Molecular Variability in North Indian Isolates of *Cylindrocladium quinqueseptatum* Causing Eucalyptus Leaf and Seedling Blight

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Abstract *Cylindrocladium quinqueseptatum* has been considered as the most destructive pathogen of *Eucalyptus* nurseries and plantations in north India. Genetic resistance has not been determined against this disease in *Eucalyptus* and genetic diversity among the fungal population in northern India is not known. Seventy three isolates from infected leaves and twigs of *Eucalyptus* were collected from different northern Indian state and analyzed through RAPD-PCR for screening genetic diversity. The UPGMA cluster analysis score of 284 loci permitted identification of 11 population lines and an outlier. This molecular variability prevalent among the north Indian population of the pathogen can be used in identifying *Cylindrocladium* leaf and seedling blight resistant *Eucalyptus* germplasm.

Keywords RAPD · *Cylindrocladium quinqueseptatum* · *Cylindrocladium* leaf and seedling blight · *Eucalyptus*

Introduction

Cylindrocladium leaf and seedling blight is a serious disease of *Eucalyptus* nurseries and plantations in north India jeopardizing the plantation programs of state forest department, farmers and paper and board industries depending heavily on *Eucalyptus* as raw material.

Cylindrocladium quinqueseptatum Boedjin and Reitsma though prevalent throughout India, is a major cause of concern among the nursery managers who cater the planting stock requirements of industries in north India specially Punjab, Haryana and Uttarakhand. The pathogen over winters in soil and the *Eucalyptus* trees surrounding nurseries leading to increased inoculum levels in the subsequent rainy season. Symptoms include chlorotic leaf spots followed by necrosis which appear on the mature leaves on the lower branches of the seedling which further spread to upper parts of the plantlets. The seedlings are killed within a week after infection in humid summer months. The plantations also show severe leaf necrosis and defoliation often exhibiting epicormic branching. Most of the *Eucalyptus* species and hybrids are severely found to be infected.

Among the foliar diseases, *Cylindrocladium* leaf and seedling blight (CLSB) caused by *Cylindrocladium quinqueseptatum* Boedjin and Reitsma is the most serious and wide spread disease of nursery and plantations [1]. *Cylindrocladium quinqueseptatum* was reported as one of the most common and destructive diseases of *Eucalyptus* spp. in India [2–4]. The disease usually attains an epidemic status in high rainfall areas during the monsoon i.e. from June to September resulting in large scale mortality of young seedlings in nurseries and extensive defoliation of young trees and young coppices shoots in plantations. Preliminary epidemiological studies had been reported on CLSB in Australia by Bolland et al. [5] and Sharma et al. [6] in Kerala. Sharma and Mohanan [7] reported a wide range of pathogenic variation among the five isolates of *Cylindrocladium quinqueseptatum* which were distinguished as different physiologic strains on 11 different states. Analysis of variance showed that CLSB severity of a state is mainly due to genetically different isolates. This

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was the first evidence for the existence of physiologic strains in *Cylindrocladium quinqueseptatum* from South India. To ascertain phylogenetic status, comparative morphology, pathology, characterization of micro-satellite loci and species with clavate vesicles, different molecular techniques were used by Wright et al. [8], Henricot and Culham [9], Uchida and Aragaki [10], Norman et al. [11] and Crous et al. [12].

In the present study different isolates of *Cylindrocladium quinqueseptatum* were subjected to RAPD-PCR for identifying different population lines. This pathogen repository will help in screening durable disease resistant *Eucalyptus* germplasm which can be later propagated and utilized by the industries, farmers and forest managers of north India for obtaining better survival and productivity.

Materials and Methods

Collection of Fungal Isolates

Eucalyptus leaves and twigs were collected from four state of northern India (Table 1). Two isolates from Orissa were incorporated in the study for comparison. Each sample was thoroughly washed and dried at room temperature. The samples were sterilized in alcohol (30 s), transferred to 2.5% sodium hypochlorite (30 s) and washed in sterile water for 1 min. Isolation was performed on potato dextrose agar (PDA) plates containing streptomycin (0.1 mg/ml). Leaves were incubated at $25 \pm 1^\circ\text{C}$ in the darkness for

4 days in damp chambers and then the growing mycelia were transferred on PDA slants. Seventy three stock cultures were prepared using this method. The mycelia from the slants were transferred after 10 days to a modified medium [sucrose-15 g, sodium nitrate-2 g, magnesium sulphate-0.05 g, potassium chloride-0.05 g, ferrus sulphate-0.02 g and agar-15 g in 1 l double distilled water (pH 7.0 ± 2)] and incubated on laboratory bench at 24°C to induce sporulation. Isolates were single spored and sub-cultured weekly on PDA.

Fungal DNA Extraction

Isolates were grown in 50 ml of potato dextrose broth (PDB) medium for 10 days at $25 \pm 1^\circ\text{C}$ in an environmental shaker. Mycelium was harvested by filtration through Whatman no. 1 filter paper, lyophilized and stored at -20°C . For each fungal isolate 5 g of lyophilized mycelium was ground in liquid nitrogen to make a fine powder. Then 25 mg of powdered mycelium was taken for DNA isolation. DNA was extracted by C-TAB protocol [13], with slight modification. DNA was dissolved in TE-8 buffer (10 mM Tris-Cl, pH 7.5 and 1 mM EDTA) and the final concentration of DNA was adjusted to 20 ng and stored at -20°C until used.

RAPD-PCR, Electrophoresis and Data Analysis

PCR was carried out in a final volume of 25 μl containing $1 \times$ taq buffer (2.5 μl), 0.25 mM of each dNTPs, 2.5 mM MgCl_2 , 0.5 mM oligonucleotide primer, 1 unit of amplitaq (Applied biosystems) and 20 ng of template DNA. Initially 40 primers were screened for polymorphism, reproducibility and amplification consistency. Out of these forty primers four RAPD primers viz. OPE-2, OPE-3, OPE-5 and OPE-6 (Operon technology, USA) were selected for the amplification of the genomic DNA as they showed polymorphic and reproducible bands (Table 2). The reaction mixture over laid with 10 μl of mineral oil and the tube was microfused at 10,000 g for 10 s. Amplification was carried out in gradient thermal cycler (Eppendorf) with initial denaturation at 95°C for 2 min and 45 cycles at 94°C for 1 min, 37°C for 45 s, 72°C for 1 min and a final extension at 72°C for 8 min.

20 μl of PCR product were mixed with 6 μl of gel loading buffer and subjected to electrophoresis at 65 V for 3 h in 1.2% agarose gel prepared in $1 \times$ TAE buffer. Ethidium bromide was added to agarose gel at 6 μl per 100 ml for staining. A medium range ladder (GeNei, Bangalore) was used as a size standard. DNA was visualized and photographed by gel documentation system (UVP). For different fungal isolates, individual RAPD bands were scored for their presence (value = 1) or absence (value = 0).

Table 1 *C. quinqueseptatum* isolates collected from different north Indian states

States	Isolates of <i>Cylindrocladium quinqueseptatum</i>
Uttarakhand	FCQ-135, FCQ-136, FCQ-137, FCQ-159, FCQ-160, FCQ-162, FCQ-163, FCQ-164, FCQ-165, FCQ-171, FCQ-172, FCQ-174, FCQ-175, FCQ-176, FCQ-177, FCQ-178, FCQ-179, FCQ-180, FCQ-181, FCQ-182, FCQ-183, FCQ-190, FCQ-191, FCQ-192, FCQ-247, FCQ-248, FCQ-249, FCQ-268, FCQ-269, FCQ-270, FCQ-280, FCQ-281, FCQ-282, FCQ-284, FCQ-309, FCQ-285, FCQ-288, FCQ-289, FCQ-310, FCQ-311, FCQ-313, FCQ-314, FCQ-294
Haryana	FCQ-119, FCQ-138, FCQ-139, FCQ-140, FCQ-141, FCQ-142, FCQ-143, FCQ-144, FCQ-145, FCQ-204, FCQ-205
Punjab	FCQ-225, FCQ-226, FCQ-227, FCQ-228, FCQ-229, FCQ-230, FCQ-231, FCQ-232, FCQ-233, FCQ-234, FCQ-235, FCQ-236, FCQ-237, FCQ-238
Uttar Pradesh	FCQ-105, FCQ-106
Orissa	FCQ-305, FCQ-306

Two isolates from south eastern Indian state Orissa was used for comparison

Table 2 Operon RAPD primer sequences which showed high polymorphism in different isolates of *C. quinqueseptatum*

Primer name	Sequence
OPE-2	5'GGTGCGGGAA3'
OPE-3	5'CCAGATGCAC3'
OPE-5	5'TCAGGGAGGT3'
OPE-6	5'AAGACCCCTC3'

The banding patterns by all the different primers were scored as presence (1) and absence (0) of band of a particular molecular size to compile a binary matrix which was then subjected to cluster analysis. Both faint and intense bands were scored if shown to be reproducible in separate runs. The relationships between the isolates were more clearly represented by a similarity matrix. The similarity matrix was constructed using simple matching coefficient according to the formula shown below [14]:

$$GD(XY) = (N_x + N_y) / (N_{xy} + N_x + N_y)$$

where N_x is the number of bands in line X and not in line Y, N_y is the number of bands in line Y and not in line X, N_{xy} is the number of bands in lines X and Y.

Data analysis was done by Gene Profiler software. A dendrogram was constructed using UPGMA (unweighted pair group method with arithmetic averages) cluster analysis to infer the relationship between the 73 isolates by Treecon software. For further genetic analysis the RAPD data was treated haplotypic comprising combinations of alleles at one or several loci. The data was tested for genetic diversity in intra-population isolates by analysis of genetic structure by Arlequin software [15].

Internal Transcribed Spacer Amplification and Sequencing

ITS-I, ITS-II along with 5.8S rDNA of three isolates were amplified using primers ITS1 and ITS4 [16]. All the PCR products were sequenced at Genomic lab of Axygen India pvt. Ltd, New Delhi, India. The ITS region was sequenced with primer ITS1. DNA sequences obtained from forward strands were directly submitted to GenBank (NCBI, USA) (FJ601699, FJ601698 and FJ601688).

Result

All the fungal isolates were identified as *Cylindrocladium quinqueseptatum* based on the microscopic characters as described in Mycobank. Conidia were hyaline, cylindrical, straight having 5 to 6 septa and $560\text{--}1040\text{ }\mu\text{m} \times 45\text{--}70\text{ }\mu\text{m}$ in length and diameter (Fig. 1). In old cultures more than 6 septa also found. Conidiophore were Hyaline, non septate or

Fig. 1 Five septa conidia of *C. quinqueseptatum*

may be one septa arising from lateral stipe and stipe extension terminating with a clavate shaped vesicle. Phialides are hyaline, $2.5\text{--}3\text{ }\mu\text{m}$ wide.

RAPD-PCR analysis is much simpler and faster to carry out. It is relatively an easy means to identifying fungi at the sub species level. RAPD analysis has been widely used for establishing phylogenetic relationship between isolates of fungal pathogens. Forty decamer operon primers were tested initially from which only four primers viz, OPE-2, OPE-3, OPE-5 and OPE-6 were taken for molecular variability study as these primers produced consistent and reproducible polymorphic bands with all fungal isolates. The amplified product range for OPE-2, OPE-3, OPE-5 and OPE-6 varied from 0.192 to 2.198 kb, 0.362 to 2.3 kb, 0.32 to 2.64 kb and 0.295 to 2.489 kb respectively (Fig. 2). The RAPD profile of 73 isolates grouped in 11, 9, 10 and 11 respectively for primers OPE-2, OPE-3, OPE-5 and OPE-6 respectively. Banding patterns for all the four primers was considered as monomorphic if a particular band was present in more than 90% of total isolates and polymorphic if the band was present in less than 90% of total isolates. A total number of 284 bands were scored in case of OPE-2 primer from which 72 bands were monomorphic and 212 bands were polymorphic.

Intra population genetic diversity analysis showed that a frequent diversity exists for a specific allele between the isolates. Maximum diversity was found in case of sub population A8 (0.7685 ± 0.0260) i.e. approximately 76% diversity for a particular allele. The isolates in A4 clustered together representing homogenous alleles. Intra population genetic diversity of all the sub population lines are given in Table 3.

ITS region of three isolates was sequenced and submitted to GenBank (NCBI). ITS regions including 5.8S rDNA yielded 492 characters which is similar to sequence submitted by different researchers to GenBank.

Discussion

Several workers have grouped fungal population of different regions by using RAPD analysis [16–18]. RAPD profile showed maximum population groups incase of OPE-2.

Fig. 2 Random amplified polymorphic DNA (RAPD) finger prints of isolates of *C. quinqueseptatum* amplified with primer OPE-2

Table 3 Genetic structure analysis showing intra-population genetic diversity among *C. quinqueseptatum* isolates

Population group	Gene copies	Polymorphic loci	Genetic diversity at intra population level
Population-1 (A1)	30	3	0.7241 ± 0.0421
Population-2 (A2)	18	3	0.7386 ± 0.0696
Population-3 (A3)	12	2	0.4848 ± 0.1059
Population-4 (A4)	6	0	0.0000 ± 0.0000
Population-5 (A5)	37	3	0.6907 ± 0.0538
Population-6 (A6)	39	5	0.7287 ± 0.0441
Population-7 (A7)	12	5	0.7121 ± 0.0691
Population-8 (A8)	29	11	0.7685 ± 0.0260
Population-9 (A9)	34	7	0.7451 ± 0.0533
Population-10 (A10)	66	2	0.3021 ± 0.0608
Population-11 (B)	11	5	0.5455 ± 0.0722

Clustering of isolates through OPE-2 resulted in two major population lines and one divergent outlier (Fig. 3), which were further divided into eleven sub groups and an outlier. Major population line (A) consisted of seventy isolates, whereas only two isolates were grouped in population line (B) and one isolate (FCQ-205) segregated as an outlier. The sub groups (A1 to A10) were established at a similarity index of approximately 62, 53, 62, 100, 57, 40, 38, 40, 58 and 75% respectively. The major population line (B) consisted of two isolates, FCQ-268 and FCQ-269 with a

similarity index of approximately 40%. Group 12 consisted only of one isolate (FCQ-205).

From the similarity matrix based on simple matching coefficient, the similarity between the clusters ranging from 62 to 100%, 53 to 100%, 62 to 100%, 100%, 57 to 100%, 40 to 100%, 38%, 40 to 77%, 58 to 100% and 75 to 100% respectively for first 10 clusters. Similarity between isolates of cluster number 11 i.e. population line (B) was 40%.

The four primers used in the present study produced specific patterns that could differentiate between *C.*

Fig. 3 Dendrogram of 73 isolates of *C. quinqueseptatum* amplified by OPE 2 showing two major population lines

quinqueseptatum isolates according to their geographical origin. In OPE 2, 85% isolates clustered in accordance with their geographical location, however, 15% isolates clustered irrespective of their geographical location. The isolates clustering in the subpopulation line A7, A8, A9 and A10 were from the same geographical origin (Uttarakhand). However two isolates viz., FCQ-306 and FCQ-106 appearing in the subpopulation line A7 and A8 respectively

were from Orissa and Uttar Pradesh. The Uttar Pradesh isolate was from the location adjoining Uttarakhand hence the clustering is as per the geographical location. In population line A6 all the isolates of Punjab state clustered together. However, three isolates of Haryana state viz., FCQ-136, FCQ-138 and FCQ-145 clustered in subpopulation A6 with isolates of Punjab as these two are neighboring states. It might be possible that air borne fungal

spores would have disseminating across the neighboring state boundaries. Sub population lines A4 and A5 consisted of isolates from Uttarakhand except FCQ-238. In case of subpopulation line A1 and A2, different isolates of three states viz. Haryana, Uttarakhand and Punjab clustered close to each other. Two isolates from high altitude region of Uttarakhand viz. FCQ-268 and FCQ-269 clustered together in major population line B. High intra-population variation was observed after the genetic structure analysis of all the major population lines and sub groups (Table 3). Maximum intrapopulation genetic variability within the isolates was observed in subpopulation A8 (0.7685 ± 0.0260) consisting of isolates from Uttarakhand and Uttar Pradesh. Minimum intrapopulation genetic variability was found in A4 consisting of isolates from Uttarakhand. Since these states are neighbors there is a high likelihood of introduction or sharing and dissemination of pathogen isolates as there is continuous flow of planting material and propagules between these states apart from air mediated conidial transmission. The clusters having isolates from all these three states or any of the two could be on account of the exchange of diseased propagules, planting stocks or industrial raw materials. In order to cater the raw material and planting stock requirements the farmers in the neighboring state act as an important supply link often disseminating the pathogen strains. Apart from this reason the high genetic variability in A8 could be because many parts of Uttar Pradesh are separated from Uttarakhand by mountain ranges and though some of the genetic material could have been exchanged but due to climatic variation and geographical barriers, different evolutionary directions taken by the pathogen isolates could have caused high variability. The low genetic variability among the isolates of A4 was found as they had been collected from the same valley in Uttarakhand.

The molecular variability can be of immense help in creating a repository of the population line representatives which can be utilized for identifying CSLB resistant *Eucalyptus* germplasm.

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