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Morphological and Molecular characterization of *Bipolaris oryzae* isolates in Ariyalur district of Tamil Nadu, India

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ABSTRACT
The present studies were undertaken to investigate the morphological and molecular characterization of Bipolaris oryzae native isolates of major rice cultivating places in Ariyalur district of Tamil Nadu, India. The brown spot pathogenic B. oryzae isolates were collected from Thirumalapadi, Ammakulam, Elakuruchi, Poyyur and Kallur in Ariyalur district. All the brown spot pathogenic isolates mentioned olivaceous, branched hyphae with brown to black coloured septate mycelium. B. oryzae conidial colour was brown to brownish white colour. Also, the number of conidial septations was ranged from four to six and maximum septation was observed in Ammakulam isolate. The BLAST search results illustrated that the ITS region sequence of all the brown spot pathogen isolates matched to B. oryzae with high homology of 97%.
Key words: *brown spot, rice, morphology, molecular characterization*

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INTRODUCTION

Rice crop has been affected by many diseases incited by fungi, bacteria, viruses, *Candidatus* Phytoplasma and nematodes. Among the various mycotic infections in rice, brown spot pathogenic agent *Bipolaris oryzae* (Breda de Haan) Shoemaker (Syn: *Helminthosporium oryzae* (Breda de Haan) Subram. and Jain) (Teleomorphic stage is *Cochliobolus miyabeanus* (Ito and Kuribayashi) Drechs. ex Dastur) is found to occur in almost all rice cultivating areas [1]. The disease is also mentioned as sesame leaf spot, fungal blight, Helminthosporium blight, Glume blotch, Sesame leaf blight, seedling blight and Helminthosporiose. The present studies were undertaken to investigate the morphological and molecular characterization of *Bipolaris oryzae* native isolates of major rice cultivating places in Ariyalur district of Tamil Nadu, India.

MATERIAL AND METHODS

Isolation and identification of brown spot pathogen *B. oryzae* (*in vitro*)

Isolation and maintenance of *B. oryzae* culture

The brown spot inciting agent *B. oryzae* was isolated from disease infected leaves of rice collected from Ariyalur district of Tamil Nadu which showed the maximum PDI by the tissue segment method [8]. The infected leaf areas were cut into minor bits, surface sterilization with 0.1 % $HgCl_2$ solution for sixty seconds. Then, the samples washed with sterile dist. water and placed on Rice Polish Agar Medium and incubated at room temp. conditions for a week. *B. oryzae* was further purified by single spore isolation method [2] and the auxenic culture was maintained on PDA slants for subsequent *in vitro* experiments. Microbial cultures were observed periodically for pathogenicity test of *B. oryzae* isolates. The pathogenic *B. oryzae* isolates were collected from Thirumalapadi, Ammakulam, Elakuruchi, Poyyur and Kallur in major rice growing areas in Ariyalur district of Tamil Nadu, India.

Morpho-pathological characteristics of *B. oryzae* isolates

15 ml of sterilized PDA medium was placed into the Petri plate and allowed to solidification. An individual isolate of nine mm *B. oryzae* pathogenic culture disc was aseptically placed at the center of the Petri plate and maintained at room temp. for a week. After that, the fungal growth, mycelium, colour and sporulation characters were observed. Micrometric measurements of *B. oryzae* spore was done after temporary

mounting medium (i.e., lactophenol cotton blue) and observed under the microscope by using ocular and stage micrometers.

Molecular characterization of *B. oryzae* isolates

Isolation of total genomic DNA

Cetyl Trimethyl Ammonium Bromide (CTAB) method was adopted to extract the total genomic DNA from the mycelial mat of all the isolates of *B. oryzae* (Doyle and Doyle 1987)**.** Actively growing mycelial disc (9mm) of each isolate was transferred into 250 ml conical flasks containing 150 ml PDA broth and the cultures were grown at room temperature (27 ± 1 °C) for 15 days. Mycelial mat was blot dried and utilized for the DNA extraction after the removal of their respective culture filtrates. Mycelium (1.0 g) was ground to fine powder in a pestle and mortar using liquid nitrogen. Powdered mycelia were mixed in pre-warmed (65°C) extraction buffer (500µl) consisting of 100 mM Tris (pH 8.5), 250 mmol l^{-1} NaCl, 0.5 mmol l^{-l} EDTA and 0.5% SDS. Half of their content was transferred to another eppendorf tube and added with 750 µl of chloroform: isoamyl alcohol (24:1) then the centrifugation was done at 10,000 rpm for 10 minutes. Thereafter the supernatant (300 µl) was carefully taken out without any admixture and transferred to another tube with the addition of 150 µl of 5M NaCl, ice cold ethanol, mixed gently and incubated at 20°C (overnight) and the content was centrifuged at 4°C for 10 minutes at 13,000 rpm (RNase (15 µl) was added to the supernatant).

The supernatant was transferred to another tube and add 510 µl of isopropanol for DNA precipitation and centrifuged at 10,000 rpm for 5 minutes. The pellet was air dried after the supernatant was expelled out and rinsed the pellet with 70 per cent ethyl alcohol twice to remove the impurities. DNA pellet was re-suspended with milliQ water. The final concentration of DNA in the extract was tested for the amount of DNA by 0.8 per cent agarose gel electrophoresis. Finally, the purified DNA was stored at –20 °C for subsequent studies.

PCR amplification of ITS gene region

ITS1 - 5′ TCCGTAGGTGAACCTGCGG 3′ (Forward primer)

ITS4 - 5′ TCCTCCGCTTATTGATATGC 3′ (Reverse primer)

ITS 1 and ITS 4 primers were used for the amplification of intermediate 5.8S ribosomal gene along with ITS1 and ITS2 region (White *et al*.,1990). PCR reaction mixture volume was made up to 20 µl by the addition of genomic DNA-2 µl, 10 µl of dNTP's (dATP, dGTP, dCTP and dTTP), 1 µl of forward primer, 1 µl of reverse primer, 1 µl of Taq DNA polymerase and 5 µl of sterile water. Samples were transferred quickly in Eppendorf mastercycler gradient PCR machine which was already programmed for 94°C for 5 mins (preheating) followed by 35 cycles (denaturation- 94 °C for 1 minutes, annealing at 58 °C for 1 minutes, extension- 72 °C for 1 minute and final extension at 72°C for 10 minutes. The resultant PCR products were analyzed using 1.2 per cent agarose gel electrophoresis. Required amount of agarose was weighed and melted in 1x TAE buffer. Ethidium bromide (1 µl) was added and cooled. The mixture was poured into a preset template with 6x loading buffer. Bromophenol blue (0.25 %) was added into the mixture and comb was fitted in the template to make wells then loaded the mixture in the well. Electrophoresis was carried out at 80V. The result was visually observed under UV light and documented through Alpha imager [3].

Sequencing of ITS and identification of *B. oryzae* by bioinformatics analysis.

The acquired DNA sequences were trimmed at 5' and 3' region wherever the sequence chromatogram was not clear. After trimming of the DNA sequence, Fasta format was used for obtaining the clear chromatogram which was used as a query sequence (Input sequence) in the nucleotide BLAST analysis program at NCBI database. The output information retrieved from the bioinformatics was analyzed and the organism with major score was considered as closely related species to the test fungus used in this study. The sequence was submitted and got published in the NCBI domain.

Phylogenetic diversity analysis

The samples were subjected to dendrogram analysis to study the divergence patterns and their evolutionary distances were computed using the Maximum Composite Likelihood method. Evolutionary studies were performed using MEGA X. (Molecular Evolutionary Genetic Analysis) software and bootstrap analysis using 100 replicates. The identified *B. oryzae* isolates were mentioned as $AUBo_1$ (Thirumalapadi), $AUBo_2$ (Ammakulam), $AUBo_3$ (Elakuruchi), $AUBo_4$ (Poyyur) and $AUBo_5$ (Kallur).

RESULTS

Morphological characters of *B. oryzae* isolates

In the present experiment, the villages from major rice cultivating areas of Ariyalur District which showed the maximum Percent Disease Index (PDI) were chosen for observing the morphological characters of *B. oryzae*. Hence, five isolates of *B. oryzae* were identified based on morphological characters and they were designated as $AUBo_1$, $AUBo_2$, $AUBo_3$, $AUBo_4$ and $AUBo_5$ (Table 1). The

mycelial characters of the *B. oryzae* isolates (AUBo1, AUBo2, AUBo3, AUBo4 and AUBo5) were observed visually and the morphological characters are presented in table 1. All the brown spot pathogenic isolates mentioned olivaceous, branched hyphae with brown to black coloured septate mycelium. *B. oryzae* conidial colour was brown to brownish white colour. When they were fully matured, the colour was changed into dark brown. All conidia are sightly curved with a bulge in the central portion and tapering towards the polar region with bipolar germination.
Among the isolates, the conidial size significantly varied from 31.0 to 33.6 μm length and 13.6 to 14.5 μm width. The maximum conidial length was observed in AUBo3 isolate followed by AUBo2, AUBo4, AUBo5 and AUBo1 isolates in the decreasing order of merit. Whereas, the maximum conidial width was observed in AUBo3 isolate followed by AUBo4, AUBo1, AUBo2 and AUBo5 isolates in the decreasing order of merit. Also, the number of conidial septations was ranged from four to six and maximum septation was observed in AUBo2 isolate.

Molecular identification of the brown spot pathogen *B. oryzae*

Isolation of genomic DNA

Genomic DNA isolated from the auxenic culture of all the effective five isolates through CTAB method and molecularly characterized by ITS primers ITS 1 and ITS 4 (Fungal universal primers). Single band of intact high molecular weight DNA was visualised through the agarose gel electrophoresis. The size of the PCR fragments was approximately amplified at 550bp (Fig. 1.)

Sequencing analysis of ITS region

ITS region of all the isolates acquired by thermocycler was cleaned with PCR clean up kit to exclude the residual primers, polymerase and salts in the PCR product. Cleaned up product was sequenced. The sequence was used for homology search in NCBI Blast program. The BLAST search results illustrated that the ITS region sequence of all the brown spot pathogen isolates matched to *B. oryzae* with high homology of 97%.

Phylogenetic analysis of *Bipolaris*

The evolutionary history was inferred using the Neighbour-Joining method. The optimal tree with the sum of branch length = 1.14960606 was shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) were showed next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 16 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated (complete deletion option). There was a total of 362 positions in the final dataset.
The nucleotide sequence of the study isolates were compared with sequence of the isolates of *B. oryzae* pathogen retrieved from the NCBI database through phylogenetic analysis using MEGA X software. The phylogenetic analysis-based nucleotide sequence of the ITS region indicated that all the five *B. oryzae* isolates were clustered into two groups. Among the study isolates, the isolates AUBo1, AUBo2 and AUBo4 were clustered under same clade with 63 % similarity whereas, the isolates AUBo3 and AUBo5 clustered in the same group with 63 % similarity. Based on the observations of nucleotide sequences and their evolutionary distance among the species, it was apparent that *B. oryzae* study isolates (AUBo1, AUBo2, AUBo3, AUBo4 and AUBo5) (Table 2) had minimum evolutionary distance with closely related species and shared a common ancestor, pointed out that the present isolates belonging to the same genotype of *Bipolaris* sp.

DISCUSSION

Morphological characters of *B. oryzae*

The fungal colony characters of all *B. oryzae* isolates exhibited olivaceous brown, light to dark brown or black in colour, presence of cross walls in hyphae, branched mycelium and abundant aerial/submerged mycelium. *B. oryzae* conidial colour was always dark brown to light brown or brownish white colour. The fungal culture characters of isolates similar to *Bipolaris* and the special morphological character, conidial colour and septations are similar to *B. oryzae* [11]. Sakamoto [9] mentioned that brown spot pathogen had branched hyphae, septate mycelium and conidia. A similar report was made in the present study with reference to the mycelium and conidia of AUBO3 isolate. The isolates of *B. oryzae* were divided into several groups based on spore morphology and pathogenic culture characters [13].
In the present research, the size of *B. oryzae* conidia significantly varied among the various isolates from 31.0 to 33.6 μm length and 13.6 to 14.5 μm width with cross walls in the conidia ranging from four to six. Similar to the present reports, the variation in conidial morphology of an individual

isolates were reported by earlier mycologists [5, 7]. These earlier *B. oryzae* conidial morphological reports lend support to the present results.

Molecular confirmation of *B. oryzae* isolates

Molecular characterization and validation play a major role in the detection of an inciting agent upto the species and sub species level. The partial sequence of *B. oryzae* pathogenic isolates *viz.,* AUBo$_1$ to AUBo$_5$ were confirmed as *Bipolaris oryzae* through BLAST search in the website of NCBI (https://blast.ncbi.nlm.nih.gov/). Molecular grouping and their variability of twenty-seven *B. oryzae* isolates from India were analysed by Singh *et al.* [10]. Similarly, Kumar *et al.* [6] mentioned that the molecular diversity of 116 *B. oryzae* isolates were reported from major rice growing areas of India. Those earlier *B. oryzae* sequence analytical reports lend support to the present findings.

Table 1. Morphological characters of *B. oryzae* isolates of selected villages

Sl. No	Characters	Thirumalapadi	Ammakulam	Elakuruchi	Poyyur	Kallur
1.	Isolates name	AUBo$_1$	AUBo$_2$	AUBo$_3$	AUBo$_4$	AUBo$_5$
2.	Colony characters	Olivaceous brown, aerial mycelium, fine texture, many branches with septate mycelium	Dull brown, aerial mycelium, branched with septate hyphae	Fluffy mycelium, Olivaceous brown, profuse, septate mycelium, fusoid or obclavate / canoe shaped conidia	pale olive in maturity level, septate mycelium	Dark brown, profuse aerial hyphae, branched with septate mycelium
3.	Conidia – Length (μm)	31.0	33.2	33.6	32.4	31.8
4.	Conidia – Width (μm)	14.0	13.8	14.5	14.2	13.6
5.	Colour of the conidium	Brown	Dull brown	Brown	Brownish white	Dark brown
6.	No. of septations per conidium	4	6	4	5	4
7.	NCBI Accession Number	ON845213	ON845214	ON601149	ON845215	ON845216

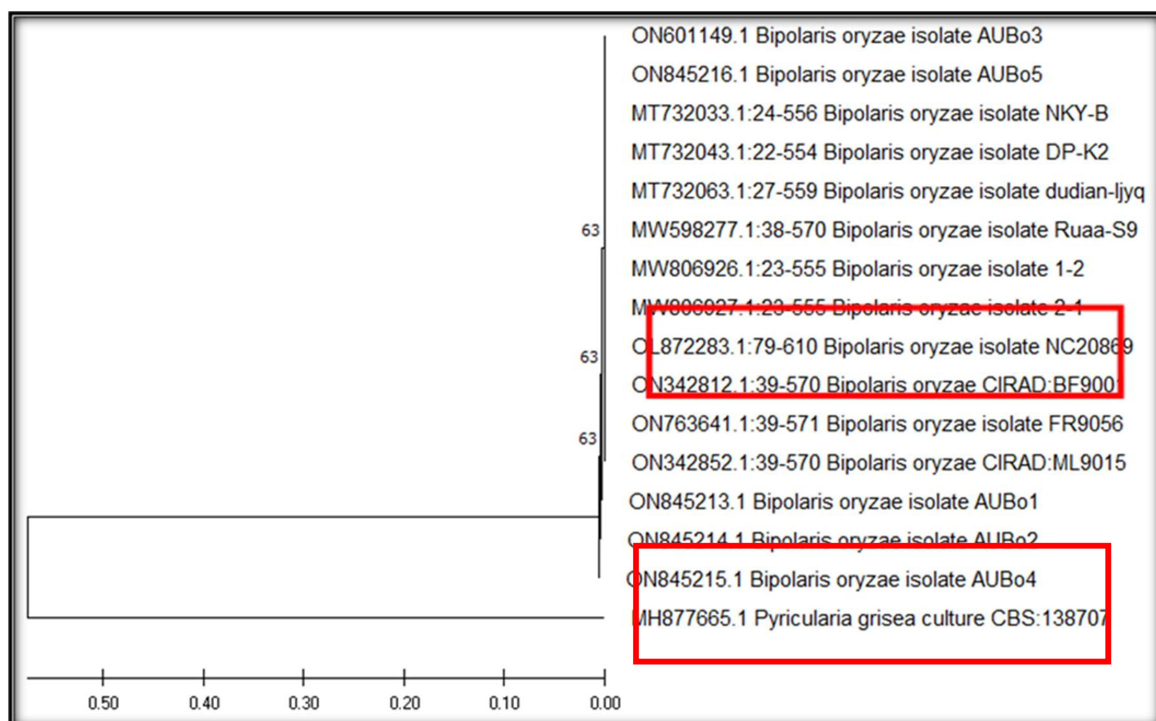

Fig. 1. Dendrogram analysis of *B. oryzae* isolates

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