International Journal of Plant Breeding ©2009 Global Science Books

Nuclear rDNA Sequence-based Identification and Relative Efficiency of ISSR and RAPD Markers for Genetic Diversity of *Fusarium* sp. Associated with Mango Malformation in India

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ABSTRACT

Inter Simple Sequence Repeat (ISSR) and Random Amplified Polymorphism DNA (RAPD) analysis were used to assess genetic variations amongst 30 isolates of *Fusarium* sp. isolated from malformed tissues of mango (*Mangifera indica* L.) collected from different locations of Pantnagar, India. *Fusarium* sp. causes one of the most serious diseases of mango, mango malformation. All isolates were identified using two taxon selective primers, ITS-Fu-f and ITS-Fu-r. Scoreable loci 149 and 128 were obtained with 16 ISSR and 16 RAPD primers, respectively. Out of 149 loci, 106 were polymorphic with ISSR markers. Percentage polymorphism ranged from 14.3% (P-48) to a maximum of 100% (P-46 and 54), with an average of 71.1%. The 16 RAPD primers produced 102 polymorphic bands. Percentage polymorphism ranged from 50% (P-78 and 114) to 100% (P-71, 73 and P-79), with an average percentage polymorphism of 79.7% among all isolates of *Fusarium* sp. Results of present investigation revealed that relatively RAPD markers were more efficient than ISSR assay. Clustering of isolates remained more or less same in RAPD and combined data of RAPD and ISSR. The similarity coefficient ranged from 0.57 to 0.92, 0.47 to 0.98 and 0.57 to 0.90 with ISSR, RAPD and combined dendrogram, respectively.

Keywords: genetic diversity, fingerprinting, molecular characterization, molecular marker, ITS

INTRODUCTION

All the disease management strategies based on host resistance require the knowledge of variability in pathogens. The development and use of molecular markers for the detection and exploitation of DNA polymorphism is one of the most significant developments in the field of molecular plant pathology. The presence of various types of molecular markers, and differences in their principles, methodologies, and applications require careful consideration in choosing one by researchers. ISSR and RAPD have been used extensively to study the genetic variability and the present study is a step towards the analysis of genetic diversity in *Fusarium* sp. isolated from malformed mango tissues. Malformation, arguably the most important disease of mango (*Mangifera indica* L.) globally, is of growing concern not only because, it is widespread and destructive and because its etiology and control are not well understood. Malformation is not only well known in India but has also been confirmed in most mango-growing areas/countries viz., Pakistan, Egypt, South Africa, Brazil, Israel, Central America, Mexico and USA (Kumar *et al*. 1993). There is a lot of confusion in the literature about the etiology of the disease because research efforts made hitherto, have not been able to ascertain its etiology; the complexity of the disorder is attributed by biotic and abiotic factors including mites, fungal, viral, physiological factors and nutritional deficiencies. However, in recent years *Fusarium* sp. is finding wide acceptability in scientific community as a causal agent of this disease. Recent advances in the detection of plant pathogens using immunological and nucleic acid-based techniques has enabled the major genera and species of disease-causing organisms to be quickly and reliably identified. Methods that are based on the use of the polymerase chain reaction (PCR) are highly sensitive and specific and have the potential to replace traditional technologies, so two taxon selective primers based on Internal Transcribed Spacer Regions (ITS-Fu-f and ITS-Fu-r) were used for quick identification of *Fusarium* sp. (Abd-Elsalam *et al*. 2003).

The recent use of molecular markers has revolutionized the analysis of population biology of plant pathogens. Molecular markers have been used to characterize genetic diversity of different *Fusarium* sp. (Alves-Santos *et al*. 1999, 2002; Bogale *et al*. 2006; Prasad *et al*. 2007). The development of RAPD markers (Welsh and McClelland 1990; Williams *et al*. 1990) has provided a powerful technique for investigating intraspecific genetic variation in fungi although it does suffer from a certain lack of reproducibility due to mismatch annealing (Karp *et al*. 1997). Inter Simple Sequence Repeat (ISSR) technique is also a PCR based method, which involves amplification of DNA segment present at an amplification distance in between two identical microsatellite repeat regions oriented in opposite direction. The technique combines most of the benefits of AFLP and microsatellite analysis with the universality of RAPD. In addition, ISSR markers are more reproducible than RAPD markers (Goulão and Oliveira 2001).

Keeping in view the above said facts, the aim of the present investigation was isolation, identification and assessment of relative efficiency of ISSR and RAPD markers for the analysis of genetic diversity among 30 isolates of *Fusarium* sp. isolated from malformed mango tissues.

Received: 1 August, 2008. Accepted: 7 January, 2009.

Original Research Paper

MATERIALS AND METHODS

Thirty samples of malformed panicles and seedlings were collected from various orchards from different locations at Pantnagar, Uttranchal, India. Infected samples were cut into small pieces and surface sterilized with 0.2% sodium hypochlorite solution for 2 min. Thereafter the samples were washed with sterilized distilled water before placing them into Petri dishes seeded with Potato Dextrose Agar (PDA) medium. The plates were sealed and kept in a BOD incubator at 28 ± 2°C for 3-4 days until fungus growth appeared. Fresh fungal growth from the plated samples was then transferred on PDA. Finally every isolate was further purified by single spore culture. Potato Dextrose Broth (PDB) medium was used for harvesting the mycelium for DNA extraction. DNA was extracted from the fungal isolates by using the protocol of DNA isolation as given by Lee and Tayler (1990). The concentration of DNA was determined by UV visible spectrophotometer (Biomate, Thermo Spectronic, Cambridge, UK).

PCR amplification of ribosomal DNA regions

Each PCR reaction mixture contained 30 ng of genomic DNA, 25 ng each of primers ITS-Fu-f and ITS-Fu-r, 2.5 µl of reaction buffer, 2.5 mM $MgCl_2$, 250 µM of each dNTPs and 2.5 U of *Taq* DNA polymerase (Life Technologies, India) in a total volume of 50 µl. The nucleotide sequences of these primers were 5′-CAACTCCC AAACCCCTGTGA-3′ (ITS-Fu-f) and 5′-GCGACGATTACCAG TAACGA-3′ (ITS-Fu-r) (Abd-Elsalam *et al*. 2003). The PCR profile was denaturation at 95°C for 4 min, followed by 35 cycles of 94°C for 1 min, 54°C for 30 sec, and 72°C for 1 min with final extension of 72°C for 5 min. The two bands were eluted from gel using QIAGEN MinElute kit and sequenced from Genei, Bangalore, India.

ISSR amplification

PCR Amplification (Zeitkiewicz *et al*. 1994) was performed with long primers obtained from Life Technologies, India (**Table 1**). ISSR amplification reactions were carried out in 20 µl volume containing 2 µl dNTP (250 µM each dNTP), 1 µl primer (30 $ng.\mu L^{-1}$), 1 µl template DNA (50 $ng.\mu L^{-1}$), 2.5 µl reaction buffer (10X), 2.0 µl $MgCl_2$ (25 mM), 0.3 µl *Taq* DNA polymerase (5 $U.\mu L^{-1}$) and deionized water 11.2 µl. PCR reactions were performed with PTC-200 Peltier Thermal Cycler (MJ Research Inc., Watertown, MS, USA). Amplification conditions were: An initial denaturation at 94°C for 5 min and 40 cycles at 1 min, denaturing at 94°C, 1 min annealing at 51°C, 2 min polymerization at 72°C and 15 min final extension at 72°C.

RAPD amplification

PCR amplification (Williams *et al*. 1990) was performed with arbitrary decamer primers obtained from Life Technologies, India. Amplifications were performed in 20 µl volume containing 2 µl

Table 1 Primers list of ISSR and RAPD used for the characterization of *Fusarium* sp. associated with mango malformed tissues associated with mango malformation disease of mango.

S.No	Primer code used in study	Primer sequence	Amplified product range (bp)	Total bands	Monomorphic bands	Polymorphic bands	% Polymorphism
ISSR Primers							
1	P-46	AGAGAGAGAGAGAGAGT	420-1800	14	-	14	100.0
2	P-48	AGAGAGAGAGAGAGAGG	500-1680	7	6	1	14.3
3	P-49	GAGAGAGAGAGAGAGAT	345-2010	11	3	8	72.7
4	P-51	GAGAGAGAGAGAGAGAA	380-1460	11	1	10	90.9
5	P-52	CTCTCTCTCTCTCTCTG	580-1470	7	1	6	85.7
6	P-54	TCTCTCTCTCTCTCTCG	520-1520	6	-	6	100.0
7	P-55	ACACACACACACACACT	410-1480	8	1	7	87.5
8	P-59	GAGAGAGAGAGAGAGAYT	270-1650	7	1	6	90.9
9	P-61	ACACACACACACACACYG	400-1400	8	1	7	87.5
10	P-62	HBHAGAGAGAGAGAGAG	400-1700	8	5	3	37.5
11	P-63	BHBGAGAGAGAGAGAGA	430-1500	12	6	6	50.0
12	P-64	VHVGTGTGTGTGTGTGT	300-1410	13	8	5	38.5
13	P-65	HVHTGTGTGTGTGTGTG	170-1610	17	5	12	70.6
14	P-67	ATGATGATGATGATGATG	400-1500	8	1	7	87.5
15	P-68	CTCCTCCTCCTCCTCCTC	470-1300	7	2	5	71.4
16	P-69	GGGTGGGGTGGGGTG	500-1620	5	2	3	60.0
		Total	-	149	43	106	-
		Average	393-1570	9.3	2.7	6.6	71.1
RAPD Primers							
1	P-71	TGCCGAGCTG	245-2250	10	-	10	100.0
2	P-72	AGTCAGCCAG	280-2160	11	2	9	81.8
3	P-73	AATCGGGCTG	470-1400	6	1	5	83.3
4	P-76	GTGACGTAGG	370-2010	8	-	8	100.0
5	P-78	GTGATCGCAG	420-1510	6	3	3	50.0
6	P-79	CAACGCCGT	470-2010	9	-	9	100.0
7	P-80	CAGCACCCAC	325-1980	9	4	5	55.6
8	P-90	GTGAGGCGTC	400-1710	6	1	5	83.3
9	P-96	TTGGCACGGG	360-1600	8	1	7	87.5
10	P-97	GTGTGCCCCA	300-2100	13	1	12	92.3
11	P-110	GGACTGCAGA	360-2150	5	2	3	60.0
12	P-112	GTCAGGGCAA	400-2100	7	1	6	85.7
13	P-113	AGACGTCCAC	600-1540	5	1	4	80.0
14	P-114	GACGCCACAC	500-1600	6	3	3	50.0
15	P-118	TCAGAGCGCC	385-1780	9	3	6	66.7
16	P-171	GGACCCAACC	310-2100	10	3	7	70.0
		Total	-	128	26	102	-
		Average	387-1875	8.0	1.63	6.4	79.7
Combined (RAPD + ISSR) primers analysis							
		Total	-	277	69	208	-
		Average	390-1723	8.66	2.2	6.5	75.1

Single letter abbreviation for mixed base positions: Y=(C,T); B=(C,G,T i.e. not A); H=(A,C,T *i.e.* not G); V=(A,C,G, *i.e.* not T).

dNTP (250 µM each dNTP), 1 µl primer (30 ng.µL^{-1}), 1 µl template DNA (50 ng.µL^{-1}), 2.5 µl reaction buffer (10X), 2.0 µl $MgCl_2$ (25 mM), 0.3 µl *Taq* DNA polymerase (5 U.µL^{-1}) and deionized water 11.2 µl. PCR reactions were performed with PTC-200 Peltier Thermal Cycler (MJ Research Inc., Watertown, MS, USA). Amplification was carried out at initial denaturation 94°C for 5 min, for 35 cycles denaturation 94°C for 1 min, annealing at 42°C for 1 min and extension at 72°C for 2 min and final extension at 72°C for 7 min.

After completion of amplifications, 4 µl of gel loading dye was added to each sample (20 µl) of rDNA, ISSR and RAPD and resolved on 1.5, 1.8 and 1.4% agarose gel in 0.5X TBE buffer, respectively. The gels were visualized under UV using gel documentation system (Bio Rad, USA).

Data analysis

DNA fingerprints were scored for the presence (1) or absence (0) of bands of various molecular weight sizes in the form of binary matrix. Data was analyzed to obtain Jaccard's coefficients among the isolates by using NTSYS-pc (version 2.11W; Exeter Biological Software, Setauket, NY, Rohlf, 1993). The SIMQUAL program was used to calculate the Jaccard's coefficients. A common estimator of genetic identity was calculated as follows:

Jaccard's coefficient = N_{AB}/ (N_{AB}+ N_A+ N_B)

where N_{AB} is the number of bands shared by samples, N_A represents amplified fragments in sample A, and N_B represents fragments in sample B. Similarity matrices based on these indices were calculated. Correlation between the two matrices obtained with two marker types (ISSR and RAPD) was estimated by means of Mantel test (Mantel 1967). Coefficient correlation (r) is provided one measure of relatedness between the two matrices. Similarity matrices were utilized to construct the UPGMA (unweighted pair-group method with arithmetic average) dendrograms. In order to estimate the congruence among dendrograms, cophenetic matrices for each marker were computed and compared using Mantel test. Principal coordinate analysis was performed in order to highlight the resolving power of the ordination. The relative efficiency of two markers system was also calculated using Pearson correlation coefficient. To determine robustness of the dendrogram, the data were bootstrapped with 2000 replications along with Jaccard's coefficient by the computer programme WINBOOT (Yap and Nelson 1996).

RESULTS

ISSR band pattern

Using primers ITS-Fu-f and ITS-Fu-r, amplification of the small subunit ribosomal DNA of *Fusarium* species isolated from malformed tissues resulted in formation of 400bp bands. The PCR amplification using 3′ and 5′ anchored dinucleotides repeat primers gave rise to reproducible amplification products. ISSR primers produced different numbers of DNA fragments, depending upon their simple sequence repeat motifs (**Fig. 1**). The 16 primers on an average produced 149 bands across 30 isolates, of which 106 were polymorphic, accounting for 71.1% polymorphism. The number of bands varied from 5 (P-69) to 17 (P-65) and varied in size from 170 to 2010 bp. Average number of bands and polymorphic bands per primer were 9.3 and 6.6, respectively. Percentage of polymorphism ranged from 14.3% (P-48) to maximum of 100% (P-46 and 54). The ISSR bands were scored for presence or absence among the genotypes and used for UPGMA analysis. A dendrogram based on UPGMA analysis with ISSR data is shown in **Fig. 3A**. Jaccard's similarity coefficient ranged from 0.571 to 0.92. The 30 isolates were clustered into four clusters. Cluster I comprises F-1, 3, 5, 6, 8, 9 and 10. Cluster II consisted of F-16, 17, 18, 20, 21 and F26. Cluster III comprises F-2, 4, 11, 14, 15 and F25. Cluster IV comprises F-19, 22, 23, 24, 28, 29 and F-30. The isolates F-7, F-12, F-13 and F-27 were separated among the all isolates. The results of PCA analysis were not exactly comparable to the cluster (**Fig. 4A**). The F-12 isolate had the minimum similarity coefficient (0.571) with F-29. The first three most informative PC components clarify 33.05% of total variation.

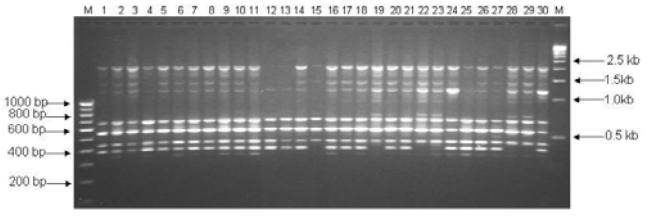

Fig. 1 Inter Simple Sequence Repeats (ISSR) polymorphism in *Fusarium* sp. isolated from malformed tissues of mango using ISSR primer P-49. Lane M: 100 and 500 bp ladder (Fermentas Life Sciences Ltd.); Lanes 1-30: *Fusarium* isolates.

RAPD band pattern

Amplification of genomic DNA of the 30 isolates, using 16 primers for RAPD analysis, yielded 128 fragments that could be scored. Number of amplified fragment ranged from 5 (P-110 and P-113) to 13 (P-97), with size ranging from 245 to 2250 bp. Out of 128 amplified bands, 102 were polymorphic, with an average of 6.4 polymorphic bands per primer. Percent polymorphism ranged from 50% (P-78 and P-114) to 100% (P-71, 73 and P-79), with an average percentage polymorphism of 79.7% across all isolates of *Fusarium* sp. (**Fig. 2**) is the representative of the extent of polymorphism observed among the all *Fusarium* isolates as revealed by primer P-72. A dendrogram based on UPGMA analysis grouped the 30 isolates into three main clusters, with Jaccard's similarity coefficient ranged from 0.467 to 0.979 (**Fig. 3B**). Four isolates F-14, F-25, F-26 and F-27 appeared to be distinct from all others. Cluster I, II and III comprises 11 isolates (F-1 to F-11), 5 (F-16, 17, 18, 20 and F-21) and 10 (F-12, 13, 15, 19, 22, 23, 24, 28, 29 and F-29), respectively. The isolates F-6 and F-7 appeared to be closer to each other, with a 0.979 similarity coefficient. The isolates F-14 and F-28 had least similarity to each other, with a similarity coefficient of 0.467. The results of PCA analysis were comparable with cluster 1 and cluster 2 but not with cluster 3 to the cluster analysis (**Fig. 4B**). The first three most informative PC components explained 37.82% of total variation.

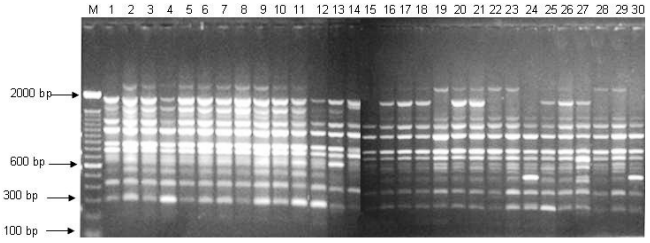

Fig. 2 Random Amplified Polymorphic DNA (RAPD) profile of *Fusarium* sp. isolated from malformed tissues of mango, using RAPD primer P-72. Lane M: 100 ladder; Lanes 1-30: *Fusarium* isolates.

ISSR and RAPD data

The ISSR and RAPD data were combined for UPGMA cluster analysis. Four clusters (**Fig. 3C**) were formed which were to some extent similar to RAPD cluster. Jaccard's similarity coefficient ranged from 0.571 to 0.90. The three dimensional ordination (**Fig. 4C**) was exactly same and confirms the cluster analysis results demonstrating that F-12 is separated with the others. The pattern of clustering of the genotypes remained more or less same in RAPD and combined (ISSR and RAPD) data, whereas dendrogram based on ISSR showed some variation in the clustering of genotypes. The matrices for ISSR and RAPD markers were also compared by using Mantel's test (Mantel 1967) for matrix correspondence. The correlation between the matrices of

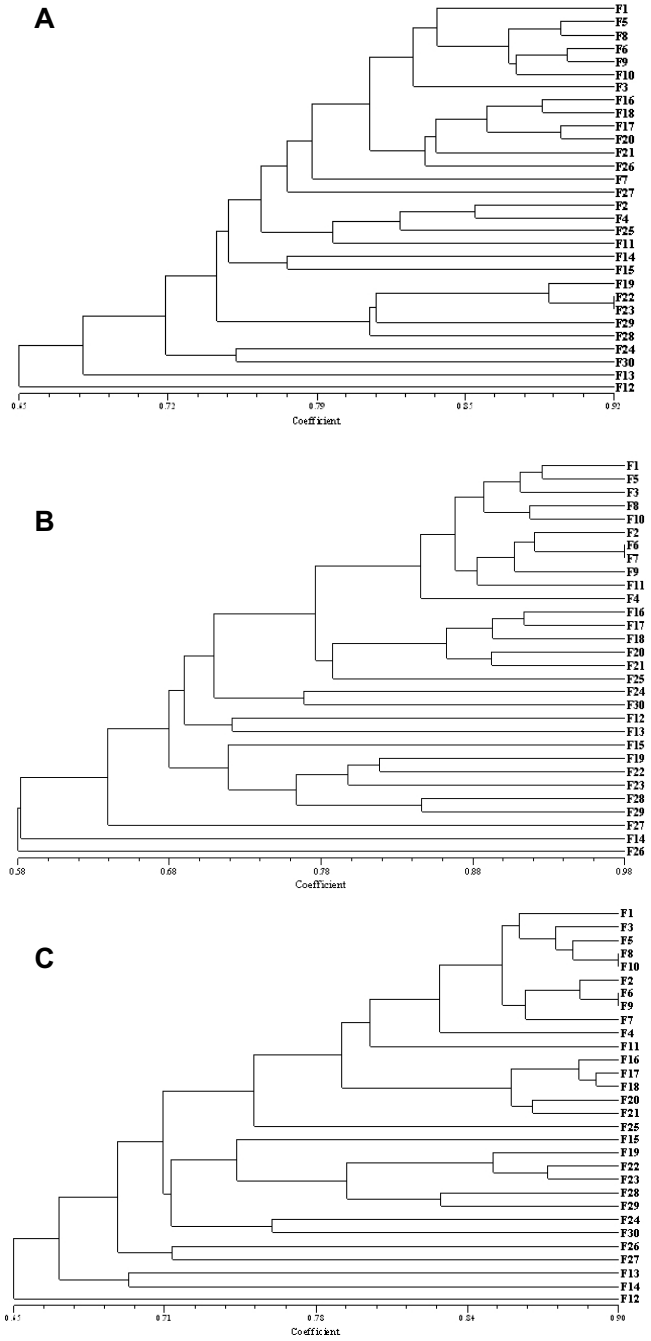

Fig. 3 Dendrograms obtained from 30 isolates of *Fusarium* sp. with UPGMA based on Jaccard's coefficient. Scale bar indicates the similarity index. (**A**) ISSR data based dendrogram. (**B**) RAPD data based dendrogram. (**C**) Combined (ISSR and RAPD) data based dendrogram.

cophenetic correlation values for the ISSR, RAPD and combined dendrogram showed r = 0.8115, 0.9283 and 0.9013, respectively. The first three most informative PC components explained 32.89% of total variation.

Estimation of relative efficiencies of ISSR and RAPD markers

The relative efficiency of a marker was assessed by correlating the genetic similarity measures with different markers. The similarity coefficients of 30 isolates through ISSR and RAPD markers were subjected to calculate the Pearson's correlation coefficient (**Table 2**). Highest Pearson correlation value (r = 0.9068) was recorded between Combined and RAPD, which revealed the closeness of RAPD markers with combined (ISSR and RAPD) data (**Fig. 5**). Consequently, the RAPD marker was found to be the most influential type of molecular marker to study the genetic diversity *Fusarium* sp. isolated from malformed tissues of mango.

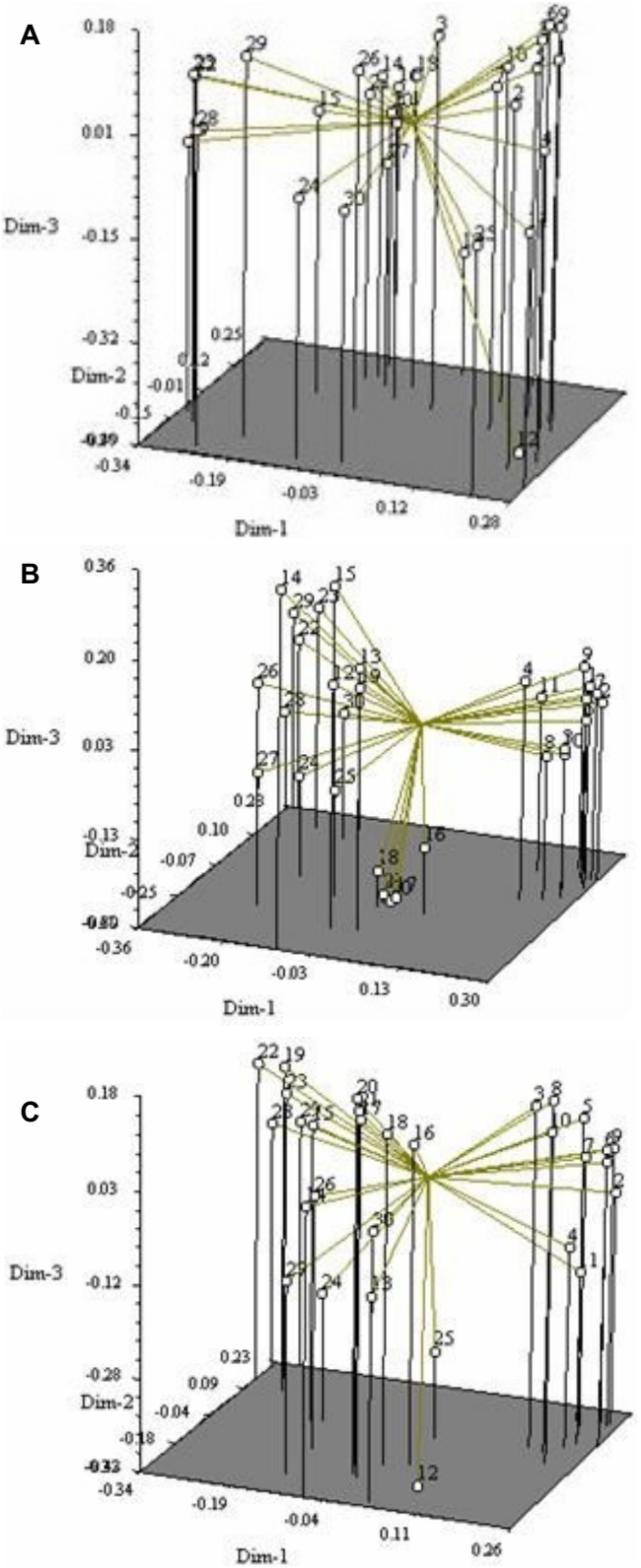

Fig. 4 Three-dimensional plot (with vectors) of 30 isolates of *Fusarium* sp. obtained using principal coordinate analysis. The numbers plotted represents individual isolates. 3D plots based on (**A**) ISSR data, (**B**) RAPD data and (**C**) Combined ISSR and RAPD data.

Table 2 Pearson's Correlation coefficient (r) and Mentel's *t*-test of ISSR and RAPD markers data obtained from 30 *Fusarium* sp. isolates.

Particulars	*r*	*t*	*P*
ISSR Vs RAPD	0.5247	4.1979	1.00
Combined molecular markers Vs RAPD	0.9068	7.2343	1.00
Combined molecular markers Vs ISSR	0.8342	6.8969	1.00

P= Prob. random Z < obs

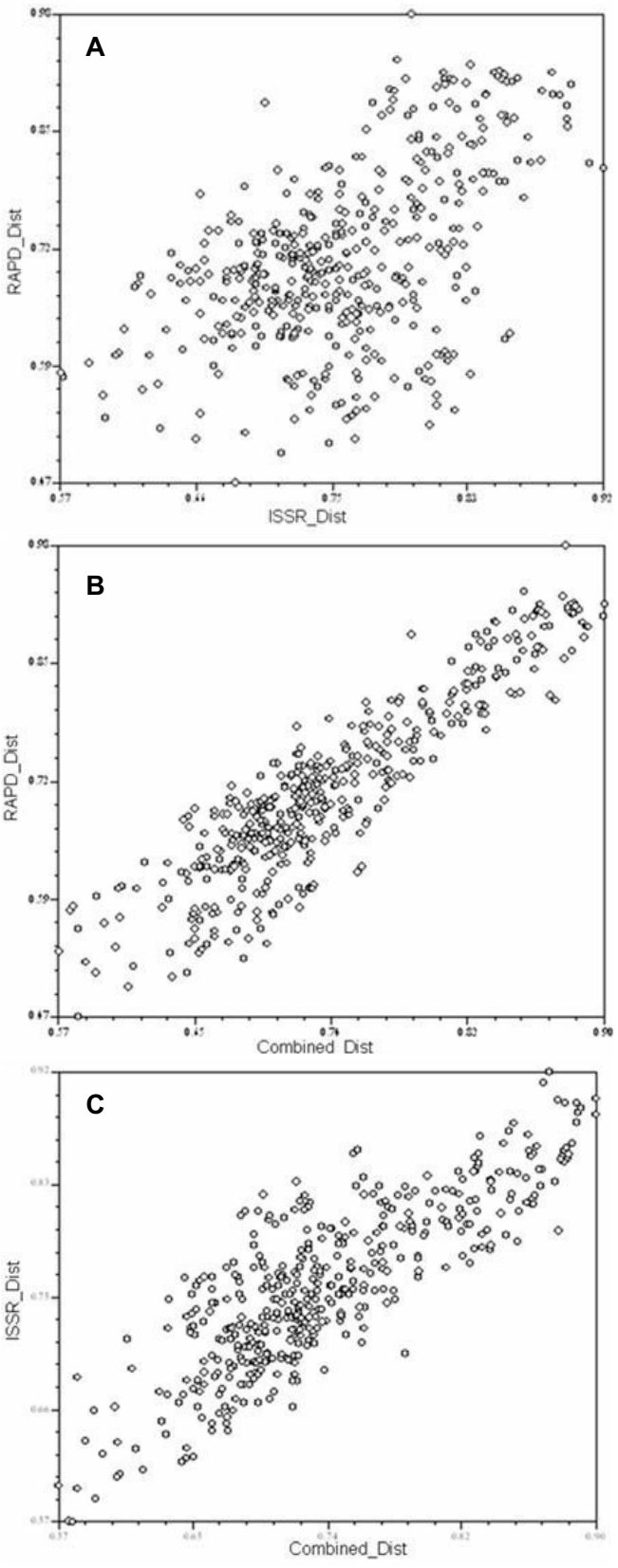

Fig. 5 Matrix comparison plot between distances obtained from different markers data of *Fusarium* isolates. (**A**) ISSR vs. RAPD data. (**B**) Combined (ISSR and RAPD) vs. RAPD data. (**C**) Combined (ISSR and RAPD) vs. ISSR data.

DISCUSSION

In the present study, the rRNA genes, commonly used in identification and taxonomic studies, was confirmed to be particularly appropriate for the purpose of providing target sequence for molecular detection. Several molecular markers and DNA based probes were developed for detecting *Fusarium* species and its different races in relation to specific hosts (Kelly *et al*. 1994; Kistler 1997; Gherbawy 1999; Wang *et al*. 2001; Jiménez-Gasco and Jiménez-Díaz 2003). Difference in the nucleotide composition of the variable ITS regions have been successfully employed to design specific primers sets that amplify selectively among and within species of plant pathogens (Moricca *et al*. 1998; Alves-Santos *et al*. 1999, 2002; Saharan 2007). Molecular markers ISSR and RAPD have been used extensively to study the genetic variability and provided important genetic information relevant to the resolution of various genetic and evolutionary properties of *Fusarium* sp. (Kini *et al*. 2002; Zheng and Ploetz 2002; Mishra *et al*. 2003;Tarakanta *et al*. 2003; Tóth *et al*. 2004; Prasad *et al*. 2007).

Researchers who have compared RAPD and ISSR methods on plant system have found that ISSR markers exhibit higher levels of polymorphism or reproducibility as compared with RAPD markers (Parsons *et al*. 1997; Esselman *et al*. 1999; Qian *et al*. 2001). But the results of the present investigation showed that the ISSR markers were not more informative than RAPD markers and showed high level of polymorphism in RAPD (79.7) and ISSR (71.1) among the 30 isolates of *Fusarium* species. The similar results were obtained in *Caldesia grandis* (Chen *et al*. 2006).

The success of the present study in identifying polymorphism is due to the use of a number of randomly selected prescribed highly informative primers. The 16 RAPD and 16 ISSR primers in the present study yielded 208 polymorphic markers that unambiguously discriminated 30 isolates into four clusters. The numbers of total polymorphic and discriminant fragments are higher for RAPD than ISSR. A possible explanation for the difference in resolution of RAPDs and ISSRs is that the two markers techniques target different portions of the genome. The ability to resolve genetic variation among different genotypes may be more directly related to the number of polymorphisms detected with each marker technique rather than a function of which technique is employed (Souframanien *et al*. 2004).

Clustering of isolates within groups was not similar when RAPD and ISSR derived dendrograms were compared. Dendrograms did not indicate any clear pattern of clustering according to the location in which they were collected. Our results indicate the presence of high genetic variability among isolates of *Fusarium* species. RAPD markers were found more polymorphic than ISSR markers but bands per primer (9.3) were more in ISSR than RAPD. RAPD also exhibited higher relative efficiency than ISSR marker with combined data and came out with best discrimination among all the isolates studied for genetic diversity analysis. But low Pearson correlation coefficient (0.5247) between distance matrices obtained from ISSR and RAPD data indicate that these two markers are giving polymorphism at different loci, so these markers should be used in combination to remark on genetic variability of different *Fusarium* isolates.

Future work is being focused on these aspects along with pathogenicity test to assess the relative ability of each isolate to infect different plants. In addition to understanding the etiology of this disease, this information will improve the basic understanding of the genetic variability of this pathogen and will be of importance to many other agriculture systems.

ACKNOWLEDGEMENTS

The authors are thankful to Department of Biotechnology, Government of India, for providing financial support for conducting the investigation and fellowship to first author.

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