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THE USE OF DNA PROFILING

FOR DISTINCTNESS, UNIFORMITY AND STABILITY TESTING

Document prepared by experts from the United Kingdom

THE USE OF DNA PROFILING FOR DISTINCTNESS, UNIFORMITY AND STABILITY TESTING.

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Introduction.

The use of gel electrophoresis for analysis of proteins and subsequent variety identification is well established. The incorporation of electrophoresis into the UPOV Guidelines for Testing of Wheat and Barley has been discussed for some years and is now nearing acceptance. Thus this is an appropriate time to be considering the possible use of DNA profiling techniques in DUS testing.

Details of the different types of DNA profiling techniques have been described previously (UPOV paper TC/28/4, 1992) and will not be repeated here. However, in terms of variety identification work, there are basically two types of methods that have been used - probe-based technologies and amplification technologies.

Probe-Based Technologies

Restriction fragment length polymorphisms (RFLPs) are the most widely reported means of revealing DNA sequence variations in a diverse range of organisms, including plant varieties. The potential of RFLPs for identification purposes is clear and the large number of available restriction enzyme/probe combinations make this a powerful approach.

Conventional RFLP analysis utilising randomly selected single-copy or low-copy genomic and/or cDNA clones as probes is an effective way of revealing differences between varieties and there are reports of successful RFLP analysis in many species, including wheat, barley, rice, maize, oats, brassicas, peppers, roses and apples. In cases where single copy probes have been found to provide only low levels of polymorphism, the use of multiple copy probes, either random or of known derivation, can be advantageous. This kind of approach has been reported in crops such as potatoes and wheat.

There are alternative sources of probes for RFLP analysis. For instance, some of the probes used for DNA 'finger-printing' in humans and other animals reveal useful polymorphisms in plants and can be used for variety identification purposes. The M13 repeat probe has been particularly used to distinguish between varieties of several horticultural species such as apples, blackberries and raspberries. Synthetic repetitive oligonucleotides such as $(GATA)_n$ have also proven to be effective and are applicable to a range of species, although there are few detailed reports of the use of such oligonucleotides for plant variety identification as yet.

Amplification Technologies

Amplification technologies based on the polymerase chain reaction (PCR) are becoming widespread. The discovery that arbitrarily chosen primers can amplify several fragments of genomic DNA has lead to the diagnostic techniques known as

RAPDs (random amplified polymorphic DNA) and AP-PCR (arbitrarily primed-PCR). The nature of the amplified fragments depends upon the primer sequence and on the target DNA. Different primers give rise to different amplified bands and polymorphisms at the priming sites result in the disappearance of an amplified band. Thus RAPDs/AP-PCR are methods for detecting polymorphisms distributed throughout the genome, with a primer usually amplifying several bands, each of which will probably originate from a different locus.

Whilst the preliminary work with these techniques showed only the existence of differences between a few rice and soybean genotypes, there has recently been a huge increase in the use of RAPDs for variety discrimination and identification. Thus there are reports of the successful application of RAPDs/AP-PCR to distinguish between varieties, genotypes, lines and species of barley, maize, various brassica species, apples, papaya, cocoa, sugar beet and onions, amongst others.

The remainder of this paper describes some of the work on DNA profiling being undertaken at NIAB and indicates areas where further deliberations and decisions are required, particularly in the context of DUS testing.

The UK Project

The UK research programme aims to develop and evaluate suitable methods for assessing the extent of DNA polymorphism in crop species. It will provide valuable information on the usefulness of various methods of DNA analysis for the molecular characterisation and identification of crop varieties. Without these data recommendations as to the most appropriate technology for further development into more discriminating, efficient and cost-effective variety testing systems cannot be made.

Concentrating initially on oilseed rape and subsequently on barley varieties, the research is investigating RFLP analytical methods and examining the level of polymorphism revealed by various enzyme/probe combinations to assess the ability of this method to distinguish between and identify varieties. This has required the establishment of in-house procedures which efficiently produce consistent results. Non-isotopic probe labelling will be evaluated. The project is also investigating PCR-based techniques, primarily RAPDs, examining the level of polymorphism demonstrated, and assessing the ability of this method to distinguish between and identify varieties.

In addition the project has examined and will continue to examine oilseed rape and barley for any DNA minisatellites, determine the level of polymorphism of these and assess their potential for use for variety identification purposes.

All these techniques will be examined for their usefulness for both non-specific application to variety identification and characterisation and for incorporation into statutory schemes for assessing Distinctness, Uniformity and Stability (DUS) of varieties, including the development of appropriate statistical methods as necessary.

Materials and Methods

DNA Extraction

DNA was extracted from around 100 seedlings of 63 different varieties of *Brassica napus* representing much of the UK National List at the start of the project. DNA was isolated by grinding the leaves in liquid nitrogen and performing chloroform extraction, RNase treatment and phenol extractions followed by isopropanol precipitation and ethanol wash. These DNA preparations were the basis of the DNA used for both RAPD and RFLP analyses.

RFLP analysis

DNAs were digested using *Hin*dIII and a sample of the digests (~0.5 ug of DNA) was electrophoresed and checked for complete cutting by hybridization to a heterologous ribosomal gene probe.
Genomic digests were fractionated through 1% agarose gel electrophoresis in TAE buffer (40 mM Tris:acetate pH 7.9, 5 mM Na acetate, 1 mM EDTA) at 2 V/cm for 16 hours.
DNA fragments were visualized by UV irradiation after ethidium bromide staining. The DNA in the gel was transferred to a nylon membrane by capillary blotting (Southern, 1975) and the DNA fixed onto the membrane by UV crosslinking.
The DNA probes were labelled using ^{32}P-αdCTP by the random oligo-labelling method (Feinberg and Vogelstein, 1984) using a commercial kit (Amersham). Hybridization was carried out in 4 X SET, 0.15% (w/v) BSA, 0.15% (w/v) ficoll (MW 400000), 0.15% (w/v) PVP (MW 360000), denatured herring sperm (10 mg/ml), 0.1% (w/v) sodium pyrophosphate, 0.1% SDS and 10% (w/v) dextran sulphate (MW 500000). The hybridization reaction was carried out for 16-20 hours (overnight) in a Techne rotating hybridization oven at 65^{o} C. The membranes were washed in 0.1 X SSC, 0.05% SDS at 50^{o} C and exposed to X-ray film.
Oligonucleotides were end labelled using T_4 kinase and γ^{32}P-ATP. Hybridizations with the oligonucleotides were the same as above, except the temperature of the reaction was 42^{o} C and the membranes were washed in 3 X SSC, 0.05% SDS at 37^{o}C.
Bands on the autoradiogram were labelled and scored manually.

RAPD analyses

RAPDs were performed in a MJ Research 16 or 60 sample programmable thermal cycler. Conditions for PCR were 0.5-1 mM primer, 0.2 mM dNTPs, 3-4 mM $MgCl_2$ in 1 X reaction buffer [supplied with the enzymes: Taq polymerase(Promega); Amplitaq, Stoffel fragment and Taq polymerase (Perkin Elmer)]. Cycling parameters were: 2 mins. initial denaturation at 92^{o}C, followed by 50 cycles of 30s at 92^{o}C, 30s at 35^{o}C and 60s at 72^{o}C. The samples were stored at 4^{o}C following a final extension time of 10 mins. at 72^{o}C.
Amplified DNA was fractionated by electrophoresis through 1% agarose gel in TBE buffer (0.045 M Tris-borate; 0.001 M EDTA pH 8.0) at 5-20 V/cm and the gels stained with ethidium bromide after the run.

Results

RFLP

Two approaches were adopted for detecting RFLPs: the use of multicopy genomic sequences and the use of simple sequence repeats as probes.
Hybridization to genomic DNAs with multicopy genomic probes has shown the following:

pN180: this probe produced about 22 different bands (6 common to all varieties) with each variety possessing 11-15 bands. These bands were spread between 1-20 kb in size and were well separated making them easy to score. However, relative band intensities varied between the varieties, especially for the smaller fragments. 60 varieties produced 46 different patterns of which 39 were unique.

pN216: this probe produced about 16 different bands (3 common to all) when probed against *Hin*dIII digested genomic DNAs. The bands were sharp and most fell within the size range of 6-20 kb creating a clustering of some bands. Of the 63 varieties tested, 26 recognizably different banding patterns could be detected, with 13 varieties in the largest group: 16 varieties had a unique set of bands with this probe; only one variety, Bristol, possessed a unique DNA fragment band.

pR36: this probe produced more than 20 separate bands with around 10 of these grouped between 4-9 kb. A complete breakdown of the banding pattern has not been done for this probe although five of the bands appeared to be common to all the varieties and polymorphism was clearly visible between the varieties.

pN107: data from this probe suggested that more than 18 separate bands were produced with up to 12 bands for each variety: only one or two were common to all varieties. The probe produced a few strong bands, the majority of which were of a weaker nature which made them difficult to score. The bands were well spread out (500 bp-20 kb) making their identification easier.

By using the presence of a band as a phenotypic marker (see table 1) it was possible to discriminate between most of the varieties using the two probes pN180 and pN216. All the varieties tested could be distinguished except for two groups:
i) Capricorn/Silex/Falcon/Zeus
ii) Eurol/Idol
Group i) could be resolved using pR36 but neither probe appeared capable of distinguishing within group ii).

A few simple sequence repeats were tested for their suitability as fingerprinting probes. The repeat $(GAAA)_5$ did not give a very strong signal whilst $(GACA)_5$ had too many bands to be useful. $(GATA)_5$ produced a few highly polymorphic intense bands with a background of many faint signals suggesting it may be useful for varietal identification. $(GATA)_5$ can clearly distinguish the four varieties in group i) but is unable to distinguish Eurol from Idol.

This is only a small number of all potential simple sequence repeats so there is scope for more sequences to be tested.

RAPD

We have investigated the use of RAPD for varietal identification. As has been reported by other groups we have demonstrated profiling difference(s) between varieties of oilseed rape (fig.1). However, we have encountered problems with reproducibility; the success of amplification varies from worker to worker with a correlation between experience and reproducibility. Equally the use of different enzymes has shown a high degree of variability (fig. 2).
However, many groups have examined the problems of reproducibility of RAPDS (Smith and Chin, 1992; Weeden *et al.*, 1992). Their results suggested that the method of DNA extraction plays an important part in reproducibility and we will continue future work taking into account the importance of this variable.

Discussion

It is clear that RFLP and RAPD can be used to show distinctness between different varieties of oilseed rape. Whilst the technique of RFLP relies on the detection of a prexisting sequence or sequences, RAPD relies on the *in vitro* synthesis of DNA *de novo* using primer-directed DNA polymerization. Consequently it is not unexpected that data using RFLP will be more reproducible between laboratories than those from RAPDs. However for any technique to be adopted for varietal identification, reproducibility between laboratories need not be a prerequisite so long as the data have "equivalent meaning" across laboratories (Smith and Chin, 1992). Although RAPDs appears to be a less intrinsically robust technique than RFLPs this does not necessarily imply that equivalent meaning will be more difficult to achieve between (and within?) laboratories than with RFLPs. The experience in the UK has been that it is not a trivial undertaking to develop a standardised RAPD protocol which gives reliable data 100% of the time, particularly if extreme demands of reproducibility are made on the interpretation of the profiles. Nevertheless we believe the technique has a potential application in variety identification and registration and that protocols can be developed which will provide equivalence of meaning between operators. However this will require a realistic widescale evaluation of the technique and that unreasonable demands on the data and requirements for its acceptance are not made.

Most of the proposals outlined by Smith and Chin (1992) as prerequisites for the use of DNA profiling techniques for varietal protection are equally valid for varietal identification. The essence of these is as follows:
i) The descriptors must have been shown publicly to have a high power of discrimination.
ii) The descriptors should exhibit no, or consistent, interaction with the environment.
iii) The same class of descriptors should be capable of generating data of equivalent meaning across laboratories.
iv) Preferably the descriptors should allow distances to be calculated between inbred lines or varieties.

v) Preferably the genetic location and control of each genomic site that is surveyed should be known.
vi) The methodology used to generate and translate banding profiles into discrete varietal identifiers must be publicly available.

Points iv) and v) are less important in varietal identification than for varietal protection. Even so, it is important that the descriptors are well scattered throughout the genome so that the technique is not surveying a restricted region(s).

The advantage of analysing the data using a +/- scoring is its simplicity but the scoring of faint bands can be subjective and perhaps does not exploit all the information on the gel or autoradiograph. For instance relative band intensities, assuming that these are reproducible characteristics of the varieties, may be used to differentiate between varieties with the same +/- band scoring profile (fig. 3). We have acquired software, Gelcompar (Applied Maths), which is capable of analysing gel or autoradiograph images, thereby removing much of the subjectiveness in band scoring. This software is currently being evaluated.

The use of computer programmes such as this allows the creation of a database of profiles as a means of varietal identification which could assist in two main objectives:
a) The registration of new varieties using DNA profiling in DUS testing.
b) The determination of genetic relatedness between existing and new varieties, which has clear implications for Plant Breeders' Rights and discussions relating to essential derivation.

Distinctness between oilseed rape varieties has been demonstrated but the question of uniformity can only be examined using DNA isolated from individual plants of the same variety. This problem is being addressed using both RFLP and RAPDs for it is only by assessing the genetic variability within varieties that are already on the National List that a framework for the use of these molecular techniques can be established in respect of uniformity.

The difficulty we have found in discriminating between Eurol and Idol is interesting in view of the fear expressed by some regarding the erosion of minimum distance between varieties. Eurol is morphologically distinct from Idol since both are registered varieties on the UK National List. It is probable that if we looked at more probes, or different restriction enzymes, we would find differences between the two varieties using these techniques. It is equally true that if enough morphological or other population metrics are measured it could be possible to distinguish all varieties of oilseed rape, even those which have presently failed DUS testing for lack of distinctness. This suggests that DNA profiling techniques can be tuned so that the level of discrimination is similar to that of the present tests.

This research project is continuing and future work will :

1) Provide data on the level of genomic variation measured using DNA polymorphisms. We intend to examine both synthetic oligonucleotide probes and RAPDs in more detail, particularly with regard to the repeatability of the latter.

2) Allow this variation to be assessed in relation to the genetics and botany of the crop species under investigation and in the context of currently accepted concepts of variety within those species. Variation within varieties (uniformity) will be examined both for oilseed rape and barley.

3) Require the investigation of appropriate biometrical techniques with which to analyse data of these kinds commensurate with the existing requirements of varietal characterisation.

4) Make recommendations on the most appropriate of these techniques for further development for use in varietal registration systems.

The project will provide fundamental data concerning the DNA polymorphism of the genomes of the species studied. By determining the potential offered by modern methods it forms an essential precursor to the development of molecular techniques which will provide methods of characterising plant varieties with a suitable degree of discrimination in line with desired genetic distances. Perhaps it is now the right time to propose that UPOV initiate a more widely based technical evaluation of these DNA profiling techniques based on the experience from comparative testing in participating laboratories with a view to establishing a framework for their introduction into routine use. With UPOV support funding from within the EU or other international agencies could be sought to allow this.

Acknowledgements

We wish to thank D. Lydiate (Cambridge Laboratory, Norwich) for providing the four genomic clones, N. Ellis and J. Peng (John Innes Institute, Norwich) for the gift of the simple sequence repeats oligonucleotides. Assistance from the MBD section within NIAB and Mr Simon Kightley is gratefully acknowledged. This work is supported by a grant from MAFF.

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Southern, E.M. (1975). Detection of Specific Sequences Among DNA Fragments. J. Mol. Biol. 15: 503-517.

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pN216 Bands	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. English Giant	+	*	-	+	-	-	+	+	+	-	-	*	-	+
2. Barsica	+	*	+	+	-	+	+	+	+	-	-	*	-	+
3. Br. Leaf Essex	+	*	+	-	-	+	+	+	+	-	-	*	-	-
4. Emerald	+	*	+	+	-	-	+	+	+	-	-	*	-	-
5. Hungry Gap	+	*	+	+	+	-	-	-	+	+	-	*	+	-
6. Bienvenu	+	*	+	+	-	+	+	+	+	-	-	*	-	-
7 Caron	+	*	+	+	-	+	-	+	+	-	-	*	-	+
8. Hobson	+	*	-	+	-	+	+	+	+	-	-	*	-	-
9. Topas	+	*	+	+	+	-	-	-	+	+	-	*	+	-
10. Optima	+	*	-	+	+	-	-	-	+	+	-	*	+	-
11. Sparta	+	*	+	-	+	-	-	-	+	+	-	*	+	-
12. Lirawell	+	*	-	+	+	-	-	-	+	+	-	*	+	-
13. Libravo	+	*	+	+	-	+	+	+	+	-	-	*	-	+
14. Diamant	+	*	+	+	-	+	+	+	-	-	-	*	-	-
15. Global	+	*	+	+	+	-	-	-	+	+	-	*	+	-
16. Aztec	-	*	+	+	-	+	+	+	+	-	-	*	-	-
17. Doublol	+	*	+	-	-	+	+	+	+	-	-	*	-	-
18. Lecor	+	*	+	-	-	+	-	+	+	-	-	*	-	-
19. Capricorn	+	*	+	-	-	+	+	+	+	-	-	*	-	-
20. Tapidor	+	*	+	+	+	-	-	-	+	+	-	*	+	-
21. Ember	+	*	+	+	-	+	+	+	+	-	-	*	-	-
22. Galaxy	+	*	-	+	-	-	+	+	+	-	-	*	-	-
23. Puma	+	*	+	+	-	-	-	-	+	+	-	*	+	-
24. Link	+	*	+	-	-	+	+	+	+	-	-	*	-	-
25. Silex	+	*	+	-	-	+	+	+	+	-	-	*	-	-
26. Rocket	+	*	+	-	-	+	+	+	+	-	-	*	-	-
27. Falcon	+	*	+	-	-	+	+	+	+	-	-	*	-	-
28. Mari	+	*	+	+	+	-	-	-	+	+	-	*	+	-
29. Golda	+	*	-	+	+	-	+	+	+	+	-	*	+	-
30. Eurol	+	*	-	+	-	+	+	+	+	-	-	*	-	-
31. Idol	+	*	-	+	-	+	+	+	+	-	-	*	-	-
32. Envol	+	*	-	+	-	+	+	+	+	-	-	*	-	-
33. Lincoln	+	*	+	+	+	+	+	+	+	-	-	*	+	-
34. Samurai	+	*	+	-	-	+	+	+	+	-	-	*	-	-
35. Forte	+	*	+	+	+	-	-	-	+	+	-	*	+	-
36. Starlight	+	*	-	+	+	-	-	-	+	+	-	*	+	-
37. Ibis	+	*	+	+	+	-	-	-	+	+	-	*	+	-
38. Tanto	+	*	+	+	-	+	+	+	-	-	-	*	-	-
39. Bingo	+	*	+	+	+	-	-	-	+	+	-	*	+	-
40. Bristol	+	*	-	+	-	+	-	+	+	-	+	*	-	+
41. Askari	+	*	+	-	-	+	-	+	+	-	-	*	-	+
42. Zeus	+	*	+	-	-	+	+	+	+	-	-	*	-	-
43. Lirajoy	+	*	+	-	-	+	+	+	+	-	-	*	-	-
44. Cobol	+	*	-	+	-	+	+	+	+	-	-	*	-	-
45. Inca	+	*	-	+	-	+	-	+	+	-	-	*	+	-
46. Apache	+	*	-	+	+	+	-	+	+	-	-	*	+	-
47. Briol	-	*	-	-	-	-	-	-	+	+	-	*	+	-
48. Evita	+	*	-	+	+	-	-	-	+	+	-	*	+	-
49. Apex	+	*	-	+	-	+	+	+	+	-	-	*	-	-
50. Mandarin	+	*	+	+	-	+	-	+	+	-	-	*	-	+
51. Tiger	-	*	+	+	-	+	+	+	-	-	-	*	-	-
52. Prestol	+	*	-	+	-	+	+	+	-	-	-	*	-	-
53. Liberty	+	*	+	-	-	+	+	+	+	-	-	*	-	-
54. Honk	+	*	+	+	-	+	+	+	+	-	-	*	-	-
55. Maya	+	*	+	+	+	+	-	+	+	-	-	*	+	-
56. Logo	+	*	-	+	+	-	-	-	+	+	-	*	+	-
57. Sponsor	+	*	+	+	+	-	-	-	+	+	-	*	+	-
58. Nimbus	+	*	+	+	+	-	+	+	+	+	-	*	+	-
59. Dallas	+	*	+	-	-	+	+	+	+	-	-	*	-	-
60. Express	+	*	+	-	-	+	+	+	+	-	-	*	-	-
61. Lictor	+	*	+	+	-	+	+	+	+	-	-	*	-	-
62. Score	+	*	+	-	+	+	-	+	+	-	-	*	+	-
63. Winfred	+	*	+	+	+	-	+	+	+	+	-	*	+	-

Table 1. Scoring of RFLP bands using +/- system. +/- denote the presence/absence of a particular sized band; * denotes a band which is present in all the varieties. Increasing band numbers represent decreasing band size.

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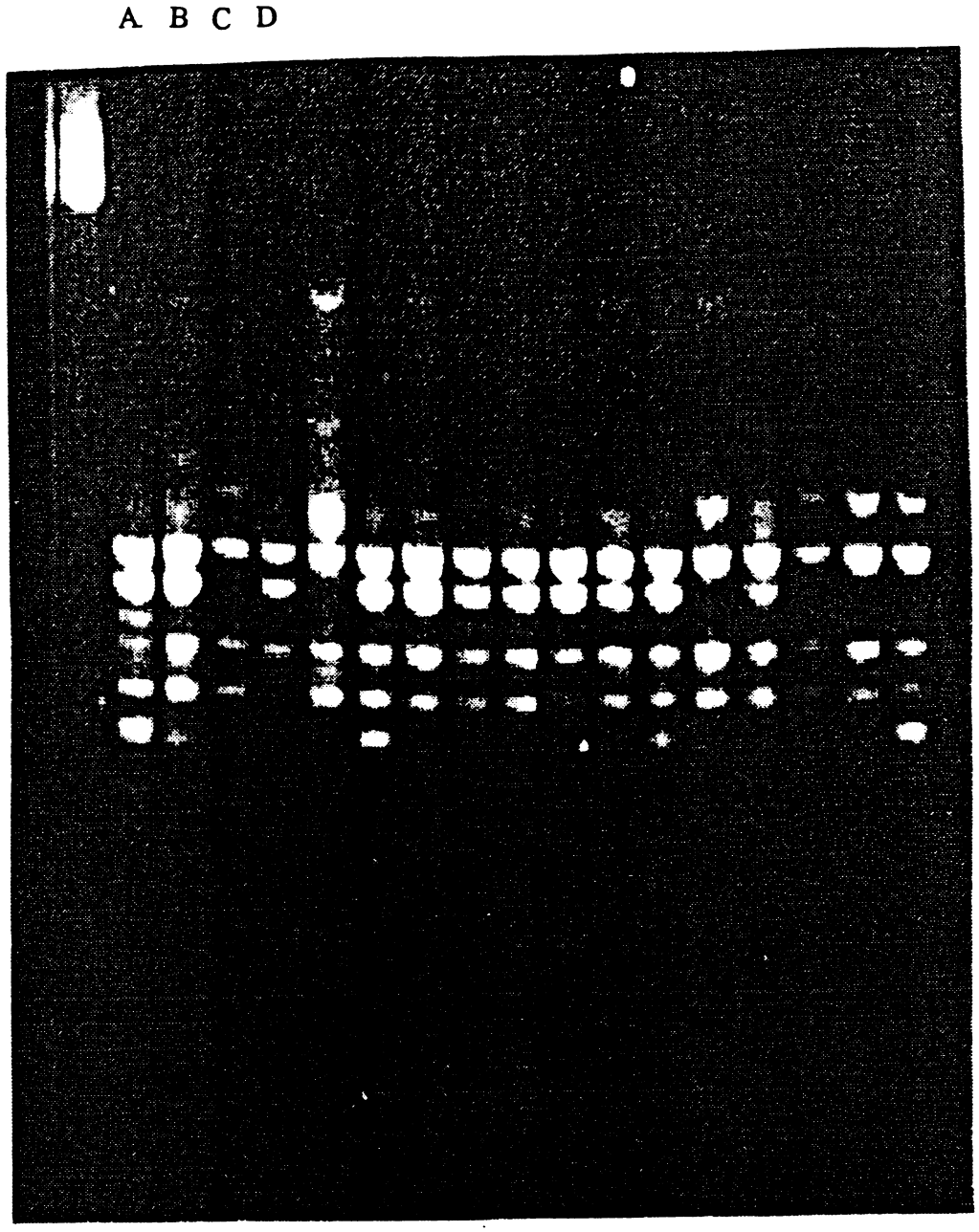

Fig. 1. Polymorphic bands between different varieties of oilseed rape amplified by RAPDs. Seventeen different varieties amplify four clearly distinct profiles A, B, C and D.

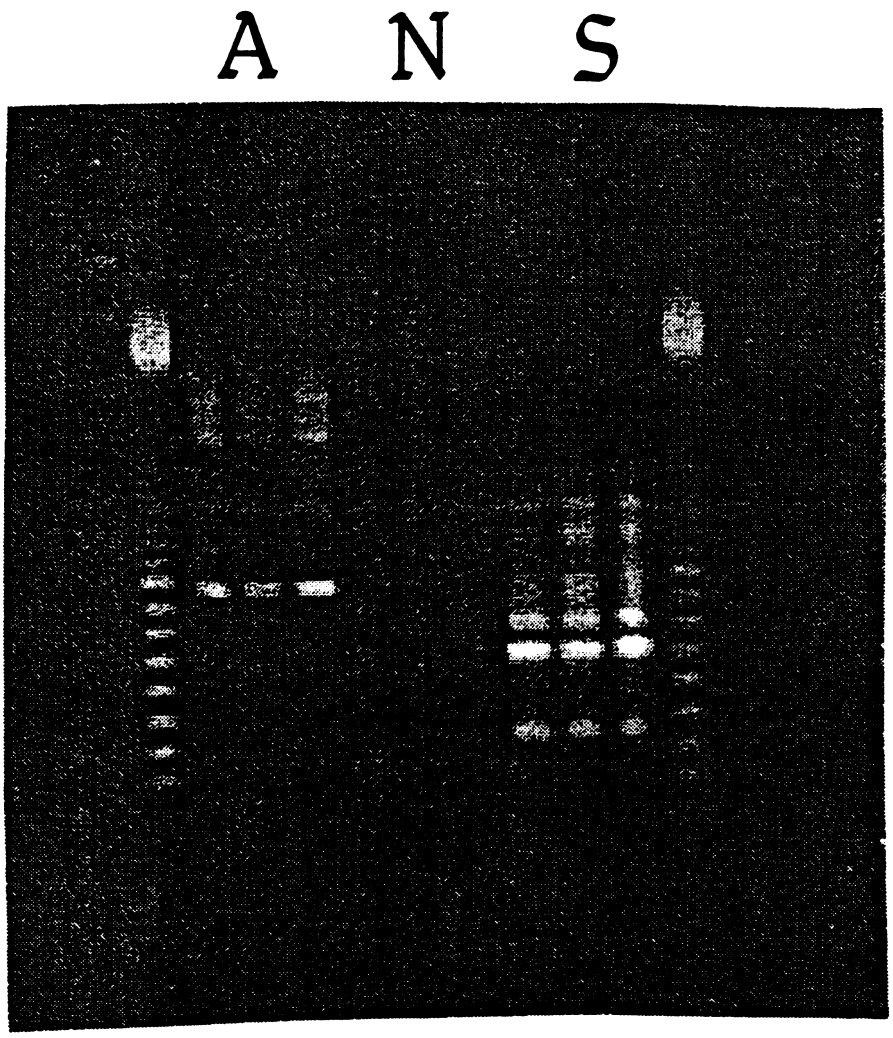

Fig. 2. Reproducibly different. The same DNA preparation was amplified under the same conditions, in triplicate, using A amplitaq, N native Taq polymerase and S Stoffel fragment (all supplied by Perkin Elmer).

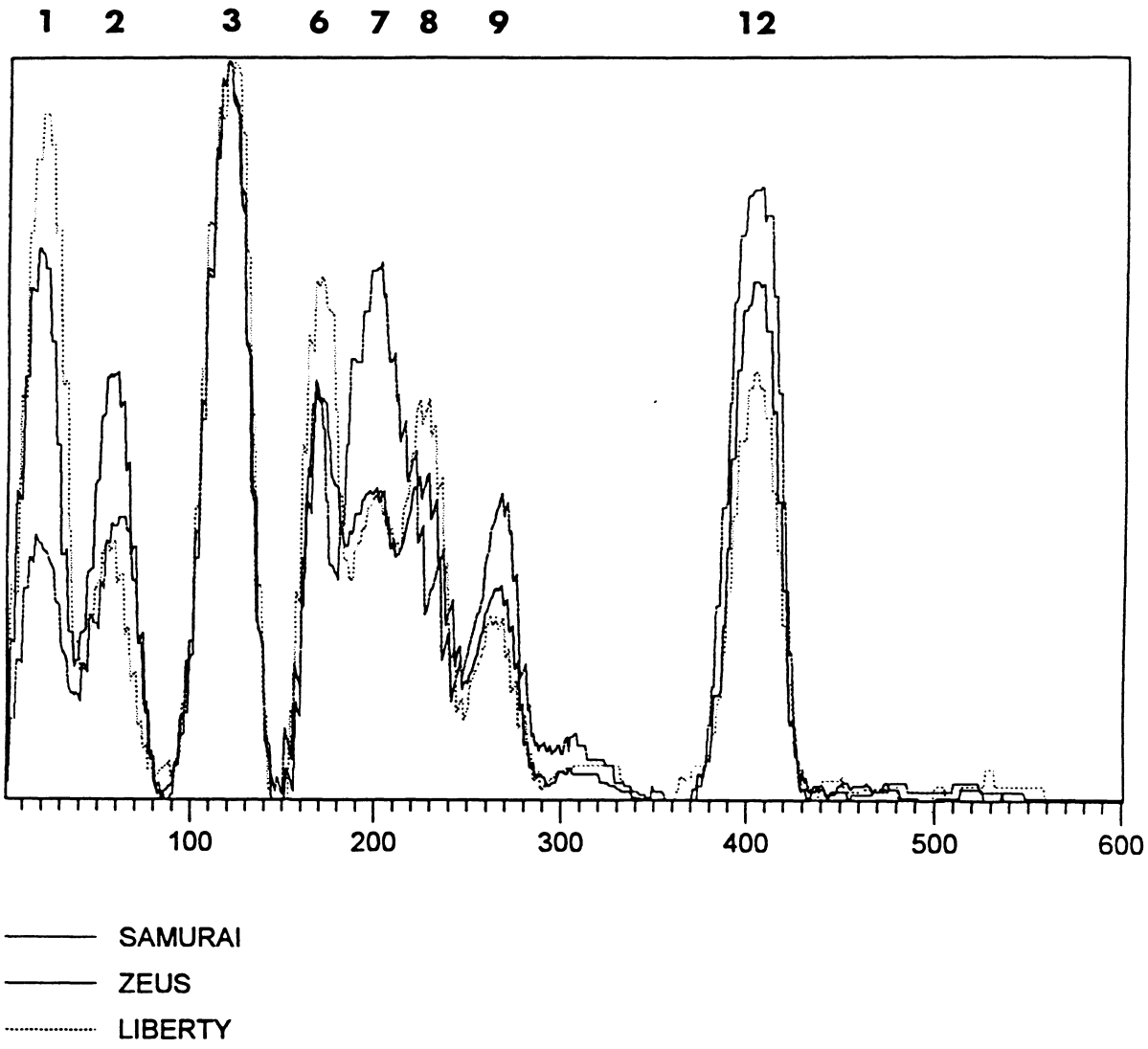

Figure 3. Densitometric analysis of similar profiles. Three varieties with the same +/- profile are shown. The numbers above the peaks denote the bands, as scored in table 1. Gelcompar has "normalised" the tracks so that the darkest band in each track are of the same intensities. The relative intensities of the bands may provide extra data for the identification of the different varieties.

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