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STUDIES ON GENETIC CHANGES IN RYE SAMPLES (*Secale cereale* L.) MAINTAINED IN A SEED BANK

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Abstract: The aim of this study was to identify genetic changes in rye seeds induced by natural ageing during long-term storage and consecutive regeneration cycles under gene bank conditions. Genomic DNA from four rye samples varying in their initial viability after one and three cycles of reproduction was analyzed by AFLP (amplified fragment length polymorphism) fingerprinting. Seven *Eco*RI/*Mse*I primer combinations defined 663 fragments, and seven *Pst*I/*Mse*I primer combinations defined 551 fragments. The variation in the frequency of the seventy-four *Eco*RI/*Mse*I bands was statistically significant between samples. These changes could be attributed to genetic changes occurring during storage and regeneration. However, the *Pst*I/*Mse*I fragments appeared to be uninfluenced by seed ageing, regeneration and propagation. A combined Principle Coordinate Analysis revealed differences between samples with different initial viability. We showed that materials with low initial viability differ in their response from highly viable ones, and that the changes exhibited in the former case are preserved through regeneration cycles.

Key words: Rye, *Secale cereale* L., Seed storage and propagation, AFLP

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Abbreviations used: AFLP – amplified fragment length polymorphism, DZ – Dańkowskie Złote; PCA – principal component analysis; RAPD – random amplified polymorphic DNA; STS – sequence-tagged sites

INTRODUCTION

The conservation of rye germplasm is still mainly achieved through long-term storage of seed under controlled moisture and temperature regimes typical of seed banks. To maintain a high level of seed viability, periodic regeneration is needed. Long-term storage may lead to a build-up of DNA changes (point mutations, chromosome rearrangements, etc.), but these are usually selected against during germination and growth [1]. However, regeneration can eliminate rare genotypes if the sample size is suboptimal (genetic shift), or may favor others due to selection processes [2, 3].

Very little work has been done to analyze the influence on population structure of long-term maintenance of germplasm under gene bank conditions. Methods based on phenotypic observations [4] or protein markers [5-7] have been employed to investigate such events. Variation at the DNA level has been investigated using markers such as RAPDs [8, 9], AFLPs [10], STSs [11] and microsatellites [12, 13]. RAPD and AFLP analyses have indicated that both storage time and the number of regeneration cycles can influence the genetic structure of a population [8, 10]. The same pattern was displayed for the fragment of repetitive DNA element R173, but could not be demonstrated for certain seed storage protein genes [11]. Moreover, studies conducted with microsatellites suggest that neither storage nor regeneration generate *de novo* variation [12, 13].

The aim of this study was to verify our previous results conducted with AFLPs, extending the genome coverage by employing more *Eco*RI/*Mse*I and *Pst*I/*Mse*I primer combinations. These enzyme combinations were chosen since it is recognized that the former amplifies DNA fragments that map by preference to the centromeric and telomeric regions of rye chromosomes, while the latter loci show a more evenly distributed pattern [14, 15]. It has been suggested that the former target largely consists of non-coding regions, and the latter of coding regions. Based on this assumption, we were also interested in exploring the extent to which the two approaches complement one another.

MATERIALS AND METHODS

Plant material

A seed sample of cv. Dańkowskie Złote (DZ) with high viability was divided into two sub-samples: one (DZ1.0) was stored for 14 years without regeneration, and the other (DZ4.0) underwent three consecutive cycles of regeneration during those 14 years (Tab. 1). The viability of DZ1.0 had dropped to 5% after 14 years of storage, while the viability of DZ4.0 had been maintained at a high level (ca 90%). Those samples were taken through one reproduction cycle in the field and marked respetively as DZ1.1 and DZ4.1, followed by three reproduction cycles, marked as DZ1.3 and DZ4.3. Samples marked as DZ1.1, DZ4.1, DZ1.3 and DZ4.3 were used for further studies (for the breeding scheme, see Fig. 1). Seeds of each treatment (88 individuals in each sample) were germinated in sterile,

Tab. 1. Rye samples used for this study.

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

watered sand at 20ºC in germinating chambers. Fresh leaves were collected from seven-day old seedlings and used for total genomic DNA isolation.

DNA extraction

DNA was extracted from about 100 mg fresh leaf tissue following the manufacturer's instructions (Qiagen: DNeasy Plant Mini Handbook for DNA isolation from plant tissue). The purity and quantity of the samples were determined spectrophotometrically. The DNA integrity and lack of RNA impurities were tested in 1.4% agarose gels (1xTBE buffer and ethidium bromide (0.5 ug/ 1ml) at 20 V/cm). For routine purposes, standard dilutions of 10 μg/ml were prepared.

AFLP analysis

The AFLP technique was performed according to the procedure described by Vos *et al.* [15] with a minor modification for rye [17, 18]. Briefly, 250 ng of genomic DNA was digested with *Eco*RI and *Mse*I, or with *Pst*I and *Mse*I, and ligated to the appropriate oligomers. A (weakly selective) pre-amplification step was performed in the presence of primers with one selective nucleotide ("A" for *Eco*RI, "C" for *Mse*I and "G" for *Pst*I). For the selective amplification, we used fourteen primer combinations (E-AAA/M-CAA, E-AAG/M-CAA, E-AAT/M-CCA, E-ACT/M-CGG, E-ACT/M-CTT, E-AGG/M-CAG, E-AGG/M-CGG, P-GAA/M-CAA, P-GAC/M-CGG, P-GCA/M-CCC, P-GCT/M-CGC, P-GGA/M-CCG, P-GGC/M-CTA, and P-GGG/M-CAG) with two additional nucleotides at the 3'-ends. The *Eco*RI and *Pst*I compatible primers were labeled at their 5'-ends with gamma-^{32}P ATP. PCR products were separated on 5% PAGE and exposed to X-ray film (FOTON) at -70ºC overnight. To check its reproductibility, the experiment was repeated twice.

Data analysis

Only reproducible and polymorphic bands were scored for all the samples as presence (1) or absence (0), and the data was arranged in the form of matrices for further evaluation. Several statistical analyses were applied to detect similarity among the samples. First, the differences between DZ4.1, DZ4.3, DZ1.1 and DZ1.3 were tested using the chi-square test (at $\alpha = 0.001$) with respect to frequency of all the individual markers. Similarly, the significance of

differences between pairs of samples was tested. Then, based on Euclidean distances between samples, similarity coefficients were calculated from the frequency data. Finally, the binary presence/absence data for individual plants was subjected to principal component analysis (PCA) [19], allowing a graphical representation to be generated of the differences between samples and the variability of plants within samples.

RESULTS

Fourteen primer pair combinations generated individual AFLP profiles with 1214 clearly visible and scorable signals shared among all four rye samples: respectively, 663 and 551 bands were derived from the *Eco*RI/*Mse*I and *Pst*I/*Mse*I primer combinations. Of these, 481 *Eco*RI/*Mse*I bands and 521 *Pst*I/*Mse*I bands were polymorphic.

The number of bands per *Eco*RI/*Mse*I primer combination ranged from 58 to 111 (average 95), of which between 49 and 82 (average 69) were polymorphic. A chi-square test showed that, in total, the differences between the samples were significant with respect to the occurrence of 236 of the polymorphic fragments. A large number of bands differentiated the low viability seeds (DZ1.1 and DZ1.3); by contrast, the viable samples DZ4.1 and DZ4.3 had a low similarity coefficient: 0.31 between DZ1.1 and DZ4.1, and 0.33 between DZ1.3 and DZ4.3 (Fig. 1A). The differences between DZ1.1 and DZ1.3 were less pronounced, although significant for 61 markers, and the differences between samples DZ4.1 and DZ4.3 were low for only 13 markers (Fig. 1A). The results are summarised in Fig. 2, in which all the individual plants belonging to samples DZ1.1, DZ1.3, DZ4.1 and DZ4.3 are shown in the space defined by the first three principal components. The PCA clearly divided the individuals into progeny of low viability seeds and progeny of high viability seeds along the first axis, V1, and differentiated DZ1.1 from DZ1.3 along the second axis, V2 (Fig. 2A, left panel). No further explanation was added by the third principal component, V3 (Fig. 2A, right panel.).

A similar analysis with the *Pst*I/*Mse*I primer combinations generated a range of 55-109 (average 79) bands per primer combination, of which 48-101 (average 70) were polymorphic. The chi-square test showed that the differences between samples were significant with respect to the occurrence of 168 bands. The similarity analysis gave a different outcome from the one based on *Eco*RI/*Mse*I: the similarity coefficients between all the pairs of samples were very close to one another. The PCA gave a more diffuse representation of the relationships between the samples (Fig. 2B, left and right panels), in that only the separation of individuals belonging to DZ1.1 was provided by the first axis, V1, and the third, V3, differentiated DZ1.3 from rest of the samples.

An interesting result was obtained when the analysis combined both the *Eco*RI/*Mse*I and *Pst*I/*Mse*I data (Fig. 1C). The structure based on the two systems (Fig. 1C) resembled to some extent the ones for the *Eco*RI/*Mse*I-type

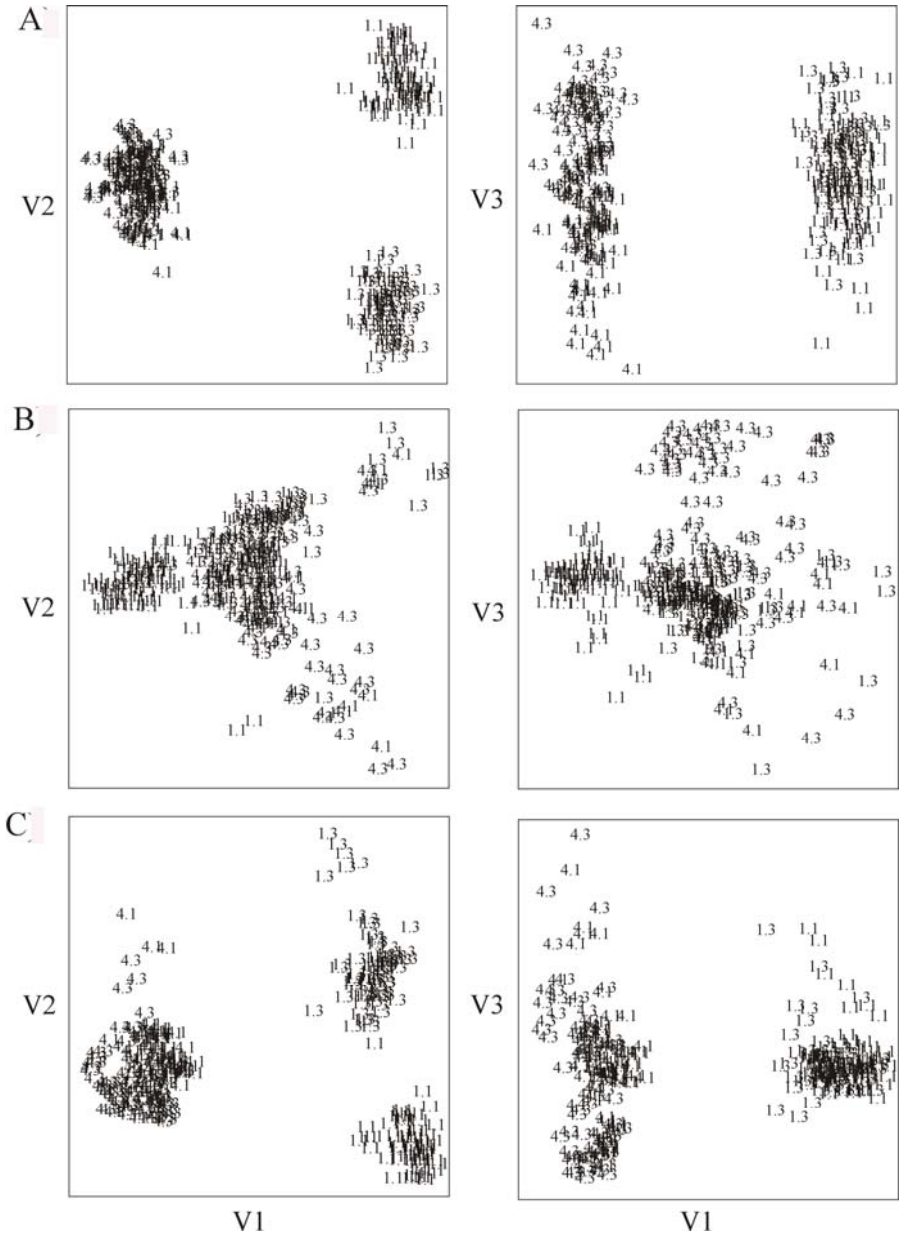

Fig. 1. The similarity structure of the analysed samples described by the number of markers with frequencies significantly different in the corresponding populations (in bold) and by the similarity coefficient based on the Euclidean distances between the populations (in italics). Panel A shows results for *Eco*RI/*Mse*I digests, Panel B those for *Pst*I/*Mse*I digests, and Panel C those for both digestion enzyme systems (*Eco*RI/*Mse*I and *Pst*I/*Mse*I).

marker system (Fig. 1A), except for the similarity between DZ4.1 and DZ4.3, which was lower (0.62). The PCA analysis provided a reliable separation between progenies of low viability seeds and high viability seeds, as seen along the first principal axis, V1, as well as between DZ1.1 and DZ1.3 in the second axis, V2 (Fig. 2C, left panel), and between DZ4.1 and DZ4.3 in the third axis, V3 (Fig. 2C, right panel).

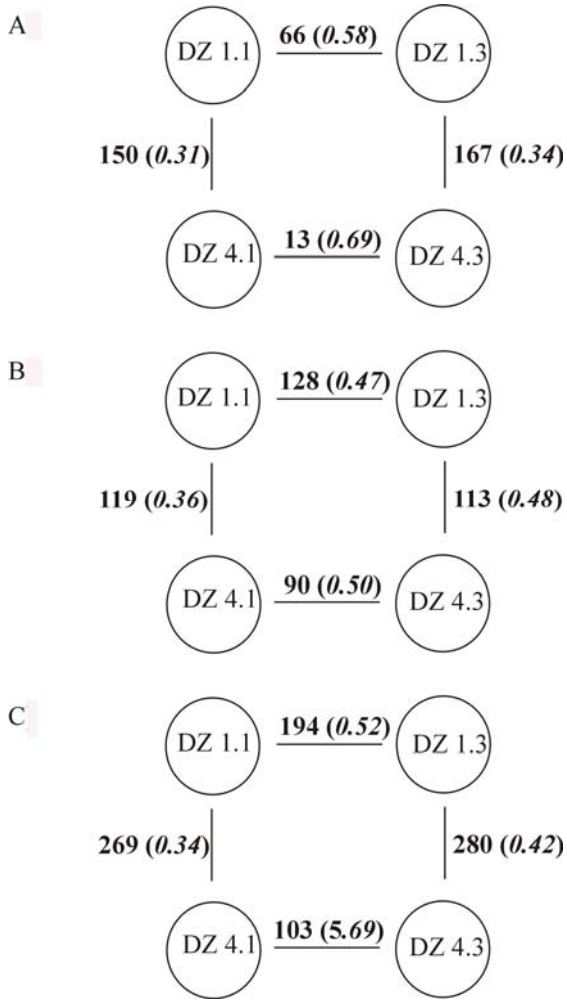

Fig. 2. Plants belonging to DZ1.1, DZ1.3, DZ4.1 and DZ4.3 in the planes spanned by the the principal component numbers V1 and V2 (left) and V1 and V3 (right), calculated from the data describing the presence/absence of all polymorphic fragments. Panel A shows results for *Eco*RI/*Mse*I digests, Panel B those for *Pst*I/*Mse*I digests and Panel C those for both digestion enzyme systems (*Eco*RI/*Mse*I and *Pst*I/*Mse*I).

DISCUSSION

Previously, we studied similar materials, using a limited number of individuals (44 per sample), and fewer primer combinations, specifically only *Eco*RI/*Mse*I [10]. We showed that material with low initial viability differs in its response from related, but highly viable seed, and that the changes induced are preserved through regeneration cycles. Although the variation observed for an initially viable sample following different numbers of regeneration cycles were not substantial, they were still observable, and are probably the result of genetic drift. Our conclusions based on RAPD profiling of the same plant material [8] were consistent with these conclusions. This investigation involved the analysis of the same samples using additional restriction enzymes and more primer pair combinations. From AFLP fingerprints generated with seven selective *Eco*RI/*Mse*I primer combinations, we were able to make a wide-scale observation of the genetic changes exhibited by different samples. The statistically greatest significant differences in the frequencies of appearance of some polymorphic DNA fragments were observed between DZ1.1 and DZ4.1, the progenies of seed lots of strongly different viability. This may suggest that at least some of the changes in population structure were associated with the loss of viability caused by the natural seed-ageing process. Differences were also observed in the comparison between DZ1.3 and DZ4.3, demonstrating that successive regeneration cycles did not preserve the initial genetic structure of the population. The smaller but still significant differences between DZ1.1 and DZ1.3 showed probable genetic drift during three cycles of regeneration. The smallest differences were observed between samples DZ4.1 and DZ4.3 (Figs 1 and 2). Differences between these series probably originated from the natural genetic variability of allogamous rye. In this case, differences between the two samples were much greater than those observed in our previous study. We interpret this as suggesting that the number of both individuals and selective primer pair combinations were insufficient in the earlier study.

Fingerprint analysis derived from the seven *Pst*I/*Mse*I selective primer combinations over all four seed samples did not show any significant influence of seed ageing on the genomic areas examined. Numerous mapping studies have demonstrated that *Eco*RI/*Mse*I AFLP fragments are not randomly distributed along chromosomes, but preferentially locate to non-transcribed regions such as the centromeric region, the nucleolus organizer region, and the telomeres. Very similar results were also demonstrated for rye [20]. By contrast, *Pst*I/*Mse*I AFLPs show a preference to gene-rich transcriptionally-active regions [21]. We therefore suggest that changes induced in non-coding regions seem to be inherited more often, and are seldom eliminated by natural selection because they have no significant influence on genome function. This result is consistent with our previous data, in which we detected some inherited changes in fragments derived from the non-coding genome region in material of low viability (same plant material) [11]. Thus, our results support the hypothesis of

Shatter *et al.* [9] that DNA alternations induced by ageing are not randomly dispersed throughout the genome, but are rather concentrated into hot-spots. It seems likely that these hot-spots are present in non-coding DNA regions.

Comparison of the *Eco*RI/*Mse*I and *Pst*I/*Mse*I data demonstrates that the first system was more successful in identifying changes in population structure after regeneration cycles and those resulting from ageing. This could be attributed to the fact that *Eco*RI endonuclease is recognized as an enzyme insensitive to methylation, which thus can cut even methylated DNA sites usually associated with unexpressed sequences. As *Pst*I is methylation sensitive, the generated AFLPs seem to be associated mostly with coding regions, which can explain the lower number of polymorphisms identified in the system. Our results concur with those presented by Börner *et al.* [12] and Chebotar *et al.* [13], who used microsatellites to demonstrate the presence of a genetic shift that most probably originated from reproduction cycles. Moreover, since microsatellites are recognized as the markers tightly linked mostly to non-coding regions [22], it is evident why a lower level of polymorphic signals was identified with *Pst*I/*Mse*I AFLPs and SSRs than with *Eco*RI/*Mse*I fragments. It should be stated that application of both enzyme combinations allowed for better separation between samples of strongly different viability seed lots (also the differences between the progeny of low viability seed (DZ1.1 and DZ1.3) are better visible). Moreover, since they most probably recognize different DNA regions, the data generated should more closely reflect changes that may take part during seed ageing and regeneration under gene bank conditions.

Our results indicate that long-term storage of seeds in gene banks, causing their low viability due to natural ageing, may lead to genetic changes in the preserved germplasm. This suggests that most of these changes and especially those generated in coding regions are eliminated from the genome due to natural selection. If this is true, then the germplasm preserved under gene bank conditions is mostly maintained unchanged with respect to coding region DNA. However, further analysis devoted to sequencing DNA fragments should be carried out to investigate the extent of silent mutations that do not influence protein function.

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