmol/g seed)	Saturated fatty acid (%)
2005	F_3	N[x]	88	-	-	-	55	55	55	-
		Range	42 – 65[y]	-	-	-	43.7 - 50.2	20.4 - 28.2	8.6 - 19.2	-
		Mean ± SE	51.1 ± 0.6	-	-	-	47.4 ± 0.2	24.9 ± 0.2	11.3 ± 0.3	-
	Conquest	N	8	-	-	-	8	8	8	-
		Range	48 - 51	-	-	-	47.9 - 50.6	23.8 - 26.8	8.3 - 9.3	-
		Mean ± SE	49.4 ± 0.5	-	-	-	49.1 ± 0.3	25.5 ± 0.4	8.9 ± 0.2	-
2006	F_4[a]	N	149[z]	149	115	115	83	83	83	68
		Range	41 - 52	2 – 8	17.5 - 59.5	63.0 - 215.0	44.2 - 53.5	26.3 - 28.6	10.0 - 16.5	6.1 - 7.3
		Mean ± SE	46.0 ± 0.3	5.9 ± 0.1	34.9 ± 0.5	125.8 ± 1.9	48.6 ± 0.2	25.7 ± 0.2	12.8 ± 0.1	6.8 ± 0.03
	A03-3NR	N	6	6	6	6	5	5	5	2
		Range	41 - 42	7 – 8	17.9 - 31.7	75.2 - 114.2	47.5 - 49.0	25.8 - 27.2	11.5 - 12.2	7.02-7.03
		Mean ± SE	41.3 ± 0.2	7.7 ± 0.2	24.1 ± 2.2	86.8 ± 7.9	48.4 ± 0.3	26.2 ± 0.3	11.9 ± 0.1	7.03± 0.01
	SP Banner	N	6	6	6	6	-	-	-	-
		Range	41 - 44	7 – 8	26.2 - 33.3	94.5 - 120.1	-	-	-	-
		Mean ± SE	42.3 ± 0.6	7.7 ± 0.2	31.4 ± 1.1	113.2 ± 3.9	-	-	-	-

[x]Number families from where data collected

[y]Correlation for days to flowering between the F_2 plants in greenhouse and F_3 family in field = 0.667*** (df = 85).

[z]Derived from 35 F_3 families

[a]One F_4 family selected which was tested in 2007 trials as F_5 line; selection intensity = 0.67%

N.B. throughout the Tables 2 to 4, seed yield was not recorded for the late flowering and maturing plots; and the families/lines selected based on earliness and seed yield were subjected to seed quality analysis.

Table 2. Agronomic and seed quality data of the winter x spring F_3 and F_4 families of Express-IMI (winter) x Cougar (spring) cross for the development of Clearfield herbicide tolerant spring canola *Brassica napus*

Year	Genotypes		Herbicide tolerance	Days to flowering	Days to maturity	Seed yield (hkg ha^{-1})	Relative seed yield (%)	Seed oil (% DM)	Seed protein (% DM)	Glucosinolate (µmol/g seed)	Saturated fatty acid (%)
2005	F_3	N[x]	53	41[y]	-	-	-	33	33	33	-
		Range	2 - 8	41 - 63[y]	-	-	-	43.0 - 48.8	24.6 - 29.9	8.8 - 16.7	-
		Mean ± SE	5.3 ± 0.3	54.6 ± 0.7	-	-	-	45.7 ± 0.2	27.2 ± 0.2	11.6 ± 0.3	-
	Cougar	N	6	6	-	-	-	4	4	4	-
		Range	7 - 8	43 - 47	-	-	-	47.2 - 49.8	25.7 - 28.0	10.5 - 11.9	-
		Mean ± SE	7.3 ± 0.2	45.0 ± 0.7	-	-	-	48.4 ± 0.6	27.2 ± 0.5	11.0 ± 0.3	-
2006	F_4[a]	N	149[z]	149	149	127	127	76	76	76	29
		Range	7 - 8	46 - 58	3 – 8	12.2 – 57.9	46.8 – 222.1	43.3 - 48.4	25.8 - 30.5	9.4 - 25.9	6.2 - 7.9
		Mean ± SE	7.7 ± 0.04	53.3 ± 0.2	5.1 ± 0.1	24.0 ± 0.8	92.0 ± 2.9	45.8 ± 0.1	28.6 ± 0.1	14.5 ± 0.3	6.7 ± 0.04
	Cougar	N	5	5	5	4	4	-	-	-	-
		Range	7 - 8	46 - 49	7 – 7	21.3 - 32.7	81.8 - 125.3	-	-	-	-
		Mean ± SE	7.8 ± 0.2	48.4 ± 0.6	7.0 ± 0.0	25.0 ± 2.7	95.7 ± 10.2	-	-	-	-
	45A71	N	4	4	4	4	4	3	3	2	1
		Range	7 - 8	49 - 51	5 – 7	21.2 - 38.8	81.5 - 148.7	46.5 - 46.9	27.3 - 27.9	13.8 - 15.2	6.7
		Mean ± SE	7.3 ± 0.3	50.0 ± 0.6	6.3 ± 0.5	27.2 ± 4.1	104.3 ± 15.8	46.8 ± 0.1	27.6 ± 0.2	14.4 ± 0.4	6.7
	72P01CL	N	2	2	2	2	-	2	2	2	1
		Range	7 - 8	46 - 49	7 – 7	18.3 - 18.9	-	47.1 - 47.5	27.8 - 28.0	11.1 - 11.7	6.4
		Mean ± SE	7.5 ± 0.5	47.5 ± 1.5	7.0 ± 0.0	18.6 ± 0.3	-	47.3 ± 0.2	27.9 ± 0.1	11.4 ± 0.3	6.4

[x]Number families from where data collected

[y]Correlation for days to flowering between the F_2 plants in greenhouse and F_3 family in field = 0.294 NS (df = 26)

[z]Derived from 35 F_3 families

[a]Four F_4 families selected which were tested in 2007 trials as F_5 lines; selection intensity = 2.68%

Table 3. Agronomic and seed quality data of the DH lines of Pollen (winter) x A99-13NR (spring) cross for the development of RoundUp herbicide tolerant spring canola *Brassica napus*

Year	Genotypes		Days to flowering	Days to maturity	Seed yield (hkg ha^{-1})	Relative seed yield (%)	Seed oil (% DM)	Seed protein (% DM)	Glucosinolate (µmol/g seed)	Saturated fatty acid (%)
2006	DH[a]	N[x]	113	113	100	100	79	79	79	33
		Range	44 - 54	4 - 8	25.8 - 55.1	82.6 - 176.1	43.6 - 50.0	22.3 - 29.4	11.0 - 19.5	6.0 - 6.6
		Mean ± SE	49.7 ± 0.2	5.1 ± 0.1	40.4 ± 0.6	129.0 ± 2.0	46.8 ± 0.2	26.1 ± 0.2	14.1 ± 0.2	6.3 ± 0.03
	A03-3NR	N	10	10	10	10	5	5	5	4
		Range	41 - 42	8 - 8	23.8 - 30.5	76.2 - 109.7	48.5 - 49.3	25.5 - 26.4	10.9 - 12.4	6.9 -7.1
		Mean ± SE	41.1 ± 0.1	8.0 ± 0.0	28.4 ± 0.9	90.7 ± 2.9	48.9 ± 0.2	25.9 ± 0.2	11.9 ± 0.3	7.02± 0.03
	SP Banner	N	2	2	6	6	-	-	-	-
		Range	43 - 44	7 - 7	29.1 - 40.7	92.9 - 130.1	-	-	-	-
		Mean ± SE	43.5 ± 0.5	7.0 ± 0.0	34.9 ± 5.8	111.5 ± 18.6	-	-	-	-

[x]Number lines from where data collected
[a]Three DH lines selected for test in 2007 trials; selection intensity = 2.65%

plants. Therefore, a low selection pressure was applied in this generation primarily to include a greater number of spring growth habit plants. Based on herbicide tolerance, days to flower, seed oil, protein and glucosinolate contents, a total of 61 F_3 families of the two crosses were selected.

Two hundred ninety eight F_4 families, derived from self-pollination of herbicide tolerant early flowering F_3 plants of Aviso x A03-3NR and Express-IMI x Cougar (Table 1 and 1), and 113 DH lines of Pollen x A99-13NR (Table 3) were grown in field nursery in 2006. In general, the F_4 families and the DH lines were late flowering and maturing compared to the check cultivars/lines. When selection for days to flowering and maturity comparable to the check was applied, only 0 to 6% of the families/lines could be selected. Therefore, less stringent selection was applied with the view of improving these traits in the next cycle of breeding, if needed.

A selection criterion was developed for the F_4 families and DH lines which was: days to flowering $\leq$ 50 or 53, maturity score $\geq$ 6, oil and protein contents $\geq$ check or check mean, glucosinolate content $\leq$ check or check mean, and saturated fatty acid = 6.4% ($\simeq$72P01CL, a recently registered cultivar, Rahman *et al*. 2011). For seed yield, wide variation was recorded between plots of the same check cultivar suggesting that non-genetic factors, e.g. experimental errors associated with yield estimation from small nursery plots, contributed significantly to this variation. Selection of the F_4 and DH's was, therefore, done based on relative seed yield of the check cultivar in the neighbouring plots.

The three populations derived from winter x spring crosses varied significantly for seed quality traits. For example, more than 50% of the F_4 families from Aviso x A03-3NR met the selection criteria for oil content; while less than 15% of the families/lines from the other two crosses could meet this criterion (Table S2). In case of saturated fatty acid, more than 70% DH lines from Pollen x A99-13NR had the content $\leq$ 6.4%. Several families/lines meeting selection criteria for an individual trait could be found in all three populations (Table S2); however, none of the families/lines could be selected when

Table S2. Percentages of F_4 families/DH lines meet selection criteria individually for different agronomic and seed quality traits. Selection criteria: days to flowering $\leq$ 50 (Aviso x A03-3NR and Pollen x A99-13NR) or 53 (Express-IMI x Cougar), maturity score $\geq$ 6, oil and protein contents $\geq$ check or check mean, glucosinolate $\leq$ check or check mean, and 6.4 for saturated fatty acid content.

Cross	Days to flowering†	Days to maturity†	Seed oil	Seed protein	Glucosi nolate	Saturated fatty acid
Aviso x A03-3NR	85.9	71.1	57.8	34.9	25.3	7.3
Express-IMI x Cougar	64.4	28.9	13.2	84.2	31.6	13.8
Pollen x A99-13NR	60.2	10.6	8.9	50.6	5.1	72.2

†If selection criteria for days to flower $\leq$ check mean and for maturity $\geq$ check mean are applied, 0 to 6% of the families depending on the crosses meet the criteria, and therefore, less stringent selection was applied for these traits. Selection criteria for Express-IMI x Cougar was even less stringent as this population flowered 4-7 days later than the other two populations.

selection for multiple traits was applied. Therefore, less stringent selection for some of the traits was needed. For example, in case of Aviso x A03-3NR, 36 F_4 families met the selection criteria for days to flowering, maturity and oil content; however, only 2 of these 36 met the selection criteria for protein content, and none of these 2 met the criteria for other traits. By changing selection criteria for protein content 1% less than the check, 15 of the 36 families were selected, where 3 met the criteria for glucosinolate content but none of these 3 could meet the criteria for saturated fatty acid. Therefore, less stringent selection was again needed for glucosinolate content; and finally one family was selected for multi-location yield trials in 2007 (Table S3). Following similar approach of selection, 4 and 3 lines respectively from Express IMI x Cougar and Pollen x A99-13NR crosses were selected for 2007 trials.

Data from 2007 trials for the F_5 and DH lines, 8 from the above-mentioned three crosses and 3 from other crosses, is presented in Table 4. Of the total 11 lines, nine (82%) produced seed yield either comparable or greater than the checks 46A65 and Q2, and 1 line (9%) yielded significantly higher than the hybrid cultivar 45H21. In general, the lines from winter x spring crosses were late in flowering and maturity and had lower oil content compared to the spring checks. However, variation for these traits was present among these lines, where one line (A07-29NI) met all criteria for a cultivar to be registered in Canada.

Genetic diversity

The six parental lines, A01-20694, A03-3NR, Cougar, Aviso, Express and Pollen, were screened with 60 publicly available SSR primer pairs covering 19 linkage groups (LG) of *B. napus*. Sixteen SSR markers from 11 linkage groups were found to be polymorphic, and these markers produced a total of 33 alleles (Table S4). The occurrence of winter alleles in the lines derived from winter x spring crosses ranged from 15.0 to 50% (Table S4).

The dendrogram depicted distinct groups, where the three winter parents, Aviso, Express and Pollen, were found to be genetically quite distinct (Fig. 2). This is not unexpected based on breeding history of these three cultivars. The cultivar Pollen bred in France, while Express is a German cultivar. On the other hand, Aviso bred in Denmark but registered in France; however, this cultivar has a German and a French cultivar in parentage. Among the spring parents, Cougar and A03-3NR grouped very closely, while A01-20694 seems to be quite distinct from the other two. Most of the lines derived from winter x spring crosses showed significant genetic diversity from the spring parents; however, two lines from Aviso x A03-3NR cross showed >80% genetic similarity with the spring parent A03-3NR.

The principal coordinate analysis (PCoA) indicated the first and the second coordinates explained 22.5% and 14.1% of the variation, respectively (Fig. 3); while the third coordinate explained 12.0% of the

Table 4. Seed yield, agronomic and seed quality traits of the F_5 and DH lines derived from winter x spring canola *Brassica napus* crosses evaluated in replicated field trials in seven locations in Canada

Entry	Generation	Seed yield Hkg ha^{-1}	Seed yield % of Q2 & 46A65	Days to flower	Plant height (cm)	Lodging[z]	Days to maturity	BL score[y]	BL % of Westar	Oil (% whole seed)[x]	Protein (% whole seed)[x]	Glucosinolates (µmol/g seed)[x]	Saturated fatty acid (%)
A07-29NI	F_5	30.00	119.9	50.9	118.0	4.1	100.0	1.22	30.7	48.7	25.2	14.3	6.10
A07-30NI	F_5	25.73	102.8	51.5	127.4	3.9	101.0	0.92	23.1	45.9	27.7	16.0	6.37
A07-31NI	F_5	26.34	105.2	51.7	123.5	4.3	100.8	0.99	24.9	46.8	27.5	14.7	6.18
A07-32NI	F_5	27.85	111.3	51.5	123.2	4.1	100.6	1.13	28.4	46.9	27.2	15.2	6.26
A07-38NR	F_5	27.34	109.2	51.1	125.4	4.4	100.4	0.75	18.8	46.4	27.2	15.5	6.26
A07-42NR	DH	23.55	94.1	48.8	120.2	3.8	98.8	1.06	26.6	46.7	26.9	14.4	6.28
A07-43NR	DH	22.78	91.0	50.3	126.3	3.9	100.7	1.48	37.2	48.3	26.0	14.5	6.29
A07-44NR	DH	25.14	100.4	51.1	128.4	3.6	100.3	0.59	14.8	47.8	26.6	13.8	6.27
A07-45NR	DH	32.02	127.9	50.9	118.6	4.3	102.0	1.47	36.9	46.8	25.4	15.7	6.16
A07-46NR	DH	27.93	111.6	51.7	119.5	4.0	101.1	3.98	100.0	45.4	27.1	15.1	6.22
A07-47NR	DH	26.87	107.4	53.3	125.0	4.9	102.4	2.55	64.1	46.1	26.3	18.3	6.27
45H21	Hybrid	28.73	114.8	47.8	112.6	2.9	97.4	0.80	20.1	48.6	26.3	14.1	6.46
46A65	Check	24.28	97.0	47.4	104.6	3.3	98.0	0.95	23.9	48.3	26.7	19.0	6.18
Q2	Check	25.78	103.0	48.5	110.0	2.8	98.0	0.64	16.1	46.8	26.6	15.1	6.24
Westar	Check							3.98	100.0				
LSD (0.05)		2.30	9.2	1.2	6.7	1.84	1.1	0.67	17.0	1.1	1.0	1.1	0.21
C.V. (%)		8.2	8.3	2.3	5.4	22.67	1.0	30.31	30.36	2.2	3.6	6.9	3.2
No. locations		7	7	7	7	2	7	1	1	7	7	7	7

[z] 1 = no lodging, 5 = completely lodged

[y] 0 = no lesions, 5 = plant completely girdled.

[x] Oil and protein contents are expressed on a zero moisture basis, and glucosinolate content on 8.5% moisture basis.

Table S3. Number F_4 families (in brackets, per cent) meet criteria for selection for multiple agronomic and seed quality traits. Less stringent selection criteria applied for the traits are indicated in the column 'modified selection

Selection for multiple traits	Aviso x A03-3NR		Express-IMI x Cougar		Pollen x A99-13NR	
	Number families	Modified selection	Number families	Modified selection	Number families	Modified selection
Flowering + maturity	104 (70%)	-	10 (7%)	-	12 (11%)	-
Flowering + maturity + oil	36 (24%)	-	1^x (0.7%)	-	0^x (0%)	-
Flowering + maturity + modified oil	-	-	8 (5%)	1% less oil	4 (4%)	1% less oil
Flowering + maturity + oil + protein	2^x (1%)	-	6 (4%)	-	1^x (1%)	-
Flowering + maturity + oil + modified protein	15 (10%)	1% less protein	-	-	4 (4%)	2% less protein
Flowering + maturity + oil + protein + GLS	3^x (2%)		4 (3%)	-	2^x (2%)	-
Flowering + maturity + oil + protein + modified GLS	8 (5%)	0.5 higher GLS	-	-	3 (3%)	0.5 higher GLS
Flowering + maturity + oil + protein + GLS + Sat FA	1 (0.7%)		4 (3%)	-	3 (3%)	-

^{x}None of these families met selection criteria for the other traits, and therefore, less stringent selection was applied

total variation. The PCoA analysis results also in agreement with the results presented in Fig. 2; where, the three winter parents again conformed to be genetically distinct, and most of the lines from winter x spring crosses found to be genetically distinct from their spring parents.

Results demonstrate usefulness of the European winter canola *B. napus* gene pool for the improvement of spring canola *B. napus* cultivars. One of the advantages of using this exotic gene pool is that all spring type lines derived from winter x spring crosses are expected to be of canola quality type, i.e. zero-erucic acid in oil and low glucosinolate in seed meal; and therefore, selection pressure for these two traits would not be needed in the breeding programs. The use of European winter canola gene pool in the present study resulted significant improvement in seed yield in one cycle of breeding. The line A07-29NI from winter x spring program was tested in Canadian Public Coop trials in 2008 and got approved by the Western Canada Canola/Rapeseed Recommending Committee (WCC/RRC) for registration in Canada.

One of the important tasks in winter x spring breeding program is to make effective selection for spring growth habit and earliness of flowering. In the present study, evaluation of the same F_2 population, seeded in greenhouse with 19 days interval, displayed significant difference for flowering including recovery of early flowering F_2 plants. Flowering time in plants is under the control of endogenous genetic factors and external environmental signals photoperiod and temperature. Molecular and genetic analysis of flowering time in Arabidopsis showed that distinct but linked pathways are involved for detecting these environmental signals (Putterill *et al*. 2004). In *B. napus*, genetic analysis of flowering time based on segregating population derived from crossing of spring and semi-winter type, disclosed 42 QTLs and 63 interacting pair of loci involved in the control of this trait (Long et al. 2007). Involvement of such a large number of QTLs and interacting pair loci might be one of the reasons of occurrence of this significant difference in flowering pattern in the F_2 population due to seeding dates. The results indicate that caution need

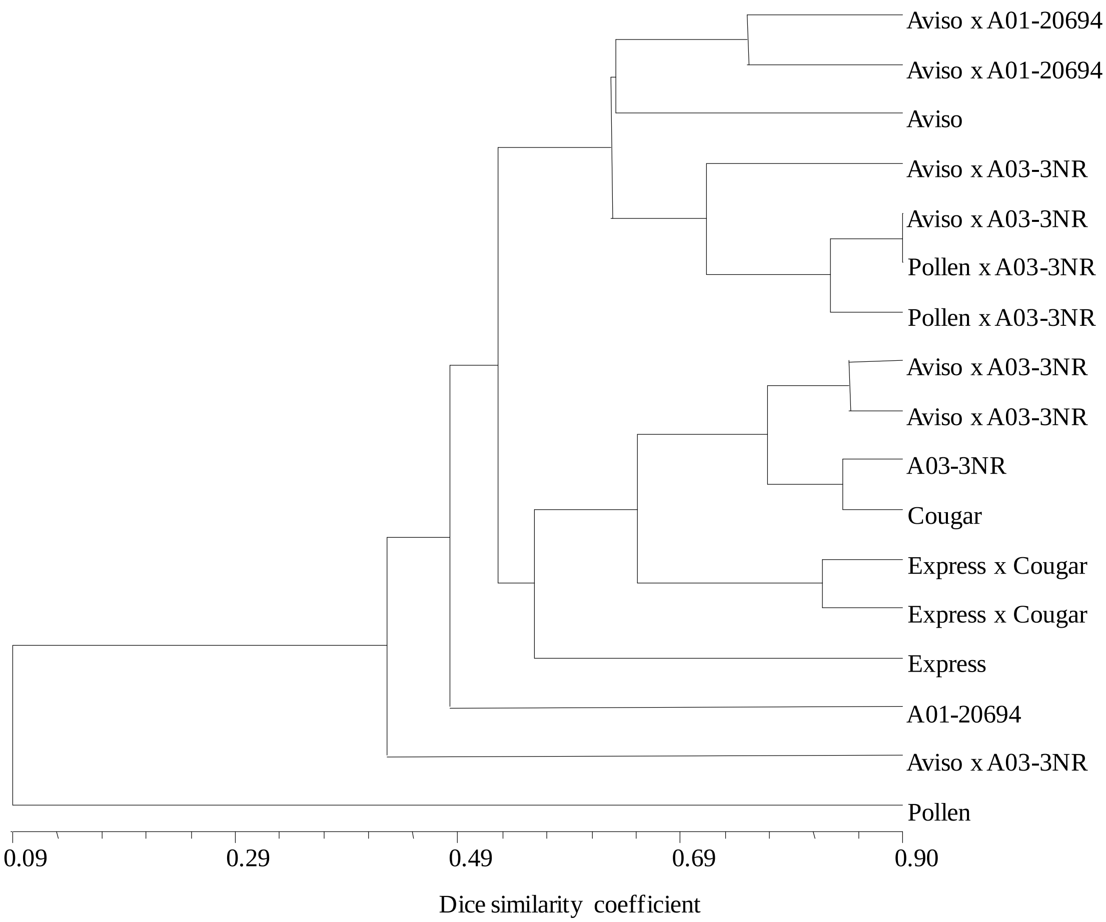

Fig 2. Dendrogram showing genetic similarity for a set of 11 spring *Brassica napus* lines derived from crosses between winter (Aviso, Express, Pollen) and spring type (Cougar, A03-3NR, A99-13NR, A01-20694) and six parental lines, as revealed by UPGMA clustering based on genetic fingerprint using polymorphic simple sequence repeat markers.

to be taken while selecting for early flowering spring growth habit plants in a winter x spring breeding program.

In *B. napus*, winter growth habit primarily differs from spring habit for vernalization requirement, as well as for survival under freezing temperature condition (winter hardiness). QTL mapping of vernalization response in *B. napus*revealed that 4 to 5 loci from the linkage groups A2, A3, A10 and C3 are involved in the control of this trait (Osborn et al. 1997, Kole et al. 2002). Molecular analysis of the genes affecting flowering time in *B. oleracea* (one of the progenitor species of *B. napus*) revealed that the spring growth habit in this species is due a non-functional allele which arose from a frameshift (1 base deletion) in exon 4 of the flowering gene *BoFLC2* (Okazaki et al. 2007). In the present study, selection against the vernalization genes was quite effective from growing the F_2 populations in a heated greenhouse and selecting the plants that flowered without the need of vernalization. However, a great majority of the spring type pedigree and DH lines derived from this program flowered and matured later than the Canadian spring canola cultivars/lines. Mei et al. (2009) identified six QTLs from A3, A7, A10 and C2 in *B. napus*which affect flowering time without the need of vernalization. According to Kole *et al.* (2002), some of the alleles causing lateness of flowering increases winter hardiness in

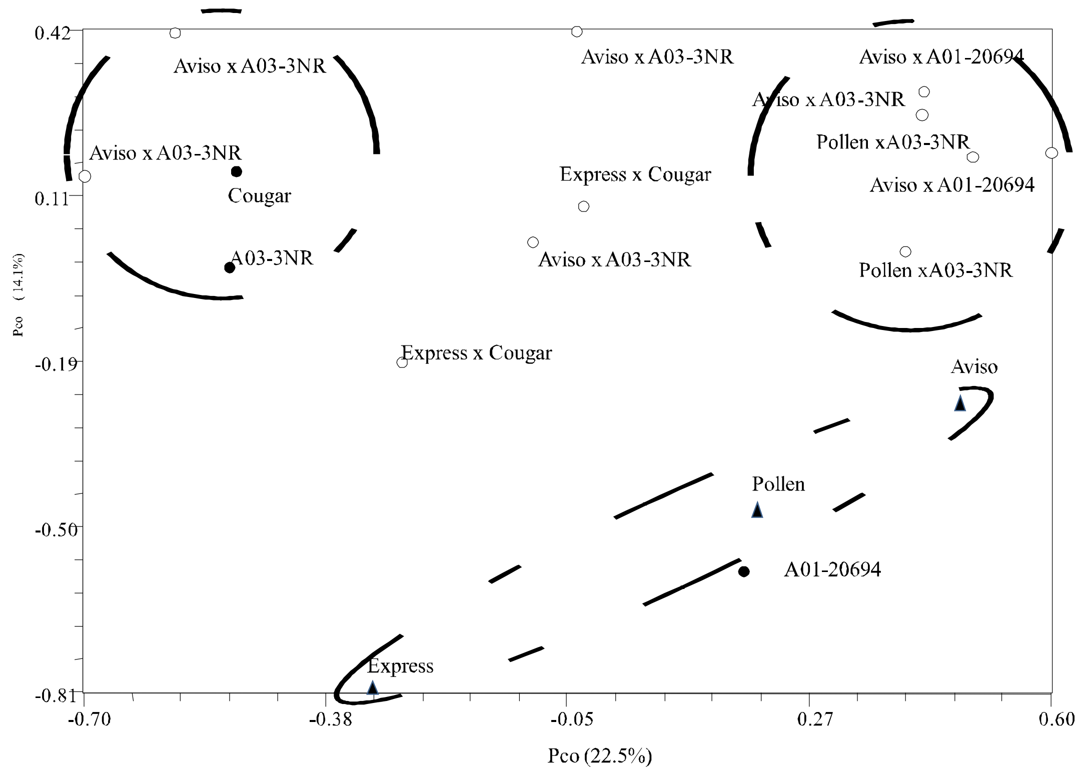

Fig 3. Plot of the first and second principal coordinates for 11 spring *Brassica napus* lines, derived from crosses between winter (Aviso, Express, Pollen) and spring type (Cougar, A03-3NR, A99-13NR, A01-20694), and six parental lines based on polymorphic bands derived from simple sequence repeat markers.

winter *B. napus*. Increased winter hardiness is an important objective of the European winter canola breeding programs. Therefore, it can be assumed that, European breeders have indirectly been selecting the late flowering alleles while selecting for increased winter hardiness, and introduction of these alleles apparently have occurred in the winter x spring breeding populations. Further investigation on genetic control of flowering and maturity using the spring canola lines derived from winter x spring crosses would be needed to explain this.

In addition to days to flowering and maturity, intensive effort was also needed for the improvement of oil content while maintaining protein content similar to the checks. A great majority of the lines had lower oil content compared to the spring check cultivars/lines. Oil content in *B. napus*is a polygenic trait controlled by several gene loci, largely by additive effect of the genes (Grami and Stefansson 1977; Engqvist and Becker 1991; Delourme *et al*. 2006, Weselake *et al*. 2009) as well as additive x additive interactions of loci (epistasis) (Zhao *et al*. 2005, Wang *et al*. 2010). QTL mapping for oil content disclosed up to 27 genomic regions to be involved in the control of this trait in *B. napus*(Ecke *et al*. 1995, Burns *et al*. 2003, Zhao *et al*. 2005, 2006, Delourme *et al*. 2006, Qiu *et al*. 2006, Zhao *et al*. 2008, Yan *et al*. 2009, Chen *et al*. 2010), of which majority of the loci show genotype x environment interaction (Zhao *et al*. 2005, 2008, Delourme *et al*. 2006, Qiu *et al*. 2006, Yan *et al*. 2009, Chen *et al*. 2010). Many of the QTL alleles with positive and negative effects on oil content are often dispersed between genotypes (Zhao *et al*. 2005, Delourme *et al*. 2006, Yan *et al*. 2009). This suggests that accumulation of the positive alleles from different genetic background could eventually lead to the development of genotype with higher oil content. Lines with high oil content was found in the segregating generations of the present winter x spring breeding program (Tables

Table S4. Number of alleles detected in winter and spring parents of *Brassica napus*

Entry	Generation	Seed yield		Days to flower	Plant height (cm)	Lodging[z]	Days to maturity	BL score[y]	BL % of Westar	Oil (% whole seed)[x]	Protein (% whole seed)[x]	Glucosinolates (µmol/g seed)[x]	Saturated fatty acid (%)
		Hkg ha^{-1}	% of Q2 & 46A65										
A07-29NI	F_5	30.00	119.9	50.9	118.0	4.1	100.0	1.22	30.7	48.7	25.2	14.3	6.10
A07-30NI	F_5	25.73	102.8	51.5	127.4	3.9	101.0	0.92	23.1	45.9	27.7	16.0	6.37
A07-31NI	F_5	26.34	105.2	51.7	123.5	4.3	100.8	0.99	24.9	46.8	27.5	14.7	6.18
A07-32NI	F_5	27.85	111.3	51.5	123.2	4.1	100.6	1.13	28.4	46.9	27.2	15.2	6.26
A07-38NR	F_5	27.34	109.2	51.1	125.4	4.4	100.4	0.75	18.8	46.4	27.2	15.5	6.26
A07-42NR	DH	23.55	94.1	48.8	120.2	3.8	98.8	1.06	26.6	46.7	26.9	14.4	6.28
A07-43NR	DH	22.78	91.0	50.3	126.3	3.9	100.7	1.48	37.2	48.3	26.0	14.5	6.29
A07-44NR	DH	25.14	100.4	51.1	128.4	3.6	100.3	0.59	14.8	47.8	26.6	13.8	6.27
A07-45NR	DH	32.02	127.9	50.9	118.6	4.3	102.0	1.47	36.9	46.8	25.4	15.7	6.16
A07-46NR	DH	27.93	111.6	51.7	119.5	4.0	101.1	3.98	100.0	45.4	27.1	15.1	6.22
A07-47NR	DH	26.87	107.4	53.3	125.0	4.9	102.4	2.55	64.1	46.1	26.3	18.3	6.27
45H21	Hybrid	28.73	114.8	47.8	112.6	2.9	97.4	0.80	20.1	48.6	26.3	14.1	6.46
46A65	Check	24.28	97.0	47.4	104.6	3.3	98.0	0.95	23.9	48.3	26.7	19.0	6.18
Q2	Check	25.78	103.0	48.5	110.0	2.8	98.0	0.64	16.1	46.8	26.6	15.1	6.24
Westar	Check							3.98	100.0				
LSD (0.05)		2.30	9.2	1.2	6.7	1.84	1.1	0.67	17.0	1.1	1.0	1.1	0.21
C.V. (%)		8.2	8.3	2.3	5.4	22.67	1.0	30.31	30.36	2.2	3.6	6.9	3.2
No. locations		7	7	7	7	2	7	1	1	7	7	7	7

[z] 1 = no lodging, 5 = completely lodged

[y] 0 = no lesions, 5 = plant completely girdled.

[x] Oil and protein contents are expressed on a zero moisture basis, and glucosinolate content on 8.5% moisture basis.

2, 3 and 4); however, these families/lines generally had lower protein content due to negative correlation between these two traits (Grami *et al.* 1977). The general tendency of having lower oil content in the progenies of winter x spring crosses may also partly be due to their late maturity. Synthesis of oil and accumulation in *B. napus* seed starts in early stage of embryo development, and most of the oil accumulation in embryo occurs between 3rd and 7th week after pollination (Fowler and Downey 1970; Perry and Harwood 1993, Murphy and Cummins 1989; Murphy *et al.* 1989); and after that period, oil accumulation continue at a steady but somewhat at lower rate until maturity (Fowler and Downey 1970, Murphy *et al*. 1989). In this context, late maturing lines from winter x spring crosses may have been penalized due to harvest of the crop at the same time when other spring canola was harvested.

In conclusion, the European winter canola offer an important and genetically diverse gene pool for use in the spring canola breeding programs for the development of improved open-pollinated spring canola cultivars as well as parental lines for hybrid cultivars. Genetic diversity analysis based on SSR markers showed that most of the lines derived from winter x spring crosses were quite distinct from their spring parents. One of the major constraints of the use of this gene pool is that lateness of flowering and maturity generally introduced from winter type; however, development of elite breeding lines with fairly acceptable flowering and maturity is still feasible. Intensive selection for other traits, e.g. high oil and protein content, saturated fatty acid, will also be needed. The elite spring canola lines developed from this breeding research is an important gene pool for broadening the genetic diversity of spring canola.

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