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Development and Application of SINE Multilocus and Quantitative Genetic Markers To Study Oilseed Rape (*Brassica napus* L.) Crops

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A genetic marker system based on the S1 Short Interspersed Elements (SINEs) in the important commercial crop, oilseed rape (*Brassica napus* L.) has been developed. SINEs provided a successful multilocus, dominant marker system that was capable of clearly delineating winter- and spring-type crop varieties. Sixteen of 20 varieties tested showed unique profiles from the 17 polymorphic SINE markers generated. The 3′ or 5′ flank region of nine SINE markers were cloned, and DNA was sequenced. In addition, one putative pre-transposition SINE allele was cloned and sequenced. Two SINE flanking sequences were used to design real-time PCR assays. These quantitative SINE assays were applied to study the genetic structure of eight fields of oilseed rape crops. Studied fields were more genetically diverse than expected for the chosen loci (mean $H_T = 0.23$). The spatial distribution of SINE marker frequencies was highly structured in some fields, suggesting locations of volunteer impurities within the crop. In one case, the assay identified a mislabeling of the crop variety. SINE markers were a useful tool for crop genetics, phylogenetics, variety identification, and purity analysis. The use and further application of quantitative, real-time PCR markers are discussed.

KEYWORDS: SINEs; *Brassica napus*; molecular markers; crop purity; volunteers; Taqman; real-time PCR; quantitative genetic markers; QSINEs

INTRODUCTION

Oilseed rape (*Brassica napus* L.) is an increasingly important crop for food, animal feed, and fuel/industrial oil production. Oilseed rape varieties are known to contain relatively high genetic variation. International systems for distinctiveness and uniformity (DUS) testing are applied to new varieties, but they may not detect genetic nonuniformity (*1*). Genetic mixing of varieties may also occur by crop-to-crop pollination (*2*), seed drop and persistence of volunteers in the soil (*3*), and farm-saving of seed. This genetic mixing may not currently be a significant problem in agriculture, but future developments in genetically modified, nonfood, or industrial (e.g., biofuel) crops will mean that such mixing will need to be prevented or minimized and monitored. In current oilseed rape production in the United Kingdom, high erucic acid (HEAR) varieties are required to be separated from food/feed production crops (low erucic acid, LEAR). This coexistence system provides a potential model for the development of monitoring methods.

Molecular genetic markers have been used in oilseed rape breeding and genetic mapping (*4, 5*) to study genetic diversity within the species and varieties (*6–10*) and introgression from other *Brassica* species (*11*). They also have potential use in variety identification and seed purity assessment (*1*). We have adapted Short Interspersed Elements (SINE) markers to study oilseed rape variety genetic diversity. SINEs are common retroposon sequences present in a wide range of organisms, including humans, insects, and plants. The *B. napus,* $S1_{BN}$ family of SINEs was first described by Deragon et al. (*12*). They have several properties that make them ideal candidates as molecular markers to study genetic diversity and variety identification: (i) a reasonably high copy number, ~500 per haploid genome; (ii) a simple biallelic nature; (iii) no homoplasy (retroposon insertions do not “revert”); (iv) high polymorphism; and (v) a consistent consensus sequence in *Brassica* spp. The adaptation of SINEs to a sequence-specific amplified polymorphism (SSAP) (*16*) system has been described previously (*13*). SSAP analysis can be a complex procedure, requiring fluorescence or radiolabeled retrotransposon/transposon anchored primers and denaturing acrylamide gel electrophoresis to achieve the required specificity in PCR product detection. The suppression PCR (SPCR) system described here is simpler, using a nested PCR approach (*14*). It requires no primer labeling and uses standard agarose gels. This system is cheaper and quicker than SSAP, but the characteristics of the markers generated are the same, that is, dominant, multilocus markers. However, a lower number of scorable markers is obtained per run.

Single SSAP markers can be isolated, sequenced, and exploited as codominant marker loci by amplifying the retroposon flank and/or the unoccupied insertion site in one or more PCRs (*15*). This approach could also be applied to SINEs. In fact, they are particularly amenable due to their short length (<200 bp), which allows for amplification of the entire element and flanks in a single PCR. However, disruptions of the insertion site during retroposon integration mean that amplification of

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the unoccupied SINE allele may not be possible using the extant SINE flank sequences (*13*). Codominant markers are more useful in population genetic applications than dominant ones because they directly provide allele frequencies, whereas dominant markers can only do so by assumption of Hardy–Weinburg (H-W) equilibrium or by measurement of H-W deviations (F_{is}) by other methods. SINE loci are always theoretically biallelic; therefore, the quantification of one allele, that is, the $SINE^+$ allele, is all that is required to give the locus allele frequencies. We have used Taqman real-time PCR to quantify $SINE^+$ alleles with reference to the frequency of an internal control allele. These quantitative markers can be used to measure allele frequency in large pools of samples, for example, harvested seed pools. To our knowledge, marker alleles have not been quantified in this way before. These quantitative molecular markers have potential application in many population genetic studies, particularly where there is a high density of individuals and the small-scale spatial and temporal structure is of interest, for example, the study of plant species' hybrid zones, the competition and spread of genets in clonal populations, identification of rare management units in conservation genetics, and local-scale phylogeography.

In this paper we describe a multilocus SINE marker system, which we have applied to study oilseed rape genetic variation. Two of these SINE markers were adapted to a single locus quantitative (QSINE) marker system. We have applied these markers to address questions of variety and crop purity and spatial genetic structure in oilseed rape fields with respect to high and low erucic content.

Table 1. Oligonucleotides Used in This Study[a]

name	sequence 5′–3′
SINE suppression PCR primers	
sine5	TACAGRCAMAGGCTGGCGCC
sine6	CTGGCGCCGGGCCTAGGTGG
sine7	CCACCTAGGCCCGGCGCCAG
sine8	GGCGCCAGCCTKTGYCTGTA
suppression PCR adapter	
GATC short oligo	GATCGACACGGCAAA
GC short oligo	CGGACACGGCAAA
SPCR long oligo	AGCGTCCAGCTGAGCTGACCTGGTTC ATGCCGTCGTGCGCCGTGTC
suppression PCR adapter primers	
P3	AGCGTCCAGCTGAGCTG
P4	CCTGGTTCATGCCGTCG
Taqman primers and probes	
HEARsine1F	CGCCGGGCCTAGGTGGA
HEARsine1R	CGGACCAAAATTGTCTAACGACAATG
HEARsine1P	fam-CGCAACGTCAACACCTGGTTAAT AAAAAAAAAAAATTTATGTCAATATCCTTGA-tamra
HEARsine3F	TCCCCAAGGAGATAGTACCACT
HEARsine3R	CTTGCAATTTTTTCTAACCGTCGAGA
HEARsine3P	fam-CTACGAGGTCCTGGGTGGTTGCCAA-tamra
OSR2AF	TTGACACAAAGCGAGATTGAAAC
OSR2AR	CCGTCCTCGTGGCTGTTG
OSR2AP	fam-CTACTCCCGAAATTGCGTGTTGGACAA-tamra

[a] fam and tamra are the 5′ and 3′ Taqman reporter and quencher dyes, respectively.

EXPERIMENTAL METHODS

SINE Suppression PCR. The consensus *B. napus* SINE $S1_{BN}$ sequence of Deragon et al. (*17*) was used to design two pairs of nested primers, directed toward the 5′ and 3′ flanks of the SINE sequence (**Table 1**). When necessary, degenerate nucleotides were incorporated to maximize the number of products. It was not investigated in this study whether nucleotide differences or increased specificity in these primers could have been used to amplify different subfamilies of SINEs, but this would appear to be a logical future approach to increasing the number of independent markers obtained.

DNA was extracted from leaf and seeds using the DNeasy Plant Mini kit (Qiagen, GMbH, Hilden, Germany; part 69104) following the manufacturer's instructions. Genomic oilseed rape DNA was digested with one of four restriction enzymes: *Bst*YI, *Bgl*II, *Bam*HI, or *Cla*I, giving 5′ overhangs of GATC or CG. Suppression PCR adapters complementary to the overhangs (**Table 1**) were made by combining 50 μM solutions of two oligonucleotides, heating at 95 °C for 5 min, and allowing the mixtures to cool to room temperature over 30 min (50× stock). Restriction digestions were carried out in a 20 μL total volume (10 μg of DNA; 1 unit of enzyme; 1× supplied reaction buffer) and incubated at the enzyme's specified optimum temperature for 2 h. Ligation components were then added directly to the digestion reaction (80 pmol of adapter, 1× supplied ligase buffer; 1 unit of T4 DNA ligase) and incubated at 20 °C overnight. All enzymes and buffers used were supplied by New England Biolabs (U.K.) Ltd.

Suppression PCR consisted of two PCRs with nested primer pairs (**Table 1**). The primary PCR contained in a total volume of 10 μL 2 μL of restriction/digestion reaction, 1× NH^{4+} reaction buffer IV (ABgene, U.K.), 2.5 mM $MgCl_2$, 200 μM dNTPs, 10 pmol of primer P3, 10 pmol of primer sine5 (for 3′ flanks) or sine7 (for 5′ flanks), and 1 unit of Thermoprime plus DNA polymerase (ABgene). Reaction conditions were as follows: 2 min at 95 °C followed by 25 cycles of 1 min at 95 °C; 1 min at 60 °C; and 1 min at 72 °C. Secondary PCRs were performed in the same way as primary PCRs except that 2 μL of a 1:100 dilution (in water) of the primary PCR was used as target and primers used were P4 and sine6 or sine8. The number of cycles was also increased to 35. All PCRs were performed on an ABI9700 thermocycler. Secondary PCR products were run on 2% agarose gels and stained with ethidium bromide.

To study genetic diversity among varieties, 20 oilseed rape varieties were selected from the Home-Grown Cereals Authority (HGCA) recommended lists as representative of varieties commonly grown in the United Kingdom in recent years (seed kindly supplied by National Institute of Agricultural Botany, NIAB, U.K.). **Table 2** gives the details of the study samples. Seven varieties were duplicated, using seed samples supplied by NIAB on two separate occasions (2002 and 2004). Four varieties were spring-sown and 16 were winter-sown types. *Arabidopsis thaliana*, four *Brassica oleracea*, and one *Brassica rapa* sample were also included. Plants were grown in a glasshouse and leaf samples used for DNA extractions. Multilocus SINEs were scored visually among these samples, and a pairwise simple Jaccard distance (*19*) was calculated. PHYLIP software (*18*) was used to construct a neighbor-joining dendrogram.

To isolate single-locus, quantitative QSINE markers, for the quantification of HEAR and LEAR varieties, a dilution series of HEAR seeds (variety Hearty) was made in LEAR seeds by mass: 100, 10, 5, 1, 0.5 0.1 0.05, and 0% HEAR. DNA was extracted from each dilution and used in SINE suppression PCR as described above. Seven restriction digestions (*Asu*I, *Spe*I, *Nhe*I, *Xba*I, *Bam*HI, *Msp*I, and *Cla*I) were screened with 3′ (sine5, sine6) and 5′ (sine7, sine 8) SPCR primers. Two SPCR bands were isolated, which were present in HEAR dilutions down to 0.5% but absent from the 100% LEAR DNA.

SINE Cloning and Sequencing. SPCR bands were excised from agarose gels of different variety samples (**Table 2**) and were purified using a Nucleospin kit (Machery Nagel, part 635961). A 60 μL eluate was precipitated with 60 μL of isopropanol, centrifuged at full speed in a benchtop minifuge for 5 min and vacuum-dried. The DNA pellet was ligated into the vector pGEM-T according to the manufacturer's instructions (Promega, part A3600). Ligations were used to transform DH5α cells (Invitrogen). Eight colonies grown on ampicillin L-B plates for each transformation were screened using a colony PCR technique: a micropipet tip was touched onto the colony and inserted in a 10 μL PCR reaction using standard M13 sequencing primers for the pGEM-T vector (forward, CGCCAGGGTTTTCCCAGTCACGAC; reverse, GTGAGCGGATAACAATTTCACACAGG). Colony PCR products with

Table 2. *Brassica* Species and Oilseed Rape Varieties Analyzed with Multilocus SINE Markers[a]

no.	variety	type
1	Canberra	spring C
2	Cohort	winter 3H
3	Concept	winter VA
4	Disco	spring RH
5	Dorothy	winter C
6	Elan	spring RH
7	Escort	winter C
8	Fortress	winter C
9	Herald	winter C
10	Lipton	winter C
11	Madrigal	winter C
12	Mendel	winter RH
13	Mendel	winter RH
14	Pronto	winter RH
15	Pronto	winter RH
16	Recital	winter C
17	Recital	winter C
18	Royal	winter RH
19	Royal	winter RH
20	Senator	spring C
21	Shannon	winter C
22	Shannon	winter C
21	Spirit	winter TC
24	Tambora	spring C
25	Winner	winter C
26	Winner	winter C
27	*Arabidopsis thaliana*	Colombia
28	*B. oleracea* var. *alboglabra*	Chinese kale
29	*B. oleracea* var. *green duke*	broccoli
30	*B. oleracea* var. *nedcha*	cauliflower
31	*B. oleracea* var. *gower*	Brussels sprout
32	*B. rapa*	Kenshin-402

[a] *B. rapa* Kenshin-402 was a mapping line provided by the *Brassica* IGF Project. Seed production method: C, conventional; RH, restored hybrid; 3H, three-way hybrid; VA, varietal association; TC, top-cross hybrid (as defined by HGCA, http://www.hgca.com).

the expected insert size were excised from agarose gels, purified as above, and submitted for DNA sequencing using M13 primers (University of Dundee Sequencing Service, http://www.dnaseq.co.uk/). All sequences were obtained using joined forward and reverse sequences. For one SINE locus, 168-169, the 3′ flank sequence was used to design primers for an SPCR from the flank toward the SINE, thus obtaining the entire SINE element sequence and the 5′ flank. For one other SINE locus, 186-187, which was polymorphic, the 5′ and 3′ flank primers were used to amplify by PCR the “empty” locus in a pool of varieties for which the SINE was absent.

Real-Time PCR. Primers and probes for Taqman PCR were designed for two cloned SINE loci, which were present in HEAR and absent in LEAR certified seeds (**Table 1**). Relative quantification of the QSINEs was done using the ΔC_T (cycle threshold) method. A *B. napus* real-time PCR reference sequence, OSR2A, was used, which was selected from the International Brassica Genome Project probe sequence database because it is a single-copy sequence (probe accession, At1g53101_0). ΔC_T equalled the difference between the SINE and reference C_T value. A dilution series of HEAR variety DNA diluted in LEAR variety DNA (Hearty and Winner, respectively) was used for calibration in each real-time PCR run. The linear regression of ΔC_T versus log percent HEAR DNA was used to calibrate the relative quantification in the dynamic range from 100 to 0.5%.

As part of the Defra funded project, RG0125, 64 samples were taken in a regularly spaced grid pattern from each of 8 oilseed rape fields. The variety of sown seed in each field was as follows (CS, certified seed; FS, farm-saved seed): fields 1, 3, and 4, Winner CS; field 2, Courage CS; fields 5, 7, and 8, Winner FS; field 6, Expert CS. Each field was planted as a LEAR crop; however, it was suspected that HEAR was present at a low level from volunteer plants of previous HEAR crops. From each sample point, 200 leaf samples were taken from 200 separate plants. A 5 mm diameter disk was cut from each leaf, and a single pool of 200 disks was made for each field sample point. DNA was extracted as above from a 400 μL aliquot of slurry from each homogenized, lysed sample. Two SINE loci, HEARsine1 and HEARsine3, were quantified in each sample.

Real-time PCR reactions were run in an ABI7900 machine with the following conditions: 20 μL total volume; 1× ABgene QPCR ROX mix (Abgene, part AB-1209); 0.25 μM forward and reverse primer; 0.125 μM probe; and 5 μL of sample DNA. Cycling conditions were as follows: 15 min at 95 °C (ABgene enzyme activation), followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C.

RESULTS AND DISCUSSION

SINEs as Multilocus Markers. Multilocus SINE SPCRs performed well as a potential method for the identification of oilseed rape varieties. Among the 20 varieties analyzed, using the 17 polymorphic markers that were generated, two pairs could not be distinguished (Royal and Recital; Winner and Shannon). **Figure 1** shows a neighbor-joining dendrogram illustrating the SINE marker relationships among the 20 varieties. Winter varieties form a single, relatively genetically similar clade, whereas spring varieties are more genetically distant. *B. oleracea* and *B. rapa* are the most genetically distant; however, only three $S1_{BN}$ SINEs were observed in *B. rapa* and four in *A. thaliana* among the polymorphisms that were scored, compared to an average of 8.15 and 9 in *B. napus* and *B. oleracea*, respectively. Given older genetic divergence, SINEs are not expected to resolve relationships efficiently because the number of markers of the same origin (i.e., in this case the $S1_{BN}$ family) will be low. The more distant genetic relationships shown are therefore less reliable. Although only one *B. rapa* variety was studied, these data suggest that $S1_{BN}$ proliferation occurred predominantly in the *B. oleracea* “C” genome of *B. napus,* although in a previous study a similar number of $S1_{BN}$ were suspected in *B. rapa* and *B. oleracea* (*20*).

Genetic diversity was estimated among all oilseed rape varieties as $H_e = 0.31$ (falling to 0.25 when spring varieties were excluded). This was lower than observed in other studies, $H_e = 0.56$ (*1*). Other than the clear division in diversity between spring and winter varieties, no other known factors appeared to contribute to genetic similarity. Factors examined were seed company of origin, date of first release, and type of breeding method used (conventional, hybrid, or varietal association).

All bands that were excised from gels and sequenced were found to be SINE retroposon in origin. **Table 3** gives the details and GenBank accession numbers of the 10 sequences obtained. All loci were found to be new and different from SINE loci described in previous studies. One sequence was obtained of an entire SINE insertion (168-169) by performing SPCR using primers in the 3′ flank obtained from an earlier sequence. Similarly, one sequence was obtained from the unoccupied SINE locus (152-153). On the basis of the 3′ SINE flank and the unoccupied locus, there was no apparent deletion or alteration of the insertion site, although 5′ SINE flank sequence would also be needed to be sure of this. **Figure 2** shows an alignment of the SINE sequences obtained within the 5′ end (*17*) and up to the variable length poly A region. The 5′ region of locus 214-215 was found to be truncated by 94 bp.

Compared to previous systems that used fragment labeling for detection, the SINE marker system described here produced fewer markers. In applications that would require a higher density (e.g., gene mapping), it is possible that increased resolution with acrylamide systems could increase the number of scorable markers per enzyme/primer combination. Alternatively, variations on the degenerate primary SPCR primer could be used to generate markers from other SINE families.

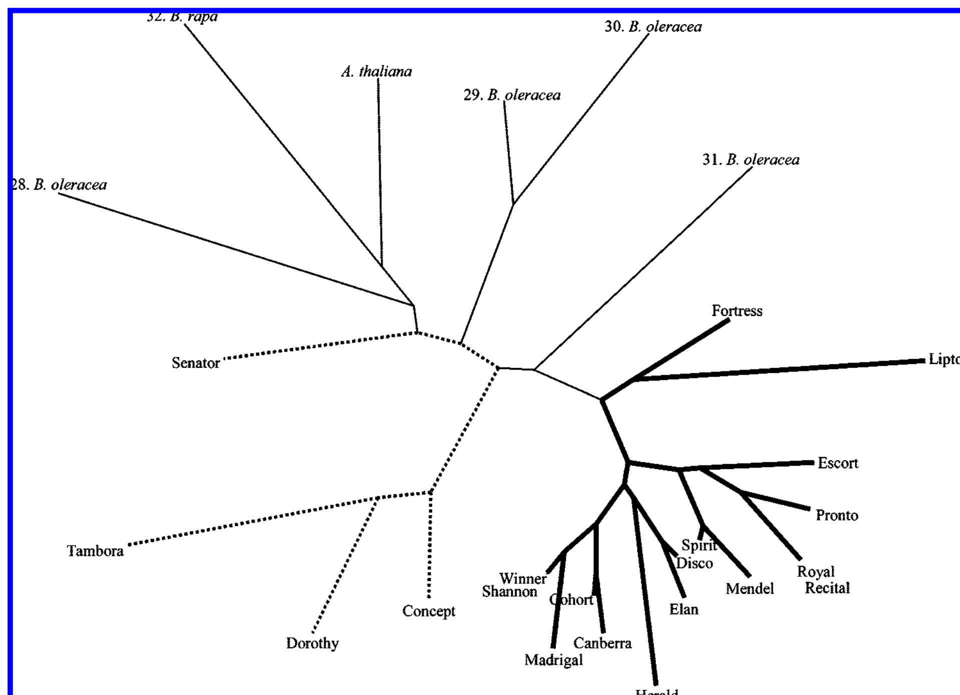

Figure 1. Neighbor-joining tree of pairwise Jaccard's genetic distances among *B. napus* varieties, progenitor species, and *A. thaliana*. Dashed branches are the spring-sown variety clade, and bold braches are the winter-sown variety clade. Branch lengths are all on the same relative scale.

Table 3. Sequenced SINE SPCR Fragments

clone	type	GenBank accession no.	length (bp)	source variety
216-217	5′ SINE and flank region	DQ825762	246	Hearty and Maplus pool Taqman assay, HEARsine3 target
214-215	5′ SINE and flank region	DQ648736	133	Maplus
220	5′ region of SINE only	DQ825763	140	Winner
154-155	3′ SINE, poly A and flank region	DQ825764	96	Hearty Taqman assay, HEARsine1 target
186-187	empty allele of SINE 152-153	EF121826	552	Canberra, Cohort, Disco, and Dorothy pooled
168-169	5′ and 3′ flanks including complete SINE insertion	DQ855619	419	Canberra, Cohort, Disco, Dorothy, Elan, Escort, and Fortress pooled
160-161	3′ SINE, poly A and flank region	DQ916280	200	Mendel and Pronto
156-157	3′ SINE, poly A and flank region	DQ916281	146	Concept, Recital, Royal
152-153	3′ SINE, poly A and flank region	DQ836225	187	Winner
KR16	3′ SINE, poly A and flank region	DQ916282	192	Recital and Royal

QSINEs in Oilseed Rape Fields. The two QSINE alleles, HEARsine1 and HEARsine3, were expected to be rare (<5%) in the studied fields and present only where LEAR seed was contaminated with HEAR seed or HEAR volunteers were present in the fields. However, a complex picture of their distribution emerged (see **Figure 3**). It was evident that the two SINE alleles selected were not specific to HEAR varieties. Two LEAR varieties, Courage and Expert, contained high levels of both alleles in their fields (more than would be expected from contamination). All fields were found to have some level of both SINEs present, except field 5, which contained no detectable HEARsine1. In four fields (1, 3, 4, and 8), the HEARsine alleles were predominantly present in a low background level (<5%) but showed structured clusters of high frequencies often at the edges or corners of the fields, areas where HEAR volunteers could be expected to persist. We would

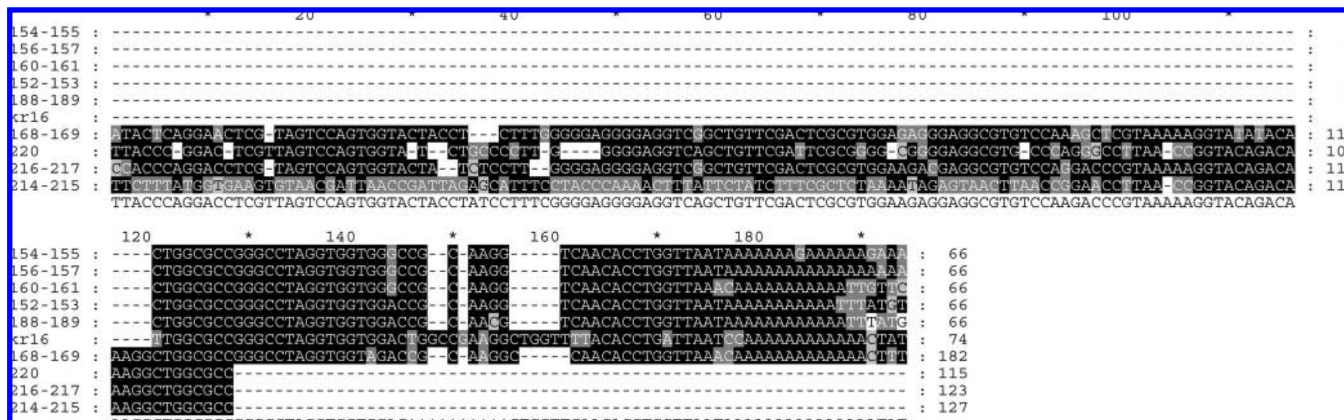

Figure 2. Alignment of sequenced SPCR SINE fragments.

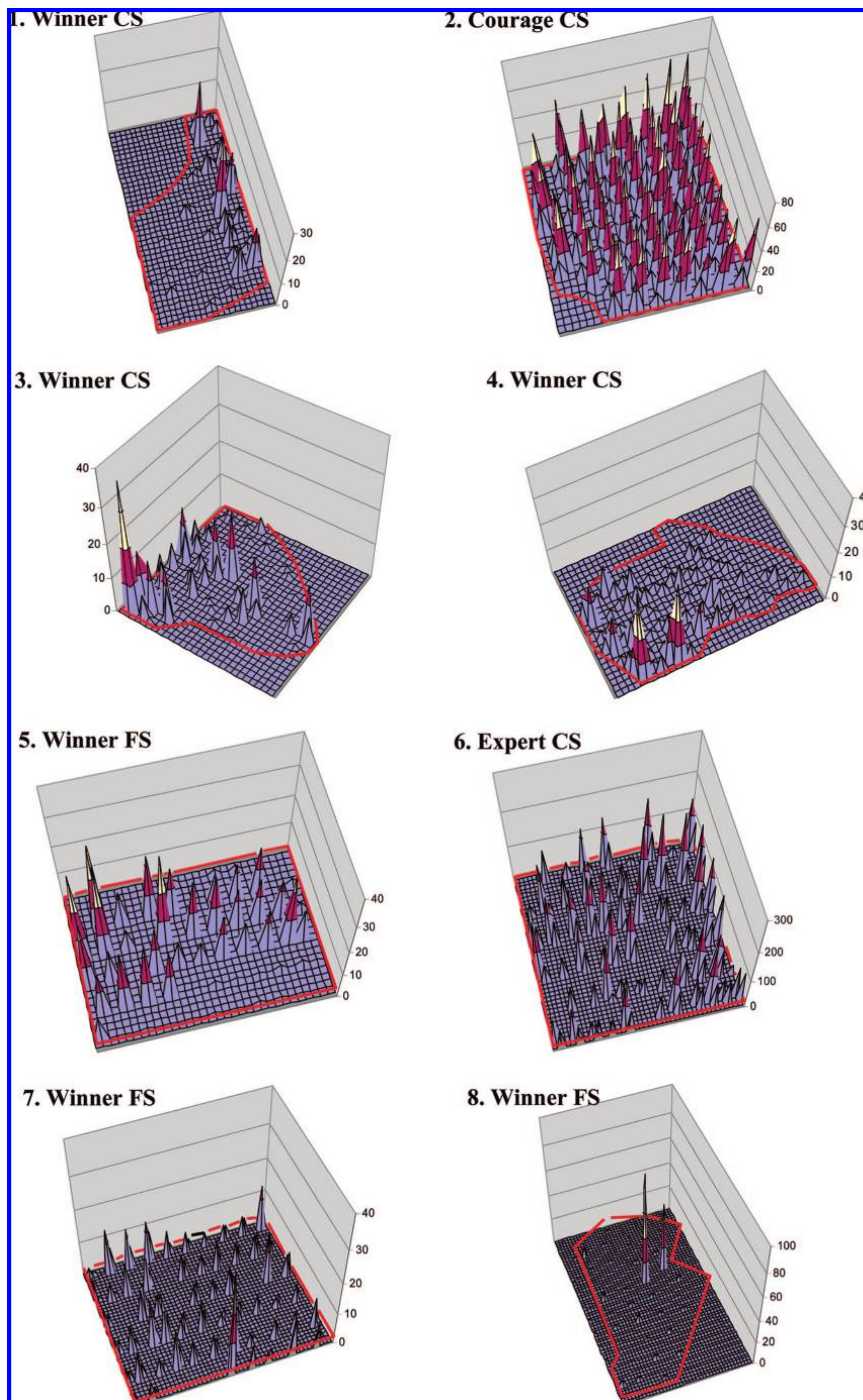

Figure 3. Spatial plots of combined HEARsine1 and HEARsine3 allele frequencies (plotted alongside each other at each sample point) measured by Taqman in eight fields from 2005 experiments. *Z*-Axes are percent SINE alleles compared to cv. Hearty leaf DNA. Note that each *Z*-axis is a different scale. Each graph square is approximately 15 m^2. Field borders are shown in red. Seed sown: CS, certified seed; FS, farm-saved seed.

Table 4. Mean Gene Diversity, H_e, of QSINE Markers, HEARsine1 and HEARsine3, Calculated for Each Studied Oilseed Rape Field[a]

	1	2	3	4	5	6	7	8
H_e	0.068	0.370	0.044	0.041	0.074	0.230	0.107	0.037
RSD	1.17	0.383	1.56	1.434	1.724	0.841	0.643	1.757
H_T	0.29							

[a] H_T = total heterozygosity over all fields. RSD = relative standard deviation of H_e.

normally expect processes up to crop sowing to effectively homogenize impurities within the seed. Field 8 in particular has two isolated, high HEARsine1 and HEARsine3 sample points. A high concentration of volunteers is the most likely explanation for this distribution.

The levels of HEARsine1 and Hearsine3 in comparable CS fields (1, 3, and 4) were significantly lower than in FS seed fields (5, 7, and 8) (ANOVA df = 339, $P = 0$; these fields could be compared because they contained the same variety). This indicates that farm saving of seed contributes to a less genetically uniform crop. However, other factors such as geographic origin could be responsible for this effect, because all CS fields were in a different region from the FS fields. Field 2, variety Courage, contained very high frequencies of HEARsine1 and HEARsine3, 17 and 51%, respectively, with no obvious spatial structure. Subsequent tests have shown that Courage certified seed normally contains a high frequency of these alleles (not shown). However, the alleles were not fixed, and diversity as measured by H_e was high (0.37, **Table 4**). This shows that at least some oilseed rape varieties (Courage is a "conventional", i.e., nonhybrid variety) maintain high genetic diversity, probably as a result of open pollination in their production. Field 6 (Expert) also showed high levels of both SINE alleles (133 and 65%) and high diversity (0.23, **Table 4**). Initially this field was identified as containing the variety Winner, but these frequencies prompted the reconfirmation of this information, and its mislabeling was subsequently confirmed by the farmer's records. It is not known whether the relative diversity in these two varieties was observed in U.K. DUS testing, which currently uses only morphological traits.

In this study, the SPCR of SINE loci successfully provided polymorphic molecular markers that could be used for variety identification using simple nonlabeled reactions. The use of a nested PCR with degenerate primary PCR primers was successful in amplifying the targeted SINE family. The method is easily adaptable to other SINE families or retroposons (or retrotransposons) in any species.

Using QSINEs, genetic diversity within varieties Courage and Expert was higher than expected. All other varieties possessed a low but significant level of diversity that prevented two SINE alleles from being used to distinguish HEAR from LEAR varieties. Due to the high diversity in varieties, it is likely that no single SINE could be used to achieve this. A multilocus approach could be more successful, but this was not attempted in this study because it was not suitable for quantification from large sample sizes. The QSINE quantitative markers developed in this study revealed genetic structure at small distance scales within the oilseed rape fields, possibly due to volunteer and/or seed contamination. Such markers could be useful to monitor genetic integrity of crops in, for example, nonfood crop coexistence and farm-saved seed scenarios. Quantitative genetic markers, such as QSINEs, have potential applications in many areas of population genetics, particularly in plant species for which fine spatial genetic structure may be of interest. The ability to accurately quantify an allele frequency from a single multi-individual pooled sample (e.g., leaf samples or seeds pools) enables many sample points to be examined simultaneously and factors such as fine-scale gene flow and extent of clones to be resolved. Temporal genetic diversity changes could also be more accurately resolved. The method could assist the study of the molecular ecology of any high population density species. The dynamics of hybrid zones and processes such as genetic drift and selective sweeps could be revealed within populations.

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