Morphological and molecular taxonomy of *Pythium longisporangium* sp. nov. isolated from the Burgundian region of France

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Received 16 March 2005; received in revised form 6 April 2005; accepted 7 April 2005

First published online 19 April 2005

Edited by R.C. Staples

Abstract

During the course of an investigation on the Pythiaceous oomycetes occurring in the Burgundian vineyards, some species of *Pythium* possessing mainly hypogynous antheridia were found. These had been classified as oomycetes belonging to the “*Pythium rostratum*” group for a long time. Three of these isolates, having similar structures and growth, are very closely related to a recently described species, *Pythium bifurcatum* Paul. A close look at these, however, underlines some fundamental differences with the latter. Not all of them produce zoospores but have very large sporangia. The type specimen is F-1200 (B 76a) which is a medium-slow growing saprophyte. The sequence of the ITS region of the rDNA also shows a very close relationship with *P. bifurcatum*. On the basis of morphological and molecular analysis, we now describe this species as *Pythium longisporangium* sp. nov. Morphological features of this new species, the sequences of the ITS region of its nuclear ribosomal DNA, and its comparison with related species are discussed.

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Key words: *Pythium longisporangium*; Sporangia; Oogonia; Antheridia; Oospores; ITS region; rRNA

1. Introduction

The genus *Pythium* is a very common oomycete found all over the world. In 1990, it was known to have more than 130 species [1]. The first author of this manuscript has also added more than 20 species to this genus since that date. Many of the isolates belonging to this genus are currently being studied for their structural and molecular diversity. Some of these are also being studied for their mycoparasitic activities on fungal pathogens like *Botrytis cinerea* [2]. Most of the members of the genus *Pythium* live in soil or aquatic environments as saprophytes, however, some of them are known to be very destructive plant pathogens, inflicting serious economic losses of crops by destroying seed, storage organs, roots, and other plant tissues [3]. Although, the majority of these organisms produce biflagellate zoospores, they are no longer considered as “aquatic fungi” and unlike most of the eumycetes, the members of this group remain diploid throughout their life cycles with meiosis occurring in the gametangia before fertilization. The entire group of the oomycetes are now supposed to be closer to algae (Phaeophyta and Chrysophyta) and higher plants. These are now classified in the Kingdom Stramenopila, one of the eukaryotic Kingdoms which includes water molds and brown algae. The position of the oomycetes as a unique lineage of stramenopile eukaryotes, unrelated to true fungi but closely related to heterokont (brown) algae, has been well established.
using molecular phylogenies that are based on ribosomal RNA (rRNA) sequences [4–6].

The taxonomy of the genus Pythium was mainly based on the morphological descriptions like the size and shape of oogonia, antheridia, and sporangia. Keys provided by Middleton [7], Plaats-Niterink [8] and Waterhouse [9] are all based on these morphological characters and are still indispensable. However these are now being supplemented with molecular characteristics. Comparative studies of the internal transcribed spacer (ITS) regions of the ribosomal RNA genes (rDNA) have become a useful tool in fungal taxonomy as these regions evolve sufficiently rapidly to distinguish different species within a genus [10,11]. The ITS sequence data can provide valuable information on “new” or undescribed taxa, as sequence diversity may support the erection of new species [12].

The morphology of Pythium longisporangium is typical for the genus Pythium. It is a slow growing oomycete, having large spherical, globose, to cylindrical sporangia; smooth-walled oogonia and mostly hypogynous antheridia. Sexual structures are readily formed in water and on solid media within a week. The morphological details of the new species together with the sequences of its ITS region of the ribosomal nuclear DNA, comparison of morphological and molecular characteristics with related species are discussed in this article.

2. Materials and methods

2.1. Fungal and oomycetous material

P. longisporangium (Type strain, F-1201/B76a) was isolated from soil samples taken in a vineyard in Marsannay situated in the outskirts of the French city of Dijon in the Burgundian region by using the usual baiting techniques [8,13]. It occurred thrice out of 65 such samples and was purified by repeated washing with sterile distilled water and sub-culturing on solid media like potato carrot agar (PCA) and corn meal agar (CMA). All these isolates are maintained at “Institut Jules Guyot”, in Dijon, France. P. longisporangium was identified with the help of keys provided by Middleton [7], Plaats-Niterink [8] and Waterhouse [9] and also by its ITS sequences using the BLAST search.

2.2. DNA isolation and PCR

All three isolates of P. longisporangium were grown in PDB (potato dextrose broth) The culture conditions, DNA isolation and the PCR of the ITS of the ribosomal nuclear DNA was done using the procedures described earlier [14,15]. Universal primers ITS1 (TCC GTA GGT GAA CCT GCG G) and ITS4 (TCC TCC GCT TAT TGA TAT GC) were synthesised and the DNA sequence was realised by Oligo Express (Paris). ITS1 is at the 3' end of the 18S rDNA gene and ITS4 is at the 5' end of the 28S rDNA gene. The sequences obtained were compared with the ITS1 sequences of related species of Pythium: Pythium bifurcatum (Genbank accession number AY083935), Pythium longandrum (AY039713), P. sp. strain F-74 (AY455695), Pythium hypogynum (AY455804), Pythium terrestris (AY039714), and Pythium segnitium (AY149173). Pythium sp. strain F-1216 (AY455697), Pythium canariense (AY065661) and Pythium rostratum (AJ233456). The sequence of the ITS region of the nuclear ribosomal DNA of P. longisporangium (F-1200) has been deposited in Genbank.

3. Results

3.1. Morphological descriptions

P. longisporangium PAUL sp. nov. (Figs. 1–3).


Etymology: The oomycete is being named as P. longisporangium because of the form of its sporangia which often are pyriform to lemoniform and are formed in abundance.

Mycelium hyaline, well branched. Main hyphae up to 6–8 μm wide. Colonies on PCA are submerged and show a narrow chrysanthemal pattern. Average radial growth of the oomycete at 25 °C on PCA is 11 mm/ day. It grows well in water on hemp-seed halves and produces asexual and sexual structures at room temperatures (18–25 °C).

Sporangia (Fig. 1, (a)–(d)) are globose to somewhat cylindrical, oval, and at times peanut shaped mostly catenulate and intercalary (Fig. 1(b)); measuring 15–55 μm in diameter (av. 32.6 μm) and up to 65 μm in length. These structures are densely granulated and the larger ones have a clear hyaline central zone which is sparsely granulated (Fig. 1, (c)). Zoospores were not observed despite repeated flooding of the cultures by distilled water, tap water or pond water. The sporangia germinate directly through germ tubes to produce a new mycelium (Fig. 1, (a, d)).
The oomycete reproduces sexually by forming antheridia and oogonia plentifully in water cultures on hemp-seed halves and also on solid media like PCA. Oogonia are smooth walled, spherical, terminal, sub-terminal and intercalary (Fig. 1, (e)–(h)), measuring 17–36 μm in diameter (av. 19.5 μm) and filled with dense, coarsely granulated protoplasm.

All the oogonia are supplied with antheridia which are usually hypogynous (Fig. 1, (e)–(h)) or monoclinous sessile (Fig. 1, (c)). Each oogonium is supplied by 1–3 antheridial cells which are at times borne in a catenulate fashion on one antheridial branch (Fig. 1, (f), 2, (c, d)). Very rarely diclinous antheridia are also present (Fig. 2, (a, b)). After fertilization the oogonia is found attached with one or two balloon-shaped antheridial cells (Fig. 1, (h), 2, (c, d, f)).

Oospores are plerotic or nearly so (Fig. 2, (e)–(h)), spherical, usually one per oogonium (Fig. 2, (e)), occasionally two per oogonium (Fig. 2, (f)) and rarely three in a single oogonium (Fig. 2, (g)). In the intercalary oogonia these can be aplerotic (Fig. 2, (h)); smooth-walled, measuring 12–22 μm in diameter (av. 18.1 μm). The oospore wall is relatively thin, measuring 1–1.5 μm in thickness.
3.2. Internal transcribed spacer region

The ITS region of the nuclear ribosomal DNA of *P. longisporangium* (Genbank accession number AY455693) is comprised of 890 bases which are:

1 ccacacctaa aaacctctca cgtgaactgt ttgtatcaga ttagcgc-
61 cgtgttttgtg gtatcactat gtattcgtac gtggtgttag caag-
121 cattgt atggagcttg
gctgatcgaa ggtcggtgcg cacctgtgt gtgtattggc tgat-
181 ttaaatatat actgattata ctgtaaggac gaaagtcttt gctttatat
241 cgcgtcgttg ggatgtctag gctcgcacat cgatgaagaa cgctgcgaac tgcgatacgt
301 aatgcgaatt gcagaattca gtgagtcatc gaaattttga acg-
361 catattgc cactttcggg tatacctgg aagtatgtct gtatcagtgt ccgta
421 tgtagtccga ttgagagtat ggcagacgtg aggtgtctcg cgactcgtat atcattgtgt
gtgtaaatcg taagagatac atacataagg tattatattaa
ttgttgccgactttttaa
541 aagcacacgt ttttttttatt gtagttatat ggagcttgta
ttgaacacgtgacttttaag tcgtggctct
601 gcgactctgg cagacgctgacttttaag tcgtggctct

Fig. 2. Sexual reproduction of *Pythium longisporangium*. (a, b): Oogonia provided with diclinous antheridia; (c): oogonia provided with hypogynous antheridia, antheridial branch bearing intercalary antheridial cells; (d): oogonia provided with hypogynous antheridia and bulbous antheridial cells; (e): plerotic, single oospores; (f): two oospores per oogonium; (g): three oospores per oogonium; (h): intercalary oogonia having a single aplerotic oospore. (a, c) bar = 50 μm, (b, d, e–h) bar = 25 μm.
Fig. 3. CLUSTAL W, multiple sequence alignment of ITS1 regions of the rDNA of *Pythium longisporangium*, *P. bifurcatum*, *P. longandinum*, *P. hypogynum*, *P. terrestris*, *P. segniitum*, *P. rostratum*, *P. canariense*, *P. sp. (F-74)*, and *P. sp. (F-1216)*.
Table 1

<table>
<thead>
<tr>
<th>Characters</th>
<th>Pythium longisporangium</th>
<th>Pythium bifurcatum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sporangia</td>
<td>Intercalary and catenulate, 15–23 μm (av. 19.5 μm)</td>
<td>Intercalary and terminal, 25–50 μm (av. 35 μm)</td>
</tr>
<tr>
<td>Oogonia diameter</td>
<td>13–23 μm (av. 19.5 μm)</td>
<td>17–36 μm (av. 26.7 μm)</td>
</tr>
<tr>
<td>Antheridia</td>
<td>Hypogynous, monokinous sessile, diclinous, antheridial cells at times catenulate</td>
<td>Hypogynous, monokinous sessile</td>
</tr>
<tr>
<td>Oospore</td>
<td>Usually one, frequently 2 and rarely 3 per oogonium, 12–22 μm (av. 18.1 μm)</td>
<td>Usually 1, rarely 2, per oogonium, 16–35 μm (av. 24.9 μm)</td>
</tr>
</tbody>
</table>

The comparison of the ITS1 sequences of *P. longisporangium* and related species is given in Fig. 3 in the form of CLUSTAL multiple alignments.

4. Discussion

*Pythium longisporangium* (F-1200) is a slow growing species that falls within the category of species having spherical non-proliferating, catenulate sporangia, smooth walled, globose oogonia which are provided by hypogynous antheridia. These characters bring this species very close to a recently-described species, *P. bifurcatum* PAUL from northern France. However the ITS sequences of these species are very different, and a close look at the morphological characters shows that they are distinct but closely related (Table 1). The presence of triple oospores and intercalary chained-antheridial cells are unique for the taxon.

The ITS region of the nuclear ribosomal DNA of *P. longisporangium* is comprised of 890 bases and a BLAST search gives the closest resemblance of this oomycete to recently-described species with hypogynous type of antheridia that comes from northern France. However there are enough structural as well as molecular differences between the two (Table 1) to justify the creation of a new taxon.

The morphological and molecular characteristics indicate that the closest relative of *P. longisporangium* is *P. bifurcatum*, a new species recently described from northern France. However there are enough structural as well as molecular differences between the two (Table 1) to justify the creation of a new taxon.

References