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Research article

Molecular identification based on the sequence of internal transcribed spacer (ITS) of the ribosomal nuclear DNA (rDNA) of pathogenic fungus *Pythium aphanidermatum* (Edson) Fitzp. isolated from soil and its morphology

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Abstract: Soil-borne fungi *Pythium aphanidermatum* causes damping-off of cucumber, coriander and other economically important plants in India and presumably in many other countries. The objective of the present investigation was to characterize the pathogen morphologically *in vitro* and to confirm its molecular identity by the sequence of ITS region of rDNA. *P. aphanidermatum* was isolated from soil and cultured in PDB broth and PDA solid media. The characteristic of growth was monitored and the reproductive structures were analyzed. Hyphae were grown with a daily increment of 2.5-3.0 cm on PDB. Sporangia were swollen, multinucleate and usually measure 10-50 µm in diameter. Oogonia were found mostly terminal, spherical, 22-27 µm in diameter. Fungus was also characterized using molecular methods based on ITS-PCR. The amplified sequence was compared with the available sequences in the NCBI GenBank. The sequence showed 99% similarity with other species of *Pythium*. Based on the morphological and molecular characters isolate was confirmed as *Pythium aphanidermatum*. The isolation, morphological characterization and sequencing of ITS region of rDNA will add knowledge to the scientific community for proper identification and in-depth research on the management of this plant pathogen.

Keywords: ITS sequence - Morphological characteristics - Oomycete - Plant pathogen - *Pythium aphanidermatum*.

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INTRODUCTION

The fungus *Pythium* is a facultative parasite and lives saprophytically on the moist humus in soil and attacks seedlings at the soil level. It causes damping-off, soft-rot, crown-rot, wheat-rot or foot-rot diseases and sometimes causing death (van der Plaats-Niterink 1981, El-Tarabily *et al.* 2009). One of the species of *Pythium*, *P. aphanidermatum* (Edson) Fitzp. causes extensive damage to economically important crops worldwide (Martin & Loper 1999). It causes considerable damage to cucumber crops worldwide by damping-off disease of seedlings and root and Crown-rots of mature plants (Zitter *et al.* 1996, Chen *et al.* 2000, Al-Sa'di *et al.* 2007). It is also a causal pathogen of damping-off disease of coriander in India (Ashwathi *et al.* 2017). Several studies have also been carried out in India. Muthukumar (2016) isolated twelve different species of *Pythium* from different soil types, water types, vegetables and ornamental plants of Delhi.

The genus *Pythium* was first created by Pringsheim in 1858. Taxonomic details of the genus had been described by the end of the 19th century and many new species had been added. Furthermore, the genus was included in a new family, Pythiaceae, by Schroter in 1897 under the kingdom of Stramenopila, subdivision of Mastigomycotina, class of Oomycetes and order of Peronosporales (Domsch *et al.* 1980). Afterwards, there are almost 307 species of *Pythium* were morphologically and taxonomically described by different scientist and

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submitted in www.mycobank.org (Middleton 1943, Waterhouse 1968, Plaats-Niterink 1981). An account of this genus in India was given by Butler (1907).

Rapid and safe identification is crucial to implement proper diagnosis and effective treatment to the diseased plants (Schurko *et al.* 2003). Though the identification and classification of *Pythium* species are based on morphological and physiological characteristics; but taxonomists faced problems due to a lack of sexual structures and failure to induce *in vitro* zoosporogenesis (Schurko *et al.* 2003). Thus the use of molecular technologies has become an indispensable tool for accurate identification of this pathogen (Grooters & Gee 2002, Schurko *et al.* 2003, Pannanusorn *et al.* 2007, Ashwathi *et al.* 2017). The polymerase chain reaction (PCR) for the amplification of the ribosomal genes are used for the genetic identification of many organisms because they comprise both highly conserved sequences during evolution.

In the present study, we have isolated the fungal pathogen *Pythium aphanidermatum*. We have identified the pathogen morphologically based on its reproductive structures. We have also obtained the sequence of the internal transcribed spacer (ITS) of the ribosomal nuclear DNA (rDNA) with the PCR method using universal primers (Chen *et al.* 1992) for the molecular identification and deposited the sequence in Genbank (NCBI).

MATERIALS AND METHODS

Collection and isolation of the fungus

The Fungus was isolated from Lucknow (26º 50′ N latitude, 80º 56′ E longitude, 128 m above the sea level), the capital of Uttar Pradesh, is spread over an area of 310 Km^2 in the central plain of the Indian subcontinent. The sample was collected from soil samples together with plant root debris in sterile capped bottles. The fungus was isolated from these samples by the usual baiting techniques (Middleton 1943, Paul *et al.* 1998). The collected sample was purified in sterile distilled water and maintained on solid media like PDA (potato dextrose agar) at 25ºC (Booth 1971). The isolate was identified with the help of keys provided by Middleton (1943), Waterhouse (1967), Plaats-Niterink (1981) and also by the sequence obtained from ITS-PCR.

DNA isolation and PCR

The culture conditions, DNA isolation and the PCR amplification of the ITS of the rDNA were the same as described earlier (Paul *et al.* 1999, Paul 2000). The fungus was grown in potato dextrose broth medium, incubated at 25ºC on a rotary shaker for 5 days. The mycelium was then washed in TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA) and was kept at 320ºC for 24 h and DNA was extracted. Polymerase chain reactions were performed in 50 µl volumes containing 100 pmol of each of the universal primers ITS1 (TCC GTA GGT GAA CCT GCG G) and ITS4 (TCC TCC GCT TAT TGA TAT GC); 200 µM of each of the four dNTPs; 1.5 U of Taq polymerase (Invitrogen) and 300 ng DNA template in a PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl). The temperature cycling parameters were 95°C for 3 min for denaturation the first cycle and 1 min for subsequent cycles, primer annealing for 1 min at 55°C and primer extension at 72°C for 1 min with a total of 35 cycles and a final extension at 72°C for 3 min 25.

Sequencing and phylogenetic analysis

The PCR product was purified and sequenced (Chromous Biotech Pvt. Ltd., Bangalore). The sequence was compiled by ApE software (A plasmid Editor). The rDNA sequence was submitted to NCBI gene bank. The sequence was also blasted (NCBI) to determine the percentage of similarity with related sequences. The BioEdit sequence alignment editor was used to obtain multiple alignments of nucleotides with related sequences for ITS. The blast output was processed for generating the phylogenetic tree in software MEGA7 by using 'Maximum Likelihood' method based on the Tamura-Nei model (Tamura & Nei 1993, Kumar *et al.* 2016). ITS sequence was aligned with other reported sequence of *P. aphanidermatum* (Genbank accession no. MF040822.1, KY646468.1, MF347709.1) to obtained the percentage of similarity using the online software 'Multalin', which creates a multiple sequence alignment by progressive pairwise alignments (Corpet 1988).

RESULTS AND DISCUSSION

Morphological descriptions

Pythium is a representative of class Oomycetes, which bear large spherical Oogonia or female gametangia (Fig. 1) (van der Plaats-Niterink 1981). The class comprises mostly water molds. In the present study, *P. aphanidermatum* was collected from soil and their hyphae were grown with a daily increment of 2.5–3.0 cm at 25ºC on PDA. Asexual reproduction occurred under favourable environmental conditions through sporangia which developed into vesicles containing zoospores; which were liberated to give rise to hyphae (Stanghellini & Burr 1973). Sporangia were swollen, multinucleate and measured 10–50 µm in diameter (Fig. 1). The structure

of sporangia differs from species to species it can be spherical, filamentous slightly inflated and filamentous inflated. In this study, sporangia were found inflated filamentous, readily produced zoospores on transfer to water at 20–30ºC, which is a typical feature of *P. aphanidermatum*. After maturation, the sporangia get separated from the rest of hyphae by means of a septum as observed here (Fig. 1). When they were matured, a tubular structure called Papilla was developed at the apex or laterally which soon developed into a sac-like thin walled vesicle (Fig. 1). Papilla helps in the dispersal of uninucleate, naked, pyriform, biflagellate zoospores (Stanghellini & Burr 1973). As *Pythium* is homothallic, the male and female sex organs were developed in close proximity of each other either on the same or different hypha embedded in the host tissue (Fig. 1). Oogonia were found mostly terminal, spherical, 22–27 µm diameter; oospores aplerotic, 17–19 µm diameter, moderately thick-walled; antheridia were barrel or dome-shaped or cylindrical with a dimension of 11–19 µm long and 10–14 µm wide monoclinous, intercalary or terminal and found 1–2 per oogonium. Ashwathi *et al.* (2017) observed the similar structure of oogonia (terminal, globose and smooth 20–25 µ diameter) and antheridia (broadly sac-shaped 10–14 µ long and 10–14 µ wide, 2 per oogonium) in *P. aphanidermatum* isolated from coriander growing regions of Tamil Nadu, India. *Pythium* species have been traditionally identified and classified based on the morphology of asexual and sexual structures (Van der Plaats-Niterink 1981) as structures of sporangia and oospore vary between the species (Schroeder *et al.* 2013). Considering the reproductive structures morphologically the isolated fungus was identified as *P. aphanidermatum* as the similar morphology found in earlier reposts (Ashwathi *et al.* 2017) and with the help of keys provided by Middleton (1943), Waterhouse (1967), Plaats-Niterink (1981).

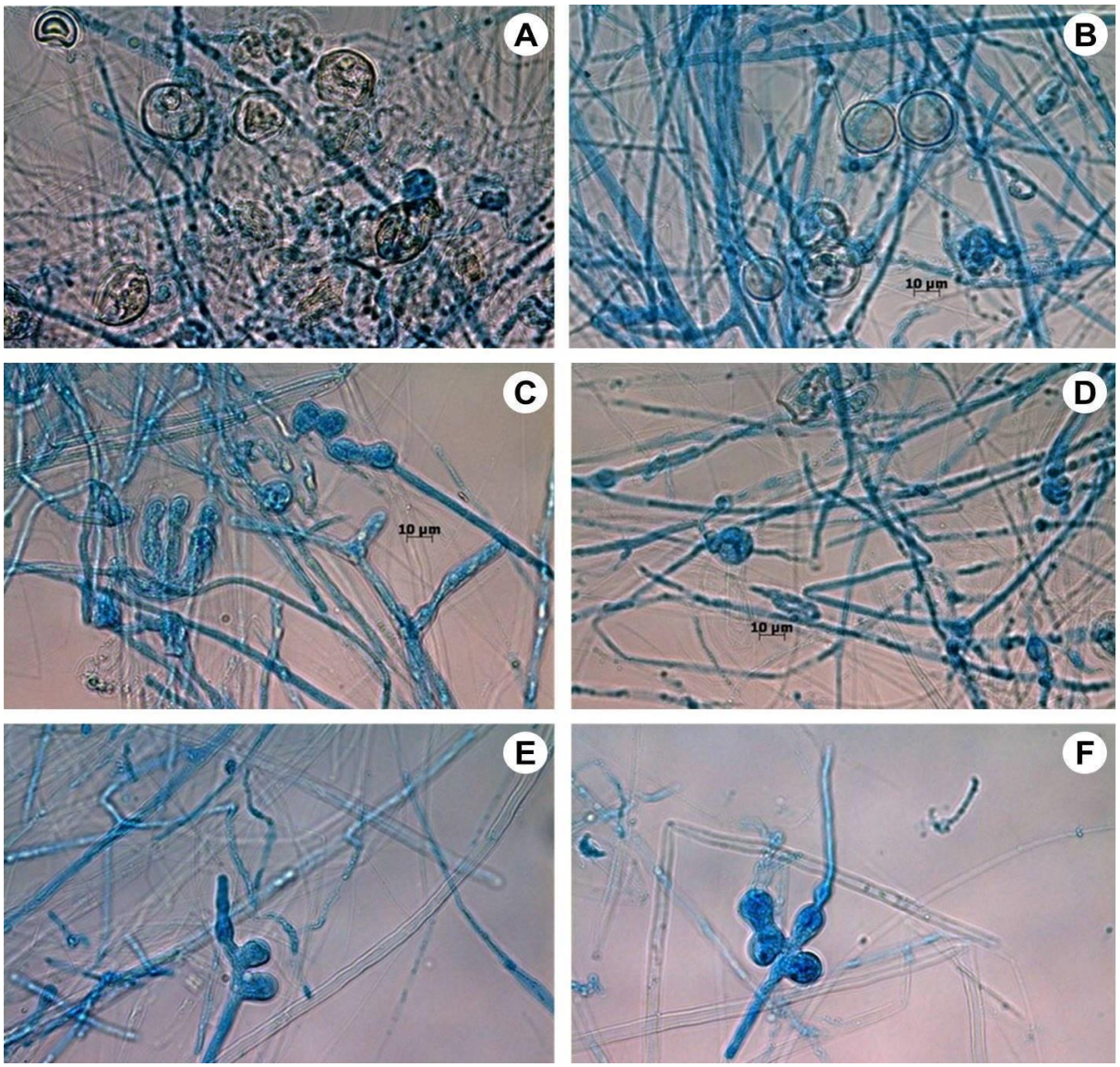

Figure 1. Vegetative and reproductive structures of *Pythium aphanidermatum* (Edson) Fitzp.: **A–B**, Oogonia; **C–D**, Inflated filamentous Sporangia; **E**, Sporangia with laterally placed Papilla; **F**, Papilla was developed at the apex of Sporangia.

Molecular identification based on the sequence of ITS region of rDNA

In the present study, the partial ITS sequence of the flanking regions of the rDNA of *P. aphanidermatum* was PCR amplified using the specific forward and reverse primers with an amplicon size 820 base pairs. The sequence was submitted to NCBI GenBank (Accession number MK158217). The sequence of amplified ITS region is given below.

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  1 tttcgtatcc gattcgcgcc gggtttcgag cgtgtttgta ttcgttactg tgtaatgcag
 61 tgatagtgca agcaatgcga ggagctttgg ctgatcgaag gtcgttgcgc aagtatttat
121 atgcgcgctt cggctgactt atactttcaa acccccttact ttaaaaactg atcaatactg
181 tgaggacgaa agtctttgct ttaaaactag ataacaactt tcagcagtgg atgtctaggc
241 tcgcacatcg atgaagaacg ctgcgaactg cgatacgtaa tgcgaattgc agaattcagt
301 gagtcatcga aattttgaac gcatattgca ctttcgggtt atacctggaa gtatgtctgt
361 atcagtgtcc gtacatcaac cttgcctctc tttgtcggtg tagtccggtt tgtagcatgt
421 gcagatgtga ggtgtctcgc ggcgtgtgtg tgtgctgtaa aatgcatacg cttgctgcga
481 gtccctttaa aacgacacga tctttctatt tgctttctat ggagcgcgta tctcgaacgc
541 ggcggtcctc ggatcgctcg cagtcgacag cgacttcagc ggagacatat ggaagaaacc
601 actattcgcg gtacgttagg cttcggctcg acaatgttgc gttttagtgt gtggattccg
661 ttttcgcttt gaggtgtact gttcggttgt gagcttgaac cttgtgtctc gctttgttag
721 tagaggtgtg tcgatttctg tggtttgatt ccgcacttta tgtgtgggta gagagactcc
781 atttgggaaa cattgtactg cgcgtacgct ttcgggtgt
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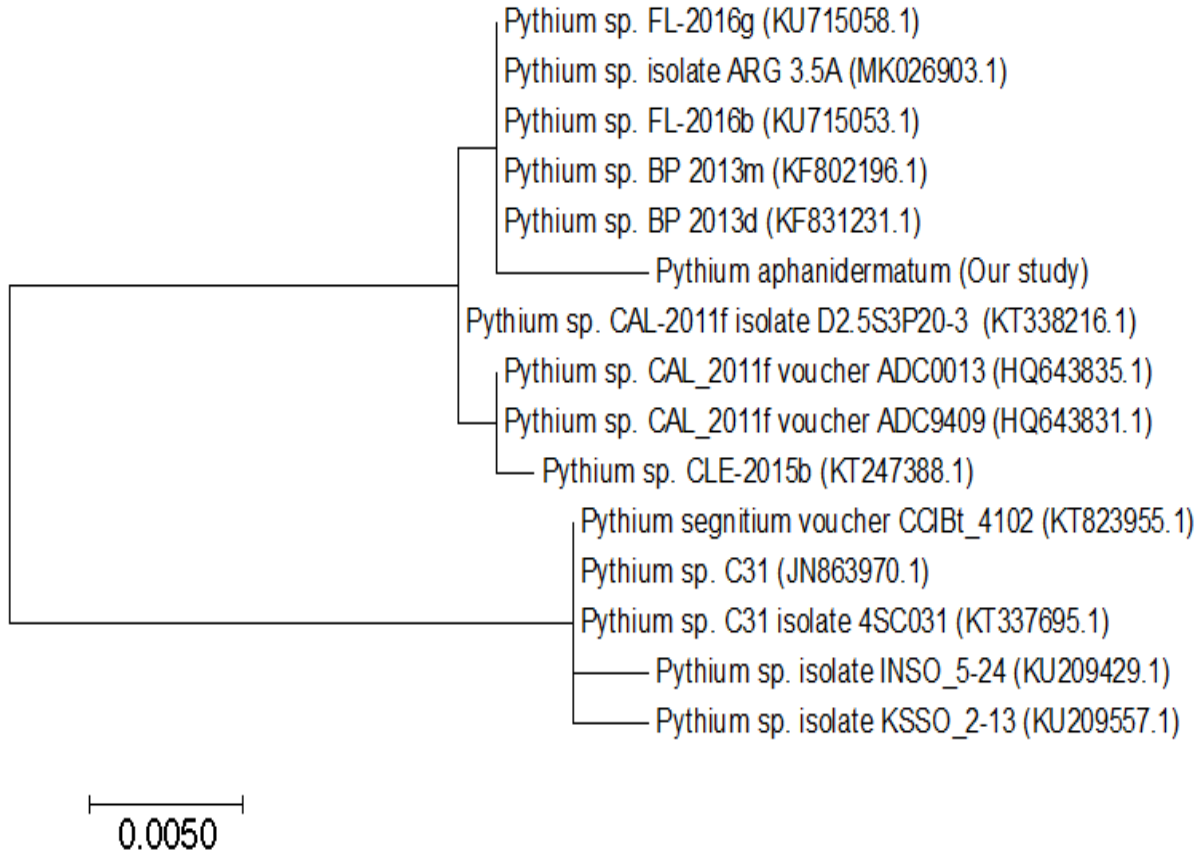

Figure 2. Molecular Phylogenetic analysis of *Pythium aphanidermatum* (Edson) Fitzp. based on the Tamura-Nei model by Maximum Likelihood method. The analysis is based on 14 nucleotide sequences from NCBI BLAST result.

The amplified sequence was blasted in the NCBI. The sequence showed 99% similarity to partial sequence of ITS-1 of 5.8S ribosomal RNA gene and ITS-2 of large subunit ribosomal RNA gene (MK026903.1, KF831231.1, KF802196.1, KU715058.1 etc.) of different isolates of *Pythium* sp. (Fig. 2). Phylogenetic analysis based on NCBI Genbank database confirmed the genus as *Pythium* and morphological observation predicted the species as *P. aphanidermatum*.

The ITS sequence was aligned with other reported sequence of *P. aphanidermatum* (Genbank accession no. MF040822.1, KY646468.1, MF347709.1) and found maximum 90% of similarity (Fig. 3).

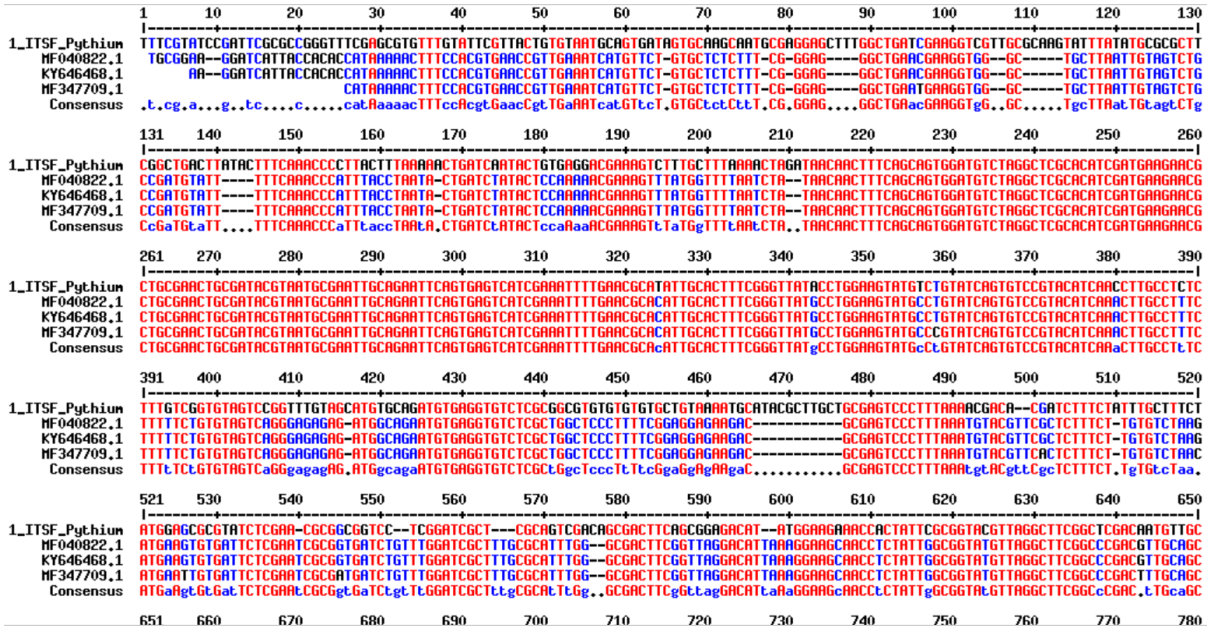

Figure 3. Multiple sequence alignment of the ITS sequence of *Pythium aphanidermatum* (Edson) Fitzp. with previously reported different isolates of *Pythium aphanidermatum* (Genbank accession no. MF040822.1, KY646468.1, MF347709.1).

The small difference in the ITS region is significant for the genus *Pythium*. The results of the sequence alignment support the morphological observations as found in earlier reports (Grooters & Gee 2002, Schurko *et al*. 2003, Pannanusorn *et al*. 2007, Ashwathi *et al*. 2017).

CONCLUSION

Pathogenic fungus *Pythium aphanidermatum* was isolated from Lucknow, India. The taxonomic identification was carried out by morphological diagnosis and molecular analysis. After the analysis of the characteristics and dimensions of hyphae, sporangia and sexual reproductive structures, this fungal pathogen was identified as *Pythium aphanidermatum*. The partial ITS sequence of the rDNA of *P. aphanidermatum* was obtained and submitted to NCBI GenBank (Accession number MK158217). Phylogenetic analysis revealed its similarity with other *Pythium* species. The isolation and identification of this fungus will help in further research on accurate biological control for this plant pathogen.

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