
Morphology, taxonomy, and phylogenetic analysis of a new species of *Pythium* isolated from France

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During the course of investigation on pythiaceous fungi occurring in the burgundian vineyards, a new species of *Pythium* has been isolated. This oomycete is characterised by its non-proliferating and non sporulating type of sporangia (hyphal bodies), smooth walled oogonia that have hypogynous, monoclinal or at times declinal antheridia, and smooth walled oospores that can have up to 3 per oogonia. The oomycete produces appressoria from which sexual structures may originate which is a rare feature for the genus. Sequence analyses of its ITS regions of rDNA show a close relationship with *P. debaryanum* and *P. violae* but has its own distinguishing characteristics. Morphological and molecular features of this isolate justify its description as a new species: *Pythium viniferum*. When grown together with *Botrytis cinerea*, the causal agent of the grey mould disease of grapevine, this oomycete shows a pronounced antagonism and suppresses its growth. Since it is not pathogenic to the grapevine it can be used as a bio-control agent. Morphology, antagonism with *Botrytis cinerea*, and the phylogenetic position of the new species are discussed here.

Key words: antheridia, biological control, *Botrytis cinerea*, ITS region, oogonia, *Pythium viniferum*, rRNA

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Introduction

The genus *Pythium* is a complex genus containing over 200 described species that occupy a variety of terrestrial and aquatic ecological habitats (Dick, 2001). Perhaps the most economically important members of this genus are plant pathogens (Hendrix and Campbell, 1973), many of which have a broad host range and cause losses by both pre- and post-emergence damping-off (Erwin and Ribeiro, 1996), as well as by reduction in plant growth and yield due to root rot (Plaats-Niterinck, 1981). Members of this genus can be pathogens on a range of organisms, including algae, fish, shrimp, or mosquito larvae (Saunders *et al.*, 1988). One species is a mammalian pathogen and can cause human disease (De Cock *et al.*, 1987). Some species in the genus can be beneficial, functioning as biological control agents protecting against

pathogenic fungi (Abdelghani *et al.*, 2004, Paul, 2004) or for production of polyunsaturated fatty acids for use as human dietary supplements.

A range of morphological criteria has been used to classify members of this genus (Plaats-Niterinck, 1981, Dick, 2001), however, the high variability among different structures and considerable overlap of some of these features among different species complicates accurate species identification (Lévesque and De Cock, 2004). In addition, these features have not been useful for determining the evolutionary relationships among the disparate species. Studies investigating the evolutionary relationships in the genus became available and are adequate to evaluate the genus as a whole (Matsumoto *et al.*, 1999, Lévesque and De Cock, 2004). These investigations used molecular data to determine evolutionary relationships among species and determine the

relationships between shared morphological characteristics and evolutionary relatedness. This molecular data also is useful for taxonomic purposes and identification of unknown isolates with greater accuracy (Lévesque and De Cock, 2004).

The phenomenon of mycoparasitism is very widespread in nature. This term is generally used to include parasites that coil around the host hyphae or overgrow other colonies on agar. This may involve either penetration of the host hyphae or antagonism by the production of antibiotics (Denis and Webster, 1971), toxic radicals (Kim *et al.*, 1990) or wall lytic enzymes (Chet, 1987). This antagonistic behaviour is known for some species within the genus *Pythium*. Most of the well known mycoparasites of this genus possess spiny oogonia such as *Pythium oligandrum*, *P. acanthicum*, *P. periplocum* and *P. acanthophoron* (Paul, 1999). Few species having smooth-walled oogonia such as *P. mycoparasiticum* (Jones and Deacon, 1995), *P. contiguanum* (Paul, 2000) and *P. paroecandrum* (Abdelghani *et al.*, 2004) have also been reported to behave as mycoparasites.

In this study, morphological details of *P. viniferum*, its phylogenetic affinities based on rDNA sequence data (ITS+5.8S) and its behaviour towards *B. cinerea*, the grapevine pathogen, are discussed.

Materials and methods

Fungal strains and DNA extraction

Pythium viniferum (Type strain, F-1201) was isolated from soil samples taken in different vineyards situated in the outskirts of the French city Dijon in the Burgundian region by using traditional baiting techniques in which the soil samples are mixed with sterile distilled water and baited with boiled hemp seed halves (Plaats-Niterinck, 1981, Paul 2004). It occurred four times 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). Since then all these isolates have been maintained in the author's personal collection of pythiaceous species at "Institut Jules Guyot", in Dijon, France. The grey mould pathogen, *B. cinerea* (BC-03) was obtained from the culture

collection of the first author maintained at the same institute on PDA (Potato dextrose agar). *Pythium viniferum* was identified by the help of keys provided by Middleton (1943), Plaats-Niterinck (1981) and Waterhouse (1967).

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 (HindIII/EcoRI DNA Marker, Biofinex, Switzerland) in 1% agarose (Biofinex) gel in 1 × TBE, subjected to 100 V for 1 hour and stained with ethidium bromide (0.5 mg/ml⁻¹). Concentrations were assayed in a S2100 Diode Array spectrophotometer (WPA Biowave, Cambridge, UK).

Amplification, sequencing and phylogenetic analysis

ITS amplifications of *Pythium* isolates were carried out using previously described universal primers ITS4 and ITS6 (White *et al.*, 1990; Belbahri *et al.*, 2006a). Reaction mixture contained 1 × PCR buffer (75 mM Tris-HCl (pH 9.0), 50 mM KCl, 20 mM (NH₄)₂ SO₄), 0.1 mM dNTPs, 0.25 μM of each primer, 1.5 mM MgCl₂, 1 U of Taq Polymerase (Biotools, Spain) and 1 μl of mycelial DNA in a total volume of 50 μl. Amplifications were carried out in a Master Gradient thermocycler (Eppendorf, Germany) according to the following amplification programme: an initial denaturation step of 95°C for 2 minutes followed by 30 cycles including denaturation for 20 seconds at 95°C, annealing for 25 seconds at 55°C and extension for 50 seconds at 72°C. Amplification was terminated by a final extension step of 10 minutes at 72°C (Belbahri *et al.*, 2006b; Paul *et al.*, 2006). 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. Purified amplicons were sequenced directly in both sense and antisense directions (Microsynth AG, Switzerland). *Pythium* samples were sequenced twice and a consensus sequence was created from the duplicates. DNA sequences have been

deposited in Genbank under the accession number AY455694.

Sequences were aligned manually using Seaview (Galtier *et al.*, 1996). The maximum likelihood (ML) trees were obtained using the PhyML program (Guindon and 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 alpha parameter of gamma distribution (G), with 8 rates categories, were estimated by the program and taken into account in all analyses. Non parametric ML bootstraps (with 100 replicates) were calculated using PhyML. Bayesian inferences (BI) were obtained with MrBayes v.3.0 (Huelsenbeck and Ronquist, 2001), using the same models of DNA evolution as for the ML analyses. The program was run for 2,000,000 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.

Interaction between B. cinerea and P. viniferum

Inter-hyphal interactions between *B. cinerea* and *P. viniferum* were studied by placing both organisms on the same PDA plate on opposite ends and also on a thin film of PDA on a glass slide as described earlier (Paul, 1999, Jones and Deacon, 1995). After two weeks of incubation at 25°C, a small square of the PDA film from the zone of contact, was aseptically cut and placed on a fresh slide in a drop of sterile water. A cover slip was placed on the film and this was sealed with nail polish to avoid dehydration. The slide was then observed microscopically.

Results

Morphological descriptions

Pythium viniferum B. Paul, **sp. nov.** (Figs 1-7)
Mycobank: CBS 499736

Etymology: The oomycete is named as *Pythium viniferum* because it was isolated from a vineyard.

Sporangio et zoosporis non observata. *Corporibus hypharum* globosa, cylindrosa, intercalaria, vel terminalia, 7-25 µm diam., zoosporae non observata. *Oogonia* globos vel cylindrosa, 17-29 µm diam. *Antheridia* 1-5, hypogynata, monoclinata raro diclinata. *Oogonia* continentia unam, duas, interdum trias oosporas, pleroticas vel apleroticas 15-22 µm diam, globosas vel cylindrosas, paries 1-2 µm crassus. *Incrementum* radiale quotidianum 25 mm 25°C in agaro Solani tuberosi et Dauci carotae (PCA).

Holotypus in herbario Universitatis Bourgogne conservatus (F-1201).

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 7 µm wide and bearing some short lateral branches resembling like spines. *Colonies* on PCA are submerged, and show a radiate to arachnoids pattern on this medium. Average radial growth of the oomycete at 25°C on PCA is 25 mm/day. The oomycete reproduces in water on hemp-seed halves and on PCA giving plenty of asexual and sexual structures.

Sporangia or *hyphal swellings* are produced plentifully (Fig. 1 A-F; 2 A-B). These are of various sizes and shapes - spherical, ovoid to elongated (Fig 2 A-B). The spherical ones measure between 7-25 µm (\bar{x} = 11.8 µm) in diameter. These are terminal and intercalary. The hyphal bodies germinate through 1-6 germ tubes (Fig. 1 F). Zoospores were never observed in spite of repeated re-culturing, at different temperatures, in sterile distilled, pond, and soil extract water.

Female gametangia (oogonia) are mostly intercalary (Fig. 1 K) occasionally terminal, sometimes catenulate, at times formed inside appressoria (Fig. 1 G; 2 C). They measure between 17-29 µm (\bar{x} = 22.3 µm). Usually these are spherical but at times these can be elongated or even dumbbell shaped and can measure up to 45 µm in length.

Male gametangia (antheridia) are hypogynous (Fig. 1 H, J; 2 F), monoclinous sessile (Fig. 1 I) or monoclinous on short branches (Fig. 1 K; 2 D), at times diclinous (Fig. 1 L; 2 E), 1-5 per oogonia. *Antheridial* cells are conspicuous and at times bi-lobed. The monoclinous stalked antheridia make a broad apical contact with the oogonia.

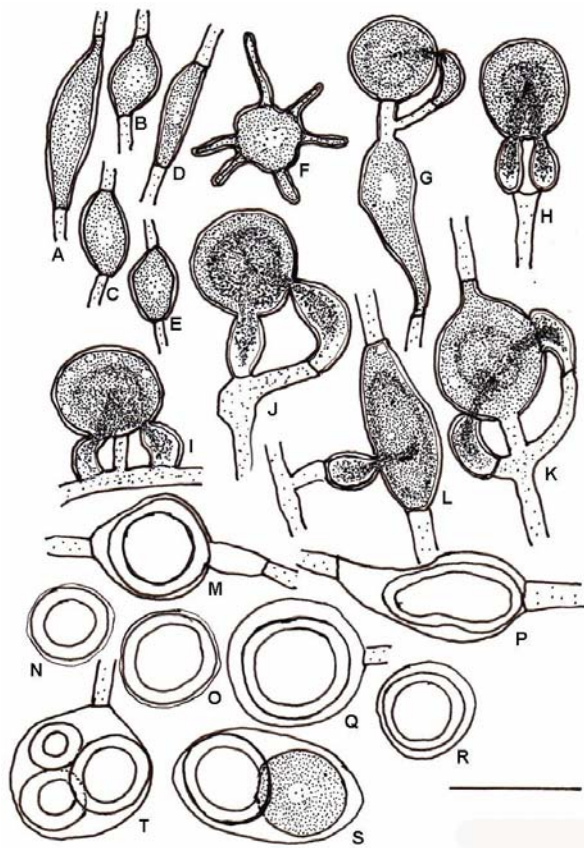


Fig. 1. *Pythium viniferum*. A-E. Hyphal bodies. F. Germinating hyphal bodies. G. Antheridia and oogonia borne on a sickle shaped appressoria. H. Double hypogynous antheridia applied to an oogonium. I. Sessile monoclinal antheridia. J. Monoclinal and hypogynous antheridia. K. Intercalary oogonia supplied with two monoclinal stalked antheridia. L. Diclinous antheridia. M-O. Plerotic single oospores. P-R. Aplerotic single oospores. S. Double oospores in an oogonium. T. Triple oospores in an oogonium. Bar = 25 μ m.

Zygote (oospores) usually plerotic (Fig. 1 M-O; 3 A) but at times aplerotic specially in the cylindrical oogonia. The oospores are generally spherical but in cylindrical (Fig. 3 C-D) or peanut-shaped oogonia these can take the shape of the oogonia. Oospores are generally single (Fig. 1 P-R; 3 B) but at times there are double (Fig. 1 S; 3 E) and triple oospore (Fig. 1 T; 3 F) formation. Spherical oospores measure 15-22 μ m (\bar{x} = 17.2 μ m). Oospore wall ranges from 1-2 μ m in thickness (\bar{x} = 1.2 μ m). The oomycetes produces spherical to sickle shaped appressoria in plenty. These appressoria are usually associated with sexual structures like those found in *P. abappressorium* (Fig.1G; 2C)

Phylogenetic position of Pythium viniferum

The ITS regions (+5.8S) of the nuclear rDNA of *Pythium viniferum* consists of 913 bases (GenBank accession number AY455694). BLAST search reveals a high homology to ITS sequences of *P. debaryanum*, *P. violae*, *P. irregulare*, *P. sylvaticum*, *P. spinosum* and *P. kunmingense*. The closest matches were *P. debaryanum* (GenBank accession AY598704) with 99.6% similarity over a sequence run of 913 bp; *P. violae* (AJ233463) with 98.4% similarity over a sequence run of 916 bp; *P. irregulare* (AB107998) with 96.9% similarity over a sequence run of 916 bp; *P. sylvaticum* (AF452139) with 95% over a sequence run of 907 bp; *P. spinosum* (AB108006) with 91.9% similarity over a sequence run of 916 bp; and *P. kunmingense* (AY598700) with 91.4% similarity over a sequence run of 916 bp. A clustal multiple alignments of all these species is given in Fig. 5. The new species presented here is believed to belong to clade F according to Lévesque and De Cock (2004). The position of the *Pythium viniferum* sequence (AY455694) and additional sequences of other *Pythium* species belonging to the “clade F” is illustrated in the ML tree (Fig. 6). The tree was rooted with *P. attrantheridium*, based on previously published *Pythium* phylogenies (Lévesque and De Cock 2004)

In the ML (Fig. 5) and Bayesian trees (data not shown), the new sequences form a sister group to *P. debaryanum*. Lévesque and De Cock (2004) noticed that *P. debaryanum* (CBS752.96) and an isolate identified as *P. violae* (GI6468680, Matsumoto *et al.*, 1999) were closely related. They suggested that this group of strains needs to be compared with species from clade F to see if it is a new species, or if the name *P. debaryanum* should be used for this unique cluster.

Antagonism with B. cinerea

In the hyphal interaction experiment, the colonies of *P. viniferum*, which are whitish in colour spread into the greyish colonies of *B. cinerea* (Fig. 4). The grey mould pathogen fails to spread into the zone of *P. viniferum* even after prolonged incubation (Fig. 4). Microscopic observation of the mycelium in

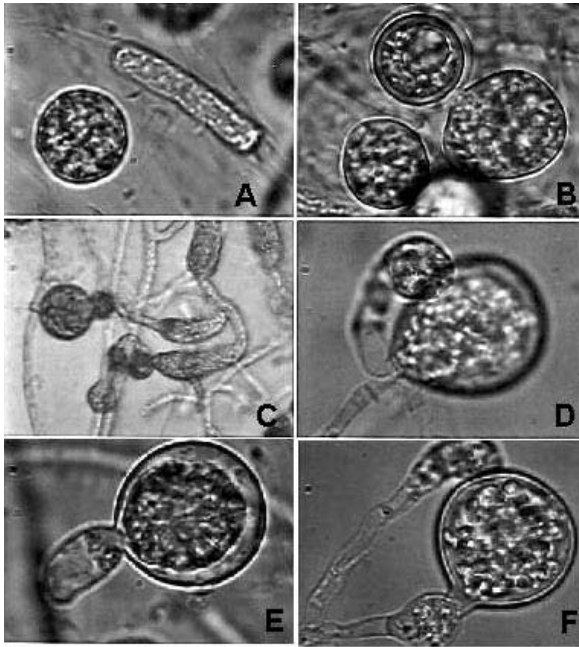


Fig. 2. *Pythium viniferum*. A-B. Spherical and elongated hyphal bodies. C. Sickle shaped appressoria bearing sexual structures. D. Oogonia with monoclinous stalked antheridia. E. Diclinous antheridia. F. Monoclinous and hypogynous antheridia. Bars: A-B and D-F = 60 μ m; C = 25 μ m.

the zone of contact revealed that *P. viniferum* does not coil around the mycelium of *B. cinerea* but its presence brought about a coagulation of the host cytoplasm which eventually empties (Fig. 7).

Discussion

Pythium viniferum is characterised by its non-proliferating and non sporulating type of sporangia (hyphal bodies), smooth walled oogonia that are supplied with hypogynous, monoclinous or at times diclinous antheridia, and smooth walled oospores that can be up to 3 per oogonia. The oomycete produces appressoria plentifully and at times the sex organs arise from these appressoria, a rare feature of the genus.

ITS sequence comparison of *Pythium viniferum* showed high similarity to two *Pythium* sequences in Genbank *P. debaryanum* (AY598704) and *P. violae* (AJ233463). However, these three species are different in their morphological characteristics.

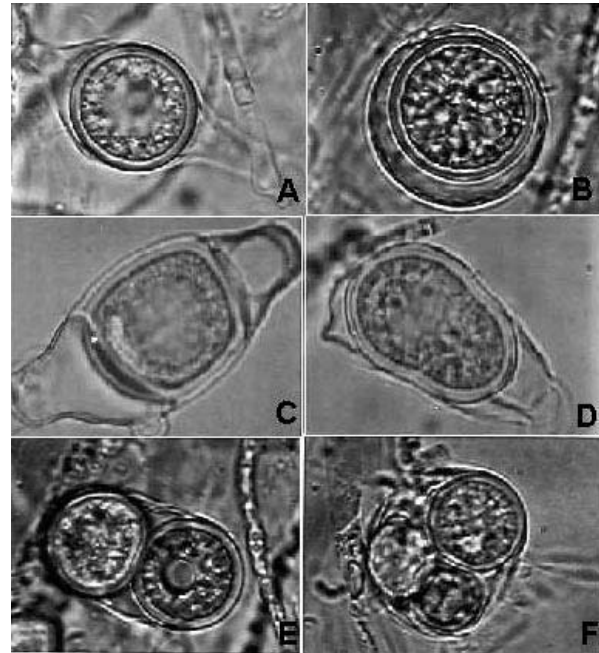


Fig. 3. *Pythium viniferum*. A. Plerotic oospore. B. Aplerotic oospore. C-D. Cylindrical plerotic oospores. E. Double oospore. F. Triple oospore. Bar = 25 μ m.

In all analyses, *P. viniferum* clusters with *P. debaryanum* and has a sister group relationship with *P. violae*. The phylogenetic association with *P. debaryanum*, however, did not receive any statistical support (Fig. 5). *Pythium viniferum* displays a higher sequence homology with *P. debaryanum*. *Pythium viniferum* clusters within clade F, a clade that consists of important plant pathogens with a world-wide distribution. *P. viniferum* from this study was close to *P. debaryanum* (CBS 752.96) which is in turn the closest relative of *P. violae* sequence (GI 6468690, Matsumoto *et al.*, 1999). However the situation within clade F and G concerning *P. violae* is not clear. Lévesque and De Cock (2004) suggested that the morphological concept of *P. violae* evidently needs revision. CBS 159.64, chosen by Van der Plaats Niterink (1981) as representative of the species, is probably an unfortunate choice and morphological characters for identification and species boundaries of isolates belonging to clade G needs an in depth investigation (Lévesque and De Cock, 2004). The morphological differences between these three

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P. viniferum          CCACACCTAAAAAACTTTCCACGTGAAGTGTCTGTATTTGTTTTGTGTCTGCGCGTTGCT
P. spinosum         CCACACCTAAAAAACTTTCCACGTGAAGTGTCTGTATTTGTTTTGTGTCTGCGCGTTGTT
P. kunmingense     CCACACCTAAAAAACTTTCCACGTGAAGTGTCTGTATTTGTTTTGTGTCTGCGCGTTGTT
P. sylvaticum      CCACACCTAAAAAACTTTCCACGTGAAGTGTCTGTATTTGTTTTGTGTCTGCGCGTTGCT
P. irregulare     CCACACCTAAAAAACTTTCCACGTGAAGTGTCTGTATTTGTTTTGTGTCTGCGCGTTGCT
P. debaryanum     CCACACCTAAAAAACTTTCCACGTGAAGTGTCTGTATTTGTTTTGTGTCTGCGCGTTGCT
P. violae         CCACACCTAAAAAACTTTCCACGTGAAGTGTCTGTATTTGTTTTGTGTCTGCGCGTTGCT
*****

P. viniferum          GGCCTGCGTTTGCTTTCGCGCTTCGGCGTTTCGCTAGTGCCTGCTGGCGGTGCGCGGACTGAA
P. spinosum         GGCATGCATTTGCTTACACTTTGGTGTGTTGCGAGTGCCTGTTGGCAGTGTGCGGACTGAA
P. kunmingense     GGCCTGCGTTTGCTTTCGCGCTTCGGCGTTTCGCTAGTGCCTGCTGGCGGTGCGCGGACTGAA
P. sylvaticum      GGCCTGCGTTTGCTTTCGCGCTTCGGCGTTTCGCTAGTGCCTGCTGGCGGTGCGCGGACTGAA
P. irregulare     GGCCTGCGTTTGCTTTCGCGCTTCGGCGTTTCGCTAGTGCCTGCTGGCGGTGCGCGGACTGAA
P. debaryanum     GGCCTGCGTTTGCTTTCGCGCTTCGGCGTTTCGCTAGTGCCTGCTGGCGGTGCGCGGACTGAA
P. violae         GGCCTGCGTTTGCTTTCGCGCTTCGGCGTTTCGCTAGTGCCTGCTGGCGGTGCGCGGACTGAA
*** ** *

P. viniferum          CGAAGGTTGTGT--TGCTGTGTGCCTGCTGCATTGCTGACTTTGCATTAAGTTGTATCGT
P. spinosum         CGAAGGTTGTGTGTTTATGTGTCTGCTGCATTGCTGACTTTGCATTAAGTTGTATCGT
P. kunmingense     CGAAGGTTGTGTGTTTATGTGTCTGCTGCATTGCTGACTTTGCATTAAGTTGTATCGT
P. sylvaticum      CGAAGGTTGTGT--TGCTGTGTGCCTGCTGCATTGCTGACTTTGCATTAAGTTGTATCGT
P. irregulare     CGAAGGTTGTGT--TGCTGTGTGCCTGCTGCATTGCTGACTTTGCATTAAGTTGTATCGT
P. debaryanum     CGAAGGTTGTGT--TGCTGTGTGCCTGCTGCATTGCTGACTTTGCATTAAGTTGTATCGT
P. violae         CGAAGGTTGTGT--TGCTGTGTGCCTGCTGCATTGCTGACTTTGCATTAAGTTGTATCGT
*****

P. viniferum          CTTGGCGGAGCGGCGGGTGTG-ATGCGACCAGCTGACTTATTTTTTCAAACCCCATACC
P. spinosum         CTTGGCGGAGTGGCGGGTACTGTGCATGCACAGCTGACTTATTTTTTCAAACCCCATACC
P. kunmingense     CTTGGCGGAGTGGCGGGTACTGTGCATGCACAGCTGACTTATTTTTTCAAACCCCATACC
P. sylvaticum      CTTGGCGGAGCGGCGGGTGTG-ATGCGACCAGCTGACTTATTTTTTCAAACCCCATACC
P. irregulare     CTTGGCGGAGCGGCGGGTGTG-ATGCGACCAGCTGACTTATTTTTTCAAACCCCATACC
P. debaryanum     CTTGGCGGAGCGGCGGGTGTG-ATGCGACCAGCTGACTTATTTTTTCAAACCCCATACC
P. violae         CTTGGCGGAGCGGCGGGTGTG-ATGCGACCAGCTGACTTATTTTTTCAAACCCCATACC
*****

P. viniferum          TAACTTACTGATTATACTGTGAGAACGAAAGTTCTTGCTTTTA-CTAGATAA---
P. spinosum         TAAATGACTGATTATACTGTGAGAACGAAAGTTCTTGCTTTAACTAGATAA---
P. kunmingense     TAAATGACTGATTATACTGTGAGAACGAAAGTTCTTGCTTTAACTAGATAAACA
P. sylvaticum      TAACTTACTGATTATACTGTGAGAACGAAAGTTCTTGCTTTTA-CTAGATAA---
P. irregulare     TAACTTACTGATTATACTGTGAGAACGAAAGTTCTTGCTTTTA-CTAGATAA---
P. debaryanum     TAACTTACTGATTATACTGTGAGAACGAAAGTTCTTGCTTTTA-CTAGATAACA-
P. violae         TAAATGACTGATTATACTGTGAGAACGAAAGTTCTTGCTTTTA-CTAGATAA---
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Fig. 4. CLUSTAL W multiple sequence alignment of ITS 1 regions of the rDNA of *P. spinosum* (AB108006), *P. kunmingense* (AY598700), *P. sylvaticum* (AF452139), *P. irregulare* (AB107998), *P. debaryanum* (AY598704) and *P. viniferum* (AY455694).

Table 1: Differences between *P. viniferum*, *P. debaryanum* and *P. violae*.

Characteristics	<i>P. viniferum</i>	<i>P. debaryanum</i>	<i>P. violae</i>
Growth on PCA	25 mm/day – 25°C	30 mm/day	15 mm/day – 25°C
Appressoria	Present	Present	-
Zoospores	Not present	Present	-
Oogonia	Mostly intercalary	Mostly terminal	Terminal or intercalary
Oogonial size	17-29 µm (\bar{x} = 22.3 µm)	13-22 µm (\bar{x} = 17.1 µm)	25-38 µm (\bar{x} = 29.5 µm)
Oospores	Spherical, elongated and irregular, up to 3 per oogonia,	Mostly spherical	Spherical
	Plerotic, rarely aplerotic	Plerotic, rarely aplerotic	Aplerotic
Oospore size	15-22 µm (\bar{x} = 17.1 µm)	12-21 µm (\bar{x} = 16.1 µm)	22-32 µm (\bar{x} = 27 µm)
Wall	1-2 µm thick	1 µm thick	Up to 3 µm thick

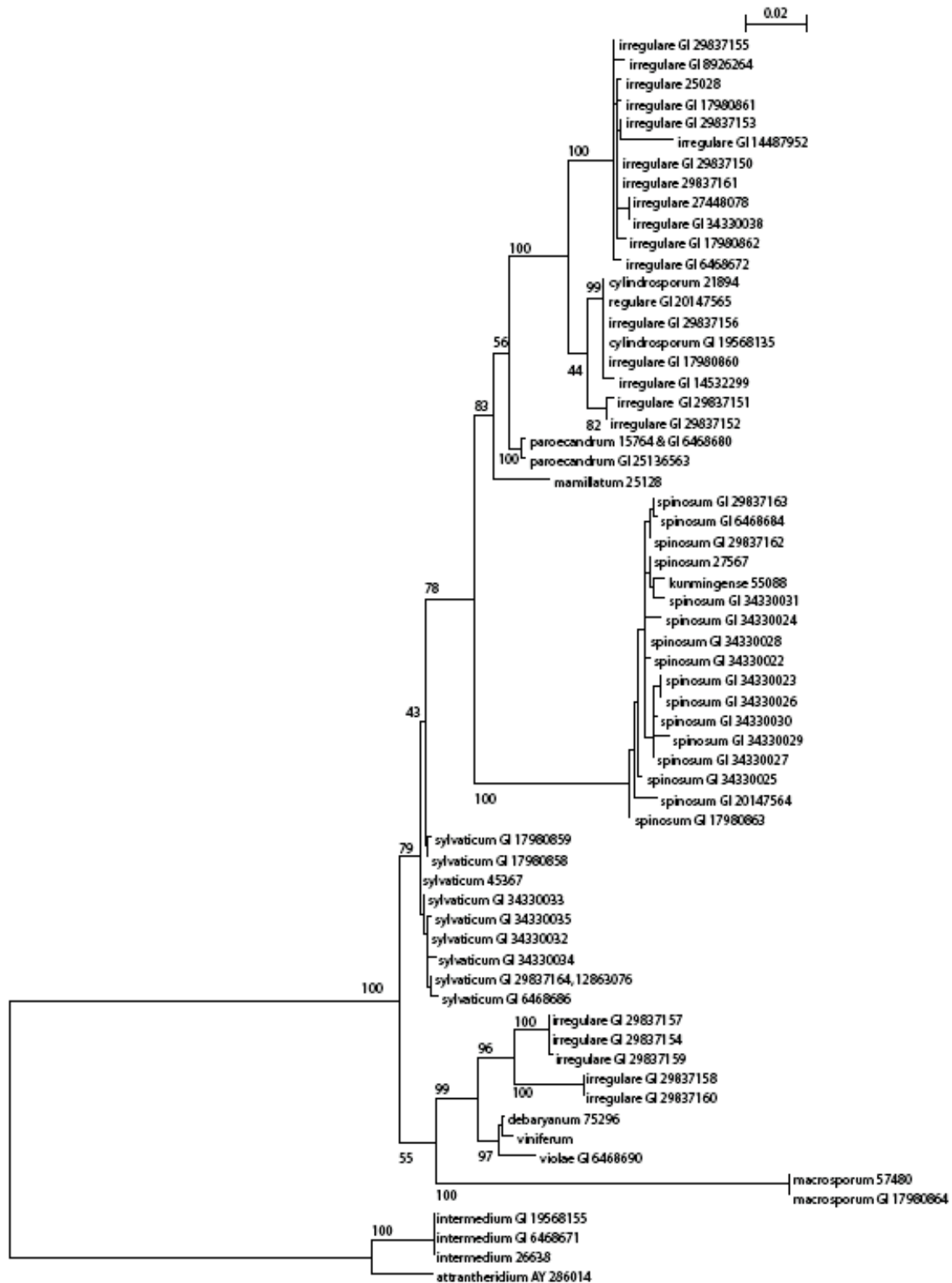


Fig. 5. Phylogenetic position of *Pythium viniferum* isolates inferred from ITS sequences by using ML method with GTR + G + I model. The numbers at nodes are non-parametric bootstrap values. The length of branches is proportional to the number of substitutions per site as indicated in the scale.

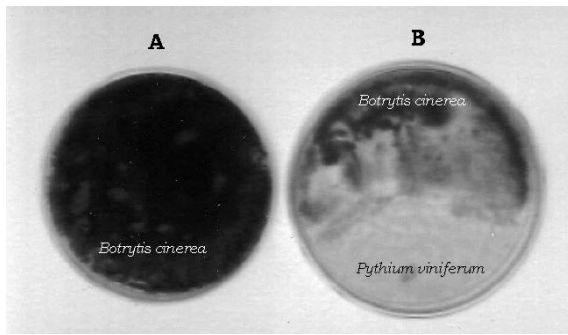


Fig. 6. Antagonism between *P. viniferum* and *B. cinerea*. **A.** Normal growth of *B. cinerea* on PDA. **B.** Overgrowth of *P. viniferum* on *B. cinerea*.

closely related species, *P. viniferum*, *P. debaryanum*, and *P. violae* are summarized in Table 1.

The hyphal interaction experiments show that the mycelium of *P. viniferum* can rapidly bring about the coagulation and ultimately death of *B. cinerea*. The oomycete does not physically enter the host hyphae or entwine it. The widespread destruction caused may be due to enzymatic activities of the oomycete. Thus the action of *P. viniferum* on the mycelium of *B. cinerea* is supposed to be a mycoparasitic one, and since the former is not a pathogen of the grapevine, it may be used as a bio-control agent against the latter.

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