1 **Molecular evidence of outcrossing rate variability in *Brassica napus***

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ABSTRACT

A leafy crop of *Brassica napus* L. called nabicol has been grown by farmers in northwestern Spain for many years, being an important horticultural product during the winter season. A collection of landraces of a *Brassica napus* leafy crop called nabicol is kept at 'Misión Biológica de Galicia' (CSIC-Spain) which can be used to search for desirable characteristics or to produce new commercial varieties to release in the market. The assessment of the mating system of nabicol landraces is particularly important to carry on adequate breeding and genetic conservation programs. The objective of this work is to estimate the outcrossing rate in nabicol under controlled pollinator conditions using SSRs. Pairs of flowering plants taken from nabicol landrace MBG-BRS0039 were placed in separated isolation cages and bumble bees (*Bombus* sp) were released for facilitating the crosses between plants. A seed sample from each plant in the cross was analyzed by SSRs that were polymorphic in the parental population. We found that the crop is partially allogamous and that there is genetic variation for the outcrossing rate among individuals. Several consequences for the maintenance and genetic improvement of the crop are discussed. The existence of genetic variability for this trait is a valuable tool that will allow us to study the genetic mechanisms underlying the mating system of this crop.

Keywords: *Brassica napus*, nabicol landraces, mating system, outcrossing rate, microsatellites, SSRs

INTRODUCTION

Brassica napus includes economically important crops, such as oilseed rape (*B. napus* L. group *oleifera* DC.), rutabaga (*B. napus* group *napobrassica* L. Reichenb.) and leaf rape (vegetable and fodder crops) (*B. napus* L. group *pabularia* (DC.) Reichenb.). Traditionally *B. napus* has been considered an autogamous species, although some authors have found that in field conditions, it can show a variable level of outcrossing. Estimates in oilseed rape were found to vary between 20-40% (Olsson 1960; Rakow and Woods 1987; Becker et al. 1992; Damgaard and Loeschcke 1994). In rutabaga, the percentage of outcrossing varies between 20-45% (Gowers 1981). As far as we know, no studies have been done in leafy *B. napus* crops. Breeding of partially allogamous crops is very difficult. Therefore, in *B. napus* selfing is widely used in early generations and standard methods like pedigree selection or single seed descendent are applied. The high demand for uniformity, distinctness and stability strongly favor pure lines or F₁ hybrids as the desired type of cultivar (Becker et al. 1999).

A leafy crop of *B. napus* called nabicol has been grown by farmers in northwestern Spain for many years, being an important horticultural product during the winter season (Cartea et al. 2005). The leaves are used for human consumption (in soups and in stews) and fodder. Local nabicol landraces from northwestern Spain were collected since the 1980s until the present and they are currently kept as an active collection at the 'Misión Biológica de Galicia' (CSIC, Spain). They are stored at 4 °C and less than 50% humidity. The molecular, morphological and agronomical characteristics of this collection have been previously studied (Cartea et al. 2005; Rodriguez et al. 2005; Soengas et al. 2006). Some landraces were considered promising for fresh production because they showed better or similar yield than commercial

controls (Rodriguez et al. 2005) and they could be included in breeding programs to obtain improved varieties. Nabilcol landraces represent a valuable resource to obtain resistant varieties to pest and diseases and to produce new commercial varieties.

In order to be able to manage germplasm effectively, some basic information is required. This includes information on the nature of mating system (Hamilton et al. 2003), meaning the extent of self-pollination and the degree of outcrossing. To design breeding programs it is necessary to determine the mating system of the crop under study. Currently, accurate estimates of outcrossing rates can be achieved using neutral molecular markers, such as microsatellites (SSRs). The advantage of using this kind of markers is that they are not subjected to the action of natural or human selection, like morphological traits (for example flower color, which can be biased because of pollinator preferences). Estimates of outcrossing rates can also be obtained using several SSRs at the same time. Compared with single-locus methods, multi-locus methods have lower statistical variance and higher robustness against the violation of model assumptions (Ritland and Jain 1981). The multi-locus method proposed by Ritland and Jain is an iterative procedure which uses the Newton-Raphson method to compute outcrossing rates. The program is based on the mixed-mating model of Brown and Allard (1970) that has several assumptions: 1) mating events are due to either selfing or random outcrossing, 2) maternal individuals sample pollen from an homogeneous pollen pool, 3) outcrossing rates are uniform over all maternal individuals within a population and 4) there is no selection between fertilization and time of assay.

The objective of this work was to estimate the outcrossing rate in a leafy crop of *B. napus* under controlled pollination conditions using the SSR multi-locus method of Ritland and Jain (1981).

MATERIAL AND METHODS

To determine the outcrossing rate of nabicol, the 35 nabicol landraces currently kept at ‘Misión Biológica de Galicia’ (CSIC, Spain) were initially studied. Thirty plants of each landrace were screened using 40 SSRs (www.ukcrop.net). Most landraces were monomorphic for the SSRs studied, so they were discarded. From the remaining landraces, MBG-BRS0039 was chosen to carry on the experiments because it was the only one whose individuals showed a synchronic flowering. MBG-BRS0039 had shown good early vigor, good leaf and seed yield and intermediate growing cycle compared to other landraces from the collection in a previous characterization carried out by Rodriguez et al. (2005). Eight pairs of synchronously flowering plants from MBG-BRS0039 were placed in separate isolation cages, where bumble bees (*Bombus sp*) were released to facilitate pollination of both parents.

A seed sample from each plant was harvested and all the seed harvested in each plant was considered as a ‘family’. Thus, 16 families were obtained to perform the statistical analysis. Approximately 100 seeds of each family were sown in seedbeds and the 4 or 5 youngest leaf of each individual plant were collected 40 days after sowing. Individual plants from each family were genotyped by those SSRs that had been polymorphic in the parental population.

DNA of each individual was extracted following the method of Liu and Whittier (1994) with minor modifications. Amplifications were performed by using a PTC-100TM Thermal Cycler (MJ Research, Watertown, MA). The amplification consisted of a denaturing step at 95 °C for 5 min followed by 35 cycles at 95 °C for 30 sec, 56 °C for 30 sec, and 72 °C for 30 sec. The program ended with an extra elongation period of 10 min followed by a continuous cycle at 4 °C. PCR reactions were carried out

in a volume of 25 µl containing 50 ng of each primer, 0.6 unit of Taq polymerase (ECOGEN), 200 µM each dNTP, 1 X reaction buffer, 2.0 mM MgCl₂, 50 ng DNA template, in distilled and autoclaved water. After amplification, SSR products were separated by electrophoresis on 6% non-denaturing acrylamide gels run in 1 × TBE buffer. Gels were stained with Ethidium bromide, run for approximately 2 h at 250V, and then were visualized under UV light.

Estimates of outcrossing rate were computed using the iterative procedure of Ritland and Jain (1981). This method assumes a ‘mixed mating model’ of a population in which, the progeny of any plant is the result of self-fertilization and outcrossing. Maximum likelihood estimates of outcrossing rates were obtained at population level with the Newton-Raphson method (Ritland and Jain 1981). Standard errors were calculated by bootstraps (1000) using the families as the resampling unit. Using this method, single-locus outcrossing rates (t_s) and multi-locus outcrossing rates (t_m) were estimated. The difference between t_m and the t_s averaged across loci, and the correlation of outcrossing rate estimates among loci were also computed. Estimates of outcrossing rates at a family level were computed with a method of moments, as described by Ritland and Jain (1981).

Two different statistical approaches were used to analyze the data. The method of Ritland and Jain (1981) can be implemented for both diploid and tetraploid species. When a microsatellite amplified only one locus or several loci that were clearly distinguishable, then they were scored and analyzed using the method for diploid species. The program MLTR version 3.2 was used to analyze the data (<http://genetics.forestry.ubc.ca/ritland/programs.html>). If a SSR amplified two loci, whose alleles could not be assigned to any of both loci the method of Ritland and Jain (1981) for tetraploid species was used to score and analyze the data. The program

140 MLTET (<http://genetics.forestry.ubc.ca/ritland/programs.html>) was used in this case
141 (Ritland 1990; Ritland 2002).

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143 RESULTS AND DISCUSSION

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145 Results from amplification in the parental population showed that three SSRs, Na12-A02,
 146 Na14-D07 and Na10-A08 were polymorphic (Table 1). SSRs primer sequences and
 147 annealing temperatures are reported by Lowe et al. (2004). SSR Na10-A08 amplified two
 148 different, polymorphic and independent loci: Na10-A08a, which was localized in a
 149 linkage group of *B. napus* (N15), and equivalent to the *Brassica rapa* linkage group R15
 150 and Na10-A08b (N9) which was mapped in the equivalent *Brassica oleracea* linkage
 151 group 09 (Piquemal et al. 2005). The SSR Na12-A02 amplified two different loci but this
 152 primer showed polymorphic banding for only one of them. SSR Na14-D07 amplified two
 153 different loci with their corresponding alleles running at the same height.
 154 SSR Na14-D07, SSR Na10-A08a and Na10-A08b amplified two alleles each, while
 155 Na12-A02 amplified four alleles in the parental population. Allele frequencies for each
 156 SSR marker are presented in Table 1. Frequency of heterozygote genotypes were among
 157 16% for SSR Na12-A02 and 39% for SSR Na14D07.

158 The outcrossing rate of MBG-BRS0039 was computed using two different
 159 approaches. The MLTET program was using to compute the outcrossing rate with SSR
 160 Na14-D07 while for the remaining loci MLTR program was employed (Ritland 1990;
 161 Ritland 2002). The idea of using two different approaches comes from the difficulty of
 162 scoring some SSRs, when more than one locus is being amplified with the same primers.
 163 This problem has been described before and is a result of the complex structure of the
 164 *Brassica* genome. *B. napus* (AACC) is an amphidiploid between *B. rapa* (AA) and *B.*
 165 *oleracea* (CC). Sometimes, homologous loci can be found inside the *B. oleracea* and *B.*
 166 *rapa* genomes and the assignment of alleles to loci is difficult and, in some cases,

impossible. Furthermore, homologous *B. napus* SSR loci can display identical alleles that cannot be resolved (Hasan et al. 2006).

The outcrossing rate computed using the MLTET program, was 0.30 ± 0.13 (Table 2). The estimation of the outcrossing rates at the loci Na12-A02, Na10-A08a and Na10-A08b were computed with the program MLTR. The program produces an estimator of the single-locus outcrossing rate (t_s), and the multi-locus outcrossing rate (t_m). The values of t_s were very similar for the three loci examined, but were more precise when the estimate was computed with the loci Na10-A08a than with the other two (Table 2). The combined value of t_m for the loci Na12-A02, Na10-A08a and Na10-A08b was equal to 0.33 ± 0.10 , which is not significantly different from that detected at the locus SSR Na14-D07. Similar estimates of t_s were obtained with the methods implemented for diploid and tetraploid species, indicating that both were adequate to analyze the genome of *B. napus*.

The t_m value found in the nabicol landrace MBG-BRS0039 is in agreement with those detected in other *B. napus* crops. Olsson (1960) found that on average, there is about one-third cross-fertilization and two-thirds self-fertilization in rapeseed using morphological traits. Becker *et al.* (1992) found similar values of outcrossing in rapeseed (an average outcrossing rate of 34%) using isozyme analysis. In rutabaga, the level of outcrossing determined by Gowers (1981) using morphological traits was similar to those found in oilseed rape.

This work demonstrates that nabicol crop has a similar level of outcrossing that other *B. napus* crops. The crop can be therefore considered as partially allogamous. The accessions of the nabicol collection of the MBG are regenerated from time to time in order to get enough seed to allow us to carry out different trials and send seed to other researchers or growers when requested. In order to maintain the genetic integrity of the

accessions as much as possible, they must be multiplied by using the same methods used for allogamous *Brassica* crops. In this case, isolation conditions are necessary to avoid gene flow and the minimum number of plants multiplied for each accession must be higher than in the case of self-pollinating crops to preserve the variability present.

Estimates of t_s and t_m were also computed at a family level. The number of individuals analyzed by family varied depending on the loci studied. This variation in the sample size was due to missing data for some of the loci when PCR amplifications for some individuals did not performed well and to the presence of possible null alleles. Even using large sample sizes, some estimates of t_s in the four loci examined were above 1.00. For example, in families 14 and 15 for the locus Na14-D07 (Table 2) estimates were over 1.00. Averaged t_s across loci or t_m estimates were below or close to 1.00, proving that multi-locus estimates are more accurate than single locus estimates. The t_m computed at a family level gave estimates that ranged from values close to 0.00 (families number 2 and 13) to values close to 1.00 (families 8 and 15). Most families showed outcrossing rates between 0.20 and 0.40 (Table 2). The biparental inbreeding was estimated as the difference between t_m and t_s averaged across loci (0.04 ± 0.02). When the biparental inbreeding is present, it can cause a bias in the estimation of selfing, but, in our case, this bias was almost negligible.

The correlation among outcrossing rates estimates obtained with different loci and computed within progeny arrays (r_t) was 0.24 ± 0.18 . This correlation is low, indicating that outcrossing rate is dependent on the locus employed, proving again that multi-locus approaches give more accurate results than single-locus approaches.

Variability for outcrossing rate could be found among individuals of MBG-BRS0039. With the experimental design employed, we could not separate the genetic and the environmental components of the phenotypic variability. We only could make

some assumptions on the causes of this variability in the present work. Phenotypic variation is due in part to environmental factors, such as the position of the flowers in the plant (Becker et al. 1992) or the preference and number of visits of pollinators (Suso et al. 2005). However, in our experiment, part of the environmental variation was reduced by placing pairs of plants in isolation cages. Different isolation cages were used for each pair of plants, and within these cages, beehives with approximately the same number of bumble bees were placed. These steps were taken to avoid variation based on pollinator preference for a particular type of plant. It is likely that part of the variation for outcrossing rate is under genetic control. Daamgard and Loeschke (1994) estimated that the narrow-sense heritability of the rate of selfing in rapeseed was 0.41. Previously reported genetic variability of outcrossing rate in rapeseed populations of *B. napus* could have been based on differences in morphological parameters such as the spatial relationship between stigmas and anthers (Syafaruddin et al. 2006) and the presence of male-sterile plants (Song et al. 2006) or auto-incompatible plants (Mohring et al. 2005). Differences in outcrossing rates among progeny arrays can be also overestimated since a limited number of biparental crosses were chosen to estimate the outcrossing rate. Finding variability for this trait is the first step to establishing an in-depth study on the mating system of the crop.

Our results showed that nabicol is a partially allogamous crop, and that there is variability for outcrossing rates among individuals. Part of this variation could be under genetic control although an exhaustive study on this subject is necessary. The variability found for this trait, as it was shown in this work, will allow for the study of the genetic mechanisms controlling the mating system of this crop; will allow knowing how heterosis and inbreeding depression are affected by the outcrossing rate, and the effect on production. If the causes of the outcrossing rate and the consequences in heterosis for

242 yield components could be determined, breeding of the crop could also exploit heterosis
243 for traits related to the production to obtain improved varieties or F₁ hybrids. To study
244 in depth causes of the outcrossing rate it is necessary to find variability for this trait. The
245 selection of self-sterile or auto-incompatible plants will become a genetic resource that
246 could be used for breeding purposes.

247

248 Table 1. Allelic frequencies; homozygotes and heterozygotes frequencies of the three
 249 SSRs analyzed in the parental population MBG-BRS0039.

Locus	Alleles	Allelic frequency	Homozygotes	Heterozygotes
			frequency	frequency
Na14-D07	1	0.750	0.612	0.388
	2	0.250		
Na10-A08a	1	0.796	0.794	0.206
	2	0.203		
Na10-A08b	1	0.783	0.776	0.224
	2	0.217		
Na12-A02	1	0.754	0.844	0.156
	2	0.167		
	3	0.010		
	4	0.069		

250 Table 2. Estimates of single-locus outcrossing rate (t_s) and multi-locus outcrossing rate (t_m). Number of individuals of each family employed in
251 the statistical analysis using four different loci.

Family	Na14-D07		Na10-A08a		Na10-A08b		Na12-A02		Average t_s^x	t_m^y	No. z
	t_s^x	No. z	t_s^x	No. z	t_s^x	No. z	t_s^x	No. z			
1	0.32 ± 0.17	81	0.25 ± 0.27	81	1.82 ± 0.09	79	0.00 ± 0.00	95	0.57 ± 0.09	0.82 ± 0.09	95
2	0.20 ± 0.06	94	0.26 ± 0.11	94	0.05 ± 0.05	95	0.00 ± 0.00	95	0.12 ± 0.05	0.14 ± 0.06	95
3	0.50 ± 0.09	76	1.28 ± 0.23	84	0.80 ± 0.05	84	0.00 ± 0.00	84	0.76 ± 0.14	0.66 ± 0.16	84
4	0.02 ± 0.00	94	0.57 ± 0.16	94	0.17 ± 0.30	92	0.00 ± 0.00	95	0.17 ± 0.10	0.33 ± 0.17	95
5	0.67 ± 0.10	84	0.76 ± 0.19	90	0.00 ± 0.01	95	1.63 ± 0.29	87	0.66 ± 0.09	0.78 ± 0.11	95
6	0.34 ± 0.07	94	0.45 ± 0.27	81			0.69 ± 0.22	78	0.79 ± 0.20	0.38 ± 0.21	82
7	1.19 ± 0.67	73	0.18 ± 0.29	73	0.19 ± 0.35	64	0.56 ± 0.04	72	0.54 ± 0.04	0.60 ± 0.06	73
8	0.24 ± 0.08	70	0.58 ± 0.19	68	0.00 ± 0.01	70	1.05 ± 0.06	70	0.93 ± 0.05	1.03 ± 0.06	70
9	0.43 ± 0.08	85	0.46 ± 0.28	78	0.00 ± 0.00	84	0.00 ± 0.00	85	0.15 ± 0.09	0.29 ± 0.17	85
10	0.00 ± 0.14	72	0.62 ± 0.19	71			0.00 ± 0.00	71	0.35 ± 0.11	0.31 ± 0.12	71
11	0.00 ± 0.00	83	0.24 ± 0.12	81	0.23 ± 0.30	76	0.49 ± 0.19	76	0.31 ± 0.12	0.31 ± 0.18	83

12	0.15 ± 0.06	81	0.22 ± 0.06	76			1.92 ± 1.37	72	0.42 ± 0.06	0.48 ± 0.06	76
13	0.53 ± 0.11	52	0.30 ± 0.13	81	0.16 ± 0.33	78	0.00 ± 0.01	81	0.06 ± 0.12	0.04 ± 0.20	81
14	1.99 ± 0.05	94	0.51 ± 0.16	86	0.10 ± 0.07	88	0.00 ± 0.00	88	0.29 ± 0.08	0.33 ± 0.09	88
15	1.99 ± 0.01	88	0.00 ± 0.01	54	0.56 ± 0.35	50	1.15 ± 0.15	54	2.57 ± 1.76	0.91 ± 0.20	56
16	0.00 ± 0.00	94	0.10 ± 0.10	48	0.00 ± 0.01	48	0.26 ± 0.13	95	0.23 ± 0.11	0.24 ± 0.11	95
Total	0.30 ± 0.13	800	0.31 ± 0.07	1240	0.30 ± 0.14	1003	0.28 ± 0.16	1298	0.29 ± 0.10	0.33 ± 0.10	1324

252 ^x: single locus outcrossing rate.

253 ^y: multilocus outcrossing rate.

254 ^z: number of individuals.

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