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UPOV

BMT/5/2
ORIGINAL: English
DATE: August 12, 1998

INTERNATIONAL UNION FOR THE PROTECTION OF NEW VARIETIES OF PLANTS
GENEVA

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

Fifth Session
Beltsville, United States of America, September 28 to 30, 1998

THE USE OF MOLECULAR MARKERS FOR VARIETY AND SEED TESTING: A SUMMARY OF RESEARCH AT NIAB

Document prepared by experts from the United Kingdom

THE USE OF MOLECULAR MARKERS FOR VARIETY AND SEED TESTING : A SUMMARY OF RESEARCH AT NIAB.

Robert J. Cooke

1. Introduction.

In previous reports to the BMT, we have described research at NIAB into the uses of (a) RAPDs and RFLPs (BMT/2/8, BMT/3/4), and (b) sequence tagged site microsatellites (STMS) (BMT/4/20), for variety discrimination and identification in oilseed rape. We have also considered some aspects of the uniformity of certain DNA profiles and ways in which the questions of uniformity and stability might be addressed (BMT/3/4).

This research forms part of a larger programme at NIAB and the current paper reviews parts of this programme that are relevant to variety and seed testing.

2. Variety and Seed Testing Research at NIAB.

There are three principal and interacting approaches to the use of DNA profiling in variety and seeds work that form the core of the research at NIAB - (i) variety identification and/or discrimination; (ii) assessment of distances and diversity; (iii) testing of genetic purity.

2.1 Variety Identification and Discrimination.

(i) As demonstrated in BMT/2/8 and BMT/3/4, molecular markers provide a powerful way of discriminating between varieties. For instance, RFLP analysis using one restriction digest plus two multi-copy probes gave a separation rate of 99.2% between 62 varieties of *Brassica napus* (Lee *et al.*, 1996a). RAPDs can be equally discriminating, with individual primers providing separation rates well above 95% for the same collection of varieties, and usually of 100% when used in combination (Lee *et al.*, 1996b). However, it has become increasingly recognised that for various reasons these two methods are probably not suitable for DUS testing (e.g. Cooke and Reeves, 1998), and consequently attention has turned to the so-called 'second generation' profiling methods. Two of these in particular - the analysis of DNA microsatellites as sequence tagged sites (STMS) and amplified fragment length polymorphisms (AFLPs) - appear to be potentially especially useful.

(ii) Both of these approaches are being investigated at NIAB. Preliminary work with STMS in oilseed rape was presented previously (BMT/4/20), where it was reported that separation rates of 97% could be readily achieved for the same set of 62 varieties as above, by combining data from three STMS primer pairs. The development and use of the STMS approach in oilseed rape has continued, with more primer pairs being produced and evaluated. These have come from databases, from published sources and from enrichment, cloning and sequencing experiments (as outlined in BMT/4/20). There have been some difficulties with these latter procedures in oilseed rape, as in many of the clones, the microsatellite has been located at the end of the insert and hence flanking primers could not be designed. Even when primers have been produced and amplification of the microsatellite successfully achieved, more than 60% of the microsatellites have proved to be monomorphic when tested against a collection of

about 90 varieties. Nevertheless, a number of polymorphic STMS primer pairs have been produced in oilseed rape and these are now being evaluated for their discrimination rates, both alone and in combination. The same primer pairs are being used to examine the uniformity of different varieties, and their transportability to other Brassica species is also being tested.

(iii) Microsatellites are also being developed and tested for application in chrysanthemum varieties, using a somewhat different approach. A technique known as inter-SSR PCR (or anchored PCR, first described by Zietkiewicz *et al.*, 1994) has been used, with a range of different primers of varying lengths of repeat and anchor, to generate profiles of varieties (e.g. Figure 1). This is a useful approach, requiring no prior sequence knowledge and, with the appropriate primer(s), allowing high levels of discrimination between even closely related varieties. In addition, polymorphic fragments can be used as the source of single locus primers, a number of which are currently being cloned, sequenced and tested.

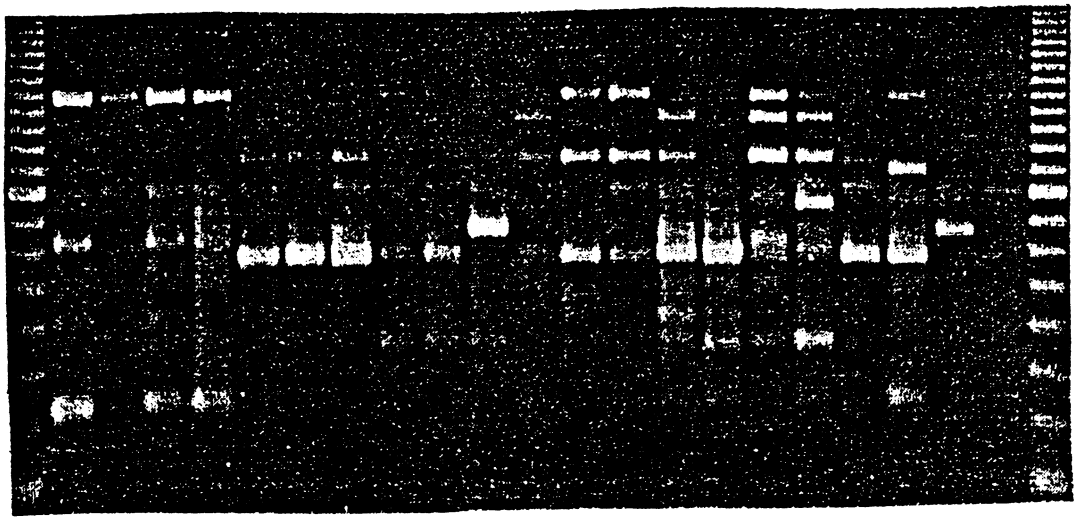

Figure 1. The analysis of chrysanthemum varieties using the inter-SSR PCR approach with a 7bp 5'- anchored primer containing a (CT) repeat. The PCR products have been separated on a 3% agarose gel and stained with ethidium bromide.

Inter-SSR PCR is also being used in a separate project to generate a database of DNA profiles of potato varieties.

(iv) In a project funded by the EU and the subject of a separate presentation (Vosman *et al.*, this meeting), existing microsatellite primer pairs are being used to demonstrate the application of molecular markers to varietal identification and discrimination in wheat and tomatoes. An important part of this project is the production of a manual for the standardised analysis of the microsatellites and identification of alleles. This will include the analysis of a number of microsatellite loci in the same PCR, a process known as multiplexing (Figure 2). Another important objective is the production of a database of DNA profiles of at least 500 varieties of both crops. The precise format for this has yet to be determined, but could be as in Table 1.

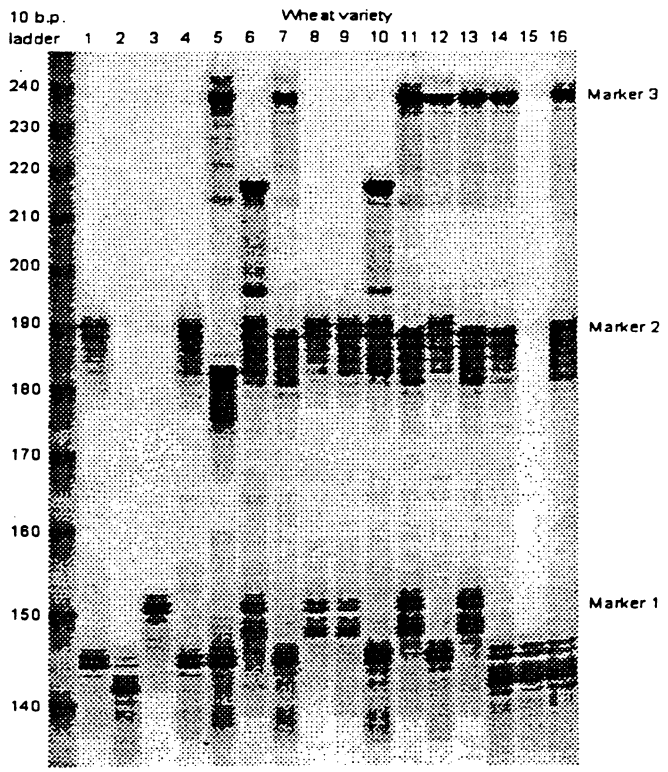

Figure 2. An example of multiplexing of microsatellites in wheat. For each variety, three different STMS markers have been amplified in the same PCR, using radioactively labelled primers followed by analysis on a polyacrylamide sequencing gel and autoradiography.

Table 1. Possible format for database of wheat variety DNA profiles.

		MICROSATELLITE LOCUS									
	Allele	1 null	1 a	1 b	2 null	2 a	2 b	2 c	3 a	3 b	3 c
	Band	-	213	238	-	182	188	190	142	145	151
VARIETY	**size (bp)**										
001		1	0	0	0	0	0	1	0	1	0
002		1	0	0	1	0	0	0	1	0	0
003		1	0	0	1	0	0	0	0	0	1
004		1	0	0	0	0	0	1	0	1	0
005		0	0	1	0	1	0	0	0	1	0
006		0	1	0	0	0	0	1	0	0	1
007		0	0	1	0	0	1	0	0	1	0
008		1	0	0	0	0	0	1	0	0	1
009		1	0	0	0	0	0	1	0	0	1
010		0	1	0	0	0	0	1	0	1	0

2.2 Distances and Diversity.

Wheat, barley and oilseed rape varieties are being utilised in projects assessing distances and genetic diversity between varieties. The overall objective of this research is to quantify and compare distances between plant varieties as determined by pedigree, morphology and

different molecular techniques, and to assess their potential in defining and measuring distances in the context of DUS testing. STMS, AFLP and RFLP data are being analysed as measures of genetic distance, along with pedigree information and data relating to morphological characteristics (phenotypic distance).

Results from this research will be discussed in more detail elsewhere (Law, Cooke and Smith, this meeting). It is hoped that the data will indicate: (a) the degree to which genetic distances reflect pedigree relationships; (b) the degree to which phenotypic distances reflect pedigree relationships; (c) the degree of correlation between genetic and phenotypic distances. It will then be possible to begin to investigate the nature of the relationship between the differences recorded from the current type of DUS examination and the differences between varieties which arise from their known degrees of genetic relatedness. Such information will be useful in elucidating the nature of the concept of minimum distance between varieties of different species in a meaningful way, and will provide data on the relationships between varieties, important for the rational discussion of essential derivation and how to measure it. Issues such as the number of markers needed to replicate the level of discrimination achievable by morphological characters (e.g. Law *et al.*, 1998) and the ways in which molecular markers might be used in DUS testing, other than as additional characters, are also being examined.

2.3 Testing of Genetic Purity.

NIAB is co-ordinating an EU-funded project entitled 'The assessment of genetic purity in hybrid varieties of crops'. This project aims to develop and evaluate DNA profiling techniques (STMS and AFLPs), and to assess their usefulness for determining the levels of hybrid purity in four crops of major interest to European agriculture (maize, oilseed rape, sunflowers and white cabbage). The six European partners involved (NIAB, UK; CPRO-DLO, The Netherlands; LUFA, Germany; INSVP, Spain; PGS, Belgium and UHOH, Germany) are comparing the efficiency of molecular markers with other testing methods such as protein electrophoresis where applicable and assessment of morphological characters in field trials, with the objective of enabling standard procedures to be developed.

Work at NIAB has so far focused on microsatellite analysis of sunflower hybrid varieties using published primer pairs (Huestis, G. published on the WWW, 1997), although we are also developing further primer pairs. In addition, analysis of these varieties will be carried out with AFLPs. AFLP profiles are being generated, but optimum primer combinations have not yet been selected.

Analysis of hybrid sunflower varieties and the appropriate parental lines is now underway. Preliminary data (Table 2) show variation between the varieties tested for the different microsatellite primer pairs, and also some evidence for variation within varieties. These primers are being used to assess genetic (hybrid) purity (Figure 3).

Screening of sunflower varieties is continuing, and will be expanded to include hybrid oilseed rape varieties, analysed using the primer pairs being developed and tested above. The other partners within the project are conducting similar research with the other crops. In all cases, the molecular markers will eventually be used to analyse 50 individual plants of 5 chosen hybrid varieties, along with 20 individuals from the parent lines, to determine the level of hybrid purity at the various loci. In addition to the molecular analyses,

Table 2 Analysis of sunflower varieties with STMS markers.

Microsatellite locus	Frequency of alleles between 25 hybrid varieties		Variation within two hybrid varieties*	
	Allele	Frequency	Variety A	Variety B
ORS 2	a	0.06	16	26
	b	0.94	25	2
	c	0	2	0
ORS 3	a	0.95	30	-
	b	0.05	40	-
	c	0	1	-
ORS 4	a	0.73	36	-
	b	0.23	0	-
	c	0.05	17	-
ORS 5	a	0.27	-	-
	b	0.73	-	-
ORS 7	a	0.16	0	0
	b	0.57	40	21
	c	0.05	0	1
	d	0.11	0	4
	e	0.11	0	0
ORS 8	a	0.89	24	-
	b	0.11	0	-

* number of individuals with a particular allele.

Figure 3. Analysis of STMS in sunflower varieties for hybrid purity testing. Amplification was performed in a 'LightCycler' rapid air thermal cycler, and the PCR products visualised on a 'Licor' automated DNA sequencer. F = female parent, M = male parent, S = self-pollination.

the same seed lots will be sown out for morphological analysis of hybrid purity in field trials conducted in Spain (sunflowers and maize) and the UK (oilseed rape).

3. Conclusions.

The research outlined in this paper forms a co-ordinated programme investigating the potential applications of molecular markers in variety and seed testing. The future directions for such work include:

- the use of low volume rapid PCR and automated, non-radioactive genotyping, including multiplexing where possible, for cost-effective data production;
- the development and use of standardised methodologies for analysis and data recording;
- the construction of variety databases.

In addition, there remain a number of areas where further research is required, including:

- the number of markers needed;
- whether the markers need to be mapped;
- whether the distribution of the markers is important;
- whether the markers need to relate to 'expressed' regions of the genome;
- the uniformity and stability of the markers and how to handle these;
- the assessment of distances in the DUS testing context.

This will then enable the central issue to be addressed, i.e.:

- how to use molecular markers for DUS testing and associated activities - should they be additional characters or part of a revised system, maintaining the current levels of discrimination and 'distance'? This will require, *inter alia*, a deeper understanding of the concept of distance and a comparison between the results obtained from the use of molecular markers with those from the current testing systems, over a number of years.

4. References.

Cooke, R J and Reeves, JC (1998) *In*: Kelly, AF and George, RAT (eds.) *Encyclopedia of Seed Production of World Crops,*). John Wiley & Sons, London pp 88-102.
Law, JR, Donini, P, Koebner, RMD, Reeves, JC and Cooke, RJ (1998) Euphytica (in press).
Lee, D, Reeves, JC and Cooke, RJ (1996a) Plant Varieties & Seeds 9, 181-190
Lee, D, Reeves, JC and Cooke, RJ (1996b) Electrophoresis 17, 261-65
Zietkiwicz, E, Rafalski, A and Labuda, D (1994) Genomics 20, 176-183.

Acknowledgements.

It is a pleasure to acknowledge the contributions of the staff of the Applied Technology Department, NIAB, to the work outlined in this paper, especially Emily Taylor, Susan Freeman, Sarah Rendell, Fiona Leigh, Jennifer Jackson, Gerard Corbett, David Lee, Derek Matthews and Vince Lea, and also John Law and James Reeves for many useful discussions. Much of this work is supported by the Ministry of Agriculture, Fisheries and Food and/or the European Commission.

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