Characterisation of *Pythium capillosum*– A new pathogen of *Xiphinema pachtaicum* (Nematoda: Longidoridae)

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Short Report

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Abstract

Isolation on culture media followed with macroscopic, microscopic, molecular and phylogenetic analyses and pathogenicity test allowed us to identified *Pythium capilosum* found to be capable of destroying certain dagger nematode through endozoic parasitism from ingested oomycete zoospores. Although endoparasitism of free-living nematodes is found to be common among Oomycetes in nature, this is the first finding of such occurrence in Bosnia and Herzegovina soils. The obtained results could give rise to initiate a study for the new biological approach for nematode control promoting environmentally safe and sustainable control measures.

Main Text

Nematodes are common parasites of plants causing severe damage to crops. Control of nematodes usually considering use of nematicides, the chemicals with often toxic compounds which have influence on environmental and health problems (Jansson and Lopez-Llorca, 2004). One of the ways to avoid these problems is development of new methods to control nematodes. The use of natural enemies is one of the alternative approaches to managing plant-parasitic nematodes. Among them, useful group of natural enemies comprise a group of soil-living fungi that are natural enemies of plant-parasitic nematodes (Devi, 2018). There are reports of parasitism of several species of Lagenidium, Phytophthora and Pythium fungi observed on populations of nematodes from Alphelenchus, Meloidogyna Cephalobus, Plectus, Rhabditis and *Xiphinema* genus, respectively (Tzean and Estey, 1981; Bileva et al. 2009). So, the aim of this small-scale study was to characterize the Oomycete isolate observed to infect and digest nematode from *Xiphinema* genus.

Soil samples were collected from depth of 30 cm from a vineyard from Trebinje municipality, Bosnia and Herzegovina (BiH). Nematode extraction was done by decanting and sieving method (Brown and Boag, 1988). Nematodes were observed on dissection microscope seven days after extraction. Additionally, molecular nematode identification was done analysing the D2D3 region of 28S rDNA (Orlando et al 2016). Initially, an Oomycete-infected nematode was transferred to 2% water-agar in a petri dish and incubated for 24-48 h at room temperature (23-28°C). Then the isolated Oomycete was grown on half-strength cornmeal agar at room temperature for 2 weeks. After that, morphological studies were made under an inverted microscope (Nikon Eclipse Ti-U). The Oomycete was identified with keys proposed by Hendrix and Papp (Hendrix and Papp, 1974) and Plaats-Niterink (Plaats-Niterinkn, 1981). To induce the production of oogonia by the Oomycete from the dead nematode, the Oomycete sporangia are commonly formed in water culture consisting of a colonized wheat grass blade in water. Mycelium-bearing agar blocks cut from the periphery of the resulting colonies were floated in 10 ml of distilled water in a petri dish with wheat grass and incubated for 48 h at 10° C (Plaats-Niterink, 1968). The zoosporangia were collected individually under a reverse microscope (×100) with the cone of a micro-pipette (10 µL) and then placed on a leaf disk in a drop (30 µL) of permuted water. Pathogenicity test was performed by adding the nematodes to the Oomycete cultures during their growth on half-strength cornmeal agar. Infected nematodes were transferred to water on glass slides for viewing under inverted microscope

(Nikon Eclipse Ti-U). DNA was extracted from 100 mg of mycelia from the obtained LT4 isolate using the DNAeasy Plant Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Five regions including internal transcribed spacer (ITS) region, b-tubulin (*b-tub*), cytochrome c oxidase subunit 2 (*cox2*), 18S small subunit (SSU), and 28S large subunit (LSU) ribosomal RNA genes were amplified by PCR. Details on cycling conditions and a number of PCR cycles specific for each primer pair are reported in Supplementary Table 1, S1. All PCR products were evaluated for successful amplification using gel electrophoresis on a 1 % agarose gel. PCR products were directly sent for Sanger sequencing to Macrogen Europe BV. All sequences were blasted at http://ncbi.nlm.nih.gov/blast/Blast.cgi, using the megablast algorithm and default search parameters (Altschul et al. 1990) to confirm their identity. The resulting sequences were deposited in the GenBank (ITS Accession No. MW699625; b-tub Accession No. OQ256244; cox2 Accession No. OQ291216; SSU Accession No. OQ359880; LSU Accession No. OQ359882). A sequence comparison was performed with sequences available in GenBank by using the BLAST software package (www.ncbi.nlm.nih.gov). Phylogenetic analyses were carried out by means of the Maximum Likelihood method. Reference strain sequences of each species (*Pythium* spp. and *Lagenidium* spp.) which showed a high sequence identity during the BLAST sequence comparisons were included: CBS 118.80 (P. aphanidermatum), CBS 772.81 (P. apleroticum), CBS 215.80 (P. aquatile), CBS 584.85 (P. caudatum), CBS 22294 (P. capillosum), CBS154.64 (P. coloratum), CBS 664.79 (P. diclinum), CBS166.68 (P. dissotocum), CBS234.72 (P. flevoense), CBS 574.85 (P. insidiosum), CBS 124053 (P. oopapillum), CBS 382.34 (P. oligandrum), CBS 227.88 (P. pachycaule), CBS 122643 (P. pectinolyticum), CBS 110030 (Pythium sukuiense) and ATCC 6680 (Lagenidium myophilum. Maximum Likelihood analysis was performed by MEGA 11 software (Tamura et al. 2021). A 2874 bp concatenated data set was obtained with the ITS, *tub2*, *cox2*, SSU and LSU sequences. Findmodel was used to select the best-fit nucleotide model for each partition (http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html) and incorporated into the analysis. Maximum-likelihood trees were then constructed with 1000 bootstrap replications.

The nematode population was identified as species *Xiphinema pachtaicum* based on morphological and morphometrical characters and analysis of the sequence of the D2D3 region of 28S rRNA. The sequence submitted to GenBank (Accession No. OR237446) was blasted and revealed maximum score of 99.49% with Spanish isolate of *X. pachtaicum* (HM921355.1). On cornmeal agar, the LT4 isolate formed a well-branched, dense and aerial white mycelium (Supplementary 2, S2). Microscopic observations of this isolate showed the formation of zoosporangia on hyphae that grew from a naturally infected nematode indicating that this endozoic fungus was the Oomycete. A subculture of the LT4 isolate in liquid culture formed Oogonia with a prevalent barrel shape with one papilla measures 16-32 µm in diameter (av. 23 µm) (Supplementary S3a). Moreover, filamentous sporangium-like hyphae were formed on an agar medium. Based on the studied characteristics and comparing it with those published with Paul (1989) and on the results obtained from molecular analyses of the ribosomal DNA the isolate was preliminarily identified as *Pythium capillosum*. Microscopic observation of the free-living nematodes infected with *Pythium capillosum* revealed the presence of ingested germinating zoospore located in the esophagus and on the body of infected nematode (Supplementary S3b).

For the purpose of molecular identification, five genomic regions (ITS, b-tub, cox2, SSU and LSU) were successfully amplified and sequenced. A BLASTn analysis of these sequences showed following nucleotide identity with the reference strain CBS 222.94 of P. capillosum: 95.15% (ITS), 99.79% (b-tub), 99.42%(cox2), 98.10%(SSU) and 98.10% (LSU) (AY598635, KJ595485, KJ595360, AY598635 and AY598635, respectively). Nucleotide identities were same or slightly lower when the LT4 isolate was compared with the reference strain CBS 122643 of closely related species P. pectinolyticum: 95.15% (ITS), 98.97%(b-tub), not available (cox2), 98.10%(SSU) and 98.10% (LSU) (HQ643739, KJ595469, N/A, HQ643739 and HQ643739, respectively). Phylogenetic analyses based on concatenated ITS, b-tub, cox2, SSU and LSU gene sequences of the LT4 isolate and reference isolates of 15 phylogenetically related Pythium spp. and 1 phylogenetically close Lagenidium species (Lagenidium miophylum) grouped the LT4 isolate with P. capillosum (Fig. 1). Two isolates of P. capillosum (LT4 and CBS22294) were positioned with another Pythium spp. within the Pythium phylogenetic clade B (Hyde et al. 2014). On the other hand, when phylogenetic analyses were carried out for five single-locus sequences analyses grouped the LT4 isolate with P. capillosum beside some other isolates (e.g. CBS 122643 P. pectinolyticum, CBS234.72 P. flevoense, ATCC 6680 L. myophilum) indicating the higher robustness of concatenated phylogenetic analyses.

Figure 1. Phylogenetic analysis of *Pythium capillosum* LT4 isolate and reference isolates of other *Pythium* spp. based on ITS, *b-tub*, *cox2*, SSU and LSU sequences. The concatenated phylogenetic tree was obtained by Maximum Likelihood analysis using the General Time Reversible plus Gamma model. The strain designation, host affiliation and fungal species are shown for each strain. The reference isolates of *Pythium capillosum* and phylogenetically close *Pythium* species (Hyide et al. 2014) are shown in regular, while the LT4 isolate characterized in this study is shown in bold.

Overall, based on the morphological identification, BLASTn and concatenated phylogenetic analyses the LT4 isolate from *X. pachtaicum* was identified as *P. capillosum*. *Pythium capillosum* sp. nov. is described and illustrated by Paul (1987) from cultivated soil from Algeria. The fungi species was also detected in Poland developing on eggs of percids in water from the Biala river (Czeczuga and Muszyńska, 1999). This is the first observation of the *Pythium* species parasitized nematode in the BiH soil and also such Oomycete species in the country. However, it is a common phenomenon, and one is tempted to speculate that endozoic parasitism may have some significance for the survival of these Oomycetes (Timper, 2011; Tranier et al., 2014). Since most plant-parasitic nematodes attack plant roots, the rhizosphere biology of nematophagous fungi and oomycetes is important from a biological control point of view. Plant parasitic nematodes are difficult pests to control since their habitat is in the soil. Utilizing natural enemies already present in the soil may offer environmentally safe and sustainable measures of nematode control. Further studies are necessary to exploit practical application of this nemathophagous oomycete in the control of dagger nematodes.

Declarations

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Compliance with ethical standards: Hereby we confirm and declare that in the work done and present in this paper there is no any potential conflict of interest, also in the research any human and/or animals' participant wasn't used and there is no any disagreement with informed consent.

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Figures

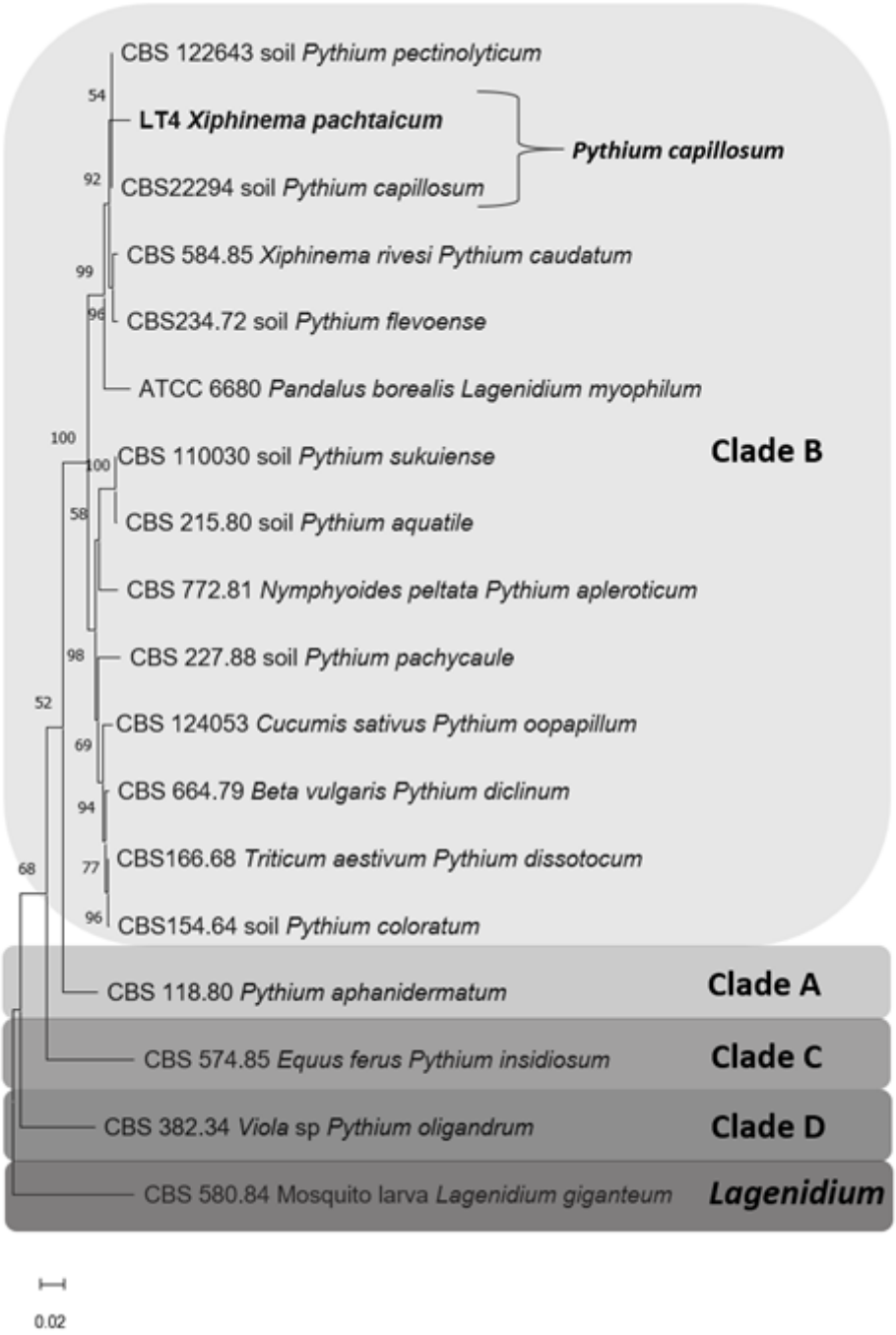

Figure 1

Phylogenetic analysis of *Pythium capillosum* LT4 isolate and reference isolates of other *Pythium* spp. based on ITS, *b-tub*, *cox2*, SSU and LSU sequences. The concatenated phylogenetic tree was obtained by Maximum Likelihood analysis using the General Time Reversible plus Gamma model. The strain designation, host affiliation and fungal species are shown for each strain. The reference isolates of *Pythium capillosum* and phylogenetically close *Pythium*species (Hyide et al. 2014) are shown in regular, while the LT4 isolate characterized in this study is shown in bold.

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