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PCR-based molecular markers for the fragrance gene in rice (Oryza sativa. L.)

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Abstract The genomic DNA clone RG28, linked to the major fragrance gene of rice (*fgr*), was assessed for polymorphism in order to produce a PCR-based marker for fragrance. A small mono-nucleotide repeat, that was polymorphic between a pair of fragrant and non-fragrant cultivars, was identified and developed into a co-dominant PCR-based marker. The polymorphism-information-content determinations for three microsatellite markers, that have been genetically mapped near RG28, are also presented. These PCR-based markers will be highly useful in distinguishing fragrance-producing alleles from non-fragrance-producing alleles at the *fgr* locus.

Keywords Fragrance · Rice, PCR marker · *Oryza sativa*

Introduction

Several chemical constituents are important to the aroma of cooked rice (Grosch and Schieberle 1997). However, 2-acetyl-1-pyrroline (AP) is regarded as the most important component of aroma in the basmati- and jasmine-style fragrant rices (Lorieux et al. 1996). AP is found in all parts of the rice plant, except for the roots (Lorieux et al. 1996), and is also found, at concentrations up to 100-times lower, in non-fragrant varieties (reviewed by Grosch and Schieberle 1997).

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Lorieux et al. (1996) reviewed the genetics of aromatic fragrance and concluded that a single recessive gene was responsible for the production of fragrant rice plants. This single recessive fragrance gene (*fgr*) was linked to the RFLP clone RG28 on chromosome 8, at a genetic distance of 4.5 cM (Ahn et al. 1992). Lorieux et al. (1996) confirmed the close linkage between RG28 and *fgr* (5.8 cM) and also identified two quantitative trait loci for fragrance, one on chromosome 4 and the other on chromosome 12.

Fragrance can be detected by tasting the associated flavour in individual seeds or assessing the aroma of leaf tissue or grains after either heating in water or reacting with solutions of KOH or I_2-KI (reviewed by Tragoonrung et al. 1996).

Tasting individual grains has been the preferred method for the quality selection of aromatic rice varieties within the Australian breeding program (Reinke et al. 1991). However, there are problems with the sensory detection of fragrance. There is considerable variation between analysts in their ability to detect fragrance or the associated flavour. Some individuals have difficulty in detecting the aroma or taste. A rapid decline of an individual's ability to distinguish between fragrant and non-fragrant samples with each analysis performed is also experienced. An analyst's ability declines as the senses become saturated or actual physical damage occurs. Abrasions to the tongue, causing bleeding, often result from chewing numerous seeds. Caustic substances such as KOH would also cause damage to the nasal passages. Sensory methods are therefore not suitable for processing large numbers of samples.

The chemical detection of AP is also possible but is time consuming and requires large samples (e.g. hundreds of grams, Lorieux et al. 1996; Widjaja et al. 1996). The development of a PCR-based molecular marker for the major component of fragrance (AP), for use within breeding programs, would have many advantages over sensory or chemical detection methods. Many more plants could be processed and sample sizes of 0.1 g or less could be analysed.

Plants from a breeding program for fragrant lines, in the early stages of cultivar development, could be assessed before maturity from small amounts of leaf tissue to reduce the number of seed samples tested by sensory methods. Non-fragrant or heterozygous individuals would be avoided for the sensory or chemical assessment of aroma or flavour quality, or tests for other grain-quality characteristics. As the recessive gene could be detected in a heterozygous state, the marker would be useful for the identification of offspring possessing *fgr* after backcrossing with homozygous non-fragrant plants.

We tested for polymorphism in homologous regions of the marker RG28 to develop a PCR-based marker for *fgr* for use in rice breeding. In addition, microsatellite markers previously mapped in the vicinity of *fgr* were assessed for polymorphism information content to provide alternative markers for fragrance.

Materials and methods

Test for monogenic inheritance of *fgr*

A population of 215 F_2 individuals derived from a cross between Kyeema (Pelde//Della/Kulu) (tall, jasmine-style fragrant, long-grain, Australian cultivar) and Doongara (Bluebelle/Calrose//Jojutla) (semi-dwarf, non-fragrant, long-grain, Australian cultivar) was transplanted to the field at Yanco Agricultural Institute, NSW Agriculture, Yanco, as glasshouse-grown seedlings in November 1997. Leaf material was collected in January 1998 and frozen for storage and DNA extraction at a later date. One hundred and twenty four F_2 plants were classified as fragrant, segregating, or non-fragrant by tasting de-hulled F_3 seed (ground between the front teeth before tasting). If five F_3 seeds were tasted and all were fragrant, the F_2 individual was considered fragrant, otherwise at least 12 seeds were tasted to separate segregating from non-fragrant F_2 individuals. The segregation ratio, of fragrant: segregating: non-fragrant, was tested by χ^2 analysis against the expected ratio for a single gene (1:2:1).

DNA extraction

DNA was extracted from approximately 0.6 g of frozen leaf tissue, as generally described by Weining and Henry (1995), for 50 F_2 individuals and parent cultivars (Kyeema and Doongara). The following modifications were included; 2 ml of 2.5% sarkosyl extraction buffer and 2 ml of phenol/chloroform/isoamyl alcohol (25:24:1) were used; DNA was precipitated with 1.3 vol of isopropanol in addition to 0.13 vol of 3 M sodium acetate (pH 4.8) and then washed twice with 70% ethanol, and the DNA was re-suspended in 200 µl of Tris-EDTA (TE) buffer. DNA preparations were diluted with TE buffer to a final concentration of approximately 50–100 ng per µl.

Supply and investigation of the RFLP probe, RG28

The probe RG28 (rice-etiolated-leaf genomic library, *Pst*I-restricted, pUC9 vector, cultivar IR36) was supplied by Dr. Susan McCouch and the Cornell Research Foundation Inc., New York. The clone was bi-directionally sequenced using commercial pUC DNA sequencing primers and the ABI Prism, BigDye Terminator Cycle Sequencing Ready Reaction Kit. Gel separation and electropherogram production were performed by the Australian Genome Research Facility, University of Queensland.

Probe-specific PCR primers were designed with the aid of MACVECTOR 6.0, Sequence Analysis Software, Oxford Molecular Group Inc. Primers were synthesised by Pacific Oligos Pty. Ltd., SCU, Lismore, NSW, Australia. PCR reactions using template DNA from Kyeema and Doongara were carried out on a Perkin Elmer, Gene Amp PCR System 9700. The reaction volume was 20 µl containing 1× Boehringer Mannheim PCR Buffer (1.5 mM $MgCl_2$), 50–100 ng of genomic DNA and 200 µM of dNTPs. The PCR reaction mix also included 150 nM of each primer, 2 mM $MgCl_2$, and 0.5 units of *Taq* DNA Polymerase (Boehringer Mannheim). The temperature cycling conditions were 1 min at 94°C; followed by 35 cycles of 94°C for 30 s, 54°C for 30 s and 72°C for 1 min; with a final hold at 72°C for 5 min.

PCR products were purified with a QIAquick PCR Purification Kit (QIAGEN) and sequenced as discussed above. Sequence polymorphism between Kyeema and Doongara, the parents of the mapping population, was identified. Primers flanking the polymorphic region were designed and synthesised as discussed above, except that the forward primer was 5′end-labelled with the fluorescent phosphoramidite dye, HEX (Perkin-Elmer). Sizing of the PCR products was performed on an ABI Prism 310 Genetic Analyser.

Linkage assessment

Fifty F_2 individuals from the mapping population were genotyped for the polymorphic marker. The genetic distance between *fgr* and the linked marker was determined as the percentage of recombinant chromosomes (cM).

Homozygous bulks of fragrant and non-fragrant F_2 plants derived from a cross between the breeding lines YRL106 [(Bluebonet50/Calrose//C.1.9187)/M7]//[(Bluebelle/Calrose)//Jojutla] and YRF203 (Basmati 370//Pelde*2/M9) were also produced. Bulked-segregant analysis was undertaken to obtain further evidence for a single recessive gene, *fgr*, being responsible for fragrance originating from a basmati source. Homozygous bulks were produced by the identification of non-segregation for the presence or absence of fragrance in the F_3 seed. DNA extractions, as described above, were prepared for the parent lines (each containing five bulked individual plants) and two bulks, one of five homozygous fragrant F_2 individuals and one of eight homozygous non-fragrant F_2 individuals.

The F_2 population (YRL106/YRF203) was part of the general rice-breeding program and one of many such plots planted in the field. No phenotypic or genetic assessment was performed on the parent or the F_1 plants. Bulked segregant analysis of a marker not linked to *fgr* was performed to test that the population was segregating at other genome regions.

Two alleles were amplified for the marker RM163 (Wu and Tanksley 1993) in both bulks (131 bp from YRF203 and 164 bp from YRL106), indicating that the plants in the bulks were segregating as expected for a sample from an F_2 population (Fig. 1).

Microsatellite markers and assessment of polymorphism

Markers RM42 and RM223 (Chen et al. 1997) and RM33 and RM85 (Cho et al. 1998) were selected in addition to the marker developed from RG28, for the assessment of polymorphism across 24 rice samples. Markers RM33 and RM85 have been genetically mapped between the probes RG28 and RG1 (Cho et al. 1998). RG28 and RG1 are linked at a map distance ranging from 10 cM (Causse et al. 1994) and 12 cM (Lorieux et al. 1996) to 25.5 cM (Cho et al. 1998). RG28 and RG1 flank the genome region that contains *fgr*; therefore, RM33 and RM85 are closely linked to the fragrance gene.

RG28 was not mapped by Chen et al. (1997) and Xu et al. (1998); however, the location of RG28 and *fgr*, in relation to RM42 and RM223, can be estimated based on the relative position of RG1 and other nearby markers, in these genetic maps, and in the genetic maps of Cho et al. (1998), Lu et al. (1996) and Causse et al. (1994).

RG28 probably lies between RM42 and RM223. RM42 may therefore be a few cM further from *fgr* than RG28, and RM223 is

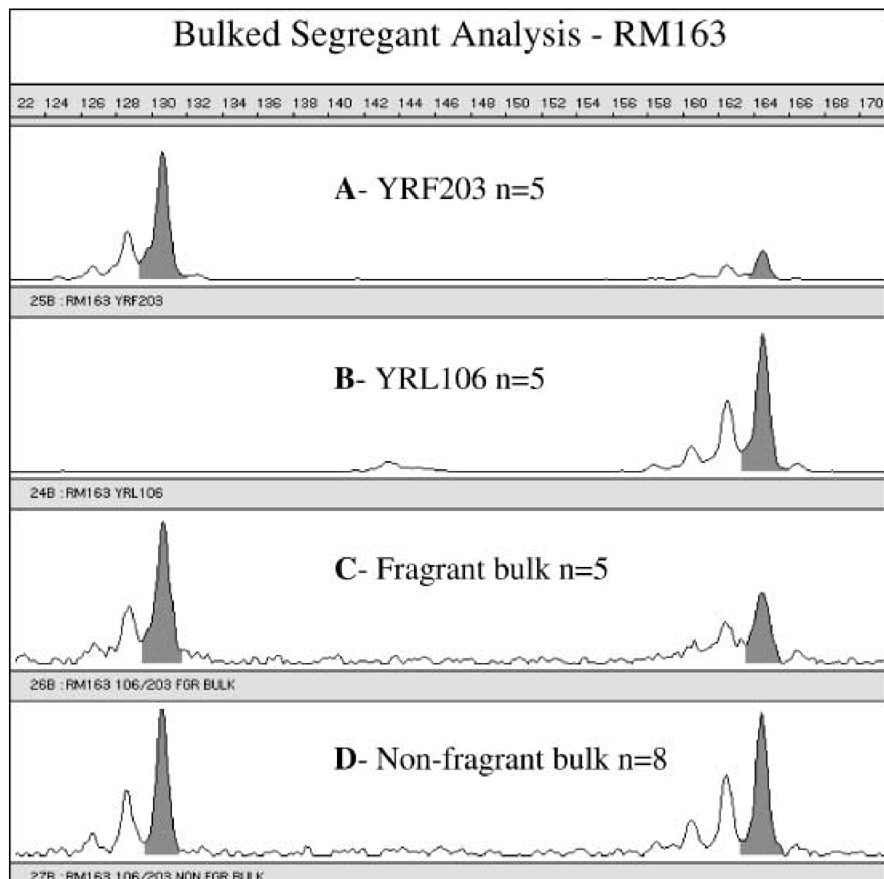

Fig. 1A–D Capillary electrophoresis output demonstrating the results of bulked-segregant analysis for the unlinked marker RM163 (scale in bp). *n*=the number of individual plants used to produce the DNA preparation. **A** The alleles present in the fragrant breeding line YRF203 (131 and 164 bp), **B** the allele present in the non-fragrant breeding line YRL106 (164 bp), **C** alleles from both breeding lines amplified in the bulk of five fragrant homozygous F_2 individuals (YRL106/YRF203), **D** alleles from both breeding lines amplified in the bulk of eight non-fragrant homozygous F_2 individuals (YRL106/YFR203)

possibly closer. In most cases these microsatellite markers are likely to be within 10 cM of *fgr* and therefore useful for gene-tagging within a breeding program.

The 24 rice samples analysed included 16 Australian breeding lines or Australian commercial cultivars. Half of the 24 samples were fragrant lines. Rice seed samples were supplied by Yanco Agricultural Institute (YAI), New South Wales Agriculture, Yanco, Australia [see Ko et al. (1994) for some of the Australian pedigrees; others are available through the YAI if required]. Five plants for each variety were grown in a glasshouse at Southern Cross University, Lismore, NSW, Australia. Approximately 1–3 cm of mature leaf was sampled from each of the five plants for each cultivar. The leaf material from the five plants for each cultivar was bulked producing a total weight of about 0.3 g. The DNA was then extracted as described above.

Primer sequences for RM42 and RM223 are available from Chen et al. (1997). Primer details for RM33 and RM85 were kindly supplied by Professor Cho Yong-Gu, Department of Agronomy, Chungbuk National University, Korea. Primers were synthesised by Pacific Oligos Pty. Ltd., SCU, Lismore, NSW, Australia. PCR reactions were carried out on a Perkin Elmer, Gene Amp PCR System 9600 or 9700. The reaction volume was 20 µl containing 1× Boehringer Mannheim PCR Buffer (1.5 mM $MgCl_2$), approximately 50 ng of genomic DNA, 200 µM of dNTPs and 200 nM of [R110] dUTP (Perkin Elmer). The PCR reaction mix also included 100 nM of each primer, 3 mM MgCl2, and 0.5 units of *Taq* DNA Polymerase (Boehringer Mannheim). The temperature cycling conditions for the microsatellite markers were 1 min at 94°C followed by 30 cycles of 94°C for 15 s, 54°C for 15 s and 72°C for 1 min.

Sizing of microsatellite alleles was performed on an ABI Prism 310 Genetic Analyser with the following run and analysis conditions (Module GS STR POP4 C, 20-min run time, size standard GENESCAN 500 – TAMRA, Local Southern Sizing method, Capillary-length-to-detector 30 cm). The sizing of DNA fragments was relative and not necessarily an absolute measure of size.

Data analysis

Polymorphism information content values (PIC) were calculated for each marker using Nei's Gene Diversity measure H_S (Nei 1973)

$$H_s = 1 - \sum_{k=1}^{n} x_k^2,$$

where x_k is the frequency of the kth allele.

Results

Development of a PCR-based marker from RG28

The sequence of the clone RG28 is presented in Fig. 2. The probe-specific primers SCU-Rice-STS-RG28.F (5′-TGCCAAGTATCCCCTGATTCC-3′) and SCU-Rice-STS-RG28.R (5′-TTTGTGCCTCCTTTGCAGATTC-3′) were designed and found to amplify a fragment of appropriate size (similar to the 754 bp as determined in the probe RG28) for Kyeema and Doongara, the parent cultivars of the mapping population (see Fig. 2 for primer locations).

The sequences for the clone RG28 and the genome regions homologous to RG28 in Kyeema and Doongara

Fig. 2 DNA sequence for the clone RG28 and homologous regions for the rice varieties of Kyeema and Doongara. Note the polymorphic T repeat at positions 727 to 735. (–) represents a deletion or no data. (.) represents the same base as RG28. The *bold sequence* represents primer-binding regions and in order of increasing base position represent the primers SCU-Rice-STS-RG28.F, SCU-Rice-SSR-1.F, SCU-Rice-SSR-1.R (reverse complement) and SCU-Rice-STS-RG28.R (reverse complement). The *lower case sequence* is in some doubt

Source	Base Position	DNA Sequence
RG28	121-180	ACTAAACTACTCCAGTGGTATTAAT**TGCCAAGTATCCCCTGATTCC**CCTACAATTCAAAA
Kyeema		--
Doongara		--
RG28	181-240	TATGTACTCCAATTCTAGGACTTAGCACTATGACCAAAAGCAGAGGGTATACAAGGTTGA
Kyeema		-----------...
Doongara		--..
RG28	241-300	AAATGGATAAGGATCTGAATATAAATTGATGGTGTTTTTCATACTTAAAAAGGAAGAAGA
Kyeema		....
Doongara		.................a..
RG28	301-360	CACAGCATGGCTAAACCAAAACAGAAAAGAAGCTAATACACATATGCATGGTGACAAAAA
Kyeema		....
Doongara		....
RG28	361-420	TTTCACATTGCAATGATAACCTCTTCTCTGATTGGTAGATTGGTAAATAGCAGCACTAGT
Kyeema		....
Doongara		....
RG28	421-480	AGTGGAAGTACAAGCATGAAAACATACCTTCCATCTTGGAGATATGCTTCCTCACTTTCC
Kyeema		....
Doongara		....
RG28	481-540	CTGCACACCCCTGGCAGTGAATAGCCACCTTCATGACCACCACCTGCAAATACATACATA
Kyeema		....
Doongara		....
RG28	541-600	ATGCACGCTGTCAACCGCCAAGCAAAGCCCAATATCAAATCAAGAAGATTCCCAGCAAGA
Kyeema		....
Doongara		....
RG28	601-660	ACAGTGTGAGAGTGCGTGAGTGAGTAGCTGTGAGAGCAACCAAAGAAACAGCATCATC**GA**
Kyeema		....
Doongara		....
RG28	661-720	**TCTCACTCCAAGTAAACTCTGAC**TTACACAGTTACACAGTGATTCTATCCCTGCATATAT
Kyeema		....
Doongara		....
RG28	721-780	ATATAATTTTTTT-ACACAGTGGTTTTTATCCTTCTTTTTCAATAC**GAGAACAGAAGCA**
Kyeema		.............--...
Doongara		..............T...
RG28	781-840	**ATGGCAGT**AACAGACTAACAGTCGTAACTCGTAACAGCAACAGCAACAATGGAGTAGAGA
Kyeema		....
Doongara		....
RG28	841-900	GAAACAGAGAGAGATGGCGATGCATCAGTGGCTAGTCT**GAATCTGCAAAGGAGGCACAAA**
Kyeema		..............--
Doongara		.............---
RG28	901-916	GTCTCCATTATCTCTT
Kyeema		----------------
Doongara		----------------

were identical except for a small microsatellite-mono T repeat (Fig. 2). Doongara had nine tandem-T repeats, RG28 had eight and Kyeema seven.

The primers SCU-Rice-SSR-1.F (HEX labelled) (5′-GATCTCACTCCAAGTAAACTCTGAC-3′) and SCU-Rice-SSR-1.R (5′-ACTGCCATTGCTTCTGTTCTC-3′) were designed to flank the microsatellite region producing an expected product of 130 bp for Doongara (marker SCU-Rice-SSR-1). The primers amplified a single product that was determined as 125 bp in Kyeema and, as expected, was 2 bp larger in Doongara (Fig. 3) (note, the sizing of the 310 Genetic Analyser is not necessarily an absolute measure when using a general size standard). PCR conditions for SCU-Rice-SSR-1 were the same as those for the RM prefixed markers without the addition of fluorescent dUTPs.

Segregation ratios and linkage assessment

Segregation ratios for the fragrance genotypes were found to be consistent with that of a single fragrance gene (Table 1). The microsatellite marker SCU-Rice-SSR-1 was closely linked to the fragrance gene in the mapping population (See Fig. 3 for a display of the genotypes observed). Four recombinants were found in the 50 F_2 plants (4 cM). The four recombinants included one individual homozygous for *fgr* and heterozygous for SCU-Rice-SSR-1, and three homozygous non-fragrant individuals that were heterozygous for SCU-Rice-SSR-1.

Table 1 Observed segregation ratio for 124 F_2 individuals (Kyeema/Doongara) and the expected segregation ratio for a single gene; χ^2 (0.05, *df*=2) analysis indicated no significant difference between observed and expected ratios, P=0.12

Number	Fragrant	Segregating	Non-fragrant	Significance
Observed	28	55	41	Not significant
Expected	31	62	31	

The microsatellite marker RM85 did not amplify and may require further optimisation or else the design of new primers. RM33, RM42 and RM223 were not polymorphic between Kyeema and Doongara and could not be assessed for linkage to *fgr* in the mapping population.

Bulked segregant analysis suggested linkage of SCU-Rice-SSR-1 (Fig. 4) and RM223 (Fig. 5) to *fgr* in the F_2 population of YRL106/YRF203 (Basmati source of *fgr*).

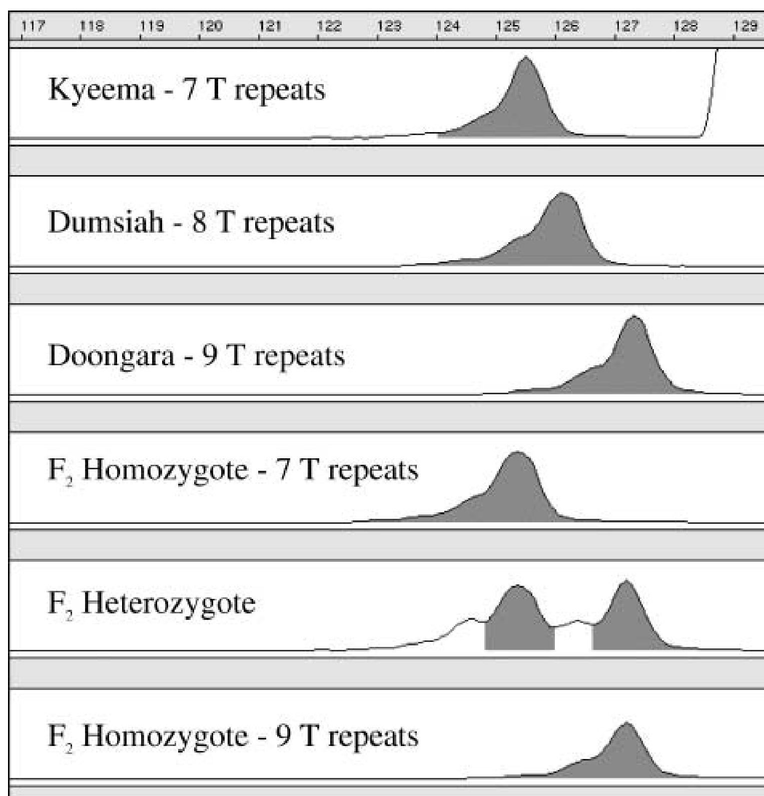

Fig. 3 Capillary electrophoresis output, demonstrating alleles for the marker SCU-Rice-SSR-1 for the varieties Kyeema, Dumsiah and Doongara. The horizontal scale is in bp. The peak to the right of the Kyeema allele is an artefact. Results are also presented for two homozygous individuals and one heterozygous F_2 individual from the mapping population (Kyeema/Doongara)

Only the allele from YRF203 amplified for the fragrant bulk (n=5, SCU-Rice-SSR-1 127 bp, RM223 151 bp) and only the allele from YRL106 amplified for the non-fragrant bulk (*n*=8, SCU-Rice-SSR-1 125 bp, RM223 145 bp). Sensitivity testing indicated that a specific allele could be detected in a DNA sample with the allelic proportions of one specific allele from one parent of the mapping population to nine alleles from the other parent (Figs. 4 and 5). This result indicates that no recombinants between *fgr* and SCU-Rice-SSR-1 or RM223 were detected for the bulk of five fragrant individuals (ten alleles sampled) and at most one or none for the bulk of eight non-fragrant individuals (16 alleles sampled). In total that represents, at most, one recombinant in 13 individuals or a genetic distance of about 4 cM (one recombinant chromosome in 26 or approximately four in 100). Although the sample size is small, close linkage is indicated.

RM42 and RM33 were not polymorphic between YRL106 and YRF203, and could not be analysed.

Polymorphism information content

Alleles for the markers SCU-Rice-SSR-1, RM223 and RM42, for a range of fragrant and non-fragrant rice samples, are presented in Table 2. RM33 was not polymorphic (PIC=0). The three alleles found for SCU-Rice-SSR-1 in the fragrant cultivars represented seven, eight and nine T repeats (PIC=0.48). Two alleles were found in the non-fragrant cultivars, the 9 bp allele predominated and only the breeding line YRK4 possessed the sec-

Table 2 Microsatellite alleles in bp (based on 310 Genetic Analyser results) for the markers SCU-Rice-SSR-1, RM42 and RM223 for a range of fragrant and non-fragrant cultivars. PIC, polymorphism information content; * more alleles may be present

Fragrant cultivars	SCU-Rice-SSR-1	RM42	RM223
Kyeema	125	167	151
Goolarah	125	167	145
Khao Dawk Mali 105	125	167	145
Azucena	125	167	147
Millagrossa	126	160, 169	145, 153*
Dumsiah	126	160	147
Moosa Tarom 110	126, 127	160	149
Dumsorkh	127	160	149
Basmati 370	127	160	149
YRF203	127	167	151
YRF204	127	167	151
Della	127	165, 167	149, 151
Non-fragrant cultivars			
Doongara	127	167	151
Amaroo	127	165	149
Echuca	127	165	149
Illabong	127	165	147
Inga	127	165	149
Jarrah	127	165	149
Langi	127	165	149
Millin	127	165	147, 149
Namaga	127	165	149
Pelde	127	165	149
YRW4	127	165	149
YRK4	126	165	140, 149
Number of alleles	3	4	6
PIC value	0.48	0.64	0.66

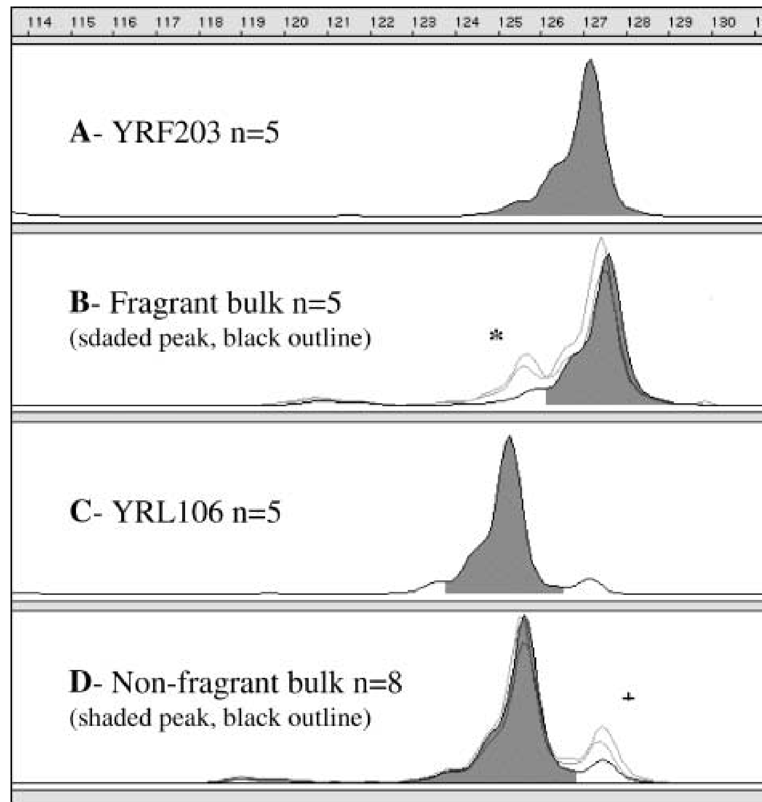

Fig. 4A–D Capillary electrophoresis output demonstrating the results of bulked segregant analysis for the marker SCU-Rice-SSR-1. The horizontal scale is in bp. *n*=the number of individual plants used to produce the DNA preparation. **A** The allele present in the fragment breeding line YRF203 (127 bp). **B** Only the allele from YRF203 appears to have amplified in the bulk of five fragrant homozygous F_2 individuals (YRL106/YFR203), (*) the superimposed peaks (faint) are the amplification products for a DNA preparation containing approximately one 125-bp allele to four alleles of 127 bp (the higher faint peak at 125 bp) or nine alleles of 127 bp (the lower faint peak at 125 bp). The 125-bp allele can be distinguished by PCR in the proportions of one at 125 bp to nine at 127 bp. **C** The allele present in the non-fragrant breeding line YRL106 (125 bp), **D** only the allele from YRL106 appears to have amplified in the bulk of eight non-fragrant homozygous F_2 individuals (YRL106/YFR203), (+) the superimposed faint peaks are the amplification products for a DNA preparation containing approximately one 127-bp allele to four alleles of 125 bp (higher-faint peak at 127 bp) or nine alleles of 125 bp (lower faint peak at 127 bp). The 125-bp allele can be distinguished by PCR in the proportions of one at 127 bp to nine at 125 bp

ond allele of 8 bp. The markers RM223 (PIC=0.66) and RM42 (PIC=0.64) were more polymorphic. Forty eight percent of the possible 144 pair-wise comparisons between the fragrant and non-fragrant cultivars for SCU-Rice-SSR-1 did not produce a distinguishing allelic difference. For this same assessment, values of 12.5% for RM42, 33.3% for RM223 and 8% for all three markers combined were determined. The rice samples that could not be comprehensively distinguished by allelic differences were Della from Doongara, Amaroo, Echuca, Inga, Jarrah, Langi, Millin, Namaga, Pelde and YRW4; and YRF203 and YRF204 from Doongara.

Discussion

The source of fragrance in Kyeema is most likely *fgr*, as similar linkage values were identified between *fgr* and RG28 in this investigation, and by Ahn et al. (1992) and Lorieux et al. (1996). The gene *fgr* was expected as Della is the source of fragrance for both Kyeema and Aromatic Lemont. Aromatic Lemont was the fragrant cultivar used by Ahn et al. (1992) to create the mapping population employed to identify the linkage between RG28 and *fgr*. Della is also the source of fragrance for the Australian lines Goolarah and YRF204.

Lorieux et al. (1996) concluded that fragrance in all varieties of aromatic rice, both jasmine and basmati style, is determined by alleles of the single recessive gene, *fgr*. However, they indicated that contradictory results were common in the literature, with the genetic model for fragrance reported as monogenic to polygenic with a dominant or recessive character. Lorieux et al. (1996) suggested that the contradictory results were due to incorrect assessment of the endospermic character in F_2 populations and problems associated with segregation distortion in double-haploid or backcross populations. In addition, all studies that used molecular markers (enabling problems with segregation distortion to be detected) identified a single recessive gene.

Further evidence for the single recessive gene *fgr* being responsible for fragrance originating from a basmati source was obtained in this investigation by bulked segregant analysis. Given that SCU-Rice-SSR-1, RM223

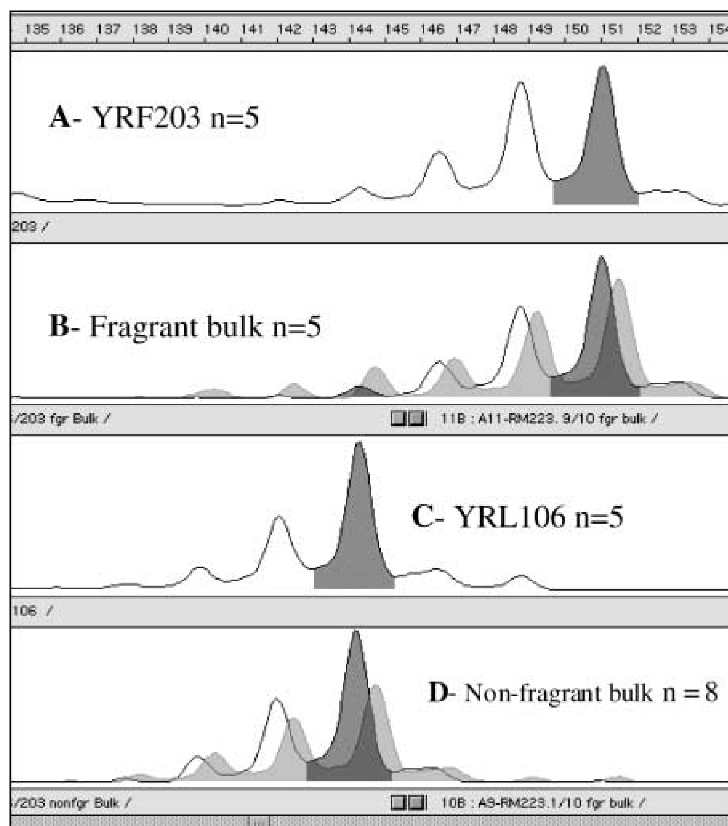

Fig. 5A–D Capillary electrophoresis output demonstrating the results of bulked segregant analysis for marker RM223. The horizontal scale is in bp. *n*=the number of individual plants used to produce the DNA preparation. **A** The allele present in the fragment breeding line YRF203 (*dark shading*, 151 bp). **B** Only the allele from YRF203 appears to have amplified in the bulk of five fragrant homozygous F_2 individuals (YRL106/YFR203), The *lightly shaded and offset product* represents the amplification from a DNA preparation containing approximately one 145-bp allele to nine alleles at 151 bp. The 145-bp allele can be distinguished by PCR in the proportions of one at 145 bp to nine at 151 bp based on the relative increase in height for the 145-bp peak and the presence of distinct peaks smaller than 145 bp. **C** The allele present in the non-fragrant breeding line YRL106 (*dark shading*, 145 bp), **D** only the allele from YRL106 appears to have amplified in the bulk of eight non-fragrant homozygous F_2 individuals (YRL106/YFR203). The *lightly shaded and offset product* represents the amplification from a DNA preparation containing approximately one 151-bp allele to nine alleles at 145 bp. The 151-bp allele can be distinguished by PCR in the proportions of one at 151 bp to nine at 145 bp, based on the presence of distinct peaks at 149 bp and 151 bp

and RM42 are most-likely suitable markers for all sources of fragrance (*fgr*) and the large proportion of fragrant and non-fragrant lines distinguished, these markers will be highly useful in the Australian rice breeding program and in rice breeding programs in general. Della was the only fragrant variety tested with low levels of separation from the non-fragrant lines. This was due to the identification of two common alleles for the markers RM223 and RM42. Markers for *fgr* in Della individuals will be distinguishable from alleles in non-fragrant lines in some cases, depending on which allele(s) for the marker is present for the individual specimen. The heterogeneity in the Della sample was not unexpected as it is susceptible to out-crossing (Garland et al. 1999). As heterogeneity was detected in several samples, it is recommended that further screening be performed for different accessions or seed samples to make the allelic characterisation comprehensive. In addition, it would be wise to verify polymorphisms between parents of a cross before a program of marker-aided selection is undertaken. The fragrance gene is likely to be characterised in the future due to the international effort to sequence the rice genome. Although a marker for the actual gene will then be available, the markers discussed here will still have utility in marker-assisted excerises to incorporate *fgr* into a more-desirable genetic background. Progeny from such an exercise of repeated backcrossing with a superior cultivar, could be selected to minimise the chromosome region from the varietal source of *fgr* that contains the fragrance gene, by the selection of individuals possessing *fgr* that also contain alleles for the linked markers that originate from the recurrent parent.

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