E

BMT/6/4
ORIGINAL: English
DATE: February 7, 2000

UPOV

INTERNATIONAL UNION FOR THE PROTECTION OF NEW VARIETIES OF PLANTS

GENEVA

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

Sixth Session
Angers, France, March 1 to 3, 2000

UNIFORMITY AND STABILITY OF MICROSATELLITE MARKERS IN WHEAT AND OILSEED RAPE

prepared by experts from the United Kingdom

UNIFORMITY AND STABILITY OF MICROSATELLITE MARKERS IN WHEAT AND OILSEED RAPE.

R J Cooke, S D Freeman, C Lowe, J Jackson, D Lee, V J Lea, P Donini, J Batley* and K J Edwards*

NIAB, Cambridge CB3 0LE, UK ; *IACR-LARS, Long Ashton, Bristol BS41 9AF.

1. Introduction.

In previous reports to the BMT and elsewhere, we have described the use of different molecular markers, including RFLPs, RAPDs, AFLPs and sequence-tagged site microsatellites (STMS, also known as simple sequence repeats, SSRs) for variety discrimination and identification in oilseed rape, barley, wheat and other crops (BMT/2/8, BMT/3/4, BMT/4/20, BMT/5/2, Lee *et al.*, 1996a, b, c, Law *et al.*, 1998). This work, as well as much other research (see, for example, references in Cooke and Reeves, 1998), has clearly demonstrated that molecular markers can readily discriminate between varieties in a wide range of species and offer many considerable potential benefits for DUS testing.

However, it has to be recognised that there remain reservations in some quarters about the use of molecular markers for variety registration and the granting of PBR. These centre around a number of issues, including the alleged potential erosion of minimum distance. Another of the principal difficulties with the adoption of molecular markers for DUS testing has been that whilst Distinctness is readily demonstrated in many crops, the questions of Uniformity and Stability have been largely overlooked. There are limited indications that in both barley and wheat, cases can be found where some varieties exhibit a degree of non-uniformity for some microsatellite loci (e.g. Plaschke *et al.*, 1995, Donini *et al.*, 1998). This is also the general picture that is emerging from the EU-funded "Molecular Markers for Variety Testing" project, which is examining SSR markers in tomatoes and wheat (BMT/5/8). In those cases where uniformity has been examined in anything like a systematic way, it has again been demonstrated that non-uniformity exists in some varieties with certain markers. An example is given in Table 1, which shows the results of analysing 10 individuals from five oilseed rape varieties (numbered 1-5) with two different microsatellite primer pairs (designated A and B):

Table 1 – uniformity of SSR markers in oilseed rape varieties (data from David Lee *et al.*, unpublished).

Variety	PRIMER PAIR	**No. of alleles**	**Ratio**
1	A	1	-
2	A	1	-
3	A	6	3:2:2:1:1:1
4	A	2	9:1
5	A	1	-
1	B	3	-
2	B	1	-
3	B	2	6:3:1
4	B	1	9:1
5	B	1	-

Hence variety 1, for instance, appears 'uniform' if examined with SSR primer pair A, but contains three allelic forms if analysed with primer pair B.

Whilst this type of result is not unexpected, given the various types of oilseed rape variety that exist, it does raise potential difficulties for the application of molecular markers in DUS testing, if the criteria presently used for assessment of uniformity were maintained.

In many ways, this situation is analogous to the existence of protein electrophoretic 'biotypes' in crop varieties. We have previously suggested (BMT/3/4, Lee *et al.*, 1996c) a number of ways of approaching this problem:

(i) it could be decided that this lack of uniformity precludes the use of certain molecular markers for DUS testing purposes; (ii) it could be accepted that the level of non-uniformity exhibited by currently registered varieties (which would need to be determined systematically and empirically) represented a baseline, which candidate varieties in the future would not be allowed to exceed; (iii) it could be suggested that from a certain date, all future candidates would have to be uniform for a particular selected marker or set of markers; (iv) it could be suggested that from a certain date, those candidates for which the marker data was the distinctness criterion would have to be uniform for that particular character; (v) it could be accepted that the repeatability (i.e. stability) of the differences between varieties is more important than the insistence on plant to plant uniformity. Thus if the variability within a variety, as estimated either by single plant analysis or by a bulk analysis, is maintained from generation to generation (and therefore is stable) then this could be accepted as evidence of sufficient uniformity within that variety.

DNA profiling techniques are ideally suited to the rapid assessment of stability, since different generations can be screened and compared side by side on the same gel.

However, an enduring problem is the lack of detailed evidence on the uniformity of plant varieties with respect to the currently favoured molecular markers. Hence we are undertaking a research programme, one of the objectives of which is to address this problem and then, on the basis of the data obtained, to suggest strategies for the use of markers in DUS testing.

2. Experimental Approach.

The main objectives of our research programme are to continue to evaluate the potential of molecular markers (DNA profiling methods) in DUS testing of plant varieties, to study the uniformity and stability of varieties with respect to selected markers and to develop an operational system for their use. Wheat and oilseed rape are being used as 'model' crops and sequence tagged site DNA microsatellites (referred to hereafter as simple sequence repeats, SSRs) are the profiling method of choice. There are wheat and oilseed rape SSRs available to NIAB from previous research projects and more oilseed rape primer pairs are being produced by LARS. In summary, we are:

(i) obtaining and optimising a number of SSR markers for wheat and oilseed rape. Although it is not clear what a 'suitable' number of markers might be for DUS testing purposes, a useful objective is to have at least one marker on each arm of each chromosome/linkage group. The target is thus to examine for both wheat and oilseed rape a total of 40-50 SSRs that either are already mapped, or will be mapped during the course of this work.

(ii) testing the polymorphism of these markers in a small group of varieties,
(iii) assessing the uniformity of these varieties with regard to SSRs, by analysis of 48 individuals,
(iv) developing a test set of suitable primer pairs that could be used in DUS testing,
(v) evaluating this test set. This evaluation will be achieved by analysing a larger number of current varieties, including oilseed rape hybrids (for both D and U), by analysing candidate varieties (past and present, for both D and U) and by undertaking a parallel running exercise in which the results obtained from testing as carried out using the current system will be compared with those that would be obtained if the SSR test set were used,
(vi) examining aspects of stability by analysing different seed lots of a number of varieties for both D and U.

The markers will be analysed using automated separation and detection systems, and the efficient throughput of analysis will be maximised through multiplexing of PCRs, multiple and sequential gel loading etc.

3. Results.

The programme started in summer 1999 and so only preliminary results are available to date for both species.

3.1 Oilseed rape.

At the moment, the selection of suitable primer pairs from those developed within the BBSRC Brassica programme is underway. Candidate primer pairs are being evaluated at LARS and NIAB using common DNA samples of 10 varieties. The levels of polymorphism detected by the SSRs so far within these 10 varieties are rather low, and further SSR selection may be necessary.

Some data are available from on-going projects at NIAB, using other microsatellites (i.e. not derived from the LARS programme) to examine hybrid oilseed rape varieties. In summary, the uniformity of 5 ‘pure’ (based on morphological examination) hybrid varieties has been examined by the analysis of 50-60 individuals, using 5 microsatellites (Table 2).

Bearing in mind that the actual purity of these hybrid samples is by no means certain (the level of hybrid purity is very unlikely to be 100%), these results confirm that (i) varieties differ in the degree of uniformity that can be detected depending on the marker used, and that (ii) markers can be ‘uniform’ in some varieties and not in others. With this limited data set, there does not seem to be a correlation between the number of alleles for a SSR locus, or its PIC value, and the detection of non-uniformity (cf. primer pair 1 in varieties A-D and E, primer pair 5 in varieties C and D, for instance). More information of this nature will emerge from the work using the larger set of LARS markers and more detailed uniformity assessments.

Table 2. – uniformity of hybrid varieties of oilseed rape using different SSR primer pairs (data from Vince Lea *et al.*, unpublished).

Variety	Primer pair	Source	No. of alleles *	PIC*	No. of profiles in variety	Proportions
A	1	NIAB	6	0.74	2	48:9
	2	Lagercrantz et al	3	0.34	1	-
	3	Kresovich et al.	11	0.69	2	45:12
	4	Kresovich et al.	3	0.59	1	-
	5	Kresovich et al.	3	0.06	Nd	Nd
B	1	NIAB	6	0.74	2	43:9
	2	Lagercrantz et al	3	0.34	Nd	Nd
	3	Kresovich et al.	11	0.69	1	-
	4	Kresovich et al.	3	0.59	1	-
	5	Kresovich et al.	3	0.06	Nd	Nd
C	1	NIAB	6	0.74	3	52:1:1
	2	Lagercrantz et al	3	0.34	Nd	Nd
	3	Kresovich et al.	11	0.69	2	56:1
	4	Kresovich et al.	3	0.59	1	-
	5	Kresovich et al.	3	0.06	2	50:8
D	1	NIAB	6	0.74	2	53:1
	2	Lagercrantz et al	3	0.34	1	-
	3	Kresovich et al.	11	0.69	1	-
	4	Kresovich et al.	3	0.59	1	-
	5	Kresovich et al.	3	0.06	1	-
E	1	NIAB	6	0.74	1	-
	2	Lagercrantz et al	3	0.34	1	-
	3	Kresovich et al.	11	0.69	1	-
	4	Kresovich et al.	3	0.59	1	-
	5	Kresovich et al.	3	0.06	Nd	Nd

*from previous analysis of 50 varieties; Nd = not determined

3.2 Wheat.

Attention has been focussed on selection of SSR primer pairs, using mainly the information given in Röder *et al.* (1998). The provisional list of primer pairs for evaluation is given in Table 3. There is mostly at least one marker per chromosome arm, although there are, for instance, no satisfactory markers for the long arm of chromosome 6D. These primer pairs are now being optimised for their analysis, preferably in multiplexes. Some information is already available for some of the markers in this respect, which indicates that it should be possible to analyse multiplexed microsatellites at least in certain cases. The markers will then be evaluated for their polymorphism in a small variety collection, before being used for uniformity screening. For all analyses, separation of PCR products is being carried out using a

Li-Cor DNA Analyser 4200, which requires that one of each primer pair is labelled with an IR dye. Two separate dyes are available.

Table 3 – provisional list of wheat microsatellite primers.

Locus	Location	Locus	Location	Locus	Location	Locus	Location
WMS 357	1AL	WMS 445	2AL	WMS 155	3AL	WMS 160	4AL
Taglut	1AS	WMS 095	2AS	WMS 674	3AS	WMS 004	4AS
WMS 259	1BL	WMS 619	2BL	WMS 247	3BL	WMS 513	4BL
Taglgap	1BS	WMS 257	2BS	WMS 389	3BS	WMS 538	4BL
WMS 018	1BS	WMS 261	2DS	WMS 003	3DL	WMS 634	4BS
WMS 458	1DL	WMS 102	2DS	WMS 161	3DS	WMS 609	4DL
WMS 106	1DS	WMS 539	2DL			WMS 624	4DL
Secalin	1RS					WMS 165	4DL/4AS

Locus	Location	Locus	Location	Locus	Location
WMS 186	5AL	WMS 169	6AL	WMS 276	7AL
WMS 415	5AS	WMS 459	6AS	WMS 631	7AS
WMS 408	5BL	WMS 626	6BL	WMS 577	7BL
WMS544	5BS	WMS 680	6BS	WMS 046	7BS
WMS 212	5DL	WMS 469	6DS	WMS 635	7AL/7DL
WMS 190	5DS	WMS 325	6DS	WMS 437	7DS

Preliminary information on the uniformity of 4 wheat (A-D) varieties for 5 markers (Table 4) confirms the findings from the previous studies with oilseed rape – varieties and markers can both vary with respect to their heterogeneity.

Table 4 – provisional uniformity data in wheat. The numbers indicate the proportions of different alleles in 4 varieties for 5 microsatellite loci. Data from Susan Freeman (unpublished).

VARIETY	**Taglut**	**WMS 018**	**WMS 261**	**WMS 325**	**Secalin**
A	15:10:3	27	27	26:1	28
B	20:6:2	28	23:3:2	28	28
C	27:1	Nd	27	28	28
D	11:1	12	12	12	12

Nd = not determined

4. Conclusions.

Molecular markers have the potential to contribute towards improved, efficient and cost-effective DUS testing procedures, but this potential needs to be assessed systematically. This research will assess the uniformity of varieties with respect to a range of microsatellites and will indicate whether it is possible to select markers that are both usefully polymorphic between varieties and homogeneous within varieties. It will also help to establish baselines of intra-varietal uniformity for two important species and will compare results obtained from currently used procedures with those obtained using molecular markers. Hence the results from this programme will contribute towards a coherent assessment of the application of DNA profiling to plant variety testing and allow its potential to be evaluated objectively.

5. References.

Cooke, R. J. and Reeves, J. C. (1998). Cultivar identification - a review of new methods. In: Kelly, A.F. and George, R.A.T. (eds.) *Encyclopedia of Seed Production of World Crops,*). John Wiley & Sons, London pp 88-102.

Donini, P., Stephenson, P., Bryan, G.J. and Koebner, R.M.D. (1998). The potential of microsatellites for high throughput genetic diversity assessment in wheat and barley. *Genetic Resources and Crop Evolution* **45**: 415-421.

Law, J.R., Koebner, R.M.D., Donini, P, Reeves, J.C. and Cooke, R.J. (1998). DNA profiling and plant variety registration. 3: The statistical assessment of distinctness in wheat using amplified fragment length polymorphisms. *Euphytica* **102**: 335-342.

Lee, D., Reeves J.C. and Cooke, R.J. (1996a). DNA profiling and plant variety registration: 1. The use of random amplified DNA polymorphisms to discriminate between varieties of oilseed rape. *Electrophoresis* **17**, 261-265.

Lee, D., Reeves J.C. and Cooke, R.J. (1996b). DNA profiling and plant variety registration: 2. Restriction fragment length polymorphisms in varieties of oilseed rape. *Plant Varieties & Seeds* **9**, 181-190.

Lee, D., Reeves J.C. and Cooke, R.J. (1996c). DNA profiling for varietal identification in crop plants. *Proceedings BCPC Symposium No. 65: Diagnostics in Crop Production*. pp 235-240.

Plaschke, J., Ganal, M. W. and Röder, M. S. (1995). Detection of genetic diversity in closely related bread wheat using microsatellite markers. *Theor. Appl. Genetics* **91**, 1001-1007.

Röder, M.S., Korzun, V., Wendehake, K., Plaschke, J., Tixier, M-H., Leroy, P and Ganal, M.W. (1998). A microsatellite map of wheat. *Genetics* **149**, 2007-2023.

Acknowledgements

The financial support of the Ministry of Agriculture, Fisheries and Food and of the European Union is gratefully acknowledged.

[End of document]