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GENOMIC DIFFERENTIATION AMONG *CHAETOMIUM GLOBOSUM* ISOLATES BY USING RAPD AND ISSR MARKERS

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ABSTRACT
Chaetomium globosum the potential biocontrol agents showing high antifungal activity was screened against *Pythium aphanidermatum* the incitant of rhizome rot of turmeric. Forty five isolates of *Chaetomium* spp. from that, effective 10 isolates were selected based on the *in vitro* antagonist activity and plant growth promotion activity. In order to study genetic differentiation the following work has been framed. For ten isolates, we studied their genetic variation using RAPD and ISSR markers. Nearly, 10 RAPD and ISSR primers used in the study, all the primers were effective in producing polymorphic fingerprint patterns (100 percent) from genomic DNA of *Chaetomium* spp. each isolates was clearly identified and separated from others based on the similarity index values indicating the presence of genetic diversity at molecular level. The isolates of TNAU-Cg6 and TNAU-Cg11; TNAU-Cg26 and TNAU-Cg36; TNAU-Cg12 and TNAU-Cg15 are clustered in the Non-metric Multi-dimensional scaling (MDS) plot. The Hierarchical Cluster Analysis (HCA) based clustering of strains and clustering of isolates showed 65-85 % similarity with reference to RAPD and ISSR fingerprints. Among the RAPD markers the OPA03 and OPA10 was found to be best which recorded 74.71% and 70.32% similarity in TNAU-Cg6 and TNAU-Cg11 respectively. In case of ISSR $(TCC)_5$ and AG_8C was 76.23% and 71.35 % similarity in TNAU-Cg6 and TNAU-Cg11 respectively. Finally, our results indicate that RAPD and ISSR are sensitive and give reproducible results for assaying the genetic variability in *Chaetomium* spp. and these two markers were reliable and could be utilized to establish the genetic relatedness of *Chaetomium* spp.

INTRODUCTION

Chaetomium is a genus belonging to the class Pyreno mycetes, order Sordariales and family Chaetomiaceae. It can produce an Acremonium-like state (imperfect stage) on medium and it is characterized by superficial flask-shaped perithecia, which are surrounded by dark and stiff hairs. *Chaetomium* has been reported to be a potential biocontrol agent against many plant pathogens *viz*., seed rot and damping off, due to several seed borne and soil borne plant pathogens like *Pythium ultimum*, *P. aphanidermatum*, *Fusarium* spp., (Harman *et al*., 1978, Vannacci and Harman, 1987), *Rhizoctonia solani* (Bayer, 1968), *Pyricularia oryzae* in rice (Soyton and Quimio, 1989) and also for *Cochliobolus sativus* (Aggarwal *et al*., 2004). *Chaetomium globosum* has a great potential as a biological control agent and has been classified based on morphological descriptions of colony growth and perthiecia (Millner *et al*., 1997; Ahammed *et al*., 2004, 2005a).

Studying the genetic diversity of plant fungal pathogens has its essential and indispensable impact on plant disease resistance breeding schemes. DNA (Deoxyribonucleic acid) markers have been extensively used to measure genetic relatedness, intra and interspecific phylogenetic relationships and the identification of races and pathotypes (Mullins and Fallona, 1987; Batista, 1993). Of these techniques, the random amplified polymorphic DNA, RAPD (Williams *et al*., 1990) has several advantages, such as the simplicity of use, low cost, and its requirement for small quantities of sample DNA. Similarly, the inter-simple sequence repeat (ISSR) (Zietkiewicz *et al*.,1994), which involves PCR (Polymerase Chain Reaction) amplification using anchored or nonanchored primers composed of a microsatellite sequences, could be used to assess genetic diversity (Qian *et al*., 2001). Molecular assays such as RAPD, ISSR markers and other techniques have elucidated genotype variation within and among these species. Molecular phylogenetic techniques were used with the hope that they could more readily elucidate the phylogenetic relationships within this species (Mahmodi *et al*., 2014).

The aim of this paper was to study the genetic relatedness among *Chaetomium* species by using genetic markers *viz*., RAPD and ISSR.

MATERIALS AND METHODS

Chaetomium isolates

Forty five isolates of *Chaetomium* were collected from various location of Tamil Nadu. Effective 10 *Chaetomium globosum* isolates (Table 1) were selected based on antagonist and plant growth promotion activity. All the cultures of ten isolates were maintained on liquid culture media (Potato Dextrose broth, PDB) for 7 days at 28 ± 1^{o}c in a shaker incubator.

Genomic DNA extraction

Mycelia of seven days old culture were filtered through

Whatman no. 1 filter paper and DNA was extracted using cetyltrimethyl ammonium bromide (CTAB) method (Murray and Thompson, 1980). Mycelium was grounded in liquid nitrogen transferred to DNA extraction buffer (0.1M Tris, 1.5M Nacl, 0.01M EDTA) and kept at 65 ºC for one hour followed by addition of 15ml of chloroform: isoamyl alcohol (24:1). Samples were centrifuged at 10,000 rpm for 10 minutes at room temperature. Upper aqueous layer was precipitated with 2 volume of ice cold isopronal and 0.1 volume of 3M sodium acetate (pH 5.2) and centrifuged at 15,000 rpm for 15 minutes. Then pellet was washed with 70% ethanol and DNA pellet dissolved in TE buffer and stored at -20 ºC for further use.

RAPD amplification

Random amplification of polymorphic DNA was conducted using single stranded oligonucleotides based on the results of initial screening of Operon decamer primers (designed by Operon Technologies, Alameda, USA) against a set of representative studied isolates. A set of 10 RAPD primers were used and listed in Table 2. Each primer was used to attempt to amplify the DNA from a set of 10 isolates of *Chaetomium* spp., and reagent concentrations and amplification conditions were adjusted empirically until amplification was achieved and different amplicons between the isolates were observed. Each PCR reaction contained genomic DNA 25 ng/ml -2.00 ml, dNTPs (2.5 mM) (Bangalore Genei Ltd., India) -0.25 ml, Primer - 1.00 ml, 10x assay buffer - 2.00 ml, *Taq* polymerase (3 units/ mL) - 0.20 ml, Magnesium chloride - 0.20 ml, Sterile distilled water -14.35 ml in a total reaction volume of 20.00 ml. PCR amplification was performed as described by Williams *et al*., 1990 with some modifications. Polymerase chain reactions (PCR) were carried out with an initial denaturation for 4 min at 94 ºC followed by 45 cycles of 1 min of denaturation at 94 ºC, 1 min of annealing temperature at 36 ºC, 2 min of extension at 72 ºC, and the final extension at 72 ºC for 10 minutes.

ISSR amplification

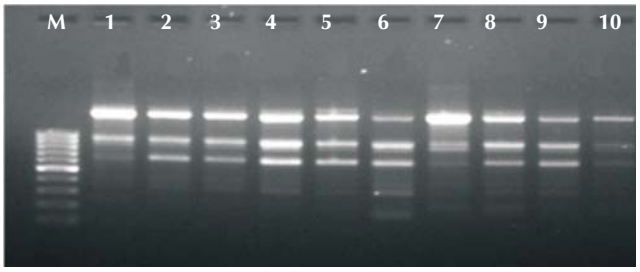
The ISSR primers used in this study were dinucleotides with Ten repeat units and one anchoring base, e.g., $(AG)_8T$; $(AG)_8C$ and $(GA)_8T$ and trinucleotides with three repeat units $(CAG)_3$ or five repeat units such as $(CAG)_5$; $(GTG)_5$; $(AAG)_5$ and $(TCC)_5$ and tetranucleotides with four repeat e.g. $(ACTG)_4$ and $(TGTC)_4$. Ten set of ISSR primers were listed in table 2 with details of nucleotide sequence and PCR conditions. DNA amplification reactions were performed according to (Bornet and Branchard, 2001) with some alternations. Each cycle consisted of a 35 cycles of denaturation step 1 min at 94 ºC, an annealing step for 52 ºC, and an extension step at 72 ºC for to 2 min, followed by extension cycle for 7 min at 72 ºC in the final cycle.

Gel electrophoresis

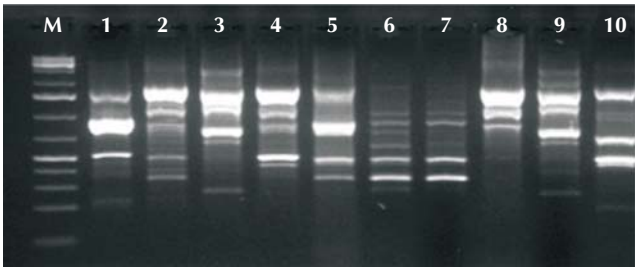
RAPD and ISSR PCR products were electrophoresed on a 1.2% agarose gel in 1X TAE buffer, visualized by staining with ethidium bromide and photographed using gel documentation unit (Alpha Innotech Corporation, San Leandro, California) (Fig 1a, 1b, 1c and 1d).

RAPD and ISSR data analysis

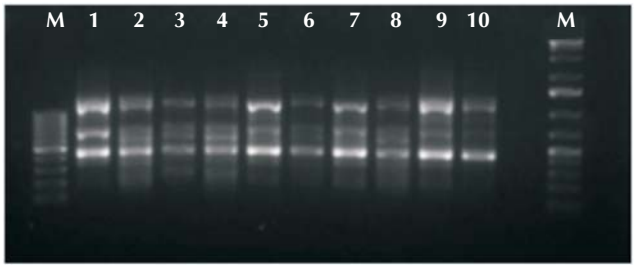
Data from the presence/absence of bands in the fingerprint profiles were imported into PRIMER 7 statistical software (Plymouth Routines in Multivariate Ecological Research, version 7.0.9; PRIMER-E, Plymouth, UK). A similarity matrix



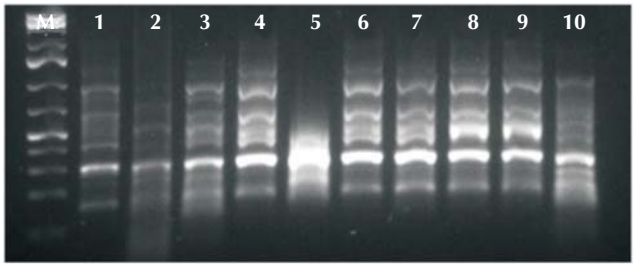
a. RAPD primer – OPA03



b. RAPD primer – OPA10



c. ISSR primer – $(TCC)_5$



d. ISSR primer – $(AG)_8C$

Figure 1: Polymorphism resultant for *Chaetomium globosum* isolates TNAU- Cg1, Cg6, Cg11, Cg12, Cg15, Cg20, Cg24, Cg26, Cg36 and Cg42; Lane M, DNA marker 100 kb and 1 kb

was constructed by calculating similarities between each pair of samples using the Bray–Curtis coefficient (Clarke, 1993). Non-metric multi-dimensional scaling (MDS) was used to ordinate the similarity data. The result of MDS ordination is a map where the position of each sample is determined by its distance from all other points in analysis. The stress of the plot is a measure of how much distortion was introduced to allow the representation of the data in the specified dimensions. A stress value greater value than 0.2 indicates that the plot is close to random, stress less than 0.2 indicates a useful two-dimensional picture, and less than 0.1 corresponds to an ideal ordination with no real prospect of misinterpretation (Clarke, 1993). In present, stress was calculated as described by

Kruskal,1964 within PRIMER 7. To visualize the relationship between different isolates of *Chaetomium globosum* using RAPD and ISSR markers, the similarity matrix using the Bray–Curtis coefficient was also analyzed by Hierarchical Cluster Analysis (HCA), a classification method that aims to group discrete clusters based on similarity. HCA was performed by a weighted, group average linkage agglomerative method and a dendrogram was constructed from the ranked similarities using PRIMER 7 software.

RESULTS AND DISCUSSION

Two-dimensional Non-metric Multi-Dimensional Scaling (MDS) plot using the Bray-Curtis similarity index ordinating the samples using the data from RAPD and ISSR marker fingerprints discriminated *C. globosum* from each isolates (Fig. 2a). The different isolates of *C. globosum* were evenly distributed in the MDS plot with less Kruskal's stress (0.07). The isolates of *C. globosum* Cg24 and Cg42 are slackly different placed in the MDS plot, which shared different ordination. Some isolates of *C. globosum viz*., Cg1, Cg6, Cg11 and Cg20 shared the same line of similarity of coordination in the plot. Likewise, the isolates of Cg6 and Cg11; Cg26 and Cg36; Cg12 and Cg15 are clustered in the MDS plot. The HCA-based clustering of strains and isolates showed 65-85 % similarity (according to Bray-Curtis similarity index) with reference to RAPD and ISSR fingerprints (Fig. 2b).The isolates of *Chaetomium globosum* (TNAU-Cg1, TNAU-Cg24 and TNAU-Cg42) show separate cluster similarity of 64%. Isolates of TNAU-Cg6 and TNAU-Cg11 shows 82% similarity followed by TNAU-Cg20, TNAU-Cg26 and TNAU-Cg36 showed 78% similarity cluster. Genetic diversity using AFLP markers in this fungus was also explored earlier (Aggarwal *et al*., 2003), showing that five *C. globosum* isolates formed two distinct clusters, one comprising isolates Cg6, Cg7 and Cg8, and the other encompassing isolates Cg1 and Cg5. Molecular characterization of *C. globosum* using RAPD primers, nine isolates were grouped into two distinct clusters, with isolates Cg2, Cg3 and Cg4 in one cluster and the remaining isolates in another (Ahammed *et al*., 2005b). Hynes *et al*. (2006) have developed strain specific AFLP-derived markers to detect various fungi such as *Aspergillus niger*, *A. oryzae* and *C. globosum* in soil by qualitative and quantitative PCR. Genetic variabitly of *C. globosum* isolates showed 60 per cent similarity coefficient between all the isolates by RAPD analysis (Shanthiyaa, 2010). Jayaprakash (2011) reported the RAPD analysis of ten *C. globosum* isolates showed similarity coefficient in the range of 12 to 55 per cent. Ashu Singh *et al*., 2015 showed that the analysis of RAPD and ISSR markers, indicated that ISSR expressed maximum resolving power of

Table 1: Details of isolates of *Chaetomium globosum* used for molecular analysis

S. No.	Isolates	Location	Source
01.	TNAU-Cg1	Coimbatore	Soil
02.	TNAU-Cg6	Erode	Soil
03.	TNAU-Cg11	Kodaikanal	Decomposed paper waste
04.	TNAU-Cg12	Thenkasi	Soil
05.	TNAU-Cg15	Ottanchattram	Soil
06.	TNAU-Cg20	Mudukulathur	Decomposed cotton waste
07.	TNAU-Cg24	Thanjavur	Coconut rhizosphere soil
08.	TNAU-Cg26	Ooty	Soil
09.	TNAU-Cg36	Thondamuthur	Grapes rhizosphere soil
10.	TNAU-Cg42	Coimbatore	Grape vein yard soil

Table 2 : List of RAPD and ISSR primers

S. No.	Primers	Nucleotide sequence 5′ to 3′	References
1.	OPA-01	5′CAGGCCCTTC3′	Bhat K.V. and Jarret R.L. (1995)
2.	OPA-11	5′CAATCGCCGT3′	
3.	OPE-02	5′GGTGCGGGAA3′	
4.	OPE-04	5′GTGACATGCC3′	
5.	OPF-06	5′GGGAATTCGG3′	
6.	OPF 7	5′CCGATATCCC3′	
7.	OPF 8	5′GGGATATCGC3′	
8.	OPF10	5′GGAAGCTTGG3′	Erdogan *et al*. (2011)
9.	OPF11	5′ACGGTACCAG3′	
10.	OPF12	5′GGCTGCAGAA3′	
11.	ISSR1	$(CAG)_5$	Gupta and Filner, 1991
12.	ISSR2	$(GTG)_5$	
13.	ISSR3	$(AGG)_5$	
14.	ISSR4	$(ACTG)_4$	Weising *et al*. (1989)
15.	ISSR5	$(TGTC)_4$	
16.	ISSR6	$(TCC)_5$	Gupta and Filner, (1991)
17.	ISSR7	$(CAG)_3$	
18.	ISSR8	$(AG)_8$ T	Dibakar Pal *et al*. (2013)
19.	ISSR9	$(AG)_8$ C	
20.	ISSR10	$(GA)_8$ T	

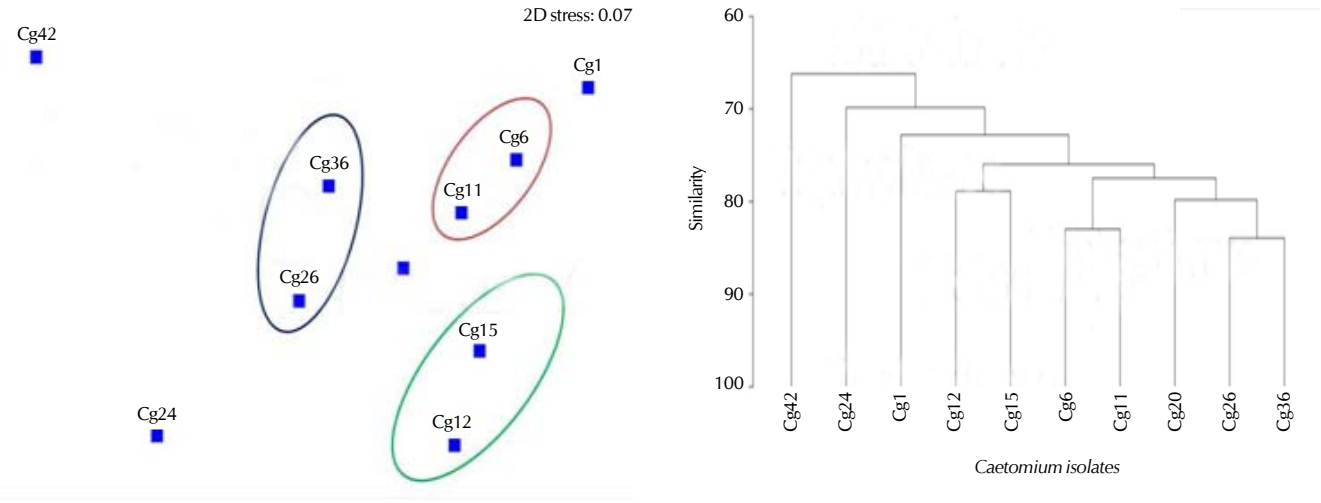


Figure 2 : Non-metric MDS plot (a) and HCA dendrogram (b) constructed from RAPD and ISSR fingerprint data of *Chaetomium globosum* isolates. The similarity matrix using the Bray-Curtis coefficient was applied for both the analyses in Primer 7.0 software

8.336 and RAPD gave maximum PIC values of 0.9925. RAPD primer OPF-13 gave the maximum accessions coverage (depending on the value of PIC) in the rice genome. Rakhonde *et al*., 2015 reported that genetic variability (RAPD primers) among eighteen Indian isolates of *Fusarium oxysporum* f. sp. *ciceri* causing wilt in chickpea. The coefficient value ranged from 0.5048 to 0.9260 across eighteen isolates of *Fusarium oxysporum* f.sp. *ciceri* indicating high degree of variation in respect to genetic similarity.

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