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EVALUATION OF RAPD-MARKERS FOR THE IDENTIFICATION OF RYEGRASS VARIETIES

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EVALUATION OF RAPD-MARKERS FOR THE IDENTIFICATION OF RYEGRASS VARIETIES

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1. INTRODUCTION

The genus Lolium is an important group of temporate forage grasses, including Italian (Lolium multiflorum), Westerwoldicum (Lolium multiflorum westerwoldicum) perennial (Lolium perenne) and hybrid (Lolium x hybridum) ryegrasses. These are self incompatible wind-pollinated species.

The synthetic varieties are often produced by recurrent systems. They consist of improved populations that are composed of up to 15 motherplants in the original polycross. Careful isolation and limitation of the number of generations during multiplication are then also necessary to maintain genetic stability and varietal identity (Camlin, 1995; Reheul et al., 1992; Van Bockstaele, 1993).

The genetic heterogenity in the genepools of cross-fertilized crops such as ryegrasses, is an important difference distinguishing these from clones and self-pollinators. Since a ryegrass variety is composed of several genotypes, it is impossible to characterize a variety with the DNA-pattern of only one genotype of the variety.

Until now, the classification of ryegrasses is only based on morphological or physiological characteristics and requires extensive observations during the whole growing season of the plants. Moreover, in some situations diagnoses lacks definition and objectivity (Wringley et al., 1987), as not all morphological traits can serve as unambiguous markers because of environmental influences.

Furthermore due to DUS-rules and due to breeder efforts, the genetic distance between the new varieties is becoming smaller and smaller. This makes the identifications by traditional techniques more difficult. For these reasons, it is generally accepted that the development of biochemical and molecular based identifications techniques will be very

useful (Yang and Quiros, 1993).

The last decades considerable interest has been focused on the use of biochemical methods for reliable plant variety discrimination and identification. Since Lolium is the most important European agricultural grass, it has generated much effort aimed at developing suitable electrophoretic methods for discrimination between varieties (Hayward and McAdam, 1977; Gilliland et al., 1982).
Although this technic offers a reliable and reproducible method, not all (unknown) varieties can be discriminated (Möller and Spoor, 1993) and a more sensitive genetic identification directly on DNA-level can probably solve this problem. This can be obtained by using DNA-markers.
In this paper we evaluate the possibilities of Random Amplified Polymorphic DNA (RAPD)-markers for the identification of ryegrass varieties.

2. RAPD-technique

The RAPD-marker technique has been developed by Williams et al. in 1990, and is based on the Polymerase Chain reaction (PCR).
For the detection of RAPD's, short DNA fragments (primers) of ten nucleotides are used as starting points for DNA synthesis. Each newly synthetisized DNA strand is used as a template for a next round of DNA-synthesis. After 30 to 50 PCR cycles, specific DNA fragments are sufficiently amplified to be visualised. When genotypes differ in the DNA sequence at the site where the primer bind, different DNA-fragments will be amplified. Consequently, differences between genotypes will be detected as the presence or absence of amplified bands. Generaly, when a primer of 10 nucleotides is used, about three to five DNA fragments are amplified, representing three to five loci (De Vries et al., 1992).
Previous experiments on Lolium cultivars have shown the potential of RAPD-markers as a rapid, reproducible and useful method for distinguishing among different varieties and clustering of genotypes in the Lolium complex (De Loose et al., 1993: De Loose and Van Bockstaele, 1994).

In further experiments, additional aspects of the RAPD-technique, such as sampling method, uniformity of the patterns obtained from individual plants and the potential of

identifying (unknown) samples were evaluated.

3. RESULTS

3.1. SAMPLING METHOD AND UNIFORMITY

In order to control the genetic uniformity and the sampling method, seeds of the L. perenne variety Vigor and seeds of an experimental variety based on Vigor were sown in the field.

The plants (30-40) were harvested in three different ways : (a) as individual plants, (b) as bulked leaf sample and (c) as bulked leaf sample but with an equal amount from each plant.

The DNA was prepared by a standard phenol extraction method as described by Dellaporte et al. (1983). RAPD-analysis was done with the primer OPC-07 (OPERON). The obtained DNA-fragments were run in 2% agarose gels, were visualised after staining the gel with ethiduim bromide and illuminated with UV-light. The pictures with the DNA-profiles were scanned with a HP-deskscan IIp and analyzed by the GELCOMPAR software programme (Vauterin and Vauterin, 1992).

The RAPD-patterns of the individual plants were checked on uniformity (Fig. 1). As could be expected, the plant by plant uniformity does not exist. Some common major bands can be detected; but a lot minor fragments differ from plant to plant.

A comparison is also made between the patterns of individual plants and those of the bulked leaf samples. Tracks 1-36 show the RAPD-pattern of the DNA prepared from the plants of the experimental variety; tracks 37-63 represent the plants descended from Vigor. The RAPD-pattern of the DNA prepared from the bulked leaf sample is presented in track a; in track b the bulked leaf sample with a equal (balanced) amount of leaf material per plant is shown.

The comparison between the patterns of track a and b learns that there's almost no difference in sampling method of mixture of individual plants.

When the patterns obtained from the bulked leaf samples are compared with those of the individual plants, a large simularity can be noticed; some major bands are present in all

patterns; but in the patterns of the bulked samples less fragments were visualized. This is not surprising because many factors can influence the equilibrium of which fragments will be amplified in th PCR-reaction. By using bulked samples possible polymorphisms in these group can be masked.

When the uniformity of Vigor and the derivated variety is considered, common fragments for each plant were detected. The frequency of those different fragments were analyzed. Only two major bands are present in both individual sample groups. One major fragment (1100 bp), present in almost all Vigor samples, was absent in the experimental variety samples.

In conclusion the genetic background can only be verified when individual ryegrass plants are analyzed. No difference can be observed when bulk leaf samples were used.

3.2. IDENTIFICATION

Several experiments are set up to evaluate the possibilities of RAPD-markers as an identification technique. For those experiments individual plants are grown in the field and DNA was prepared as described earlier.

3.2.1. Identification between species

RAPD's can be used to be diagnostic on species level. Seven primers revealed different and complex patterns for a perennial and Italian ryegrass type. One primer resulted in only one major DNA-fragment that differed in length between Italian and perennial ryegrass (Fig. 2). This RAPD-reaction was repeated on 15 individual plants from 5 Italian and 5 perennial ryegrasses. All Italian ryegrasses revealed in this RAPD reaction a DNA fragment of 1000 bp. Four of the perennial ryegrasses showed a fragment of 1550 bp, while in the variety Phoenix the two fragments were observed. Phenotypical observations and information of the breeder confirmed that the perennial ryegrass variety Phoenix was created with 'some Italian ryegrass blood' in it. Restriction digest analysis confirms that the DNA fragments (1000 aan 1550 bp) have the same offspring. This may suggest an extraction - contraction polymorphism.

3.2.2. Identification within species

The GELCOMPAR program allows to classify all patterns from a variety in one group (Fig. 3).

Plants descended from prebasic seed were used. A clear separation between two Italian ryegrass varieties which are phenothypically and agrotechnically similar could be obtained.

In a following experiment, basic seed was used. The cluster capacity can be increased by combining multiple RAPD-profiles. After scanning the gels, the profiles were normalized based on a molecular weight marker.

Also after two multiplication cycli after the original polycross, RAPD-profiles allowed us to cluster individual plants from different varieties (Fig. 4).

Finaly an attempt was made to identify unknow varieties, by using certified (commercial, R1) seed. Three known very similar old Italian ryegrass varieties and two unknow Italian ryegrass varieties were evaluated.

A polymorphism analysis on all the samples is carried out on the combined RAPD-patterns. Cluster analysis was performed using the UPGMA clustering algorithm and the simularity is calculated using the DICE-coëfficient (Gelcompar).

The strong relationship between all the tested varieties is reflected in the diagram. Fig. 5 shows the combined pattern resulting from RAPD reactions with different primers. In contrast with the experiment carried out with basic seed, no clear clustering in groups per variety could be obtained. Individual plants of variety x are more linked to variety 3 and the patterns of variety y are more clustered with variety 2. On the contrary an unequivocal identification of the unknown varieties could not be reached. This is not surprising, since at least three multiplication cycli after the original polycross were needed to produce the commercial seed. The production of these R1-seeds of the varieties happened at different locations and the number of generations were totaly unknown. In addition the essential variety purity for certified seed is not so strickt as for basic seed. As a consequence all these characteristics of these certified seed are reflected in the presented diagram : geneticaly wide overlapping varieties. The possible shift in genetic (in)stability and the varietal identity during the multiplication cycli will be studied under known

conditions in further experiments.

4. CONCLUSION

By combining multiple RAPD profiles in the GELCOMPAR software analysis, we were able to produce reproducible dendrogams for different varieties of ryegrasses. The identification of plants descending from geneticaly wide commercial seed is not perfect.
In order to optimize the method for cross fertilized crops, the characterisation of a variety has to be based on allele frequencies, rather then on their presence or absence. In addition, more reproducible markers, distributed at regular intervals over the chromosomes, are necessary to identify the varieties with much greater accuracy. With these experiments we have shown the potential of DNA markers obtained with PCR-amplification.

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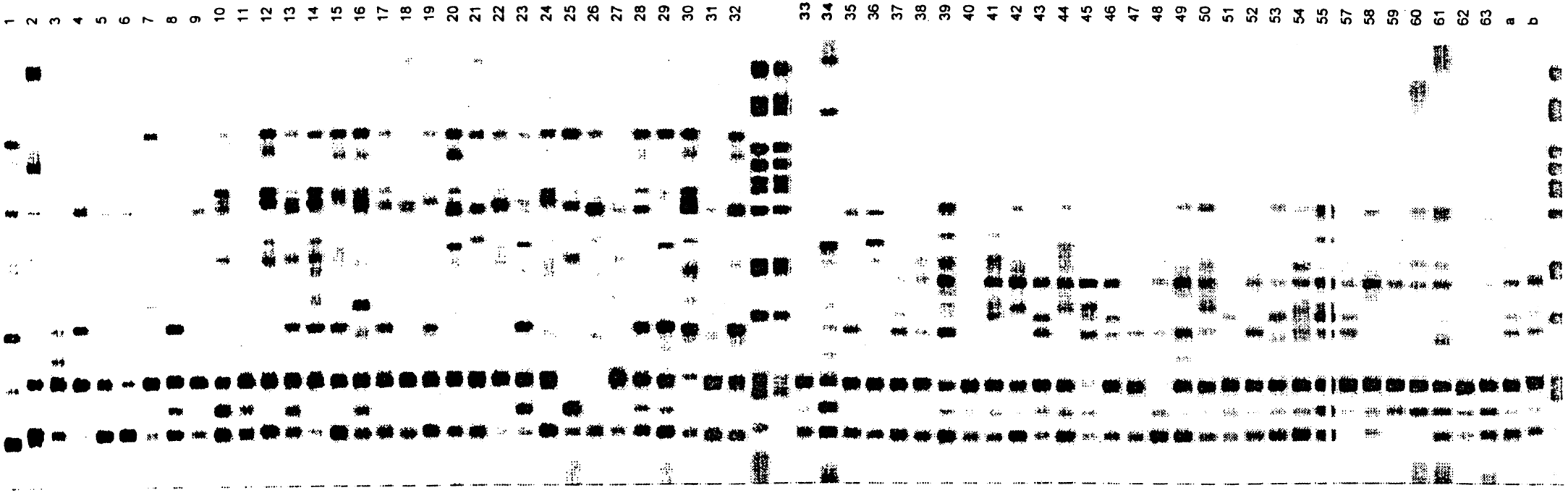

Fig. 1 . RAPD-profile of Vigor and an experimental variety based on Vigor. Track 1 - 36 represents RAPD-profiles of the experimental variety; Track 37 - 63 : Vigor; Track (a) and (b) represent a RAPD-profile of an unbalanced bulked leaf sample and of a balanced bulked leaf sample, respectively.

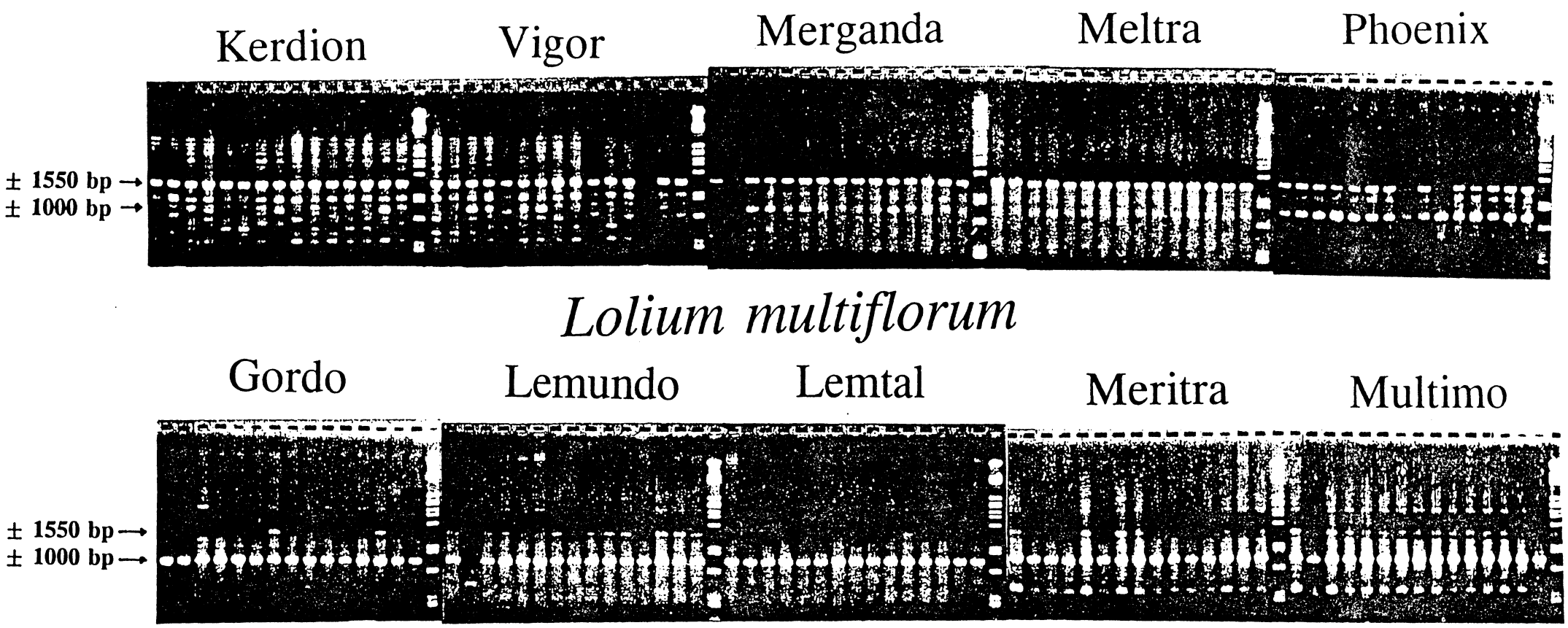

Fig. 2. RAPD-analysis on 15 individual plants of 5 Lolium perenne and 5 Lolium multiflorum varieties.

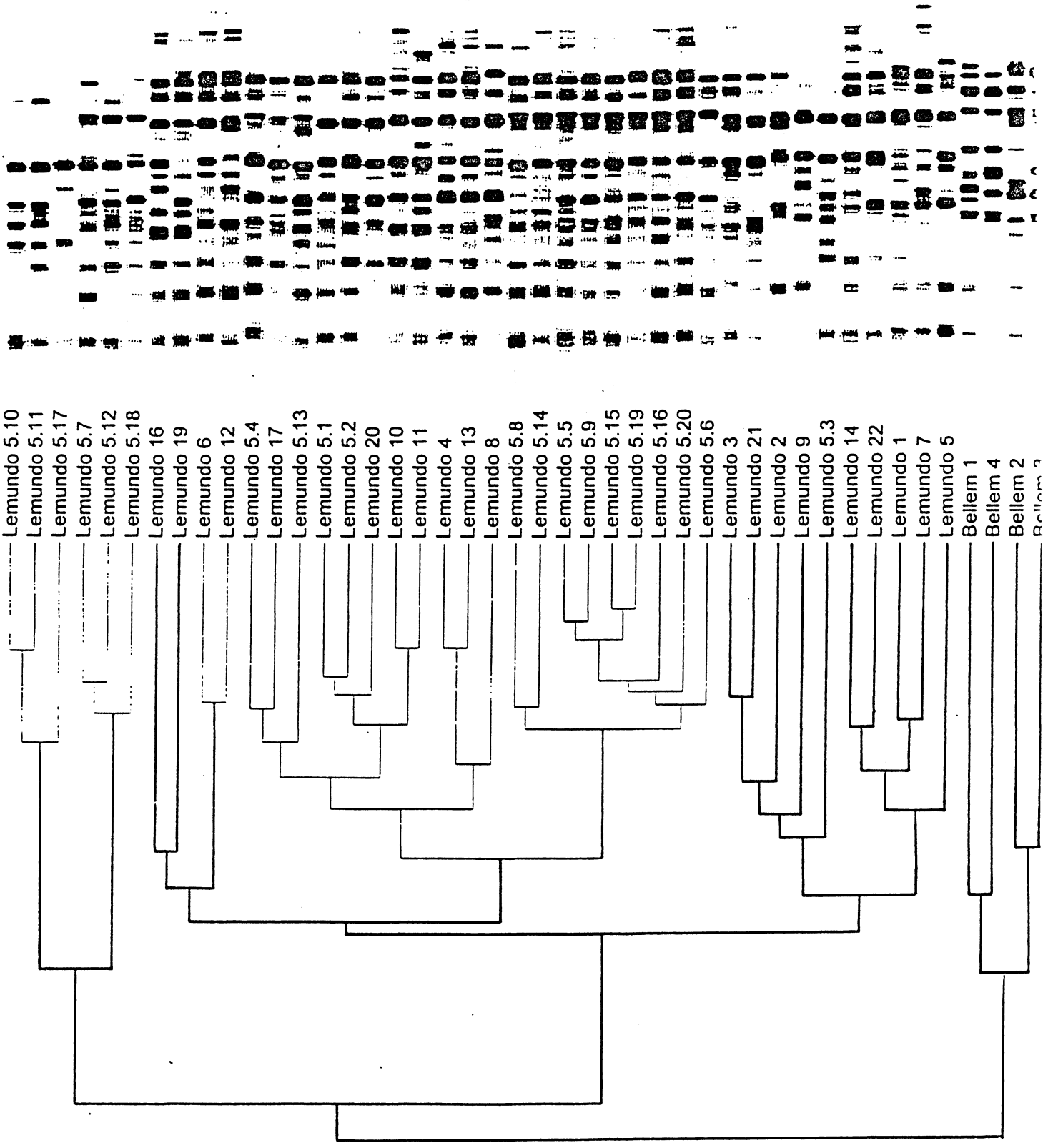

Fig. 3. Relationship between different ryegrass varieties. RAPD-profiles of 2 Italian ryegrass varieties obtained with one primer (OPC-9) and DNA isolated from bulked leaf samples indicated with '5.-' or from individual plants and on the left the resulting dendrogram obtained by UPGMA of correlation values of normalized profiles (GELCOMPAR).

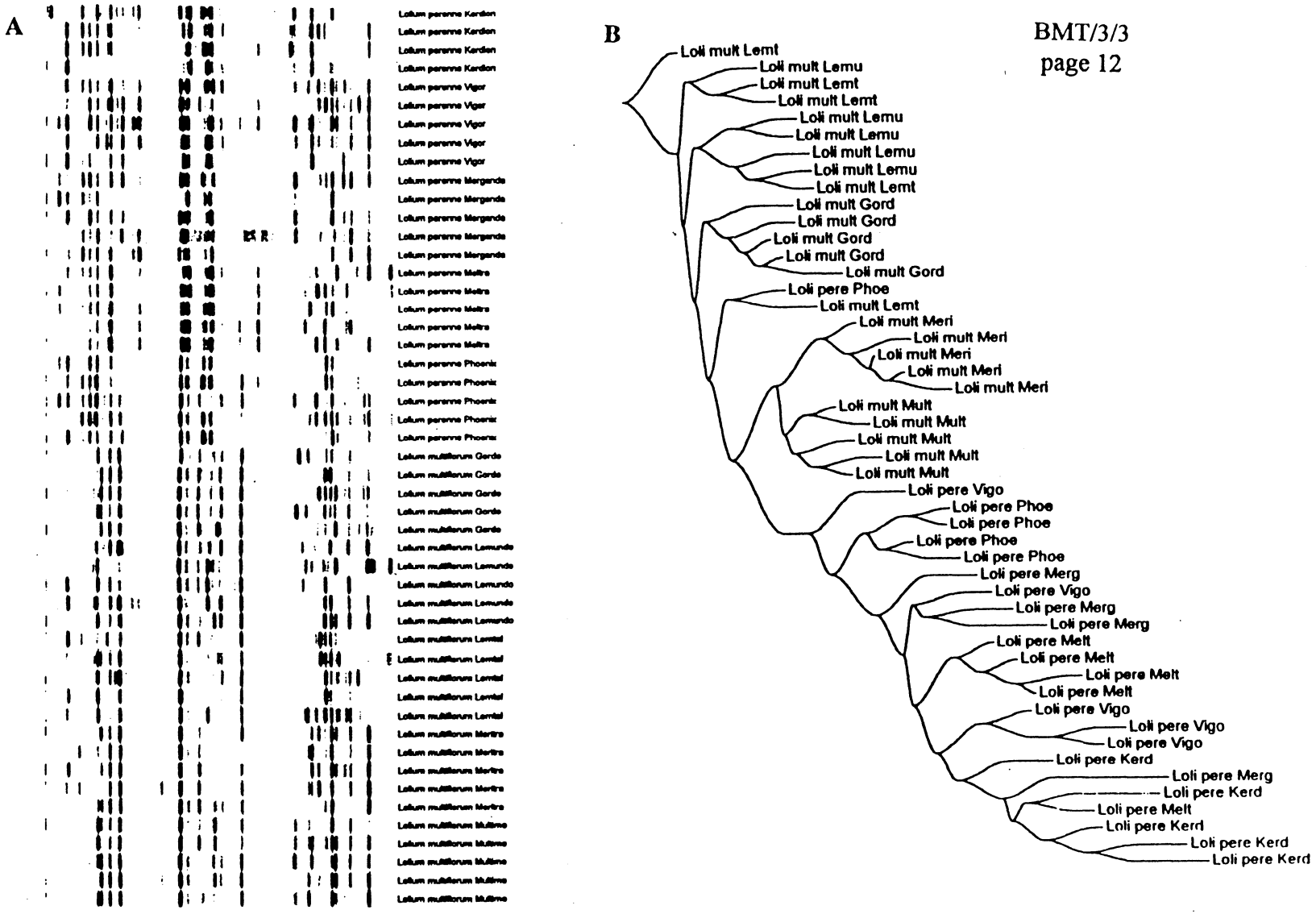

Fig. 4. A. DNA-fingerprint obtained by the combination of 3 RAPD-profiles using the software program GELCOMPAR. The analysis was performed on 5 individual plants of 5 L. perenne and 5 L. multiflorum varieties.

B. Dendrogram obtained after analysis of the combined RAPD patterns using 'neigbour joining' clustering method (GEL-COMPAR)

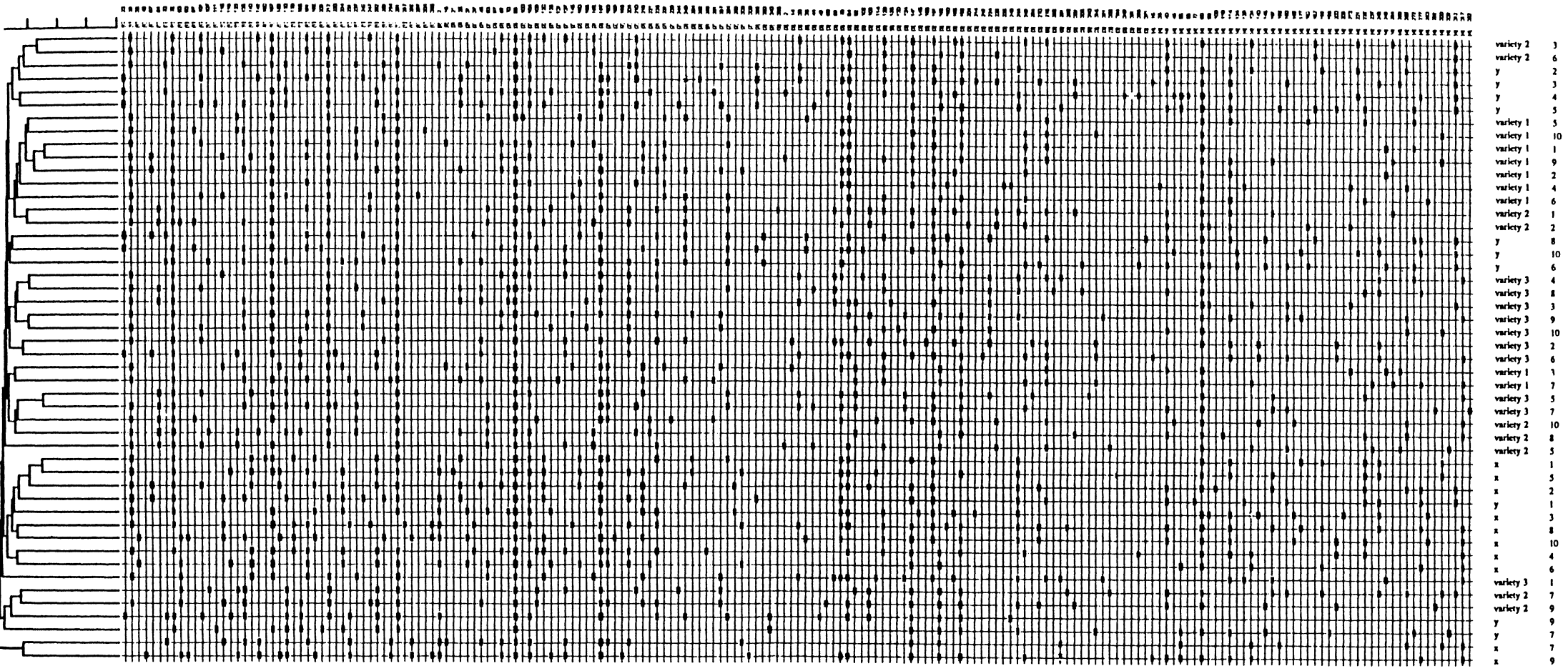

Fig. 5. Combined and reorganised RAPD-profiles from 10 individual plants of 5 L. multiflorum varieties. In the resulting dendrogram the phylogenetic relationship is shown.

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