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UPOV

BMT/3/16
ORIGINAL : English
DATE : September 25, 1995

INTERNATIONAL UNION FOR THE PROTECTION OF NEW VARIETIES OF PLANTS

GENEVA

WORKING GROUP ON BIOCHEMICAL AND MOLECULAR TECHNIQUES AND DNA-PROFILING IN PARTICULAR

Third Session

Wageningen, Netherlands, September 19 to 21, 1995

AN ANALYSIS OF GENETIC VARIABILITY WITHIN AND AMONG LOLIUM VARIETIES: IMPLICATIONS OF DNA-PROFILING FOR THE IDENTIFICATION OF OUTCROSSING VARIETIES

Document prepared by experts from Australia

An analysis of genetic variability within and among *Lolium* varieties: implications of DNA profiling for the identification of outcrossing varieties.

Document prepared by Australia for the third session of the UPOV working group on biochemical and molecular techniques and DNA-profiling in particular, Wageningen, Netherlands, Sept 1995

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Introduction

Since the last UPOV working group on DNA profiling meet in Paris in 1994, many new studies have shown that DNA profiling methods can distinguish among varieties of a range of important crops (Morell et al. 1995). However, most of the varieties studied to date have been either clonal, inbred or predominantly selfing taxa and therefore little is known about the resolving power of DNA profiling for outcrossing varieties. Clearly the potential to distinguish among varieties is dependant on the way genetic variability is apportioned within vs among taxa. Inbred, selfing and clonal varieties often will exhibit little variation within, but fixed and therefore diagnostic genetic differences among varieties. On the other hand, patterns of genetic variation in outcrossing varieties will be more complex: genetic variation being found both within and among taxa. Furthermore, only a minor portion of the total genetic variation is likely to be apportioned among varieties. It is only within this minor fraction of the total genetic diversity that differences among populations/varieties will be found. In addition, most of these genetic differences are likely to be frequency differences rather than fixed differences. The identification of outcrossing varieties by DNA profiling methods will thus require analysis of many more individuals than for other varieties as well as appropriate statistical analysis.

In this document we report the findings of our analysis of genetic variability within and among varieties of *Lolium perenne* using the PCR based RAPD technique. Based on our findings we discuss the implications for plant variety protection.

Recently, work has also commenced on the development and use of Sequence Tagged Site (STS) markers for this species, and if time permits our preliminary findings will be discussed in Wageningen.

Study species and varieties

This study was initiated for Groupe Limagrain and full findings have been presented in a confidential report by Morell et al. (1995b). Limagrain have kindly allowed the information we present here to be made public.

Lolium represents an interesting example of an economically important plant which is an outcrosser. "Varieties" of *Lolium* are polymorphic and this polymorphism may in many cases make the unambiguous identification of a variety on the basis of morphological comparisons problematic. There has therefore been interest in the application of cytogenetic, biochemical and molecular techniques to describe and identify *Lolium* varieties.

Allozymes and protein banding patterns have proven to be useful for varietal identification in many plant species and their use has been investigated in *Lolium* (Lallemand 1991; Murphy *et al.* 1990; De Prins *et al.* 1983). However, while allozyme systems have proven utility, their use is restricted by the limited number of enzyme systems that can be visualised and by the possibility that allozyme expression can be influenced by environmental conditions or management practices. The former consideration is particularly limiting when examining the degree of variability within a variety.

DNA based markers offer a number of advantages over allozymes and other biochemical methods for identifying distinctness. Firstly, the DNA sequence of an organism is independent of environmental conditions or management practices. Secondly, the presence of the same DNA in every living cell of the plant allows tests to be conducted on any tissue at any stage of growth (provided that DNA of sufficient purity can be isolated). Thirdly, new DNA profiling techniques enable us, for the first time, to quickly and easily scan large sections of the genome in search for polymorphisms that can be used to demonstrate distinctness. For a full discussion of the potential advantages of DNA profiling refer to Morell et al. (1995a).

Materials and methods

DNA extraction and PCR

At total of 220 individuals representing 11 varieties of *Lolium* identified by Groupe Limagrain and 2 Australasian varieties were analysed in this study. DNA was extracted from the leaves of plants that were 3-4 weeks old using a Sarkosyl buffer followed by phenol/chloroform extraction. The DNA was finally purified by RNAse and Protease treatments. Based on an initial screening of some 25 RAPD primers, 3 primers that yielded 10-20 reproducible and polymorphic products were used under optimised PCR conditions. See appendix 1 for further detail.

PCR products were separated by agarose gel electrophoresis and visualized by staining with Ethidium Bromide under UV light. The bands were scored manually from photographs of gels based on a comparisons with a standard sample run on each gel.

Banding patterns were converted to a vector of 1's and 0's, where 1 represents the presence of a band at a particular molecular size and 0 represents the absence of the band. Band intensities were not considered in this analysis.

Statistical Analysis

Pairwise genetic distances among all individuals from the 13 varieties were calculated from the table of scored data by the computer program RAPDistance (freely available from the first author) using the algorithm of Excoffier (1992) and Huff et al. (1993):

$$E = n\left[1 - \frac{2n_{xy}}{2n}\right]$$

(where $2n_{xy}$ equals the number of shared bands and n equals the total number of banding positions). This Euclidean metric is equivalent to a tally of band differences between individuals. The pairwise genetic distance matrix formed the basis for subsequent cluster, AMOVA and multivariate analyses.

Methods for calculating individual pairwise genetic distances have been available for multilocus data (eg. RAPDs) for some time. Recently, Peakall et al. (1995) have also developed procedures for calculating genetic distances among individuals based on single locus data such as allozymes or microsatellites. This approach enabled explicit comparison of allozyme (single locus data) and RAPD (multilocus data) variation in a turf grass using common procedures and statistics (Peakall et al. 1995). This development makes it possible for the first time to standardize data analysis and presentation irrespective of the type of genetic marker used.

An Analysis of Molecular Variance (AMOVA) [Excoffier et al. 1992] was performed with the program WINAMOVA to examine the pattern of genetic variation within vs among the varieties following the procedures used by Huff et al. (1993) and Peakall et al. (1995) for RAPD data. With this procedure significance values based on random permutation at all levels of analysis are calculated avoiding the restrictive assumption of normality required for standard analysis of variance.

Results

Analysis of band patterns

The final data set for the 220 individuals consisted of 25 band positions for primer A4, 24 band positions for the Histone primer and 22 band positions for the 18S primer, giving a total of 71 band positions, and a total of 15,620 data points. Every one of the 220 individuals analysed had a unique profile. When considering the full set of bands, only one of the 71 bands scored (1.4%) was monomorphic across all 220 individuals. No diagnostic bands were found for any varieties. Thus genetic differences among varieties reflect more subtle frequency differences in the data.

Clustering of individuals

In order to reveal the genetic relationships among individuals, a dendrogram was prepared based on a UPGMA cluster analysis of the genetic distance data. Given the large data set it was not surprising to find that multiple equivalent dendrograms were produced, reflecting the limitation of both the data itself and the clustering algorithm. However, inspection of some 25 equivalent trees revealed that the major groups remain the same, although some individual placements changed when the differences between individuals were equivalent or small. Regardless, a clear pattern emerged from this analysis: individuals for some varieties always clustered strongly together while individuals in other varieties did not necessarily cluster with individuals of their own variety. Thus some varieties were both more distinct and genetically more uniform than other varieties.

Analysis of Molecular Variance

An analysis of molecular variation (AMOVA) enabled the total genetic variance to be apportioned within and among the varieties. At the same time, it was also possible to determine the contribution of each of the three primers to the total variability. Overall, it was apparent that most of the genetic variation was represented in every variety, although 20% of the variation accounted for the differences among the varieties. The resolution of varietal relationships is therefore dependent on this differentiation among the varieties. The results of this analysis also showed that the RAPD primer A4 was the most effective primer for detecting variation among varieties since it showed less variation within varieties.

Clustering of Varieties Based on Average Genetic Distances

The AMOVA procedure also enabled the calculation of average genetic distances among the varieties. This analysis condensed the complex pairwise genetic matrix for the individuals (220 x 220) into a more manageable pairwise genetic distance matrix (13 x 13). Based on 1000 random permutations of the data set, all pairwise genetic distances among the varieties were found to be significantly different from random expectations ($P<0.001$). The significant differences among varieties were also reflected in the finding that for any given clustering algorithm there was only one

possible tree. However, different clustering algorithms did produce slightly different outcomes.

An outstanding feature of the dendrograms based on the average genetic distance among the varieties was that the relationships depicted were consistent with the known pedigrees for 9 out of the 13 varieties. For example: progenitor and derived varieties typically clustered together; Australasian varieties clustered together but within European varieties; morphologically distinct varieties were also genetically distinct. However, one inconsistency was noted, while information for the remaining cultivars was lacking. A notable additional finding was that even closely related varieties exhibiting little morphological differentiation were genetically distinct.

Summary of the key findings

- Individuals of *Lolium perenne* varieties were found to be highly variable for the RAPD markers studied with only 1 out of 71 bands being monomorphic. All individuals exhibited a unique DNA profile.
- Genetic variation was found both within (80% of the total) and among varieties (20%). No diagnostic genetic markers were found for any variety. Differences among varieties therefore reflected subtle frequency differences.
- Cluster analysis based on a pairwise genetic distance among individuals revealed that individuals for some varieties clustered consistently together while individuals of other varieties did not necessarily cluster. Therefore, some varieties were more distinct and/or genetically uniform than others. It follows that assignment of an unknown individual to its correct variety may not be possible with this set of genetic markers. On the other hand, as noted below, when 15-20 individuals per variety are analysed, varietal identification is possible.
- Cluster analysis based on the average genetic distances among varieties generally revealed genetic relationships consistent with known pedigrees. Even closely related varieties showing little morphological differentiation were found to be distinct. Thus, average genetic distances were informative revealing both the distinctiveness of varieties and their genetic relationships.

Implications for Plant Breeders Rights

- The key finding of this study is that DNA profiling combined with appropriate statistical analysis can reveal genetic differences among varieties of *Lolium*. This points to the utility of DNA profiling for quantifying distinctiveness in outcrossing varieties. At the same time, the data suggest it may be feasible to measure uniformity and stability as well. However, the assignment of unknown individuals to a variety represents a different and more difficult problem.
- As demonstrated by this study, the RAPD technique provided a useful entry point for the DNA profiling of a species which was poorly know at both the population genetic and DNA levels. Furthermore, this type of DNA profiling was performed with standard PCR laboratory facilities and without radioactive methods. However, we advocate the development of STS markers, such as microsatellites, for the routine and more extensive study of outcrossing species for PBR purposes. Compared with RAPDs, STS markers offer the advantage of simpler genetic interpretation, in turn enabling automation of the analysis and scoring with automatic genotyping systems. STS markers are also likely to be more easily transferred among laboratories. Microsatellites may also prove to be more variable than RAPDs although it remains to be seen if they provide more discrimination among outcrossing varieties. Nevertheless, our RAPD study has demonstrated the feasibility of investing further in the development of these more time consuming and expensive profiling methods for outcrossing species such as *Lolium*
- Statistical procedures are now available that will allow the standardization of data analysis and presentation irrespective of the type of genetic marker used. We suggest that a standardized statistical approach is more likely to be of value in the process of PBR than standardizing the DNA profiling method itself.

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Appendix 1

Methods for the genetic study of *Lolium* by RAPDs

DNA Extraction

DNA was extracted from the leaves of plants that were 3-4 weeks old. The plants were approximately 8-10 cm in height and contained greater than 3 leaves, usually about 15 leaves. DNA was extracted separately from 17 individual plants from each variety (apart from the Australian variety Brumby, for which only 16 individuals were studied) using a method modified from Appels, R., (1992). Approximately 15 x 10 cm long leaf segments were collected. The leaves were cleaned, and transferred into a mortar pre-cooled with liquid nitrogen. They were then ground to a fine powder and transferred to another mortar containing 5 ml of DNA extraction buffer (4% sarkosyl, 100 mM Tris-HCl pH 8.5, 100 mM NaCl, 10 mM EDTA). The material was homogenised by gentle grinding, transferred to a 15 ml polypropylene centrifuge tube, and incubated at 65°C for 15 minutes. Five ml of phenol/chloroform (1:1) were then added, and mixed by inversion for 30 minutes. The phases were separated by centrifugation at 3000 RPM for 10 min and the aqueous phase transferred to a new tube. An equal volume of chloroform was then added, and mixed by inversion for 15 minutes. The phases were separated by centrifugation at 3000 RPM for 10 min and the aqueous phase transferred to a new tube. DNA was precipitated by adding 0.1 volume of 3M NaOAc pH 4.8 and 2 volumes of 99% ethanol and incubating at -20°C for at least 30 minutes. DNA was recovered by centrifugation at 3-10,000 RPM for 10-20 min. The supernatant was discarded and the DNA pellet washed twice by adding 2 ml of 70% ethanol, gently inverting the tube and decanting the ethanol. The DNA pellet was dried under vacuum, resuspended in 400 µl TE and transferred to a 1.5 ml tube. To enhance the purity of the DNA, it was treated with RNAseA (25µg/ml of DNA) for 30 minutes at 37°C followed by the addition of SDS (0.5%) and proteinase K (50µg/ml of DNA). The sample was then extracted with phenol/chloroform (1:1) twice (mixed for 10 minutes, spun at 10,000 RPM for 5 minutes) followed by a chloroform extraction (as for phenol/chloroform) and ethanol precipitation (as described above). Finally the purified DNA was resuspended in 500µl of double distilled water. The quality and purity of samples were analysed by agarose gel electrophoresis and spectrophotometric analysis.

Primers

The primers screened in this study were A1 to A20 and M19 (supplied by Operon Technologies, Alameda, California), and SSU, Histone and 18S, which were synthesised at the CSIRO Division of Plant Industry. The primers A4, Histone and 18S were selected as the initial primers to be used on the basis of their ability to amplify a sufficient number (10-20) of bands, including a number of bands that show polymorphism both within and among varieties. A1, A7, A8, A9, A11, and A12 were also judged to be clearly suitable for RAPD analysis. Several other primers showed potential in preliminary experiments but were not investigated further due to the availability of other suitable primers. The Histone, 18S and SSU primers are 13 base primers that were originally designed to target sites in highly conserved genes (namely histone, 18S and the rbc*S* gene) that typically exist in multigene families. However, other studies in our lab show that these primers used singly at the low stringency conditions used in RAPD analysis, act as arbitrary primers that amplify from regions outside the targeted genes and have proved to be useful in a number of plant species.

PCR Conditions

PCR reactions contained 10 mM Tris-HCl buffer (pH 8.8), 3.5 mM $MgCl_2$, 7.5 mM KCl, 200 µM each of dATP, dGTP, dCTP, dTTP, 500 nM primer, 20 ng/10 µl reaction template and 1U of Boehringer DNA polymerase. PCR was conducted in a volume of 10 µl in sealed positive displacement capillary tubes in a Corbett Research (Mortlake, NSW) FTS-1 capillary thermal sequencer. The programme was for 41 cycles, with an initial cycle of 2 min strand separation incubation at 92 °C, 2 min annealing at 35 °C and 1.5 min extension at 72 °C. Four cycles of 10 s at 92 °C, 2 min at 35 °C and 1.5 min at 72 °C were followed by 35 cycles of 10 sec at 92 °C, 20 s at 40 °C and 1.5 min at 72 °C. A final cycle of 10 s at 92 °C, 20 s at 40 °C and 5 min at 72 °C followed and then samples were maintained at 25 °C until analysed by electrophoresis.

Data Scoring

The banding patterns were manually converted to a vector of 1 and 0 records, where 1 represents the presence of a band at a particular molecular size and 0 represents the absence of a band at a particular molecular size. Band intensities were not considered in this analysis. Band positions were standardised between gels by running standard *Lolium* RAPD reactions on each agarose gel.

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