ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

# Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 7 (3): 475-480, 2008
ISSN 1682-296X


# Genetic Changes Between $C_0$, $C_3$, $C_5$ Cycles of Selection and $S_4$ Lines of Two Cabbage (*Brassica oleracea* L. var. *capitata* L.) Open Pollinated Populations Based on RAPD Markers

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**Abstract:** Genetic diversity during selection process (starting material, material after 3 and 5 cycles of mass-selection and breeding lines produced by selfing and 4 cycles of pedigree selection) obtained from two local Greek cabbage Open-Pollinated (OP) populations was investigated, using RAPD (Random Amplified Polymorphic DNA) markers, on 12 individual plants selected from each population. A total number of 24 random primers were employed and in the entire material studied 277 bands were detected with an average of 11.54 bands/primer. Some of the bands (80 or 72 out of all the bands) revealed polymorphism in selection products of the cabbage populations at an average of 3.47 and 3.00 polymorphic bands/primer for each population. Nei's standard genetic distances between the selected products were grouped by the Unweighted Pair Group Method and Arithmetic Average (UPGMA) clustering method. Average genetic diversity within and between the selection cycles and S-lines was evaluated. It was concluded that RAPD markers could be used to monitor changes occurring during the breeding process in the two cabbage populations.

**Key words:** *Brassica oleracea*, breeding, genetic changes, molecular marker, RAPD, selection process

## INTRODUCTION

Cabbage (*Brassica oleracea* L. var. *capitata* L.) is a cultivar or a subspecies of the species *Brassica oleracea* L. (2n = 2x = 18) of the Cruciferae family (Chiang *et al*., 1993). It was cultivated for the first time by ancient Greeks in 600 BC (Thompson, 1974; Peirce, 1987). At that time there were no cultivars of the species *Brassica oleracea* to form heads (var. *acephala*). The var. *capitata* appeared in Germany in 1150 AD (Thompson, 1974). At present, it is being cultivated in five continents (Peirce, 1987) and is considered as one of the most important vegetables (Chiang *et al*., 1993). All local cultivars of cabbage, whose cultivation in Greece is being reduced year by year, comprise Open-Pollinated (OP) populations that are less productive compared to commercial hybrids as their heads lack uniformity and field durability. However, they appear with thinner, more crispy and juicy head leaves (Koutsos and Koutsika-Sotiriou, 2001a, b). These local populations are a valuable genetic resource for cabbage breeding and consequent commercial exploitation.

RAPD (Random Amplified Polymorphic DNA) markers described in 1990 (Williams *et al*., 1990) they have been widely used for a number of applications in plant breeding and genetic studies (Kumar, 1999), for their simplicity and inexpensiveness. In *Brassica* species, RAPD markers were employed to estimate intraspecific genetic relationships among genotypes (Lazaro and Aguinagalde, 1998; Geraci *et al*., 2001; Cartea *et al*., 2005; Fu *et al*., 2006), to estimate genetic distances and to study the genetic diversity between accessions (Rabbani *et al*., 1998; Divaret *et al*., 1999; Chuang *et al*., 2004; Teklewold and Becker, 2006; Okumus and Balkaya, 2007). RAPD markers have been also used to assess seed purity of commercial hybrids (Crockett *et al*., 2000) and to discriminate among cultivars (Cansian and Echeverrigaray, 2000; Astarini *et al*., 2004). Since

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molecular markers are assumed to be phenotypically neutral and environmentally independent, it usually appears easier to work with them in comparison to agronomically important traits under field conditions. The use of RAPD markers to monitor changes occurring at the molecular level during the selection process has been reported earlier for a Greek snap bean cultivar (Tertivanidis *et al*., 2003).

The objectives of this study were to investigate the genetic changes occurred after 5 cycles of mass-selection and four cycles of consecutive selfing and pedigree selection in two local Greek Open-Pollinated (OP) populations of white cabbage, by using RAPD (Random Amplified Polymorphic DNA) markers. The increase of homozygosity or the decrease of heterozygosity in genotypes, which reaches its maximum rate by selfing were worth studying. The genetic changes, because of the selection, were assessed by analyzing genetic diversity within and between the breeding materials.

## MATERIALS AND METHODS

**Plant material:** The materials studied were obtained from two local cabbage OP populations Aeginiou (A) and Neas Magnesias (NM). These OP populations ($C_0$) firstly were improved by three cycles of mass selection under nil-competition and controlled pollination. Then, on the $C_3$ genetic material two more cycles of mass-selection ($C_5$) and four cycles ($S_4$) of successive selfing and pedigree selection were applied. The selection criteria were heading, no bolting, fewer head cover leaves and fewer frame leaves relative to head size. Individual plants from this material were grown at the stage of three to four leaves in the greenhouse. At that time, 12 plants from each generation were sampled (2-3 leaves from each plant) and stored in a deep freezer at -80°C. The number of plants chosen is considered an adequate sample to study diversity in a population of outbreeding species such as cabbage (Divaret *et al*., 1999).

**DNA extraction:** Total genomic DNA of 200 mg treat healthy tissue of starting material was isolated from each individual plant by a slightly modified CTAB method (Rogers and Bendich, 1988). The explant was frozen in liquid nitrogen and ground into a fine powder that was transferred to a micro tube. One milliliter of preheated CTAB buffer was added and the mix was incubated for 30 min at 65°C. Following the incubation, the mix was centrifuged for 10 min at 12000 g and 500 μL of supernatant was transferred to a fresh micro tube. Two microliter of RNase A solution (10 mg $mL^{-1}$) were added and incubated for 15 min at 37°C. Chloroform extraction was carried out as follows: 200 μL chloroform/isoamylalcohol (24:1) were added and the mixture was shaken vigorously for 30 sec. The extract was centrifuged for 10 min at 12000 g and the upper phase was transferred to a fresh microtube. Two volumes of CTAB precipitation buffer were added and the mixture was incubated for 60 min at room temperature, followed by centrifugation at 12000 x g for 5 min. The supernatant was discarded and the pellet was dissolved in 350 μL of 1.2 M NaCl solution for 15 min at room temperature. A chlorophorm/isoamylalcohol extraction step was performed and 0.6 volume of isopropanol was added to the supernatant. DNA was collected by centrifugation for 10 min at 11500 x g. The DNA pellet was washed with 70% (v/v) ethanol and dissolved in 100 μL TE buffer solution. DNA concentration was estimated by agarose gel stained with ethidium bromide.

**Generation and analysis of RAPD:** For RAPD analysis, 10-mer primers random (Operon Technologies Inc., Alameda, CA) were used. Amplification was carried out in 25 μL reaction mix containing about 25 ng genomic DNA, 0.2 mM of each dNTP, 0.2 μM of primer, 2 mM of $MgCl_2$ and 1 U of Taq DNA polymerase (Biotools) with supplied buffer. DNA amplification was performed in a PTC 200 thermal cycler (MJ Research) according to the following thermal profile: initial denaturation at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 45 sec, annealing at 35°C for 1.5 min and extension at 72°C for 1.5 min, followed by final extension at 72°C for 3 min. Amplification products were separated in 1.5-2% agarose gel and detected by staining with ethidium bromide (Sambrook *et al*., 1989). The gels were photographed under UV light. Each PCR reaction was run in duplicate and only well defined and reproducible bands were analyzed.

**Data analysis:** Each polymorphic band in every sample was scored as a binary character with the value of 1 for the presence and 0 for the absence of the band and a data matrix was created. DNA bands of identical gel migration were assumed to represent the same allele at a locus. Only reproducible and clearly visible bands were scored. The bands detected in all plant samples at the same position (monomorphic) as well as not directly comparable bands between individuals because of very similar migration, were excluded from the analysis. POPGENE software (Yeh *et al*., 1997) was used to calculate gene frequencies and genetic diversity within and between different genetic materials. The tabulated gene frequencies were interested as input into the program NTSYS pc version 2.1 (Rohlf, 2000) for calculation of Nei's (1972) genetic distances and construction of a dendrogram based on the Unweighted

Pair Group Method and Arithmetic Average (UPGMA) clustering procedure. The cophenotic correlation for each dendrogram was also computed as a measure of goodness of fit for the method of cluster analysis used for each respective data set.

## RESULTS AND DISCUSSION

A set of 24 random primers, showing detectable polymorphism in a previous test (data not shown), was used in PCR amplification reactions with all 96 individuals tested. A total number of 277 bands ranging in size from 250 to 2200 bp were detected. The number obtained for each primer ranged between 5 to 17, with a mean of 11.82 bands/primer for individuals of population A and between 6 to 17, with a mean of 11.04 bands/primer for individuals of population NM. Excluding all monomorphic bands from analysis, a total of 80 and 72 polymorphic bands were finally scored for selection products of the populations A and NM, respectively. The primer OPAL6 showed polymorphic band only for individuals of population NM. The relatively high number of loci analysed was chosen to balance against the number of individuals per population, as recommended by Nei (1987). The percentage of polymorphic bands, averaged over all markers, was 31.36% for all selection products of population A and 27.17% for all selection products of population NM (Table 1). Earlier reports demonstrate consistency of 22.41% in RAPD polymorphic bands detected in commercial F1 hybrids and 16.9% in DH lines of cabbage, respectively (Crockett *et al*., 2000; Kaminski *et al*., 2003). Selfing gives the most rapid increase in homozygosity and also the most rapid decrease in fitness due to inbreeding depression (Miranda, 1999).

Gene frequencies were determined from the presence or absence of a particular amplified fragment across the different categories of the genetic material studied. Fragments of identical migration pattern on the electrophoretic gel were assumed to represent the same allele at a locus. Some of the alleles were present only in one type of genetic material studied. Average genetic diversity within each of the selection product of the two populations, was calculated by Nei's (1973) index. Genetic diversity ranged from 0.307 to 0.235 for selection products of population A (Table 2) and from 0.226 to 0.192 for selection products of population NM (Table 3). As expected, in both cases, breeding lines manifested the lowest diversity level.

The breeding material resulting from either three or five cycles of selection showed very little or no reduction in average genetic diversity. The absence of diversity reduction might be due to quite mild selection imposed and elevated buffering capacity of landraces

Table 1: Polymorphism detected by the use of 24 random primers on selection products from two local Greek cabbage OP populations

| | A | | | NM | | | |
|---|---|---|---|---|---|---|---|
| Primer | No. of amplified products* | No. of polymorphic bands** | Percentage of polymorphic bands | No. of amplified products* | No. of polymorphic bands** | Percentage of polymorphic bands | Products length (bp) |
| OPAL7 | 11 | 5 | 45.4 | 11 | 4 | 36.4 | 450-2000 |
| OPAL8 | 10 | 3 | 30.0 | 10 | 3 | 30.0 | 650-2200 |
| OPAH12 | 11 | 4 | 36.4 | 10 | 4 | 40.0 | 250-2000 |
| OPAL5 | 13 | 4 | 30.8 | 11 | 3 | 27.3 | 500-2000 |
| OPAK12 | 8 | 2 | 25.0 | 9 | 3 | 33.3 | 350-2000 |
| OPAM10 | 11 | 4 | 36.4 | 11 | 3 | 27.3 | 600-2200 |
| OPAH15 | 14 | 4 | 28.6 | 11 | 3 | 27.3 | 450-2000 |
| OPAB4 | 13 | 4 | 30.8 | 12 | 4 | 33.3 | 400-2200 |
| OPR6 | 11 | 3 | 27.3 | 12 | 3 | 25.0 | 500-2200 |
| OPAL12 | 9 | 3 | 33.3 | 8 | 2 | 25.0 | 400-1500 |
| OPAB5 | 16 | 5 | 31.2 | 16 | 3 | 18.7 | 400-2000 |
| OPR12 | 12 | 3 | 25.0 | 12 | 4 | 33.3 | 350-2000 |
| OPAK4 | 14 | 4 | 28.6 | 12 | 3 | 25.0 | 400-2200 |
| OPAK5 | 10 | 5 | 50.0 | 9 | 3 | 33.3 | 350-1500 |
| OPAK15 | 16 | 5 | 31.2 | 15 | 7 | 46.7 | 450-2200 |
| OPAL14 | 17 | 4 | 23.5 | 17 | 4 | 23.5 | 350-2000 |
| OPAG15 | 11 | 3 | 27.3 | 11 | 2 | 18.2 | 300-1500 |
| OPAG20 | 11 | 3 | 27.3 | 11 | 3 | 27.3 | 500-2000 |
| OPAB9 | 13 | 3 | 23.1 | 13 | 3 | 23.1 | 500-2000 |
| OPAG5 | 5 | 1 | 20.0 | 6 | 1 | 16.7 | 550-1500 |
| OPAL17 | 9 | 2 | 22.2 | 9 | 1 | 11.1 | 550-2000 |
| OPAL6 | 11 | - | - | 11 | 1 | 9.1 | 500-2000 |
| OPAH18 | 5 | 3 | 60.0 | 7 | 3 | 42.9 | 450-2200 |
| OPAK2 | 11 | 3 | 27.8 | 11 | 2 | 18.2 | 500-2200 |
| Total | 272 | 80 | | 265 | 72 | | |
| Mean | 11.82 | 3.47 | 31.36 | 11.04 | 3.00 | 27.17 | |

*Total number of scorable bands detected, **Total number detected as polymorphic in at least one sample

Table 2: Average genetic diversity within four selection products of population A for 80 polymorphic RAPD markers

| Genetic material | Gene diversity | SE* | Gene identity** |
|---|---|---|---|
| A $C_0$ | 0.28 | 0.09 | 0.72 |
| A $C_3$ | 0.31 | 0.09 | 0.69 |
| A $C_5$ | 0.28 | 0.09 | 0.72 |
| A $S_4$ | 0.23 | 0.10 | 0.76 |

*Standard error within populations, **1-gene diversity

Table 3: Average genetic diversity within four selection products of population NM for 72 polymorphic RAPD markers

| Genetic material | Gene diversity | SE* | Gene identity** |
|---|---|---|---|
| NM $C_0$ | 0.22 | 0.09 | 0.78 |
| NM $C_3$ | 0.22 | 0.08 | 0.78 |
| NM $C_5$ | 0.23 | 0.09 | 0.77 |
| NM $S_4$ | 0.19 | 0.09 | 0.81 |

*Standard error within populations,**1-gene diversity

Table 4: Partitioning of mean genetic diversity within and between different breeding materials of the cabbage populations A and NM

| | Breeding stages | Ht* | Hs** | Gst*** |
|---|---|---|---|---|
| A | $C_0$-$C_3$ | 0.30 (0.03)**** | 0.29 (0.033) | 0.03 |
| | $C_0$-$C_3$-$C_5$ | 0.30 (0.03) | 0.29 (0.03) | 0.05 |
| | $C_0$-$C_3$-$C_5$-$S_4$ | 0.31 (0.03) | 0.28 (0.03) | 0.10 |
| NM | $C_0$-$C_3$ | 0.22 (0.04) | 0.22 (0.04) | 0.01 |
| | $C_0$-$C_3$-$C_5$ | 0.23 (0.04) | 0.22 (0.04) | 0.03 |
| | $C_0$-$C_3$-$C_5$-$S_4$ | 0.24 (0.03) | 0.21 (0.03) | 0.12 |

*Total genetic diversity over all genetic materials, **Genetic diversity within genetic material, ***(Ht-Hs)/Ht-proportion of genetic differentiation between genetic materials, ****Standard deviation

(Frankel *et al*., 1995), which caused no significant gene frequency changes. By contrast, a significant reduction of genetic diversity was evident in the germplasm resulting after four cycles of selfing and pedigree selection. This most intensive kind of inbreeding converts variation among the individuals of a population into variation between resulting lines, while the pedigree approach further minimizes genetic differentiation.

These results are further substantiated by partitioning the mean genetic diversity observed. As shown in Table 4, mean diversity over all genetic materials of population A (Ht) is 0.307 and is partitioned in an average of 0.276 within genetic material diversity (Hs) and a mean of 0.101 between genetic materials differentiation (Gst). The latter means that only about 10% of the diversity is observed between breeding materials compared to the remaining 90% of within genetic materials diversity. For the selection products of population NM, mean overall diversity was equal to 0.122, showing that only about 12% of the diversity is observed between breeding materials. As expected, there was a gradual increase of diversity between NM selection cycles and S-lines.

Genetic distances (Nei, 1972) among the selection products of populations A and NM, calculated on the basis of gene frequencies are showed in Table 5 and 6. These genetic distances were used to construct the respective dendrograms, based on the UPGMA method for different materials of populations A and NM (Fig. 1, 2).

Table 5: Nei's (1972) Genetic distances among four breeding materials of the cabbage population A

| Breeding material | $A_0$ | $A_3$ | $A_5$ | AS |
|---|---|---|---|---|
| $A_0$ | - | | | |
| $A_3$ | 0.09 | - | | |
| $A_5$ | 0.08 | 0.18 | - | |
| AS | 0.32 | 0.34 | 0.31 | - |

Table 6: Nei's (1972) Genetic distances among four breeding materials of the cabbage population NM

| Breeding material | $NM_0$ | $NM_3$ | $NM_5$ | NMS |
|---|---|---|---|---|
| $NM_0$ | - | | | |
| $NM_3$ | 0.02 | - | | |
| $NM_5$ | 0.09 | 0.12 | - | |
| NMS | 0.28 | 0.32 | 0.32 | - |

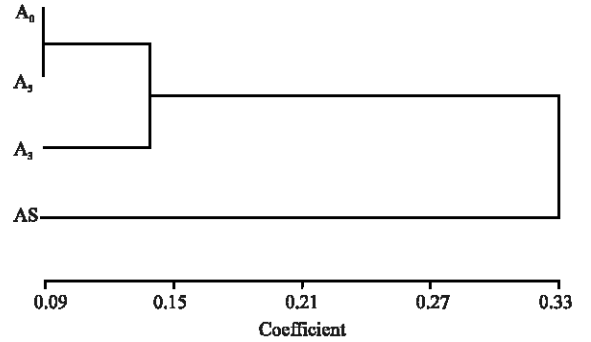


Fig. 1: Dendrogram based on data of the Table 5 showing the Nei's (1972) genetic distance coefficient on four selection products of cabbage populations A (cophenotic value r = 0.95)

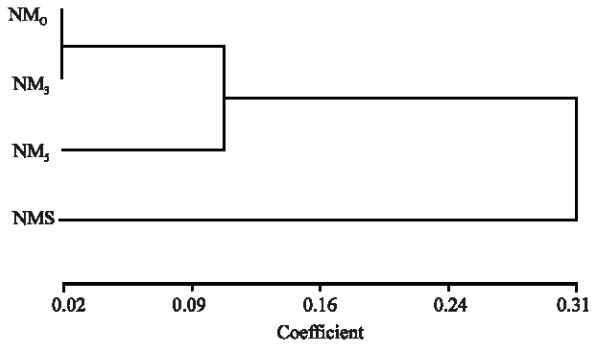


Fig. 2: Dendrogram based on data of the Table 6 showing the results of clustering using Nei's (1972) genetic distance coefficient on four selection products of cabbage populations NM (cophenotic value r = 0.99)

Studying the dendrograms, it is concluded that the three and even the five cycles of mass selection in nil competition conditions of both (A and NM) local populations of cabbage did not change the gene frequencies to such an extent so as to change cluster in the dendrograms. On the contrary the inbred lines AS and NMS are grouped in different cluster than both those of

the starting material ($C_0$) and the materials obtained from mass selection ($C_3$ and $C_5$). In conclusion, a wider change of the gene frequencies was revealed between inbred lines and mass selection materials. The above result was anticipated, as Koutsos and Koutsika-Sotiriou (2001a) reported remarkable progress in the $C_3$ selection product in all traits (heading, no bolting, fewer head cover leaves and fewer frame leaves relative to head size). The phenotypic changes due to further selection were also recorded. The most worth mentioned are: in the $C_5$ the percentages of heading and no bolting reached 100%; the seed germination in the $S_1$ selection product of A and NM populations was 8 and 21%, while in the $S_4$ was 44 and 70%, respectively; the mean head weight in the $C_0$, $C_3$ and $C_5$ was roughly 5 kg but in $S_4$ reduced to 2.5 kg.

It is undoubtful that selfing gives some combinations of genes, which do not result in viable embryos. It is well known that limitations for studying inbreeding that do not contribute to the performance of progenies, as when seeds are planted in excess and the lethal will not appear in the harvest (Fisher, 1965). However, the use of inbred lines could result in a biased estimate of the inbreeding depression effects (Sing *et al*., 1967). Consequently, some genes are excluded from the generations obtained through selfing, which in turn resulting in a wide change of genes frequencies in every single generation.

Conclusively, the results of the present study showed that RAPD markers could be used to follow DNA changes occurring during a selection process. In particular, the data obtained demonstrate that mass selection, which is based on phenotypic characters evaluation, caused a gradual change on allele frequencies. As anticipated, these changes were dramatic in the lines produced by self-pollination, although some of them are not detected in $S_4$ generation. Despite the evidences of a proportionally high contribution of deleterious genes to minor and not visually detected effects, a better knowledge of their actual proportion on the net inbreeding depression would be helpful for understanding the phenomenon and planning breeding strategies (Miranda, 1999).

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