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PCR analysis of oilseed rape cultivars (*Brassica napus* L. ssp. *oleifera*) using 5′-anchored simple sequence repeat (SSR) primers

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Abstract Primers complementary to simple sequence repeats (SSRs) and with variable three-base 'anchors' at their 5′ end, were used in PCR analyses to compare pooled DNA samples from various *Brassica napus* and *B. rapa* cultivars. Amplification products were resolved on polyacrylamide gels and detected by silver-nitrate staining. The resulting banding patterns were highly repeatable between replicate PCRs. Two of the primers produced polymorphisms at 33 and 23 band positions, respectively, and could each discriminate 16 of the 20 cultivars studied. Combined use of both primers allowed all 20 cultivars to be distinguished. The UPGMA dendrogram, based on the cultivar banding profiles, demonstrated clustering on the basis of winter/spring growth habit, high/low glucosinolate content, and cultivar origin (i.e. the breeder involved). Intra-cultivar polymorphism was investigated using a minimum of ten individuals for each cultivar and was found to vary considerably between cultivars. It is concluded that anchored SSR-PCR analysis is a highly informative and reproducible method for fingerprinting oilseed rape populations, but that intra-cultivar variation should be investigated before using banding profiles from pooled samples for the identification of individuals.

Key words *Brassica napus* · Cultivar identification · DNA fingerprinting · Anchored SSR · PCR · Silver staining

Introduction

Cultivars of oilseed rape (*Brassica napus* L. ssp. *oleifera*) are difficult to distinguish on the basis of their morphology. Descriptive lists compiled from DUS (distinctness, uniformity and stability) tests do not provide useful morphological markers. Performance traits recorded in VCU (value for cultivation and use) trials, such as yield, earliness of flowering and standing power, are of limited value for the identification of small populations (such as stands of crop volunteers or feral plants) and cannot be used to identify individuals. In addition, performance traits have comparatively low discrimination and high cost. These difficulties are exacerbated by the large number of cultivars available and by their relatively short commercial lifespan.

Several biochemical methods have been investigated as alternative means of identifying *B. napus* cultivars. These include the comparison of seed oil fatty acid profiles by GLC analysis (White and Law 1991), HPLC analysis of leaf glucosinolates (Adams et al. 1989), and starch-gel electrophoresis of cotyledon isozymes (Mundges et al. 1990). Such techniques detect only a limited degree of polymorphism and may be sensitive to environmental and developmental variation. In contrast, nuclear DNA is believed to be unaffected by external influences and has been used to distinguish cultivars of many crop species including those belonging to the genus *Brassica*. Restriction fragment length polymorphism (RFLP) analysis was the first molecular technique to be used for this purpose. This approach is renowned for its reliability but generally produces low numbers of discriminating loci (Waugh and Powell 1992). Hallden et al. (1994) used RFLP band profiles to compare three *B. napus* breeding lines and found that a minimum of 30 probe/enzyme combinations were required to distinguish reliably between them and to describe their relationship. In addition to the often protracted process of identifying suitable probe/enzyme combinations, the procedure also requires relatively large quantities of sample DNA. Randomly amplified polymorphic DNA (RAPD) analysis is a simple, quick and convenient procedure requiring much smaller quantities of template DNA. Mailer et al. (1994) used RAPD analysis to discriminate between 23 cultivars of *B. napus* and *B. rapa*. Polymorphic band positions were noted from 22 of the 100 primers screened and six of these were required to identify all

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of the cultivars. The use of a more informative PCR-based method would reduce the need to screen such large numbers of primers.

PCR analysis using anchored simple sequence repeat (SSR) primers has gained attention recently as an alternative means of characterising complex genomes (Wu et al. 1994; Zietkiewicz et al. 1994). Anchored SSR primers are complementary to genomic microsatellites (1–4 nucleotides occurring in tandem repeats) and contain short oligonucleotide 'anchor' sequences that ensure the primers anneal to either the 5′ or 3′ end of the genomic repeat. Microsatellite regions are abundant throughout the eukaryotic genome (Tautz and Renz 1984) and are highly polymorphic in length (Levinson and Gutman 1987). SSR primers, therefore, target highly variable and numerous loci. Lagercrantz et al. (1993) reported the occurrence of $(GT)_n$ and $(CT)_n$ repeats in oilseed rape, and Poulsen et al. (1993) confirmed the occurrence of at least six microsatellite classes, namely $(GATA)_4$, $(GACA)_4$, $(GGAT)_4$, $(GTG)_5$, $(CA)_8$ and $(CT)_8$. Inter-cultivar polymorphism was detected when the SSR $(GATA)_4$ was used as a probe for Southern analysis of eight oilseed rape cultivars (Poulsen et al. 1993). Intra-cultivar variability was also revealed by the analysis and only 3 of the 11 plants of cv Topas possessed identical banding profiles.

In the present study 5′-anchored SSR primers are used in the PCR analysis of 20 *Brassica* cultivars. The amplification products were resolved on pre-cast polyacrylamide gels and detected non-radioactively using silver-nitrate staining. Banding profiles were compared to identify variation at the inter- and intra-cultivar levels.

Materials and methods

Plant material

Seed samples of oilseed rape cultivars were obtained from the suppliers listed in Table 1. The turnip rape cv Kova (*B. rapa*) was included for comparative purposes. All cultivars except those marked (*) were obtained from certified seed stocks and cvs Comet and Libravo were obtained from more than one source (Table 1).

Twenty seeds of each cultivar were sown in a glasshouse and harvested after the emergence of the fourth leaf. Four sets of 20 seedlings of the cultivars Envol, Samourai and Libravo were grown to compare banding profiles of pooled DNA samples. Two leaves were collected from each plant, one being stored individually and the second being pooled with leaves from other plants of the same cultivar. All leaves were placed on ice until collection was complete (30 min or less) and stored at –80°C until DNA extraction.

PCR amplifications

DNA was extracted from 150 mg of leaf material according to the procedure of Hu and Quiros (1991). The 14 primers screened during the present study are described in Table 2. Eight primers were obtained from UBC (University of British Columbia) set 9, and the remaining six were synthesised by the Chemistry Department at SCRI (Scottish Crop Research Institute).

PCR reaction mixtures (20 μl) contained the following components/concentrations: one unit of *Taq* DNA polymerase, 1.5 mM $MgCl_2$ buffer (supplied with the enzyme, Boehringer Mannheim), 0.2 mM of each dNTP (all Boehringer Mannheim), with 0.3 μM of a single primer and 20 ng of genomic DNA. PCR amplifications were performed on a Hybaid Omnigene Thermocycler using the following programme: 30 cycles of (1 min at 94°C, 2 min at 55°C, 30 s at 72°C) with a final 5-min extension at 72°C.

Table 1 *Brassica* cultivars raised for DNA extraction and fingerprinting

Cultivar[a]	W/S[b]	Type[c]	Breeder	Supplier
Askari*	W	DH	Lembke, Germany	Semundo, UK
Bienvenu	W	SL	Serasem, France	SCRI, UK
Bristol	W	DL	Cargill, France	British Seed House, UK
Cobra	W	DL	Lembke, Germany	SCRI, UK
Comet	S	DL	Svalof Weibull, Sweden	(1) West Crop, UK (2) Sharpes, UK
Envol	W	DL	Cargill, France	Cargill (UK)
Express	W	DL	Lembke, Germany	Twyford Seeds, UK
Falcon	W	DL	Lembke, France	Twyford Seeds, UK
Forte	S	DL	KWS, Germany	Dalgety Agriculture, UK
Hobson	W	DH	Sharpes, UK	Sharpes, UK
Idol	W	DL	Cargill, France	Cargill (UK)
Kova	S	DL	Svalof Weibull, Sweden	Dalgety Agriculture, UK
Libravo	W	DL	DSV, Germany	(1) Nickerson Seeds, UK (2) SCRI, UK
Lineker	W	DL	DSV, Germany	Nickerson Seeds, UK
Martina*	W	DH	Semundo	West Crop, UK
Pasha	W	SL	Thomas, UK	SCRI, UK
Rafal	W	SL	Serasem, France	SCRI, UK
Rocket	W	DL	Twyford Seeds, UK	Twyford Seeds, UK
Samourai	W	DL	Serasem, France	Twyford Seeds, UK
Tapidor	W	DL	Serasem, France	Twyford Seeds, UK

[a] *, seed not obtained from certified stocks
[b] W, winter cultivar (i.e. biennial); S, spring cultivar (i.e. annual)
[c] DH (double high), high glucosinolate, high erucic acid in seed oil; DL (double low), low glucosinolate, low erucic acid; SL (single low), low erucic acid. Cultivar classifications were obtained from listings compiled by the National Institute of Agricultural Botany (NIAB), Cambridge, UK

Table 2 List of primer sequences screened with pooled oilseed rape cultivar DNA

I.D. no.	Sequence	Source	I.D. no.	Sequence[a]	Source
882	VBV-[AT]7	UBC#9	1417	BDT-[CA]7	SCRI
884	HBH-[AG]7	UBC#9	1418	BVB-[GATA]4	SCRI
886	VBV-[CT]7	UBC#9	1419	G-[GATA]4	SCRI
887	DVD-[TC]7	UBC#9	1423	HVH-[TGT]5	SCRI
888	BDB-[CA]7	UBC#9	1424	BDB-[CAC]5	SCRI
889	DBD-[AC]7	UBC#9	1425	BDV-[CAG]5	SCRI
880	VHV-[GT]7	UBC#9	891	HVH-[TG]7	UBC#9

[a] Each primer comprises a repeat sequence preceded by a 5′ anchor. All anchors, except those for primers 1417 and 1419, consist of three variable base positions. These are designated as follows: B=C,G or T (i.e. not A). Similarly, D=not C; H=not G; V=not T

Electrophoresis of PCR products

Amplification products were resolved on the Multiphor II (Pharmacia Biotech) flatbed system using pre-cast polyacrylamide gels (Cleangel 48S, Pharmacia Biotech) re-hydrated in 112 mM Tris-acetate (pH 6.4) with 0.2 M Tris base, 0.2 M tricine, 0.55% (w/v) SDS, pH 8.0, as the electrode buffer. Electrophoresis was conducted using a three-stage programme: (1) 20 min at 200 Vmax, 20 mAmax, 10 Wmax; (2) 50 min at 380 Vmax, 30 mAmax, 20 Wmax; (3) about 30 min at 450 Vmax, 30 mAmax, 20 Wmax, and was deemed complete when the blue dye front ran to the gel margin.

Silver staining

Bands were detected using the method of Bassam et al. (1991) with modifications as follows: (1) 30 min fixing in 250 ml of 10% acetic acid; (2) 3×2 min washings in 250 ml of distilled water; (3) 30 min silvering in 200 ml of freshly prepared 0.1% (w/v) $AgNO_3$, with 200 μl of 40% w/v formaldehyde added immediately prior to use; (4) 20 s rinse in 250 ml of distilled water; (5) about 10 min developing in 200 ml of freshly prepared 2.5% (w/v) Na_2CO_3 with 200 μl of 2% (w/v) Na_2SO_3 and 200 μl of formaldehyde added immediately prior to use at 10°C in a pre-chilled dish; (6) 10 min stop/de-silver in 200 ml of 2% (w/v) glycine, 0.5% (w/v) EDTA-Na_2; and (7) 10 min gel impregnation in 250 ml of 5% (v/v) glycerol. Glassware was used throughout the procedure.

Gels were laid flat and air-dried at room temperature prior to examination on a light box.

Gel scoring and cluster analysis

A cluster analysis was performed to confirm the distinction of cultivars on the basis of their banding profiles. Band positions and sizes were scored from pooled-sample profiles with the aid of a BioImage System (Millipore UK Ltd) gel scanner including Kodak Megaplus CCD camera and Visage (4.2) software. Bands were recorded as present (1) or absent (0) at 67 positions and scores assembled in a data matrix. Pairwise comparisons, similarity matrices and the UPGMA cluster analysis were performed using the Genstat 5 software package (Genstat 1987).

Individual plant banding profiles were scored by recording bands at 21 comparable positions.

Results

Detection of inter-cultivar variation using pooled DNA samples

Cultivars Askari and Rocket differ in a range of biochemical and agronomic traits and were used to identify potentially useful primers selected from UBC set 9. These consisted of eight di-nucleotide repeat sequence primers, anchored at their 5′ end and totalling 17 bases in length (Table 2). Banding profile reproducibility was assessed through a side-by-side comparison of independent PCR product profiles in all analyses (lane pairs indicated by brackets in Figs. 1, 2 and 4). No differences were ever detected between repeat pairs. On the basis of these initial analyses, four primers (888, 889, 890, 891) were screened further to compare the following ten cultivars: Askari, Bienvenu, Cobra, Envol, Falcon, Forte, Libravo, Rafal, Samourai and Tapidor. Primer 888 revealed the greatest variation in banding profiles and was selected for PCR analysis of all 20 cultivars. These reactions produced unique banding profiles in 16 of the cultivars (Fig. 1). The remaining four cultivars divided into two indistinguishable pairs (Idol-Envol and Pasha-Bienvenu). A further six primers (Table 2) were then screened without success in an attempt to type all 20 cultivars using a single primer. Primer 1423

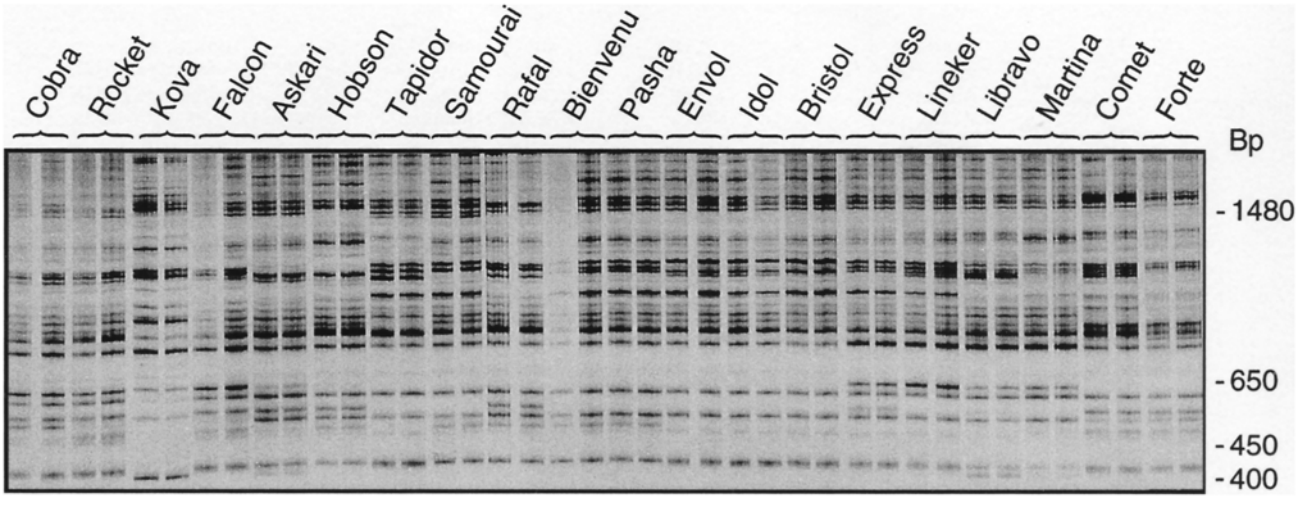

Fig. 1 Inter-cultivar variation. Banding profiles from pooled DNA samples of 20 cultivars using primer 888 (gel shown is approximately 90% life size). *Lane brackets* indicate profiles produced by independent PCRs

(a tri-nucleotide repeat) could be used to identify 16 cultivars and could distinguish between the like-pairs unresolved by primer 888 (Fig. 2). The combined use of primers 888 and 1423 therefore enabled all 20 cultivars to be distinguished. Not all bands scored were informative for the purpose of visual discrimination of the cultivar banding profiles. This could be achieved by considering only 17 major bands produced by primer 888 and three major bands produced by primer 1423. The fidelity of this system was investigated by scoring the banding patterns produced by six independent PCR products resolved on three independent gels for each cultivar. All scores remained constant.

Cluster analysis

The banding profiles obtained using primers 888 and 1423 were scored and compiled as a data matrix. The majority of bands (56 out of 67 scored) were variable between cultivars. Disregarding the turnip rape (*B. rapa*) cultivar, 44 bands were polymorphic for the 19 *B. napus* cultivars. The UPGMA dendrogram confirmed that all 20 cultivars could be distinguished following reference to their banding profiles (Fig. 3). The turnip rape (cv Kova) separated from all the oilseed cultivars at 59.5%. The two spring (annual) oilseed rapes (cv Comet and cv Forte) formed a distinct group although they had been produced by different breeders. This was also true of the double high cultivars Askari and Martina. The 11 double low, winter (biennial) cultivars showed a tendency to associate according to their breeder origin: cultivars Envol, Idol and Bristol, bred by Cargill, grouped together, as did the two DSV cultivars, Lineker and Libravo. Although cultivar parentage was not investigated here, Bienvenu was very closely associated with its orange-flowered variant, Pasha, suggesting additional clustering on the basis of pedigree.

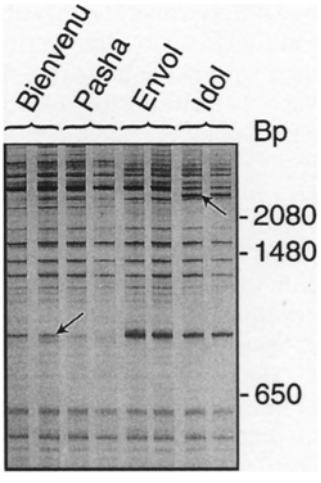

Fig. 2 Banding profiles obtained using primer 1423 from pooled DNA samples of the cultivars Bienvenu, Pasha, Envol and Idol. *Arrows* indicate bands distinguishing the cultivar pairs: Bienvenu/Pasha and Envol/Idol

Variation between seed samples

Triplicate pooled DNA samples were prepared from the cultivars Envol, Samourai and Libravo. Each sample was composed of a different set of 20 individual plants. No variation was detected between banding patterns of pooled samples using either primer 888 or 1423. Cultivars Comet and Libravo were similarly investigated using pooled DNA samples from two different seed suppliers (Table 1). The banding profiles of cv Comet were identical from both sources using either primer. Profiles of the different seed sources of cv Libravo were polymorphic at one band position using primer 888 [i.e. absent from the banding profile for seed source (2), Table 1] although no variation was detected using primer 1423.

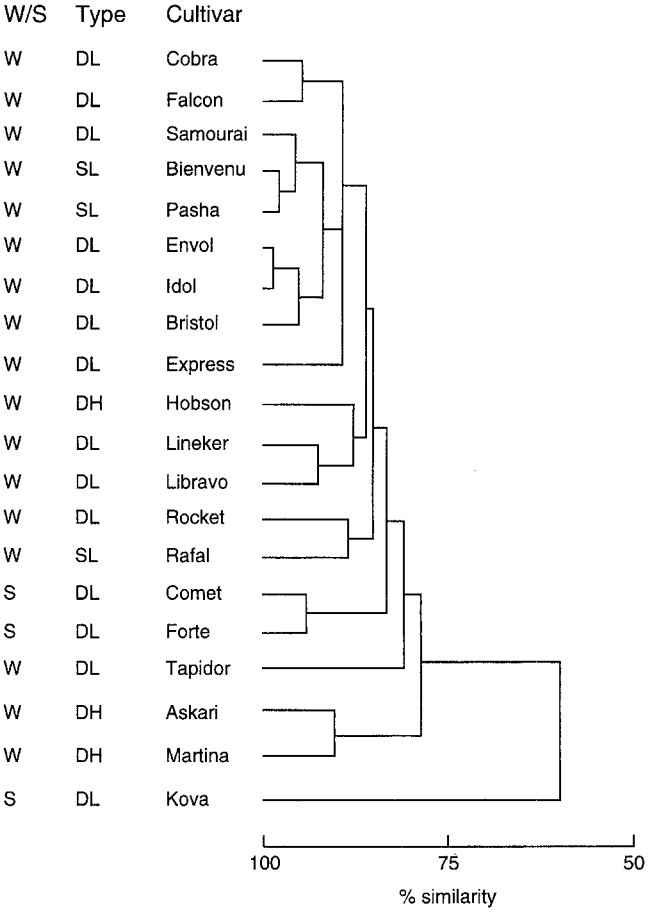

Fig. 3 Dendrogram comparing 20 cultivars of *Brassica napus* and *B. rapa* on the basis of banding profiles generated using primers 888 and 1423. Abbreviations as for Table 1

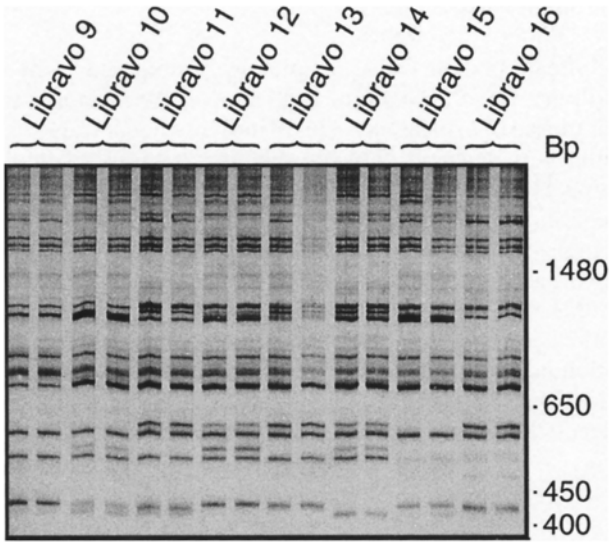

Fig. 4 The comparison of eight individuals of cv Libravo using primer 888

Table 3 Number of intra-cultivar polymorphic bands detected using primer 888 for the 20 cultivars studied

Cultivar	No. bands	Cultivar	No. bands
Bienvenu	0	Idol	2
Bristol	0	Tapidor	2
Falcon	0	Askari	3
Hobson	0	Comet	3
Pasha	0	Forte	3
Rafal	0	Lineker	3
Envol	1	Cobra	5
Rocket	1	Kova	9
Samourai	1	Martina	9
Express	2	Libravo	10

Variation between individuals

Profiles of 20 individuals representing the cultivars Envol, Samourai and Libravo were compared using primer 888. Banding profiles of all samples were identical between PCR repeats for the same plant. Profiles of cultivars Envol and Samourai were invariable between all individuals except for a single polymorphic band. In contrast, the banding profiles of cv Libravo individuals (Fig. 4) varied in ten of the 21 band positions scored. The remaining 17 cultivars were similarly investigated using ten individuals from each. The majority of cultivars exhibited little or no variation between individuals. Six cultivars showed no polymorphism in the 21 band positions and ten cultivars contained three or less polymorphic bands (Table 3). Individuals of cv Cobra were more variable with five bands being polymorphic and three of the 20 cultivars were extremely variable and contained at least nine polymorphic bands.

Discussion

The variability revealed using these techniques with primers 888 and 1423 was far greater than reported previously using RAPD primers (Mailer et al. 1994) or RFLP probes (Hallden et al. 1994; Diers and Osborn, 1994) and was sufficient to enable either primer to distinguish 16 of the 20 cultivars studied. The combined use of both primers allowed identification of all 20 cultivars.

There was no variation between replicate DNA samples or between independent PCR amplifications during the course of the study. Several features of the protocol contribute to this repeatability, including the use of comparatively long primers (17 bases compared to the 10–12 generally used in RAPD analysis), the standardisation of band resolution and the use of a highly sensitive silver staining procedure for DNA detection. There were also no differences between pooled samples originating from the same cultivar and very little variability between samples taken from different seed sources. In consequence, the repeatability of band profiles, the speed of analysis (PCR, product resolution and band detection in under 10 h), and the high levels of inter-cultivar polymorphism revealed, all suggest that this approach has considerable potential for the rapid identification of seed batches and populations of oilseed rape cultivars.

The UPGMA dendrogram (Fig. 3) confirmed the distinction of all 20 cultivars on the basis of their banding profiles. The *B. rapa* sample gave a very distinct banding pattern and was clearly separated from the *B. napus* cultivars. Thus the variation between the two species exceeded that found between cultivars of oilseed rape. Similar results were obtained by Mailer et al. (1994) using RAPD analysis. It is also interesting to note that cultivars sharing such traits as seed oil composition (e.g. double high, single low etc.) and spring/winter habit, showed a tendency to associate together. Groupings were also found to contain cultivars with a common origin (cvs Lineker and Libravo bred by DSV, Germany, and cvs Envol, Idol and Bristol bred by Cargill, France) although these were also invariable for their agronomic traits (i.e. winter sown, double low cultivars). In addition, the cultivars Bienvenu and Pasha, which have a common parentage, associated very closely, differing in only one band. The close associations of cvs Samourai with Bienvenu and Cobra with Falcon are in agreement with the more extensive survey of Diers and Osborn (1994) using 161 RFLP fragments. These associations, together with the excellent repeatability of the PCR profiles, suggest that the banding patterns genuinely reflect the cultivar genotypes.

The applicability of the approach for the identification of individual plants required the investigation of variability within oilseed rape cultivars. Analysis of the banding profiles of over 200 individual plants revealed variability in 14 of the 20 cultivars studied. The degree of intra-cultivar polymorphism detected ranged between cultivars composed of individuals possessing identical banding profiles (e.g. cv Rafal) to one which exhibited variability at

10 of the 21 bands scored (cv Libravo). There are several potential causes of such high levels of intra-cultivar polymorphism. One possible explanation is that cultivars which show acceptably low phenotypic variability for the agronomic traits scored during DUS trials (distinctness, uniformity and stability) may nevertheless contain substantial levels of cryptic genetic variability as a result of the breeding methods used in their development. Equally, whilst stocks submitted to DUS trials might be largely homogeneous, inter-cultivar hybridisation events subsequent to release may increase the levels of intra-cultivar genetic variability depending on the source of seed. The variable levels of intra-cultivar polymorphism detected indicate that fingerprints derived from pooled samples do not necessarily provide a method of identifying individuals or small populations.

The data presented here demonstrates that PCR analysis using 5′-anchored SSR primers can be used to identify pooled samples of oilseed rape cultivars and also has the potential to identify individual plants.

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