Pythium sterilum sp. nov. isolated from Poland, Spain and France: its morphology and molecular phylogenetic position

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Abstract

In a survey of *Phytophthora* species associated with forest decline in Spain, Poland and France, we found three *Pythium* isolates, which have been characterized with internal transcribed spacer rRNA gene sequences and with classical morphological descriptors for *Pythium* spp. These isolates showed unique internal transcribed spacer sequences, different enough from those of any described species to justify new species status. These three distinct isolates failed to produce any sex organs with an entirely asexual reproduction and were found to represent a new species for which the name *Pythium sterilum* is proposed. This paper describes and illustrates the morphology of *P. sterilum* and presents its taxonomic position and relationships with other, related *Pythium* species belonging to clade K.

Introduction

The genus *Pythium*, belonging to the family of *Pythiaceae*, order *Perenosporales*, class Oomycetes, phylum *Heterokonta* and lineage *Stramenopila* is widely distributed throughout the world (Dick, 2001). More than 200 species of this genus have been described (Paul, 1999). Members of the genus *Pythium* are ubiquitous and occupy several ecological niches (Plaats-Niterinck, 1981). While most of these species are saprophytes in various types of soils and aquatic environments, many others are important plant pathogens. Some can also behave as parasites of mosquito larvae (Saunders *et al*., 1988) and one is known to be a mammalian pathogen (de Cock *et al*., 1987). The most comprehensive taxonomic account with descriptions of species was provided by van der Plaats-Niterink (1981), whose key was updated by Dick (2001). The historical lack of consensus on the most important morphological characteristics for identification (Levesque & de Cock, 2004), the high variability within the most important structures and considerable overlap among species, and the absence of diagnostic morphological structures for many isolates or species, all contributed to potential errors in identification, especially for those lacking many years of experience working with this genus (Levesque & de Cock, 2004).

Morphology-based taxonomy is increasingly being supplemented by molecular characteristics of a given species (Paul, 2003). The internal transcribed spacer (ITS) region of the rRNA gene sequences has become a useful tool in *Pythium* taxonomy and can be used for identifying or detecting different *Pythium* spp. (Paul, 2002a, b; Levesque & de Cock, 2004). Variability seems appropriate for studies at the species level as demonstrated by numerous works (Allain-Boule, 2004). Molecular phylogenies based on the ITS region have been recently produced for the genus *Pythium* (Matsumoto *et al*., 1999; Levesque & de Cock, 2004) and these ITS sequences have been made available in the GenBank database, (National Centre for Biotechnology Information, Bethesda, MA). This information can be used to determine the identity or phylogenetic position of unknown *Pythium* isolates. We collected a number of isolates of which the ITS sequence was significantly different from all recognized species. These isolates failed to produce any sex organs and the reproduction was entirely asexual. In the

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present paper, this new species, for which the name *Pythium sterilum* has been proposed, is described and its phylogenetic position within clade K of *Pythium* is discussed.

Materials and methods

Isolates

Pythium sterilum (PE101) was isolated from soil samples taken from a *Quercus ilex* stand in the vicinity of Madrid, Spain in the year 2001. Since then it has been maintained in the author's personal collection of pythiaceous species, at University of Cordoba, Spain. UASWS0186 has been isolated from soil samples from an alder stand in Raszyn, Poland and was maintained at School of Engineering of Lullier culture collection. UASWS0189 was isolated from soil samples from a vineyard in the Burgundian region in France and maintained in the same culture collection. Other oomycete materials were also taken from the same collection for comparison. The oomycetes were maintained on solid media like potato dextrose agar (PDA), potato carrot agar (PCA) and in water on boiled hemp-seed halves as described earlier (Paul *et al.*, 1998, 1999). Morphological description was done according to Paul *et al.* (1998, 1999).

DNA extraction

DNA was purified from mycelia with the use of the DNA-Easy Plant Mini kit (Qiagen, Basel, Switzerland), according to manufacturer's specifications. Quality was checked by visualization under UV light following electrophoretic separation with a molecular mass standard (*Hin*dIII/*Eco*RI DNA Marker, Biofinex, Praroman, Switzerland) in 1% agarose (Biofinex) gel in 1 × TBE, subjected to 100 V for 1 h and stained with ethidium bromide (0.5 mg mL^{-1}). Concentrations were assayed in a S2100 Diode Array spectrophotometer (WPA Biowave, Cambridge, UK).

DNA amplification

ITS amplifications of *Pythium* isolates were carried out using previously described universal primers ITS4 and ITS6 that target conserved regions in the 18S and 28S rRNA genes (White *et al.*, 1990, Cooke *et al.*, 2000). Reaction mixture contained 1 × PCR buffer (75 mm Tris-HCl (pH 9.0), 50 mM KCl, 20 mM $(NH_4)_2$ SO_4), 0.1 mM dNTPs, 0.25 μM of each primer, 1.5 mM $MgCl_2$, 1 U of Taq Polymerase (Biotools, Madrid, Spain) and 1 μL of mycelial DNA in a total volume of 50 μL. Amplifications were carried out in a Master Gradient thermocycler (Eppendorf, Basel, Switzerland) according to the following amplification programme: an initial denaturation step of 95 °C for 2 min followed by 30 cycles including denaturation for 20 s at 95 °C, annealing for 25 s at 55 °C and extension for 50 s at 72 °C. Amplification was terminated by a final extension step of 10 min at 72 °C (Cooke *et al.*, 2000). Amplicons were purified using a Minelute PCR Purification Kit (Qiagen), according to manufacturer's specifications. Quantity and quality were checked as described above for DNA extraction.

DNA sequencing and phylogenetic analysis

Purified amplicons were sequenced directly in both sense and antisense directions (Microsynth AG, Balgach, Switzerland). All pathogen samples were sequenced twice and a consensus sequence was created from the duplicates. DNA sequences have been deposited in GenBank under the accession numbers DQ217603 for *Pythium sterilum* (PE 101), DQ217604 for the polish isolate UASWS0186 and DQ220744 for the French isolate UASWS0189.

Sequences were aligned manually using Seaview (Galtier *et al.*, 1996). The maximum likelihood (ML) trees were obtained using the PhyML program (Guindon & Gascuel, 2003) with the HKY (Hasegawa *et al.*, 1985) model allowing transitions and transversions to have potentially different rates and general time reversible (GTR) model allowing all rates to be different (Lanave *et al.*, 1984, Rodriguez *et al.*, 1990). In order to correct the among-site rate variations, the proportion of invariable sites (I) and the α parameter of γ distribution (G), with eight rate categories, were estimated by the program and taken into account in all analyses. Nonparametric ML bootstraps (BSs) (with 100 replicates) were calculated using PhyML. Bayesian inferences (BI) were obtained with MrBayes v.3.0 (Huelsenbeck & Ronquist, 2001), using the same models of DNA evolution as for the ML analyses. The program was run for 1 700 000 generations, sampled every 100 generations, with four simultaneous chains. The trees, sampled before the chains reached stationarity, were discarded. NJplot and Treeview were used to view ML and Bayesian trees, respectively.

Results

Pythium sterilum, BELBAHRI & LEFORT sp. nov. (Figs 1 and 2) isolated from Spain, Poland and France.

Sporangia globosa, subglobosa, terminalia, interdum intercalaria, 16–32 μm diam., zoosporae incapsulate 7–12 μm diam. Oogonia, antheridia non observata. Incrementum radiale quotiadianum 12 mm 25 °C in agaro Solani tuberosi et Dauci carotae (PCA). Holotypus in herbario Universitatis Helvaticae Occidentalis conservatus (PE 101, UASWS0186, UASWS0189) *Distractum*.

Etymology

The oomycete is being named as *P. sterilum* because it fails to produce any sex organs. The reproduction is entirely asexual.

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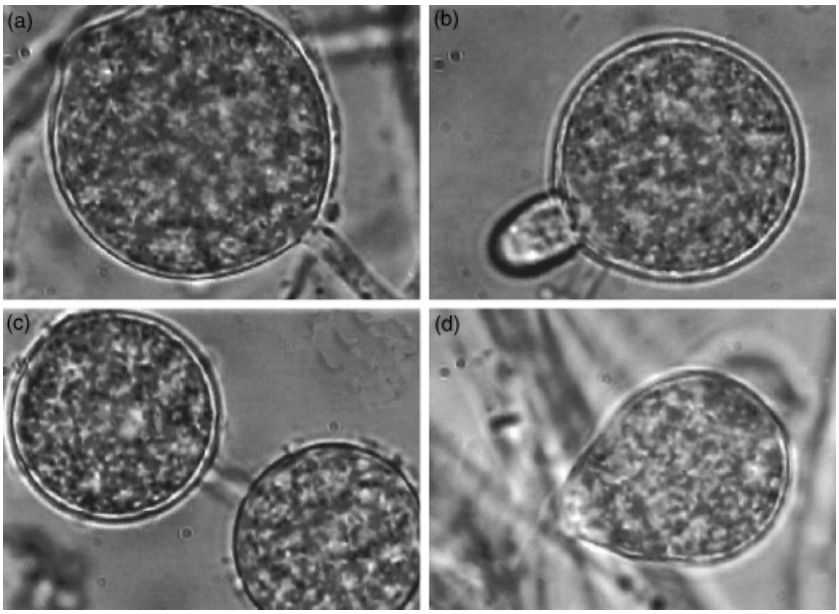

Fig. 1. (a) Terminal sporangia; (b) sporangia with an apical papilla; (c) intercalary sporangia and (d) terminal pyriform sporangia. Scale bar = 30 μm.

The oomycete grows well both on solid media as well as hemp seed halves in water. Its mycelium in water is hyaline, well branched with the main hyphae measuring up-to 5–6 μm wide. Colonies on PCA are submerged, and show a broad chrysanthemal pattern on this medium. Average radial growth of the oomycete at 25 °C on PCA is 12 mm/day. The oomycete reproduces in water on hemp-seed halves and on PCA giving plenty of asexual structures. Sexual structures were never observed in spite of repeated culturing in pond water, tap water, or soil extract water. Even after prolonged incubation and treatment by different sterols the oomycete failed to produce sex organs or oospores.

Asexual reproduction is in abundance through sporangia and zoospores. Sporangia (Fig. 1a–d) are usually globose, at times pyriform; terminal, sub terminal, or intercalary; measuring 16–32 μm in diameter (average 25.7 μm). Most of these are provided with a very conspicuous apical papilla (Fig. 1b). Sporangia are of proliferating type. Encysted zoospores measure from 7–12 μm (average 9.3 μm). measuring 18–35 μm in diameter (average 27.5 μm). Formation and dehiscence of the sporangial vesicle in *P. sterilum* is shown in Fig. 2.

The ITS region of the nuclear ribosomal DNA of the oomycete comprises of 835 bases (GenBank accession numbers DQ217603, DQ217604 and DQ220744). The three sequences for the three distinct isolates showed a very high similarity. Two sequences for isolates PE101 (Spain) and UASWS0186 (Poland) were identical and the third sequence for isolate UASWS0189 (France) displayed only three single nucleotide differences with the others over a sequence run of 835 bp. BLAST searches through the GenBank database content revealed a unique pattern different enough from those of any described species to justify a new species status. The closest matches were *P. citrinum* (GenBank accession AY197328.1) with a 97% similarity over a sequence run of 648 bp and an oomycete isolate named *Phytophthora cinnamomi* (GenBank accession L76535) with 95% similarity over a sequence run of 427 bp. The new species presented here is believed to belong to clade K according to Lévesque & de Cock (2004). The comparison of the ITS1 sequences of

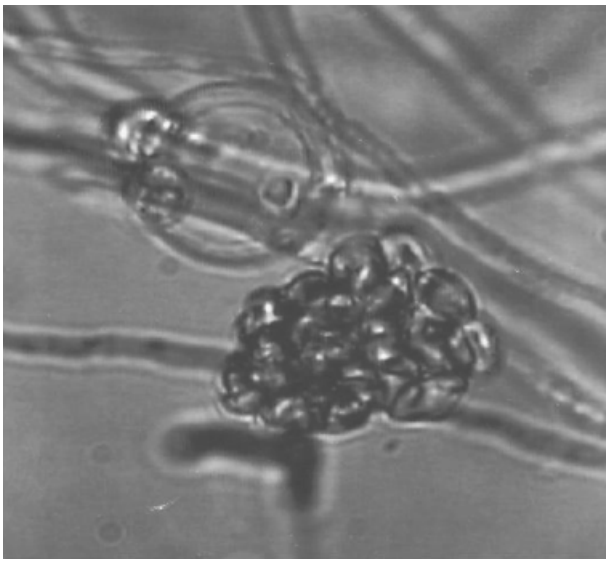

Fig. 2. Formation and dehiscence of the sporangial vesicle in *Pythium sterilum*. Bar = 60 μm.

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Phytophtora cinnamomi CR6B      CCACACCTAAAAAA--CTTTCCACGTGAACTGTTTTACTGTACCTTTGGGCCTCGCC--- 55
Pythium sterilum                CCACACCTAAAAAT--CTTTCCACGTGAATTGTTTTGCTGTACCTTTGGGCTTCGCC--- 55
Pythium carbonicum              CCACACCTAAAAAAATCTTTCCACGTGAACTGTTTTT--GTAAATTTGGGCTTCGCCTGC 58
Pythium montanum                CCACACCTAAAAAT--CTTTCCACGTGAACTGTTTT---GTAAATTTGGGCTTCGCCTGC 55
Pythium citrinum                CCACACCTAAAAAT--CTTTCCACGTGAATTGTTTTGCTGTAATTTAGGGGCGTGT---- 54
                                ************   ************ ******   ***  ** ***    *

Phytophtora cinnamomi CR6B      ---GTGGTCTTGTCCTTTGGTAAG--------------AGAAAGGGAAAGGCGCGGTTGG 98
Pythium sterilum                ---GTTGTCTTGTTCTTTTGTAAG--------------AGAAAGGGGGAGGCGCGGTTGG 98
Pythium carbonicum              TGCGTTCTTTTACCATTTAATTATTTATTTTTTCTTTGGTGAGGGAATGCGACGGCTTG 118
Pythium montanum                TGCGC-GCTTTGCTTTTTCGTGAA--------------------GAGTGCGACGGCTTG 94
Pythium citrinum                ----TCGTCGTGCGTTTT---------------------------------ATGATTCT 82
                                          *    ***                                  *  *

Phytophtora cinnamomi CR6B      AGGCCACCAGGG---GTGTGTTCGTCGCGGGTTGTTTCTTTTGTTGGAACTTGGGGGCCC 155
Pythium sterilum                AGGCCATCAGGG---GTGTGTTCGTCGCGGTTTGTTTCTTTTGTTGGAACTTGCGCGC-- 153
Pythium carbonicum              AGGCCATCAGGGTGCGCTTGCTTGGTTACTTTTGTTTGTACCATTCAAAAAAATGTAATC 178
Pythium montanum                AGGCCATCAGGG------------TGCGCTTGCTTGATTTTTTTTGGAATCGGGCGA-- 139
Pythium citrinum                A-----------------------------TGATTCTATGATTCACGCGCGAGTGC-- 109
                                *                                *  *    **         *

Phytophtora cinnamomi CR6B      GAGTAGTCCCTCTCTTT-CCAACCCATTTTTTGAATGAAAAACTGAACATACTGGGGGGA 214
Pythium sterilum                --GGATGCGTCCTTTTG-TCAACCCATTTTTTGAATGAAAAACTGATCATACTGTGGGGA 210
Pythium carbonicum              GAGCGAGTGCGCTCTTTGTCAACCCATTTCTTT----TGAAACTGATTATACTGTGGGGA 244
Pythium montanum                ------GTGCGCTCTTTGTCAACCCATTTCTTT----TGAAACTGATTATACTGTGGGGA 189
Pythium citrinum                ---------GTCCTTTTGTCAACCCATTTCTTT--TGAAAAACTGATCATACTGTGGGGA 158
                                          *  **   ********** **       *******  ****** *****

Phytophtora cinnamomi CR6B      CGAAAGTCTCTGCTTTTAACTAGATAG 241
Pythium sterilum                CGAAAGTCTCTGCTTTTAACTAGATAG 237
Pythium carbonicum              CGAAAGTCTCTGCTTTTAACTAGATAG 271
Pythium montanum                CGAAAGTCTCTGCTTTTAACTAGATAG 216
Pythium citrinum                CGAAAGTCTCTGCTTTTAACTAGATAG 185
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Fig. 3. CLUSTAL W (1.81) multiple sequence alignment of the internal transcribed spacer 1 sequences of *Pythium sterilum* (DQ217603) with those of *Pythium carbonicum* (AY191003), *Pythium montanum* (AY162278), *Pythium citrinum* (AY197328) and *Phytophtora cinnamomi* CR6B (L76535).

P. sterilum and related species within clade K is given in Fig. 3.

The position of the *Pythium* isolates sequences (DQ217603, DQ217604 and DQ220744) and additional sequences of clade K *Pythium* species (Matsumoto *et al.*, 1999) was illustrated in the BI trees represented in Fig. 4. The tree was rooted according to the tree of Lévesque & de Cock (2004) on the phylogeny of the genus *Pythium*.

In all analyses, the sequences of *Pythium sterilum* used form a monophyletic group supported by high values of BS and posterior probabilities (PP) (100% BS and 100% PP). In the ML (data not shown) and BI trees (Fig. 4), the new sequences form a sister group to *Pythium citrinum*. Lévesque & de Cock (2004) noticed that *P. citrinum* and an isolate identified as *P. ostracodes* (GI 27448074) were not similar to anything else in clade K. Thus, *P. sterilum* consists of a new species clustering with *P. citrinum* in the subclade of *P. boreale*, *P. ostracodes*, *P. oedochilum*, *P. chamaehyphon* and *P. helicoides*.

Discussion

Pythium sterilum grows well in solid media as well as hemp seed halves, it fails to form any sexual organs and was named accordingly. Since its isolation in 2001 in Spain and its re-isolation from Poland and France in 2004 it was maintained in the author's collection of Fungi and *Pythium* spp. ITS sequence analysis of the three isolates showed unique patterns and the sequence was similar to a sequence in GenBank named as *Phytophthora cinnamomi* CR6B (L76535). Morphological examination of this isolate is necessary in order to assign it to the genus *Phytophthora* or to *Pythium*.

Phylogenetic analysis of the ITS region consistently indicates that *Pythium* isolates described in this study shared a common ancestor with all *Pythium* sequences used in the present study. In all analyses, the sequences of *Pythium sterilum* isolates of the present study form a monophyletic group supported by high values of BS and posterior probabilities confirming the species status of these isolates. In our study, *Pythium sterilum* displays a higher sequence homology with *P. citrinum*. Thus molecular evidence indicates that *P. sterilum* consists of a new species clustering with *P. citrinum* in the subclade of *P. boreale*, *P. ostracodes*, *P. oedochilum*, *P. chamaehyphon* and *P. helicoides*.

Pythium sterilum clusters within clade K, a clade subjected to some debate over whether or not the species belonging to clade K are appropriately classified in *Pythium*

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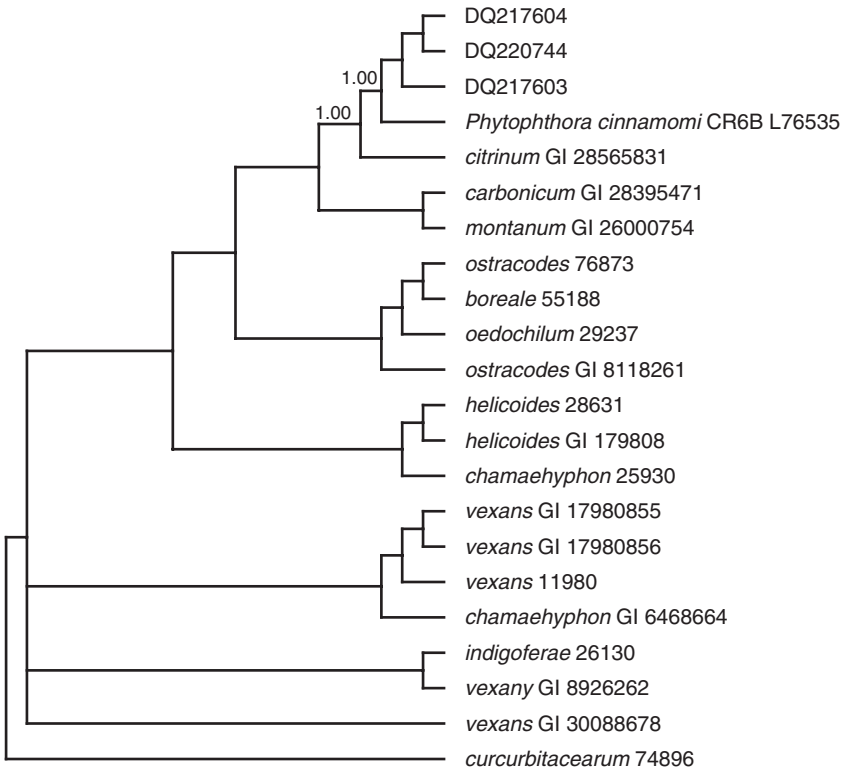

Fig. 4. Phylogenetic position of DQ217603, DQ217604 and DQ220744 inferred from internal transcribed spacer sequences by using Bayesian inferences method. The number at nodes *Pythium citrinum – P. sterilum*-L76535 and L76535-*P. citrinum* represent posterior probabilities.

(Briard *et al.*, 1995; Panabieres *et al.*, 1997; Dick, 2001). Moreover, there are some similarities between these *Pythium* clade K species and *Phytophthora* spp. such as the presence of elicitin genes (Panabieres *et al.*, 1997). As mentioned earlier by Lévesque & de Cock (2004), we could also conclude that, when using all available *Pythium* ITS sequences for analysis of the taxonomic position of clade K, *Pythium* becomes polypheletic because of this clade. Using all available oomycete sequences in a new phylogenetic study should be useful to solve the generic status of clade K.

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