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C. Cekic · N.H. Battey · M.J. Wilkinson

The potential of ISSR-PCR primer-pair combinations for genetic linkage analysis using the seasonal flowering locus in *Fragaria* as a model

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Abstract ISSR-PCR has been widely used for genetic distance analysis and DNA fingerprinting but has been less well utilised for mapping purposes. A key limitation lies in the small number of primer designs available to generate useful polymorphisms. In this study, the potential of paired combinations of ISSR primers is evaluated using a test cross mapping population of 168 BC_1 individuals between *Fragaria vesca* f. *vesca* and a closely related line *F. vesca* f. *semperflorens*. Ten ISSR primers and all possible pairwise combinations between them were used to generate markers potentially linked to the locus controlling seasonal flowering in *F. vesca*. Band profiles of individual primers were found to be highly reproducible for band position and intensity, and only minor variation was noted in band intensity (but not in position) between different constituent mixes of primer-pair combinations. Overall, ISSR primers used in isolation produced 85 markers of which only five were specific to *F. vesca*. None of these markers were linked to the seasonal flowering locus. In contrast, the primer-pair combinations yielded 493 markers, including 14 specific to *F. vesca*. These markers included two located within 2.2 cM of the seasonality locus. The strengths and limitations of using pairs of ISSR primers in combination for mapping and other genetic analyses are briefly explored.

Keywords *Fragaria vesca*, Seasonal flowering · ISSR-PCR · Bulked segregant analysis · Genetic linkage

Introduction

Simple sequence repeats (SSRs), also known as microsatellites, consist of tandem arrays of short oligonucleotide sequences 2–6 bases in length. SSRs are extremely common throughout the eukaryotic genome (Tautz and Renz 1984) and are highly polymorphic in length (Levinson and Gutman 1987). Inter simple sequence repeat PCR (ISSR-PCR) exploits the abundance of SSRs to generate complex banding profiles that vary both within and between species. ISSR primers contain sequences complementary to a SSR motif and also a 1–3 base 'anchor' at either the 3′ or 5′ end. Base positions within the anchor may contain any nucleotide other than that needed to continue the repeat sequence. Thus, the first base of an anchor for a 'CA' repeat could be G, T or A but not C. The anchor thereby ensures that the primer binds only to one end of a targeted SSR and not in the middle. The many amplification products generated by ISSR-PCR consist largely of the region between neighbouring and inverted SSR sites. Zietkiewicz et al. (1994) first used this approach to distinguish between closely related plant genotypes but it has since been applied for cultivar identification (Wolff et al. 1995; Kantety et al. 1995; Charters et al. 1996), to detect gene-flow and introgression (Timmons et al. 1996; Allainguillume et al. 1997) and for mapping purposes (Kojima et al. 1998). The utility of this approach stems from its simplicity and reproducibility, the high number of polymorphisms revealed and the fact that there is no prior need for DNA sequence information from the organism under study. A key limitation lies in the relatively small number of primers that generate useful polymorphisms. For example, Nagaoka and Ogihara (1997) screened 100 primer designs but found polymorphisms had been generated in only 33. Of these, 90% were comprised of primers targeting CA/GT or GA/CT motifs. Other workers have also found that primers targeting these types of motif account for almost all of the variation revealed (e.g. Esselman et al. 1999). The other dinucleotide motif types (AT/TA, GC/CG) have usually yielded no or only small amounts of variation, partly because of the strong tendency of these primers to form dimers and 'hair-pin' structures. Primers complementary to longer motifs such as tri- or tetra-nucleotide repeats, target sequences that are less abundant within the genome and therefore tend

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C. Cekic · N.H. Battey · M.J. Wilkinson (✉)
School of Plant Sciences, The University of Reading, Whiteknights, P.O. Box 221, Reading, RG6 6AS, U.K.
e-mail: m.j.wilkinson@reading.ac.uk

to generate fewer amplicons. There is also considerable scope for redundancy and duplication of the loci amplified. For example, a primer targeting CT repeats with a one-base G anchor at the 3′ end would target exactly the same sites as one targeting GA repeats with a C anchor at the 3′ end. Sites targeted by primers with a 3' anchor may duplicate some of those targeted by primers anchored at the 5′ end. There is also scope for more cryptic duplication of amplification products. Tolerance of a base mismatch in the third position of the anchor (5′ end), even when high annealing temperatures are used, means that two different anchors can generate amplicons from the same locus. Whilst it might be argued that these considerations have only marginal importance for applications such as DNA fingerprinting or genetic distance analysis, they are of far greater significance for mapping purposes. The generation of extra variability using ISSR primers may therefore enhance the ability of the technique to distinguish between closely related genotypes and, at the same time, reduce the need to screen large numbers of primers. Furthermore, the ability to produce additional amplicons deriving from different loci would greatly enhance the value of the technique for mapping purposes.

Fragaria vesca L. is a diploid wild species closely related to the octaploid *F. X ananassa* (cultivated strawberry). There are two forms of the diploid: one exhibits seasonal flowering (*F. vesca* f. *vesca*), the other is continually in flower (*F. vesca* f. *semperflorens*). A single gene controls the trait and the dominant allele confers seasonal flowering in *F. vesca* f. *vesca* (Brown and Wareing 1965). The identification of markers that are closely linked to this gene represents the first step towards its ultimate isolation by map-based cloning. In this study, we first examine the ability of ten ISSR-PCR primers to generate polymorphisms between the two forms of *F. vesca* and of a F_1 hybrid between them. Polymorphisms are screened on a BC_1 generation by bulk ed segregant analysis to identify markers putatively linked to the seasonality locus. ISSR primers are then used in pair-combinations to establish whether additional polymorphisms can be generated. The origins of some amplicons are verified by cloning and sequence analysis. Finally, the genetic map distances are calculated between all selected markers and the locus controlling seasonal flowering.

Materials and methods

Plant material

F. vesca f. *vesca* was obtained from the breeders' germplasm collection at Horticulture Research International, East Malling, U.K. The other form of *F. vesca* (*F. vesca* f. *semperflorens*) was received from the private collection of Dr. J.K. Jones held in the Department of Agriculture Botany, The University of Reading, Berkshire, U.K. Both lines are highly homozygous (unpublished data).

F_1 hybrids were produced by hand-pollination of emasculated *F. vesca* f. *vesca* using pollen from *F. vesca* f. *semperflorens*. Pollinated flowers were bagged to prevent wind pollination and allowed to set seed. The F_1 hybrids were then backcrossed to *F. vesca* f. *semperflorens* to generate a BC_1 testcross progeny of 168 plants.

DNA extraction and ISSR-PCR analysis

Genomic DNA was isolated from unexpanded leaves of both parental lines and the F_1 hybrid using the method described by Doyle and Doyle (1987). Extracted DNA was quantified using a DyNA 200 fluorimeter (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

ISSR-PCR analysis was performed according to the protocol described by Charters et al. (1996) using one or two of the primers listed in Table 1. All possible pairwise combinations of primers were used on all three genotypes studied. In brief, 10 ng of genomic DNA were added to a reaction mixture containing 1 unit of *Taq* polymerase, 1 × 5mM $MgCl_2$ buffer, 0.2 mM of each dNTP (all Boehringer Mannheim) and 0.2 μM of primer. DNA was amplified on a Hybaid Omnigene thermocycler using the following programme: 1 min at 94°C and 30 cycles of (1 min at 94°C, 2 min at 55°C and 30 sec at 72°C) followed by a final 5-min extension at 72°C. Amplification products were loaded onto a pre-cast polyacrylamide gel comprising a 5% w/v polyacrylamide stacking gel and a 10% w/v pre-cast polyacrylamide gel (Amersham Pharmacia Biotech, St Albans, U.K., Cleangel 48S). They were then fractionated and silver-stained according to the procedure described by Charters et al. (1996). Two to four replicates were performed of each PCR on each genotype. The effect of primer competition on the reproducibility of band profiles when two ISSR primers were used in paired combinations was investigated empirically. Mixes were made containing each primer in the following concentration ratios: 0: 0.2 μM; 0.1 μM: 0.2 μM; 0.2 μM: 0.2 μM; 0.2 μM: 0.3 μM; 0.2 μM: 0.4 μM. PCR master mixes were made using the protocol described by Charters et al. (1996) but, where necessary, the final volume was adjusted to 20 μl by changing the amount of water used.

Cloning and DNA sequencing protocol

Novel ISSR products to be cloned were first fractionated on a 10% pre-cast polyacrylamide gel (48S Cleangel, Amersham Pharmacia

Table 1 Primers used in ISSR-PCR analyses

No.	Sequence	Repeat	Annealing temp.
808	AGA GAG AGA GAG AGA GC	$(AG)_8$ C	52
810	GAG AGA GAG AGA GAG AT	$(GA)_8$ T	50
811	GAG AGA GAG AGA GAG AC	$(GA)_8$ C	52
819	GTG TGT GTG TGT GTG TA	$(GT)_8$ A	50
820	GTG TGT GTG TGT GTG TC	$(GT)_8$ C	52
821	GTG TGT GTG TGT GTG TT	$(GT)_8$ T	50
835	AGA GAG AGA GAG AGA GYC	$(AG)_8$ YC	54/56
836	AGA GAG AGA GAG AGA GYG	$(AG)_8$ YG	54/56
841	GAG AGA GAG AGA GAG AYC	$(GA)_8$ YC	54/56
842	GAG AGA GAG AGA GAG AYG	$(GA)_8$ YG	54/56

Biotech, St Albans, U.K.) and silver nitrate-stained according to Charters et al. (1996). Selected bands were then excised and re-amplified by PCR using the same ISSR primers and PCR conditions described above. Re-amplified products were cleaned using the PCR SELECT-III Spin Column (5 prime→ 3 prime, Inc., Boulder, USA). Purified and re-amplified products were then inserted into the *Escherichia coli* vector pNoTA/T7 using the PRIME PCR CLONER SYSTEM according to the manufacturer's instructions (5 Prime→ 3 prime, Inc., Boulder, USA). Cycle sequencing reactions (Sanger et al. 1977) were performed on cloned fragments using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with Ampli*Taq* DNA polymerase (Perkin Elmer Corp.) according to the manufacturer's instructions. Samples were fractionated in an automated DNA Sequencer (ABI 373A, Applied Biosystems) and data collected on a Macintosh platform. Sequencing data were edited and assembled using the program ''LaserGene 2'' (DNASTAR, USA).

Bulked segregant analysis

Plants in the BC_1 progeny were classified according to whether they exhibited seasonal or perpetual flowering. DNA was extracted from 84 'seasonal' plants and from 84 plants showing perpetual flowering. The extracted DNA was quantified as above and 100 ng of each sample from each group mixed to create two pooled DNA stock samples containing 33.3 ng/µl. The stock DNA samples were used as a template for ISSR-PCR using the ten primers identified in Table 1 and all 45 paired combinations of these primers according to the method given above.

Segregation analysis and mapping

ISSR primer and primer-pair combinations that generated markers identified as putatively linked to the seasonality locus were applied to 168 individuals from the BC_1 progeny. The genetic distance between each of the markers and the seasonal flowering locus was determined using the Joinmap 2.0 software package (Stam 1993).

Results

Reproducibility

No variation was observed between the replicate band profiles generated by any ISSR primer when used alone on DNA from any genotype (data not shown). When pairs of ISSR primers were used for the PCR, changes in primer-mix constitution had a variable effect on the appearance of bands in the profile. The majority of bands from either ISSR primer used alone remained present in the profile of the 'primer pair' for all mix combinations of primer concentration. A small proportion of bands, however, became faint or disappeared when the concentration of one primer increased relative to the other (e.g. Fig. 1). It was also noted that whilst the intensity of bands that only appeared in pair-combinations varied with primer-mix composition, their presence was nevertheless constant in all profiles from PCR mixes containing both primers. Profiles generated using equal concentrations of both ISSR primers contained the most amplicons and were also the most reproducible between replicates.

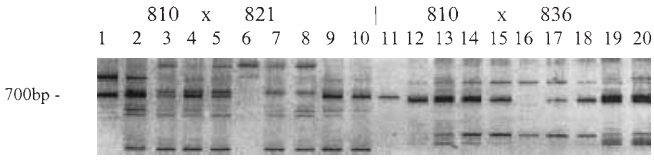

Fig. 1 Variation in band profiles generated by two pairs of ISSR primers used in different mix combinations. Primer pair 810 × 821 (*lanes 1–10*) and 810 × 836 (*lanes 11–20*). For each combination set, concentration of primer 810 precedes that for the second primer and are given in the following order: *lanes 1 and 11* 0.2 µM: 0 µM; *lanes 2 and 12* 0.2 µM: 0.1 µM; *lanes 3 and 13* 0.2 µM: 0.2 µM; *lanes 4 and 14* 0.2 µM: 0.3 µM; *lanes 5 and 15* 0.2 µM: 0.4 µM; *lanes 6 and 16* 0 µM: 0.2 µM; *lanes 7 and 17* 0.1 µM: 0.2 µM; *lanes 8 and 18* 0.2 µM: 0.2 µM; *lanes 9 and 19* 0.3 µM: 0.2 µM; *lanes10 and 20* 0.4 µM: 0.2 µM

Generation of polymorphisms

Band profiles generated by ten ISSR primers (Table 2) were screened for the presence of polymorphisms between two closely related forms of *F. vesca* (form *vesca* and form *semperflorens*). There were 85 bands generated across all primers, with primer 835 yielding the highest number of products (15 amplicons) and primer 821 the least (two amplicons). The number of polymorphic bands also varied between primers, with primers 835 and 842 each generating four polymorphisms and 819 yield-

Table 2 Number of *F. vesca*- and *F. vesca* f. *semperflorens*-specific markers generated by ten ISSR primers when used alone (column A) and in all possible pairwise combinations. In all cases, the two figures given represent the number of *F. vesca*-specific and *F. vesca* f. *semperflorens*-specific markers respectively. The cumulative number of extra markers produced by each primer when used in pairwise combinations is given at the base of the column for each primer

A	Primers	808									
0 2	808		810								
0 1	810	1 2		811							
0 1	811	1 1	0 2		819						
0 0	819	0 1	1 0	0 1		820					
0 1	820	1 0	0 0	1 0	0 0		821				
1 0	821	1 0	0 1	0 0	0 0	0 0		835			
1 3	835	1 2	1 0	0 1	2 0	1 0	0 1		836		
1 0	836	0 1	0 1	0 1	0 1	0 0	1 1	0 1		841	
0 2	841	0 1	0 1	0 0	0 2	1 0	0 0	0 2	2 0		842
2 2	842	0 2	1 1	0 1	1 1	1 2	0 2	0 1	1 0	0 1	

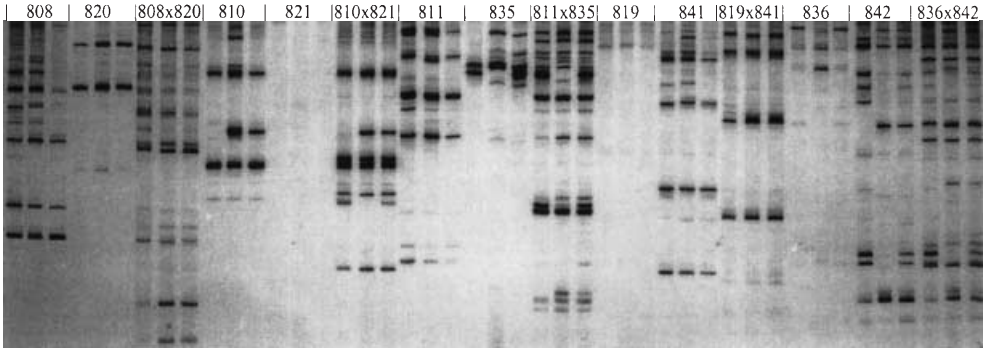

Fig. 2 Band profiles generated by individual ISSR primers when used in isolation and in pairs. For each primer/primer-pair combination, the three profiles are in the order of *F. vesca*, *F. vesca* f. *semperflorens* and the F_1 hybrid between the two

ing none. Overall, there were 17 polymorphisms generated between the two plant forms. Five of these were specific to form *vesca* and 12 were found only in form *semperflorens*.

The combined use of ISSR primers in all 45 pairwise combinations (Table 2) generated new, distinct band profiles (Fig. 2) that collectively contained 493 bands. This included a further 14 amplicons specific to form *vesca* and an extra 23 bands diagnostic of form *semperflorens*. The number of new polymorphisms generated by the combined use of two ISSR primers (as opposed to the same primers used individually) varied from 0 to 2. The mean yield of novel polymorphisms revealed in profiles generated by paired primer combinations was 1.04. This value compares with a mean of 1.7 for new ISSR primers when used in isolation (ten primers yielded 17 novel markers). Thus, whilst the use of ISSR primers in pairs increased the total number of polymorphisms, on average, the frequency of new polymorphisms obtained was lower than would be generated by using a new ISSR primer design.

Nature of the extra polymorphisms

The use of the ten ISSR primers in isolation yielded a total of 85 bands compared with the 493 amplified from the 45 pairwise combinations made between them. On the basis of an individual profile, fewer bands were generated when ISSR primers were used in isolation ($\bar{x}$ = 8.5) than when the same primers were used in paired combinations ($\bar{x}$ = 10.95). Furthermore, band profiles generated by pairs of ISSR primers did not correspond to the sum of those generated by each primer used in isolation. The difference was accountable largely by bands that were present in the 'paired' profile but absent from profiles of either ISSR primer when used in isolation (novel bands), although a minority of bands that were present in the 'individual' ISSR profiles were absent in the 'paired' profiles (lost bands). Overall, the majority of the 578 bands (54%) were conserved between the profiles of *F. vesca*, *F. vesca* f. *semperflorens* and the F_1 hybrid whether the primer was used individually or included as part of a pair. There were also 25% of (novel) bands that appeared only in 'primer pair' profiles of one or more genotypes, and so not in the profile generated by either primer used individually. In addition, 10% of (lost) bands were present in profiles of at least one genotype using a single primer but were missing from one or more corresponding profiles yielded by primer pairs. The remaining bands were present in the profile of only one parent and the hybrid, and were found using individual primers and primer pairs. Disparity was noted between the mean size of the novel bands ($\bar{x}$ = 350 bp) and those lost from the 'paired' ISSR band profiles ($\bar{x}$ = 850 bp). This meant that the mean size of amplicons generated by individual primers ($\bar{x}$ = 500 bp) was larger than that of bands produced by pairs of primers ($\bar{x}$ = 450 bp).

Source of novel bands

The presence of different primer-binding sites at either end of an amplicon represents the most plausible source of the novel bands found in the ISSR primer-pair profiles. The validity of this explanation, as opposed to artifacts of the PCR, was tested by reference to the DNA sequence of four novel amplicons isolated from the profiles of the following primer combinations: 808 $(AG)_8$ C × 811 $(GA)_8$ C; 819 $(GT)_8$ A × 836 $(AG)_8$ YA; 820 $(GT)_8$C × 808 $(AG)_8$C and 820 $(GT)_8$C × 836 $(AG)_8$YC. In all cases, partial or complete DNA sequences corresponding to different primer binding sites were detected at either end of the products (Table 3). One sequence [820 $(GT)_8$C × 808 $(AG)_8$C] also included a further microsatellite motif (AT_8) not targeted by either of the ISSR primers.

Genetic linkage to the seasonality locus

Amplicons found to be polymorphic between *F. vesca* f. *vesca* and f. *semperflorens* were screened for linkage to the seasonality locus using a segregating testcross progeny. Bulked segregant analysis was performed on pooled subpopulations comprising 84 seasonal and 84 perpetual flowering individuals as an initial screen to identify markers putatively linked to the seasonality locus. Two of the 35 *F. vesca* f. *vesca*-specific markers generated by the combined use of two ISSR primers (both from 835

Table 3 DNA sequence information generated from cloned amplicons of four primer pair combinations [808 $(AG)_8$ C × 811 $(GA)_8$ C; 819 $(GT)_8$ A × 836 $(AG)_8$ YA; 820 $(GT)_8$C × 808 $(AG)_8$C and 820 $(GT)_8$C × 836 $(AG)_8$YC]. Primer binding sites are given in bold and italics are used to indicate plasmid sequences. A non-targeted motif (AT_8) is underlined in the sequenced amplicon from the 820 $(GT)_8$C × 808 $(AG)_8$C primer combination

808 $(AG)_8$ C × 811 $(GA)_8$ C

*AAACGTC***AGAGAGAGAGAGAGAGC**CACCGATCTGCTCTCGAAGATCGGAATCGACATAATCTCCTACGCCGGCGAGAAGA
AGCTGTTTCCTGGATTTAAGCATCGCGAGGTCAAAAACCGCCTGTGAGGTATCGGCTCCAGCTTTGGA**GTCTCTCTCTCTC**
TCT*ACGGATCCC*

819 $(GT)_8$ A × 836 $(AG)_8$ YA

*AAACGTC***AGAGAGAGAGAGTXAGC**ACCAGATCGATCGGGAGGAGAGGATAAGAAGAAAGAGATAAGATTTGTGTGGGTA
TAATTTACAAAAGATAATAACGTCTTTTTTTAATGAATAATTGGAATGGGCATAAGGATAAAATTTTCATTAGAATGAGGTTTA
GTTTTACTGTTTTTGTGCAAATGGCATTTTTCCTTATTCTTATGTCTCTTCTCCATAACACC<u>ATATATATATATATATAT</u>GTATA**TACA**
CACACACACACAC*GACGGATCC*

820 $(GT)_8$C × 808 $(AG)_8$C

*AAACGTC***GAGAGAGAGATAGAGAC**GAGGACCCAATTCCATXTXCGGGXAAGGTGTCCCGXTTTTCGGAXTAGAGATGTXC
GCAAAGATCGATTGAGAGAAAGTCGATAGACGAXAGACXAGGACCCAACTTCATTTCTGACAAAAGTGTCCGGTTTCTCG
GATCGGATATGTCXGTTGAGATCAATCGXCGXCGCTCAGTCTCATGTAAGTATGTG**GCTCTCTCTCTCTCTCT***ACGGATCC*

820 $(GT)_8$C × 836 $(AG)_8$YC

*AAACGTC***GTC**ATAAAACGAGTTTCGAGGGATGAAAAAAGCTGACTGAGACGTTGAGACGACGAATGGCTTTCTGACGGAT
CCTCTCT*GGATCC*

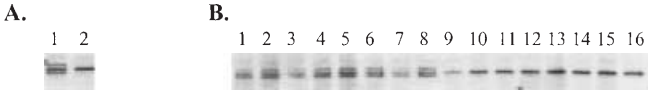

Fig. 3A, B ISSR-PCR profiles using primer combination 835 and 841. **A** Bulked samples of BC_1 progeny exhibiting seasonal flowering (*lane 1*) and perpetual flowering (*lane 2*). **B** Profiles of 16 individual plants showing seasonal flowering (*lanes 1–8*) and perpetual flowering (*lanes 9–16*)

and 841) were observed in the 'seasonal' pool but were absent from the 'perpetual flowering' pool (Fig. 3a). In contrast, all 12 amplicons specific to *F. vesca* f. *vesca* yielded by individual ISSR primers appeared in the profiles of both pools. The 835 × 841 primer-pair combination was then applied to 168 segregating individuals of the BC_1 progeny (Fig. 3b). Neither marker deviated from the expected 1:1 ratio ($\chi^2 = 0.29$, $p < 0.05$) and both shared a position 2.4 cM from the seasonality locus with a LOD value of 43.2, corrected to 2.2 cM using Kosambi's map function.

Discussion

The results presented here are concordant with those of previous studies that demonstrate the reproducibility of band profiles generated by ISSR-PCR when fractionated by PAGE (e.g. Albani and Wilkinson 1998). The use of two ISSR primers in combination, however, creates additional scope for PCR artefacts. These may arise through primer competition, differential efficacy of primer extension at the chosen annealing temperature or the formation of primer-primer heteroduplexes. Consistency in the presence of bands after the relative concentration of constituent primers was varied, however, suggests that these primer designs exhibited similar annealing kinetics and did not compete for binding sites. The reliability of amplification and the absence of low-molecular-weight fragments of the appropriate size are similarly indicative that the primers used here do not have a strong tendency to form heteroduplexes and so prevent, or inhibit, amplification by PCR.

Differences between the ISSR profiles generated by two primers, to those seen when the same primers are used individually, are typically manifest as the loss of a few larger bands from the individual profiles and the appearance of additional, smaller bands in the combined profile. The presence of a binding site for one ISSR primer within an amplicon generated by the second is the most-likely cause of the failed amplification from the combined profile. The internal, smaller product yielded by the two different ISSR primer sites would be preferentially amplified over the larger product arising from the two identical sites. This, in turn, would lead to the loss of a larger fragment and the production of a smaller band in the combined profile that originates from the same locus. Change in product size may, or may not, alter the level of polymorphisms exhibited between genotypes. For instance, should the binding site made redundant by the amplification from the internal site be the cause of polymorphism between genotypes, then this polymorphism may be lost. Conversely, the internal site may introduce a fresh source of polymorphism that was absent in the original, larger product. Thus, overall, changes of this nature will tend to enhance the scope for distinguishing between genotypes.

A further source of additional amplicons in the combined profile may arise whenever binding sites for the two different ISSR primers are sufficiently close to permit their amplification by PCR. These new products will be distributed randomly throughout the genome, and so are extremely unlikely to derive from the same locus as other bands represented in the profiles of either primer

when used alone. The generation of any additional bands in combined primer profiles increases the capacity of the technique to distinguish between genotypes. In contrast, co-migrating bands that are conserved in all profiles provide no scope for increasing the power of diagnosis. Under the conditions used here, such bands accounted for approximately half (53.7%) of those bands in the combined profiles. It is therefore intriguing to note that the use of a primer-pair combination yields, on average, approximately half as many new polymorphisms compared with the use of a completely new ISSR primer design in isolation (1.04 compared to 1.7 new polymorphic bands). The implication is that the value of combining pairs of ISSR primers lies principally in maximising the total yield of polymorphisms generated from any given set of primer designs, rather than of identifying a particular primer or primer-pair combination with optimal power of diagnosis.

The scope for accumulating additional polymorphic bands is considerable. In the present study, the ten primers used in isolation produced 85 bands of which 12 were polymorphic between the study organisms. This compares with 35 bands from the 45 pair-wise combinations possible between them. The number of combinations possible (c) between *n* primers increases according to the simple relationship: $c = (n^2\text{-}n)/2$. Should the trend observed here extend to other primers applied to the group, an increase from 10 to 12 primers would yield another three or four polymorphic bands from the use of each primer in isolation (1.7 bands per primer). In comparison, there would be another 21 additional combinations from this change, collectively producing an expected 22 extra polymorphic bands (1.04 bands per combination × 21 combinations). The relative size of this benefit can be crudely gauged as the difference between the number of polymorphisms generated by primer-pair combinations and that created from the use of the primers in isolation. This relationship is described by the following formula:

Benefit (B) = [mean polymorphic bands per combination (a) × number of combinations (c)] – [mean polymorphic bands per primer (b) × number of primers (*n*)],

but $c = (n^2\text{-}n)/2$, thus $B = a([n^2\text{-}n]/2) - bn$.

In practical terms, the factor limiting the number of polymorphisms generated is likely to be the time required to perform PCRs for all possible combinations. Inevitably, a balance should be made between the introduction of new primers and exploitation of the possible pairwise-combinations between primers already held. This decision will be influenced by the relative performance of paired combinations for generating new polymorphisms, cost constraints and the time required to synthesise and optimise PCR primers for new designs. Cognisance should also be taken of the possibility of incompatible primer pairings. For example, a primer comprising a GT repeat would have a strong tendency to form heteroduplex dimers or concatamers with a primer that incorporates a CA repeat. Considerations such as this would limit the total number of combinations that are likely to be of practical value.

The locus from which a polymorphic band originates has great importance for its value for genetic-linkage mapping and map-related purposes. Novel bands originating from the truncation of an amplicon present in a profile from an individual primer may replicate existing data and, therefore, be of little value for the construction of a map. This would not be the case, however, should the polymorphism only manifest itself in the smaller amplification product, thereby restricting any segregation analysis to the primer combination. In contrast, novel bands generated from the juxtaposition of binding sites for each of the paired primers would represent new loci and so be of direct value for map construction or the identification of amplicons for marker-assisted breeding. In the present work, the use of pair combinations of the ten ISSR primers and bulked segregant analysis permitted the identification of two markers linked to within 2.2 cM of a gene controlling seasonal flowering in *F. vesca*. The failure to identify any linked markers when the same ten primers were used in isolation suggests that the combination markers arose from novel loci and so illustrates the value of using primer-pair combinations for map-based purposes as well as for genetic distance analysis and fingerprinting. Data presented in this study could also be of direct value for marker-assisted introgression of seasonality and runnering from *F. vesca* and into the cultivated strawberry.

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