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THE USE OF PCR MARKERS FOR VARIETY TESTING IN ALFALFA

Document prepared by experts from France

The use of PCR markers for variety testing in alfalfa

Joëlle Lallemand, Stephane Fouilloux and Mireille Bourgoin-Grenèche
Laboratoire de biochimie - GEVES
BP 52 17700 Surgères, France

Distinction of alfalfa (*Medicago sativa*) varieties is difficult because of :
The limited genetic basis of the commercial varieties
The heterogeneity of the varieties due to the cross- pollination of the species

In France, a first study using isozymes was initiated by GEVES and continued by the company LOCUS to investigate the variability within the species and wether it was possible to distinguish the varieties whith these markers. This study indicated that the polymorphism present was high but the maximum variability occurs within the varieties. The « between varieties » Variability was not sufficient to distinguish the varieties. This study was conducted on 4 varieties and 6 enzymes.
A second attempt to distinguish the varieties was conducted in INRA Toulouse with a grant from CTPS using RAPD primers on 4 varieties, Oro Europe, Euver and Rival. 5 primers were tested and showed polymorphic patterns. The statistical method used was AMOVA or Analysis of Molecular Variance (Excoffier *et al.*, 1992). Only Rival could be distinguished from the others (this distinction is also obvious using the morphological markers). More over, the method gives a global index and does not enable to determine which bands from which primers have been efficient for distinguishing the varieties.
Consequently it was found necessary to go further in the investigation. This paper presents the results obtained in GEVES on 8 varieties.

Materials and methods

DNA was extracted from individual 3 weeks old seedlings using the CTAB extraction procedure (Tai and Tanksley, 1990). For each variety 50 individual plants were studied.
The repeatability of the method was studied using the same DNAs of the same plants in 3 series of amplifications : one amplification in INRA Toulouse, two amplification in GEVES.
Choice of the bands :
The bands chosen had to meet 3 criterias :
a clear position : bands too close to each other were not considered
a clear state of expression : either present or absent- no faint band
a good repeatability : same state of expression for the 3 amplifications

Results

One of the main objective of the work was to study the repeatability of the method.

We obtained a good repeatability between the 3 sets of amplifications. This was achieved only because we made a severe screening of the bands. Even so, a few bands did not give consistent results and were eliminated. However, in most cases clear bands with a clearly defined position are likely to be repeatable. Examples of such bands are given in figure 1.

Using only the clear repeatable and discriminant bands (i.e. bands for which the percentage of occurrence was different for at least one variety), we obtained only 6 markers among the primers tested (B1, B6, B7, B8, B10 and C7). Each primer yielded 5 to 15 bands but most of them were not studied further either because they were monomorphic or if polymorphic, not discriminant or because they did not seem reliable enough.
For this reason, we also tried specific amplification using two pairs of 20 bp primers corresponding to the extremities of the genes Early nodulin and Leghemoglobin (Kiss *et al.*, 1993). Figure 2 shows the results obtained for Early nodulin. Leghemoglobin gave more complex patterns but in both cases the bands obtained were clear and repeatable.

Using the 6 RAPD markers and the Early nodulin marker (Leghemoglobin is still under investigation), we described our 8 varieties on 50 individuals. The results are presented in the following table.

	B1-740	B6-450	B6-1350	C7-653	C7-1033	C7-1500	ENOD12
BOJA	4	6	14	8	20	8	34
EUVER	0	2	4	12	16	12	50
EUROPE	0	0	6	18	26	4	26
RIVAL	42	0	64	34	18	0	34
DIANE	0	24	6	0	20	12	28
ALIZE	0	6	16	15	20	0	44
ORO	2	4	4	6	16	10	26
CARMEN	0	0	10	10	14	12	38

*The figures indicate % occurrence of bands

Only Rival and Diane were different from all others. Not taking into account these 2 varieties, the range of variation between the varieties is low :
0 to 4% for B1-740
0 to 6% for B6-450
4 to 16% for B6-1350
6 to 18% for C7-653
14 to 26% for C7-1033
0 to 12% for C7-1500
26 to 50% for ENOD12
from which only the last, ENOD 12 is significant (with n=50).
This means that, although the DNA polymorphism is very high in alfalfa, much of the variation is encountered within the varieties and is not useful for distinguishing between the varieties. In this study, 16 comparisons out of 28 give a significant difference at the 5% level (13 out of 28 at the 1% level). For comparison, in the case of rye-grass, with the same number

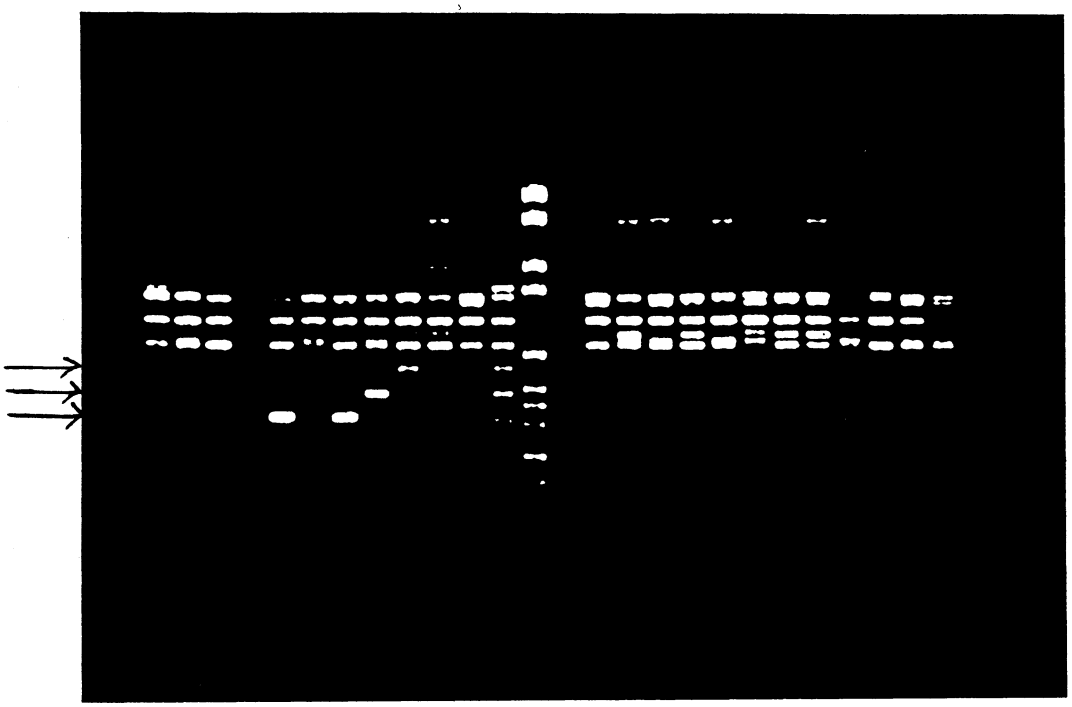

FIGURE 1

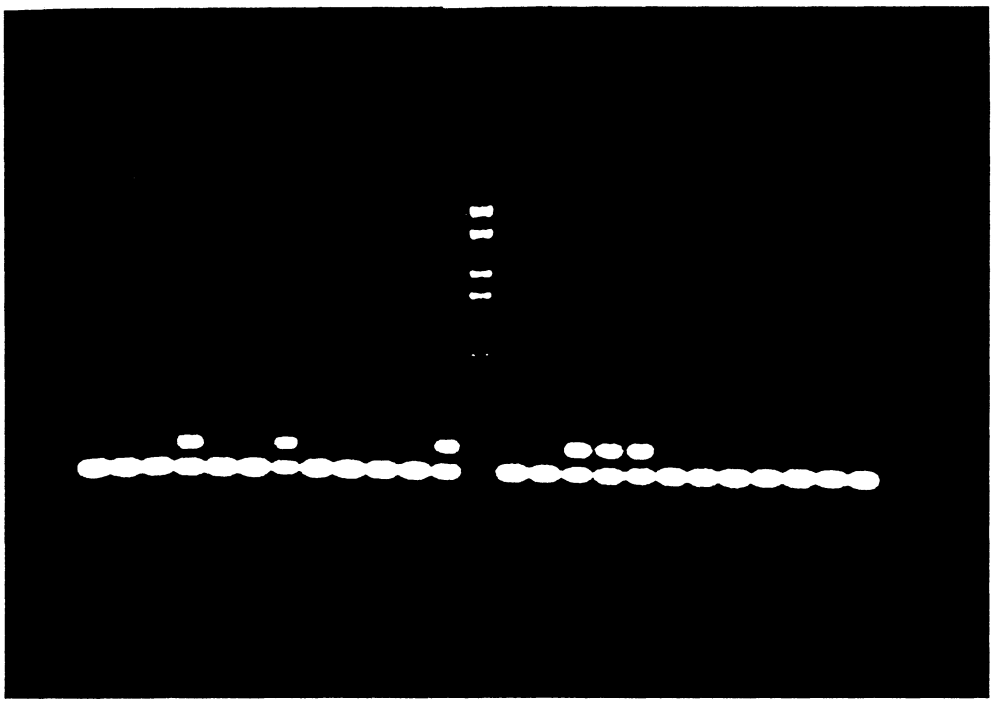

FIGURE 2

of varieties and 3 isozyme markers, a 100% rate of distinction (at the 1% level) can be achieved.
This is not too surprising, given the genetic basis of the commercial varieties. Kidwell *et al.* (1994), using 35 RFLP markers were not able to distinguish among 7 accessions. Since they had only 12 individuals per variety we can expect to achieve better results, but still, it is likely that a great number of distinction problems will remain even if we increase the number of markers.
An other difficulty arise from the dominance of the markers we used and from the tetraploïdy of alfalfa. Most polymorphism in RAPDs (but not all) is expressed as presence versus absence of a particular band. Genotypes +/+ and +/- produce one band, genotype -/- does not produce the band. In a tetraploïd species, 5 genotypes may occur : +/+/+/+, +/+/+/-, +/+/-/-, +/-/-/- and -/-/-/- among which only the last will not be able to produce one band. It means that the expected distribution of the phenotypes observed in the progeny of a selfed heterozygote will be ¼ absence ¾ presence for the diploïd species and 1/16 absence 15/16 presence for the tetraploïd species. The following table gives the probability of the occurrence of the phenotype absence of band for different frequencies of the « - » allele in the population.

% allele « - »	% phenotype « absence »	% phenotype « presence »
0	0	100
25	0.39	99.61
50	6.25	93.75
75	31.64	68.36
90	65.61	34.39
100	100	0

This table shows that an important increase of the « - » allele from 0 to 50 will result in no detectable change in phenotype frequency (for n=50). A better distinction will be achieved for higher frequencies of the « - » allele i.e. low frequencies of occurrence of bands. This was the case in the present study.

Conclusion

The polymorphism observed with RAPDs in M. sativa is high. All 20 primers tested gave polymorphic patterns and more than half the bands observed showed presence/absence polymorphism. Other markers such as isozymes, RFLPs (Kidwell *et al.*, 1994) and spots from two dimensionnal electrophoresis (Picard, 1995) also show a high level of polymorphism. However, there is more « within varieties » variability than « between varieties » variability which makes the description of a high number of individuals per variety necessary. Therefore the use of PCR derived techniques is desirable. Using 7 markers we obtained a poor distinction of our 8 varieties which is not very surprising.
Hopefully, increasing the number of markers will improve the situation even if it is likely that a lot of distinction problems will remain. The next step is to try PCR specific markers and if possible codominant ones. This is the case of the Leghemoglobin marker which gives codominant expression after digestion by Taq 1 (Kiss et al., 1993). This work is undergoing now and will be presented soon.

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