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Estimation of Outcrossing Rate in Ethiopian Mustard (*Brassica carinata*) using RAPD Markers

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ABSTRACT

Breeders regard Ethiopian mustard (*Brassica carinata*) as a predominantly self-pollinated crop and employ breeding methods for self-pollinated crops. However, the proportion between self- and cross-pollination is not experimentally quantified. The aim of this study was to estimate the outcrossing rate of *B. carinata* and its variation across environments. Outcrossing rate was estimated by analysing the banding pattern of random amplified polymorphic DNA (RAPD) markers of offspring of two parental lines grown in open-pollinated isolation plots at Holetta (normal and delayed planting condition), Kulumsa and Debrezeit Research Centers in Ethiopia and Cordoba, in Spain. The analysis of polymorphic banding pattern based on visual inspection of gels yielded three variable loci in the parents that helped to distinguish progenies obtained from self-pollination and outcrossing. Outcrossing rate in *B. carinata* was on average 29.6% and varied between environments from 21.8 to 39.2%. Factors such as the abundance of pollinating agents seem to affect the rate of outcrossing more than variation in geographic location, as the lowest and highest outcrossing rates were recorded in the same location, Holetta, from normal and one month delayed planting date, respectively. This study demonstrates that *B. carinata* possesses a substantial amount of outcrossing. The outcrossing rate observed could be an opportunity for breeders to exploit heterosis through synthetic and/or hybrid cultivar breeding.

Keywords: Cross-pollination, molecular marker, pollen parent, seed parent, self-pollination

INTRODUCTION

Flowering plants possess variation in their mating system from complete self-pollination to complete cross-pollination (Barrett 2003). A plant's mating system has a large impact on population genetic structure (Dje *et al*. 2004) in determining the spatial and temporal patterns of genetic diversity (Barrett 2003). Predominantly selfing species exhibit lower genetic diversity within and higher diversity among populations, compared to outcrossing species (Hamrick and Godt 1990). Since long time, plant breeders have recognized the importance of mating system for breeding, germplasm management and conservation (Jain 1979). The proportion of selfing to outcrossing determines the feasibility of maintaining pure lines, influences formulation of optimal strategies for hybridization, and recombination of genotypes, genetic sampling (Ritland 1983) and the way germplasm collection should be maintained *ex situ* (Brown and Marshall 1995) and *in situ* (Dje *et al*. 2004). The amount of outcrossing is a crucial characteristic in the exploitation of heterosis through synthetic cultivars and for seed multiplication, and variety maintenance (Becker 1988; Jain 1979).

Outcrossing rate in plants is genetically controlled but is also influenced by environmental conditions (Abdel-Ghani *et al*. 2004). Meteorological factors like rainfall (Parzies *et al*. 2000; Abdel-Ghani *et al*. 2004), temperature (Abdel-Ghani *et al*. 2004), light intensity (Demotes-Mainard *et al*. 1995; Li *et al*. 1996) and geographical factors like elevation (Rita 1983) affect the rate of outcrossing in different crop species.

The diversity, abundance, activity, and behaviour of pollinating agents also affect the genetics of outcrossing of a plant species (Suso *et al*. 2001). Agronomic practices such as plant population density have been shown to influence the rate of outcrossing (Krueger and Knapp 1991). Therefore, it is wise to conduct outcrossing experiments in more than one environment.

Studies to determine selfing and outcrossing rates have usually been conducted using easily identifiable morphological markers, but the development of biochemical and molecular markers paved the way for easier and reliable techniques (Becker *et al.* 1992; Gaiotto *et al.* 1997; Dje *et al*. 2004; Antonio *et al*. 2011; Soengas *et al.* 2011; Sousa *et al*. 2012). Molecular markers are stable and detectable in all tissues regardless of growth, differentiation, development or defence of the cell and are not confounded by the environment, pleiotropic and epistatic effects (Agarwal *et al.* 2008); Paterson *et al*. 1996; Melchinger *et al*. 1994; Melchinger 1999; Agarwal *et al.* 2008; Kumar *et al.* 2009).

Breeders regard *Brassica carinata* as a predominantly self-pollinated crop. To date, cultivar improvement in *B. carinata* has been mostly done by employing breeding methodologies used for self-fertilized crops. The mode of pollination of *B. carinata* is believed to be similar to *Brassica napus* and *Brassica juncea*, where the flowers open and stigma is receptive for some time before pollen is released (Salisbury 1991 as cited by Mendham and Salisbury 1995). So far, there is no report that describes the level of outcrossing and selfing in *B. carinata*. Thus, our objectives were to determine the rate of outcrossing and its variability between environments using random amplified polymorphic DNA (RAPD) markers.

MATERIALS AND METHODS

Selection of plant material

Two S_5 inbred lines of *B. carinata* selected as described below were used in the experiment. Initially 22 inbred lines (S_5) developed from accessions collected from eight different geographic areas of Ethiopia (**Table 1**) were screened for their RAPD polymorphism. Forty-three ten-mer oligonucleotides primers from

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Table 1 Inbred lines of Ethiopian mustard, their parental accession code along with area of collection and altitude included in the study.

Inbred line code	Parental acc. No‡	Area of collection	Altitude (m)	Inbred line code	Parental acc. No	Area of collection	Altitude (m)
G 1	21278	Wolo	2290	G 12	21071	Bale	2640
G 2	21369	Kefa	1772	G 13	21182	Welega	2120
G 3	21245	Gonder	1860	G 14	21182	Welega	2120
G 4	21080	Arssi	2390	G 15	21253	Gojam	1740
G 5	21068	Bale	2500	G 16	21265	Wolo	1950
G 6	21069	Bale	2450	G 17	21316	Shewa	2430
G 7	21005	Arssi	2450	G 18	21276	Wolo	2290
G 8	21209	Welega	2460	G 19	21007	Arssi	2900
G 9	21224	Kefa	1750	G 20	21192	Welega	2090
G 10	208404	Gojam	1960	G 21	21289	Wolo	2570
G 11	21071	Bale	2640	G 22	43/79	Sweden	-

‡ G stands for genotype; Accession No. refers the accession identification number of the Institute of Biodiversity Conservation, Ethiopia

Operon technologies (Almada, CA, USA) were screened on replicate over six genotypes at the Institute of Agronomy and Plant Breeding laboratory, Goettingen, Germany. Those primers that gave clear and consistent amplification (Teklewold and Becker 2006, 2010) were ultimately used for PCR amplification. One primer that showed clear polymorphism in pairs of inbred lines that can serve as potential parents in the outcrossing study was identified. Since synchrony in the flowering period is important for effective pollination (Soengas *et al.* 2011), flowering time of the inbred lines was also another criterion used to select parental genotypes. Based on combined criteria, two inbredlines: inbred 247 and 327 that produced reproducably polymorphic band when amplified by one primer (OPQ-15) and synchornized in flowering were selected as parent for the outcrossing study.

DNA extraction, PCR amplification and band scoring

DNA was isolated using Nucleon PhytoPure DNA extraction kit (Amersham International PLC 1997) from 0.1 g of young leaves taken from a single plant per genotype. Leaves were frozen in liquid nitrogen and stored at -20°C. DNA extraction was carried out using a mini DNA isolation method following Nucleon extraction kit. DNA quality was assessed by electrophoresis on a 1% agarose gel and concentration was determined by fluorescent spectroscopy using Hoechst 33258 calf thymus DNA as a standard (following BIO-RAD's catalogue number 170-2480). The DNA was diluted to 5 ng/µl of working sample with TAE buffer before use.

PCR reactions were carried out in a 25 µl volume containing 1× reaction buffer [200 mM Tris-HCL, pH 8.55, 160 mM $(NH_4)_2SO_4$ 0.1% (v/v)], 3.0 mM $MgCl_2$, 0.4 mM of dNTP (dATP, dCTP, dGTP and dTTP), 0.16 µM primer, 1.0 units of *Taq* DNA polymerase and 25 ng of genomic DNA template. The remaining volume was filled by distilled, filtered and sterilized water. To protect from evaporation during amplification two drops of mineral oil were added in each sample tube. DNA amplifications were performed in a Perkin Elmer 480 thermocycler programmed for a preliminary step of 1 min at 94°C, 45 cycles of 30 s at 92°C, 60 s at 35°C and 2 min at 72°C and a final step of 6 min at 72°C. This was followed by a hold time at 4°C until samples get removed from the machine. The RAPD fragments were separated by electrophoresis using 1.8% agarose gel and visualized with ethidium bromide and photographed under UV light using a computer printer. Images of DNA profiles on the gels were captured into the PC files using a Camera Module online with the computer using the program Herolab E.A.S.Y. store (Herolab GmbH Laborgeräte, Wiesloch).

Open-pollination experiment

The two inbred lines selected to be parents in the study were planted under isolation at three locations in Ethiopia (Holetta, Kulumsa and Debrezeit) and one location in Spain, Cordoba. At Holetta an additional environment was created by delaying planting by a month, so that, the experiment was carried out in five environments. Detailed eco-geographic information of the environments is given in **Table 2**. At each location, plots were isolated by other crops. The plot size was 36 m^2 (6 × 6 m). Each plot contained 21 rows spaced at 30 cm. Plants in the first three and the last three rows and plants grown on the outer 90 cm of the head and tail land of the plots were considered as border plants. Each seed parent (inbred 247) was planted on every other row at intra-row spacing of 60 cm and theoretically, 64 seed parent plants (eight plants × eight rows) were available in each isolation plot. The space between two seed parents was planted by seed of the male parent (inbred 327) with intra-row spacing of 10 cm. The seed parent (inbred 247) was planted initially at three seeds/hill and after full germination only a single seed parent plant/hill was maintained. The experiment was carried out during the rainy season. Fertilizer was applied at the rate of 46/69 kg/ha of N/P_2O_5. There was no serious disease and pest occurrence, ultimately, no pest control measure was considered. Other agronomic practices were carried out as recommended for each location. Pollination was allowed to occur through the natural pollination agents: insects and wind. Since the pollen parent was earlier by about two days, pollen was readily available for transfer before the opening of flowers on plants from the seed parent genotypes. Depending on survival of seed parental plants, seeds were harvested from 53 to 64 seed parental plants from each environment.

Estimation of outcrossing

A random sample of 50 seeds, each from 50 randomly chosen seed parental plants, from each location were grown in a greenhouse, Göttingen, Germany. Total genomic DNA was extracted from each individual progenies and amplified using the same primer that had shown polymorphism in the seed and pollen parents. RAPD pattern of the progenies along with the seed and pollen parents was scored following the methods described above under the subtitle DNA extraction, PCR amplification and band scoring. Outcrossing was determined by direct maternal and paternal allele counting for each location. Progenies obtained from pollination plot were determined as potentially the result of self pollination if only maternal allele was found at the scorable loci and potentially the result of an outcross pollination if it has an allele other than found in the maternal parent (paternal allele). Then estimates of outcrossing rate was computed as a ratio of number of offspring that showed both maternal and paternal banding patterns to the total number of offspring genotyped at each location based on the formula:

$$\text{Outcrossing (\%)} = (\text{NOMPBP}/\ \text{NOTP}) \times 100$$

where N = number of offspring that showed both maternal and paternal banding pattern and NOMBP = number of total offspring gentoyed.

χ^2 statistics was carried out to determine uniformity of heterozygous and/or homozygous progenies for the marker loci among the different environments.

As RAPD markers are typically dominant, verification of the reproducibility and inheritance of each cultivar-specific marker was essential. This has been verified by producing hypothetical F_1 plants by mixing equal amount (50:50) of the maternal and paternal plants DNA and compared the banding pattern with putative outcrossed progenies banding pattern following the scoring

Table 2 Eco-geographic information of the five environments.

Environment	Country	Location	Altitude m a.s.l	Geographical location	Weather variables for the flowering month		
					Min. Tem. ^{0}C	Max. Tem. ^{0}C	Rainfall (mm)
E1	Ethiopia	Holetta	2400	38^0 30`E and 9$^0$ 0`N	9.1	18.7	149.3
E2	Ethiopia	Holetta	2400[a]	38^0 30`E and 9$^0$ 0`N	7.8	19.7	107.4
E3	Ethiopia	Kulumsa	2190	39^0 9`E and 8$^0$ 1`N	9.4	21.0	97.2
E4	Ethiopia	Debrezeit	1900	38^0 59`E and 8$^0$ 46`N	12.2	25.2	44.4
E5	Spain	Cordoba	120	4^0 45`W and 37$^0$ 55`N	9.1	20.4	130.3

[a] Planting date one month later than the normal

method described above under the subtitle DNA extraction, PCR amplification and band scoring. The reproducibility was also checked by running DNA amplification of the selected parents in replicate.

RESULTS AND DISCUSSION

Clearly identifiable and reproducible banding patterns were obtained when the 22 inbred lines and replicates of lane 10 and 20 amplified by primer OPQ-15 (**Fig. 1**). Analysis of polymorphic banding pattern based on visual inspection of gels yielded three variable loci in the parental DNA. The 22 inbred lines can be grouped in to five based on their banding pattern across the three variable loci. Group I comprised only one inbred line (lane 18) that produced a single band at the 1st loci. Group II is with two inbred lines (lane 15 and 20) that produced two bands at the 1st and 3rd loci. Group III has seven inbred lines (lane 1, 2, 6, 11, 12, 13, and 19) that produced only one band at the 2nd loci. Group IV is with 12 inbred lines (lane 3, 4, 5, 7, 8, 9, 10, 14, 16, 17, 21 and 22) that produced two bands at the 2nd and 3rd loci. Although more than two pairs of inbred lines were polymorphic in at least one locus, two inbred lines: inbred 247 and 327 were selected for this experiment. The two inbred lines were polymorphic at the 1st and 2nd loci but monomorphic at the third loci (**Fig. 1**). Banding profile of the two inbred lines was free from doubtful bands that minimize error in scoring. The two inbred lines also showed synchrony in flowering. Inbred 247 that flowered two days later than inbred line 327 (at Holetta) was used as female parent and the later as pollen parent.

The use of dominant markers such as RAPD and AFLP can not distinguish a homozygous genotype for the dominant allele "band presence" (+/+) from the heterozygous (+/-) or (-/+). Since seed from a maternal plant could be the result of either pollen from the same plant or from cross-pollination by a different parent/genotype, to observe heterozygosity for dominant marker loci in F_1 generation, identification of maternal and paternal genotypes that produce polymorphic bands at least at one locus (one band scored for presence in the pollen parent and absence in the seed parent) is essential. By selecting cultivar and cultivar-specific markers, actual source of individual outcrossing events could be identified (Kobayashi *et al.* 2000). Such genotype and primer pre-screening also avoids application of sophisticated models to estimate outcrossing rate and is important to test the reproducibility of the assay used. Indeed Ferreira *et al.* (2010) observed congruent outcrossing rate in passion fruit using dominant (RAPD) and co-dominant (microsatellite) marker.

In most of the cases, RAPD bands are believed to represent a dominant locus with two alleles (Williams *et al*. 1993). The presence of a specific band indicates the dominant allele whereas the absence of the same band indicates the recessive allele. Offspring produced by self-pollination would have only maternal banding pattern, while those produced by cross-pollination would have both maternal and paternal banding patterns. **Fig. 2** illustrates an example of the banding pattern observed in the gels that allowed unambiguous distinction among progenies obtained by self-pollination and outcrossing. All the progeny carried the maternal allele conferring complete homozygousity of the maternal plants. Lanes 2, 11, 13, 17, 19, 22 showed both maternal and paternal banding pattern conferring heterozygosity

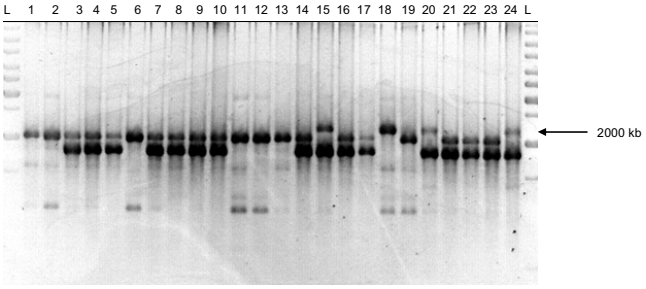

Fig. 1 RAPD amplification of 22 *B. carinata* lines with primer OPQ-15. The lanes 1-22 corresponds to the 22 inbred lines. Lanes 23 and 24 are replicate of lane 10 and 20, respectively. L denotes MBI Fermentas # Smo311, gene ruler^{TM} 1 Kb ladder. Lane 14 (inbred 247) and 18 (inbred 327) were polymorphic at three different marker loci.

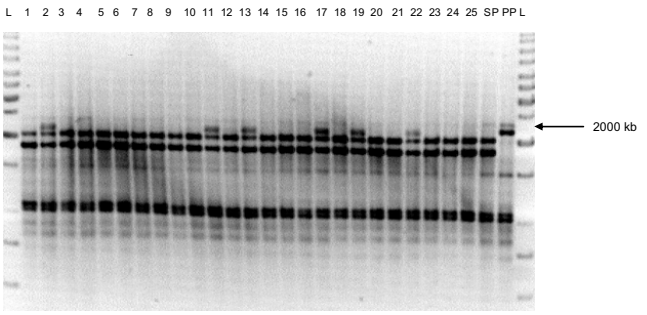

Fig. 2 An example of RAPD amplification of 25 *B. carinata* progeny from inter-crossing isolation plots with primer OPQ-15. The lanes 1-25 corresponds to 25 progenies. Lane SP and PP denote seed parent (inbred 247) and pollen parent (inbred 327), respectively. The first and last lanes are MBI Fermentas # Smo311, gene ruler^{TM} 1 Kb ladder. Lanes 2, 11, 13, 17, 19, 22 are heterozygous for SP and PP banding pattern.

resulted from outcrossing occurred in the isolation plot.

Williams *et al*. (1993) and Hallden *et al*. (1996) suggested that the outcomes of a RAPD reaction is in part determined by a competition for priming sites in the genome causing non-Mendelian marker inheritance. To confirm the reliability of the RAPD markers in detecting outcrossing, hypothetical F_1 plants were created by mixing equal amount (50:50) of the parental DNA. This artificially mixed DNA was scored for the presence of expected parental bands. The hypothetical F_1 plants were confirmed as heterozygous for the sequence arrangement of amplified parental DNA fragments (**Fig. 3**). The size and banding patterns of the hypothetical F_1s were also identical with what is observed in the heterozygous progenies harvested from seed parents. Once we were convinced to include inbreds 247 and 327 as parents (lane 10 and 20), DNA from this inbredlines was amplified in replicate (lane 23 and 24, respectively) to test the repeatability of the technique. As is shown on **Fig. 1**, the banding patterns of lane 10 and 20 are identical to their respective replicate: lane 23 and 24. This gives confidence on the reproducibility of the technique and reliability of outcome of the experiment. The reliability of RAPD pattern in the same laboratory using homozygous genetic material under a stringent reaction condition and careful data scoring has been discussed by Teklewold and Becker (2010).

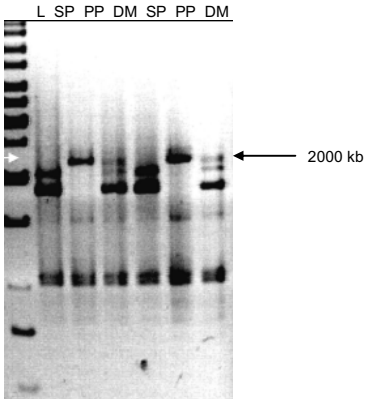

Fig. 3 RAPD amplification of seed and pollen parents as compared to the hypothetical F_1 created by mixing parental DNA (50:50), two times replicated. L corresponds to MBI Fermentas # Smo311, gene ruler^{TM} 1 Kb ladder. SP = seed parent; PP = pollen parent and DM = 50:50 DNA mixture of the two parents.

Table 3 Outcrossing rate at the different environments.

Locations/ Environments	No of offspring with heterozygous banding pattern (out of 50)	Outcrossing percentage
E1	10	21.8
E2	18	39.2
E3	14	30.5
E4	14	30.5
E5	12	26.2
Mean	13.6	29.6

With the advent of molecular biology, DNA techniques have been adapted for the study of pollination behaviour in different plant species: *Calopogonium mucunoides* Des (Sousa *et al*. 2012), olive (Souza *et al*. 2012), avocado (Borrone *et al*. 2008; Kobayashi *et al*. 2000), mango (Santos and Neto 2011), palm (Ramos *et al*. 2011), sweet passion fruit (Ferreira *et al*. 2010), *Cyperus difformis* (Merotto *et al*. 2009), rice (Gealy and Estorninos Jr. 2004), sorghum (Dje *et al*. 2005), barley (Abdel-Ghani *et al*. 2004), cassava (Silva *et al*. 2003), *Phaseolus vulgaris* (Ferreira *et al*. 2000). In this study too, the RAPD assay based outcrossing rate study has established that *B. carinata* possess a substantial amount of outcrossing (**Table 3**). The outcrossing rate varied from 21.8 to 39.2% with an average of 29.6%. The average out crossing rate, 29.6%, might have been slightly biased by the higher level of outcrossing observed from one of the environments, E2, due to delayed planting. When the 39.2% outcrossing rate observed by delaying planting is excluded from calculating the mean, outcrossing rate is reduced to 27.3%. The 39.2% outcrossing rate obtained from delayed planting by a month imply that outcrossing rate of the crop could be manipulated to some extent by manoeuvring the environments that favour either high or low outcrossing. The outcrossing rate observed in this study corroborates previous breeder speculations of the *Brassica* species as predominantly self-pollinating. In *B. napus*, Becker *et al*. (1992) reported 34% outcrossing rate averaged over five locations. Recently, a combined rate of 33% outcrossing rate was obtained from molecular marker-based outcrossing study on a leafy crop of *Brassica napus* L. called nabicol (Soengas *et al.* 2011). The maximum rate of outcrossing observed by delaying planting in same location could be explained by increase in pollinating agents visiting the crop as most of the other field crops have completed flowering by that time. Hence, when synthetic varieties are pursued, outcrossing rate could be increased through early or delayed planting to reduce competition of pollinating agents which otherwise visit other crops that flower the same time.

Molecular markers have been widely used in analyses of genetic diversity in *Brassica* crops (Teklewold and Becker 2006; Abbas *et al.* 2009; Snowdon and Friedt 2008; Li *et al*. 2009; Teklewold and Becker 2010; Faltusova *et al.* 2011). Nevertheless the majority of the works utilizing molecular markers in *Brassica* oilseed breeding have to date been based on genome analysis through genetic mapping using various DNA marker systems in segregating populations generated for specific investigations of particular traits of interest and in some cases to map-based cloning of the responsible genes (Snowdon and Friedt 2008; Li *et al*. 2009). Reports on molecular marker application in studing mating system of the *Brassica* crops are limited to Soengas *et al.* (2011) and Becker *et al*. (1992) both carried out in *Brassica napus*. This is the first reported study of its kind that detrmine experimentally the pollination behavior of *B. carinata* and estimate the out crossing rate using molecular markers.

The study showed that *B. carinata* is a partially allogamous crop and has a similar level of outcrossing to *B. napus* (Becker *et al*. 1992; Soengas *et al.* 2011). Therfore, appropriate precondition of isolation in maintaining genetic purity during accessions rejuvenation to avoid gene flow and keeping the minimum number of plants to retain original variability is important. While using seed in planting variety trial, the possibility of genetic contamination should be considered. In this case, seed for every advanced trials should come from original source.

The observed number of heterozygous and/or homozygous progenies for the marker loci at the different environments did not depart significantly from the expected (χ^2 = 3.56, df 4) implying that outcrossing rate was similar across locations. The low variation for outcrossing rate across locations observed in this study might correspond to low level of variation in type, activity and abundance of pollinators across test locations. Becker *et al*. (1992) reported the influence of environment in affecting outcrossing rate of *B. napus* that ranged from 12 to 47% among locations.

B. carinata exhibits a substantial amount of heterosis (Teklewold and Becker 2005); therefore, as is observed in *B. napus*, heterosis breeding is an option to improve yield of *B. carinata*. Either hybrids and/or synthetic varieties can be sought in exploiting heterosis. Nevertheless in the near future, exploitation of heterosis by developing hybrid varieties would be limited due to unavailability of suitable pollination control mechanism (sterility system) that ensures cross-pollination. Nevertheless, synthetic cultivars could be pursued to partially exploit available heterosis. An essential requisite for developing synthetic varieties is a high outcrossing rate (Becker 1988; Becker *et al*. 1998). *B. carinata* possessing as high as 39% out crossing, seems amenable for synthetic variety development. The level of outcrossing required for inter-crossing and multiplication of synthetics could be increased by selecting genotypes with higher rate of outcrossing and manipulating other biological and physical variables to enhance outcrossing. Becker *et al*. (1998) reported a high genetic variation for outcrossing rate in *B. napus* double haploid lines.

In conclusion, *B. carinata* has a mixed-mating system with about 30% outcrossing rate. Extent of outcrossing seems less affected by geographic location. The amount of outcrossing could be appealing to breeders in exploiting heterosis through synthetic or hybrid cultivar development. To attain a maximum level of cross-pollination required for both synthetic and hybrid cultivar development, genotypes with higher level of out-crossing should be selected in the future.

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