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THE USE OF DNA-PROFILING TECHNIQUES FOR THE IDENTIFICATION OF RYEGRASS VARIETIES

Document prepared by the experts from Belgium

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THE USE OF DNA-PROFILING TECHNIQUES FOR THE IDENTIFICATION OF RYEGRASS VARIETIES

Isabel Roldán-Ruiz[1], Jan De Riek[1], Jochen Dendauw[1], Ann Depicker[2],
Erik Van Bockstaele[1] & Marc De Loose[1].

INTRODUCTION

It has been shown for different crops that DNA profiling techniques can help to distinguish among commercial varieties (Smith *et al.* 1991, Joshi & Nguyen 1993, Zang *et al.* 1993, Morrell *et al.* 1995, Sharma *et al.* 1996). Most of the work has been done on varieties produced by clonal propagation, inbreeding or selfing. To date little is known about the applicability of these methods to outcrossing crops. In clonal, inbred or selfing crops no or little variation is expected to be found within a variety and fixed differences are expected to be found among varieties. In outcrossing crops like *Lolium spp.* genetic variation is expected to be found both within and among varieties (UPOV-BMT/3/16). It is only the among varieties variation that can be used in identification studies. An added difficulty is that the observed differences between varieties will be at the level of frequency of appearance of a number of molecular markers, rather than at the level of the presence or absence of molecular markers. This implies that to identify a variety it is not enough to analyse one individual, but a (representative) number of individuals should be analysed and appropriate statistical analyses should be used to test for significant differences between varieties.

The number of techniques to detect polymorphic DNA markers is steadily increasing. The capacity of discrimination of the different DNA molecular marker systems depends on their intrinsic capacity to reveal polymorphisms and on the genetic structure of the species under study. Therefore, before any routine work can happen, an evaluation must be done of each particular crop for the different DNA profiling techniques.

In this document we present the first results of our analysis of ryegrass varieties using AFLP (Vos *et al.* 1995). We first describe a number of technical details about the implementation of the technique in our laboratory and make a comparison of two detection systems (radioactive and fluorescent). The preliminary results presented here for ryegrass let us discuss the implications of the use of this technique for variety identification in this crop.

THE AFLP TECHNIQUE

AFLP is a PCR-based technique that combines characteristics from RFLP and RAPD. It consists in the amplification of a subset of genomic restriction fragments. The DNA is first digested by a combination of two restriction enzymes. Specific adaptors are ligated at the sticky ends and the fragments are amplified using primers which are homologous to the adaptors. By amplifying all the

[1] Department of Applied Plant Genetics and Breeding, Centre for Agricultural Research, B-9820 Merelbeke, Belgium.
[2] Laboratory of Genetics, University of Gent. K.L. Ledeganckstraat, 35, B-9000 Gent, Belgium

fragments a continuous smear is obtained. To be able to reveal only a limited number of bands, the primers are extended with one to three arbitrary bases. Each extra base that is added to a primer reduces the amount of fragments with a factor of 4. The set of fragments obtained depends on the restriction enzymes and primer extensions used. In order to make the fragments visible, the primers are labelled with radioactivity or with fluorescent dyes. The fragments are separated in a sequencing gel. This method has as advantages over other techniques that no sequence data are necessary and that large numbers of polymorphic markers can be scored in each reaction. The implementation of the technique for a given species and in a given laboratory can happen in a very short time span.

In our laboratory, the plant material (approximately 100 mg per plant) is obtained from seedlings 4-6 weeks old that are nursed in the greenhouse. After harvesting, the plant material is lyophilised, grounded in a mill and vacuum-packed. DNA is extracted using the CTAB method (Weisig *et al.* 1991).

Individual DNA samples are consecutively used to generate AFLP markers according to the procedures developed by Keygene and using commercially available kits. We use as restriction enzymes *EcoR*I and *Mse*I. It was first tested the optimal number of selective bases that revealed a workable number of fragments per reaction. Using 6 bases, around 100 fragments are detected per plant and we work with this number of selective bases for the analysis of ryegrass plants. To date 64 primer combinations composed of 6 selective bases have been tested in five *Lolium* plants, including three *L. perenne*, one *L. multiflorum* and one *L. x boucheanum.* The primer combinations that revealed a too dense pattern of bands or repetitive sequences were discarded. Thirty eight primer combinations revealed workable patterns.

RADIOACTIVE AND FLUORESCENT DETECTION SYSTEMS

The radioactive gels are handled according to one of the following procedures:

1- brought in contact with a light sensitive film during 4 days, developed and visually scored,
2- brought in contact with a light sensitive film during 4 days, developed and scanned in a 'flatbed' scanner,
3- placed in a Phosphor-Imager cassette during 24 hours and scanned using a Phosphor Imager.

Using both the scanner and the Phosphor-Imager, a image file is constructed that can be analysed using appropriate software.

It has been found that when the purpose is to score the presence or the absence of a small number of bands in a number of gels, the visual scoring of the gels is the most quick and reliable method to follow. When the purpose is to score the presence or absence of all the bands in a gel, the visual scoring of the gels is error prone and subjectivity plays a too important role. In this late case it is advisable to follow an automatic procedure as standardised as possible.

In the search of an automatic analysis procedure, five image files obtained with the scanner and five image files obtained with the Phosphor-Imager were analysed using GelCompar 4.0. The Phosphor-Imager files showed a higher resolution than the scanner files, and the bands were sharper

and therefore easier to detect for GelCompar in the first case. The greatest problem found when using GelCompar is related to the normalisation of the gels. The normalisation process is the alignment of the patterns present in a gel or in different gels via known reference bands within the patterns or the indirect alignment via a dedicated reference pattern applied at regular intervals on the gels. The alignment of the patterns via reference bands within the patterns was immediately discarded because given the high degree of polymorphism found in ryegrass varieties or populations it is sometimes very difficult to find bands which are present in all the patterns present in a gel. The normalisation is then performed using external reference patterns loaded each 5-6 samples. In all the cases analysed it has been very difficult or almost impossible to obtain a right alignment of corresponding bands in different patterns. The mis-alignment of corresponding bands is even bigger when the patterns were localised on different gels. The faults can be corrected manually but this is a very time consuming activity and in our laboratory this approach is followed only when absolutely unavoidable. The phenomenon is illustrated in figure 1. From our point of view, the way of working of GelCompar is not suitable for the analysis of AFLP gels.

GelCompar offers a second possibility which is the calculation of correlation coefficients between couples of patterns. The correlation coefficients can be interpreted as similarities between couples of patterns. The dendrogram shown in the second part of this document (comparison of two ryegrass varieties) is based on calculations of this type. From our point of view this approach, that can be useful in exploratory studies (like the one presented here) and that has been applied in some published studies, is not appropriate for an accurate comparison because it does not compare the patterns band per band, but compares the densitometric curves associated to each pattern.

A third way was investigated, and two gels were analysed using the software developed by Keygene specifically for the analysis of AFLP patterns. An appreciable improvement was achieved in comparison to the analysis performed using GelCompar and the bands were properly aligned.

Since May 1996 we are also working with a fluorescent detection system by using the ABI Prism™ 377 Sequencer of Perkin Elmer. The sequencer offers a number of advantages in comparison to the radioactive detection system: i) it uses internal lane standards for the normalisation of the gels, which results in a correct alignment of corresponding bands in different samples and in a very accurate sizing of the fragments (see figure 2), ii) no radioactive waste is produced, iii) it has a bigger capacity, which results in important time savings per reaction, iv) the analysis of the gel is done during the run. We are therefore leaving the radioactive detection system and all new experiments are being carried out using the fluorescent labelling system (See De Riek *et al.*, 1996 for more details).

At this moment the bottleneck of using the sequencer for AFLP runs is the lack of appropriate software for the analysis of the results; Genotyper was not developed for the analysis of AFLP markers and it does not offer enough flexibility for the processing of the results (GelCompar is more appropriate for this post-run analysis). A software packet appropriate for the analysis of fluorescent AFLP runs is currently being developed by Perkin Elmer. Currently we use a self-designed analysis procedure that exports the sequencer data to EXCEL and ACCESS for further processing.

COMPARISON OF TWO RYEGRASS VARIETIES

In order to illustrate the potentiality of the AFLP marker system, we present here some results obtained for the commercial ryegrass varieties Meribel and Meradonna. Meribel is a diploid *Lolium multiflorum* variety and Meradonna is a tetraploid *Lolium perenne* variety. In figures 2 and 3 are represented the patterns obtained for a number of plants of Meribel and Meradonna respectively using the primer combination E-AAC+M-CAT. When these patterns are compared using GelCompar with the correlation coefficient as similarity index and UPGMA as clustering method, the two varieties appear clearly differentiated (figure 4). This differentiation is also very clear if we compare both gels, because the degree of polymorfisme found within Meribel is much higher than the degree of polymorfisme found within Meradonna. This observation is corroborated if we compare the diversity index (Shannon & Weaver 1963) calculated for Meribel (0.2525) with that calculated for Meradonna (0.1137). This fact can be related to the fact that Meribel is diploid and Meradonna is a tetraploid. Because the markers were scored in a dominant way the presence of a 'fragment' in one of the four arms of the homologous chromosomes of a tetraploid plant was sufficient for that marker to be scored positive.

DISCUSSION

AFLP markers can be of great help for the identification of ryegrass varieties. Given the fact that in outcrossers a (representative) number of plants has to be analysed in order to characterise a variety, in our opinion any DNA profiling technique that could potentially be applied in practice should have as characteristics: i) be suitable for automatic analysis, ii) easy and quick to perform, iii) have a high efficiency in the generation of markers per reaction (be a multilocus technique) iv) be highly reproducible among laboratories v) have low costs per data point produced. As it has been demonstrated in different studies the AFLP technique suits to all these requirements.

As the AFLP markers permit a quick determination of allele frequencies for many markers distributed over the genome (when the location of the markers on a genetic map is known), they can allow the identification varieties with a high accuracy. This information could be processed using AMOVA (Excoffier *et al.* 1992), which is designed for the comparison of populations using molecular markers and which allows to give different weights to different loci or different bands. For example, loci situated on the same chromosome may be given a smaller weight, if this information is known (UPOV-TWC/14/15).

Finally, data on the genotype can also help to reduce the number of plants that has to be kept in gene banks and in reference collections for DUS (Distinction, Uniformity and Stability) trials, which can make these test less expensive. Information on the genotype based on molecular markers may be added to the DUS report as complementary.

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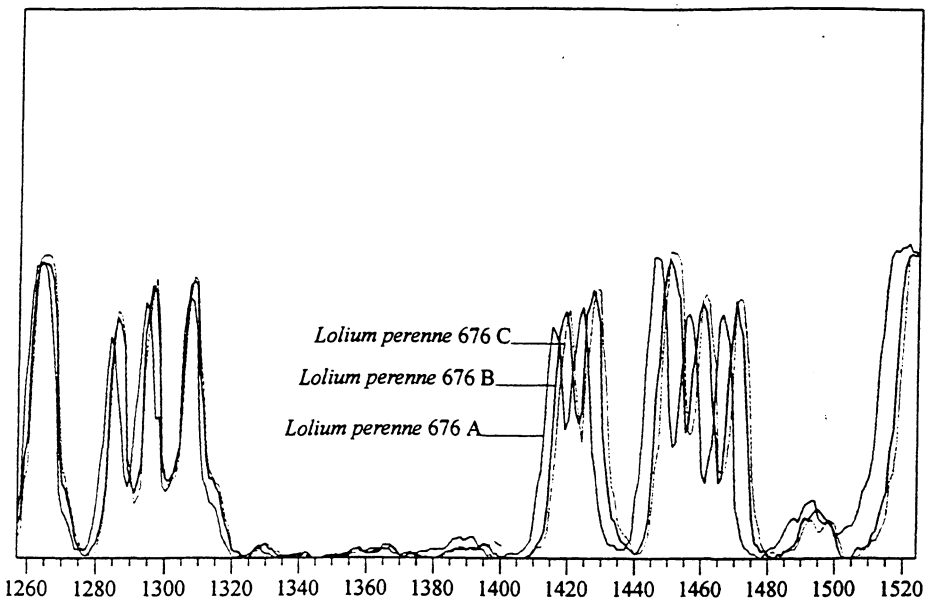
A) Scanner + GelCompar



B) PhosphorImager + GelCompar

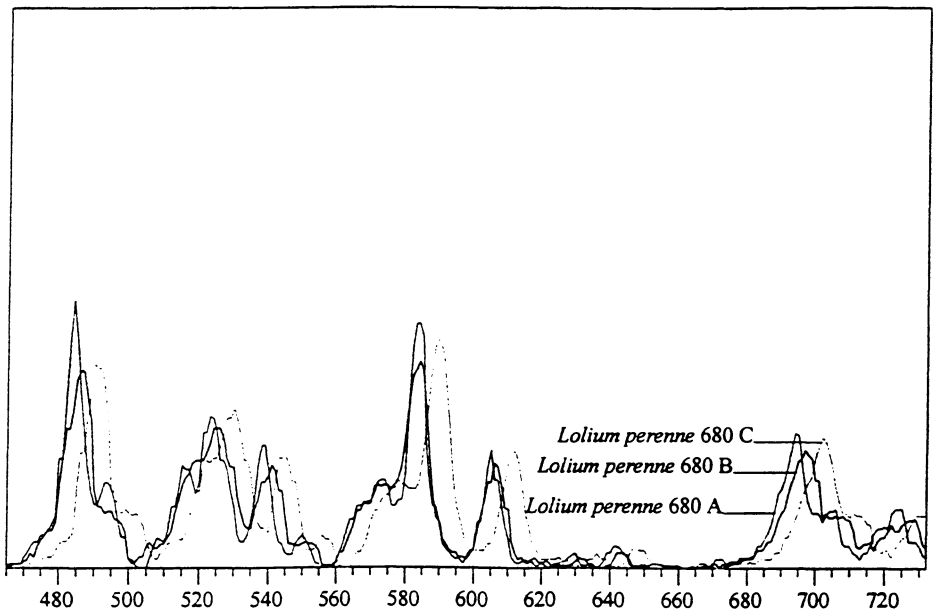


Figure 1. Comparison of the densitometric curves obtained with GelCompar when the same AFLP reaction is three times loaded on a gel and analysed. The peaks in the curves correspond with bands on the gel. A) starting from an image file obtained with the scanner; B) starting from an image file obtained with the PhosphorImager.

The problem shown here is solved when using the sequencer (see text and figure 2).

Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
30G, 75 *Lolium perenne* 680 D	63.76	198.50	1022	14951	2391
30G, 79 *Lolium perenne* 680 D	68.56	217.47	1108	20185	2571
30G, 106 *Lolium perenne* 680 D	88.59	294.92	810	10312	3322
30G, 123 *Lolium perenne* 680 D	102.51	349.15	714	8060	3844
30G, 137 *Lolium perenne* 680 D	114.40	395.88	139	1307	4290
30G, 139 *Lolium perenne* 680 D	115.73	401.19	407	5975	4340
32G, 49 *Lolium perenne* 680 E	63.92	198.18	838	18030	2397
32G, 52 *Lolium perenne* 680 E	68.89	217.63	869	18732	2576
32G, 69 *Lolium perenne* 680 E	88.80	294.79	630	13909	3330
32G, 85 *Lolium perenne* 680 E	102.83	349.04	555	9532	3856
32G, 99 *Lolium perenne* 680 E	114.83	395.83	124	1513	4306
32G, 101 *Lolium perenne* 680 E	116.16	401.08	406	6587	4356

Figure 2. Comparison of the AFLP paterns obtained when de same reaction (*Lolium perenne* 680) is two times loaded on a gel and analysed with the sequencer. Six bands spread over the electropherogram were selected to show the accuracy of the analysis procedure. As can be seen in the table, corresponding bands (peaks) are asigned the same size in both cases (within a margin of 0.5 bp).

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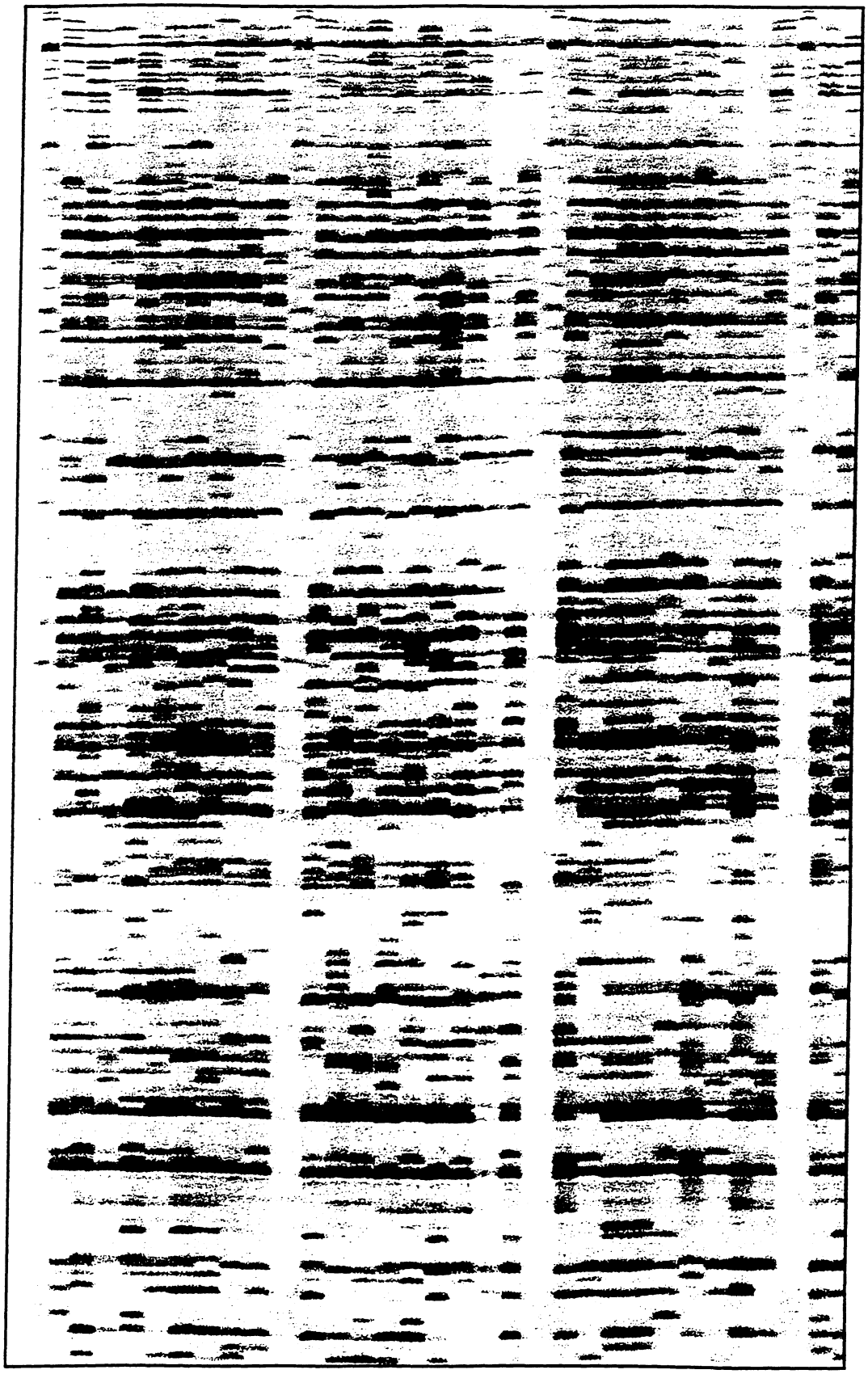

Figure 3. AFLP™ gel for plants belonging to the *Lolium multiflorum* variety Meribel.

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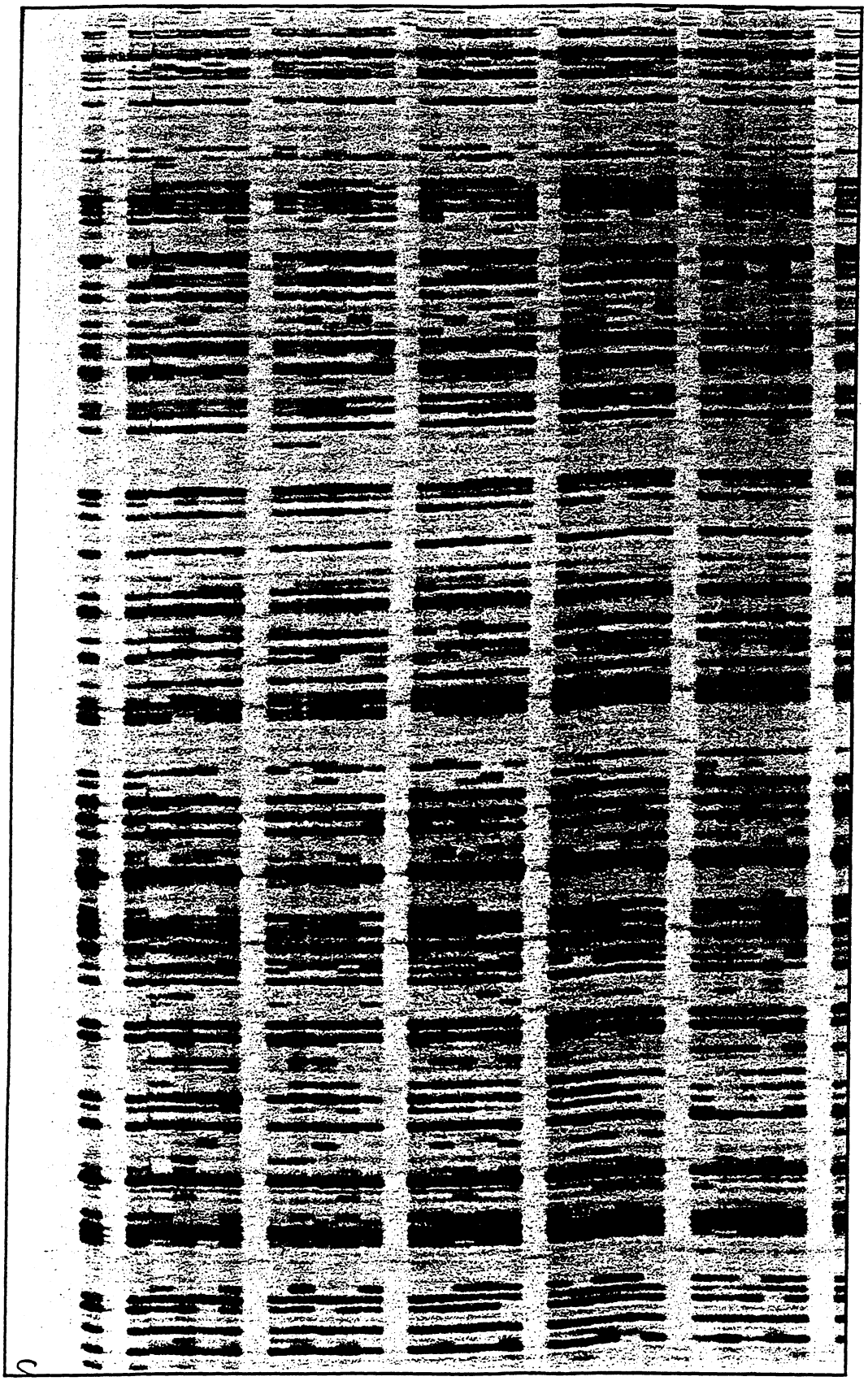

Figure 4. AFLP™ gel for plants belonging to the *Lolium perenne* variety Meradonna.

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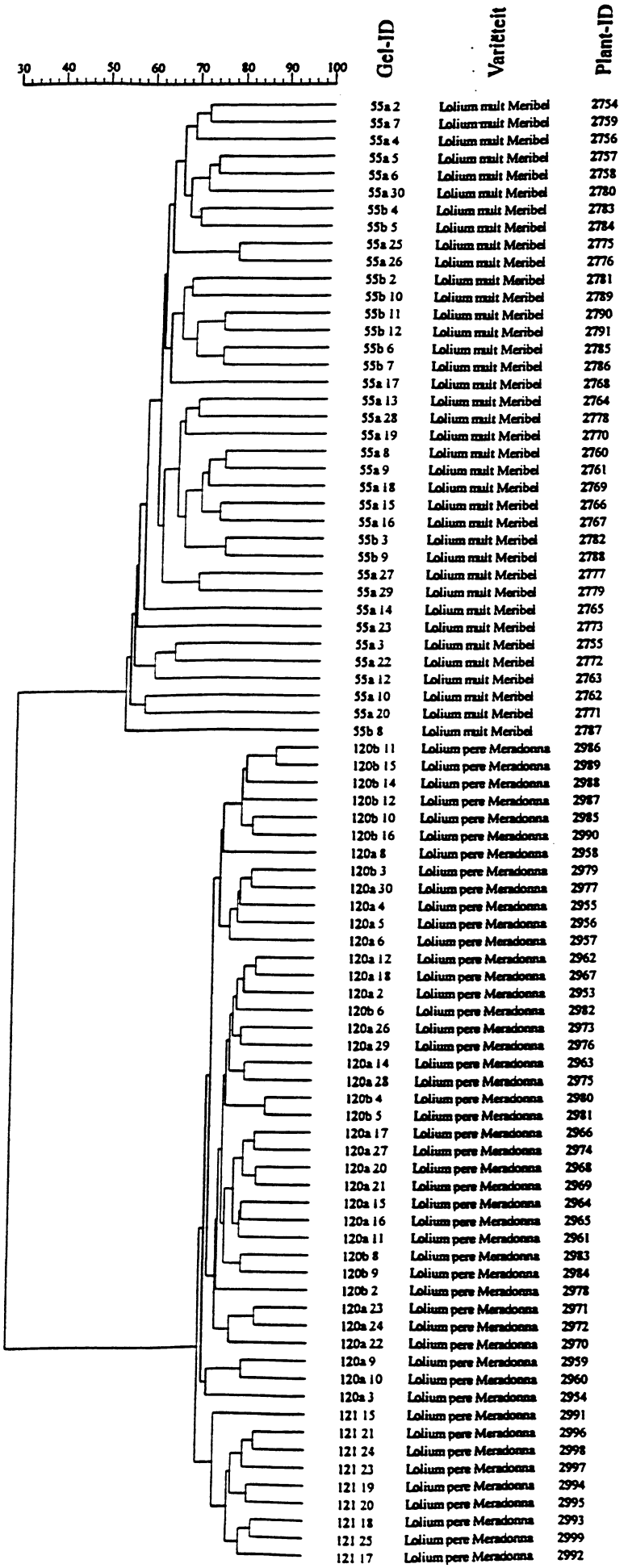

Figure 5. Dendrogram obtained for a group of plants of the varieties Meribel and Meradonna. Similarity index: correlation coefficient; Clustering method: UPGMA.

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