

Molecular variability in *Curvularia lunata* isolates causing *Curvularia* leaf spot of maize in Karnataka

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Abstract: Molecular variability of *Curvularia lunata* pathogen causing *Curvularia* leaf spot of maize was carried out by using ITS primers and subsequently subjected to sequence analyses. Results revealed that, phylogenetic analysis grouped *Curvularia lunata*, isolates into two main clusters with 0.005 similarity coefficient. The similarity coefficient ranged from 0.001 to 0.005 indicating good level of similarity. UPGMA cluster analysis based on genetic distance coefficients clearly separated all the isolates. Out of five isolates Bailhongal, Badami, Davangere and Dharwad isolates were grouped under cluster I, whereas Mundagod isolate formed separate cluster II. In cluster I, Bailhongal and Dharwad isolates were showed almost one per cent of divergence. In cluster II only one isolate Mundagod was present with nearly 5.5 per cent divergence compared to cluster I, this indicated there was 96.5 per cent of similarity among the isolates of *Curvularia lunata*.

Keywords: *Curvularia* leaf spot · Primers · Ecology · Maize

Introduction

Maize (*Zea mays* L.) is the world's leading crop with wider adaptability across agro-ecological zones with versatile uses. It is the main raw material for industrial products like glucose, sorbitol, dextrose and oil. Besides

it is used as livestock, poultry and animal feed, and for manufacturing of starch and starch based products. It can contribute to diet diversification and improve nutrition in human beings through exploitation of quality protein maize. In India, maize is the important food crop after rice and wheat cultivated in an area 9.86 million hectare with an annual production of 30.16 million tonnes and an average productivity of 3.06 t/ha (FAOSTAT, 2020). The maximum area and production of maize is in Uttar Pradesh and the highest productivity is in Andhra Pradesh (4637 kg/ha) followed by Tamil Nadu (4,134 kg/ha). In Karnataka, maize is cultivated in an area of 1.40 million hectare with a production of 3.96 million tonnes and an average productivity of 2.9 t/ha which is almost same as the national productivity.

Maize is subjected to as many as 112 diseases on global basis. In India, there is a record available for 35 diseases. The annual loss due to maize diseases in India was estimated to the tune of 13.2 to 39.5 per cent (Payak and Sharma, 1985). *Curvularia* leaf spot of maize caused by *Curvularia andropogonis* which was a minor disease earlier has emerged as an important disease all over the maize growing area in India (Choudhary *et al.*, 2011). In Karnataka, the disease was reported for the first time from Dharwad district during 2008 (Anonymous, 2008) and further the occurrence of the disease and causal organism as *C. lunata* Wakker (Boedikun) on the basis of morphological characters was reported by Harlapur *et al.* (2012 and 2014).

The symptoms of the disease start as minute, chlorotic, pinhead sized translucent spots on the leaf surface. Subsequently the spots increase in size and necrosis start from the centre. The disease severity of *Curvularia* leaf spot of maize in Karnataka ranged from 18.5 to 61.5 per cent with an estimated 22.6 per cent loss

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in grain yield (Harlapur *et al.*, 2012). In India though *C. lunata* has a narrow host range infecting maize and paddy, it extracts significant morphological as well as pathogenic variability. Although some of the earlier work had thrown light on its morphological characterization and pathogenic variability, but that does not provide a clear-cut situation in the variability existing in the pathogen. Thus, the present study was undertaken to study the molecular variability of *C. lunata* along with morphological variability.

Materials and method

Molecular variability of the pathogen was studied using ITS primers and subsequently subjected to sequence analysis. The detailed methodology followed for preparation of formulation is as below.

Curvularia lunata isolates were collected from 15 locations of major maize growing areas of northern Karnataka (Table 1) and were assessed for molecular variability. The DNA was isolated from all the isolates of *C. lunata*, by following standard CTAB method with minor modification (Cuervo-Parra *et al.*, 2011). For DNA isolation, 2-3 of fungal mat grown potato dextrose broth was taken and homogenized using pestle and mortar in liquid nitrogen. To the above solution 1 ml of lysis buffer was added. The suspension in pestle and mortar was extracted with equal volume of phenol: chloroform: isoamyl alcohol (1:1 W/V) in centrifugation tube, centrifuged at 10,000 rpm for 15–20 minutes at 4°C. Supernatant was taken in fresh centrifuge tube and 2.5 µl RNase and 2.5µl protienase-k was added and incubated at room temperature for 30 minutes. Chilled isopropanol of about 1/3rd volume (300-400 µl) was added and centrifuged @ 10,000 rpm for 15minutes at 4°C. The 500 µl buffer was added and centrifuged at 10,000 rpm for 5 min at 4°C. DNA, pellet was washed with 70 per cent ethanol; air dried and resuspended in 500 µl of T₁₀E₁. This DNA obtained was further quantified by agarose gel electrophoresis.

Polymerase chain reaction (PCR)

The ribosomal DNA (rDNA) unit contains genetic and non-genetic or spacer region. Each repeat unit consists of copy of 18S, 5.83S and 28S like rDNA and its spacer like internal transcribed spacer (ITS). The rDNA has been employed to analyze evolutionary events because it is

highly conserved, whereas ITS rDNA is more variable hence, it was used for investigation. Forward ITS-1 (5'-TCCGTAGGTGAACCTGCG-3') and reverse primers ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') were used.

Master mix for PCR

Amplification reaction mixture was prepared in 0.2 ml thin-walled PCR tubes containing following components. The total volume of each reaction mixture was 20 µl.

Reaction mixture	Quantity
Template DNA (25 ng/µl)	1.00 µl
Primer (5PM/µl)	F-1.00 µl R-1.00 µl
dNTPs mix (2.5 mM each)	1.00 µl
10 x assay buffer with 15 mM MgCl ₂	2.00 µl
Taq DNA polymerase (6.0U µl-1)	0.50 µl
Sterile distilled water	13.50 µl
Total	20.00 µl

Except template the master mix was distributed to PCR tubes (19 µl tube) and later 1 µl of template DNA from the respective isolates was added making the final volume of 20 µl. The amplification was performed in the thermal cycler by following method suggested by Cuervo-Parra *et al.* (2011).

Table 1. The details of the sites (GPS position) from where the isolates have been collected

S.No.	Location	Name of the isolate	GPS Details	
			Latitude	Longitude
1.	Kalghatagi	CI Kal	15.18	74.97
2.	Navalagund	CI Nav	15.57	75.37
3.	Dharwad	CI Dha	15.46	75.01
4.	Davangere	CI Dav	14.47	75.91
5.	Kerimattihalli	CI Ker	14.77	75.39
6.	Haveri	CI Hav	14.80	75.40
7.	Badami	CI Bad	15.82	75.59
8.	Bagalkot	CI Bag	16.18	75.69
9.	Mundagod	CI Mum	14.97	75.03
10.	Nargund	CI Nar	15.72	75.38
11.	Hulakote	CI Hul	13.26	77.35
12.	Bailhongal	CI Bai	15.82	74.86
13.	Gokak	CI Gok	16.16	74.83
14.	Naganoor	CI Nag	15.18	75.10
15.	Raichur	CI Rai	16.21	77.34

Table 2. Blast results of nucleotide sequence of *Curvularia lunata* ITS region

S.No.	Isolate code	Place	Accession number	NCBI BLAST Hit results	Location	Host	Max. Indent
1	CI Dha	Dharwad	KR633084.1	<i>Curvularia lunata</i> strain CX-3 18S ribosomal RNA gene, partial sequence; internal transcribed spacer	China	Maize	99
2	IC Dav	Davangere	KR633132.1	<i>Curvularia lunata</i> strain SD-5 18S ribosomal RNA gene, partial sequence; internal transcribed spacer	China	Maize	99
3	CI Bad	Badami	KU315128.1	<i>Curvularia lunata</i> strain CSV3, 5.8S ribosomal RNA gene and internal transcribed spacer	Jammu & Kashmir	Maize	99
4	CI Mun	Mundagod	JX256431.1	<i>Curvularia lunata</i> voucher MFLUCC 10-0706 internal transcribed spacer	Thailand	Maize	99
5	CI Bai	Bailhongal	KU215414.1	<i>Curvularia lunata</i> strain CL-1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer	China	Maize	99

Gel electrophoresis

Agarose gel electrophoresis was performed to resolve the amplified product using 1.4 per cent agarose in 1X TBE (Tris Borate EDTA) buffer, 0.5 µl/ml of Ethidium bromide and loading buffer (0.25 per cent Bromophenol Blue in 40 per cent sucrose). Four microlitre of loading dye was added to 20 µl of PCR product and loaded to the agarose gel. Electrophoresis was carried out at 75 V for 1.5 hr. The gel was observed under UV light and documented using gel documentation unit.

Sequencing of ITS region

The ITS region was sequenced for five isolates of *C. lunata* belonging to different geographical regions to confirm organism and to know the variability present in them.

Results and discussion

The full length ITS rDNA region was amplified with ITS-1 (5'-TCCGTAGGTGAACCTGCG-3') and ITS-4) 5'-TCCTCCGCTTATTGATATGC-3') primers for fifteen isolates of *C. lunata*. DNA amplicon was observed at the region 560 bp by checking the amplified products on 1.2 using the NCBI BLAST programme, these isolates were confirmed as *C. lunata*. Cuervo-Para *et al.* (2011) obtained the similar results where the DNA of three strains of *C. lunata* electrophoresis and amplicon was observed at the region of 560 bp with universal ITS fungal primers. By using the NCBI-BLAST programme confirmed the isolates as *C. lunata*. The list of isolates, accession number, per cent homology and name identified are given in Table 2.

Phylogenetic analysis

The ITS rDNA region sequence was used in this analysis because it has been shown to be more informative and closest phylogenetic relative in *Curvularia lunata*, isolates. In order to evaluate whether the grouping pattern obtained on the basis of the ITS sequences would be useful frame to identify and align, these isolates and were identified at the species level by morphological characters using the existing taxonomic criteria analysis and analysis of their ITS rDNA region gene sequences. In the present case, isolates of *Curvularia lunata* were used as an out group vice-versa to interpret the clustering of isolates as distinct or related out group of genus. Phylogenetic analysis grouped *Curvularia lunata*, isolates into two main clusters with 0.005 similarity coefficient. The similarity coefficient ranged from 0.001 to 0.005 indicating good level of similarity.

Curvularia lunata

The UPGMA cluster analysis-based of genetic distance coefficients clearly separated all isolates. The five isolates were grouped into two clusters. The cluster I included the isolates which belonged to Dharwad, Bailhongal, Badami and Davangere, whereas the isolates of Mundagod were grumped in to cluster II. The per cent divergence between the clusters was less. Further, the divergence within the clusters indicated that the isolates of Dharwad and Bailhongal showed only 1 per cent and the isolates of Badami and Davanagere showed 2.5 to 3.0 per cent divergence. (Figure 1). Form the amplification of ITS region (Figure 2) and sequencing data of ITS region, it is evident that the genetic diversity within *Curvularia lunata*, among the collected isolates is low. The presence of this

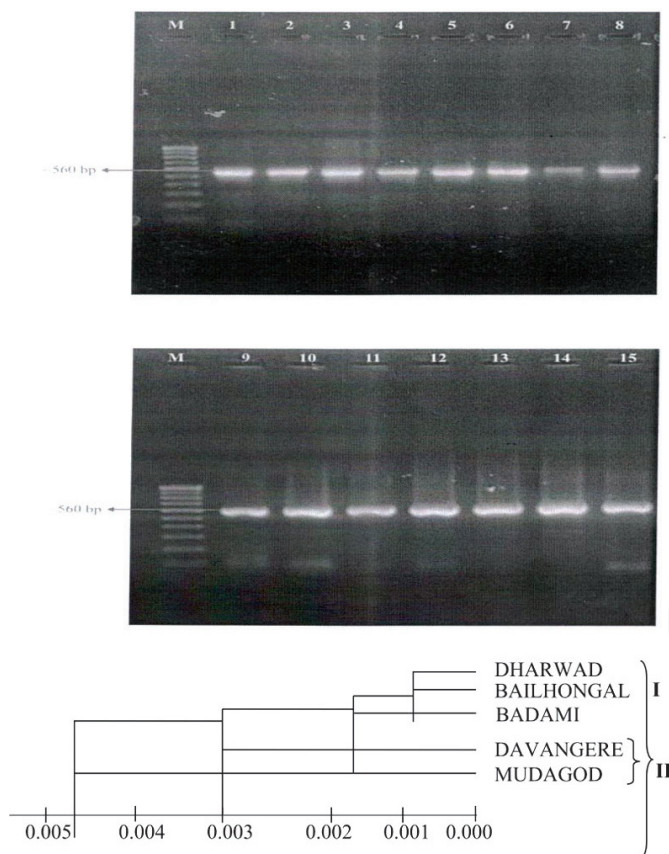


Figure 1. Phylogenetic relationship on ITS rDNA among the isolates of *Curvularia lunata* from different regions of northern Karnataka

low genetic variability of the isolates in the regions helps to manage the *Curvularia* leaf spot of maize by following similar management practices in the regions.

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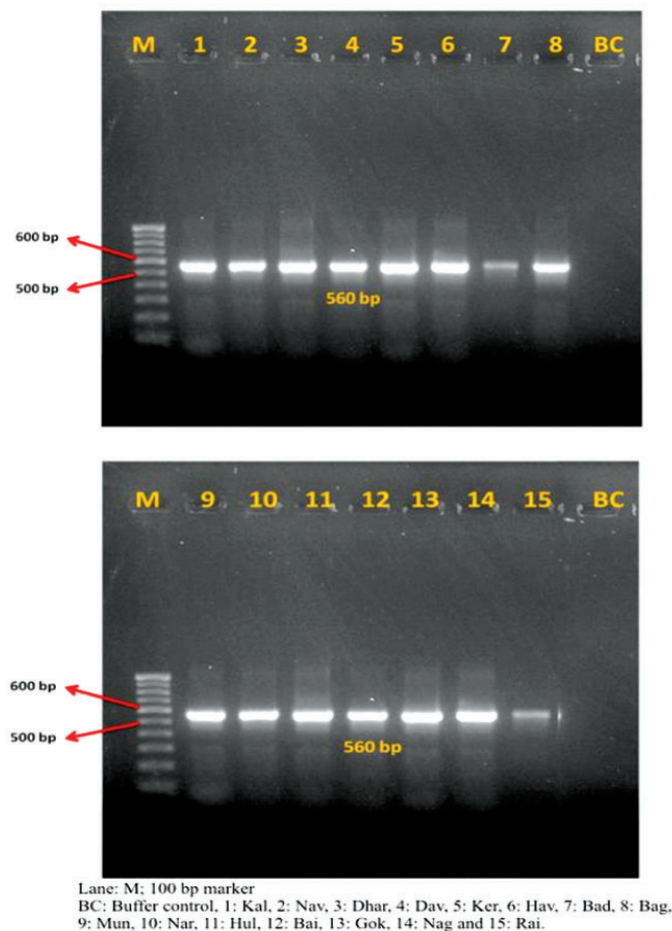


Figure 2. PCR amplification of ITS region of

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