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UPOV

TC/28/4 0121
ORIGINAL: English
DATE: September 26, 1992

INTERNATIONAL UNION FOR THE PROTECTION OF NEW VARIETIES OF PLANTS

GENEVA

TECHNICAL COMMITTEE

Twenty - eighth Session
Geneva, October 21 to 23, 1992

Determination of Distinctness, Uniformity and Stability

of Varieties Using DNA Profiling Techniques

Document prepared by experts from

Australia

(Original)

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DETERMINATION OF DISTINCTNESS, UNIFORMITY AND STABILITY OF VARIETIES USING DNA PROFILING TECHNIQUES

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I. Introduction

1. Member States and the Office of the International Union for the Protection of New Varieties of Plants ("the Union") have, by their timely development and adoption of the 1991 Act of the Union, shown both vision and adaptability to developments in biotechnology.

2. While technical and legal refinements in varietal protection embodied in the 1991 Act are the product of UPOV committees at all levels, the Technical Committee has been particularly effective in coordinating the adoption of new technology in Member States.

3. The effective coordinating role played by the Technical Committee is manifest in, for example, the electronic measurement of colour and protein electrophoresis for the determination of distinctness.

4. The Technical Committee has also given preliminary consideration to DNA characteristics as determinants of varietal distinctness(TC/27/9).

5. The purposes of this paper are to:

(a) consider briefly the nature and developments in DNA based analysis and its application to characterisation of varieties;

(b) discuss the complementary role for DNA based analysis in testing for distinctness;

(c) determine the validity of using DNA profile characteristics for establishing distinctness of a variety under the 1991 Act of the Union; and

(d) make recommendations to the Technical Committee to expedite the adoption of DNA based analysis for varietal characterisation within the Union.

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II. DNA based analysis and varietal characterisation

6. Molecular characterisation has provided a variety of tools that have potential use in the identification of plant varieties. Visual images based on DNA characteristics often referred to as DNA profiles seem to have particular merit for the determination of varietal distinctness.

DNA profiling

7. A DNA profile (or DNA 'fingerprint') is a visual product derived from an analysis of some parts of the DNA molecule. The DNA profile can be likened to a 'bar-code' or a human fingerprint. It is unique combination of identifying characteristics of the product or individual, but it bears no relationship to the appearance (description) or performance of that product or individual.

8. There are currently two major DNA profiling techniques of relevance to varietal characterisation: Restriction Fragment Length Polymorphism (RFLP) and Random Amplified Polymorphic DNA (RAPD) analysis (Attachment 1). RFLP analysis provides limited information about the genotype. RAPD analysis is based on the *in vitro* expression of some polymorphic regions of the DNA molecule. RAPD analysis neither provides information about the genotype nor is it an analysis of the genotype itself. DNA techniques allow the analysis of any area of the genome that displays polymorphism (which is most often present in non-coding portions of the DNA molecule). Details of RAPD and RFLP analytical procedures are briefly described in paragraphs 15 - 20.

Advantages of DNA profiling over other molecular techniques

9. The structure of DNA (on which DNA profiling techniques are based) is not likely to be influenced by the climate, environment, latitude or developmental stage of the plant. This is in contrast to the quantity and nature of all other molecules which are more or less influenced by the environment, latitude, developmental stage of the plant.

10. Protein techniques including isoenzyme analysis can examine only a small portion of the genome encoding for a highly specific set of soluble proteins which on the whole do not exhibit heterogeneity. DNA profiling techniques, by contrast, analyse any area of the DNA molecule that displays polymorphism (which is most often present in non-coding regions of the DNA molecule).

11. The banding patterns obtained from the electrophoresis of storage proteins are very detailed with a large number of overlapping bands produced. This makes interpretation of the gel picture complex and not readily amenable to automated

analysis.

12. Normally isoenzyme analysis requires the application of a range of different enzymatic procedures to develop a "fingerprint" profile. Often the procedures will also differ for different tissues and species. By contrast, there is only one standard RAPD analytical procedure for all tissues and species. The standard RAPD procedure is used universally and only the 'primer-set' applied is varied to enhance the resolving power of the analysis. This means fewer chemicals are required, there are fewer conditions to be standardised, there is less operator error and automation is simplified.

13. In the past DNA analysis techniques were more costly than protein analysis techniques. However, the development of RAPD analysis in 1990 has made the cost per assay comparable. Automation of DNA profiling will reduce costs further and it is likely that fewer DNA based assays will be needed for varietal characterisation.

14. In contrast to DNA based characteristics, secondary metabolites (phenolics, pigments, lipids, etc.) which are products of a complex series biochemical reactions, are normally severely affected by the environment, nutritional status, latitude and stage of plant development. They therefore provide no particular advantage for varietal characterisation.

Comparison of RAPD with RFLP analysis

15. The table below gives a comparison of the features of RAPD and RFLP with reference to those aspects related to the possible use of the two techniques for varietal characterisation.

PROPERTY	RFLP	RAPD
Application	all species	all species
Detect allelic variant	yes	no
No. loci detected	1-3	1-10
Genome surveyed	low copy regions	whole genome
Quality of DNA	pure	crude
Quantity of DNA	2-10 micrograms	10-50 nanograms
Use of radioisotopes	yes	no
Type of probe(primer)	species specific low copy DNA or cDNA	arbitrary 9-10 mer oligonucleotide
Tech. difficulty	intermediate	low
Time for analysis	3-6 days	1 day(automated) 2 days(manual)
Prior information	DNA sequencing of sp	None

Flow charts of RAPD and RFLP techniques are depicted in Attachment 1.

Random Amplified Polymorphic DNA(RAPD)

16. RAPD analysis was first developed in 1990. It is

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technically simple and can be readily automated. RAPD requires only small amounts of DNA (as little as one nanogram). Unlike RFLP analysis it does not require the use of species specific primers, radioactive probes, cDNA library construction or Southern hybridisations, nor does RAPD depend on cloning or prior DNA sequencing of the species.

17. **Methodology:** The basis of RAPD is that short oligonucleotide primers of arbitrary sequence are incubated with plant genomic DNA and allowed to combine (hybridise). The primers will bind to many different positions (loci) on the genome and they are allowed to replicate from those points of hybridisation in a thermal cycling reaction (polymerase chain reaction - PCR), producing varying lengths of DNA depending on how close together two of these primers were on opposing DNA strands.

18. This process is allowed to continue through a number of replications so that the fragments created are in sufficient quantities to be visualised on an agarose or polyacrylamide gel. For most plants, primers that are 9-10 nucleotides long will generate 2-10 amplification products (bands) per primer set. The creation of these DNA fragments is completely duplicable.

19. The products are easily separated by standard electrophoretic techniques and visualised under UV light by staining with ethidium bromide. Polyacrylamide gels can also be used and combined with silver staining of DNA to increase the resolution and detection of less amplified fragments.

20. As a rule individual amplification products represent one allele per locus and are transmitted as dominant markers. A small amount of the variation in the RAPD profile can be due to DNA sequence variation, either insertion or deletion, but this does not represent a significant problem in the DNA profile analysis.

21. **Sampling : What tissues? How many samples?** The most commonly used tissue is fresh, actively growing leaf or tuber material, although the use of other tissues is being investigated. Analysis can be carried out using less than one gram of plant material.

22. The number of samples, manner of sampling and loci per sample for different species to be assayed for varietal characterisation needs to be established and standardised.

23. **Automation and instrumentation:** RAPD analysis lends itself to automation which results in reduced laboratory operator error and analysis time and increased throughput with consequential cost reductions. Automation also decreases the variation of results between laboratories.

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Automation of RAPD can lead to the compilation of computer files of varietal DNA fingerprints and centralised data storage.

Two scientific instrument firms produce packages for the automated analysis of RAPD profiles:

Applied Biosystems International-373 DNA Sequencer using 'Genescan 672' software
Pharmacia LKB Biotechnology-ALF DNA Sequencer using 'Fragment Manager' software

24. These programs and instruments are not interchangeable and the output of the two systems is not directly comparable. Should UPOV accept RAPD analysis and automated profile analysis as a method of establishing distinctiveness of plant varieties, it is imperative that *instrumentation be standardised* as soon as possible for both national and international compatibility of DNA profile database management.

25. The estimated cost(AUD)*, excluding capital outlays, of automated and manual RAPD analysis is depicted in the following table:

ITEM	AUTOMATED RAPD	MANUAL RAPD
Primers	0.57	0.32
Taq polymerase	0.57	0.57
Buffers/dNTPs	0.26	0.26
Size standards	0.37	0.32
Gel	0.11**	0.27***
Reagent cost/locus	1.87	1.74
Labour/locus($15/h)	0.11	>0.33
Duration	1 day	1-2 days
Data storage	automatic	manual

* based on 10 - 15 loci required for identification
** Calculated using a loading of 72 samples/gel
*** Calculated using agarose gels, loading 14 samples/gel and one size standard

Applying RAPD to varietal characterisation

26. **RAPD profile storage, transmission and evaluation:** Data from automated evaluation of RAPD profiles can be stored automatically either as chromatographs (peak position and area) or fragment size tables or fragment diagrams (gel pictures).

27. Electronically stored RAPD profiles may be transmitted by standard electronic media (modem, floppy disc or CD) and software programs can be developed for electronic comparison

* Note: AUD 1 = Sfrs. 0.96 = US$ 0.74 (exchange rate 92.09.23)

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of varietal profiles with RAPD profiles in a database.

28. 'Reference DNA' of a variety can be stored permanently and/or transported for later RAPD analysis or used for alternative DNA based varietal characterisation techniques that may develop in the future.

29. **Quantification and cluster analysis:** The significance of the presence or absence of a band on a gel picture has always been a problem when using molecular techniques for varietal characterisation. The question of what comprises a variety may be addressed by the application of "cluster analysis" of bands at particular positions in combination with a defined empirical minimum distance (% similarity). The envisaged method of scoring RADP data and the construction of similarity matrices and cladograms of similarity is briefly outlined in Attachment 2.

30. Statistical evidence can be enhanced by increasing the number of primer sets analysed. 40-80% of random primers can be expected to give polymorphic markers.

31. DNA profiling techniques are most applicable to highly heterozygous, non-inbred, asexually propagated plants where the number of primer sets required to demonstrate uniquness is minimal. Varieties of this group of species are those for which applications for breeder's rights are common.

Applying RAPD analysis to DUS

32. **Distinctness:** The RAPD profile or 'fingerprint' of a variety is analogous to the human fingerprint as a unique distinguishing characteristic of an individual (variety). Increasing the number of primer sets increases the resolution of RAPD analysis. The results of RAPD analysis are quantifiable, subject to statistical analysis and 'minimum distances' can be based on percentages from similarity matrices (Attachment 2).

33. RAPD analysis can be standardised, automated and profiles can be stored and evaluated electronically. Further, DNA templates are not influenced by climate, environment, latitude or developmental stage of the plant thus it is only be necessary to compare a RAPD profile of the variety under test with stored profiles of the closest varieties to establish distinctness.

34. RAPD profiles of varieties of common knowledge can be stored in central databases, periodically updated, transmitted by CD-ROM and profiles of candidate varieties under test compared with varieties of common knowledge in an international profile database.

35. If necessary a 'reference set' of extracted DNA can be stored indefinitely and readily transported between Member

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States.

36. **Uniformity and Stability** of a variety can be readily established by RAPD profiling a number of individual plants and generations. Tolerances for variation ('off-types') of profiles dependent on modes of propagation and pollination will need to be established.

III. Complementary role of RAPD analysis with morphological and physical criteria in varietal indentification

37. While RAPD analysis can provide an objective means of establishing varietal distinctness, the RAPD profile provides no descriptive information. Description based on visual (morphological and physical) characters is of both practical significance in marketing and to minimise unintentional infringement.

38. It is therefore not envisaged that granting of rights be based only on comparative RAPD profiles, but that descriptive characters (and possibly performance data) from UPOV test guidelines complement RAPD profiles for filing purposes.

39. It may also be appropriate for RAPD profiles to complement performance data of cultivars for cultivar registration purposes.

IV. Validity of the use of DNA characteristics as distinctness criteria under the 1991 Act of the Union

40. **Article 1(vi), Article 7** and **Article 14(5)(b)** of the 1991 UPOV Convention are relevant to a consideration of the validity of the use of molecular testing procedures by Member States' breeder's rights offices.

41. **Article 1 (vi)** contains the definition of "variety" which is required to be:

- "defined by the expression of the characteristics resulting from a given genotype or combination of genotypes"
- "distinguished from any other plant grouping by the expression of at least one of the said characteristics..."

42. Some doubt has been expressed (TC/27/9) about the validity of using DNA profiles as varietal determinants in terms of the definition of 'variety' in Article 1(iv) of the 1991 Act of the Union.

43. RAPD analysis provides a unique combination of

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identifying characteristics of a variety, but a RAPD profile bears no relationship to the appearance (description) or performance of that variety. A RAPD profile is an *expression* of the physical structure of polymorphic regions of the DNA molecule and is not a pictoral representation of the genotype.

44. A RAPD profile is a combination of characteristics derived from the replication products of primed regions of polymorphic DNA. Distinctness can therefore be established by the comparison of the RAPD profiles of two or more varieties.

45. The expression of selective or primed DNA regions which DNA profiling produces is a direct analogy of the morphological characteristics a plant expresses.

46. **Article 7** contains the requirements for a variety to be distinct. The requirement that the variety be "clearly distinguishable" can be satisfied by molecular methods such as RFLP or RAPD analysis which provide unique expressions of varietal genotypes.

47. Where molecular techniques do not completely satisfy the distinctiveness requirement, a national plant breeder's rights office can supplement molecular criteria with comparative morphological tests based on UPOV-approved DUS test guidelines.

48. If UPOV Member States agree that a standardised form of RAPD analysis and evaluation could be used for the verification of distinctness, national offices could use their discretion in implementing this new technique according to their individual national priorities.

49. **Article 14(5)(b)** defines an essentially derived variety as one that retains the essential characteristics, which are an expression of the genotype, of the initial variety. The ability of DNA techniques to identify with great particularity the similarity of varieties based on the DNA molecule makes them suitable for ascertaining if the requirement for "essential derivation" is satisfied.

50. In the event of a dispute concerning essential derivation, the task of the courts would be simplified as the use of DNA techniques obviates the necessity for evidence concerning the origin and breeding history of the varieties concerned.

51. In conclusion, the 1991 Convention of the International Union for the Protection of New Varieties of Plants does not constitute any impediment to the acceptance by competent authorities of Member States of the use of the RAPD

profiling technique for establishing distinctness of a variety.

V. Recommendations

52. That the Technical Committee:

(a) accepts RAPD profiling as a method for establishing the distinctness of varieties;

(b) recommends to Council that a subgroup be formed to coordinate the development and adoption by member states of the Union of RAPD analysis for varietal characterisation;

(c) coordinates RAPD profile characterisation with the cultivar registration and varietal protection agencies of Member States.

This document has three attachments

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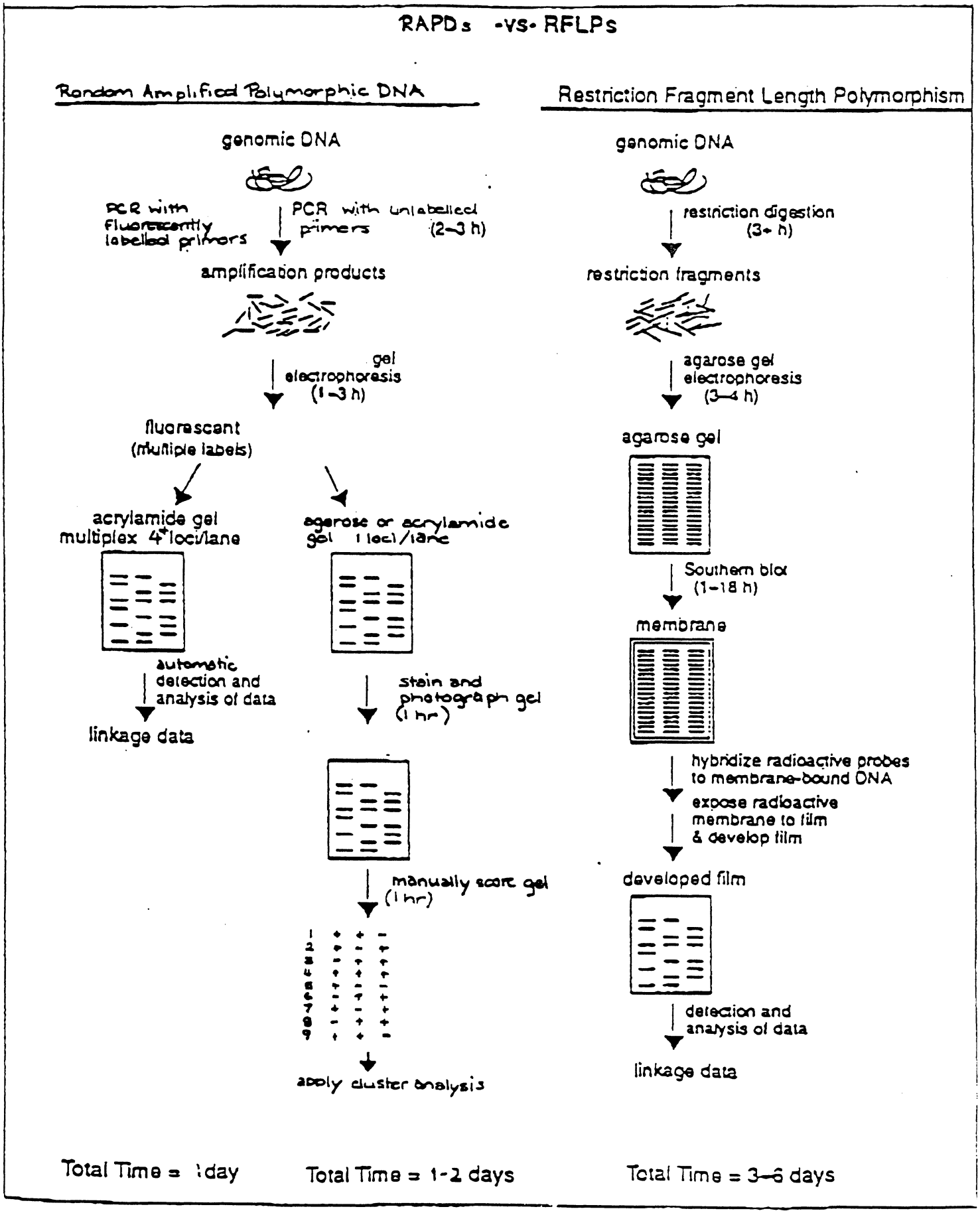
Attachment 1 Flow chart of DNA profiling techniques

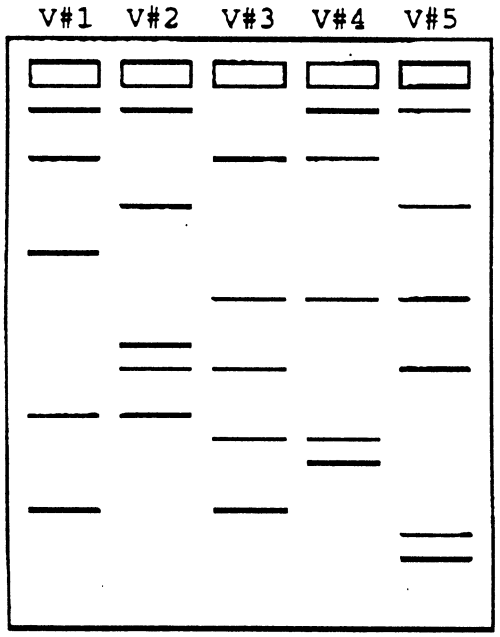


Attachment 2. RAPD profile examples

The most appropriate statistical cluster analysis is cladistic analysis. This seeks only to determine similarity or relatedness and not to define ancestry. There are 3 stages in the analysis: scoring the raw data, forming a similarity matrix, and preparing a cladogram

The raw data is the presence or absence of DNA bands (loci) on an electrophoretic gel. The presence of a band is scored as (+) and the absence as (-). The results of all of the primer sets used are combined and then made into a similarity matrix where relatedness is expressed as a percentage. This relatedness can then be expressed in graphical form.

Gel profile of RAPD fragments for primer #1



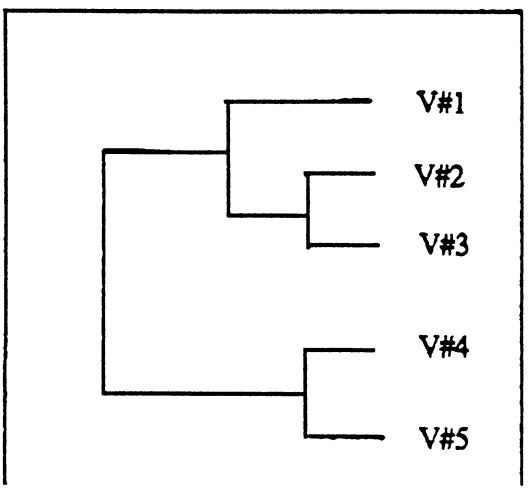
Scoring of Raw Data from Primer #1

Primer #1	V.#1	V.#2	V.#3	V.#4	V.5
Position#1	+	+	-	+	+
Position#2	+	-	+	+	-
Position#3	-	+	-	-	+
Position#4	+	-	-	-	-
Position#5	-	-	+	+	+
Position#6	-	+	-	-	-
Position#7	-	+	+	-	+
Position#8	+	+	-	-	-
Position#9	-	-	+	+	-
Position#10	-	-	-	+	-
Position#11	+	-	+	-	-
Position#12	-	-	-	-	+
Position#13	-	-	-	-	+

Similarity Matrix of Combined Primer Raw Data

	Variety#1	Variety#2	Variety#3	Variety#4	Variety#5
Variety#1	100%				
Variety#2	82%	100%			
Variety#3	46%	55%	100%		
Variety#4	12%	26%	83%	100%	
Variety#5	75%	12%	30%	27%	100%

Cladogram of Similarity Matrix



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Attachment 3 Glossary of Terms

Alleles	Different forms of a gene
Autoradiography	The visualisation of radioactivity by exposure to an X-ray film
Cloning	Patching a length of DNA into a bacterial plasmid with compatible restriction enzyme sites
Coding	Those areas of the genome which are transcribed into RNA (leading to a protein product)
DNA	Deoxyribonucleic Acid. The carrier of the genetic information in cells composed of 2 complementary chains of nucleotides wound in a double helix; capable of self replication as well as coding for RNA synthesis.
Electrophoresis	The separation by charge of nucleic acid or protein within a gel structure.
Genome	The complete set of chromosomes(DNA), with their associated genes
Hybridisation	Binding of fragments of nucleic acids to compatible regions of the genome
Loci	Defined genomic DNA positions.
Markers	Short fragments of DNA which bind to the genome at specific locations determined by their sequence
mini-satellite DNA	Small repeated units of DNA in the non-coding portions of genomes.
Non-coding	Portions of the genome which do not encode for RNA or protein products.
Nucleotides	The basic unit of nucleic acids. There are 5 types: Guanine Adenosine, Cytosine, Thymine, and Uracil. Thymine is found only in DNA, and is substituted by Uracil in RNA
Oligonucleotide	Lengths of nucleic acids
PCR	Polymerase Chain Reaction. Oligonucleotide primers are incubated with genomic DNA and allowed to hybridise. They bind to many different loci and then replicate from those points of hybridisation producing varying lengths of DNA depending on how close together 2 of these primers were on opposing DNA strands. The fragments produced are then released by increasing the temperature, and the hybridisation step repeated. This process continues through a number of cycles so that the fragments created are in sufficient quantities to be visualised on an agarose or polyacrylamide gel.
Polymorphism	The presence in a population of 2 or more phenotypically distinct forms of a trait.
Primers	Short fragments of nucleic acids which bind to the genome at specific locations determined by their sequence and act as starting points for nucleic acid replication.
Probes	Fragments of nucleic acids incorporating radioactively, enzymatically or flourescently labelled nucleotides which bind to the genome at specific locations determined by their sequence allowing the visualisation of these points of hybridisation.
RAPD	Random Amplified Polymorphic DNA
Restriction Enzymes	Enzymes that cleave the DNA double helix at specific nucleotide sequences
RFLP	Restriction Fragment Length Polymorphism
Sequence	The pattern of nucleic acids in the DNA molecules
Southern Hybridisation	The process of hybridising DNA probes with DNA bound to a membrane support
Thermal Cycling Reaction	see PCR

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