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Morphology Taxonomy DNA Barcoding and Phylogenetic Analysis of Soil Fungal isolated (JMBL-1) from Gorakhpur, Uttar Pradesh, India

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Abstract
Mycelium hyaline non septate sporangium globose mycelia grow slowly on PCA 7mm per day on 20-30 degree centigrade forming a rosette growth pattern without aerial mycelium. Sporangia look spherical to sub- spherical and seen terminal germinating by germ tubes. Spores are plerotic with 0.5-1.5 mm thick wall. Oogonia diclinous antheridium with acutely tipped tapering spines. Pythium species with a distinguishing feature of spherical sporangia with echinulate oogonia by slow growth rate distinctive rosette pattern of growth and production of oogonia bearing long, tapering acuminate spines infers to be *P. erinaceum* Robertson. The amplified product of PCR amplicons with COX II primers were subjected to BLASTn. By this, amplified products (Query sequence) shown 99% identity with the *Pythium* species. The BLAST analysis shows 99% identity with maximum score of 1014 with 94% query coverage for the species *P. erinaceum* AB362326.1. Then we did sequence homology with the wild type *Pythium erinaceum* COX II primer with our amplified product (Query sequence) through T-COFFEE algorithm alignment. The alignment shows very good score of 99%. This region shows 97% consensus region with *P. erinaceum* therefore, the fungal isolate belongs to the Pythium species. The strong homology reveals the isolate belong to the *P. erinaceum*. The similar studies performed with ITS universal primers for the characterization of our isolate JMBL-1 shows 98% identity with *P. erinaceum*. The BLAST analysis shows 98% identity with maximum score of 1559 with 95% query coverage for the species *P. erinaceum* HQ643534,1. Then we did sequence homology with the wild type *Pythium erinaceum* ITS region with our amplified product (Query sequence) through T-COFFEE algorithm alignment. The alignment shows very good score of 99%. This region shows 99% consensus region with Pythium erinaceum therefore, the fungal isolate belongs to the Pythium species. The strong homology shows *Pythium erinaceum*. This result also confirmed and augments the earlier result of COX II amplicons. So the morphological features augments the molecular analysis of PCR based COX II as well as ITS primers indicating that the fungal isolate undoubtedly is *Pythium erinaceum* Robertson. Further studied MSA using NEJ method with different other species COX ll and ITS sequences and was found out query sequence JMBL 1 showing identity with *P. erinaceum* followed by *Pythium schmitthenneri*, *P. acrogynum*, *P. hypogynum*, *P. selbyi*, *P. rostratum*, *P. pulchrum* and *P. carolinianum*. This may be the first report of *Pythium erinaceum* in Gorakhpur.

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

1. INTRODUCTION

The Oomycetes are microscopic Stremenopiles. Oomycetes are also known as "Water molds". Many year 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 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.

Pythium is a genus with over 200 species found worldwide, some of which are residents of terrestrial habitats while others

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are aquatic. In terms of nutrient acquisition, species within the genus may be saprophytes, plant or animal parasites, or mycoparasites. Pythium is a closely related to Phytophthora, famous for causing the ill fated "Famine of Ireland" in the years 1845 to 1849 in which 1 million people died.

Schoch et.al. observed DNA phylogenetic comparisons have shown that morphology-based species recognition often underestimates fungal diversity[1]. 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 mass species assignments. The present study 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 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. Our Oomycete isolate JMBL^{-1} was isolated from a soil sample taken in Korabhar crop fields in the city of Gorakhpur, Uttar Pradesh, India.

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^{-1} fungal isolate was prepared for microscopic morphological study and identification.

2.3 Growth rate measurements

The JMBL^{-1} fungal strain was inoculated on PCA, incubated at 25^{0}C and growth measurements in mm was recorded every 24 hours until the isolate covered the entire the culture plate.

2.4 Maintenance of culture

The purified cultures was inoculated in 15ml culture tubes containing PCB liquid broth, which was placed on the rotatory shaker incubated at 25ºC. The growth of pure mycelia JMBL $^{-1}$ was recorded at the interval of 24- 96h. After, 72-96h of growth, the culture media was transferred at 4ºC in refrigerator. Mycelial biomass (JMBL^{-1}) formed after 3-5 days of inoculation was collected on a muslin cloth by discarding the liquid media. Biomass was washed 5-6 times with deionized water carefully and mycelia was air dried and used for the further characterization.

2.5 Extraction of Fungal Genomic DNA

0.1g of dried JMBL^{-1} mycelium was ground in liquid nitrogen into a fine powder in a pre-chilled mortar and pestle. 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 min. 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 dissolve the pellet.

2.6 Characterization of JMBL-1

The JMBL^{-1} 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.[2] followed by PCR amplification of the ITS region of ribosomal DNA and COX II gene for oomycetes. The amplicons were sequenced by Pyrosequence analyzer and the resultant sequence were subjected to BLASTn, T-COFFEE and CLC Biomain Workbench analyses for identify the specific identification of oomycete.

2.7 PCR Amplification

The ITS region of JMBL-1 isolate was PCR amplified and sequenced using the universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [2]. PCR reaction consisted of 5.0µl of 10X amplification buffer (100mM Tris-HCl, pH 8.3; 500mM KCl, 2.5mM $MgCl_2$)), 3.0µl of 10mM dNTPs, 1µl each of 10µM ITS1 primer and ITS4 primer, 1µl of Tag Polymerase , about 100ng of sample DNA and nuclease free water to the final volume of 50µl. Prior to thermocycling, samples were heated at 94˚C for 5min., this was followed by 40 cycles of 94˚C for 1min., 55˚C for 1min. and 72˚C for 1min. using Master cycler personal (Eppendorf) the final extension was performed at 72˚C for 7 minutes. The successful amplification of the ITS region was checked by running 20µl of reaction mixture 1.5 % agarose gel in 1X Tris acetate EDTA buffer (TAE) at 100 volts for 60 minutes.

2.8 Pyrosequencing

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

2.9 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

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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 rooted phylogenetic 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

3.1 Morphological description

Isolate JMBL-1 of oomycete was isolated from soil samples taken in Khorabar area in the city of Gorakhpur, Uttar Pradesh, India. The oomycete grows well on hemp seed halves as well as on solid media like PCA, CMA and PDA. Our isolate JMBL^{-1} mycelium hyaline non septate sporangium globose mycelia grow slowly on PCA 7mm per day on 20-30 ^{o}C forming a rosette growth pattern without aerial mycelium. The asexual reproduction is achieved by the formation of sporangia and zoospores. Sporangia look spherical to sub- spherical and seen terminal germinating by germ tubes (Fig. 1). Spores are plerotic with 0.5-1.5 mm thick wall (Fig. 2). The sexual reproduction of the oomycete is a typical oogamy. The female gametangia are known as oogonia (Fig.3) diclinous antheridium with acutely tipped tapering spines (4). The oospore is a resting spore and can remain viable in the soil or in plant debris for many years. On the advent of favorable conditions of temperature, humidity and nutrition, it germinates to give a new mycelium.

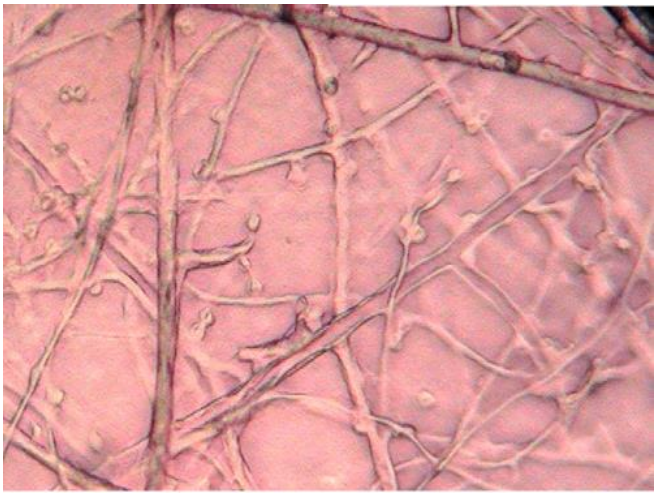

Fig.1: Vegetative Hyaline Hyphae

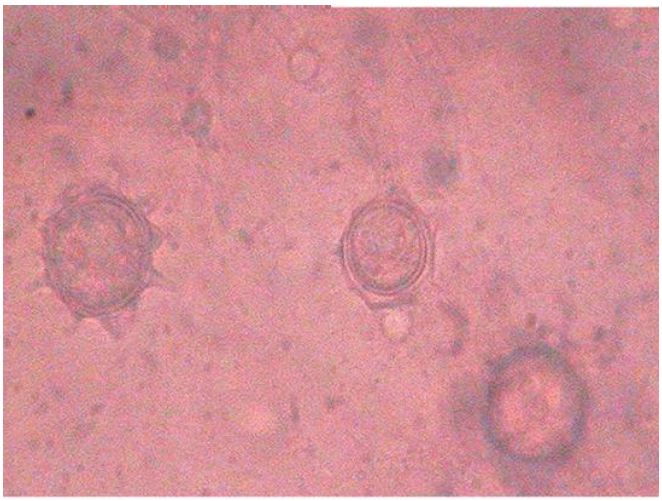

Fig.2:Sporangia germinating by Germ tubes

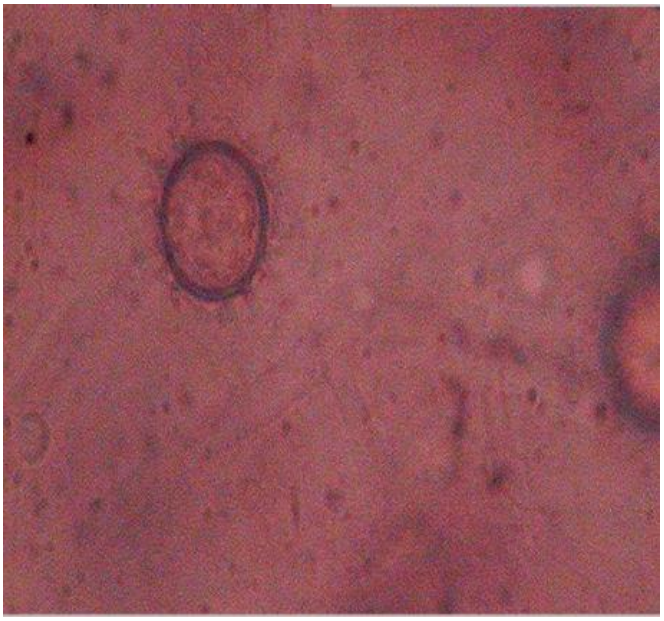

Fig.3: Plerotic oospores

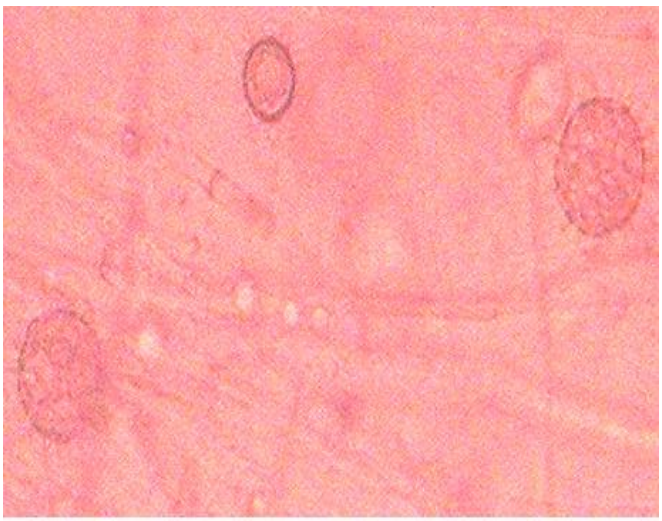

Fig.4: Immature Oogonia with acuminate spines

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The structural features of the isolate JMBL-1 as revealed by microscopy shows a typical hyaline oomyceteous mycelium. In optimal conditions this fungus like organism produces asexually through proliferating sporangia and zoospores. When the conditions becomes adverse, it produces antheridia and Oogonia abundantly. After camera goal copulation and fertilization, a Pythium species with a distinguishing feature of spherical sporangia with echinulate oogonia by slow growth rate distinctive rosette pattern of growth and production of oogonia bearing long, tapering acuminate spines infers to be *Pythium erinaceum*. This our isolate JMBL^{-1} the First report of its presence in Indian soil. Previously reported on the wheatfields in Canterbury, New Zealand [4].

3.2 Molecular characterization

3.2.1 Genomic DNA extraction

Prior to extraction, pure isolate of JMBL-1 was reactivated by sub-culturing in growth medium and inoculated at 27 0C for 3-7 days massive production of mycelia. DNA was extracted from resulted mycelia JMBL^{-1} using our own protocol developed but following the basic tenets of Saghai-Maroof et.al. [2]. Agarose gel electrophoretic analysis of the purified DNA of JMBL-1 showed approximate molecular weight of 40 kb (Fig.5).

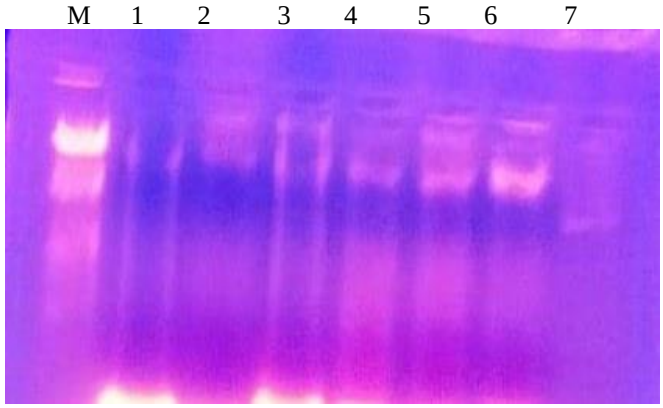

Fig.5 :Agarose gel electrophoresis of purified genomic DNA of JMBL-1.
M, 50-Kb DNA Marker; Lane 2, Isolate JMBL-1.

3.3 PCR amplification

The cytochrome oxidase II (COX II) gene of JMBL-1 was PCR amplified using forward FM 58 (5′-CCACAAATTTCACTACATTGA-3′) and Reverse FM 66 (5′-TAGGATTTCAAGATCCTGC 3′)[5]. The amplified COX II gene was approximately 600 bp (Fig.6). It is in consonance with the results of earlier reports[5-6]. The ITS region was also amplified using the universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) White et al., (1990). The amplified ITS region was approximately 800-900 bp (Fig.6). This result is agreement with that of earlier authors[7-16]. The PCR products from the gel were cut and was submitted to the Apical Scientific Sequencing Division Bhd., Malaysia for sequencing using Applied Biosystem and the same Primers use it for amplification.

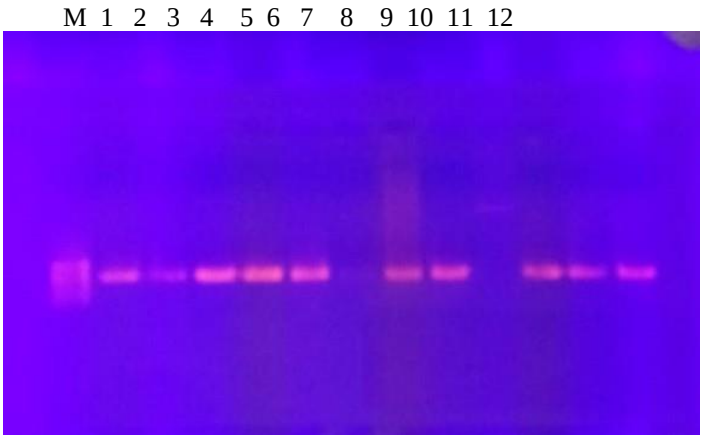

Fig. 6: Band pattern of PCR amplified COX II region
M 1 Kb DNA ladder Marker; Lane 1, Isolate JMBL-1

The PCR products from the gel were cut and was submitted to the Apical Scientific Sequencing Division Bhd., Malaysia for sequencing using Applied Biosystem and the same Primers use it for amplification. The similar studies performed with ITS universal primers for the characterization of our isolate JMBL-1 shows 98% identity with Pythium erinaceum. The BLAST analysis shows 98% identity with maximum score of 1559 with 95% query coverage for the species Pythium erinaceum HQ643534,1 (Fig.7). Then we did sequence homology with the wild type *Pythium erinaceum* ITS region with our amplified product (Query sequence) through T-COFFEE algorithm alignment. The alignment shows very good score of 99%. This region shows 99% consensus region with Pythium erinaceum therefore, the fungal isolate belongs to the Pythium species. The strong homology shows Pythium erinaceum(Fig. 24b). This result also confirmed and augments the earlier result of COX II amplicons. So the morphological features augments the molecular analysis of PCR based COX II as well as ITS primers indicating that the fungal isolate undoubtedly is Pythium erinaceum Robertson.

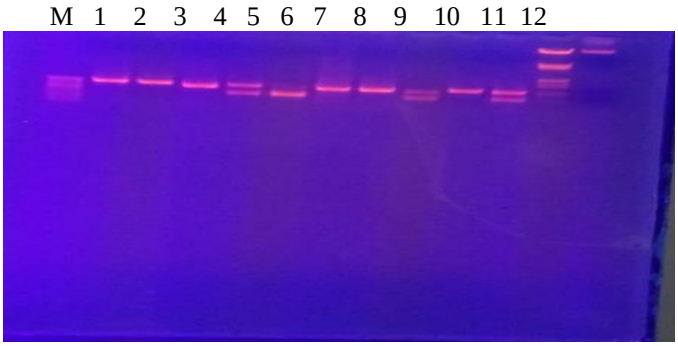

Fig. 7: Agarose gel electrophoresis of PCR amplified ITS region
M, 1kb DNA ladder Marker; Lane 1, Isolate JMBL-1

3.5 Phylogenetic tree

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The amplified COX II as well as ITS sequences of our isolate JMBL-1 was also subjected to multiple sequence alignment through CLC bio Main Workbench while constructing the phylogenetic tree using NEJ method. According to the tree our sequence has showed relationship with Pythium erianaceum with different other species COX ll (Fig. 8) and ITS (Fig.9) sequences and were subsequently found out query sequence JMBL 1 showing identity with Pythium erinaceum followed by *Pythium schmitthenneri*, *P. acrogynum*, *P. hypogynum*, *P. selbyi*, *P. rostratum*, *P. pulchrum* and *P. carolinianum*. This may be the first report of *P. erinaceum* in Gorakhpur particular and Indian soil as a whole.

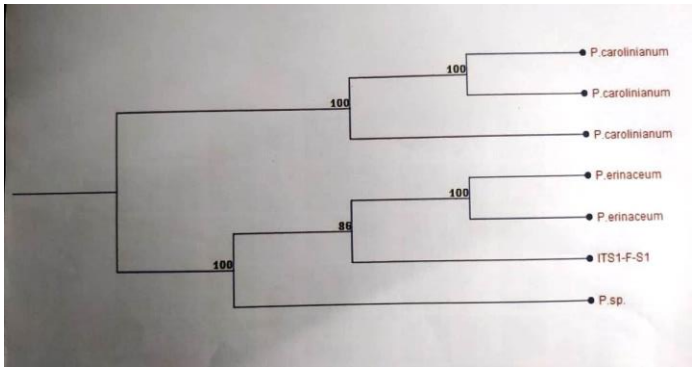

Fig.8: Phylogenetic tree relationship with Pythium erianaceum with different other species COX ll

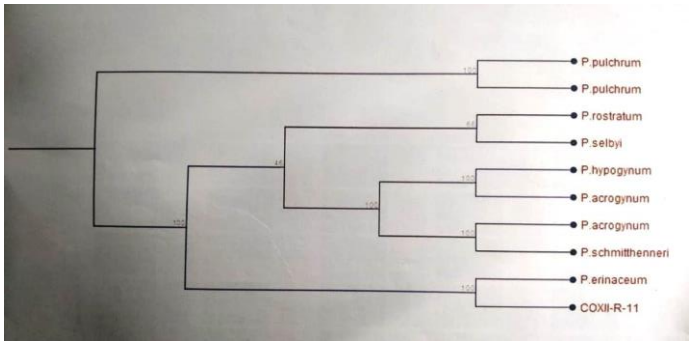

Fig.9: Phylogenetic tree relationship with Pythium erianaceum with different other species COX ll

4. REFERENCES

1. 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.
2. 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.
3. White T, Burns T, Lee S, et al. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M, Gelfand D, Sninsky JJ, et al. (eds). PCR Protocols: A Guide to Method and Applications. New York: Academic Press, Inc.; 1990: 315-322.
4. Robertson GI. *Pythium erinaceum* sp. nov. from wheatfields in Canterbury, New Zealand, New Zealand Journal of Botany, 1979; 17(3): 283-286, DOI: 10.1080/0028825X.1979.10426902.
5. 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.
6. Martin, Frank, Tooley, Paul. Phylogenetic Relationships among Some Pythium Species Inferred from Sequence Analysis of the Mitochondrially Encoded Cytochrome Oxidase II Gene. Mycologia, 2003; 95: 269-84. 10.2307/3762038.
7. Cooke DEL, Drenth A, Duncan JM, Wagels G, Brasier CM. A molecular phylogeny of Phytophthora and related oomycetes. Fungal Genetics and Biology, 2000; 30: 17–32. doi: 10.1006/fgbi.2000.1202.
8. Coince A, Cael O, Bach C, Lengelle J, Cruaud C, Gavory F, Morin E, Murat C, Marcais B, Buee M. Below-ground fine-scale distribution and soil versus fine root detection of fungal and soil oomycete communities in a French beech forest. Fungal Ecology, 2013; 6: 223-235. doi: 10.1016/j.funeco.2013.01.002.
9. Sapkota R, Nicolaisen M. An improved high throughput sequencing method for studying oomycete communities. Journal of Microbiological Methods, 2015; 110: 33-39. doi: 10.1016/j.mimet.2015.01.013.
10. Ishii K, Fukui M. Optimization of annealing temperature to reduce bias caused by a primer mismatch in multitemplate PCR. Applied and Environmental Microbiology; 2001; 67(8): 3753–5. doi: 10.1128/AEM.67.8.3753-3755.2001.
11. Acinas SG, Sarma-Rupavtarm R, Klepac-Ceraj V, Polz MF. PCR-induced sequence artifacts and bias: insights from comparison of two 16S rRNA clone libraries constructed from the same sample. Applied and Environmental Microbiology, 2005; 71: 8966–8969. doi: 10.1128/AEM.71.12.8966-8969.
12. Schmidt PA, Bálint M, Greshake B, Bandow C, Römbke J, Schmitt I. Illumina metabarcoding of a soil fungal community. Soil Biology and Biochemistry, 2013: 65: 128–132. doi:10.1016/j.soilbio.2013.05.014.
13. Innis MA, Gelfand DH, Sninsky JJ, White TJ. PCR Protocols A Guide to Methods and Applications.

Applied Biology & Biotechnology
ISSN 2320 –7825 (Print); 2320 –7833 (Online)

Academic Press, 1990; 1-482. doi: 10.1016/0167-7799(90)90215-J.
14. Bachofer M. Molekularbiologische Populationsstudien an Plasmopara halstedii, dem Falschen Mehltau der Sonnenblume Dissertation, Universitat Hohenheim Germany, 2004; 1-140.
15. Spring O, Bachofer M, Thines M, Riethmüller A, Göker M, Oberwinkler F. European Journal of Plant Pathology; 2006; 114: 309. doi: 10.1007/s10658-005-5996-9.