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Molecular characterization of primitive, elite and exotic ginger genotypes to protect the biowealth of elite ginger accessions.

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Abstract

Molecular fingerprints of elite, exotic and primitive ginger genotypes were developed using RAPD and ISSR markers to characterize and protect the accessions. Among the 30 molecular markers studied, 13 could easily discriminate the genotypes. Cluster analysis of data using UPGMA dendrogram placed the ginger genotypes into four separate groups. The grouping of elite genotype with the putative wild types in the dendrogram implies that there is some phylogenetic relationship between the putative wild types and modern cultivars. An exotic type from Japan, resembling the putative types in rhizome features, shared high similarity with the four indigenous putative types.

Key words : Exotic, ginger, improved variety, ISSR, putative wild, RAPD

Abbrevations

CTAB, Cetyl trimethyl ammonium bromide; ISSR, Inter simple sequence repeats; NTSYS, Numerical taxonomy system; RAPD, Randomly amplified polymorphic DNA; UPGMA, Unweighted Pair Group Method with Arithmetic Mean; WTO, World trade organization.

Introduction

Ginger (*Zingiber officinale* Roscoe) a perennial rhizomatous spice belongs to the family Zingiberaceae, growing annually. It is valued as a spice, flavouring agent and herbal medicine and is also employed in the perfume industry. Ginger is cultivated in many tropical and sub tropical areas, the main producers being India, China, Nigeria, Sierra Leone, Indonesia, Bangladesh, Australia, Fiji, Jamaica and Nepal. Among the different spices exported from India, ginger is one of the major items of export. It is estimated that around 5000 tones of ginger, valued about Rs. 2,340 lakh is exported during the year 2003-04 (Spices Statistics, 2004)

Ginger is believed to have originated in South East Asia probably in India (Purseglove *et al*.1981). However the existence of wild forms of ginger is not yet reported unambiguously even though putative wild forms are there (Sasikumar *et al*. 1995; Muralidharan & Velayudhan 1983). Putative wild forms of ginger such as 'Sabarimala' 'Kakkakalan', 'Kozhikalan' etc. were collected and conserved at Indian Institute of Spices Research, Calicut. Many of the putative wild type ginger accessions

though poor yielder is good in quality (Menon 2007; Anonymous). Pink ginger (*Z. officinale*) characterized by copper brown coloured rhizomes is an essential ingredient in the Naga cuisines of Nagaland. 'Kintoki' (*Z. oficinale* var. rubens) is an exotic ginger highly valued as a medicine in Japan (Tanabe *et al.*1992; Kano *et al.*1990).

Characterization of elite, exotic and primitive lines of high value and rare gingers is important for the protection of the biowealth of ginger in the present post WTO era. Though morphological characters are relatively easy to be done, molecular characterization based on DNA markers are more reliable.

Genetic diversity analysis of Zingiber officinale cultivars using RAPD/AFLP markers have been reported (Gao *et al*. 2006; Nayak *et al*. 2005; Mohd et al. 2004; Wahyuni et al. 2003 and Rout et al. 1998).

Though the putative wild types are quite distinct from the elite and exotic ginger cultivars in terms of its rhizome features and quality traits, there are no studies on the comparative molecular profiling of putative wild type vis-à-vis the improved varieties and exotic introductions. Such a study may throw some light on their genetic relatedness besides as a means to protect the material from biopiracy. The present work is an attempt in this direction.

Materials and methods

Plant material

The study was conducted at the Genetic Resources and Molecular Breeding Laboratory, Crop Improvement and Biotechnology Division, Indian Institute of Spices Research, Calicut. The experimental material comprised of seven different genotypes of ginger mainly exotic (Kintoki - Japan), elite (Varada), primitive or putative wild type varieties (Pink ginger, Kozhikalan Kakkakalan, Ellakkallan, Sabarimala) of ginger maintained in the field gene bank of Indian Institute of Spices Research, Calicut.

DNA extraction

Fresh ginger rhizomes were used for the isolation of DNA. The genomic DNA was isolated by modified CTAB method (Syamkumar et al. 2003). The extraction buffer contains 3% CTAB, 2 M NaCl, 100 mM Tris, 20 mM EDTA and 0.1% ß mercaptoethanol.

Polymerase Chain Reaction amplification

RAPD analysis

Twenty two random decamer primers (Operon Technologies, Almada, USA) were used for PCR amplification according to Williams et al. (1990). RAPD reaction was carried out in 25 µl reaction volume containing 25 ng genomic DNA, 1 U Taq DNA polymerase (Genei, Bangalore), 200 mM dNTPs, 2 mM $MgCl_2$ and 10 pmoles of random decamer primer. Standard amplification condition consisted of pre denaturation at 93 ºC for 3 min, denaturation at 93 ºC for 1 min, annealing at 37 ºC for 1 min, extension at 72 ºC for 1 min and final extension at 72ºC for 10 min and number of cycles was 35. The reaction was carried in a Master Cycler (EP gradient S, Eppendorf, Germany).

ISSR reaction

Fifteen ISSR primers (Integrated DNA Technologies, USA) were used. 20ng genomic DNA and 60 pmoles primer concentration were used for ISSR reaction. Concentrations of MgCl2, dNTP and Taq DNA polymerase were same as in the RAPD reaction. The annealing temperature in PCR condition was raised to 55 ºC and number of cycle repeats was 32.

Electrophoresis of PCR products

The amplified products were visualized in a 2% agarose gel containing 0.5 µg ml-1 of ethidium bromide and documented by a gel documentation system (Alpha Imager 2200, USA). The bands were scored based on the molecular weight marker (1 Kb DNA ladder, Biogene, USA).

Data scoring and analysis

The electrophoretic patterns were visually analysed and bands were scored as present (1) or absent (0). The matrix obtained was entered into the NTSYSpc programme (Rohlf, 1993). An UPGMA dendrogram was constructed based on the similarity coefficient.

Results and discussion

Out of 40 random decamer primers screened, 16 that gave consistent amplification pattern were selected for RAPD analysis (Fig.1). A total of 20 ISSR primers were screened and 14 were selected for the characterization (Fig.2).

The sequence of the RAPD/ISSR primers used for the genetic fingerprinting of the elite and exotic ginger genotypes and the total number of bands produced by each primer, number of polymorphic bands and percentage of polymorphism produced by each primer are represented in Table 1.

Among the 120 markers produced by 16 RAPD primers, 33 markers were polymorphic. Maximum polymorphism (40%) was observed in the case of primer OPJ05 and OPC11. Among the 16 primers utilized, only 6 primers produced unique bands. Three primers viz OPB-19, OPC-13, OPE-11 generated discrete bands in Pink ginger. OPJ-07 produced a unique band in Varada. While the primers OPA-08 and OPB-05 generated bands specific to 'Kintoki' and 'Kozhikkalan' respectively.

Whereas in the case of fourteen ISSR primers studied, maximum polymorphism (62.5%) were observed in the case of the primer (GA)8 T and 7 primers produced unique bands. The ISSR primers that discriminated Pinkginger were ISSR-1 and ISSR-6, ISSR-8 and ISSR-10. The primer ISSR-9 was discriminatory in case of Kintoki. The primer ISSR-13 and ISSR-3 produced Varada specific bands. No RAPD/ ISSR primer produced unique bands in the primitive type Ellakkallan, Kakkakalan and Sabarimala Maximum number of unique bands was observed in Pinkginger in the case of both RAPD and ISSR primers. The RAPD/

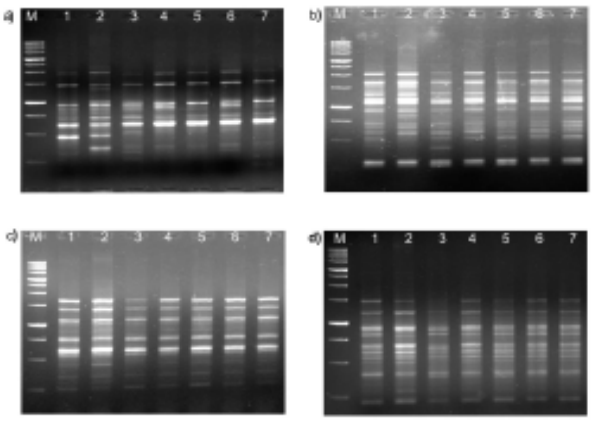

Fig. 1. RAPD profile of the DNA isolated from ginger genotypes amplified with primers
a) OPJ07 (5'CCTCTCGACA3'), b) OPA08 (5'GTGACGTAGG3'), c) OPD08 (5'GTGTGCCCA3'), d) OPD07 (5'TTGGCACGGG3') respectively. M-Marker-1Kb ladder; Lane 1-7 Varada, Pink ginger, Kintoki, Kozhikkalan, Kakkakalan, Ellakkallan and Sabarimala respectively.

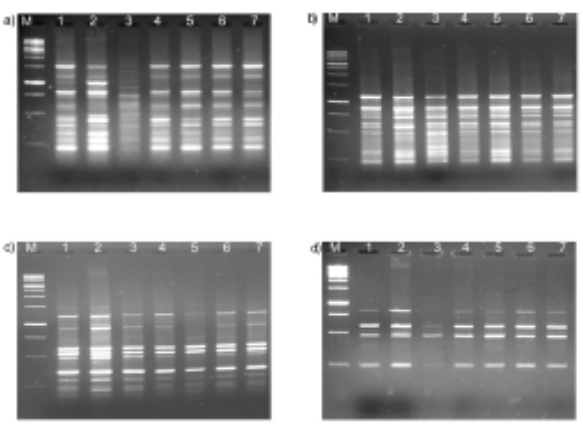

Fig. 2. ISSR profile of the DNA isolated from ginger genotypes amplified with primers
a) ISSR 5 [(AGC) 4 GT], b) ISSR 9 [(AC)8 G], c) ISSR 13 [(GA)8C], d) ISSR 8 [(CTC)3GC] M-Marker-1Kb ladder; Lane 1-7 Varada, Pinkginger, Kintoki, Kozhikkalan, Kakkakalan, Ellakkallan and Sabarimala, respectively.

ISSR primers that discriminate the ginger genotypes are shown in Table 2.

Cluster analysis

The UPGMA dendrogram (Fig.3) constructed based on the similarity coefficient showed a single cluster with 4 groups. The similarity coefficient of ginger genotypes is given in Table 3. The first and second group comprised of Pink Ginger and Kintoki, respectively. The

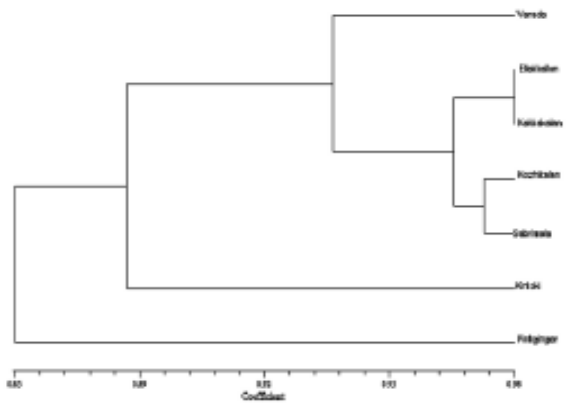

Fig.3. UPGMA dendrogram of ginger genotypes produced by RAPD and ISSR markers.

third group comprised four primitive ginger types like Ellakkalan, Kakkakalan, Kozhikalan and Sabarimala, collected from different pockets of Kerala. These primitive types showed maximum similarity (97%) between them (Ellakkalan & Kakkakalan and Kozhikalan & Sabarimala). Varada formed the fourth group. It is interesting to note that the exotic material Kintoki (*Z. officinale* var rubens) had comparatively high similarity with the indigenous primitive type, the Pinkginger (80%) and with the putative wild forms (88%). But the Pinkginger had comparatively low affinity with the four

Table 1. Sequence of RAPD/ISSR primers, number of bands generated by each primer, number of polymorphic bands and percentage polymorphism.

Sl No	Primer	Sequence (5'-3')	Total No of Bands	Number of Polymorphic band	% Polymorphism
1	OPA 8	GTGACGTAGG	14	3	21.4
2	OPA14	TCTGTGCTGG	9	1	11
3	OPB 8	GTCCACACGG	7	1	14
4	OPB 19	ACCCCCGAAG	8	2	12
5	OPC 8	TGGACCGGTG	8	2	25
6	OPC11	AAAGCTGCGG	5	2	40
7	OPC13	AAGCCTCGTC	7	1	14
8	OPC14	TGCGTGCTTG	6	1	16
9	OPD02	GGACCCAACC	6	1	16
10	OPD07	TTGGCACGGG	11	1	9
11	OPD06	ACCTGAACGG	6	2	33
12	OPD08	GTGTGCCCA	7	1	14
13	OPE 11	GAGTCTCAGG	11	3	27
14	OPJ 05	CTCCATGGGG	6	3	50
15	OPJ 07	CCTCTCGACA	12	5	41
16	OPJ 08	CATACCGTGG	6	2	33
17	ISSR-1	(CT) 8A	9	3	33
18	ISSR-2	(GA)8C	12	3	25
19	ISSR-3	(GA)8T	8	5	62.5
20	ISSR-4	(AGAC)3A	5	2	40.0
21	ISSR-5	(AGC)4GT	11	3	27
22	ISSR-6	(TCC)5AG	10	3	30
23	ISSR-7	(CT)7TG	6	5	83
24	ISSR-8	(CTC)3GC	4	2	50
25	ISSR-9	(AC)8G	12	3	25
26	ISSR-10	(CT)8G	6	2	33
27	ISSR-11	(GA)7G	11	0	0
28	ISSR-12	(CA)8G	7	3	42
29	ISSR-13	(GA)8C	10	5	50

Table 2. Discriminatory RAPD and ISSR primers and their unique bands specific to different ginger genotypes.

SI No.	Ginger genotypes	Discriminatory RAPD primers	No.of unique bands	Size and identity of bands(by)	Discriminatory ISSR primers.	No.of unique bands	Size and identity of bands (bp)
1.	Varada	OPJ-07	1	OPJ-07$_{(480)}$	ISSR-3	1	ISSR-3$_{(470)}$
					ISSR-13	1	ISSR-13$_{(490)}$
2.	Pinkginger	OPB-19	1	OPC 19$_{(1500)}$	ISSR-1	2	ISSR-1$_{(250)}$
		OPC-13	1	OPC 13$_{(500)}$	ISSR-6	3	ISSR-$_{(200)}$
		OPE-11	1	OPE 11$_{(990)}$	ISSR-8	1	ISSR-6$_{(250)}$
							ISSR-6$_{(490)}$
							ISSR-6$_{(740)}$
							ISSR-8$_{(510)}$
							ISSR-10$_{(900)}$
							ISSR-10$_{(1250)}$
3.	Kintoki	OPA-08	1	OPA08$_{(450)}$	ISSR-9	1	ISSR-9$_{(300)}$
4.	Ellakkallan	0	0	0	0	0	0
5.	Kozhikalan	OPB-05	1	OPB05$_{(500)}$	0	0	0
6.	Kakkakalan	0	0	0	0	0	
7.	Sabarimala	0	0	0	0	0	

different putative forms 83-88%. Varada showed high similarity with primitive types (92%-93%) followed by Pinkginger (86%) and Kintoki (85%).

In the present study the RAPD/ISSR markers could clearly discriminate the ginger genotypes studied. The percentage of polymorphism for the genotypes studied ranged 21-50% in RAPD, 40-62% in ISSR respectively. Similar results were also observed by Nayak *et al.* (2005). The authors assessed the genetic diversity of 16 promising cultivars of ginger using RAPD markers. The study revealed a differential polymorphism of DNA showing a number of polymorphic bands ranging from 26 to 70 among 16 cultivars. Mohd *et al.* (2004) also reported the genetic variation among the three ginger cultivars from Malaysia using RAPD marker. UPGMA dendrogram revealed a general pattern that the primitive, exotic and elite lines of ginger genotypes forming separate groups. Kintoki and the putative wild types like Ellakkallan, Kozhikalan, Sabarimala, Kakkakalan are characterized by small rhizomes, dwarf plant structure, low yield and high pungency. However, rhizomes of Kintoki have a different taste. But the Pink ginger characterized by copper brown colour and slender long rhizomes are morphological distinct from the four putative types. The high affinity observed between Varada and primitive ginger genotypes are suggestive of probable origin of the improved varieties from these progenitors.

Though the existence of wild forms of ginger is not yet established beyond doubt (Sasikumar *et al*.1995; Muralidharan & Velayudhan, 1983) the present finding implies that the so called putative types of ginger may be the progenitors of elite ginger cultivars. In a crop like ginger where there is no sexual reproduction, the evolution of elite types with bold rhizomes, high yield etc. from the small rhizome, low yielding and primitive forms may be due to mutation followed by selection over time. The specific banding pattern observed in case of the putative, rare specimen can be used as markers to protect these accessions while germplasm registration or in the event of litigation involving biopiracy.

Table 3. Similarity coefficient of ginger genotypes using RAPD and ISSR markers

	Varada	Pinkginger	Kintoki	Ellakkalan	Kozhikalan	Kakkakalan	Sabarimala
Varada	1						
Pinkginger	0.86	1					
Kintoki	0.85	0.80	1				
Ellakkalan	0.93	0.86	0.89	1			
Kozhikalan	0.92	0.83	0.88	0.96	1		
Kakkakalan	0.93	0.88	0.88	0.97	0.94	1	
Sabarimala	0.93	0.86	0.88	0.97	0.97	0.96	1

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