Molecular and Cellular Probes (2002) **16**, 67–72
doi:10.1006/mcpr.2001.0404, available online at http://www.idealibrary.com on IDEAL®

FISSR-PCR: a simple and sensitive assay for highthroughput genotyping and genetic mapping

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(Received 11 October 2001; accepted for publication 27 November 2001).

The recently developed Inter-Simple Sequence Repeat PCR (ISSR-PCR) or microsatellite primed PCR or Simple Sequence Repeat (SSR)-Anchored PCR technique detects polymorphic markers in a wide variety of genomes. Usually the ISSR primers are either 5′ end-labeled with $\gamma[^{32}P]$ATP or one of the $\alpha[^{32}P]$ labeled dNTPs is added to the PCR reaction and the PCR products are resolved on PAGE and autoradiographed. Alternatively, cold PCR products are resolved on agarose gel electrophoresis. In the present study, we show that informativity, sensitivity and speed of the ISSR-PCR can be substantially enhanced by adding fluorescent nucleotide in the PCR reaction followed by resolution of PCR products on an ABI 377 automated sequencer. The informativeness, measured as a number of detectable amplified fragments, was two-fold higher and the quantity of required template DNA is two-fold lower than the regular ISSR-PCR. We have termed this method as FISSR-PCR and show its usefulness in generating large number of species and varietal specific markers in plants, insects, parasites of insects and human and various infectious organisms. Further, we show that the FISSR markers are inherited and segregated in Mendelian fashion as demonstrated on a panel of 99 F_2 offspring derived from a cross of two divergent silkworm strains. The FISSR-PCR marker assay could be a method of choice for large scale screening of varieties/cultivars and highthroughput genotyping in mapping of genomes where microsatellite information is scanty or absent.

KEYWORDS: FISSR-PCR, *Bombyx mori*, microsatellite markers, highthroughput genotyping, genetic mapping.

INTRODUCTION

Recent progress in DNA marker technology, particularly PCR based markers, such as randomly amplified polymorphic DNA markers (RAPD),[1,2] amplified fragment length polymorphisms (AFLP),[3] and microsatellite markers[4–6] have augmented the marker resources for genetic analyses of a wide variety of genomes. As PCR technology finds increased use in various genetic analyses, additional novel variations of this technique are emerging in order to augment the high-resolution genotyping and genetic mapping of various complex animal, plant and microbial genomes. The PCR analysis using anchored simple sequence repeat primers, referred to as ISSR-PCR or anchored SSR-PCR, has gained attention recently as an attractive means of characterizing complex genomes.[7,11,13]

The ISSR-PCR approach employs oligonucleotides based on simple sequence repeats (SSR) anchored

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0890–8508/02/010067+06 $35.00/0

either at the 5′ or 3′ end with two or four purine or pyrimidine residues, to initiate PCR amplification of genomic segments flanked by inversely oriented, closely spaced microsatellite repeats.[7]

The ISSR-PCR strategy is especially attractive because it avoids the need to carry out costly cloning and sequencing inherent in the original microsatellite-based approach. As a result, ISSR-PCR has been profitably used for genetic linkage analysis of various plant species[8–12] and the silkworm, *Bombyx mori*.[13,14]

The most commonly used approach for generating ISSR-PCR markers is either 5′-end labeling of ISSR primers with $\gamma[^{32}P]$ ATP or one of the $\alpha[^{32}P]$ labeled dNTPs is added to the PCR reaction along with cold dNTPs in appropriate ratio, followed by resolution of PCR products on PAGE and autoradiographic detection of ISSR markers. Alternatively, some investigators have also resolved ISSR-PCR products on Nusieve agarose gels, of course with a marked reduction in number of markers compared to PAGE. While the former involves stringent standardization and extensive use of radioactive isotopes, the latter compromises with the number of markers generated per PCR reaction. Besides, both the methods require higher quantity (>10 ng) of template DNA per PCR reaction. These features prove to be disadvantageous in high resolution genetic mapping experiments where a large number of markers are analyzed using a single mapping population and genetic analysis of infectious organisms and parasites where DNA yield per sample may be too low for conventional PCR assays.

In the present study we demonstrate that the sensitivity, speed and informativeness of the existing ISSR-PCR method could be enhanced substantially by using fluorescent dye labeled nucleotide in the ISSR-PCR reaction, which we call FISSR-PCR, followed by separation of PCR products on an ABI automated sequencer 377, using diverse species of plants, and parasitic organisms (data not shown). We also show that the FISSR-PCR polymorphic markers could be unambiguously scored in the segregating F_2 population of silkworm thus showing that FISSR-PCR technique is a simple, sensitive and robust method for high resolution mapping of complex genomes.

MATERIALS AND METHODS

Plant materials

Eight different plant species of Wheat, Rice, Maize, Lentil, Chick Pea, Cow Pea, Pigeon Pea, and Pea and two to three varieties from each of the plant species were used for FISSR analysis.

Silkworm samples

An F_2 mapping population of 99 offspring developed by sib-mating of F_1 hybrid silk moths derived from a cross of two divergent strains of *B. mori*, $P^{50} \times C_{108}$ was used to demonstrate the inheritance and segregation of FISSR markers.

DNA extraction

Plant genomic DNA was extracted as described by Dellaporta *et al.*[15] Briefly, the seeds were ground in 200 µl of grinding/lysis buffer (50 mM Tris-HCl pH 7·2, 50 mM EDTA pH 8·0, 3% SDS, 1% 2-mercaptoethanol). The DNA was precipitated with 0·1 final volume of 3·0 M sodium acetate pH 5·2 and 2·5 volume of absolute ethanol, incubated at −70°C. DNA was quantified on 0·8% agarose gel and diluted to a uniform concentration (10 ng/µl). Genomic DNA from silk moths was isolated either from the posterior silk glands collected from day 3 fifth instar larvae or from the whole pupae.[16] Briefly, silk glands or whole pupae were ground in liquid nitrogen using a pestle and mortar. Extraction buffer (100 mM Tris-HCl, pH 8·0, 50 mM NaCl, 50 mM EDTA and 1% SDS) was added to the ground tissue and incubated at 37°C for 2 h with occasional swirling. The DNA was extracted twice with phenol-choloroform-isoamyl alcohol (25 : 24 : 1) and once with chloroform. The supernatant DNA was ethanol precipitated, resuspended in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8·0) buffer and incubated at 37°C for 1 h after addition of RNase A (100 µg/ml).

Primers

We designed and synthesized in-house six 5′-anchored primers [GRT RCY GRT R$(CA)_7$, RAY RAT RC$(GA)_7$, RYA CRY RCA R$(TG)_7$, YAY RYA CAY $(TG)_7$T, CRT RT$(GT)_9$, YGY RAY RAT $(GA)_8$] and two 3′-anchored primers [$(GA)_8$ C RG, $(RY)_8$A TCC] on an ABI DNA synthesizer, purified by PAGE and then desalted through an oligonucleotide purification cartridge (OPC).

PCR amplification and electrophoresis

FISSR-PCR

For FISSR-PCR assay, amplification was performed in 10 mM Tris-HCl, pH 8·3/50 mM KCl/2·5 mM $MgCl_2$/0·01% gelatin/0·01% Triton X-100/1 mM dNTPs/

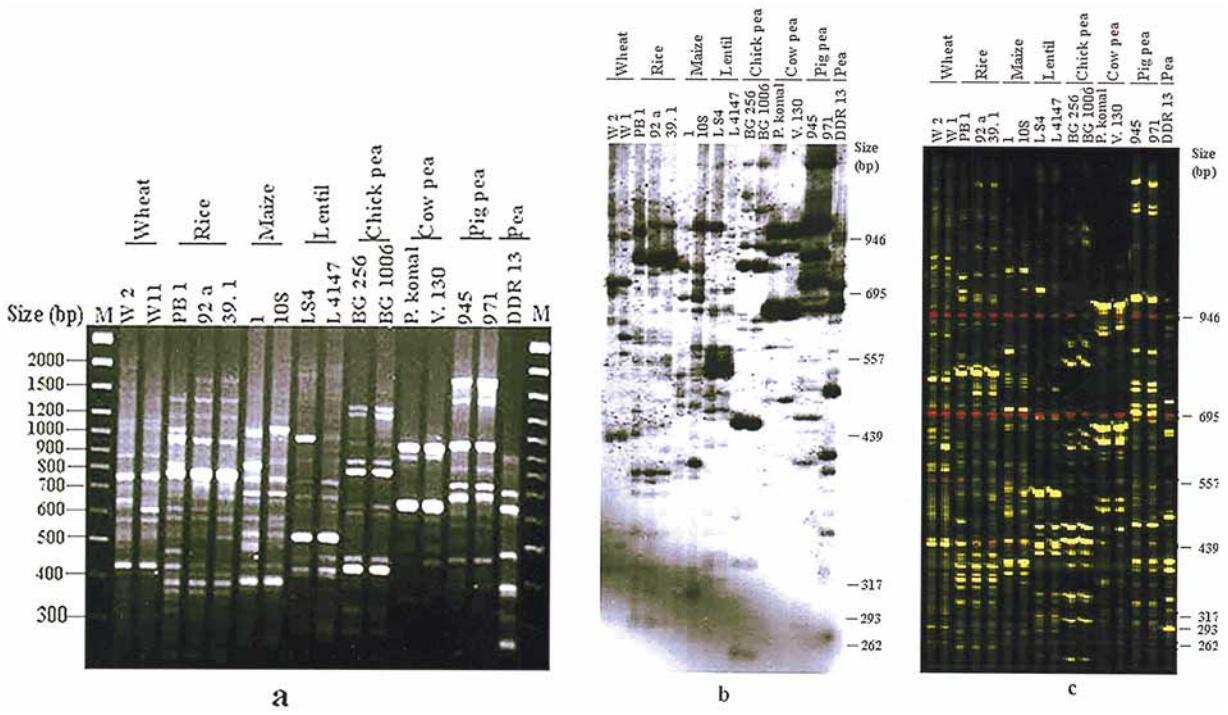

Fig. 1(a–c). Comparison of regular ISSR-PCR and FISSR-PCR assays using the primer 5′ CRT RT$(GT)_9$ 3′ in eight crop species on three electrophoretic systems showing reduced sensitivity on Nusieve agarose (a); intermediate on polyacrylamide sequencing gel with radiolabelling (b), and high sensitivity in FISSR-PCR products resolved on an ABI 377 sequencer (c).

0·4 μM Fluorescent dUTP (TAMARA Perkin-Elmer)/ 0·25 unit of Taq DNA polymerase (Ampli Taq Gold, Perkin-Elmer), 4 μM primer with 5 ng of genomic DNA per 5 μl reaction. The thermal cycling conditions were as follows: initial denaturation of 10 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 50°C and 1 min at 72°C, final extension of 10 min at 72°C. The PCR was performed on a DNA thermal cycler (Perkin-Elmer 9600). One μl of PCR products was mixed with 1·5 μl of 6 × loading buffer (1 : 4 mixture of loading buffer and formamide (Sigma) and 0·4 μl of Gene scan-1000 (ROX) size was included in the loading samples. The samples were denatured at 92°C for 1 min prior to loading on to an ABI 377 automated sequencer and electrophoresed on 5% polyacrylamide gel (Long ranger, FMC) under denaturing conditions containing 7 M urea, in 1 × TBE buffer (90 mM Tris borate, pH 8·3 and 2 mM EDTA). We also analyzed ISSR-PCR products on agarose gel electrophoresis.

ISSR-PCR

To compare the efficacy of the FISSR-PCR assay with the regular ISSR-PCR assay, the ISSR-PCR reactions were carried out. The PCR and thermal cycling conditions used were as in FISSR-PCR assay except 20 ng template DNA and 8 μM primer without fluorescent dUTP were used in 10 μl reaction. The PCR products were electrophoresed on 2% agarose (50% Sigma + 50% Nusieve) for 3 h at 100 V. The ISSR-PCR reactions were also carried out as previously reported[13] in the presence of $\alpha[^{32}P]$ labeled dCTP and cold dCTP (added in the ration of 1 : 4) in the PCR reaction. The PCR products were denatured at 75°C for 2 min, chilled on ice and electrophoresed on a standard sequencing gel (5% acrylamide, 7 M urea, 1 × TBE) and run at 900 V under constant power supply for 15 h.

RESULTS AND DISCUSSION

Each of the eight SSR-anchored primers revealed distinct PCR profiles in agarose, PAGE (PCR products incorporated with $\alpha[^{32}P]$ labeled nucleotide) and ABI 377 automated sequencer (PCR products incorporated with fluorescent nucleotide) in all the 8 plant species with characteristic species and varietal specific profiles as shown in Figure 1a–c. However, the number of markers revealed in each of the

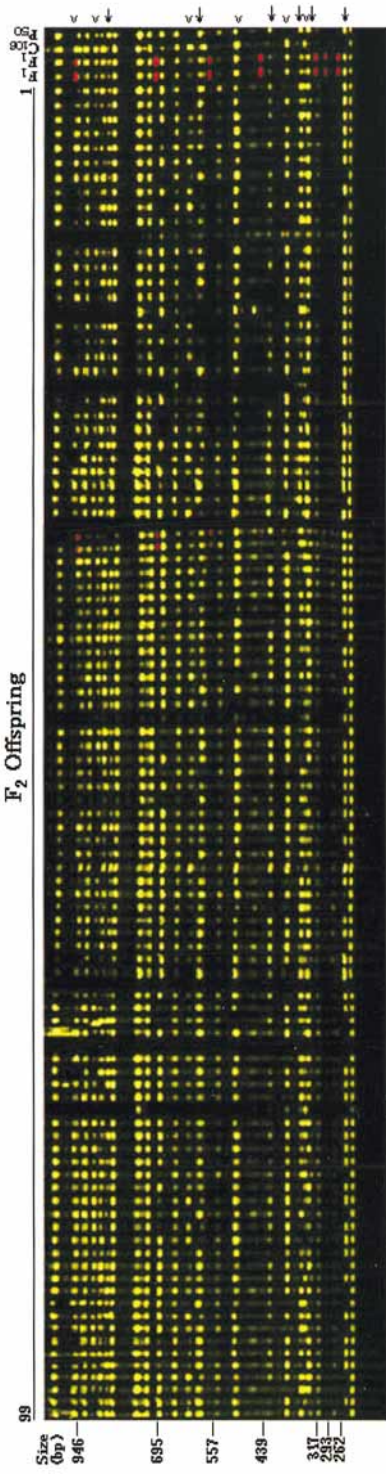

Fig. 2. The Mendelian segregation of FISSR markers in silkworm. The FISSR markers were generated using a primer 5′ RAY RAT RC$(GA)_7$ 3′ on two parental strains, P^{50} and C_{108}, and their F_1 and F_2 offspring. The arrows and arrowheads indicate markers specific to P^{50} and C_{108}, respectively.

methods differed substantially. The FISSR assay was found to be the most informative in all the species studied. It resolved almost two-fold more number of markers as compared to the existing ISSR-PCR methods based on agarose and PAGE analyses. As a result, the number of species and varietal specific markers available for scoring increased substantially (Fig. 1a–c, and Table 1a). For example, the primer CRT RT$(GT)_9$ in FISSR assay resolved 45 scorable bands in rice out of which as many as 14 markers were specific to rice as compared with the other seven plant species. Ten markers could distinguish all the 3 rice varieties (Fig. 1c and Table 1a).

Since FISSR-PCR technique is very sensitive it is a method of choice for detecting polymorphic markers in closely related varieties/populations which are otherwise difficult to discriminate by using other marker systems. As could be seen from Fig. 1 and Table 1a, the inter-varietal polymorphisms among the self pollinated and most conserved legume varieties suggest the efficiency and usefulness of this assay for varietal and cultivar identification. The FISSR-PCR assay could be successfully used as a rapid, sensitive and informative technique to quickly fingerprint a large number of genotypes of a given species in a cost-effective manner. Since FISSR-PCR assay is automated, a single assay including analysis could be completed within a day.

The method is also very useful to quickly evaluate the abundance of microsatellite repeats in different genomes using anchored primers with different microsatellite motifs. For example, $(GT)_n/(CA)_n$ is more abundant in wheat and rice genomes than other genomes studied (Table 1a). Such information is crucial in the projects aimed at microsatellite marker development.[17]

We also used this technique successfully to amplify the diverse genomes of insects, parasites of insects and human such as *Plasmodium*, *Leishmania* and *Brugia malayi* and many infectious organisms such as *Vibrio cholerae*, *Mycobacterium tuberculosis*, *Helicobacterium pylori*, *Pseudomonas aeruginosa* and other organisms (data not shown) where DNA yield per sample may be too low to be useful for conventional PCR-based marker assays. In all the cases, very clear, easily scorable and highly reproducible FISSR markers could be resolved depending on the type of core microsatellite repeat included in the anchored ISSR primers.

To demonstrate the utility of FISSR-PCR markers in genetic mapping experiments, we analyzed 99 F_2 offspring of silkworm, *B. mori* derived from a cross of two divergent silkworm strains, P^{50} and C_{108} using the markers amplified by the primer, RAY RAT RC $(GA)_7$. As could be seen from Figure 2 and Table 1b,

Table 1a. Comparison of FISSR-PCR assay using CRT RT$(GT)_9$ with regular ISSR-PCR based on agarose gel and PAGE analysis

Crop	Assay	Total number of bands	Molecular size (bp)
Wheat	Agarose ISSR-PCR	18 (06,02)	300–1900
	PAGE ISSR-PCR	22 (05,03)	290–1200
	FISSR-PCR	47 (14,03)	250–1200
Rice	Agarose ISSR-PCR	21 (06,04)	250–1700
	PAGE ISSR-PCR	24 (05,03)	260–1100
	FISSR-PCR	45 (14,10)	250–1200
Maize	Agarose ISSR-PCR	14 (08,03)	300–1000
	PAGE ISSR-PCR	21 (09,02)	270–1200
	FISSR-PCR	32 (12,05)	260–1100
Lentil	Agarose ISSR-PCR	13 (04,01)	300–1000
	PAGE ISSR-PCR	23 (07,02)	280–1200
	FISSR-PCR	32 (14,02)	250–1200
Chick Pea	Agarose ISSR-PCR	14 (08,02)	350–1200
	PAGE ISSR-PCR	16 (08,03)	250–1200
	FISSR-PCR	22 (10,—)	250–1200
Cow Pea	Agarose ISSR-PCR	08 (04,02)	350–0900
	PAGE ISSR-PCR	10 (05,03)	250–1200
	FISSR-PCR	19 (08,03)	250–1000
Pigeon Pea	Agarose ISSR-PCR	12 (04,02)	400–1700
	PAGE ISSR-PCR	17 (08,02)	260–1200
	FISSR-PCR	33 (16,02)	250–1200
Pea	Agarose ISSR-PCR	09 (05,—)	270–0900
	PAGE ISSR-PCR	20 (12,—)	250–1000
	FISSR-PCR	26 (08,—)	290–1000

Figures in the parentheses indicate species and varietal specific markers, respectively.

Table 1b. Mendelian segregation of FISSR-PCR markers amplified by RAY RAT RC$(GA)_7$ in 99 F_2 offspring derived from P^{50} and C_{108} cross

Polymorphic markers (bp)		No. of F_2 offspring scored	Expected Ratio	Observed Ratio	χ^2	*P*>
P^{50}	C_{108}					
	940	99	74·25 : 24·75	70 : 29	0·973	0·300
	850	99	74·25 : 24·75	70 : 29	0·973	0·300
835		99	74·25 : 24·75	67 : 32	2·876	0·100
	575	99	74·25 : 24·75	69 : 30	1·485	0·200
560		99	74·25 : 24·75	75 : 24	7·600	0·006
	500	99	74·25 : 24·75	70 : 29	0·973	0·300
420		99	74·25 : 24·75	74 : 25	10·943	0·001
	390	99	74·25 : 24·75	78 : 21	0·379	0·500
340		99	74·25 : 24·75	77 : 22	0·406	0·500
	335	99	74·25 : 24·75	72 : 27	0·372	0·500
330		99	74·25 : 24·75	75 : 24	7·600	0·005
260		99	74·25 : 24·75	73 : 26	0·084	0·800

the 12 markers which were polymorphic between the two parental strains inherited and segregated according to Mendelian principle. These results show that the FISSR-PCR markers are very useful for quick and highthroughput genotyping of mapping population using very small quantity of template DNA. For constructing high density linkage maps, a large number of F_2 individuals need to be analyzed using

a number of molecular markers and the template DNA requirement in the case of other conventional PCR methods is relatively high. The FISSR assay provides a large number of DNA markers per primer and allows detection of markers with as little as 2–5 ng of template DNA, on an automated sequencer obviating the necessity for using radioactive isotopes.

In conclusion, we show that this rapid, less hazardous, simple and informative assay could be used in large-scale screening of varieties/inbred lines, high resolution genetic mapping of complex genomes and quick genetic analysis of large sample sizes of various infectious organisms which yield very little quantity of DNA, with high degree of accuracy.

ACKNOWLEDGEMENTS

Financial support by the Department of Biotechnology, Government of India to J.N. is gratefully acknowledged.

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