Genetic Relationships Among *Brassica napus* Crops Based on SSR Markers

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Abstract. *Brassica napus* includes economically important crops such as oilseed rape, rutabaga, and leaf rape. Other vegetable forms of *Brassica napus*, namely nabicol and couve-nabiça, are grown in northwestern Spain and north of Portugal, respectively, and their leaves are used for human consumption and fodder. The relationship of nabicol with other *Brassica napus* leafy crops was studied before, but its origin remained unclear. The aims of this work were to study the genetic relationships among nabicol landraces and other *B. napus* crops based on microsatellites and to relate the genotypic differences with the use of the crop. The relationship among 35 *Brassica napus* populations representing different crops was studied based on 16 microsatellite markers. An analysis of molecular variance was performed partitioning the total variance into three components. The source of variation resulting from groups was defined considering the main use of the crop and accounted for a smaller percentage of variation than other sources of variation, proving that this division is not real. Populations clustered into seven different clusters using a similarity coefficient of 0.82. No clear association was evident between clusters and the main use of populations, suggesting genetic differences among populations could reflect differences in their origin/breeding or domestication. Spanish nabicol could have originated from a sample of couve-nabiças, and couve-nabiças could be used to improve nabicol landraces, because they have a narrow genetic basis that limits their potential for breeding.

Different hypothesis have been suggested for the origin of *B. napus*. Sinskaia and Schiemann (Gómez-Campo and Prakash, 1999) have proposed an origin in the Mediterranean region of southwest of Europe, where the two contributing parents (*B. oleracea* L. and *B. rapa* L.) overlapped in their natural distribution, probably in an agricultural environment. Song et al. (1988) and Song and Osborn (1992) proposed multiple origins of *B. napus* based on restriction fragment length polymorphism (RFLP) analyses. Repeated backcrosses of the interspecific hybrids to one or both parental populations have been suggested for the origin of some populations of *B. napus* (Palmer et al., 1983).

Brassica napus includes economically important crops such as oilseed rape (*B. napus* L. var. *oleifera* DC.), rutabaga (*B. napus* var. *napobrassica* L. Reichenb.), and leaf rape

(vegetable and fodder crops) (*B. napus* L. var. *pabularia* [DC.] Reichenb.). In Galicia (northwestern Spain), a cultivar of *B. napus* var. *pabularia*, namely 'nabicol', is a traditional crop grown by the farmers of the area from many years ago as an important horticultural product during the winter season (Cartea et al., 2005). The leaves are used both for human consumption and fodder. Nabicol landraces have been collected since the 1980s in northwestern Spain in those areas where the crop is important, mainly in the border with Portugal. Populations have been kept as an active collection at the 'Misión Biológica de Galicia' (MBG) until the present (Ordás and Baladrón, 1985).

The origin of the local nabicol populations grown in Galicia is unclear. Rodríguez et al. (2005) found a low level of genetic diversity among them based on agronomic and morphologic characteristics, and they concluded that could be the result of a common genetic origin of the populations studied. Cartea et al. (2005) studied the relationships among 33 nabicol populations to 16 British populations of forage rape and rape kale based on RAPD markers and concluded that Spanish and British populations have different origin.

Simple sequence repeat (SSR; microsatellite) markers may provide a useful method to characterize, conserve, and use agricultural crop diversity. Identification and characterization of SSR markers have been done in different species of the *Brassica* genus (Lowe et al., 2004; Plieske and Struss, 2001;

Saal et al., 2001; Suwabe et al., 2002; Szewc-McFadden et al., 1996; Westman and Kresovich 1998). Microsatellite markers have been useful to study the genetic diversity of *Brassica* (Westman and Kresovich 1999), to characterize resynthesized rapeseed lines (Seyis et al., 2003), to distinguish between spring/winter types or genotypes with high/low glucosinolate content (Charters et al., 1996), or to identify sources for improving heterotic potential in oilseed breeding (Hasan et al., 2005).

The genetic basis of nabicol landraces is narrow as was demonstrated by (Rodríguez et al. 2005) and (Cartea et al. 2005). Genetic improvement of nabicol could be done by introducing interesting genes from other *B. napus* crops. Information about genetic relationships of nabicol with other *B. napus* crops could be useful in designing breeding programs to obtain improved nabicol varieties.

The aims of this work are 1) to study the genetic relationships among nabicol landraces and other *B. napus* crops based on SSR markers, and 2) to relate the genotypic differences based on SSR markers with the use of the crop.

Material and Methods

Plant material. Thirty-five *B. napus* cultivars from different geographic origins and with different uses were evaluated in this study (Table 1). To facilitate the description of the material, populations were divided into four sets (leafy crops, oilseed crops, root vegetable crops, and unknown use). The first cluster was formed by nabicol landraces, couve-nabiças, and five cultivars of forage rape and rape kale from diverse origin. Nabicol landraces are from the northwest of Spain and were chosen based on previous morphologic and molecular characterizations (Cartea et al., 2005; Rodríguez et al., 2005) to represent the clusters obtained in those works. Portuguese populations of couve-nabiça are used for human consumption and were chosen to represent different geographic and climatic conditions. The second set included five oilseed *B. napus* populations, including spring and winter canola. Finally, the third and fourth sets consisted of rutabaga and wild populations, respectively (Table 1).

Simple sequence repeat analysis. Forty days after sowing, the 4 or 5 youngest leaves were collected. Leaf samples were taken from 5 plants of each accession and DNA was extracted following the method of Liu and Whittier (1994) with minor modifications. A total of 40 primer combinations were tested for their suitability. After prescreening, 16 primer pairs were chosen that gave clear, reproducible, and polymorphic amplification products at one or more loci in *B. napus*. The sequence of primers Ni2-C12, Ra2-EO4, Na12-AO2, O110-F11, Na12-EO5, Na14-DO7, Na14-EO8, Ni2-FO2, Na10-AO8, O112-FO2, O112-F11, Na10-DO9, Ni4-DO9, and Na10-FO6 was taken from the public domain: www.ukcrop.net. The sequence of primers BRMS-030 and

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Table 1. List of *Brassica napus* accessions/cultivars included in this study, number of polymorphic bands amplified, and index of polymorphic content (PICS) for the 35 populations analyzed.

Accession number/cultivar	Country of origin	Common name	Type ²	Seedsources	No. of polymorphic bands	PICS
Leafy crops (<i>Brassica napus</i> var. <i>pabularia</i> L.)						
MBG-BRS0014	Spain	Nabicol (NC)	L	MBG	29	0.06
MBG-BRS0039	Spain	Nabicol (NC)	L	MBG	38	0.14
MBG-BRS0054	Spain	Nabicol (NC)	L	MBG	30	0.06
MBG-BRS0063	Spain	Nabicol (NC)	L	MBG	35	0.16
MBG-BRS0113	Spain	Nabicol (NC)	L	MBG	29	0.04
MBG-BRS0356	Spain	Nabicol (NC)	L	MBG	29	0.09
MBG-BRS0378	Spain	Nabicol (NC)	L	MBG	32	0.12
MBG-BRS0541	Spain	Nabicol (NC)	L	MBG	35	0.09
Nabicol Rocalba	Spain	Nabicol (NC)	C	MBG	34	0.16
'Nabicol'	Spain	Nabicol (NC)	C	MBG	37	0.15
BE006	Portugal	Couve-nabiça (CN)	L	HRI	36	0.14
BE013	Portugal	Couve-nabiça (CN)	L	HRI	40	0.21
BE014	Portugal	Couve-nabiça (CN)	L	HRI	29	0.05
BE015	Portugal	Couve-nabiça (CN)	L	HRI	38	0.12
BE030	Portugal	Couve-nabiça (CN)	L	HRI	31	0.02
BE031	Portugal	Couve-nabiça (CN)	L	HRI	38	0.16
BE033	Portugal	Couve-nabiça (CN)	L	HRI	36	0.11
BE038	Portugal	Couve-nabiça (CN)	L	HRI	37	0.13
'Couve-nabiça'	Portugal	Couve-nabiça (CN)	C	MBG	36	0.15
César Santos	Portugal	Couve-nabiça (CN)	C	MBG	33	0.19
Ragged Jack kale	New Zealand	Rape kale (RK)	C	CGN	32	0.10
Russian kale	UK	Rape kale (RK)	C	CGN	32	0.05
Colza Rocalba	Spain	Forage rape (FR)	C	MBG	34	0.09
Vysokopol'skij	URSS	Forage rape (FR)	C	CGN	36	0.11
Mara	The Netherlands	Forage rape (FR)	C	CGN	35	0.08
Oilseed crops (<i>Brassica napus</i> var. <i>oleifera</i>)						
Valle del oro	Spain	Winter oilseed rape (WOR)	C	MBG	33	0.15
Express	Canada	Winter oilseed rape (WOR)	C	MBG	30	0.08
Belinda	The Netherlands	Winter oilseed rape (WOR)	C	CGN	34	0.10
Quest	Canada	Spring oilseed rape (SOR)	C	MBG	28	0.05
Petranova	Germany	Spring oilseed rape (SOR)	C	CGN	36	0.15
Root vegetable crops (<i>Brassica napus</i> var. <i>napobrassica</i>)						
Colinabo Rocalba	Spain	Rutabaga (RB)	C	MBG	33	0.12
Friese Gele	The Netherlands	Rutabaga (RB)	C	CGN	27	0.01
Lollo	Germany	Rutabaga (RB)	C	CGN	28	0.05
Unknown use						
BE061	France	Wild populations (WP)	L	CGN	32	0.04
BE062	Great Britain	Wild populations (WP)	L	CGN	27	0.02

²L, landrace; C, commercial.

³MBG, Misión Biológica de Galicia, Pontevedra, Spain; HRI, Horticulture Research International, Wellesbourne, U.K.; CGN, Center for Genetic Resources, The Netherlands.

BRMS-037 was taken from Suwabe et al. (2002). Amplifications were performed by using a PTC-100 Thermal Cycler (MJ Research, Watertown, MA). The amplification consisted of a denaturing step at 95 °C during 5 minutes followed by 35 denaturing cycles at 95 °C for 30 seconds, annealing at 56 °C for 30 seconds, and elongation at 72 °C for 30 seconds. The program ended with an extra elongation period of 10 minutes followed by a continuous cycle at 4 °C. Polymerase chain 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× reaction buffer, 2.0 mM MgCl₂, 50 ng DNA template, and distilled and autoclaved water. After amplification, SSR products were separated by electrophoresis on 6% nondenaturing acrylamide gels run in 1× TBE buffer. Gels were stained with ethidium bromide, run approximately for 2 hours at 250 V, and then were visualized under ultraviolet light.

Data analysis. Each SSR band was scored as present (1) or absent (0) for all markers

across all populations. The reason to treat codominant markers as if they were dominant is because *B. napus* is an amphidiploid and most of the primers amplified more than one locus, and it was not clear for some bands if they belong to one or the other locus. Only neat and polymorphic bands across populations were scored. Analysis of molecular variance (AMOVA) was performed by the software Arlequin version 2.0 (Schneider et al., 2000). The total variance was partitioned into three sources of variation: among groups, among populations within groups, and within populations. The source of variation among groups was defined considering the main use of the crop: leaf consumption (nabicol, couve-nabiça, forage rape, and rape kale), oleiferous (oilseed rape), root consumption (rutabaga), and unknown use (wild populations). The significance level for variance components was tested with permutation tests.

The polymorphic information content for each population and SSR was computed as: $PIC_i = 2f_i(1 - f_i)$, where PIC_i is the poly-

morphic information content, f_i is the frequency of the amplified SSR band in each population, and $(1 - f_i)$ is the frequency of the absence of band following Roldán-Ruiz et al. (2000). PIC values were averaged across all loci.

A similarity matrix with the presence-absent data were constructed by the NTSYS-PC version 2.1 (Rohlf, 1998) based on the Dice coefficient, also known as the similarity coefficient of Nei and Li (1979). A band was considered as present in a population if at least was amplified in one individual of the population. The Dice coefficient is computed as $2a/(2a + b + c)$, where a is the number of SSR bands shared by genotypes in each pairwise comparison and b and c are the number of SSR bands present in one genotype and not present in the other. Cluster analysis was performed using the unweighted pair group method with arithmetic averages (UPGMA) with the program NTSYS (Rohlf, 1998). A cophenetic correlation was calculated to test for the goodness-of-fit between similarity matrix obtained from the cluster

and the original similarity matrix with NTSYS (Rohlf, 1998).

Results and Discussion

Fifty-eight SSR bands were scored across all populations. Analysis of molecular variance showed that all three sources of variation were significant (Table 2). The source variation among populations within groups accounted for 50.49% of the total variation, followed by within populations (34.01%) and among groups (15.50%). Larger differences among groups, defined by the main use of the crop, and lower differences within populations were expected because *B. napus* is considered an autogamous species and the populations studied represented different crops. *B. napus* has been reported to have a variable outcrossing rate that depends on the genotype and environmental conditions (Becker et al., 1992), and this could be partially responsible for the high level of variability found within populations. The fact that variation among groups is the smallest source of variation provides evidence that the division made according the end use of the crop is not real and that differences among populations could be rather related to the geographic origin of populations or to their pedigree than to the use of the crop.

Part of the variability was present at the within-population level. The number of polymorphic bands varied between 27 for the wild accession BE062 and the rutabaga 'Frieze Gele' and 40 for the couve-nabiça BE013. PIC values varied between 0.01 for 'Frieze Gele' and 0.21 for BE013 (Table 1). Couve-nabiças named BE013 and BE031 showed both a high number of polymorphic bands and PIC values (Table 1).

The cophenetic correlation between the similarity matrix based on Dice coefficient and the similarity matrix obtained from the cluster was 0.72. This is not a very high correlation (Rohlf, 1998); therefore, results from the cluster analysis should be taken cautiously. All populations, apart from BE030, grouped together for a similarity coefficient of 0.76. A relatively close relationship was revealed among *B. napus* cultivars that could be explained because of its recent origin compared with other *Brassica* species (Gómez-Campo and Prakash, 1999).

Populations grouped into seven different clusters using a similarity coefficient of 0.82 (Fig. 1). The first cluster consisted on 23 populations. Most of the couve-nabiça and nabicol populations were together in cluster I,

but they were differentiated in two subclusters (Fig. 1): subcluster Ia comprised 13 populations and was formed by most of the nabicol populations from Spain, together with one Spanish commercial forage rape, one Spanish commercial rutabaga ('Colinabo Rocalba'), 2 Portuguese commercial couve-nabiças ('César Santos' and 'Couve-nabiça') and one winter oilseed rape (Fig. 1). Subcluster Ib consisted on four accessions of couve-nabiça, besides one nabicol, three oilseed rapes, and two forage rapes. Cluster II was composed of two forage rape populations and a wild accession. A single population of nabicol (MBG-BRS0054) appeared on cluster III. Cluster IV was composed of 2 couve-nabiças. In cluster V, one couve-nabiça plus a wild population and an oilseed rape were grouped together. Two rutabagas were classified into cluster VI. Finally, one single accession of couve-nabiça comprised VII.

Most nabicol populations were similar, because all of them except MBG-BRS0054 were grouped together, confirming previous studies. A low level of genetic diversity among the nabicol landraces was found at the molecular (Cartea et al., 2005) and morphologic (Rodríguez et al., 2005) levels. These authors concluded that the origin of these landraces must be in common. Population MBG-BRS0054 classified far away from the rest of the nabicol populations in this work. This population showed some alleles that were not present in the rest of nabicol populations but were in couve-nabiças.

Taking into account the main use of the populations, most the nabicol and couve-nabiça were in cluster I. All the bands present in nabicol populations were in the couve-nabiça ones, but 7 bands present in couve-nabiças were not in nabicol populations (data not shown), suggesting that nabicol from the northwest of Spain could have originated from a subgroup of couve-nabiças. Both crops are very similar morphologically and the regions where they are grown are quite close. Commercial nabicol sold by seed companies are landraces that have not been selected, except for selection carried out by growers. The variability present in couve-nabiças could be used in breeding to improve nabicol landraces, because they have a narrow genetic basis that limits its potential for breeding (Cartea et al., 2005; Rodríguez et al., 2005).

Forage rape 'Colza Rocalba' was in cluster Ia very close to the commercial population named 'Nabicol'. 'Colza Rocalba', sold as "forage rape," could be a nabicol population. The origin of the commercial seed is un-

known, but it could be possible that they were collected on the same or very close areas and sold under different names. The rest of forage rape populations ('Vysokopolskij' and 'Mara') were classified into cluster Ib; rape kale populations ('Ragged Jack kale' and 'Russian kale') were grouped apart on cluster II. They are also morphologically distinct, because rape kales present wavy leaf margins. They are used for human consumption in salads or boiled. Forage rapes are harvested in the autumn to supplement the grass feed for cattle or for sheep. Sometimes both groups are not clearly distinguished because rape kales can be also denominated forage rape. Forage rape and rape kales were also classified separately in previous works based on molecular markers (Cartea et al., 2005; Hasan et al., 2005). Rape kales, represented by the variety 'Asparagus kale,' could have an independent origin from the rest of rapeseed and rutabagas studied by Song et al. (1988) and Song and Osborn (1992), and it would be more related to *B. rapa* than other *B. napus* populations. Based on this work and previous ones, rape kale populations could have a different origin than the rest of *B. napus* crops, including nabicol and couve-nabiça.

Winter oilseed rape 'Belinda' was in cluster Ia very close to 'Couve-nabiça'. 'Couve-nabiça' could be obtained from an oilseed rape variety, because a previous work (E. Cartea, unpublished data) showed a very low level of erucic acid content in seeds, similar to commercial populations of oilseed rape. Three oilseed rape populations were in cluster Ib, 'Valle del oro' and 'Express' winter crops and 'Petranova', a spring crop. Spring and winter populations usually classified separately, because they constitute two genetically different groups (Diers and Osborn 1994; Hasan et al., 2005; Lombard et al., 2000; Seyis et al., 2003). The fact that 'Valle del oro', 'Express', and 'Petranova' classify together could be the result of a common genetic background.

Rutabaga populations, 'Frieze Gele' and 'Lollo', were in cluster VII separated from the rest of the groups and the third rutabaga accession, 'Colinabo Rocalba', was genetically closer to other nabicol populations (cluster I) than to the rest of rutabagas. Other authors have found that rutabagas cluster together (Diers and Osborn, 1994; Song and Osborn, 1992). As mentioned before, 'Frieze Gele' and 'Lollo' had a low level of variability. 'Colinabo Rocalba' had a high level of variability and probably it includes in its pedigree local germplasm from the northwest of Spain.

Wild BE061 clustered with the rape kales and BE062 with oilseed rape populations and a couve-nabiça. Following Gómez-Campo and Prakash (1999), there are not wild forms of *B. napus* in nature, although it often occurs as an escape. Wild populations BE061 and BE062 could be escapes from cultivated populations instead of truly wild genotypes, because at least BE062 has a low level of genetic diversity and both populations classified with other morphotypes.

Table 2. Analysis of molecular variance (AMOVA) for 35 populations of *Brassica napus*.^z

Source of variation	df	Sum of squares	Variance components	Percentage of variation	P value ^y
Among groups	3	61.2	0.45	15.5	<0.00001
Among populations within groups	31	257.2	1.47	50.5	<0.00001
Within populations	139	137.6	0.99	34.0	0.00098
Total	173	456.0	2.91		

^zSum of squares, variance components, percentage of variation, and P value for the sources of variation.

^yProbability to obtain a random value $\geq$ observed value using 1023 permutations.

Fig. 1. Dendrogram based on UPGMA analysis of the Dice similarity coefficient among 35 *B. napus* populations as determined by simple sequence repeat markers.

No clear association was evident between clusters formed and the main use of the populations, because different crops are mixed in several clusters. To classify populations, differences between types of cultivars are not as important as differences in the rest of the genome that does not determine the use of the crop. This could be because differences in the use of the plant are determined by few genes and the number of SSR markers used in this work is not enough to detect their effect. Zhao et al. (2005) in a molecular characterization of *B. rapa* populations found that different morphotypes are often more related to other morphotypes from the same region than similar morphotypes from different regions, suggesting an independent origin or separate breeding/domestication.

In conclusion, genetic differences based on SSR markers among the populations analyzed are not related with their use as different crops. These differences probably reflect their origin breeding/domestication instead. Spanish nabicol is closely related to the Portuguese couve-nabiças and it could have originated from a sample of these. Couve-nabiças could be used to improve nabicol landraces, because nabicol has a narrow genetic basis that limits its potential for breeding.

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