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TECHNICAL NOTE

Intersimple sequence repeat (ISSR) polymorphisms as a genetic marker system in cotton

BAO LIU and JONATHAN F. WENDEL
Department of Botany, Bessey Hall, Iowa State University, Ames IA 50011, USA

Abstract

We studied the applicability of intersimple sequence repeat (ISSR) polymorphism in cotton. We found that: (i) the resolving power of agarose gels is poor relative to that provided by sequencing gels; (ii) fluorescent labelling of ISSR amplification primers produced numerous scorable bands; (iii) primer mixing (double priming) generated more bands than the sum of fragments resulting from two single primers, although an unexplained disappearance of several larger fragments also reproducibly occurred; (iv) ISSR fingerprinting patterns are highly heritable; and (v) double priming ISSR is an easy and informative genetic marker system in cotton for revealing both inter- and intraspecific variations.

Keywords: cotton, fluorescent-labelling, *Gossypium*, intersimple sequence repeat (ISSR) polymorphism, primer mixing

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Microsatellites or simple sequence repeats (SSR) are short DNA sequence stretches consisting of motifs of one to six bases that are tandemly repeated. Owing to their ubiquity, hypervariability, abundance and genome-wide distribution, SSR loci represent a new generation of powerful genetic markers for eukaryotes. Use of this marker system, however, is hampered by the requirement for sequence information from flanking regions, from which primers are designed for polymerase chain reaction (PCR) amplification. Discovery and characterization of a large number of SSRs is therefore time-consuming and expensive for many taxa.

A recently developed modification of SSR-based marker systems, i.e. ISSR (intersimple sequence repeat) analysis, circumvents this requirement for flanking sequence information, and thus has found wide applicability in a variety of plants. Popularized largely by A. Wolfe and colleagues (Wolfe *et al*. 1998), ISSR analysis involves PCR amplification of genomic DNA using a single primer that targets the repeat *per se*, with 1–3 bases that anchor the primer at the 3′ or 5′ end. In addition to freedom from the necessity of obtaining flanking genomic sequence information, ISSR analysis is technically simpler than many other marker systems. The method provides highly reproducible results and generates abundant polymorphisms in many systems. Most applications to date have used either agarose gel electrophoresis with ethidium bromide detection (Nagaoka & Ogihara 1997; Joshi *et al*. 2000) or polyacrylamide gel electrophoresis (PAGE) separation combined with either silver staining or isotopic detection (Blair *et al*. 1999).

Cotton (*Gossypium*) provides the world's most important textile fibre. Genetic diversity in cultivated cotton has been studied using several approaches (Wendel *et al*. 1992; Brubaker & Wendel 1994; Pillay & Myers 1999; Liu *et al*. 2000). A consensus reached by these studies is that genetic variation among cotton cultivars is very low. Thus, more efficient marker systems are needed for both breeding applications and basic evolutionary/ecological studies.

A recent modification of ISSR analysis utilizes fluorescein-labelling of amplification primers and automated detection on sequencing gels (Huang & Sun 2000). Here we extend these modifications and in addition demonstrate the flexibility of ISSR analysis to incorporate data on genome size and relative SSR frequency into experimental design.

To assess the efficiency of ISSR markers in revealing polymorphisms in cotton, nine accessions of *Gossypium hirsutum* and two accessions of *G. barbadense* were selected (Fig. 1c,d). These same accessions were used in earlier studies of allozyme and restriction fragment length polymorphism (RFLP) variation, permitting a comparison of techniques. To test whether ISSR markers were stably

Correspondence: Jonathan F. Wendel. Fax: 515 294 1337; E-mail: jfw@iastate.edu

Table 1 Number of polymorphic intersimple sequence repeat (ISSR) bands detected on automated sequencing (above diagonal) and agarose (below diagonal) gels. Total number of bands per accession are shown along the diagonal for automated sequencing and agarose (in brackets) gels. Intra- and interspecific polymorphisms are shown for each pairwise comparison among accessions, in terms of number of band differences and percentages (in parentheses). The data shown summarize results from six amplifications using different primers or primer combinations: $(GA)_9C$, $(GA)_9T$, $(GA)_9A$, $(GA)_9C + (GA)_9T$, $(GA)_9C + (GA)_9A$, and $(GA)_9T + (GA)_9A$

Cotton accession	TX1	TX6	TX21	TX44	TX93	TX94	TX119	TX166	TX188	B106	B444
TX1	**84[25]**	9(5.33)	12(7.50)	8(4.73)	14(8.92)	9(5.59)	13(8.13)	13(8.13)	12(7.36)	24(14.20)	28(15.91)
TX6	3(5.66)	**85[28]**	11(6.83)	9(5.29)	16(10.13)	9(5.56)	12(7.45)	12(7.45)	12(7.32)	22(12.94)	26(14.69)
TX21	2(4.17)	6(11.76)	**76[23]**	10(6.21)	12(8.05)	13(8.50)	7(4.61)	8(5.26)	8(5.16)	21(12.50)	27(16.07)
TX44	1(1.96)	2(1.18)	3(6.12)	**85[26]**	15(9.49)	16(9.88)	13(8.07)	12(7.45)	17(10.37)	18(10.17)	23(12.99)
TX93	3(6.38)	6(12.00)	1(2.22)	4(8.33)	**73[22]**	7(4.67)	12(8.05)	9(6.04)	4(2.63)	25(15.15)	26(15.76)
TX94	3(6.25)	5(10.00)	0(0.00)	3(6.12)	1(2.22)	**77[23]**	9(5.88)	9(5.88)	9(5.77)	24(14.20)	27(15.98)
TX119	3(6.12)	4(7.49)	5(10.64)	2(4.00)	6(13.04)	5(10.64)	**76[24]**	8(5.26)	9(5.81)	20(12.42)	27(16.07)
TX166	1(2.04)	4(7.69)	3(6.38)	2(4.00)	4(8.70)	3(6.48)	2(4.17)	**76[24]**	7(4.52)	21(13.24)	28(16.67)
TX188	4(4.51)	6(12.00)	3(6.67)	5(10.42)	4(9.09)	3(6.67)	4(8.70)	3(6.52)	**79[22]**	23(14.02)	25(14.62)
B106	3(6.25)	5(9.80)	2(4.35)	3(5.77)	4(8.33)	2(4.35)	2(4.06)	1(2.13)	2(4.44)	**85[23]**	12(6.78)
B444	3(5.88)	3(5.56)	5(10.20)	4(7.69)	6(12.50)	5(4.20)	5(10.00)	6(12.00)	4(8.33)	5(10.20)	**92[26]**

inherited and to evaluate the effects of primer mixing, a synthetic allohexaploid (G350) between *G. anomalum* (G29) and *G. hirsutum* (G173) and its parental lines were used.

Genomic DNA was isolated from expanded leaves of individual plants using the Nucleon Plant DNA isolation & Purification Kit (Amersham). Three ISSR primers were synthesized, each based on the dinucleotide GA as a core sequence and terminated by a single nucleotide for 3′ anchoring: $(GA)_9T$, $(GA)_9C$ and $(GA)_9A$. The choice of the dinucleotide GA was based on its relative abundance in the cotton genome (B. Burr, personal communication). Primers $(GA)_9T$ and $(GA)_9C$ were 5′-labelled with 6-carboxyfluorescein (6-FAM) while $(GA)_9A$ was labelled with 4,7,2′,7′-tetrachloro-6-carboxyfluorescein (TET). Primers were used both singly and in all possible combinations ('double primer' ISSR). PCR components and amplification conditions were as in Blair *et al*. (1999), except a cosolvent (2% formamide) was included. Amplification products were electrophoretically separated by both agarose (NuSieve 3:1, FMC) and automated sequencing gels, the latter on an ABI Prism 373 DNA sequencer. For automated detection and band sizing, an internal size standard (GeneScan-500 Rox) was added to each sample and gel images and trace files were captured and analysed with GENESCAN version 2.0.2 and GENOTYPER version 2.0 (PE/Applied Biosystems). Gel images were also examined visually to evaluate reliability of band calling.

ISSR analysis using agarose gels led to relatively poor resolution with all primer combinations (Fig. 1a,c; Table 1). This result contrasts with reports from rice (Joshi *et al*. 2000) and wheat (Nagaoka & Ogihara 1997), where using the same primers as us and routine analytical-grade agarose gels, more informative ISSR patterns were generated. The variation among studies may reflect differences in abundance and/or distribution of the SSR motifs between the genomes of the two grasses and cotton.

As a potentially widely applicable alternative to agarose gel electrophoresis, we tested fluorescent labelling and sequencing-gel separation and found that this method produced a much larger number of resolved bands (Fig. 1b,d; Table 1). An additional advantage of using sequencing gels and fluorescein-based detection is that by using the GENESCAN AND GENOTYPER software, stutter bands could be filtered out, making automated scoring more accurate than in agarose gels. Totalled across primers and primer combinations, sequencing gels revealed approximately 3.3 times as many ISSR fragments as observed on agarose gels (Table 1).

If wide genomic spacing of specific SSR loci constitutes a limitation on amplification, as suggested, inclusion of more than one primer in amplification reactions should lead to amplification of both 'homo-ISSRs' (those flanked by the same primer site) and 'hetero-ISSRs' (those flanked on each end by a different primer). This logic suggests that the number of bands amplified may be optimized by judicious selection and mixing of different primers. This prediction should hold generally, and lead to an enhanced applicability of ISSR analysis to eukaryotic genomes. To evaluate this concept in cotton, we tested effects of primer mixing on the number of bands generated. In most cases combining two primers did not increase band number beyond that detected with single primers, when using agarose gels, but when sequencing gels and fluorescently labelled primers were combined, far more bands were observed than when individual primers were used. Moreover, as expected from the prediction of hetero-ISSRs, many novel fragments were generated, particularly in the low

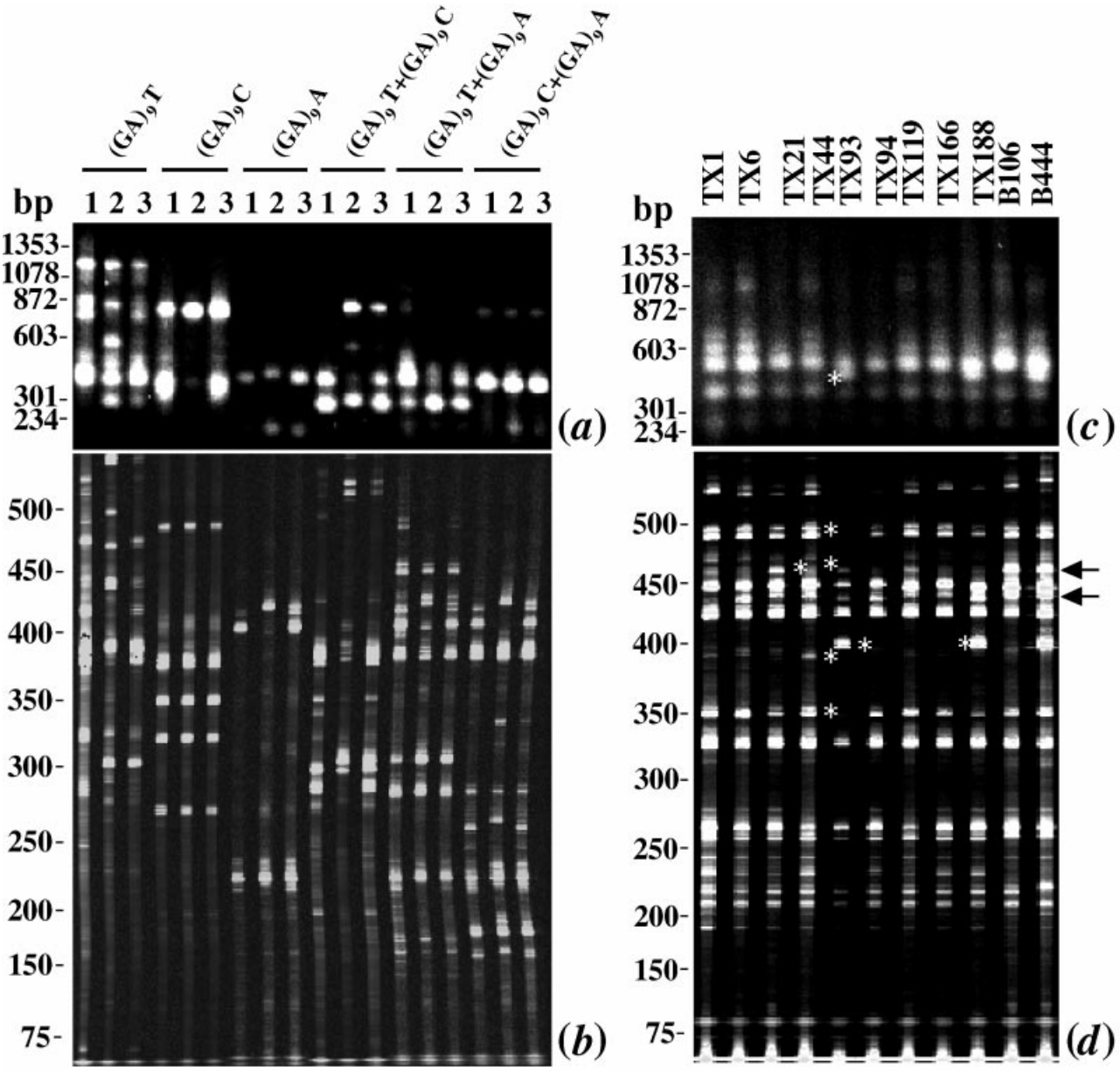

Fig. 1 Relative efficiency of different electrophoretic separation and detection methods for intersimple sequence repeat (ISSR) analysis in cotton, and Mendelian inheritance of fragments (a,b), and effects of ISSR primer mixing [$(GA)_9T + (GA)_9A$] on detecting intra- and interspecific polymorphism in cotton (c,d). (a) Agarose-gel electrophoresis and ethidium bromide staining. (b) Sequencing gel electrophoresis and flourescent detection. Note the disappearance of bands in both (a) and (b) and the appearance of novel bands in (b) as a result of primer mixing. Lanes 1, 2, and 3 are *Gossypium hirsutum* (G173), *G. anomalum* (G29) and their allohexoploid (G350), respectively. Primer combinations and sizes are given. (c) Agarose gel electrophoresis and ethidium bromide staining. (d) Sequencing gel electrophoresis and fluorescent detection. Intraspecific polymorphisms in *G. hirsutum* are indicated by asterisks, whereas *G. barbadense*-specific bands are marked by arrows. Plant designation (top) and fragment sizes (left) are shown.

molecular weight region (Fig. 1b,d; Table 1). Presumably this is due to the occurrence of two $(GA)_n$ loci that are flanked by different nucleotides at the 3′ end. A similar phenomenon was recently observed in sweet potato (Huang & Sun 2000), suggesting that primer mixing may generally provide enhanced ISSR sensitivity.

An unexpected observation was the disappearance of several larger fragments when double primers were used (Fig. 1a,b). Neither fragment cloning and sequencing, or alterations of amplification conditions and constituent concentrations yielded insight into the cause of this phenomenon. Nonetheless, double-priming is clearly advantageous,

as ISSR patterns so produced are reproducible with a gain in fragment number that far exceeds the few bands that inexplicably vanish (Table 1).

Analysis of a synthetic allohexaploid between *G. hirsutum* and *G. anomalum* along with its parents, using all single and double primers, showed that ISSR bands were additive in the synthetic allopolyploid relative to its parents, indicating Mendelian inheritance (Fig. 1a,b).

As a preliminary assessment of the utility of ISSR analysis for detecting polymorphism in cotton, we selected nine representative accessions of *G. hirsutum* and two accessions of *G. barbadense*. These accessions were studied previously using allozyme and RFLP markers (Wendel *et al*. 1992; Brubaker & Wendel 1994), and collectively exhibit low levels of genetic variation. We subjected these materials to ISSR analysis using all six primer combinations described above. Few fragment differences were observed using agarose gels, and one pair of accessions was indistinguishable (TX21/TX94). Using sequencing gels, however, polymorphism levels were higher, with three times as many polymorphic bands in all pairwise comparisons than observed with agarose gels (Table 1, Fig. 1c,d). In all cases, more polymorphic bands were detected in inter- than in intraspecific comparisons (Table 1); *G. hirsutum* and *G. barbadense* were distinguished by approximately 13% of the fragments scored, whereas mean intraspecific polymorphism levels were about 6%. These observations are congruent with both established biological context and previous results based on allozyme and RFLP analysis (Wendel *et al*. 1992; Brubaker & Wendel 1994). The relatively high level of ISSR polymorphism observed in both cotton species indicates that relative to other existing technologies, ISSR methods represent a cost-effective and efficient means of generating molecular markers in cotton.

An especially attractive attribute of ISSR analysis is its flexibility in terms of experimental design. For example, the number of amplicons generated may be optimized by primer design (e.g. the number of anchored bases) and by judicious combining of primers. Hence, the strategy we outline here may have broad applicability across plants and animals, regardless of genome size and the abundance and distribution of SSR loci. Fluorescein-based sequencing gel ISSR analysis, particularly with double priming, appears to be an easy and informative genetic marker system for diverse applications.

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