BIODIVERSITAS
Volume 19, Number 2, March 2018
Pages: 621-626

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d190233

Short Communication: Genetic diversity of fumonisin producing *Fusarium* isolates from rice using PCR-RFLP of IGS-rDNA region

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Manuscript received: 17 February 2018. Revision accepted: 19 March 2018.

Abstract. *Nayak S, Dhua U, Chhotaray A, Samanta S, Sengupta C. 2018. Short Communication: Genetic diversity of fumonisin producing* Fusarium *isolates from rice using PCR-RFLP of IGS-rDNA region. Biodiversitas 19: 621-626. Fusarium verticillioides* (Sacc.) and related species produce carcinogenic mycotoxin known as Fumonisins in several agricultural crops including rice. However, this principal food crop has been infected by genetically diverse *Fusarium* species. Odisha belongs to the coastal part of India and many popular rice varieties are in the food chain in this region. Many *Fusarium* species producing fumonisins have been found to be associated with these rice varieties. Hence, the genetic diversity of twenty eight Fumonisin producers and non producers of *Fusarium* pathogens in this region was carried out in the current study. The IGS regions of 28 *Fusarium* isolates (both fumonisin producing and non producing) were amplified and the PCR products were restriction digested with ECoRI and HhaI. The digested products were separated on PAGE and bands were visualized by Silver Nitrate Staining. The 28 isolates could be separated into 14 IGS haplotypes. The lowest similarity was detected to be of 33% between F40 and F47. A group containing 14 isolates represented the biggest haplotypes. The isolates in which the FUM gene had not been detected (fumonisin non producer) were in a separate group having 90% similarity with each other and placed consistently in separate branch from others. Presence of unique band for this group was observed at 1650bp where as absence of specific bands was observed at 380bp and 300bp. The result of this study indicated a high degree of genetic variation among 28 *Fusarium* isolates. PCR RFLP of IGS region was also found to be useful for diversity study in *Fusarium*.

Keywords: *Fusarium*, Fumonisins, IGS, ECoRI, HhaI, rice, mycotoxins, RFLP, FUM

INTRODUCTION

The acts of *Fusarium* species are mainly observed as a disease causing agent in many of crops in temperate as well as tropical regions. In addition to that many species of *Fusarium* infect stored grains and food products which gradually lead to the production of mycotoxins (Llorens et al. 2006). One such group of pathogens belonging to *Gibberella fujikuroi* mating population, Aanamorph *Fusarium verticillioides* (Sacc.) and several related species contaminate and produce Fumonisins in rice and other stored food products like corn, wheat, asparagus, cowpeas, maize, sorghum, millet, farro, black tea, beer etc. (Kushiro et al. 2008, 2009; Maheshwar et al 2009; Karthikeyan et al. 2011; Seefelder et al. 2002). Fumonisins are a series of structurally related sphingosine analog toxins (Shier 1992), the most abundant and one of the most active members of this series is Fumonisin B1 (FB1) (Abbas et al. 1993). Many animal and human health implications have been associated with fumonisins like leukoencephalomalacia in horses, pulmonary oedema syndrome in pigs, and showing nephrotoxicity, hepatotoxicity, and hepatocellular carcinogenicity in rats, human esophageal cancer and neural tube defects in human babies etc. (Kushiro et al. 2008). Eventually the International Agency for Research on Cancer (IARC) evaluated FB1 derived from *F. verticillioides* as Group 2B, i.e., a possible human carcinogen (IARC 2002).

Paddy or rice serves as the staple food for more than 65% of population in India. At the same time, globally more than 3.5 billion people depend on rice for more than 20% of their daily calories (Maheshwar et al. 2009; Hegde and Hegde 2013). Despite many such importance of rice in global food security, its contamination by *Fusarium* species and occurrence of fumonisins have consistently been reported in many countries over the globe mainly due to poor storage of harvested paddy (Bansal et al. 2011). The diversity of fumonisins producing *Fusarium* species infecting maize or corn has been discussed in many reports (Magculia and Cumagun 2011; Aiyaz et al. 2016). However the molecular genetic diversity of such mycotoxins producing *Fusarium* in rice was less studied which is very important as the extent of genetic diversity determines the potential of pathogen population towards evolution (Desjardins et al. 1997; Wulff et al 2010; Nayak et al. 2014).

The ribosomal DNA (rDNA) of fungi contains many conserved and variable regions. One of such regions is the intergenic spacer (IGS) which separates the repeated

ribosomal units and appears to be the most rapidly evolving spacer region (Hillis and Dixon 1991; Woudt et al. 1995; Edel et al. 1997; Alves-Santos et al. 1999; Kim et al. 2001). PCR amplification of IGS region followed by Restriction Fragment Length Polymorphism (RFLP) analysis has been proved to be a useful tool to study the intraspecific variation and taxonomic discrimination of *Fusarium* isolates (Martin 1990; Hillis and Dixon 1991; Fernandez et al. 1994; Appel and Gordon 1995; Miyagawa et al. 2002; Konstantinova and Yli-Mattila 2004; Kim and Min 2004; Aminnejad et al. 2009; Hsuan et al. 2010). Further PCR-RFLP pattern has also been found to have some link with the presence of fumonisin gene of *Fusarium* (Llorens et al. 2006).

In Odisha rice is synonymous with food where agriculture to considerable extent means growing rice. Rice covers about 69 per cent of cultivated area. It is the staple food of almost entire population of Odisha, therefore, the state economy is directly linked with the improvement in production and productivity of rice in the state (www.rkmp.co.in). However the infection of rice grain and seed by fungus like *Fusarium* may be of great concern. Considering the importance of rice in this part of India and the prevalence of *Fusarium* sp. the current investigation was carried out to analyse the genetic diversity of some *Fusarium* isolates having potential to produce fumonisin from popular rice varieties.

MATERIALS AND METHODS

Fusarium isolates

Twenty eight *Fusarium* cultures were studied during present investigation *viz*. i.e. F32, F36, F37, F40, F44, F45, F47, F49, F55, F90, F91, F92, F93, F94, F96, F97, F98, F110, F111, F113, F114, F115, F116, F117, F119, F121, F122 and F124. These *Fusarium* cultures were collected from Dr. (Mrs.) Urmila Dhua's laboratory, Plant Pathology Culture Collection of National (previously Central) Rice Research Institute, Cuttack, Odisha, India. Out of these cultures 24 isolates were detected to be potential as producer of fumonisins having FUM gene as detected in earlier study of Nayak et al. (2014). Passport data and culture characters are presented in Table 1.

Amplification of IGS region

The IGS region of the ribosomal DNA was amplified with the primer CNL12 and CNS1. Amplification was performed in 100µl of reaction mixture containing 10µl of 10X PCR buffer (500 mM KCl, 100 mM Tris HCl (pH 9.0), 1% Triton X-100), 2 mM MgCl2, 0.4 µg of template DNA, 200 mM of each dNTPs, 2.5 units Taq DNA polymerase (Fermentas) and 10pmol of both primers (Sigma), CNL12 (CTGAACGCCTCTAAGTCAG) and CNS1 (GAGACAAGCATATGACTACTG). The mixture was subjected to PCR in a thermal cycler (PTC-100, MJ Research). An initial denaturation step for 5 min at 94ºC was followed by 30 cycles of denaturation for 1 min at 94ºC, annealing for 1 min at 54ºC and extension at 72ºC for 2 min. A final extension of 72ºC for 5 min was incorporated into the program. PCR amplification was detected by running products in electrophoresis through 1.5% agarose gels. Gels were stained with Ethidium bromide and visualised in Syngene (G-Box) Gel Documentation system (Kim et al. 2001).

Restriction digestion of IGS and visualization of DNA fragments

Restriction digestion of amplified fragments was done using two sets of restriction enzymes: *EcoRI-HindIII* and *EcoRI-HhaI* (Fermentas). Double digestion was done with the master mix prepared as follows: Deionised water 15.97 µl, Buffer R 4.00 µl, EcoRI 1U and HindIII 1U or EcoRI 1U and *HhaI* 1U. To the master mix, 10 µl of PCR amplified product was added and kept for 4 hours at 37^0C and then thermally deactivated at 65^0C after digestion. The restricted product was differentiated by running on 8% Polyacrlamide gel (native PAGE) and visualized by Silver Nitrate staining (Barril and Nates 2012; Bassam et al. 1991).

Scoring and data analysis

Data were compiled as a binary 0/1 matrix by the presence (1) or absence (0) of a band at a particular position. All calculations were conducted by using the computer program NTSYS-PC version 2.02 (Rohlf 1990). Genetic similarity matrix was calculated based on the method of simple matching coefficient and the values were used to generate a similarity matrix using SIMQUAL programme.

Table 1. Details of *Fusarium* isolates

Isolate ID*	Source (Rice cultivar)	Fumonisin producing ability
F32	Sarala	+
F36	Sarala	+
F37	Sarala	+
F47	Sarala	-
F49	Sarala	+
F94	Sarala	+
F98	Sarala	+
F110	KMJ-1-17-2	+
F114	Akutphou	+
F115	Akutphou	+
F116	KMJ-1-17-2	+
F117	KMJ-1-17-2	+
F45	Sarala	+
F122	Meher	+
F40	Sarala	+
F113	Meher	+
F124	Meher	+
F55	Sarala	+
F111	NDR-8002	+
F44	Sarala	+
F119	Rambha	+
F90	Sarala	-
F91	Lunisree	+
F92	Sarala	-
F93	Lunisree	+
F96	Sarala	-
F97	Sarala	+
F121	Rambha	+

Note: *CRRI, Plant Pathology Culture Collection

The resulting matrix was analyzed for hierarchical clustering of the isolates by the Unweighted Pair Group Method with Arithmetic mean (UPGMA) by SAHN programme. The matrix was used to construct a dendrogram with TREE programme using the UPGMA for establishing to analyze the level of relatedness among the 28 isolates.

RESULTS AND DISCUSSION

Amplification and restriction digestion of IGS region

A total of 28 *Fusarium* cultures were analysed in the current study which were isolated from seeds of popular rice varieties of India. Four isolates had not showed amplification of FUM gene hence considered as non producer of fumonisin while the rest of the isolates were fumonisin producers as observed in earlier study (Table 1). All the 28 isolates were subjected to PCR amplification of genomic DNA with the primer CNL12 and CNS1 which resulted in the single double-stranded product of approximately 2.6kb, representing IGS region (Figure 1). PCR amplified product was double digested with two pairs of restriction endonucleases such as; *EcoRI-HindIII* and *EcoRI-HhaI*. Restriction with *EcoRI-HindIII* produced no significantly variable DNA fragments hence not considered. Restriction digestion with *EcoRI-HhaI* and subsequent separation of digested product in 8% Poly acryl amide gel with staining by Silver Nitrate resulted in the visualization of variable size fragments ranging from approximately 50bp to 1650bp (Figures 2 and 3). The number of fragments generated per isolate ranged between 7 (F122) to 19 (F40) with a total of 27 different fragment sizes.

Genetic diversity of the *Fusarium* isolates

Each combination of size of RFLP pattern was interpreted as an IGS haplotypes. The dendrogram constructed from cluster analysis separated 28 *Fusarium* isolates in to 14 haplotypes (Figure 4). Isolates F32, F44, F49, F113, F122, F45, F98, F40, F115, F124, F47 and F96 represented one haplotypes each. Isolates F90 and F92 represented one haplotype. Fourteen *Fusarium* isolates *viz* F36, F37, F55, F91, F121, F119, F117, F116, F93, F114, F94, F111, F110 and F97 were found to be genetically identical making it the largest haplotype group. The dendrogram showed that hierarchical clustering separated the isolates into two major groups according to their similarity coefficients. One group comprising of F47, F90, F92 and F96 was having approximately 90% similarity with each other containing 3 haplotypes. These isolates weren't amplified by Fum5F and Fum6R indicating those as fumonisin non producers. Rest of the isolates placed in other group which had been branched in to eight subgroups (with 11 haplotypes). The fumonisin non-producing isolates were having unique banding patterns with presence of unique bands at 1650bp, though not so prominent (Figure 4 and 5). However unique absence of bands specific to this group was observed at 380bp and 300bp. Unique absence of such band was observed at 650bp for this group along with F122. Isolate F47, F90 and F92 showed absence of band at 280bp but such fragment was observed for F96. Isolate specific unique bands were observed at 1000bp and 440bp for F40 and at 480bp for F45. Similarity matrix on simple matching coefficients was calculated for 28 isolates where the similarity ranged to the highest of 100% to the lowest of 33% (between F40 and F47, in Table 2).

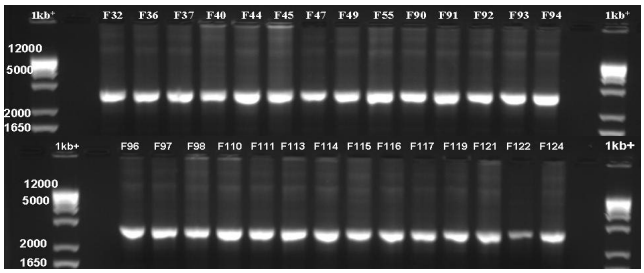

Figure 1. PCR amplification of IGS region of *Fusarium* isolates by primer pair CNL12 and CNS 1

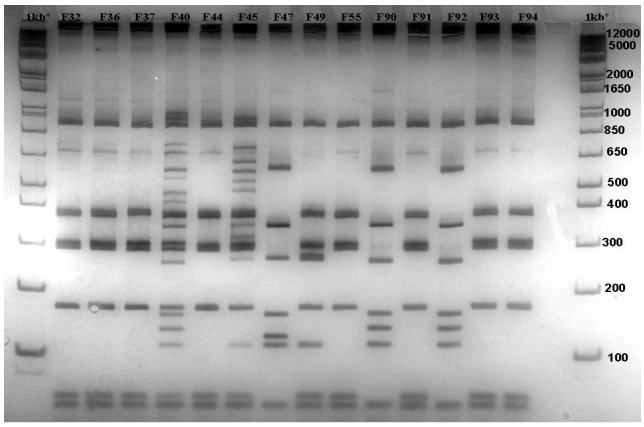

Figure 2. Separation of restriction products of IGS region, on PAGE and visualization by Silver Nitrate staining. Restriction of IGS region by *EcoRI* and *HhaI*. Isolate F32 to F94

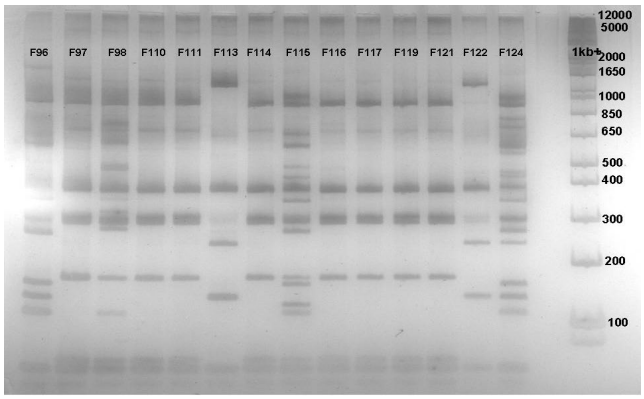

Figure 3. Separation of restriction products of IGS region, on PAGE and visualization by Silver Nitrate staining. Restriction of IGS region by *EcoRI* and *HhaI*. Isolate. F96 to F124

Table 2. Similarity index of *Fusarium* isolates inferred by restriction of IGS region

	F32	F36	F37	F40	F44	F45	F47	F49	F55	F90	F91	F92	F93	F94	F96	F97	F98	F110	F111	F113	F114	F115	F116	F117	F119	F121	F122	F124
F32	1.00																											
F36	0.96	1.00																										
F37	0.96	1.00	1.00																									
F40	0.63	0.59	0.59	1.00																								
F44	0.93	0.96	0.96	0.63	1.00																							
F45	0.74	0.70	0.70	0.59	0.67	1.00																						
F47	0.48	0.52	0.52	0.33	0.48	0.44	1.00																					
F49	0.89	0.93	0.93	0.59	0.89	0.78	0.59	1.00																				
F55	0.96	1.00	1.00	0.59	0.96	0.70	0.52	0.93	1.00																			
F90	0.48	0.52	0.52	0.41	0.48	0.44	0.93	0.59	0.52	1.00																		
F91	0.96	1.00	1.00	0.59	0.96	0.70	0.52	0.93	1.00	0.52	1.00																	
F92	0.48	0.52	0.52	0.41	0.48	0.44	0.93	0.59	0.52	1.00	0.52	1.00																
F93	0.96	1.00	1.00	0.59	0.96	0.70	0.52	0.93	1.00	0.52	1.00	0.52	1.00															
F94	0.96	1.00	1.00	0.59	0.96	0.70	0.52	0.93	1.00	0.52	1.00	0.52	1.00	1.00														
F96	0.52	0.56	0.56	0.44	0.52	0.48	0.89	0.63	0.56	0.96	0.56	0.96	0.56	0.56	1.00													
F97	0.96	1.00	1.00	0.59	0.96	0.70	0.52	0.93	1.00	0.52	1.00	0.52	1.00	1.00	0.56	1.00												
F98	0.78	0.74	0.74	0.56	0.78	0.81	0.48	0.81	0.74	0.48	0.74	0.48	0.74	0.74	0.52	0.74	1.00											
F110	0.96	1.00	1.00	0.59	0.96	0.70	0.52	0.93	1.00	0.52	1.00	0.52	1.00	1.00	0.56	1.00	0.74	1.00										
F111	0.96	1.00	1.00	0.59	0.96	0.70	0.52	0.93	1.00	0.52	1.00	0.52	1.00	1.00	0.56	1.00	0.74	1.00	1.00									
F113	0.74	0.78	0.78	0.52	0.74	0.48	0.44	0.70	0.78	0.52	0.78	0.52	0.78	0.78	0.56	0.78	0.52	0.78	0.78	1.00								
F114	0.96	1.00	1.00	0.59	0.96	0.70	0.52	0.93	1.00	0.52	1.00	0.52	1.00	1.00	0.56	1.00	0.74	1.00	1.00	0.78	1.00							
F115	0.63	0.67	0.67	0.70	0.70	0.67	0.63	0.74	0.67	0.56	0.67	0.56	0.67	0.67	0.59	0.67	0.70	0.67	0.67	0.44	0.67	1.00						
F116	0.96	1.00	1.00	0.59	0.96	0.70	0.52	0.93	1.00	0.52	1.00	0.52	1.00	1.00	0.56	1.00	0.74	1.00	1.00	0.78	1.00	0.67	1.00					
F117	0.96	1.00	1.00	0.59	0.96	0.70	0.52	0.93	1.00	0.52	1.00	0.52	1.00	1.00	0.56	1.00	0.74	1.00	1.00	0.78	1.00	0.67	1.00	1.00				
F119	0.96	1.00	1.00	0.59	0.96	0.70	0.52	0.93	1.00	0.52	1.00	0.52	1.00	1.00	0.56	1.00	0.74	1.00	1.00	0.78	1.00	0.67	1.00	1.00	1.00			
F121	0.96	1.00	1.00	0.59	0.96	0.70	0.52	0.93	1.00	0.52	1.00	0.52	1.00	1.00	0.56	1.00	0.74	1.00	1.00	0.78	1.00	0.67	1.00	1.00	1.00	1.00		
F122	0.70	0.74	0.74	0.48	0.70	0.44	0.48	0.67	0.74	0.56	0.74	0.56	0.74	0.74	0.59	0.74	0.48	0.74	0.74	0.96	0.74	0.41	0.74	0.74	0.74	0.74	1.00	
F124	0.63	0.59	0.59	0.70	0.56	0.67	0.63	0.67	0.59	0.70	0.59	0.70	0.59	0.59	0.74	0.59	0.63	0.59	0.59	0.59	0.59	0.78	0.59	0.59	0.59	0.59	0.56	1.00

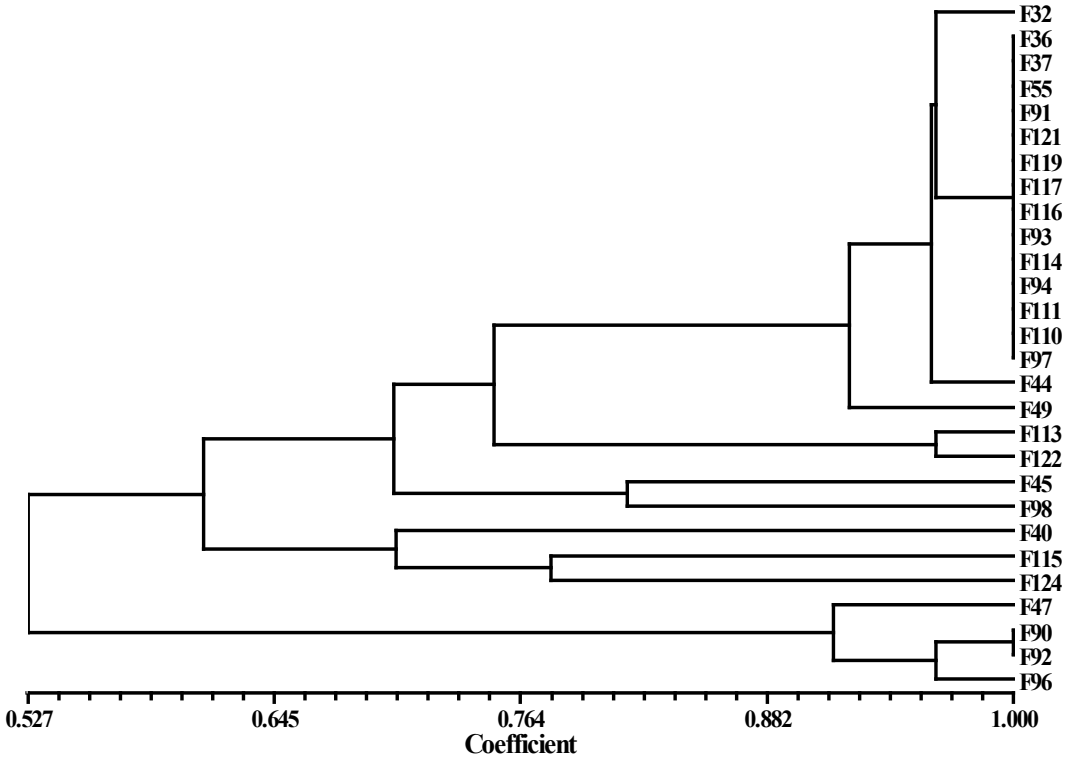

Figure 4. Dendrogram showing similarity index of IGS region of *Fusarium* isolates restriction digested by *EcoRI* and *HhaI*

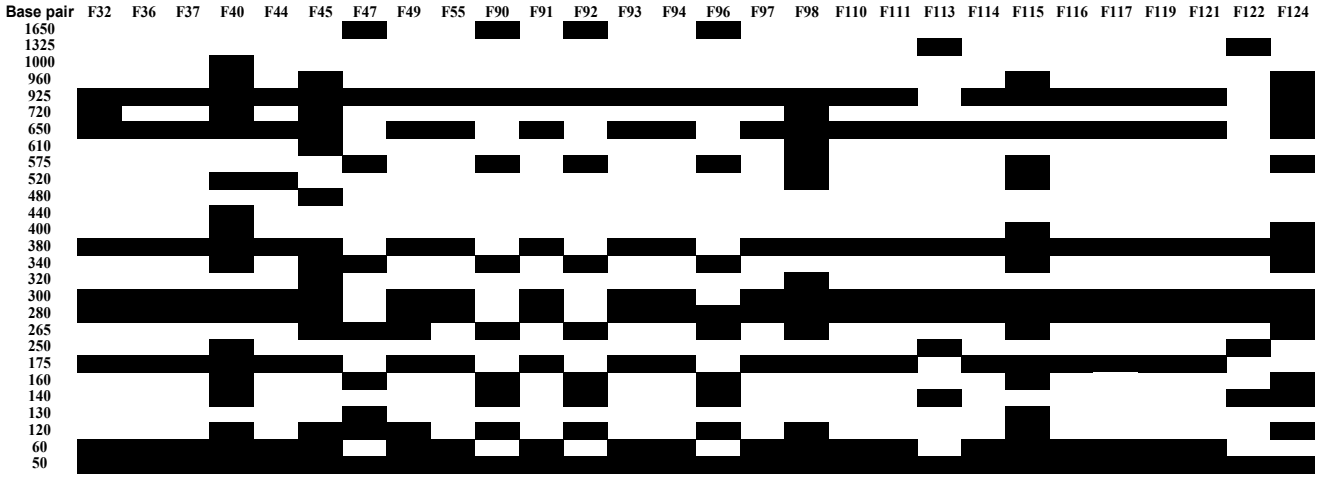

Figure 5. Schematic diagram of position of DNA fragments obtained by restriction of IGS region of *Fusarium* isolates

Discussion

The isolates analyzed in the current investigation were discriminated into producers and non-producers of fumonisin in previous study by detecting the presence of FUM gene using primer pair Fum5F and Fum6R as done by Baired et al. (2005) (Nayak et al. 2014). The genetic diversity and similarity among *Fusarium* spp isolates were inferred by restriction digestion of IGS region. This is otherwise called Non Transcribing Spacer fragment and was amplified by primer pair CNL12 and CNS1 which resulted in a double stranded product of approximately 2.6 kb for all isolates, representing IGS. The 2.6 kb corresponded with the expected size as reported by Kim et al. (2001) and Appel and Gordon (1995). But Alves-Santos et al. (1999) found that there were two IGS haplotypes in *F. oxysporum* isolates, the smaller IGS (2.55 kb) and the larger IGS (2.60 kb) following the size of the IGS fragment. The amplification of IGS region with the primer CNL12 and CNS1 and restriction of amplified product had showed some difference within and between *F. oxysporum* formae species in earlier studies of Kim et al. (2001). Appel and Gordon (1995) identified 13 IGS haplotypes among a sample of 56 *F. oxysporum* isolates collected in Maryland and California. Alves-Santos et al. (1999) found six different IGS haplotypes among one hundred and twenty-eight isolates of *F. oxysporum.* Edel et al. (1997) performed IGS analyses for 400 strains of *Fusarium oxysporum* using 7 kinds of restriction enzyme and divided them into 16 IGS types. Similarly Woudt et al. (1995) separated pathogenic and non-pathogenic strains of *F. oxysporum* in to six IGS types. In the present study fourteen haplotypes have been identified among 28 *Fusarium* isolates which represented a high degree of genetic diversity. Konstantinova and Yli-Mattila (2004) explained this variability obtained by PCR-RFLP of IGS region could be due to minor changes in the nucleotide composition which may generate different restriction patterns even within the same species. Variations could also be caused by length and sequence variations especially among closely related species (Hsuan et al. 2010; Martin 1990; Hillis and Dixon 1991).

Nuclear rDNA provides useful inter and intra-specific polymorphisms in eukaryotic organisms. The IGS, which separates rDNA repeat units, appears to be the most rapidly evolving spacer region. Closely related species may show considerable diversity in IGS, often reflecting both length and sequence variation (Kim et al. 2001). PCR-RFLP analysis of the IGS is a rapid and reproducible technique to resolve genetic variation among *Fusarium* isolates (Aminnejad et al. 2009; Miyagawa et al. 2002). Llorens et al. (2006) even correlated fumonisin production with IGS typing. In our study similar results were obtained. *Fusarium* isolates *viz* F47, F90, F92 and F96 which weren't amplified by Fum5F and Fum6R supposed to be fumonisin non producers, were placed in separate hierarchy in the dendrogram. Also level of genetic similarity was high (90%) among them.

IGS-RFLPs markers therefore proved to constitute a rapid and suitable way to group closely related *Fusarium* isolates and to estimate the genetic relationships between the groups. Restriction analysis of PCR amplified rDNA sequences has been shown to be a suitable method for taxonomic studies in *Fusarium*. Variations in rDNA among closely related taxa are found in the intergenic spacer (IGS), which separates the repeated ribosomal units (Fernandez et al., 1994). The main advantage of this technique is that the IGS region is amplified using PCR and digested to produce RFLPs, avoiding the need to time consuming probe digests of whole cell DNA. The choice of enzyme allows the screening ability of the method to be fine tuned. This method allows categorisation of groups of closely related strains of different fungi at an intraspecific level with simple banding pattern.

Conclusion

Fusarium species are not only harmful as pathogens but is also the producer of deadly mycotoxin i.e fumonisins. Higher genetic diversity possesses the chance for higher

rate of sexual recombination and evolution of new aggressive and more virulent pathogen species. Hence analysis of pathogen diversity at molecular or genetic level is very much necessary. Our results indicated a high level of genetic variation among the 28 *Fusarium* isolates which suggested that it might be possible to delimit the 28 isolates of *Fusarium* from each other. Comparison of more variable DNA regions might allow discrimination of *Fusarium* isolates that are placed in the same group such as some mitochondrial DNA regions have shown to be variable enough to allow differentiation of closely related *Fusarium* strains.

ACKNOWLEDGEMENTS

Authors are grateful the Director, ICAR-National (Central) Rice Research Institute, Cuttack for providing necessary facilities. Support by Dr. Arup K. Mukherjee, Principal Scientist of the same institute is also acknowledged.

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