Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2017; SP1: 572-576

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Molecular variability in *Curvularia lunata* isolates infecting paddy grain discoloration from different paddy growing areas of Karnataka

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Abstract
Grain discoloration disease was first recorded as minor disease of rice in Karnataka, with yield losses approaching >30% under appropriate environmental conditions. *Curvularia lunata* is plant pathogenic fungus which is one of the dominant causal organism of grain discoloration of rice. The present work was done to assess the molecular variability in twenty isolates of *Curvularia lunata*, collected from five different agro-ecological regions of Karnataka, ranging from North and South irrigated maidan area, Southern transitional area, Hilly and coastal areas of Karnataka using random amplified polymorphic DNA (RAPD) markers. The similarity values of RAPD profiles ranged from 0.39 to 0.66 among all the isolates. Maximum similarity of 66% was observed between isolates Cl-14 and Cl-3 followed by 65 per cent between isolates Cl-8 and Cl-3. Out of twenty isolates 17 isolates were in cluster A, under various sub clusters showing some degree of variability. Most of the isolates collected from similar agro-ecological location clustered together in the present study.

Keywords: *Curvularia lunata*, rice, molecular variability, RAPD

Introduction
Rice grain discolouration has gained considerable importance in rice growing areas. It is becoming more serious with the changing agricultural practices. It is a complex disease caused by a large number of fungi and also bacteria associated (Ou, 1985) [1]. The yield loss was approximately estimated from 20 to 55 per cent (Ghose *et al.*, 1960) [2]. Many fungi have been isolated from discolored grain, in which *Curvularia lunata* (Wakker) Boedign was found dominant pathogen (35.30%) of grain discolouration of rice in rice growing tracts of Tungabhadra Project Area and Upper Krishna Project Area of Karnataka state (Sumangala and Patil, 2011) [3]. The emergence of *Curvularia lunata* as an economically important rice pathogen has been attributed to the intensification of the rice-cropping systems with the development of new short-statured, high-tillering, high yielding varieties, high plant densities and an increase in nitrogen fertilization. These factors promote disease spread by providing a favorable microclimate. However, lack of adequate information on the genetic variability of the fungal population occurring in India. Till date, no reportable data exists on the genetic diversity among the grain discoloration isolates of *Curvularia lunata* in Karnataka. Diversity within rice grain discoloration isolates has been studied by morphological characterization, pathogenicity testing and also by various molecular techniques. Perhaps, evaluation of the genetic diversity in pathogen isolates has been an initial step towards understanding the population structure.

Molecular techniques have become reliable and are highly suitable tools for identifying pathogen species and for assessing genetic variation within collections and populations (Sundravadana *et al.*, 2011) [4]. Random amplified polymorphic DNA (RAPD) markers have been successfully applied to numerous filamentous fungi in different fields of experimental mycology (Pollastro *et al.*, 2000) [5]. RAPD offers a promising, versatile and informative molecular tool to detect genetic variation within population of plant pathogens (Chiochetti *et al.*, 1999) [6]. Variability in molecular characters is used for determining resistant cultivars (Thirumalasamy *et al.*, 2006) [7] and for the evaluation of the germplasm resistant line (Shekhar *et al.*, 2006) [8]. Understanding the genetic structure of pathogenic fungi is critical for developing appropriate strategies for disease management. Thus, the present study was undertaken to assess the molecular variability using RAPD markers to distinguish the isolates collected from different agro-ecological zones of Karnataka.

Materials and methods
Fungal isolates and culture maintenance
Twenty (n = 20) isolates of *Curvularia lunata* used in the study were collected from paddy

fields of different agro-ecological regions of Karnataka. Stock cultures were maintained on the potato dextrose agar (PDA) and designated as Cl-1, Cl-3, Cl-5, Cl-8, Cl-10, Cl-12, Cl-14, Cl-15, Cl-18, Cl-21, Cl-25, Cl-29, Cl-35, Cl-38, Cl-40, Cl-43, Cl-46, Cl-49, Cl-54 and Cl-59. The isolates of *C. lunata* used in the study and their geographic locations are presented in Table 1.

Isolation of genomic DNA

Actively growing mycelial plugs were inoculated in 50 ml of potato dextrose broth and incubated for 5 days at 28°C. High quality genomic DNA isolation was done for ten isolates of *C. lunata* according to Guha *et al.* (2006) [9]. Isolated DNA was quantified on 1% Agarose gel. Final working DNA concentration for setting up PCR was about 20 to 30 ng/ul.

RAPD analysis

In preliminary experiments, a total of 30 primers were screened for RAPD analysis, of which twenty were selected and used for the present study based on the reproducibility of the PCR amplification. All PCRs with these twenty RAPD primers were repeated three times to assess reproducibly. These primers: OPA08(5'-GTGACGTAGG-3'), OPA11 (5'-CAATCGCCGT-3'), OPA12(5'-TCGGCGATAG-3'), OPA14 (5'-TCTGTGCTGG-3'), OPA16(5'-AGCCAGCGAA-3'), OPA17(5'-GACCGCTTGT-3'), OPA18(5'-AGGTGACCGT-3'), OPA20(5'GTTGCGATCC-3'), OPB03(5'-CATCCCCCTG-3'), OPB05(5'-TGCGCCCTTC-3'), OPB06(5'-TGCTCTGCCC-3'), OPB08(5'-GTCCACACGG-3'), OPB09(5'-TGGGGGACTC-3'), OPB11(5'GTAGACCCGT-3'), OPB12(5'-CCTTGACGCA-3'), OPB14(5'-TCCGCTCTGG-3'), OPB15(5'-GGAGGGTGTT-3'), OPC06(5'-GAACGGACTC-3'), OPC16(5'-CACACTCCAG-3'), OPC19(5'-GTTGCCAGCC-3') were used for PCR-based amplification of the template DNA of the isolates. PCR amplifications were carried out in 0.2 ml eppendorf tubes with 20 µl reaction mixture which consists of 2.0 µl of 10x Taq buffer, 2.0 µl of 25 mM $MgCl_2$, 2.0 µl of primer (1 picomolar/µl), 1.5 µl of dNTP, 0.3 µl of Taq polymerase enzyme and 12.2 µl of sterile PCR water (Genei, Bangalore) and 2 µl of DNA sample. Amplification was carried out by 4 min of initial denaturation at 94°C followed by 40 cycles of denaturation of 94 °C for 1 min; annealing at 37 °C for 1 min; extension at 72 °C for 2 min with final extension at 72 °C for 10 min. Amplified PCR products were subjected to 1.0% agarose gel electrophoresis with 1 x TAE as running buffer. The banding patterns were visualized under UV trans-illuminator with ethidium bromide (10 mg/ml) staining. The DNA banding profiles were documented in the gel documentation system and compared with 1 kb DNA ladder.

The patterns generated were tested for their reproducibility. Each amplified band was considered as RAPD marker and recorded for all isolates. Data was entered using a matrix in which all the observed bands or characters were listed. The RAPD pattern of each isolate was evaluated; assigning character state '1' to all the bands that could be reproducible and detected in the gel and '0' for the absence of the band. The similarity coefficients were subjected to Unweighted Pair-Group Method on Arithmetic Average (UPGMA) cluster analysis to group the isolates based on their overall similarities. Statistical Package for Social Science (SPSS) package was used for the cluster analysis and subsequent dendogram preparation and per cent polymorphism was calculated by using the formula.

$$\text{Per cent polymorphism} = \frac{\text{No. of polymorphic bands}}{\text{Total number of bands}} \times 100$$

Results and discussion

Out of 60 isolates collected from different agro ecological regions, twenty isolates were selected for RAPD analysis. Banding profile of different primers for different isolates of *Curvularia lunata* is presented in Table 2 and Fig 1. Of the 20 primers used for amplification, OPA-11, OPA-14, OPA-17, OPB-14 and OPB-16 showed hundred per cent polymorphism. Information on banding pattern for all the primers was used to to construct a dendrogram. Similarity coefficient of twenty isolates of *C. lunata* based on RAPD analysis is given in Table 3. Coefficient ranged from 39 to 66 per cent. Maximum similarity of 66% was observed between isolates Cl-14 and Cl-3 followed by 65 per cent between isolates Cl-8 and Cl-3. Whereas, the least similarity of 39 per cent was observed between Cl-43 and Cl-15 and also in Cl-35 and Cl-25 isolates.

RAPD data distinguished the various isolates into major cluster A and B. Majority of the isolates (11 isolates) were in cluster A (1) and 6 isolates were in cluster A (2) under various sub-clusters showing some degree of variability. Remaining isolates fall under cluster B which has 3 isolates. In case of sub cluster B (2), comprises a single isolate CL-35. Out of twenty isolates 17 isolates were in cluster A, under various sub clusters showing some degree of variability. Remaining three isolates were under major cluster B, where sub cluster B (2) has got a single isolate Cl-35, which differed from all other isolates in the major clusters (Fig 2). These results are in consonance with of Sharma and Tenga (1996) [10], indicating high genetic variability in the pathogen population in different epidemiological regions.

Cluster analysis of the RAPD banding pattern data, however, revealed a substantial amount of genetic diversity among all the isolates examined. The high amount of genetic diversity resulted in virtually no clusters that clearly identified geographic origin or pathogenicity of the isolates.

The study also revealed that pathogen showed molecular variation over locations and also in a location depending on the genotype grown. These results are in agreement with Weikert-Oliveira *et al.* (2002) [11] who studied twenty isolates of *Helminthosporium* disease in cereals. The strains were compared by PCR-RAPD analysis. The RAPD profiles indicated an expressive level of polymorphism among different species, compared with a low level of polymorphism among isolates of the same species. These results are in conformation with Peeyush Kumar *et al.* (2011) [12] where thirteen isolates of *Bipolaris oryzae* tested for their variability with Random Amplified Polymorphic DNA (RAPD) primers. Twenty RAPD primers were screened, of which 10 showed amplification. The RAPD markers have potential as a means of identifying the rice grain discoloration pathogens and also increased our understanding of the ecology and biology of the fungus by providing measurements of genetic relatedness and variation within isolates (Neeraja *et al*., 2002) [13]. Despite this, the results of this study suggest that RAPD banding patterns are not suitable to differentiate isolates based on their pathogenicity but geographically differentiable. These results confirm those of Woo *et al.* (1996) [14], who also found that RAPDs were not useful in differentiating Fop pathogenic types.

Table 1: Passport data of *Curvularia lunata* isolates collected from different regions of Karnataka.

Designation	Isolate locality	District	Designation	Isolate locality	District
Cl-1	Yadgir	Yadgir	Cl-31	Alur	
Cl-2	Shahapur		Cl-32	Sakleshpur	
Cl-3	Shorapur		Cl-33	Chikamagalur	Chikamagalur
Cl-4	Shorapur		Cl-34	Koppa	
Cl-5	Raichur	Raichur	Cl-35	Mudigere	
Cl-6	Raichur		Cl-36	Mudigere	
Cl-7	Manvi		Cl-37	Madikeri	Kodagu
Cl-8	Sindhnur		Cl-38	Virajpet	
Cl-9	Devadurga		Cl-39	Virajpet	
Cl-10	Bellary	Bellary	Cl-40	Mangalore	Dakshina Kannada
Cl-11	Hosapet		Cl-41	Mangalore	
Cl-12	Siraguppa		Cl-42	Bentval	
Cl-13	Siraguppa		Cl-43	Udupi	Udupi
Cl-14	Koppal	Koppal	Cl-44	Karkal	
Cl-15	Gangavathi		Cl-45	Mysore	Mysore
Cl-16	Gangavathi		Cl-46	Nanjangud	
Cl-17	Davangere	Davangere	Cl-47	Nanjangud	
Cl-18	Harihar		Cl-48	T.Narshipura	
Cl-19	Harihar		Cl-49	Chamarajanagar	Chamarajanagar
Cl-20	Shimoga	Shimoga	Cl-50	Yelandur	
Cl-21	Shimoga		Cl-51	Kollegal	
Cl-22	Tirthahalli		Cl-52	Kollegal	
Cl-23	Bhadravati		Cl-53	Mandya	Mandya
Cl-24	Hosanagar		Cl-54	Pandavpur	
Cl-25	Sirsi	Uttara Kannada	Cl-55	Pandavpur	
Cl-26	Sirsi		Cl-56	Malavalli	
Cl-27	Yellapur		Cl-57	Malavalli	
Cl-28	Mundgod		Cl-58	Ramanagar	Ramanagar
Cl-29	Hassan	Hassan	Cl-59	Kankapur	
Cl-30	Alur		Cl-60	Kankapur	

Table 2: RAPD banding profile of different primers for different isolates of *Curvularia lunata* of rice.

Sl. No.	Primer	Total No. of bands	No. of Polymorphic bands	Polymorphism (%)	Sl. No.	Primer	Total No. of bands	No. of Polymorphic bands	Polymorphism (%)
1.	OPA-08	8.00	7.00	87.50	11.	OPB-6	5.00	3.00	60.00
2.	OPA-11	9.00	9.00	100.00	12.	OPB-8	6.00	4.00	66.66
3.	OPA-12	11.00	10.0	90.90	13.	OPB-9	13.00	12.00	92.30
4.	OPA-14	7.00	7.00	100.00	14.	OPB-11	9.00	8.00	88.88
5.	OPA-16	13.00	1.30	100.00	15.	OPB-12	9.00	7.00	77.77
6.	OPA-17	7.00	7.00	100.00	16.	OPB-14	8.00	8.00	100.00
7.	OPA-18	7.00	6.00	85.71	17.	OPB-15	8.00	7.00	87.50
8.	OPA-20	9.00	8.00	88.88	18.	OPB-6	9.00	8.00	88.88
9.	OPB-3	4.00	3.00	75.00	19.	OPB-16	9.00	9.00	100.00
10.	OPB-5	9.00	7.00	77.77	20.	OPB-19	6.00	4.00	66.66

Table 3: Similarity coefficient values in RAPD for *Curvularia lunata* isolates

Isolate	Cl-1	Cl-3	Cl-5	Cl-8	Cl-10	Cl-12	Cl-14	Cl-15	Cl-18	Cl-21	Cl-25	Cl-29	Cl-35	Cl-38	Cl-40	Cl-43	Cl-46	Cl-49	Cl-54	Cl-59
Cl-1	1.00																			
Cl-3	0.50	1.00																		
Cl-5	0.51	0.57	1.00																	
Cl-8	0.57	0.65	0.53	1.00																
Cl-10	0.55	0.56	0.57	0.62	1.00															
Cl-12	0.47	0.53	0.54	0.49	0.52	1.00														
Cl-14	0.48	0.66	0.56	0.65	0.60	0.55	1.00													
Cl-15	0.41	0.57	0.54	0.49	0.51	0.59	0.56	1.00												
Cl-18	0.56	0.61	0.47	0.56	0.55	0.49	0.58	0.48	1.00											
Cl-21	0.49	0.56	0.55	0.51	0.54	0.48	0.53	0.41	0.64	1.00										
Cl-25	0.50	0.52	0.52	0.50	0.55	0.40	0.49	0.44	0.47	0.49	1.00									
Cl-29	0.57	0.52	0.56	0.53	0.59	0.47	0.55	0.52	0.51	0.54	0.53	1.00								
Cl-35	0.45	0.53	0.46	0.43	0.53	0.48	0.48	0.51	0.50	0.41	0.51	0.40	1.00							
Cl-38	0.50	0.60	0.57	0.54	0.60	0.54	0.60	0.54	0.50	0.55	0.39	0.56	0.44	1.00						
Cl-40	0.47	0.55	0.50	0.41	0.53	0.51	0.58	0.51	0.51	0.53	0.51	0.52	0.52	0.50	1.00					
Cl-43	0.49	0.45	0.56	0.49	0.55	0.44	0.46	0.39	0.46	0.47	0.56	0.44	0.47	0.49	0.54	1.00				
Cl-46	0.45	0.55	0.53	0.53	0.56	0.48	0.60	0.52	0.47	0.54	0.46	0.55	0.55	0.61	0.49	0.44	1.00			
Cl-49	0.53	0.53	0.50	0.53	0.56	0.58	0.50	0.51	0.55	0.50	0.54	0.53	0.47	0.58	0.50	0.49	0.55	1.00		
Cl-54	0.51	0.59	0.62	0.59	0.54	0.57	0.54	0.58	0.51	0.58	0.47	0.47	0.46	0.53	0.47	0.52	0.52	0.48	1.00	
Cl-59	0.57	0.57	0.56	0.52	0.54	0.51	0.59	0.56	0.51	0.56	0.44	0.46	0.52	0.51	0.60	0.57	0.53	0.42	0.59	1.00

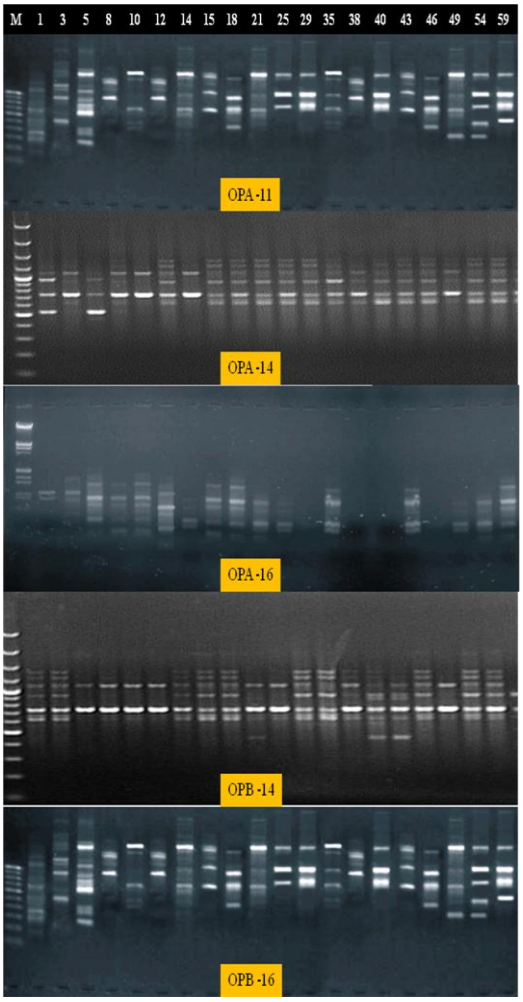

Fig 1: Gel electrophoresis of RAPD amplication of twenty *Curvularia lunata* isolates by random RAPD primers

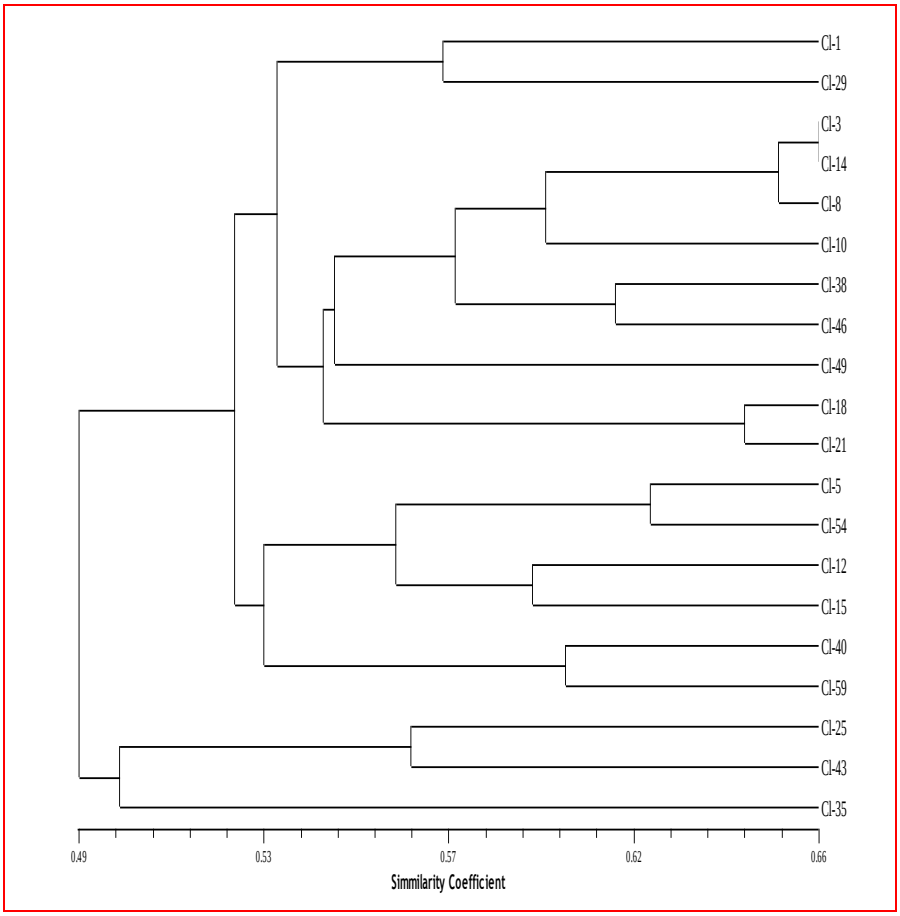

Fig 2: Phylogenetic analysis of twenty isolates of *Curvularia lunata*

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