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First Report of *Pythium rostratum* from Soil Sample JMBL-4 in Gorakhpur Uttar Pradesh, India: Morphology Taxonomy DNA Barcoding and Phylogenetic Analysis

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Abstract

Isolate JMBL-4 of Oomycete was taken from soil samples in "Jungle Dhushad" of Gorakhpur, Uttar Pradesh, India. The oomycete grows well on hemp seed halves as well as on solid media like PCA, CMA and PDA producing a chrysanthemum pattern and colony shows rosette growth of medium density. No development of aerial mycelium. The growth rate of the oomycete on soild media is 7mm per day at about 25 degree centigrade on PCA. Sporangia terminal or occasionally intercalary, globose, and sub spherical(25micrometer diameter). Oogonia are intercalary, smooth, and subglobose. Antheridia monoclinous 1-2 per oogonium originating immediately below oogonium base. Oospore is plerotic with thick wall(1-2micrometer). These characters of the oomycete isolate belongs to *Pythium rostratum.* Further molecular analysis through Agarose gel electrophoresis of the purified DNA of JMBL-4 showed approximate molecular weight of 40 kb. The cytochrome oxidase II (COX II) gene of JMBL-4 was PCR amplified and the amplicon was approximately 600 bp. The amplified product of PCR amplicons with COX II primers were subjected to BLASTn. By this, amplified product (Query Sequence) shown 99% identity with Pythium species. The BLAST analysis shows 99% identity maximum score of 1048 with 97% query coverage for the species *Pythium rostratum* EU265667.1. Then we did sequence homology with the wild type *Pythium rostratum* COXII primer with our amplified product (Query sequence) through T-COFFEE algorithm alignment. The alignment shows very good score 98% .This region shows 99% consensus region with *Pythium rostratum* therefore, the fungal isolate belong to the Pythium species. The strong homology reveals the isolate belong to *Pythium rostratum*, Butler. The amplified COX II of our isolate JMBL-4 was also subjected to MSA through CLCbio Main Workbench while constructing the phylogenetic tree by using NEJ method. According to the tree, our sequence has showed close relation with *Pythium rostratum* with different isolate sequences (accessions) that are aligned. Further studied MSA using NEJ method with different other species COX II and it was found our Query sequence JMBL-4 identity with *Pythium longisporangium* and *Pythium selbyi* followed by *Pythium segnitium* , *Pythium hypogynum, Pythium schmitthenneri, Pythium acrogynum, Pythium pulchrum, Pythium middletonii* and *Pythium multisporum.* The results of phylogenetic analysis using COX II indicated *Pythium rostratum* is very close to *Pythium longisporangium* and *Pythium selbyi.*

Keywords: *Pythium;* morphology; taxonomy; DNA barcoding; phylogeny; India

1. INTRODUCTION

The Oomycetes are microscopic Stremenopiles. Oomycetes are also known as "Water molds". Many years ago, the Oomycetes were defined as "Aquatic phycomycetes". In the latest edition of the classification of fungi, Oomycetes are defined as – *"A class within the kindom Chromista*". The Oomycetes *Pythium* and *Phytophthora* are two of the Fungi denote the highest eukaryotic diversity on earth. Approximately 1.5 million fungi species exist in our environment[1]. Regardless of the fact that fungi are important sources of both bioactive compounds and mycotoxins and that they are very ubiquitous in our environment, their species identification is hampered by incomplete and often unclear literature . Fungi identification has been primarily based on their phenotypic and physiological characteristics. However, the unique characteristics of fungi create difficulties in morphology – based identification and classification. Thus, only well- trained experts are able to correct identify fungi species solely based on fungal morphology and harmful

genera of plant pathogens worldwide. They are known to cause seed rot, root rot, seedling, damping off, rots of lower stems, tubers and soft rots of fleshy fruits in contact with the soil. Most of the Oomycetes are important sources of both bioactive compounds and mycotoxins. So, that they are very ubiquitous in our environments. Oomycetes identification has been primarily based on their phenotypic and physiological characteristics**.** Schoch et al. [2] observed DNA phylogenetic comparisons have shown that morphology-based species recognition often underestimates fungal diversity. Therefore, the need for accurate DNA sequence data, tied to both correct taxonomic names and clearly annotated specimen data, has never been greater. Furthermore, the growing number of molecular ecology and micro-biome projects using high-throughput sequencing require fast and effective methods for en masse species assignments. The present study therefore, is aimed to isolate and purify Oomycetes belonging to the stramenopiles of order peronosporales. This method is free from harmful chemical pesticides and eco-friendly approach to identify the species and further its Barcoding and phylogenetic position so as to its use in biological diversity. It's impact in our opinion, has never been studied in Eastern U.P. in particular and in India in general.

2. MATERIALS AND METHODS

2.1 Soil samples

Samples of brown decaying twigs, leaves and woods of the local dominant vegetation will be collected from Gorakhpur, India and will be brought to the laboratory in separate sterile polyethylene bags. Oomycetes will be isolated from these samples by the usual baiting techniques

2.2 Morphological characterization in water and PCA cultures

Water culture of the JMBL-4 fungal isolate was prepared for microscopic morphological study and identification(Fig.1-3).

2.3 Growth rate measurements

The JMBL-4 fungal strain was inoculated on PCA, incubated at 25^0C and growth measurements in mm was recorded every 24 hours until the isolate covered the entire plate.

2.4 Maintenance of culture

The purified cultures was inoculated in 15ml culture tubes containing PCB liquid broth which was placed on rotatory shaker incubated at 25ºC. The growth of pure mycelia JMBL–4 was recorded at the interval of 24 hr. for 96hr. After, 72-96 hours of growth, the culture media was transferred at 4ºC in refrigerator. Mycelial biomass (of JMBL-4) formed after three to five days of inoculation was collected on a muslin cloth by discarding the liquid media. Biomass was washed 5-6 times with deionised water carefully and mycelia was air dried and same was used for further characterization.

2.5 Extraction of Fungal Genomic DNA

0.1g of dried JMBL-4 mycelium was ground in liquid nitrogen into a fine powder in a pre-chilled mortar and pestle. And extracted with 600µl of extraction buffer and crushed it well using tissue grinder. Then again extracted with 50µl of 10% SDS and incubated at 65˚C for 30 minutes. After incubation added 800µl of Phenol: chloroform:Isoamylalchol (25:24:1) and centrifuged at 10000rpm for 15 minutes. The upper aqueous layer was transferred into fresh eppendorf tubes and added 10µl RNAs and incubated it 37˚C for 30 min. Then added equal volume of isopropanol and again incubated at -20˚C for 20 min. After incubation centrifuged at 10000rpm for 20min. Discarded the supernatant and added 500µl of 70% ethanol, vortexed and again centrifuged at 10000rpm for 2 min. Finally added 200µl of pre warmed nuclease free water to dissolved the pellet.

2.6 Characterization of JMBL-4

2.6.1 PCR Amplification

The JMBL-4 isolate was characterized by morphological as well as molecular biology tools. For their identification by molecular tools, DNA was extracted by our own protocol but basic tenets prescribed by Saghai-Maroof et.al.[3] followed by PCR amplification of the ITS region of ribosomal DNA and COX II gene for oomycetes. The amplicons were sequenced by Pyrosequenceanalyzer and the resultant sequence were subjected to BLASTn, T-COFFEE and CLC Biomain Workbench analyses for identify the specific identification of oomycete.

2.7 Pyrosequencing

Apical Scientific Sequencing Division Bhd , Malaysia has used ABI PRISM 3730xl Genetic Analyzer developed by Applied Biosystems, USA, for sequencing the amplions..

2.8 Phylogenetic tree

A phylogenetic tree or evolutionary tree is a branching diagram or " tree" showing the inferred evolutionary relationships among various biological species or other entities based upon similarities and differences in their physical and /or genetic characteristics .the taxa joined together in the tree are implied to have descended represents the inferred most recent common ancestor.in a rootedphylogenetic tree, each node with descendants represents the inferred most recent common ancestor of the descendants, and the edge lengths in some trees may be interpreted as time estimates. Each node is called a taxonomic unit. Internal nodes are generally called hypothetical taxonomic unit (HTUs)as they cannot be directly observed. trees are useful in field of biuologyun such as bioinformatics, systematics and comparative phylogenetics

3. RESULTS AND DISCUSSION

The oomycete grows well on hemp seed halves as well as on solid media like PCA, CMA and PDA producing a chrysanthemum pattern and colony shows rosette growth of medium density. No development of aerial mycelium. The growth rate of the oomycete on soild media is 7mm per day at about 25 degree centigrade on PCA. Sporangia terminal or occasionally intercalary, globose, and sub spherical(25micrometer diameter). Oogonia are intercalary, smooth, and subglobose. Antheridia monoclinous 1-2 per oogonium originating immediately below oogonium base.

Oospore is plerotic with thick wall(1-2micrometer). These characters of the oomycete isolate belongs to *Pythium rostratum* Butler.

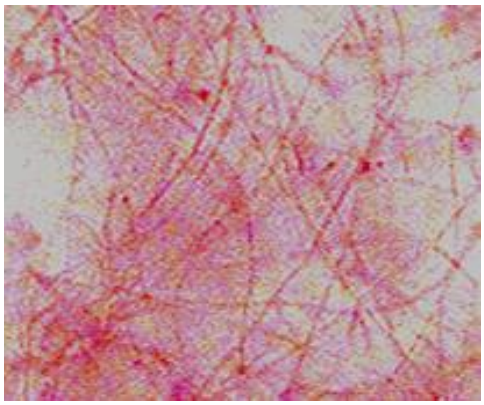

Fig.1: Aerial mycelium on PCA

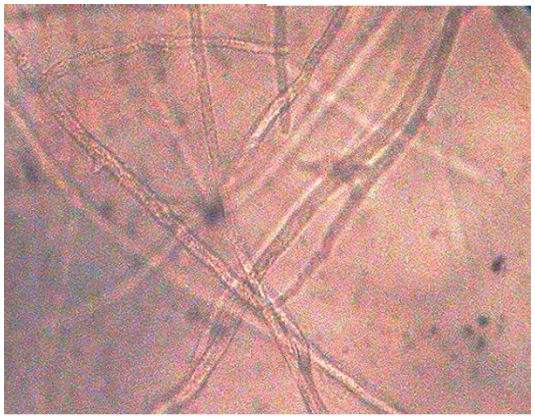

Fig.2: Vegetative hyaline mycelium

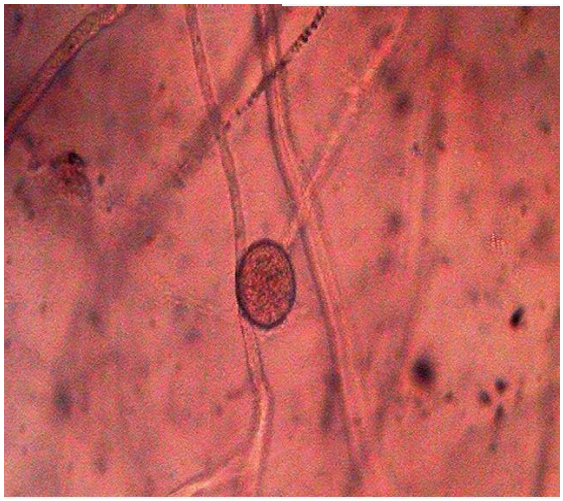

Fig.3: Mature plerotic Oospore

3.1 Molecular characterization

3.1.1 Genomic DNA extraction

Prior to extraction, pure isolate of JMBL-4 was reactivated by sub-culturing growth medium and inoculated at 25 ^{0}C for 3-7 days massive production of mycelia. DNA was extracted from resulted mycelia JMBL -4 using our own protocol developed but following the basic tenets of Saghai-Maroof et.al.[3]. Agarose gel electrophoretic analysis of the purified DNA of JMBL-4 showed approximate molecular weight of 40 kb.

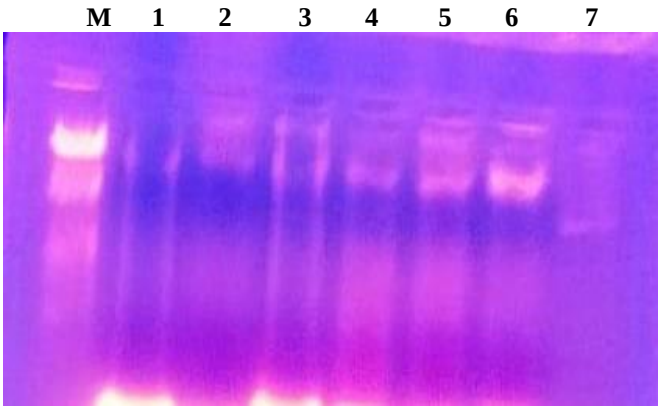

Fig.4: Agarose gel electrophoresis of purified genomic DNA of JMBL-4. M, 50-bp DNA Marker; Lane 4 -, Isolate JMBL-4.

3.2 PCR amplification

The cytochrome oxidase II (COX II) gene of JMBL-4 was PCR amplified using forward FM 58 (5′-CCACAAATTTCACTACATTGA-3′) and Reverse FM 66 (5′-TAGGATTTCAAGATCCTGC 3′) [4]. The amplified COX II gene of JMBL-4 was approximately 600 bp. It is in consonance with the results of Martin [5], Hudspeth et.al.,[6],Villa et.al.,[4].

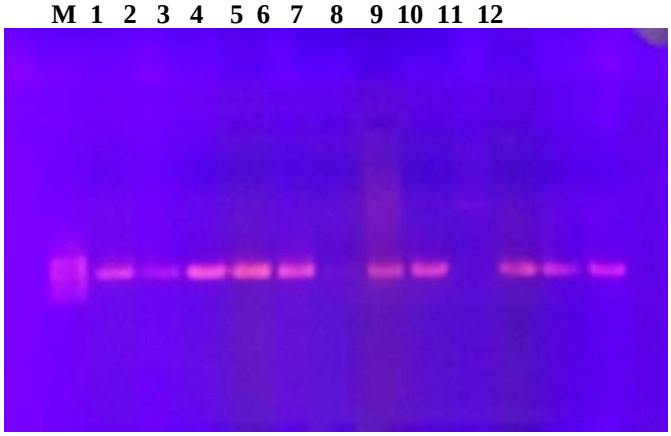

Fig.5: Band pattern of PCR amplified COX II region
M 1 Kb DNA ladder Marker; Lane 4 -, Isolate JMBL-4

3.3 Pyrosequencing

The PCR products from the gel were cut and was submitted to the Apical Scientific Sequencing Division Bhd., Malaysia for sequencing using Applied Biosystems (as mentioned under the pyrosequencing of materials and methods) and the same Primers use it for amplification.

3.4 Analysis of Sequences

The amplified product of PCR amplicons with COX II primers were subjected to BLASTn. By this, amplified product (Query Sequence) shown 99% identity with Pythium species. The BLAST analysis shows 99% identity maximum score of 1048 with 97% query coverage for the species *Pythium rostratum* EU265667.1. Then we did sequence

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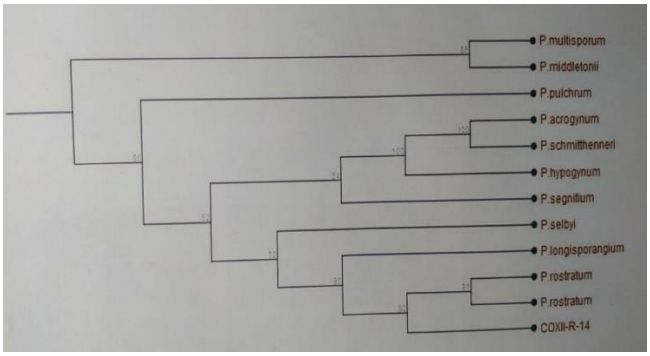

Fig.6: Phylogenetic tree

3.6 Phylogenetic tree

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4. REFERENCES

1. Hibbett DS, Ohman A, Glotzer D, et al. Progress in molecular and morphological taxon discovery in Fungi and options for formal classification of environmental sequences. Fungal Biol. Rev.,2011; 25: 38-47.
2. Schoch, Conrad, Robbertse, Barbara, Robert, Vincent, Vu, Thuy, Cardinali, Gianluigi, Irinyi, Laszlo, Meyer, Wieland, Nilsson, Rolf Henrik, Hughes, Karen, Miller, Andrew, Kirk, Paul, Abarenkov, Kessy, Aime, Mary, Aime, Ariyawansa, Hiran, Bidartondo, Martin, Boekhout, Teun, Buyck, Bart, Cai, Qing, Federhen, Scott. Finding needles in haystacks: Linking scientific names, reference specimens and molecular data for Fungi. Database The Journal of Biological Databases and Curation, 2014; 10.1093/database/bau061.
3. Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard RW. Ribosomal DNA spacer length polymorphism in barley: Mendelian inheritance, chromosomal location and population dynamics. Proc. Natl. Acad. Sci. USA, 1984; 81: 8014-8018.
4. Villa NO, Kagiyama K, Asano T, Suga H. Phylogenetic relationships of Pythium and Phytophthora species based on ITS rDNA, cytochrome oxidase II and beta-tubulin gene sequences. Mycologia, 2006; 98:410-422.
5. Martin FN. Phylogenetic relationships among some *Pythium*species inferred from sequence analysis of the mitochondrially encoded cytochrome oxidase II gene. *Mycologia*, 2000; 92:711–727 .
6. Hudspeth DSS, Nadler SA., Hudspeth MES. A COX2 molecular phylogeny of the Peronosporomycetes. *Mycologia*, 2000; 92:674-684.