Disclaimer: unless otherwise agreed by the Council of UPOV, only documents that have been adopted by the Council of UPOV and that have not been superseded can represent UPOV policies or guidance.

This document has been scanned from a paper copy and may have some discrepancies from the original document.

———

Avertissement: sauf si le Conseil de l'UPOV en décide autrement, seuls les documents adoptés par le Conseil de l'UPOV n'ayant pas été remplacés peuvent représenter les principes ou les orientations de l'UPOV.

Ce document a été numérisé à partir d'une copie papier et peut contenir des différences avec le document original.

———

Allgemeiner Haftungsausschluß: Sofern nicht anders vom Rat der UPOV vereinbart, geben nur Dokumente, die vom Rat der UPOV angenommen und nicht ersetzt wurden, Grundsätze oder eine Anleitung der UPOV wieder.

Dieses Dokument wurde von einer Papierkopie gescannt und könnte Abweichungen vom Originaldokument aufweisen.

———

Descargo de responsabilidad: salvo que el Consejo de la UPOV decida de otro modo, solo se considerarán documentos de políticas u orientaciones de la UPOV los que hayan sido aprobados por el Consejo de la UPOV y no hayan sido reemplazados.

Este documento ha sido escaneado a partir de una copia en papel y puede que existan divergencias en relación con el documento original.

BMT/4/20 446
ORIGINAL: English
DATE: February 27, 1997

UPOV

INTERNATIONAL UNION FOR THE PROTECTION OF NEW VARIETIES OF PLANTS

GENEVA

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

Fourth Session
Cambridge, United Kingdom, March 11 to 13, 1997

DEVELOPMENT OF DNA MICROSATELLITES FOR DISTINCTNESS, UNIFORMITY AND STABILITY TESTING

Document prepared by experts from the United Kingdom

DEVELOPMENT OF DNA MICROSATELLITES FOR DISTINCTNESS, UNIFORMITY AND STABILITY TESTING

David Lee, Michelle Leverington, Wendy Cooper and Robert J. Cooke
NIAB, Cambridge, UK

Introduction

We have previously reported to the UPOV BMT (BMT/2/8 and BMT/3/4) and elsewhere (Lee, Reeves and Cooke, 1996a, b, c) the results of a research project in which two DNA profiling methods - RFLPs and RAPDs- were developed and evaluated for variety discrimination and DUS testing in oilseed rape and barley. The potential advantages of molecular markers as an unlimited source of characters have been described in previous BMT documents (e.g. BMT/3/2). Not only do DNA polymorphisms directly reflect genetic differences between varieties, but testing could be done more quickly, independently of growing seasons and not be affected by environmental factors. However, both RFLP and RAPD analysis have their drawbacks. RFLP analysis requires many steps: DNA extraction, DNA restriction, separation of the fragments on agarose gels, transfer of the DNA onto membranes, hybridisation of a radioactively labelled probe and finally autoradiography to detect the bound probe. Analysis of RAPDs has the advantages of requiring smaller amounts of DNA for each reaction and no isotopic labelling, but doubts regarding reproducibility have been reported and could compromise the routine use of this approach in variety testing. Consequently, there is now increasing interest in the utilisation of methods which retain the discriminating power of RFLP and RAPD but which are less difficult technically, more robust, more amenable to automation, transportable between laboratories, employ publicly available materials and do not involve the use of radioactivity. Of these, the PCR-based analysis of simple sequence repeats (microsatellites) may prove to be particularly useful for variety identification and testing. This procedure is known as the sequence-tagged site (STS) approach.

Microsatellites are tandemly repeated DNA sequences with a basic repeat unit (or 'core' sequence) of 2-8 base pairs (e.g. GA, CTT and GATA). The polymorphism found in microsatellites is due to variations in the copy number of the basic repeat unit. In many crop species, multiple alleles have been shown to exist for many microsatellites, arising from these differences in copy number. These alleles can be separated by agarose or polyacrylamide gel electrophoresis.

The initial isolation and identification of useful microsatellite markers are labour intensive activities. However, once developed, genetic profiling should be quicker to perform than with RFLPs and the use of longer and more specific primers means that the technique does not suffer from the problems of reproducibility found with RAPDs. This paper describes progress in a research project which is developing microsatellite markers for varietal differentiation in oilseed rape. We are also employing a similar approach in projects investigating sequence-tagged site microsatellites in chrysanthemums and sunflowers.

Materials and Methods

Plant material.

Seed of *Brassica napus* L. varieties was obtained from the reference collection held at NIAB. All the varieties used were selected from the UK National List of Varieties (1993). Seedlings were grown in a greenhouse until the fourth true leaf stage.

Isolation of microsatellites.

DNA was obtained from leaves using chloroform/phenol extractions as described previously (Lee *et al.*, 1996c).

Microsatellites were cloned from oilseed rape using a modification of the method of Karagyozov *et al.*(1993). Adapters were ligated to *Taq*1-restricted genomic DNA (extracted from the variety Eurol), allowing fragment amplification. Microsatellite containing fragments were enriched by the hybridisation of PCR-amplified restriction fragments to simple sequence repeats (SSRs), immobilised on nylon membranes. Bound fragments were eluted from the membranes by boiling in TE buffer. The enriched DNA fractions were amplified using primers corresponding to the adapter. The hybridisation procedure was repeated before the enriched fraction was amplified with a primer which also added a *Xho*1 restriction site at the 5' end, facilitating cloning of the fragments.

The reaction products were cleaned using a QIAquick column (Quiagen) before digestion with *Xho*1. The digested products were ligated to *Sal*1-digested pUC19 and used to transform *E. coli* (DH5α) cells. White colonies were picked and the inserts amplified using M13 forward and reverse primers. The products of these reactions were fractionated on 1% agarose gels and the DNA transferred onto membranes. Potential clones were selected on the basis of hybridisation of the inserts to biotinylated oligonucleotides, detected using streptavidin-horseradish peroxidase conjugate and visualised using luminol and hydrogen peroxide.

DNA was prepared from the clones which gave a positive signal. The fragments were sequenced from both ends with the M13 forward and reverse Universal primers using the ABI Prism™ Dye Terminator Cycle Sequencing Ready Reaction Kit, containing Amplitaq ® DNA polymerase (Perkin-Elmer).

Microsatellites from other sources.

Information on the sequences of primer pairs for the amplification of other STS-microsatellites in oilseed rape was obtained from published data (Lagercrantz *et al.*, 1993, Kresovich *et al.*, 1995, Szewc-McFadden *et al.*, 1996). The Genebank databank was also searched for other potential repeat sequences in *Brassicas* that could provide STS-microsatellites.

449

Primer design.

Primer pairs for the amplification of microsatellites from the various sources were designed using the 'PrimerSelect' software where necessary, and then purchased from commercial sources.

Amplification and detection of microsatellites.

Microsatellites were amplified by PCR of extracted DNA using the appropriate specific primers. Various methods for the separation of the amplified products have been assessed, including agarose gel electrophoresis followed by ethidium bromide staining and polyacrylamide gel electrophoresis coupled with silver staining. The procedure most commonly used to date was PCR incorporating ^{35}S-dATP as described by Thomas and Scott (1993) or using ^{33}P end-labelled primers (Donini *et al.*, 1997), followed by separation on denaturing polyacrylamide sequencing gels. Microsatellite bands were then visualised using autoradiography.

Results.

Development of microsatellites.

Sequence data from the 'positive' clones selected as described above have shown that hybridisation to membrane-bound microsatellite oligonucleotides does enrich DNA libraries for microsatellite sequences. Different wash conditions were found to help in the determination of potential clones (see Figure 1). However, many clones which appeared to give a strong hybridisation signal were found to contain little in the way of repeat motifs following sequencing. Out of a total of 46 clones sequenced to date, four contained repeats greater than 40 nucleotides in total, three contained trinucleotide motifs between 5-7 repeats long and four contained stretches of sequence that were rich in two bases e.g. CT/GA or CA/GT. Examples of the sequences of some of the enriched clones are given in Figure 2.

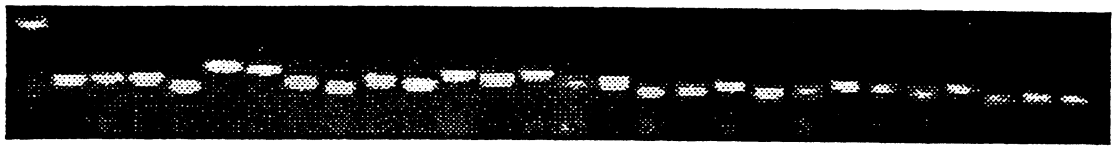
Other sources.

A search of the currently available databases for mono-, di- and trinucleotide repeats in *Brassica* species produced a few potentially useful microsatellites. Likewise, the published scientific literature contained sequence data that were reported to be useful for the amplification of microsatellites in oilseed rape and other *Brassicas*.

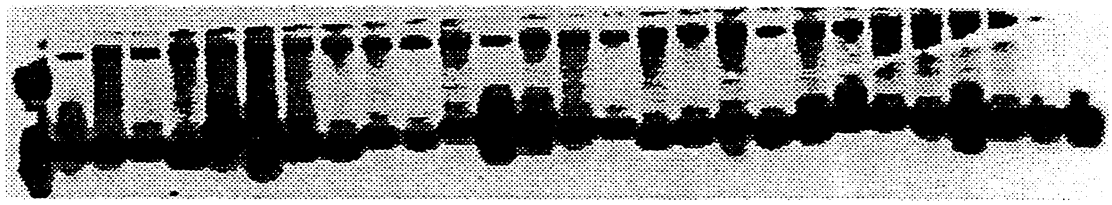
The microsatellites so far identified within this project are summarised in Table 1.

450

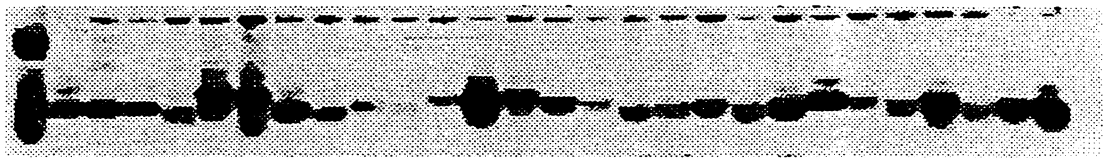
Figure 1. Screening of clones by hybridisation and effect of different wash conditions.



Inserts of white colonies amplified using M13 forward and reverse primers



Hybridisation of inserts to mixed microsatellite primers/probes: washed in 2 X SSC @ 50°C.



Hybridisation of inserts to mixed microsatellite primers/probes: washed in 0.1 X SSC @ 50°C.

Evaluation of microsatellites.

The usefulness of the microsatellites in Table 1 is being evaluated in terms of their polymorphism in a collection of oilseed rape varieties. For the initial screening, a set of 10 diverse varieties was chosen. Those primer pairs which demonstrated useful polymorphism are being taken for further analysis using 50 varieties. To date, over half of the initial screening has been carried out and eight of the primers have been screened against the total variety set.

451

Figure 2. Examples of sequences of some enriched clones: lower case letters denote regions of 'unusual' (repeat) sequence structure.

Clone GA35

TCAACGATGTCCTTATCGCCTAAACCAGCTAACTCGGTGATAACCGAGTTA
ACCCAGTGTGGATGACCCCGTAAAGGCTGAGTAGAGATCAACTTTTCATCA
TTATCACCTGGAACATGTCAAAGAAAAGGGTAATGATGCTGGAGTGAAAT
GATCAAACCAGAAACCCATGTTGTGTTCATCTCCTGTTTCACCTCTTTGCCT
CTTCACTCTGCTCAGACAAGACTCGTCCTCCTTGTCTTCAGCCAATCTCTTC
AAActcttcttctctgttgttgttgttgtcgttgttCCATGAAAACCCATCTGCATTTCCTCTTG
TAGTTGCCAAAGGTGGAAGCTTTATGCTCTGCTTAAGCGAACATCCACCAG
TTttgttgttgttgttgttgctgttgatgtt

Clone GTT91

GTCGAGTTACTCAGGACTCAACGACCACATGCCTCAACTTAGGCGGGCCTG
AACCATCCTAAATcaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaacaac
aacaacaacaacaacaacaacaacaTCCGCACCAACGACATAATTTTCACGCCGAGAAT
GAWGATGACCTCCATCGGCAAGTGTWTTTTGAGGATCTCCAGCAACGACA
CAACTATGATAAAAAGCTTTCGGGTTGATATCCCAGAATTTCACGGAGGTC
TCCGCGGCGACGAACTAATCGTCGAGTCCTGAGTAACTCG

Clone 33

GTCGAGTTACTCAGGACTCAACGGAGGCCGCAGCACAACTGTCGTCATAT
GCTACTTGGTTTGTGTTGCAATTATTCCCATTAAAAACTTTGCTTCAtctctctct
ctctctctctctctctctctctctctctctctccctctctctctctcTGATTATATTTTCGCAATGCAGG
TGCAACACAAACACATGACACCTGAAGCAGCATATGATTATGTAAAGTCA
ATCAGGCCCAGGGTTAAACTAGCTACCACCCAATGGAAGGTAATCAACAG
GGGCTCTTTACTCTGTTCTTTCTTTGTTCTTCTTCTTATTCATCCTCTGATAC
CTAATTGTGTTTGCAGGCCGTTCTTGAGTACTACCATGTCAAGGTGCTGAA
TACTCAGAGTTCCTTGCCTGATGCAACTTCAGCTTTGATCCCAAGAAATAT
GAAGCAGGTTTGCTCCGGGAATGTGGTTGTGTTTGATGATGGGTCGTTGAG
TCCTGAGTAACTCGAC

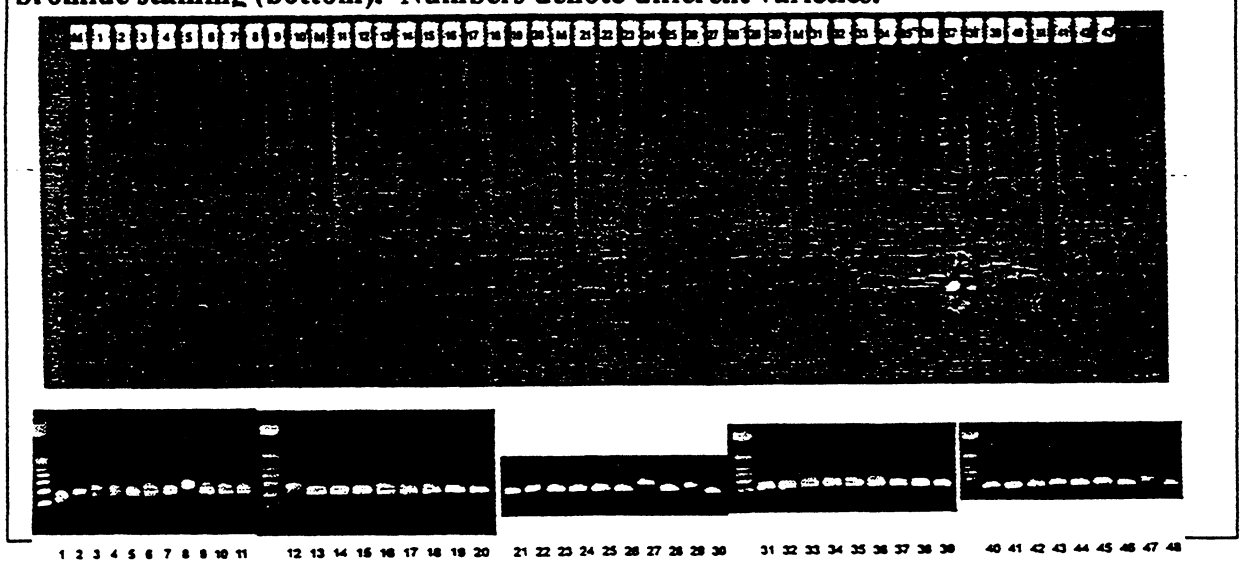
Separation of microsatellites.

All of the electrophoresis methods used were able to demonstrate polymorphisms in oilseed rape varieties with suitable microsatellite primer pairs. The results of the non-radioactive approaches are demonstrated in Figure 3. Agarose gels probably do not achieve sufficient resolution for most purposes. Non-denaturing acrylamide gels with silver staining have some advantages for this work, although the gels used to date (from a commercial supplier) gave insufficient resolution of the fragments of interest. We intend to continue to explore this system further, using other types of gel. However, denaturing acrylamide sequencing gels will resolve single base-pair differences and hence we have so far used these for primer pair evaluation.

Table 1. A list of microsatellites so far identified from oilseed rape.

Primer Name	Microsatellite Repeat	Predicted Fragment Size (Bp)
BN5a2	$(GATT)_4$	96 bp
BN9a	$(GA)_{27}$	184 bp
BN12a	$(GA)_{14}(AAG)_4$	286 bp
BN16a2	$(CT)_{28}$	267 bp
BN20a	$(GA)_{25}$	215 bp
BN25C2	$(GA)_{10}$	142 bp
BN26A	$(GA)_{13}$	100 bp
BN27b2	$(GA)_{12}$	199 bp
BN59a1	$(CA)_{11}$	430 bp
BN68/1	$(GA)_{24}(ACACA)_2$	276 bp
BN72a	$(TAA)_3(GA)_9$	247 bp
BN83b1	$(GA)_{11}$	194 bp
BN92A1	$(A)_{28}$	113 bp
MB1	$(GA)_9$	n.d
MB2	$(GA)_2(CC)(GA)_{14}(A)(GT)_2$	n.d
MB3	$(TG)_9$	n.d
MB4	$(TG)_{10}$	n.d
MB5	$(AT)_2(GT)(AT)_4(GT)_8$	n.d
33	(GA)7(CG)(GA)16	121 bp
gt91	$(CAA)_{27}$	123 bp
gt91A	$(CAA)_{27}$	132 bp
ga35	$CTT)_2(CT)_8(GTT)_4(GTC)(GTT$	304 bp
ga84	$(TCT)_{14}(ACT)_3(TCC)_{14}(TCT)_8$	251 bp
95	purine rich	365 bp
91	pyrimidine rich	357 bp
bnaccg8	$(A)_{21}$	209 bp
bnlpdna	$(TAA)_9$	165 bp
bnaccpro5	$(T)_{20}$	189 bp
bnatkl	$(TA)_{11}$	205 bp
bnsla2g	$(GA)_9$	134 bp

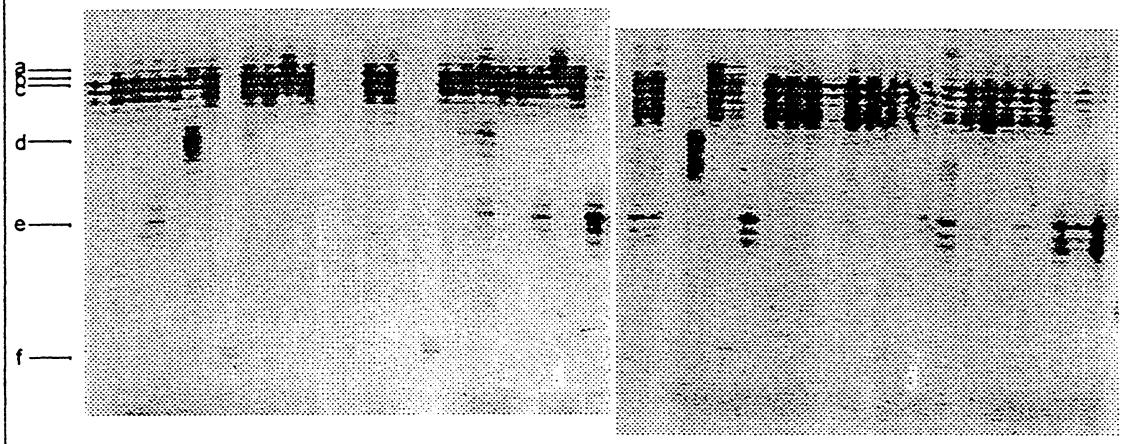
Figure 3. Microsatellites amplified using primer pair BN9a and separated on an acrylamide gel visualised by silver staining (top) or an agarose gel with ethidium bromide staining (bottom). Numbers denote different varieties.



458

In initial experiments we employed the incorporation of α^{35}S-dATP into the DNA fragments as the method of labelling (Figure 4). However, problems with contamination of the PCR thermal cycler were encountered, probably due to break-down of the α^{35}S-dATP during cycling and the production of $^{35}SO_2$. Hence we have now changed to using ^{33}P end-labelled primers in the PCR reactions to avoid this hazard. The amplified and labelled fragments produced by the two labelling methods can look somewhat different on gels, but comparison of them is possible. The intensities of the bands produced by direct incorporation reflect a number of different factors, e.g. the copy number of the target sequence and the number of A residues in the DNA fragments. End-labelled primers reflect only the copy number and the efficiency of the amplification of the target sequence.

Figure 4. Microsatellite alleles amplified by primer pair 33 in oilseed rape varieties. Fragments were labelled by direct incorporation of ^{35}S-dATP. The letters denote different sized products.

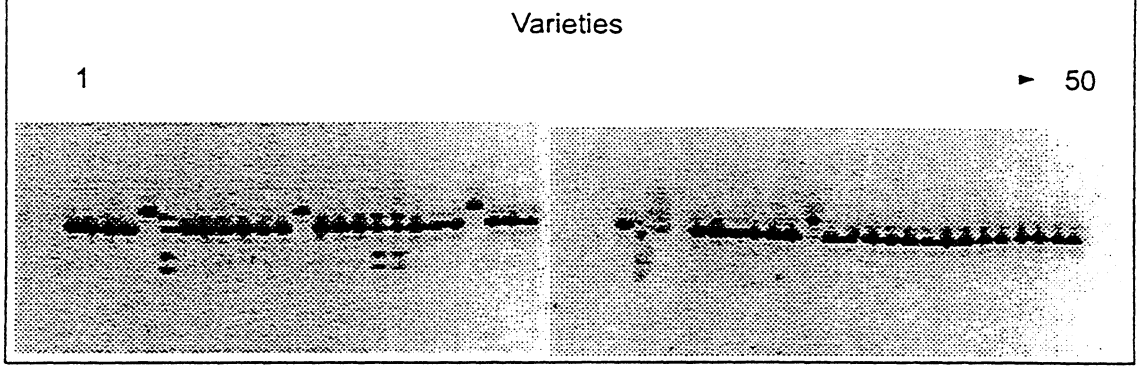


Variety discrimination.

The type of result produced by the amplification of sequence-tagged microsatellites using specific primer pairs and end-labelling with ^{33}P is shown in Figure 5.

Data such as these are relatively easy to 'score', in that each variety can be analysed and the pattern present (allele) recorded, either by numbering or by size. We are currently continuing screening the remaining primer pairs and subsequently analysing the complete variety collection. This will eventually enable us to catalogue the alleles present in the different varieties and thus begin to evaluate the usefulness of microsatellites for distinctness testing in oilseed rape.

Figure 5. An example of microsatellite analysis in oilseed rape. At least three different patterns (alleles) are detected by the primer pair MB5.



Discussion.

About 30 potentially useful primer pairs that should amplify different microsatellite sequences in oilseed rape varieties have so far been identified from the literature or developed within this project. Our work to date has indicated some of the problems likely to be encountered in the development and evaluation of STS-microsatellites, such as the choice and optimisation of separation, labelling and detection methods. Future work will concentrate on the development of further microsatellites from our own DNA libraries and more extensive evaluation of primer pairs. The objective is to obtain a 'Variety Test Set' of 10-20 primers that will allow high levels of discrimination between of the variety collection. When selecting primer pairs, attention will be given to the possibilities of multiplexing of microsatellites, i.e. the detection of a number of markers (loci) in a single PCR reaction. In addition to improving efficiency, such multiplexing is very suitable for automation, which both enhances the data gathering process and improves cost-effectiveness.

Alternative methods of microsatellite separation and detection will also continue to be pursued. For effective use of microsatellites in variety testing, a high throughput, non-radioactive system is preferable. Thus we shall pursue the use of silver stained acrylamide gels for more 'routine' applications. In addition to gel electrophoresis, other more automated and rapid methods, for example based on capillary electrophoresis and/or the fluorescent detection of microsatellites, are becoming available, especially in relation to human forensic work, and such approaches could revolutionise variety identification. The possibilities offered by such an approach for plant variety testing have already been highlighted (Kresovich *et al.*, 1995, Thomas, Cain and Scott, 1994). Of course, before being widely adopted for DUS testing, issues such as the uniformity of varieties for microsatellite profile also need to be addressed. However, the potential of sequence-tagged site DNA microsatellite analysis for cost-effective, automated variety testing cannot be ignored.

455

Acknowledgements.

This work is supported by a grant from the Ministry of Agriculture, Fisheries and Food. Dr Graham King (HRI, Wellesbourne) is thanked for the provision of some primer sequences.

References.

Donini *et al* (1997). Proc Plant & Animal Genome V, San Diego.
Karagyozov *et al.* (1993). Nucl. Acids Res. 21: 3911-3912.
Kresovich, S *et al.*, (1995). Theor. Appl. Genet. 91: 206-211.
Lagercrantz, U, Ellegren, H and Andersson, L. (1993). Nucl. Acids Res. 21: 1111-1115.
Lee, D, Reeves, JC and Cooke, RJ (1996a). Electrophoresis 17, 261-265.
Lee, D, Reeves, JC and Cooke, RJ (1996b). *Diagnostics in Crop Production*, Proc. BCPC Symposium 65, 235-240
Lee, D, Reeves, JC and Cooke, RJ (1996c). Plant Varieties and Seeds 9: 181-190.
Szewc-McFadden, AK *et al.*, 1996. Theor. Appl. Genet. 93: 534-538.
Thomas, MR, Cain, P and Scott, NS (1994).Plant Mol. Biol. 25, 939-949.

[End of document]