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USE OF DNA MARKERS FOR CEREAL LINE UNIFORMITY ASSESSMENT

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Prior to the registration of a new variety, it is required to undergo Distinctness, Uniformity and Stability (DUS) testing. Preparing a newly developed variety to meet the requirements of DUS testing is a lengthy process, particularly regarding aspects of uniformity and stability. Field testing of a large number of lines is time and resource intensive. In addition, the expression of certain traits may be influenced by environmental conditions. The use of DNA markers may allow rapid assessment of the level of genetic diversity within a particular line or variety, and to remove individuals that are genetically differentiated, thus accelerating the homogenisation of a newly developed variety. In this study, we utilised AFLP and the iPBS marker techniques to assess genetic variation within advanced breeding lines of several cereal species (triticale, wheat, barley). The combined use of molecular and morphological selection over three years of analysis and selection resulted in the reduction of genetic diversity within breeding lines.

Key words: *DUS testing, genetic uniformity, DNA markers, AFLP, iPBS.*

INTRODUCTION

One of the goals of modern breeders is not only to produce new varieties with improved properties, but also to ensure stability and uniformity of the newly bred varieties. This facilitates the use of mechanised planting, harvesting and processing of the crop. In addition, modern breeding is primarily undertaken within breeding institutes and, increasingly by private breeding companies. In order to protect the breeder's investment, the International Union for the Protection of New Varieties of Plants (UPOV), an international organisation aiming to protect new plant varieties, has been established. The UPOV Convention provides a *sui generis* form of intellectual property protection, which has been specifically adapted for plant breeding with the aim of encouraging breeders to develop new varieties of plants. One of the procedures for granting of breeder's rights to a newly developed variety is Distinctness, Uniformity and Stability (DUS) testing, which a variety is required to pass for it to be registered. Preparing a newly developed variety to meet the requirements of DUS testing is a lengthy process, particularly regarding aspects of uniformity and stability. Field testing of a large number of lines is time and resource intensive. In addition, the expression of certain traits may be influenced by environmental conditions. The use of DNA markers may allow rapid assessment of the level of genetic diversity within a particular line or variety, and to remove individuals that are genetically differentiated, thus accelerating the homogenisation of a newly developed variety.

The utilisation of DNA markers in line purity assessment has been considered (DellaVecchia *et al.*, 1998), but its successful implementation has not been widely reported. This may be partly due to the increasing role of private companies in breeding, but is also due to various technical difficulties and considerations. These include cost and technical problems (DellaVecchia *et al.*, 1998), as well as other more basic genetic considerations. The level of genetic diversity detected using DNA markers is usually higher than the phenotypic diversity, and reflects polymorphism in non-coding sequences that do not influence the phenotype (Singh *et al.*, 2004). In addition, the levels of genetic diversity detected using DNA markers can be high, particularly in out-crossing species, which can complicate analysis and discriminative power (Roldán-Ruiz *et al.*, 2000). Various DNA markers techniques, including RFLPs, have been applied to line purity assessment (Dillmann *et al.*, 1997), SSRs (Singh *et al.*, 2004) and AFLPs (Lombard *et al.*, 2002). These differ in the number of loci detected per assay and in technical aspects of utilising each DNA marker technique.

In this study, we utilised AFLP (Vos *et al.,* 1995) and iPBS (Kalendar *et al.*, 2010) marker techniques to assess genetic

variation within advanced breeding lines of several cereal species (triticale, wheat, barley, oat). AFLP markers have been employed for assessment of genetic diversity of breeding lines and for selecting inbred lines for hybrid development (Lombard *et al.*, 2002; Burton *et al.,* 2004). The AFLP technique has also been used for varietal fingerprinting, mapping and genetic diversity studies. One of the major advantages of the AFLP technique over other DNA markers techniques is that it generates a larger number of amplified products in a single reaction (Powell *et al*., 1996). The iPBS marker technique is based on retrotransposon sequences, which are ubiquitous in plant genomes, and large portions of plant genomes are comprised of retroelement sequences (Sabot and Schulman, 2006). The PCR primers utilised in the iPBS marker technique are based on highly conserved primer binding sites (PBS), and thus are applicable to a large range of species and are very sensitive in detecting genetic polymorphism (Kalendar *et al.*, 2010).

MATERIALS AND METHODS

Plant material and DNA extraction. The advanced breeding lines were provided by breeders from the State Stende Cereals Breeding Institute (barley, wheat, oat) and Priekuļi Plant Breeding Institute (triticale, barley). Plant material was obtained both from plants growing in the field and from germinated seedlings. DNA extraction was performed using a CTAB protocol (Doyle and Doyle, 1987). In most cases, breeding lines were divided into sub-lines, of which seeds from individuals were maintained separately.

Amplified fragment length polymorphism (AFLP) analysis. AFLP analysis was generally carried out according to the protocol published in Vos *et al.* (1995). A modification was that the forward selective primers were labelled with fluorescent labels 6-FAM, HEX or NED. This was done to facilitate fragment genotyping on an ABI 3130*xl* genetic analyser (Applied Biosystems, USA). The selective primers utilised were: Eco+AGG, Eco+ACG, Eco+ACA, Eco+ACT, Eco+AAC, Eco+AAG, Mse+CAA, Mse+CAG, Mse+CTG (Table 1).

PCR amplification and fragment analysis using iPBS markers. Four iPBS primers were used (2076, 2001, 2083, 2081) (Kalendar *et al*., 2010). Reactions were performed with 100 ng DNA in a 20 µl reaction mixture containing 1x *Dream Taq* buffer, 2.5 mM $MgCl_2$, 10 mM each dNTP Mix, 1 µM primer, 1 U *Dream Taq* polymerase, 0.04 U *Pfu* polymerase. The PCR thermocycler protocol was an initial denaturation at 95 ^{o}C for 3 min followed by 38 cycles of 95 ^{o}C for 30 s, 50 ^{o}C for 40 sec, and 68 ^{o}C for 1 min each, followed by 72 ^{o}C for 10 minutes. Amplified products were separated using an ABI genetic analyser 3130 *xl*.

Data analysis. AFLP and iPBS marker amplification fragments were genotyped using GeneMapper v4.0, and a binary data matrix was constructed. Fragments that were monomorphic (present in all individuals) were removed prior to analysis as they were not informative. Results were analysed using GenAlEx 6 (Peakall and Smouse, 2006). Phylogenetic trees were constructed using the Neighbour-Joining cluster analysis in MEGA version 4 software (Tamura *et al*., 2007). The distance matrices and other information are available from the corresponding author.

Table 1

SELECTIVE AFLP PRIMERS UTILISED FOR BREEDING LINE ANALYSIS

Species	Eco primer	Mse primer	Label
Wheat	Eco+ACA	Mse+CTG	6-FAM
	Eco+AGG	Mse+CAG	HEX
	Eco+ACG	Mse+CAG	NED
Barley	Eco+ACT	Mse+CTG	6-FAM
	Eco+AAC	Mse+CAG	HEX
	Eco+AAG	Mse+CAG	NED
Triticale	Eco+ACT	Mse+CAA	6-FAM
	Eco+AGG	Mse+CAG	HEX
	Eco+ACG	Mse+CAG	NED
Oat	Eco+ACT	Mse+CAA	6-FAM
	Eco+AGG	Mse+CAG	HEX
	Eco+ACG	Mse+CAG	NED

RESULTS

The analysis of the breeding lines was undertaken over three successive growing seasons (2008–2010). The results from the preceding year were utilised to select the most similar individuals for the following season. The molecular analyses were also combined with morphological analysis of the breeding lines. The AFLP marker technique was performed with three selective primer combinations for each species (Table 1) and the four iPBS markers (2076, 2001, 2083, 2081). The AFLP technique resulted in a larger number of genotyped fragments than observed using the iPBS markers (Table 2).

After genotyping with AFLP and iPBS markers, the binary matrices were analysed and Nei genetic distances between individuals as well as sub-lines were calculated. These distances were visualised via Principal co-ordinate analysis and construction of dendrograms. The most genetically differentiated individuals were identified, and seeds from these individuals were not utilised in the following growing season. The division of the breeding lines into sub-lines allowed to analyse the results according to individuals or sub-lines. One example is presented for the oat breeding line ‘Līva’. In the PCA, it is possible to identify a group of individuals that are clustered separately from the majority of individuals (Fig. 1). An alternative method for visualising the genetic distance data was construction of neighbour-joining dendrograms (Fig. 2), which enabled more precise delineation of groups of individuals from the breeding lines, as well as identification of more genetically differentiated individuals.

An analysis of the overall success of the breeding line homogenisation was undertaken by comparing the results from

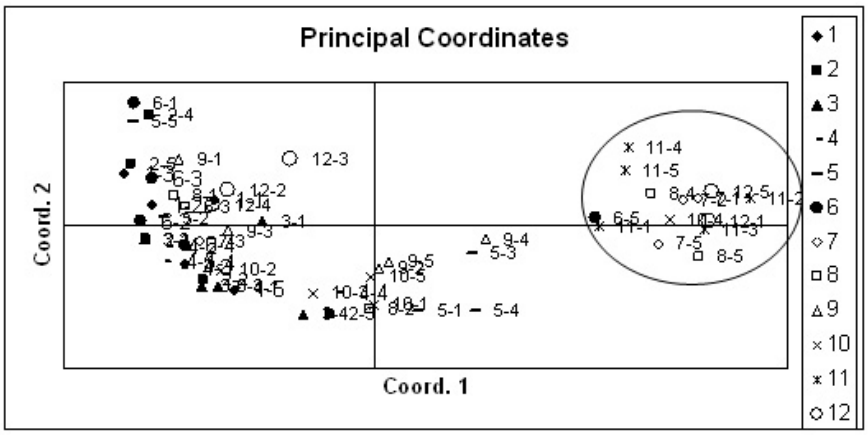

Fig. 1. Principal co-ordinate analysis of individuals from the oat breeding line 'Līva'. Individuals are grouped into sub-lines 1–12.

Table 2

CHANGES IN FRAGMENT NUMBER AND POLYMORPHISM BY YEAR

Triticale breeding line		Year		
		2008	2009	2010
9402-3	No. of individuals (AFLP)	48	64	56
	No. of AFLP fragments	208	176	37
	No. of AFLP fragments (f 5%)	208	140	14
	Polymorphic AFLP loci (%)	99.52%	93.58%	47.44%
	No. of individuals (iPBS)	49	64	64
	No. of iPBS fragments	28	78	23
	No. of iPBS fragments (f 5%)	24	66	8
	Polymorphic iPBS loci (%)	96.55%	82.98%	63.89%
9405-23	No. of individuals (AFLP)	44	60	nd
	No. of AFLP fragments	236	184	nd
	No. of AFLP fragments (f 5%)	236	162	nd
	Polymorphic AFLP loci (%)	100.00%	98.40%	nd
	No. of individuals (iPBS)	46	60	nd
	No. of iPBS fragments	21	80	nd
	No. of iPBS fragments (f 5%)	20	71	nd
	Polymorphic iPBS loci (%)	55.17%	84.04%	nd
9534-22	No. of individuals (AFLP)	nd	80	50
	No. of AFLP fragments	nd	179	47
	No. of AFLP fragments (f 5%)	nd	153	15
	Polymorphic AFLP loci (%)	nd	94.65%	60.26%
	No. of individuals (iPBS)		80	39
	No. of iPBS fragments	nd	80	17
	No. of iPBS fragments (f 5%)	nd	64	11
	Polymorphic iPBS loci (%)	nd	84.04%	47.22%
9710-4	No. of individuals (AFLP)	51	45	nd
	No. of AFLP fragments	254	163	nd
	No. of AFLP fragments (f5%)	254	103	nd
	Polymorphic AFLP loci (%)	100.00%	87.17%	nd
	No. of individuals (iPBS)	54	45	nd
	No. of iPBS fragments	23	72	nd
	No. of iPBS fragments (f 5%)	20	62	nd
	Polymorphic iPBS loci (%)	75.86%	74.47%	nd

nd, no data

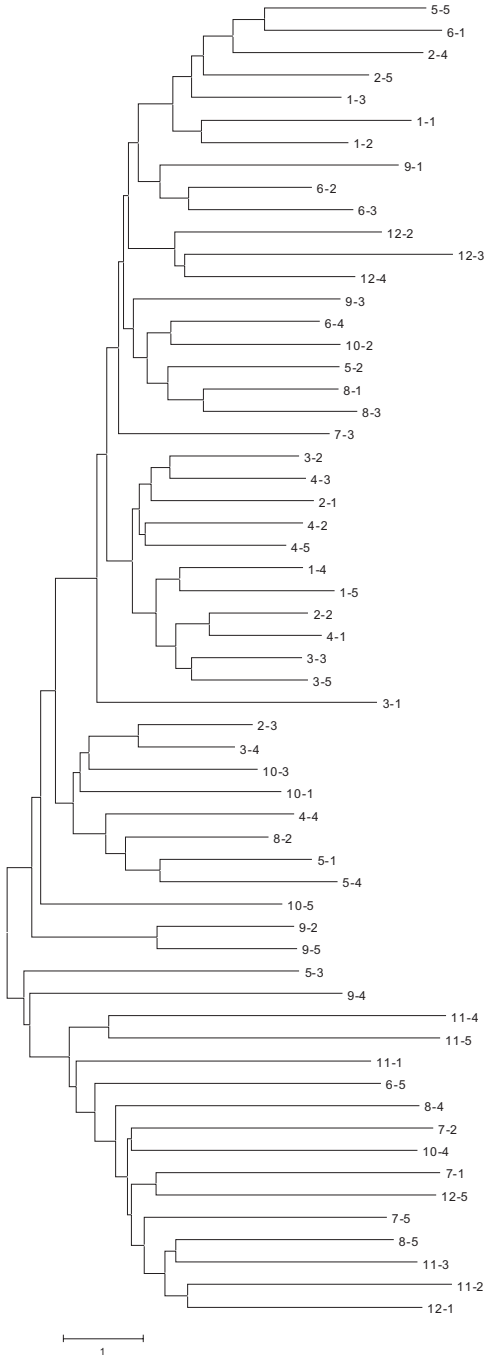

Fig. 2. Neighbour-joining dendrogram of the oat breeding line 'Līva'. Individuals are labelled according to sub-line and individual.

successive years. In those cases where it was possible to analyse one breeding line in two or three successive years, the total number of fragments, the number of fragments with a frequency over 5%, and the percentage of polymorphic fragments were compared (Table 2). The total number of fragments depended on the genotyping reaction conditions, and may varied from year to year. The number of fragments with frequency of over 5% decreased, indicating that the breeding lines were becoming more homogeneous, and that the number of individuals with polymorphic fragments was decreasing. In all cases, the percentage of polymorphic fragments decreased over the course of three years, with the greatest decrease occurring in 2010. This decrease in the number of common fragments, combined with the decrease in the percentage of polymorphic fragments, showed that the breeding lines were becoming more genetically uniform.

An additional analysis of genetic diversity was performed by calculating the Nei genetic distances between sub-lines in successive years. In all cases where this analysis was caqrried out, the average genetic distances between sub-lines decreased, as along with the maximum and minimum genetic distances. The genetic distances obtained using AFLP and iPBS markers were similar. The decrease of genetic distances between sub-lines is illustrated in the triticale line 9402-3 (Table 3). In 2008, the average genetic distance between sub-lines from the triticale line 9402-3 was 0.147 (AFLP) and 0.122 (iPBS), in 2010, the average distance had reduced to 0.013 (AFLP) and 0.025 (iPBS). Similar reductions were found for the maximum and minimum genetic distances. The minimum distance between sub-lines in 2010 calculated with the iPBS data was 0.00, indicating that some sub-lines could not be differentiated using these markers.

Table 3

NEI GENETIC DISTANCES BETWEEN SUB-LINES IN THE TRITICALE LINE 9402-3

Triticale line 9402-3	2008		2009		2010	
Nei genetic distance	AFLP	iPBS	AFLP	iPBS	AFLP	iPBS
Average	0.147	0.122	0.076	0.085	0.013	0.025
Maximum	0.295	0.311	0.253	0.194	0.031	0.068
Minimum	0.069	0.016	0.025	0.025	0.002	0.000

DISCUSSION

The combined use of molecular and morphological selection resulted in the reduction of genetic diversity within breeding lines over the three years of study. The two utilised DNA marker techniques (AFLP and iPBS) detected differing numbers of fragments, and the numbers of fragments detected in each year also differed. This variation was due to differences in the sensitivity of the genotyping reactions, which was influenced by DNA quality and other experimental factors. However, despite the differences in the numbers of analysed fragments, the estimated genetic diversity was similar when calculated for the different marker techniques. This indicates that these two marker techniques are equally efficient in the detection of genetic polymorphism within breeding lines in the grain species analysed. In addition, these genetic diversity indicators showed a decrease over the three years of the project, in particular in the final year (2010). This indicates that the use of these DNA marker techniques, in conjunction with phenotypic assessment of diversity in the field, can be efficient for reducing the level of genetic polymorphism within breeding lines.

The use the AFLP technique in breeding line purity assessment has been previously reported (Lombard *et al.*, 2002), but the iPBS marker technique has not been previously utilised for this purpose. The AFLP marker technique is technically more demanding than the iPBS marker technique, it requires a larger amount of DNA and several steps are required before the genotyping of fragments. In contrast, the iPBS technique requires only one PCR step. One of the advantages of the AFLP technique is the high multiplex ratio — the number of markers obtained from a single analysis (Vos *et al*., 1995). Given that the genetic diversity indicators were similar using both techniques, even though fewer fragments were genotyped using the iPBS marker technique, the use of this technique might be preferable due to its lower cost and technical complexity.

One consequence of using anonymous markers such as AFLPs or iPBS markers is that the genotype markers are most probably not associated with particular morphological traits. This means that evaluation of morphological traits is required in order to ensure that a potential new variety will be compliant with the DUS testing requirements. However, the use of molecular markers can accelerate the homogenisation of a breeding line, and can also be used to minimise the material that must be morphologically analysed in field conditions.

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GRAUDAUGU SELEKCIJAS LĪNIJU VIENDABĪGUMA IZVĒRTĒŠANA, IZMANTOJOT DNS MARĶIERUS

Lai reģistrētu jaunu šķirni, tai ir nepieciešams iziet atšķirīguma, viendabīguma un stabilitātes (AVS) testu. Jaunas šķirnes izveidošana atbilstoši AVS testa prasībām ir ilgstošs process, īpaši viendabīguma un stabilitātes aspektā. Lauka pārbaudes lielam skaitam līniju ir laika un resursu ietilpīgas, un vides apstākļi var ietekmēt fenotipiskās īpašības. Izmantojot DNS marķierus, ir iespējams noteikt ģenētisko daudzveidību selekcijas līnijās un atlasīt indivīdus, kuri uzrāda paaugstinātu ģenētisko diferenciāciju, tā paātrinot jaunās šķirnes ģenētisko izlīdzināšanu. Šajā pētījumā tika izmantoti AFLP un iPBS marķieri, lai noteiktu ģenētisko daudzveidību dažādu graudaugu (tritikāle, kvieši, mieži, auzas) selekcijas līnijās. Izmantojot molekulāro un morfoloģisko izlasi, trīs gadu laikā tika panākta ģenētiskās daudzveidības samazināšanās analizētajās selekcijas līnijās.