The Bioscan 10(1): 55-61, 2015
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
www.thebioscan.in

ISSR MARKER BASED DNA FINGERPRINTING IN RELEASED VARIETIES AND SELECTED SUPERIOR SOMACLONES OF GINGER (*ZINGIBER OFFICINALE* ROSC.)

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KEYWORDS
ISSR marker
DNA fingerprinting
Somaclones
Ginger

Received on :
05.05.2014

Accepted on :
10.12.2014

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ABSTRACT
Inter Simple Sequence Repeats (ISSR) assay was performed to fingerprint ginger varieties released from Kerala Agricultural University, Vellanikkara, Kerala*viz*., "Athira", "Karthika" and "Aswathi" and three selected superior somaclones of ginger viz., B3, 478R and 88R and source parent cultivars Maran and Rio-de-Janeiro. Good quality genomic DNA was extracted from ginger varieties/somaclones and source parent cultivars using CTAB method. Thirty ISSR primers were screened for amplification of genomic DNA and eleven primers were selected based on the amplification pattern. DNA fingerprints of the varieties/somaclones were developed utilizing the clear, distinct bands generated in ISSR profiles and size of the amplicons. Different colour codes were assigned for amplicons produced by the marker system in different varieties, somaclones and source parent cultivars to generate fingerprints. The fingerprints developed were unique and specific for the varieties/somaclones and source parent cultivars. The specific fingerprint data will serve as a mark for identifying the varieties/clones and could be utilized for registration and documentation of varieties, settling IPR issues and to avoid biopiracy.

INTRODUCTION

India, 'the spice bowl of the world,' enjoys a unique position in the production and export of ginger (*Zingiber officinale* Rosc.) from time immemorial. The crop is much valued as a spice, medicine and vegetable. Breeding of ginger through selection and hybridization is seriously handicapped by lack of variability, absence of natural seed set and exclusive vegetative propagation. As natural variability stands limited, broadening the genetic base through mutagenesis or tissue culture techniques pave way for exploitation of induced variability for isolation of plant types with desirable traits.

Somaclonal variation was induced in the two cultivars viz., Maran and Rio-de-Janeiro at College of Horticulture, Kerala Agricultural University, India, through various modes of regeneration and through in vitro mutagenesis. The somaclones were subjected to single plant evaluation and they were further evaluated for yield, quality and reaction to pests and diseases (Paul and Shylaja, 2009; Paul *et al.,* 2009; Paul *et al.,* 2011; Paul and Shylaja, 2012). After conducting Initial Evaluation Trials, Advanced Varietal Trials, On Farm Evaluation Trials and Multi Locational Trials, the three superior somaclones, were released as varieties under the names "Athira" "Karthika" and "Aswathi" (Shylaja *et al.,* 2010) and somaclones viz., 478R, 88R and B3 were selected as superior clones after the evaluation. The varieties Athira, Karthika and somaclone B3 were derived from the cultivar Maran and the variety Aswathi, somaclones 478R and 88R were from the cultivar Rio-de-Janeiro.

DNA fingerprinting is an efficient tool for genotype identification, assessing genetic diversity and protecting plant varieties. DNA fingerprinting was also attempted in ginger by several workers (Nayak *et al.,* 2005; Harisaranraj *et al.,* 2009; Palai and Rout, 2007; Prem *et al.,* 2008; Sajeev *et al.,* 2011). Central Seed Committee established under the Seed Act, 1996 stipulates the necessity of DNA fingerprint data for the varieties released or proposed to be released.

DNA-based molecular markers show differences in nucleotide sequences of DNA, which are now well established as powerful and versatile tools in the fields of plant breeding, taxonomy, physiology, genetic engineering (Kesawat and Das Kumar, 2009) genome mapping, and gene tagging (Bornet and Branchard, 2004). The inter-simple sequence repeats (ISSR)-PCR technique was chosen for assessing genetic variation in this study. ISSRs are randomly distributed throughout the genome and use simple sequence repeats anchored at the 5'- or 3'-end by a short arbitrary sequence as PCR primers (Zietkiewicz *et al.,* 1994). They provide a powerful

tool for genetic mapping and assessment of genetic diversity between closely related species and to detect similarities between and within species as well (Davilá *et al.,* 1998; Moreno *et al.,* 1998).

The ISSR technique has been reported as a good alternative to AFLP when tested on Curcuma species (Syamkumar and Sasikumar, 2007; Das *et al.,* 2011), roses (Jabbarzadeh *et al.,* 2010), and pea and strawberry varieties, where it is less expensive, more rapid and more reproducible. The main advantage of ISSRs is that no sequence data for primer construction are needed. Since the analytical procedures include PCR, only low quantities of template DNA are required. ISSR markers are effective multilocus markers for application such as diversity analysis, fingerprinting and genome mapping, gene tagging and marker assisted selection. As no prior sequence knowledge is required, they are more rapidly applied than SSR markers, and they are more reliable and robust than RAPD markers, mainly due to the method of detection, and possibly also to the fact that primers are longer, and hence PCR condition are more stringent. Taheri *et al.* (2012) used PCR-based molecular markers (ISSRs) to assess genetic variation and relationships between five varieties of curcuma (*Curcuma alismatifolia*) cultivated in Malaysia. Singh *et al.* (2012) studied the genetic diversity among turmeric accessions from ten different agro-climatic regions comprising five cultivars and 55 accessions using two DNA-based molecular marker techniques, viz., Random Amplified Polymorphism DNA (RAPD) and Inter Simple Sequence Repeat (ISSR).

For the newly released ginger varieties and selected superior somaclones in pipeline for release, no fingerprint data are available.Keeping this in view the present study was conducted to characterize the released varieties and selected superior somaclones of ginger along with source parent cultivars using ISSR marker and to develop a DNA fingerprint specific to each genotype.

MATERIALS AND METHODS

Genomic DNA extraction

Three KAU released varieties of ginger (Athira, Karthika and Aswathi), three superior selected somaclones (478R, 88R and B3) and two source parent cultivars (Maran and Rio-de-Janeiro) maintained at Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara, Kerela, India were used for the study. Good quality genomic DNA was extracted from one gram young leaf of the ginger varieties/ somaclones/ source parent cultivars following CTAB method reported by Rogers and Bendich (1994) and quantified in 0.8% agarose according to a standard DNA marker.

PCR amplification and fragment analysis

ISSR PCR reactions were performed as per the procedure reported by Zietkiewicz *et al.* (1994). The primers for ISSR assay were supplied by Sigma Aldrich chemicals Pvt. Ltd. and Xcelris Genomics Primex. The amplification was carried out in Veriti^{R} Thermal cycler (Applied Biosystems). Thirty ISSR primers in the series UBC, ISSR and SPS were screened with bulked DNA samples from the variety Athira and somaclones 292R and 478R. Genomic DNA at the concentration of 25 to 30 ng/μL was subjected to amplification using selected primers.

The annealing temperature of ISSR primers used in the study ranged from 43ºC to 55ºC. After an initial screening, primers were selected for ISSR assay. Primers with good resolving power were used for amplification of DNA. The PCR reaction was performed using a 20μL reaction mixture containing 30ng of genomic DNA, 10 X assay buffer (B) (pH 8.3), 10mM/mL dNTP mix, decamer primers, 2mM of $MgCl_2$ and 0.5 U of Taq (Thermophilus aquaticus) DNA polymerase (Genei). The basic PCR program to amplify DNA was as follows: an initial hot start and denaturing step at 94ºC for 4 min followed by 35 cycles of a 45 seconds denaturation at 94ºC, 1 min annealing at 43ºC and 2 min primer elongation at 72ºC. A final extension step at 72ºC for 8 min was performed.

Gel electrophoresis

15 μL aliquot of the PCR amplified samples was combined with 2μL of a loading buffer (0.4% Bromo-phenol Blue, 0.4% xylene cyanole and 5 mL of glycerol). The amplified products were loaded on two per cent agarose gel using buffer stained with ethidium bromide along with marker (100bp Invitrogen). The documented ISSR profiles were carefully examined for amplification of DNA as bands. The size of polymorphic bands in kb / bp of bases were recorded in comparison with marker and using the software Quantity One.

Out of 30 ISSR primers screened for ISSR analysis, primers which gave good amplification products for each ginger variety / clone were selected for further assay (Table 1).

Data Analysis

Scoring of bands on agarose gel was done with the Quantity

Table 1: ISSR primers used for screening ginger genotypes

Sl. No.	Primers	Nucleotide Sequence
1.	UBC 840	5'GAGAGAGAGAGAGAGAYT3'
2	UBC 844	5'CTCTCTCTCTCTCTCTRC3'
3	UBC 890	5'VHVGTGTGTGTGTGTGT3'
4	UBC 811	5'GAGAGAGAGAGAGAGAC3'
5	UBC813	5'CTCTCTCTCTCTCTCTT3'
6	UBC 815	5'CTCTCTCTCTCTCTCTG3'
7	UBC354	5'CTAGAGGCCG3'
8	UBC S 2	5'CTCTCTCTCGTGTGTGTG3'
9	UBC 866	5'CTCCTCCTCCTCCTCCTC3'
10	UBC 826	5'ACACACACACACACACC3'
11	UBC 848	5'CACACACACACACACARG3'
12	UBC 845	5'CTCTCTCTCTCTCTCTRG3'
13	UBC 868	5'GAAGAAGAAGAAGAAGA3'
14	UBC 834	5'AGAGAGAGAGAGAGAGYT3'
15	UBC 835	5'AGAGAGAGAGAGAGAGY3'
16	UBC 836	5'AGAGAGAGAGAGAGAGY3'
17	UBC 807	5'AGAGAGAGAGAGAGAGT3'
18	UBC 817	5'CACACACACACACACAA3'
19	UBC 818	5'CACACACACACACACAG3'
20	UBC 820	5'GTGTGTGTGTGTGTGTC3'
21	ISSR 04	5'ACACACACACACACACC3'
22	ISSR 05	5'CTCTCTCTCTCTCTTG3'
23	ISSR 06	5'GAGAGAGAGAGAGAGAC3'
24	ISSR 07	5'CTCTCTCTCTCTCTTG3'
25	ISSR 08	5'GAGAGAGAGAGAGAGAT3'
26	ISSR 09	5'CTCTCTCTCTCTCTCTCG3'
27	ISSR 10	5'ACACACACACACACACG3'
28	ISSR 15	5'TCCTCCTCCTCCTCC3'
29	SPS 03	5'GACAGACAGACAGACA3'
30	SPS 08	5'GGAGGAGGAGGA3'

One software. lDNA marker (EcoRI+Hind III double digest, 1000bp) and 100bp ladder were used as molecular weight size marker for each gel along with DNA samples. The bands were scored as one and zero for the presence and absence respectively and their size recorded in relation to the molecular weight markers used and with the software Quantity One.DNA fingerprint of each genotype was generated based on the presence of clear and distinct bands and size of the bands. Separate colour codes were given to highlight the presence of unique bands, bands shared with two genotypes, three genotypes etc. In fingerprints generated the presence of unique band was represented in violet colour. Navy blue was used to highlight the bands shared with two genotypes, light blue for bands shared with three genotypes, pink for bands shared with four genotypes, yellow for bands shared with five genotypes, orange was for bands shared with genotypes, red for bands shared with seven genotypes and green for bands present in all the eight genotypes.

Table 2: Amplification pattern of ISSR primers in ginger genotypes

Sl. No.	Primers	Amplification Pattern			
		No. of bands	Types of bands		Remarks
			Distinct	Faint	
1.	UBC 840	5	3	2	Selected
2	UBC 844	3	3	0	Selected
3	UBC 890	0			-
4	UBC 811	3	0	3	-
5	UBC813	0			-
6	UBC 815	2	2	0	-
7	UBC354	0			-
8	UBC S 2	1	1	0	-
9	UBC 866	7	2	5	Selected
10	UBC 826	0			-
11	UBC 848	1	0	1	-
12	UBC 845	0			-
13	UBC 868	2	1	1	-
14	UBC 834	5	2	3	Selected
15	UBC 835	6	2	4	Selected
16	UBC 836	4	0	4	-
17	UBC 807	0			-
18	UBC 817	1	0	1	-
19	UBC 818	1	0	1	-
20	UBC 820	1	0	1	-
21	ISSR 04	7	2	5	Selected
22	ISSR 05	3	3	0	Selected
23	ISSR 06	6	1	5	Selected
24	ISSR 07	2	0	2	-
25	ISSR 08	4	2	2	Selected
26	ISSR 09	2	1	1	-
27	ISSR 10	6	2	4	Selected
28	ISSR 15	2	0	2	-
29	SPS 03	5	1	4	Selected
30	SPS 08	3	0	3	-

RESULTS AND DISCUSSION

The amplification pattern produced by thirty primers belonging to different ISSR primer series viz., UBC, ISSR and SPS are provided in Table 2. Based on the presence of clear and distinct bands, eleven primers were selected for ISSR assay of ginger varieties / somaclones. The selected primers were UBC 834, UBC 835, UBC 840, UBC 844, UBC 866, SPS-03, ISSR-04, ISSR-05, ISSR-06, ISSR-08 and ISSR-10 (Table 3).

DNA fingerprinting of the ginger genotypes

Variety Athira

The amplification pattern observed for the genomic DNA of ginger variety Athira with eleven selected ISSR primers is presented in Plate 1a. Twenty nine clear and distinct loci were generated with eleven ISSR primers. The amplicons ranged in size from 200bp to 1300bp. Most of the primers amplified two and four distinct bands for the variety Athira whereas primers UBC 835 and SPS 03 amplified only one distinct band and primer ISSR 06 amplified five distinct bands. Fingerprint developed based on presence of clear and distinct bands and size of bands produced with eleven ISSR primers is presented in Fig. 1a. In ISSR fingerprint, the salient feature observed for Athira was the unique band developed by the primer ISSR 06 (900bp) which is highlighted as violet in the fingerprint (Fig. 1a).

Variety Karthika

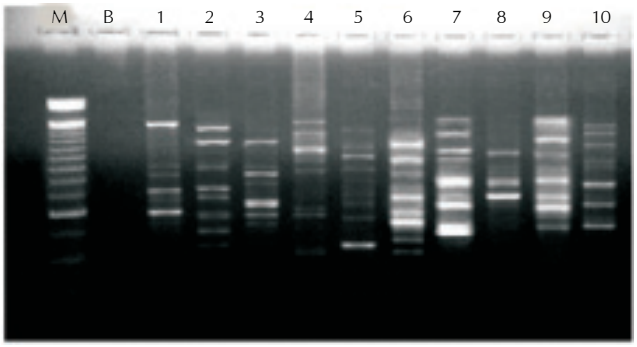
The amplification pattern observed for the genomic DNA of ginger variety Karthika with eleven selected ISSR primers is presented in Plate 1b. Twenty six clear and distinct loci were produced by eleven ISSR primers. The amplicons ranged in size from 200bp to 1300bp. Most of the primers amplified two distinct bands for the variety Karthika, while it was only one for the primers UBC 835and SPS 03. The primer UBC 840 gave four, ISSR 05 gave three and ISSR 08 gave five distinct and clear bands. Fingerprint developed based on clear and distinct bands and size of bands produced with ten RAPD primers is presented in Fig. 1b.

Somaclone B3

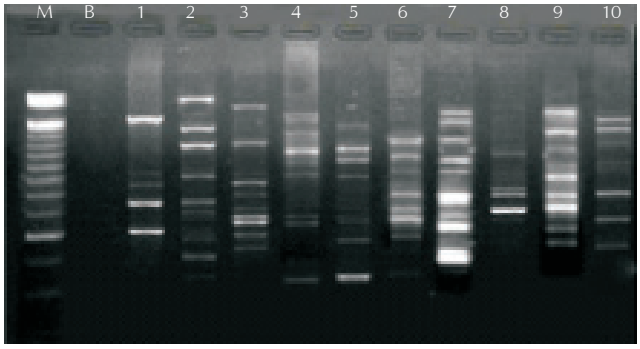
The amplification pattern observed for the genomic DNA of

Table 3: Selected ISSR primers

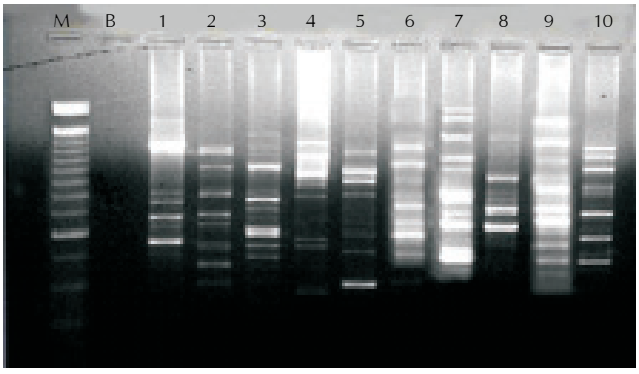
Sl. No.	Name of Primer	Sequence	Annealing temperature (°C)
1	UBC 834	5′AGAGAGAGAGAGAGAGYT3′	45
2	UBC 835	5′AGAGAGAGAGAGAGAGY3′	43
3	UBC 840	5′GAGAGAGAGAGAGAGAYT3′	45
4	UBC 844	5′CTCTCTCTCTCTCTCTRC3′	47
5	UBC 866	5′CTCCTCCTCCTCCTCCTC3′	55
6	SPS-03	5′GACAGACAGACAGACA3′	43
7	ISSR-04	5′ACACACACACACACACC3′	47
8	ISSR-05	5′CTCTCTCTCTCTCTTG3′	43
9	ISSR-06	5′GAGAGAGAGAGAGAGAC3′	47
10	ISSR-08	5′GAGAGAGAGAGAGAGAT3′	45
11	ISSR-10	5′ACACACACACACACACG3′	47



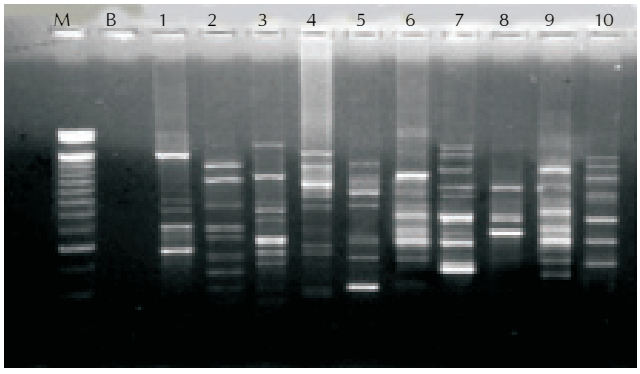
A. Athira



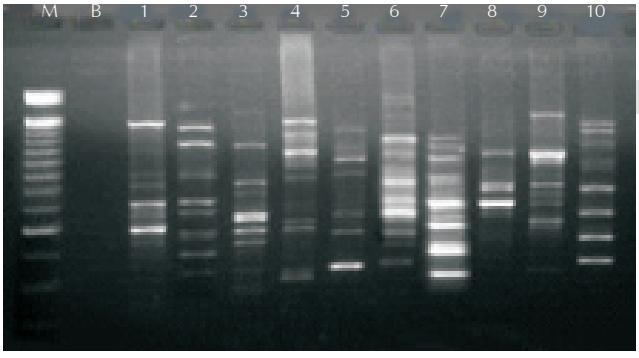
B. Karthika



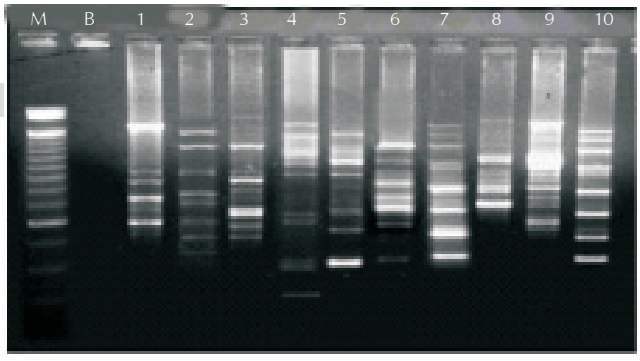
C. B3



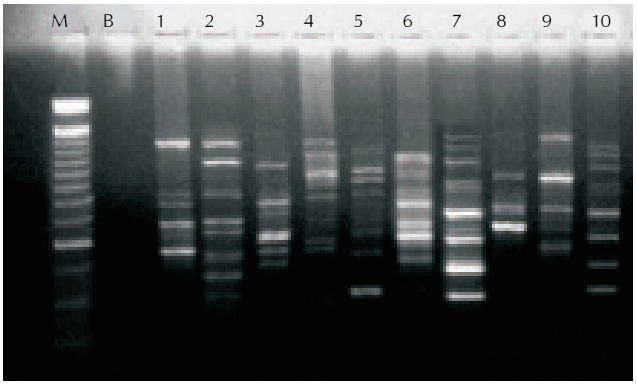
D. Maran



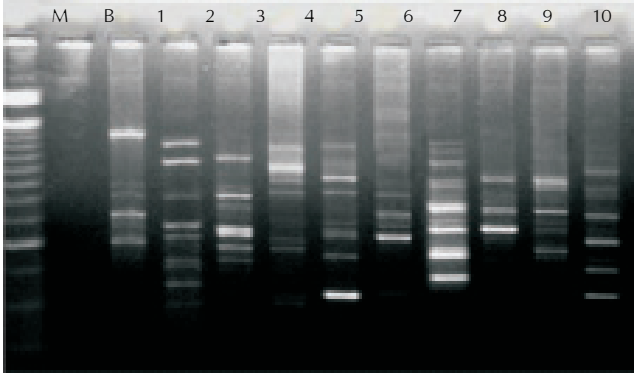
E. Aswathi



F. 478R



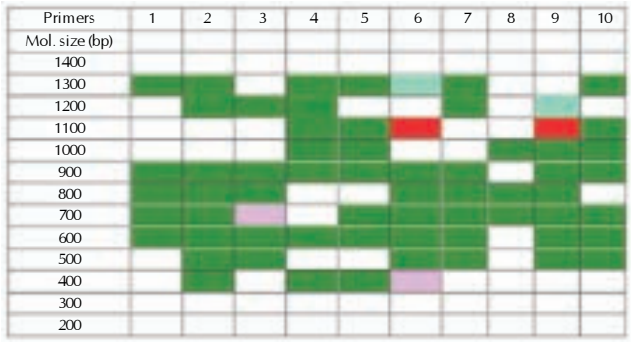
G. 88R



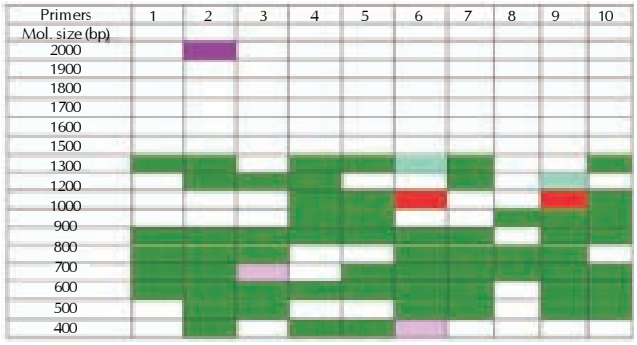
H. Rio-de-Janeiro

M: 100bp ladder, B: Blank
1: OPA 04, 2: OPA 12, 3: OPA 27, 4: OPA 28, 5: OPD 15, 6: OPD 20, 7: OPP 16, 8: OPU 03, 9: RN O8, 10: S 11

Plate 1: Amplification patterns of ginger genotypes with the selected ISSR primers

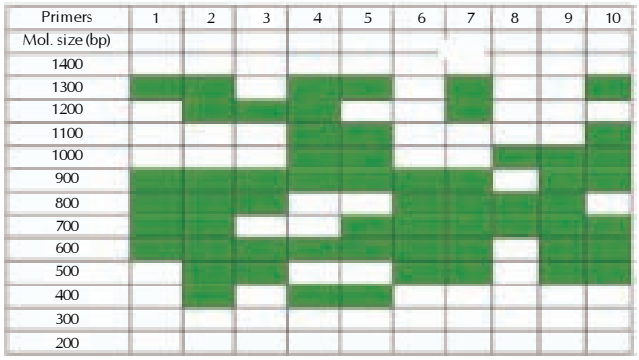


A. Athira

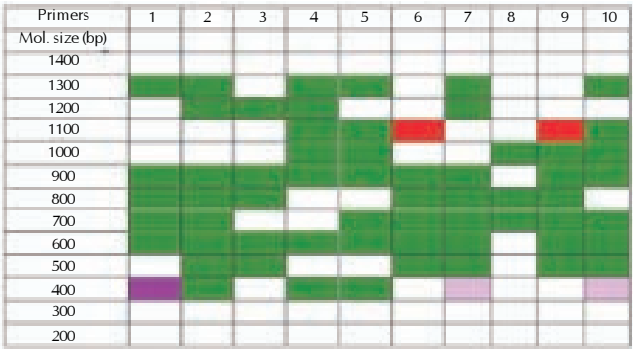


B. Karthika

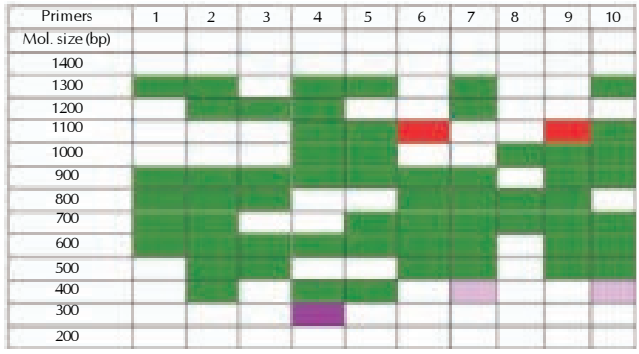
C. B3



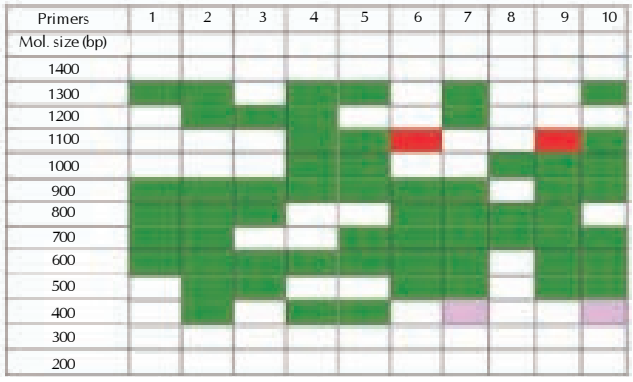
D. Maran



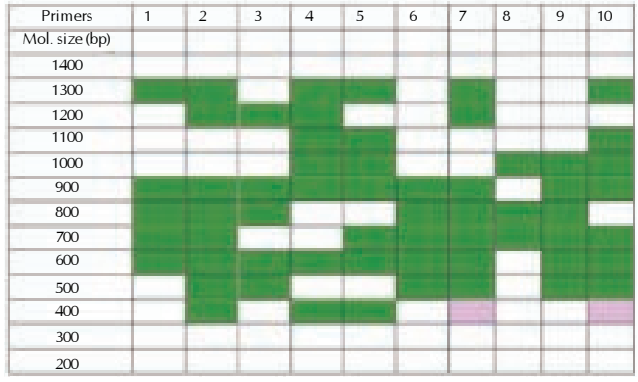
E. Aswathi



F. 478R



G. 88R



H. Rio-de-Janeiro

Legend: Colour code for sharing of bands among varieties/somaclones

- Unique band
- Shared by two
- Shared by three
- Shared by four
- Shared by five
- Shared by six
- Shared by seven
- Shared by eight

Figure 1: DNA fingerprints of eight ginger varieties/somaclones

ginger somaclone B3 with eleven selected ISSR primers is presented in Plate 1c. The number of clear and distinct loci produced with eleven ISSR primers was twenty seven. The amplicons ranged in size from 200bp to 1300bp. Primers UBC 834, UBC 844, UBC 866, ISSR 05 and ISSR 10 gave two distinct and clear bands whereas primers UBC 835 and SPS03 gave only one distinct band. Primers ISSR 04 and ISSR 06 gave three clear and distinct bands. Primer UBC 840 gave four and ISSR 08 gave five distinct bands. Fingerprint was developed based on clear and distinct bands (Fig. 1c).

Cultivar Maran

The amplification pattern observed for the genomic DNA of ginger source parent cultivar Maran with eleven selected ISSR primers is presented in Plate 1d. The selected eleven ISSR primers produced thirty one clear and distinct loci. The amplicons ranged in size from 200bp to 1300bp. The primers UBC 840, ISSR 04, ISSR 05 and ISSR 06 gave four clear and distinct bands, whereas, primers UBC 834, UBC 844, UBC 866 and ISSR 10 gave two distinct bands. Primers UBC 835 and SPS 03 gave only one clear band and primer ISSR 08 gave five distinct and clear bands. Fingerprint was developed based on clear and distinct bands produced by the selected ISSR primers (Fig. 1d).

Variety Aswathi

The amplification pattern observed for the genomic DNA of ginger with Variety Aswathi eleven selected ISSR primers is presented in Plate 1e.

Twenty six clear and distinct loci were observed with eleven selected ISSR primers. The amplicons ranged in size from 200bp to 1200bp. Primers UBC 834, UBC 844, UBC 866 and ISSR 10 produced two clear and distinct bands, whereas, primers UBC 840, ISSR 05, ISSR 06 and ISSR 10 produced three clear and distinct bands. Primers UBC 835 and UBC SPS 03 gave only one clear and distinct band and primer ISSR 04 gave four clear and distinct bands. Fingerprint was developed based on clear and distinct bands produced by eleven selected ISSR primers (Fig. 1e).

Somaclone 478R

The amplification pattern observed for the genomic DNA of ginger somaclone 478R with eleven selected ISSR primers is presented in Plate 1f. Twenty four clear and distinct loci were observed with eleven ISSR primers. The amplicons ranged in size from 200bp to 1300bp. Primers UBC 834, UBC 840, UBC 844, UBC 866, ISSR 05 and ISSR 10 gave two clear and distinct bands, whereas, ISSR 06 and ISSR 08 gave three clear and distinct bands. Primers UBC 835 and SPS 03 gave only one clear and distinct band and primer ISSR 04 gave four clear and distinct bands. Clear and distinct bands produced by eleven selected ISSR primers were used to develop the fingerprint (Fig. 1f).

Somaclone 88R

The amplification pattern observed for the genomic DNA of ginger somaclone 88R with eleven selected ISSR primers is presented in Plate 1g. Twenty six clear and distinct loci were observed with eleven ISSR primers. The amplicons ranged in size from 200bp to 1300bp. Primers UBC 840, ISSR 05, ISSR 06 and ISSR 08 gave three clear and distinct bands, whereas, primers UBC 834, UBC 844, UBC 866 and ISSR 10 gave two clear and distinct bands. Primers UBC 835 and SPS 03 gave one clear and distinct band and primer ISSR 04 gave four clear and distinct band. Fingerprint was developed based on clear and distinct bands (Fig. 1g).

Cultivar Rio-de-Janeiro

The amplification pattern observed for the genomic DNA of ginger source parent cultivar Rio-de-Janeiro with eleven selected ISSR primers is presented in Plate 1h. Twenty eight clear and distinct loci were observed with eleven ISSR primers. The amplicons ranged in size from 200bp to 1300bp. Primers UBC 834, UBC 844, UBC 866 and ISSR 10 gave two clear and distinct bands, whereas primers UBC 840, ISSR 06 and ISSR 08 gave three clear and distinct bands. Primers UBC 835 and SPS 03 gave one clear and distinct band and primer ISSR 04 gave four clear and distinct band. Fingerprint was developed based on clear and distinct bands (Fig. 1h).

DNA fingerprinting could be done for the varieties and selected somaclones using two molecular marker systems viz., RAPD and ISSR. The investigations could bring out certain unique bands for genotype identification. The studies also helped to assess variability in the selected varieties / somaclones and to assess the extent of variability from parent cultivar.

The choice of a molecular marker technique depends on its reproducibility and simplicity. The best marker for genome mapping, marker assisted selection, phylogenic studies, and crop conservation should have low cost and labour requirements and high reliability. Since 1994, a molecular marker technique called Inter Simple Sequence Repeat (ISSR) has been available and is being exploited (Zietkiewicz *et al.,* 1994). ISSRs are semiarbitrary markers amplified by PCR in the presence of one primer complementary to a target microsatellite.

The ISSR markers are found useful in the fingerprinting of cultivated and wild species germplasm, and in understanding the evolutionary relationships of various crops such as *Oryza spp.* (Joshi *et al.,* 2000), *Solanum lycopersicon* Mill., (Aguilera *et al.,* 2011) and *Mangifera indica* L. (Luo *et al.,* 2011).The present findings are in corroborate with the above findings.

ACKNOWLEDGEMENT

We are indebted to Department of Biotechnology (DBT), Ministry of Science and Technology, Government of India and Kerala Agricultural University (KAU), Vellanikkara, Kerala for providing technical and financial support.

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