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### **AFLP-PROFILING OF LONG-TERM STORED AND REGENERATED RYE GENE BANK SAMPLES**

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**Abstract:** The aim of these studies was to analyse the genetic changes induced by natural aging during long-term seed storage of rye. For this purpose, the AFLP (Amplified Fragment Length Polymorphism) technique was applied. In the experiment, DNA variation was demonstrated in seven-day-old seedlings of four seed samples of cv. Dańkowskie Złote, showing different levels of viability following long-term storage. Among the 362 AFLP fragments analysed, 73 had significantly different frequencies in at least one of the series. Principle Coordinate Analysis (PCA) based on molecular data revealed differences between the progenies of naturally aged seed samples with variable initial viability. It was clearly shown that materials with low viability differed in structure from highly viable ones, and that the population changes exhibited in the first case are preserved through regenerations. Although changes that were observed for initially viable samples were not so significant, they still occurred – probably as a result of genetic shift.

**Key words:** Rye, *Secale cereale* L., AFLP, Seed Aging, Genetic Shift, Genetic Drift

### **INTRODUCTION**

During long-term storage, as necessary for *ex situ* conservation, genetic changes are an important issue. These changes may take the form of induced mutations, such as chromosomal aberrations and point mutations, or result from natural selection within a heterogeneous population. It has long been recognized that chromosome aberrations occur more frequently in plants grown from old seed

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[1-3]. Osborne [2] indicated that with seed aging, the total number of unrepaired DNA strand breaks increased [2]. But Murata's [3] results on barley clearly demonstrated that aging-induced chromosomal aberrations were eliminated during the growth and reproductive stages of the plant's life and thus had little consequence in altering the genetic structure of the successive generations. If small changes such as point mutations are non-lethal, they could be accumulated in a given population. Information on this particular topic is limited and has generally only been observed at the phenotypic level [4, 5]. A more serious threat to the genetic structure of a seed population is the natural selection process. A heterogeneous seed population undergoes selection both during storage and regeneration. Selection within a preserved population may result in a differential survival rate during storage and in productivity changes due to environmental selection under field conditions during germplasm regeneration, including the effects of weather, pathogens, maturity stages, outcrossing, and even human error [6, 7].

However, very little work has been done so far to address the extent to which seed aging can alter DNA fingerprints. Only a couple of studies have been focused directly on genomic diversity by undertaking RAPD analysis [8, 9]. Other methods used for the investigation of such events have been based on phenotypic observations [10] or on protein markers [11, 12], but very rarely on DNA markers [9, 13]. We were interested in determining whether AFLPs could be applied to study the effects of seed aging during storage under gene bank conditions. We also investigated the possible contributions of study at the molecular level for natural selection changes in a series of rye samples of different viability and after different reproduction cycles.

## MATERIALS AND METHODS

### Plant material

In the experiment four rye seed samples – cv. Dańkowskie Złote (DZ) progenies of the initial population DZ1.0 (44 individuals in each sample) were used. Those seeds which had been stored for 14 years under seed bank conditions (viability about 5%) followed by one reproductive cycle in the field were marked DZ1.1; those seeds stored as above but followed by three reproductive cycles were

Code of sample	Period of storage (years)	Germination ability (%)	Number of reproduction cycles
DZ4.1	4	91	1
DZ4.3	4	91	3
DZ1.1	14	5	1
DZ1.3	14	5	3

Tab. 1. Rye samples used for investigation

marked DZ1.3; those seeds with an initial viability of more than 91% followed by one and three reproduction cycles were marked DZ4.1 and DZ4.3 (two control samples) (Tab. 1). Seeds of each treatment were germinated in sterile sand moistened with distilled water at 20°C in germinating chambers. Seven-day-old seedlings were cut, washed with ether and dried on filter paper.

#### **DNA extraction**

DNA extraction was performed on fresh leaf tissue (about 100 mg) ground in liquid nitrogen according to the Qiagen kit procedure (DNeasy Plant Mini Handbook for DNA isolation from plant tissue). DNA integrity was analysed on 1.4% agarose gels containing 1<sup>x</sup> TBE buffer and ethidium bromide (50 µg/100ml) under 20 V/cm. The quantity was determined spectrophotometrically (GeneQuanta, Pharmacia LKB). For routine work, standard dilutions of 10µg/ml were used.

#### **Amplified Fragment Length Polymorphism**

The AFLP technique was performed according to the procedure described by Vos [14] with our modification for rye [15]. Briefly, 250 ng of genomic DNA underwent simultaneous digestion with *EcoRI* and *MseI*. After inactivation, ligation of adapters corresponding to the sticky ends of the DNA fragments generated by both enzymes was performed. This was followed by a pre-amplification step in the presence of the primers with one selective nucleotide ("A" for *EcoRI* and "C" for *MseI*). During selective amplification, oligonucleotides with two additional selective nucleotides at the 3'-ends were used: EAAC/MCCA; EAAT/MCTT; EACG/MCGG; and EAGT/MCAA.

In order to identify the amplified DNA fragments, *EcoRI* compatible primers were labelled at the 5'-end with  $\gamma$ -<sup>32</sup>P ATP. PCR products were separated on 5% PAGE and exposed to (FOTON-XC) X-ray film overnight.

#### **Data analysis**

Visible, reproducible and polymorphic bands were scored for all the samples as presence (1) or absence (0), and arranged in the form of matrices for further evaluation. The frequencies of ones for all the markers were calculated. Several statistical analyses, aimed at detecting similarity among the samples, were applied. First, the differences between DZ4.1, DZ4.3, DZ1.1 and DZ1.3, with respect to the frequency of all the individual markers, were tested using the chi-square test (with a critical significance level of 0.001, appropriate for the large number of hypotheses tested). Similarly, the significance of differences between pairs of samples was tested. Then similarity coefficients were evaluated, based on Euclidean distances between the samples, as calculated from the frequency data. Finally, the binary presence/absence data for individual plants were subjected to principal component analysis [16], allowing the generation of a graphical representation of the differences between samples and the variability of plants within samples.

## RESULTS

For selective PCR, four different AFLP primer pair combinations were applied, generating individual profiles and allowing the identification of 362 DNA fragments among which 231 (64%) were polymorphic and 131 (36%) monomorphic. The number of DNA fragments identified by individual primer pairs ranged from 74 to 103 with an average of 90.5. All the primer pairs generated polymorphic fragments, and their number varied from 41 to 65 with an average of 57.8. The chi-square test showed that the differences between samples were significant with respect to the frequency of 73 polymorphic fragments (at the significance level  $\alpha = 0.001$ ).

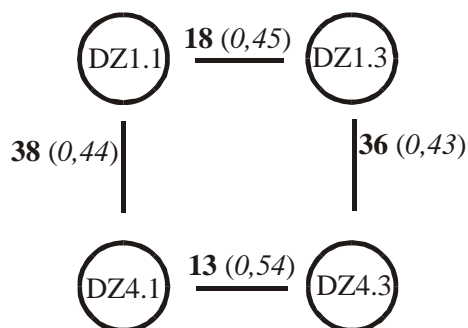


Fig. 1 Similarity structure of the analysed samples described by the number of markers with frequencies significantly different in corresponding populations (in bold) and by the similarity coefficient based on the Euclidean distances between populations (in italics).

Figure 1 shows the structure of differences between pairs of samples, measured by the number of markers with frequencies differing significantly in two corresponding samples ( $\alpha = 0.001$ ) and by the similarity coefficient of two samples. The diagram shows that the situation is dominated by two contrasts: between DZ1.1 and DZ4.1 and between DZ1.3 and DZ4.3 (with a large number of significant markers, 38 or 36, and low similarity coefficients, 0.44 and 0.43, respectively). Therefore, it can be concluded that samples of different viability have different genetic structure for many markers. The differences between DZ1.1 and DZ1.3 and between DZ4.1 and DZ4.3 are less pronounced, although significant for 18 and 13 markers, respectively. Thus, the number of regeneration cycles plays a less important role, especially for samples after 4-years of seed storage. Figure 2 shows the positions of individual plants from DZ1.1, DZ1.3, DZ4.1 and DZ4.3, in the coordinates determined by the principal components analysis. In the left panel, the most important difference between the “old” samples (DZ1.1, DZ1.3) on one side and the “young” ones (DZ4.1, DZ4.3) on the other is shown: plants differing in the length of their storage period are clearly discriminated by the first principal axis, V1, containing most

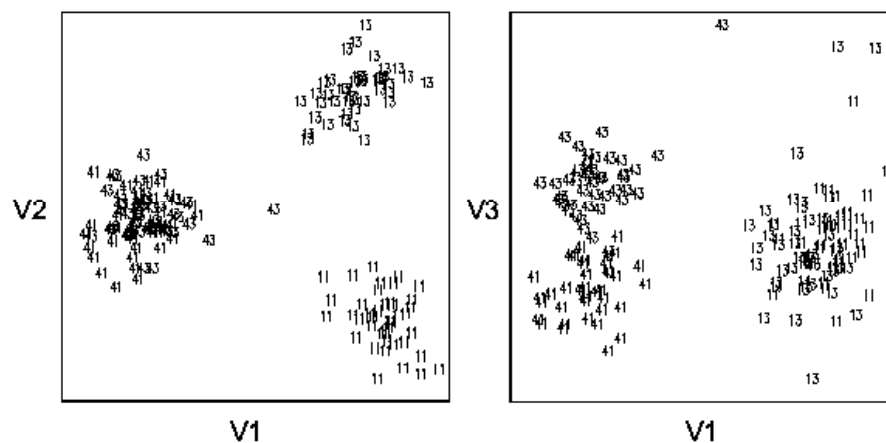


Fig. 2. Plants belonging to DZ1.1, DZ1.3, DZ4.1 and DZ4.3 in the planes spanned by the principal components numbers 1 and 2 (left) and numbers 1 and 3 (right), calculated from the data describing the presence/absence of 231 polymorphic fragments.

of the inter-plant variability. The same panel also shows the less important, but also significant, difference between DZ1.1 and DZ1.3 along the second axis, V2. In the right panel, the least important difference between DZ4.1 and DZ4.3 is shown as the dispersion of plants along the V3 axis.

## DISCUSSION

As a control for DZ4.1, the DZ4.3 was used. Such a comparison gave the opportunity to verify whether three cycles of regeneration of highly viable seeds influenced the population structure. Since only a limited number of seeds (300 kernels for rye) were taken for regeneration, and storage conditions only permitted the maintenance of small samples, there was a threat of loss of the collection's initial genetic variability. This could especially be true for an outcrossing species, which shows naturally high levels of polymorphism.

An analysis of the AFLP fingerprints generated with four selective primer pairs made observations of the genetic changes exhibited among the samples possible. The biggest statistically significant differences in the frequencies of the appearance of some polymorphic DNA fragments were observed between the DZ1.1 and DZ4.1 samples, progenies of strongly different viability seed lots.

This may suggest that at least some of the changes in population structure were associated with a loss of viability, caused by the natural seed aging process. Differences were also observed on comparison of samples DZ1.3 and DZ4.3, demonstrating that successive regenerations did not restore the initial structure of the population and differences between DZ1.1 and DZ1.3 showed the probable influence of selection processes during three cycles of regeneration.

The smallest differences were observed between samples DZ4.1 and DZ4.3 (Fig. 1, Fig. 2). The differences between these series could originate from the natural variability of allogamous rye, rather than by genetic drift during regeneration. It is also possible that the identified DNA fragments are linked to some important genome regions of unknown nature.

Although these results are consistent with those reported in our previous studies based on RAPD profiling [8], the estimated genetic distance between DZ1.1 and DZ1.3 was considerably more distinct with RAPDs than with AFLPs. This could probably be explained by the fact that total genomic DNA was extracted from the endosperm of dry seeds for the RAPD technique. By contrast with RAPD, in the AFLP approach where seedlings are used for extraction, most DNA changes should be eliminated during imbibition and germination. Those facts probably influenced our results.

Till now only Shatters [9] succeeded in monitoring changes induced by seed aging at the DNA level. It was suggested that they could be the result of damages accumulated during seed aging. The author revealed that those alterations were not dispersed throughout the genome. Our experiments performed on a large number of rye plants showed that (in the DZ4.1-DZ1.1 pair) changes of frequencies of some DNA fragments were significant and in that are convergent with Shatter's results. However, investigations performed by Marcos-Filho and McDonald [13] did not ascertain the usefulness of the RAPD approach for such purposes.

These results also concur with earlier reports, where authors observed changes induced by aging and regeneration using morphological traits [10, 17], isozymes [18] and seed storage proteins [11, 12], but, for the first time, at the molecular level it has been demonstrated that the maintenance of high viability of germplasm is important. Using the AFLP approach, it has been shown that material with low viability differs in its structure from highly viable material, and that the population changes exhibited in the first case are preserved through regenerations. Although changes that were observed for an initially viable sample were not so significant in number, they probably still occurred as the result of genetic shift.

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