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Genetic relationships between Sicilian wild populations of *Brassica* analysed with RAPD markers

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Abstract

Brassica comprises very variable species, both morphologically and genetically. Among these species, the Sicilian populations of *Brassica* sect. *Brassica*, species related to kale crops form a complex group. The genetic relationships among 15 populations occurring in Sicily and one from Calabria, representing the existing diversity, have been investigated using random amplified polymorphic DNA (RAPD) markers. This assay, carried out with 22 arbitrary primers, generated 236 polymorphic fragments, 21 of which were specific for single populations (mainly *Brassica insularis*, *Brassica incana* and *Brassica macrocarpa*). Jaccard's genetic distances were computed and the phylogenic tree was established using the UPGMA algorithm. The dendrogram obtained showed four branches grouping: (1) *B. incana* populations; (2) *B. insularis* and *B. macrocarpa,* occurring in small islands around Sicily; (3) *B. rupestris* populations; and (4) *B. villosa* populations. Within *B. rupestris*, only *B. rupestris* subsp. *brevisiliqua* was clustered in the *B. villosa* group. The classification obtained is discussed with regard to the morphological, ecological and geographical data.

Key words: *Brassica* — genetic variability — Jaccard's distance — RAPD markers — wild populations

Sicily is one of the main centres of diversification for wild species belonging to the same cytodeme of *Brassica oleracea* L. These Sicilian taxa are very important because they can hybridize with cultivated forms, thus representing a useful genetic resource for the improvement of cultivated varieties (Kianian and Quiros 1992, von Bothmer et al. 1995).

Brassica sect. *Brassica* ($2n = 18$), occurring in Sicily and in the small islands around, is represented by five distinct species: *B. incana* Ten., *B. insularis* Moris, *B. macrocarpa* Guss., *B. rupestris* Rafin. and *B. villosa* Biv. (Snogerup et al. 1990). *B. macrocarpa* is endemic to the Egadi Islands, whereas *B. rupestris* and *B. villosa* are endemic to Northwest Sicily.

On the basis of morphological characters, as well as ecological and geographical data, infraspecific taxa have been distinguished and referred to as *B. villosa* (subsp. *villosa*; subsp. *bivoniana* (Mazzola & Raimondo) Raimondo & Mazzola; subsp. *drepanensis* (Caruel) Raimondo & Mazzola; subsp. *tinei* (Lojac.) Raimondo & Mazzola) and *B. rupestris* (subsp. *rupestris*, subsp. *hispida* Raimondo & Mazzola; subsp. *brevisiliqua* Raimondo & Mazzola) (Raimondo and Mazzola 1997). Previously, the taxa referred to as *B. villosa* were described at the species level (Raimondo et al. 1991). Phenological and micro- and macro-morphological analyses confirmed the constancy of these characters in geographically distinct populations, even though some populations such as *B. rupestris* subsp. *brevisiliqua*, growing in localities where *B. villosa* and *B. rupestris* occur, show intermediate characters, e.g. large siliqua like *B. villosa* and glabrous leaves like *B. rupestris* (Raimondo 1997, Geraci 1998). Recently, Gómez-Campo (1999) agreed with this taxonomic arrangement.

Several authors differentiated and characterized *Brassica* species or cultivars with molecular markers (Quiros et al. 1987, 1991, Hosaka et al. 1990, Song et al. 1990, Demeke et al. 1992, Kresovich et al. 1992, Margalé et al. 1995, Lanner 1997). More precisely, wild forms of *Brassica* sect. *Brassica* (including some Sicilian populations) were tested using restriction fragment length polymorphism (RFLP) (Hosaka et al. 1990, Song et al. 1990, Lanner et al. 1997), analysing cpDNA (Warwick and Black 1991, Lanner 1998) and using random amplified polymorphic DNA (RAPD) markers (Lanner et al. 1996a, 1996b, Lazaro and Aguinagalde 1996, 1998b), respectively. These studies demonstrated that molecular markers are powerful tools to analyse the genetic relationships of wild taxa within the *Brassica* genus.

In the present study, the genetic diversity in Sicilian populations of *Brassica* sect. *Brassica* were assessed and the possible genetic relationships between the taxa were investigated using RAPD markers on seed bulks.

Materials and Methods

Plant materials: Seed samples were collected from natural habitats. Table 1 shows the plant material used for this study (p 1 to p 16) and its geographical origin. Seeds from seven to 10 different individuals were collected for each site. The seeds were stored, separately for each individual, under appropriate conditions.

DNA methods: DNA was extracted from a bulk of 40 seeds for each population as described by Divaret et al. (1999).

Polymerase chain reaction (PCR) was performed in a volume of 10 μl containing 67 mM of Tris-HCl pH 8.8, 16 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 2 mM $MgCl_2$, 0.15 mM of each dNTP, 0.25 μM primer, 15 ng of genomic DNA and 0.4 units of *Taq* DNA polymerase (EUROBIOTAQ). A total of 22 decamer primers from the kits A (01, 04, 05, 08, 09, 16, 18, 20), B (01, 06, 08, 12, 15), C (04, 06, 07, 09, 11, 18), D (01) and E (01, 14), of OPERON Technologies (Alameda, CA, USA) were used for PCR amplification and to produce reproducible and informative marker patterns.

DNA amplifications were performed in a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CI, USA) programmed for an initial denaturation step of 30 s at 94°C, then 45 cycles at 92°C (30 s), 35°C

Taxa	Origin	Identification
B. vs. subsp. *villosa*	M. Calcerame (Sagana)–Palermo	p 1
B. vs. subsp. *bivoniana*	M. Inici–Trapani	p 2
B. vs. subsp. *drepanensis*	M. San Giuliano–Erice–Trapani	p 3
B. vs. subsp. *bivoniana*	C.da Serbatoio (Fontanarossa)–Trapani	p 4
B. vs. subsp. *tinei*	P.lla Scalazza(Marianopoli)–Caltanissetta	p 5
B. r. subsp. *rupestris*	M. Pellegrino–Palermo	p 6
B. r. subsp. *rupestris*	Rocca Busambra–Palermo	p 7
B. r. subsp. *rupestris*	Stilo–Catanzaro (Calabria)	p 8
B. r. subsp. *rupestris*	M. Sferrovecchio (San Ciro)–Palermo	p 9
B. r. subsp. *brevisiliqua*	Capo San Vito (Isolidda)–Trapani	p 10
B. r. subsp. *hispida*	M. Pizzuta–Palermo	p 11
B. r. subsp. *hispida*	Cozzo Cicero (Borgetto)–Palermo	p 12
B. incana	Gonato–Madonie–Palermo	p 13
B. incana	Passo della Zita (Longi)–Messina	p 14
B. macrocarpa	M. Santa Caterina (Favignana)–Trapani	p 15
B. insularis	Pantelleria–Trapani	p 16

Table 1: Geographical origin of the (*Brassica*) plant material studied

(1 min), 72°C (2 min) for denaturation, primer annealing and primer extension, respectively, and a final primer extension at 72°C for 5 min. Amplification products were analysed by electrophoresis in 1.8% agarose gels run at 120 V in 1× TAE and detected by staining in ethidium bromide and illumination with ultraviolet light.

Data analysis: RAPD bands were scored as 0 for absent or 1 for present in each population. Jaccard distances, based on similarity index, were calculated using a program created with Microsoft Visual Basic for Excel, version 5.0.

A dendrogram was then generated from the distance matrices using the UPGMA option of the NEIGHBOUR program of Phylogeny Inference Package (PHYLIP 3.5c., Felsenstein 1993). The robustness of branching nodes was tested by bootstrap resampling (100 samples) using the SEQBOOT and CONSENSE programs of PHYLIP.

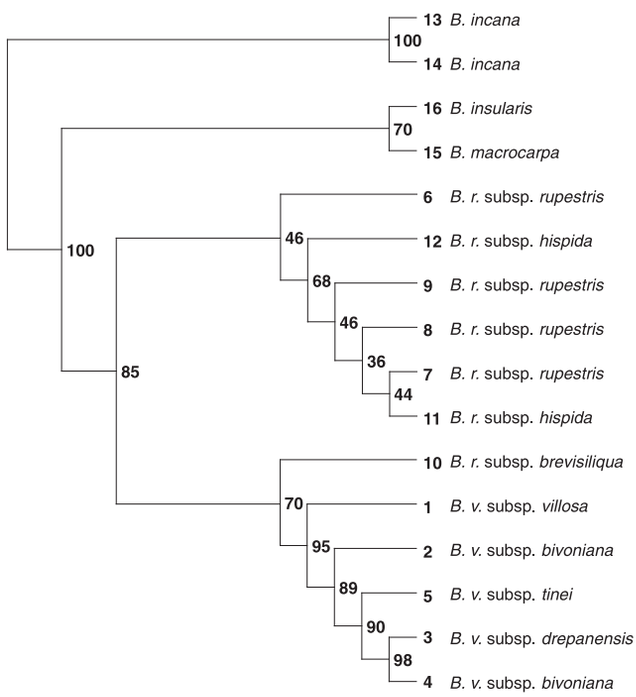

Fig. 1: Dendrogram obtained with Jaccard's coefficient of similarity using RAPD data of sixteen populations of *Brassica* sect. *Brassica* (p 1–p 16). The tree was constructed using SEQBOOT (100 samples), the UPGMA option of NEIGHBOR and CONSENSE from the PHYLIP package. The numbers at each node represent their frequency

Results

The number of polymorphic fragments varied per primer from 5 (OPC11) to 17 (OPA01) and ranged from 200 to 2300 bp. The minimum size difference between any two polymorphic products generated by a primer was approximately 10 bp. Overall, 236 polymorphic fragments were generated, among which 20 fragments were common to all taxa studied (7.8%). Twenty-one unique markers were detected (8%): eight fragments were specific for *B. insularis* p 16 (A-16, 860 bp; A-18, 1150, 1030 and 1010 bp; A-20, 2300 and 2000 bp; B-06, 1580 bp; B-12, 1550 bp), six for *B. macrocarpa* p 15 (A-16, 870 and 810 bp; B-06, 980 bp; B-12, 1360 bp; C-09, 1500 bp; C-18, 600 bp), two for *B. villosa* subsp. *tinei* p 5 (A-18, 830 bp and B-12, 1375 bp) and for *B. rupestris* subsp. *hispida* p 12 (C-04, 520 bp; C-18, 950 bp), and one for *B. villosa* subsp. *villosa* p 1 (C-06, 2030 bp) and *B. rupestris* subsp. *rupestris* p 6 (B-01, 950 bp) and p 9 (C-04, 900 bp). In the *B. incana* populations (p 13 and p 14) pattern, 12 exclusive common bands were found (A-08, 1650 and 340 bp; A-16, 550 bp; A-20, 1500 bp; B-12, 1575 bp; C-06, 1820 and 1000 bp; C-11, 830 bp; E-01, 780 and 760 bp; E-14, 1170 and 1150 bp). In *B. villosa* populations, two common specific fragments were scored (B-15, 1850 bp and C-18, 2000 bp). In addition, *B. macrocarpa* and *B. insularis* showed three exclusive common bands (A-08, 1610 and 1540 bp; C-06, 1850 bp). High heterogeneity among populations was observed in *B. rupestris* showing no characteristic polymorphic fragment.

The genetic distances calculated from these data, and represented in a dendrogram, allow the probable relationships among the populations to be evaluated (Fig. 1). Four clusters were obtained. The first regrouped *B. incana* populations; the second comprised *B. macrocarpa* and *B. insularis* populations; the third included *B. rupestris* populations, except for *B. rupestris* subsp. *brevisiliqua*, which was found in the fourth cluster together with *B. villosa* populations.

Bootstrap values were 100% for the grouping of *B. incana* and 70% for *B. macrocarpa* and *B. insularis*. *B. rupestris* and *B. villosa* originated by the same branching with a bootstrap value of 85%. In the *B. villosa* grouping, bootstrap values were greater than 89% except for *B. rupestris* subsp. *brevisiliqua* (70%), whereas in the *B. rupestris* grouping bootstrap tests

showed very low values ranging from 36% to 68%, which were not meaningful.

Discussion

DNA analysis using RAPD markers on seed bulks was a useful tool to assess the genomic complexity of Sicilian populations of *Brassica* sect. *Brassica*.

A great number of bands was obtained for each primer. More species-specific polymorphic fragments were found for two populations of *B. incana* (12), for *B. insularis* (eight) and for *B. macrocarpa* (six) than for *B. villosa* and *B. rupestris*. This difference may be because *B. incana*, *B. insularis* and *B. macrocarpa* have specific genomic zones. High DNA polymorphism was also detected by Lazaro and Aguinagalde (1996, 1998b), who obtained 129 and 151 markers, respectively, using six primers of Operon A, to test wild populations of *Brassica* sect. *Brassica* from western and eastern Europe.

The two Sicilian populations of *B. incana*, detected in the same cluster in the dendrogram, confirmed their separation from other Sicilian taxa by both geographical (east Sicily) and morphological factors. As reported by Gómez-Campo (1980, 1999), it could represent one of the lines leading to the evolution of *B. montana* and *B. oleracea* in Europe, since it shows ancestral morphological characters, i.e. hairiness and beak-bearing seeds. The fact that *B. insularis* and *B. macrocarpa* were found in the same cluster could be explained by introgressive phenomena between these two species. This regrouping is interesting because it confirms the data reported by Lanner et al. (1997) and Lanner (1998) examining RFLP and cpDNA. These authors found some affinity between *B. macrocarpa* and *B. insularis*, which were separated from the *villoso–rupestris* group, from which they could be originated. Lazaro and Aguinagalde (1998b), using a RAPD assay, also found *B. insularis* to be more closely related to Sicilian taxa and to *B. macrocarpa* in particular. Great heterogeneity was found in the cluster containing *B. rupestris*, (low values of bootstrap test), while in the *B. villosa* cluster, different populations showed remarkable genetic similarity (high values of bootstrap test). The population of *B. villosa* subsp. *bivoniana* (p 4) in particular seemed to be closely related to *B. villosa* subsp. *drepanensis* (p 3). *B. rupestris* subsp. *brevisiliqua*, which is in the *B. villosa* cluster and therefore shows more genetic affinity to taxa related to *B. villosa*, could be considered as an introgressive form between *B. rupestris* and *B. villosa* since the geographical areas of these two species overlap where *B. rupestris* subsp. *brevisiliqua* occurs.

Song et al. (1990), using RFLP, found close relationships among *B. incana*, *B. villosa* and *B. rupestris*, but not *B. macrocarpa*. In contrast, the chloroplast-based phylogeny obtained by Warwick and Black (1991) excludes *B. incana* from the *B. rupestris* and *B. villosa* group (the latter also comprising *B. macrocarpa*). Different evidence, using RAPD markers obtained from six primers, is reported by Lazaro and Aguinagalde (1996), who found *B. incana* more closely related to *B. oleracea* than to *B. rupestris*, *B. villosa* and *B. macrocarpa*, while *B. rupestris* showed more genetic similarity to *B. macrocarpa* than to *B. villosa*.

The close relationship between *B. rupestris* and *B. villosa* mentioned by all these authors and a more distant relationship with *B. macrocarpa* (in a separate clade) as reported by Song et al. (1990), was thus confirmed in this work. The results further confirmed isozyme analyses (Geraci 1998), where the genetic distances between *B. rupestris* and *B. villosa* populations were not very high, as well as the identity of chloroplast haplotypes recognized by Lanner (1998) and the RAPD and isozymes analyses carried out by Lazaro and Aguinagalde (1998a, 1998b), in which they found the smallest genetic distance between these two species.

Compared with the other studies, the present study also deals with relationships and variability at the infraspecific level among wild *Brassica* populations. Subspecies, both geographically and morphologically distinct, were not found to be appreciably distant genetically, probably because of their recent evolution. A similar case has been detected by Brunell and Whitkus (1997) on subspecies of *Eriastrum densifolium* which are very distinguishable in their morphological characters as well as being separated geographically.

In conclusion, these molecular analyses carried out using a bulk of 40 seeds were very simple and very fast in detecting diversity among populations and between different species. Until now only populations of the same species, i.e. *B. oleracea* have been tested (Divaret and Thomas 1997). Using RAPD markers, the wild Sicilian species of *B.* sect. *Brassica* have been distinguished and these data can be added to morpho-ecological data to improve the knowledge about genetic variation among these natural populations and to advance a hypothesis about their relationships and a new probable taxonomic position. The use of a RAPD assay is a useful tool in plant biosystematics to confirm morphological differences among populations and/or to differentiate apparently similar populations.

In addition to the information given on the relationships between taxa, this molecular assay also detected a high level of polymorphism among the wild Sicilian populations, which makes them valuable germplasm to preserve as a genetic resource for useful characters such as trichome-based resistance against flea beetle disease (Palaniswamy and Bodnaryk 1994) or the level of glucosinolates showing inhibitory action on the growth of cancerous cells (Faulkner et al. 1997).

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