# EVOLUTIONARY STUDY ON BRASSICA NIGRA AND RELATED SPECIES

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## INTRODUCTION

The evolutionary study of Brassica cultivated species by U (1935), established the allotetraploid origin of B. napus, B. carinata and B. juncea. His work was later confirmed by studies on chloroplast DNA (Palmer et al, 1983), serology of seed proteins (Vaughan, 1977), rubisco (Robbins and Vaughan, 1983) and molecular markers (Coulthard and Denford, 1982; Arus, 1984; Quiros et al., 1985; 1986; Fidgore et al., 1988)).

In contrast, no much is known about the origin of the cultivated diploid species involved in the U triangle. Based on studies of pachytene karyotypes (Robbelen, 1960), di-genomic and tri-genomic hybrids (Mizushima, 1980; Prakash and Hinata, 1980) a common origin for B. nigra, B. oleracea and B. campestris genomes from an ancestor of six chromosomes was proposed. Song et al.(1989) in a taxonomic study based on RFLP's, proposed a closer relationship between B. oleracea and B. campestris than B. nigra, which was found to be in the same lineage as the species of the genus Sinapis.

Several cytogenetic studies have attempted to determine the relationship between cultivated and wild species of Brassica and related genera including Sinapis, Diplotaxis, Erucastrum, Eruca and Raphanus (Mizushima, 1968; Harberd, 1972; Prakash and Hinata, 1980; Takahata and Hinata, 1983). Recently, Warwick (1989) based on chloroplast DNA proposed a phylogenetic tree for these species.

In the present study we analyze the relationships among the n=8 species B. nigra, B. fruticulosa, B. maurorum, B. spinescens and Sinapis arvensis (n=9) by cytological and molecular approaches. Although these species have been reported by previous studies to be related, their philogeny remains unclear.

## MATERIALS AND METHODS

We surveyed eight accessions of B. nigra and one accession for each of the other n=8 and n=9 (2 to 4 plants per accession). Geographical origins as well as accessions numbers are listed in Table 1.

Chromosome pairing in $F_1$ interspecific hybrids is considered to be a measure of relatedness between parental. Thus, $F_1$ interspecific hybrids were generated by crossing the species in all possible combinations. Two accessions of B. nigra listed in Table 1 were considered. Emasculation of the female parent was used to eliminate the possibility of selfing. Embryos were rescued at ten days after fertilization and allowed to mature and germinate on artificial media (Quiros et al., 1987). All $F_1$ hybrids were confirmed by isozyme analysis. Male fertility on these hybrids was estimated by the percentage of pollen grains stained with 1% acetocarmine. At least 1000 pollen grains were counted for each plant. For the pairing studies, flower buds were fixed in Carnoy's (6 parts of alcohol, 3 parts of chloroform and 1 part of acetic acid) for 24 hours and stored in 50% alcohol at 4 °C. Anthers were dissected and squashed in 1%

acetocarmine and pollen mother cells were observed under phase-contrast microscopy.

Two types of DNA markers were used for the molecular study: RFLPs (Restriction Fragment Length Polymorphism) and RAPDs (Random Amplified Polymorphism). The techniques reported by Kianian (1990) were followed for DNA extraction and Southern blotting procedures. RAPD markers were generated by using random primers from Operon Technology (Kit A) following the Polymerase Chain Reaction amplification procedure of Williams et al. (1990).

Table 1: Geographical origins and accessions numbers for the different species used in the study.

| Species | Acc # | Geo. Origin |
|---|---|---|
| B. nigra (ng) | B1154* | Pakistan |
| | B1155 | Pakistan |
| | B1157 | India |
| | B1158 | Netherlands |
| | B1160 | India |
| | B1164* | Turkey |
| | B1170 | Ethiopia |
| | B1179 | Abissinia |
| B. fruticulosa (ft) | B1707 | Spain |
| B. maurorum (bm) | B1706 | Algeria |
| B. spinescens (sp) | B1705 | Algeria |
| S. arvensis (ar) | B1679 | Spain |

*: accessions used in the crosses

## RESULTS

Reciprocal crosses between the different species generated a total of eighteen interspecific hybrids. Pollen fertility in the hybrids ranged from 0 to 43.5%. The range of male fertility as well as the number of individuals generated in each cross are listed in Table 2.

Table 2. Interspecific hybrids, range of pollen fertility and maximun number of bivalents in metaphase I.

| Hybrid | # in. | Range Fert. | Max # biv. |
|---|---|---|---|
| sp x ng | 2 | 13.9-29.9 | 4 II |
| ft x ng | 3 | 0 | 4 II |
| ma x ng | 1 | 0 | 3 II |
| ma x ar | 2 | 0 | 3 II |
| ft x sp | 4 | 11.0-30.0 | 8 II |
| sp x ft | 6 | 20.7-43.5 | 8 II |

Chromosome studies showed different frequency of bivalent formation in all the hybrids (Table 2). The hybrids between B. spinescens and B. nigra showed low chromosome pairing, however, in spite of poor pairing, the hybrids were partially fertile. On

the other hand, B. fruticulosa x B. nigra sterile hybrids, showed a maximum number of four bivalents. B. maurorum x B. nigra and B. maurorum x S. arvensis showed 3 bivalents. In most of the cases the univalents were unevenly distributed in the poles in Anaphase I. The hybrids between B. fruticulosa and B. spinescens showed the highest fertility and number of bivalents. Even though configurations of 8 II were usual it was also possible to find multivalent formation. The most frequent configuration was 5 II and 1 VI.

At the molecular level both RFLP and RAPD markers revealed large interspecific and intraspecific variation for B. nigra . It was possible to find both, common as well as species-specific fragments for each of the species. A total of 143, both RFLP and RAPD, were scored for presence/absence. The results are listed in Table 3. Examples of these markers are shown in Fig. 1 and 2.

Table 3. Percentage of coincident markers.

| | ng | ar | ft | ma | sp |
|---|---|---|---|---|---|
| ng | 68.9* | 16.9 | 25.4 | 27.1 | 30.5 |
| ar | | - | 15.3 | 15.3 | 11.9 |
| ft | | | - | 23.7 | 25.4 |
| ma | | | | - | 25.4 |
| sp | | | | | - |

*: mean value from 8 accessions.

58.7 % of the fragments were species-specific. In some cases intraspecific variability was observed.

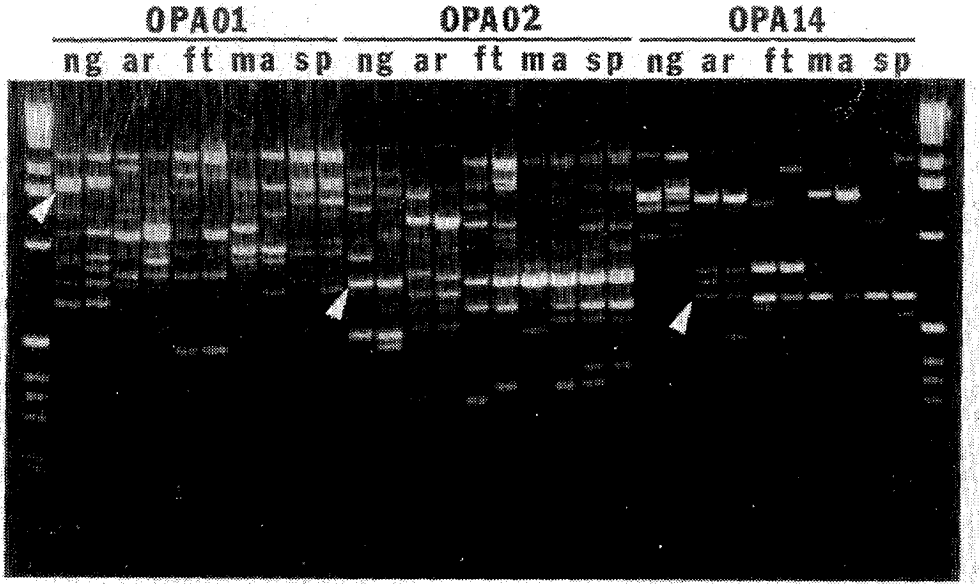


Fig.1: Example of amplification products disclosed by three different primers (OPA01, OPA02 and OPA14) for 5 species. Arrows point coincident markers.

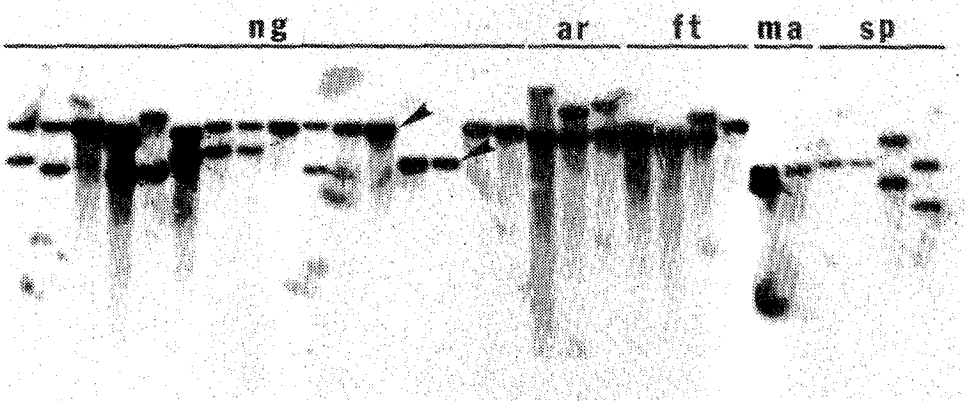


Fig.2: Example of a RFLP marker (Probe pBN33) for Eco RI digests of 5 species. Arrows point coincident markers.

## DISCUSSION

The chromosome pairing studies revealed partial homology of the genomes of the parental species. The highest homology was observed for B. fruticulosa and B. spinescens. This agrees with the hybridization studies of Harberd (1972), who classified both species in the B. fruticulosa cytodeme, along with B. maurorum. However, chromosomal rearrangements among these species have been reported by Takahata and Hinata (1983). This is in agreement with the presence of multivalents in our hybrids, due perhaps to multiple chromosomal translocations, thus resulting in low pollen fertility.

The low frequency of bivalents (3II to 4II) in the hybrids involving B. nigra and the other n=8 species indicates extensive chromosomal divergence among the genomes of these species. Complete sterility was observed in these hybrids, except for B. spinescens x B. nigra which had a pollen fertility equivalent to that observed for the B. fruticulosa x B. spinescens hybrids. Previous studies on hybrids involving B. nigra, B. fruticulosa, B. maurorum and S. arvensis (MIzushima, 1968; Prakash and Hinata, 1980; Takahata and Hinata, 1983) reported that the number of bivalents in the hybrids sometimes varies when different accessions are used as parental species (Takahata and Hinata, 1983). Thus in order to determine reliably the chromosomal homology of these species, hybrids involving more than one accession will be necessary.

At the molecular level the percentage of DNA markers coincident between B. nigra and the wild species with n=8 is similar to the values obtained among B. fruticulosa, B. maurorum and B. spinescens, which belong to the same cytodeme. Considering these results, B. nigra could be included in the same group with the other Brassicas with 8 chromosomes. On the other hand, the percentage of fragment coincidence between the different accessions of B. nigra is 68.9%. The highest value of coincidence between B. nigra and the other n=8 species is 30.5% (B. nigra and B. spinescens), far from the value observed among B. nigra accessions.

Although the present study sampled only a single accession for each of the wild species, it is possible to conclude that

the species classified in the B. fruticulosa cytodeme are quite divergent from each other in spite of the partial fertility and chromosome pairing of the hybrids. Furthermore, the species B. nigra, which is not included in this cytodeme (Harberd 1972), displayed the highest marker coincidence with B. spinescens, and similar coincidence values with the other two n=8 species, as those observed among themselves. As expected, the lowest coincidence values were observed between S. arvensis (n=9) and the species with n=8 chromosomes.

In conclusion, the level of divergence of B. fruticulosa, B. maurorum and B. spinescens is similar to that observed between them and B. nigra . Furthermore, the higher marker coincidence between B. nigra and B. spinescens and partial hybrid fertility indicates that these two species are more related than previously thought. Additional accessions for each of the wild species will be surveyed to get a more accurate determination of their phylogenetic relationships.

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