A new species of *Pythium* with ornamented oogonia: morphology, taxonomy, internal transcribed spacer region of its ribosomal RNA, and its comparison with related species

Bernard Paul[1], Kanak Bala[1], Belbahri Lassaad[2], Gautier Calmin[2], Esperanza Sanchez-Hernandez[3] & Francois Lefort[2]

[1]Laboratoire des Sciences de la Vigne, Institut Jules Guyot, Université de Bourgogne, Dijon, France; [2]Laboratory of Applied Genetics, School of Engineering of Lullier, University of Applied Sciences of Western Switzerland, Jussy, Switzerland; and [3]Agroforestry Pathology, Department of Agronomy, University of Cordoba, Cordoba, Spain

Correspondence: Bernard Paul, Laboratoire des Sciences de la Vigne, Institut Jules Guyot, Université de Bourgogne, BP 27877, 21078 Dijon, France. Tel.: +33 3 80396326; fax: +33 3 80396326; e-mail: bernard.paul@u-bourgogne.fr

Received 18 October 2005; revised 2 November 2005; accepted 3 November 2005.
First published online January 2006.

doi:10.1111/j.1574-6968.2005.00048.x

Editor: Richard Staples

Keywords
Pythium spiculum; oogonia; antheridia; oospores; ITS region; rRNA; phylogenetic tree.

Abstract

Pythium spiculum sp. nov. was isolated from soil samples taken in a vineyard in the Burgundian region of France and from different locations in Spain and Portugal. The oomycete has spiny oogonia and does not sporulate readily. It resembles *Pythium mamillatum* Meurs, but has its own distinguishing characteristics. It also exhibits sickle-shaped as well as spherical appressoria which at times are associated with sex organs like those found in *Pythium abappressorium* Paulitz and *Pythium contiguanum* Paul. Sequencing of the internal transcribed spacer region of its nuclear ribosomal DNA and a close look at its morphological characters have now enabled us to describe it as a new species. The internal transcribed spacer region of its rRNA gene sequence is comprised of 945 bases. This oomycete is closely related to the members that form ornamented or spiny oogonia like *Pythium mamillatum, Pythium spinosum* and *Pythium irregulare* but also with those producing smooth-walled oogonia like *Pythium paroecandrum, Pythium sylvaticum* and *Pythium cylindrosporum*. Taxonomic description of this new species, its comparison with related oomycetes, the sequence of the internal transcribed spacer region of its rRNA gene and the phylogenetic tree, are given here.

Introduction

The members of the genus *Pythium* are distributed throughout the world (Martin, 1995). Most of these are 'amphibians' and can be found in terrestrial as well as aquatic environments. The 'oomycetes' no longer enjoy the place of 'true fungi' but are now supposed to be closer to 'algae' than to the fungal world. They are now placed within the kingdom 'Chromista' (Cavalier-Smith, 1998). Quite a number of the members of the genus *Pythium* are known for their ornamented oogonia (female gametangia), out of which the most commonly found is *Pythium echinulatum*. The oogonial ornamentations, not only makes these members 'spectacular,' but are also of great taxonomic value. Plaats-Niterink (1981) described 21 species of *Pythium* having ornamented oogonia. Ever since that date, the first author of this manuscript has added three more: *Pythium ornamentatum*, *Pythium radiosum* and *Pythium ornacarpum* (Paul, 1987, 1992, 1999). This is the fourth to be added to the group. The name *P. spiculum* is being given to this oomycete to highlight the oogonial ornamentations (latin spiculum = spines). On the basis of morphological characters, the sequence of the internal transcribed spacer (ITS) region of its rRNA, and the phylogenetic analysis, it has been possible to erect this new taxon. In the recent past, morphological differences supplemented with sequence diversity have played an important role in the erection of new species (Paul, 2002, 2003; Timothy *et al*., 2003).

Pythium spiculum was isolated from 12 different places in France, Spain and Portugal. The isolates are: F-1022 from soil samples taken in the Burgundian vineyard; PA-53, PA-54, PA-55 and PA-56 from soil samples collected under oak trees in the Huelva province in Spain and isolates PE 154, PE 155, PE 156, PE 157, PE 158, PE 159 and PE 160 taken from soil samples under oak trees in Algrave province in Portugal. For quite a long time, these isolates were considered as *P. mamillatum* because of the presence of blunt spines on its ornamented oogonia. A close look at the sexual and asexual structures and the ITS sequences reveal that these isolates have many differences from the latter. Hence, a new taxon '*P.*

spiculum' is being created to fit in these isolates. One of these (F-1022) produces sexual structures readily and this isolate is considered as the type material for the species. The morphological and molecular features of this new oomycetes, and their comparison with related species, are discussed in this article.

Materials and methods

Oomycete isolates

Soil samples, together with plant root debris were collected in sterile capped bottles and were brought to the laboratory. Oomycetes were isolated from these samples by the usual baiting techniques as described elsewhere (Paul *et al.*, 1998, 1999; Paul, 1999).These were purified by repeated culturing in sterile distilled water and ultimately grown and maintained on solid media like potato carrot agar (PCA) and potato dextrose agar. The temperature–growth relationship of the oomycete was taken when it was grown on PCA incubated at 25 °C.

DNA extraction

The oomycetes were grown in potato dextrose broth. 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

Internal transcribed spacer amplifications of *Pythium* samples (Table 1) 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). The reaction mixture contained 1 × PCR buffer (75 mm Tris-HCl (pH 9.0), 50 mM KCl, 20 mM $(NH_4)_2SO_4$), 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 conidial DNA in a total volume of 50 μL. Amplifications were carried out in a Master Gradient thermocycler (Eppendorf, Hamburg, Germany) 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

Table 1. Genbank accession of different isolates of *Pythium spiculum*

Isolate	Country	Province	Host species	Genbank accession
F-1022	France	Bourgogne	*Vitis vinifera*	DQ205094
PA 53	Spain	Huelva	*Quercus suber*	DQ196121
PA 54	Spain	Huelva	*Quercus suber*	DQ196122
PA 55	Spain	Huelva	*Quercus suber*	DQ196123
PA 56	Spain	Huelva	*Quercus suber*	DQ196124
PE 154	Portugal	Algarve	*Quercus ilex*	DQ196125
PE 155	Portugal	Algarve	*Quercus ilex*	DQ196126
PE 156	Portugal	Algarve	*Quercus ilex*	DQ196127
PE 157	Portugal	Algarve	*Quercus ilex*	DQ196128
PE 158	Portugal	Algarve	*Quercus ilex*	DQ196129
PE 159	Portugal	Algarve	*Quercus ilex*	DQ196130
PE 160	Portugal	Algarve	*Quercus ilex*	DQ196131

(Cooke *et al.*, 2000). PCR products were separated in 1% agarose (Biofinex) gels in 1 × TBE, subjected to 100 V for 1 h, stained with ethidium bromide (0.5 mg L^{-1}) and visualized under UV light.

DNA sequencing and phylogenetic analysis

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. Amplicons were sequenced directly in both sense and antisense directions. All pathogen samples were sequenced twice and a consensus sequence was created from the duplicates. DNA sequences have been deposited in Genbank.

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 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 rates 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. Neighbor-joining plot and Tree view were used to view ML and Bayesian trees, respectively.

The ITS1 sequence of the isolate F-1022 was compared with the ITS1 sequences of related species. The sequence of

the ITS and the flanking regions of the rRNA gene of *P. spiculum* has been deposited in the GenBank (Table 1). These isolates will be deposited in the culture collection of 'Centraalbureau voor schimmelcultures' at BAARN in Holland.

Results

Morphological characteristics

Pythium spiculum Paul sp. nov. (Figs 1–4)

Sporangio et zoosporis non observata, Corporibus hypharum globosa, cylindrosa, intercalaria, catenaria, vel terminalia, 15–33 μm diam., zoosporae non observata. Oogonia terminalia, globosa, 13–22 μm diam,. ornata spiculis 3–7 μm longis. Antheridia raro, monoclinata; Oosporae, pleroticae vel apleroticae, globosae 8–18 μm diam, paries 0.5–1 μm crassus. Incrementum radiale quotiadianum 25 mm 25 °C in agaro Solani tuberosi et Dauci carotae (PCA). Holotypus in herbario Universitatis Bourgogne conservatus (F-1022).

The oomycete grows well both on the solid media as well as on 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 shows a broad chrysanthemal pattern. Average radial growth of the fungus at 25 °C on PCA is 25 mm day^{-1}.

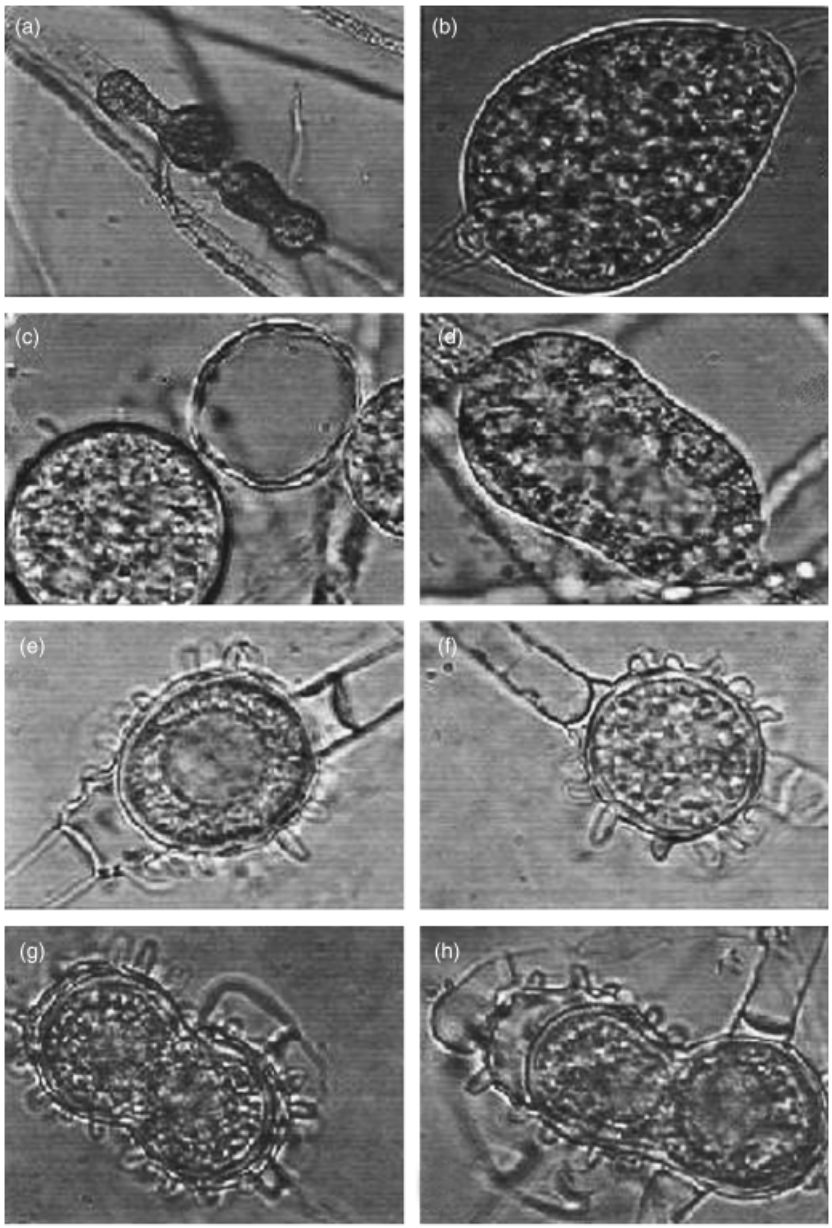

Fig. 1. Asexual and sexual reproductive bodies of *Pythium spiculum*: (a) peanut-shaped intercalary hyphal bodies; (b, d) intercalary elongated hyphal bodies; (c) spherical hyphal body; (e, f) spherical and intercalary ornamented oogonia; (g, h) elongated to peanut-shaped oogonia with dumbbell-shaped oospores and monoclinous stalked antheridia. (a) bar = 100 μm; (b–h) bar = 25 μm.

Sporangia or hyphal bodies are produced plentifully (Figs 1a–d). These are spherical, ovoid, cylindrical and at times peanut-shaped (Fig. 1a) and are mostly intercalary to catenulate, rarely terminal. The spherical ones measure from 15 to 33 μm in diameter (av. 23 μm). Zoospores were never produced by these hyphal bodies.

The female gametangia (oogonia) are mostly intercalary or formed in chains, and are provided with ornamentations which are blunt (Fig. 1e). These measure up to 7 μm in length, and are 1–1.5 μm broad at the base (Figs 1e–h). The oogonia itself measures 13–22 μm in diameter (av. 15.6 μm). These are often spherical, but at times are cylindrical to peanut-shaped. The elongated oogonia can measure up to 45 μm in length and 20 μm in breadth. These are filled with dense, coarsely granulated protoplasm (Figs 1g and h).

The male gametangia (antheridia) are strictly monoclinous and stalked, usually one per oogonium (Figs 1g and h) but at times up to three. The antheridial cells make a broad apical contact with the oogonia. These are usually persistent and remain attached to the oogonia, even after fertilization.

The zygotes (oospores) are usually plerotic but at times aplerotic especially in the cylindrical oogonia. The oospores are generally spherical, but in cylindrical- or peanut-shaped oogonia these can take the shape of the oogonia (Figs 1g and h). Spherical oospores measure between 8 and 18 μm in diameter. The oospore wall is very thin measuring 0.5–1 μm in thickness.

The oomycete produces spherical- to sickle-shaped appressoria in plenty. These appressoria are usually associated with sexual structures like those found in *P. abappressorium* (Figs 2a–d).

Internal transcribed spacer1 and ITS2 sequences and their flanking regions of *P. spiculum* is comprised of 945 bases. The Genbank accessions of different isolates are given in Table 1. The comparison of the ITS1 sequences of *P. spiculum* and related species is given in Fig. 3.

Phylogenetic analysis

The position of the *Pythium* isolates sequences (DQ196121, DQ196122, DQ196123, DQ196124, DQ196125, DQ196126, DQ196127, DQ196128, DQ196129, DQ196130, DQ196131 and DQ205094) and additional sequences of clade F *Pythium* species (Lévesque & de Cock, 2004) is illustrated in the BI trees represented in Fig. 4. The tree is rooted with *P. intermedium* sequences according to the tree of Lévesque & de Cock (2004) on the phylogeny of the genus *Pythium*.

In all analyses, the sequences of *P. spiculum* 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, the new sequences form a sister group to *P. mamillatum*.

Discussion

The isolates of *Pythium spiculum* were collected in summer 2002. Lack of zoosporangia, zoospores, presence of intercalary catenulate hyphal bodies, ornamented oogonia bearing blunt spines, very thin-walled oospores are the characteristics of *P. spiculum*. With the exception of sporulation most of these characters are common with *P. mamillatum* and it was identified as such for a long time. While doing the reculturing, for the analysis of the rRNA genes of

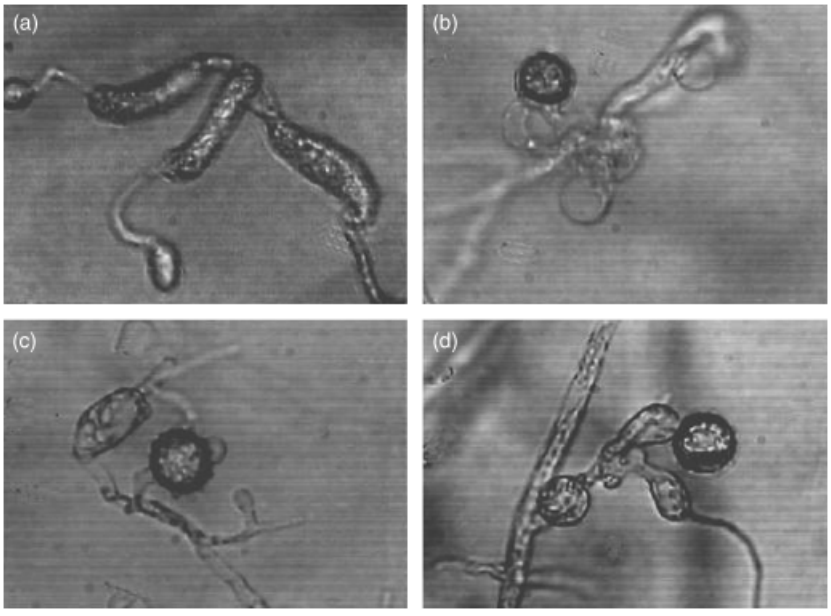

Fig. 2. Appressoria of *Pythium spiculum*: (a) sickle-shaped appressoria; (b–d) appressoria bearing sex organs. Bar = 60 μm.

```
P. cylindrosporum       CCACACCTAAAAAAACTTTCCACGTGAACTGTCGTTATTTGTTGTGTGTGTGCGCGTTGG 60
P. regulare             CCACACCTAAAAAAACTTTCCACGTGAACTGTCGTTATTTGTTGTGTGTGTGCGCGTTGG 60
P. mamillatum           CCACACCTAAAAAA-CTTTCCACGTGAACTGTCGTTATTTGTTGTGTGTGTGCGCGTTGC 59
P. spiculum             CCACACCTAAAAAA-CTTTCCACGTGAACTGTCGTTATTTGTTGTGTGTGTGCGCGTTGC 59
P. paroecandrum         CCACACCTAAAAAA-CTTTCCACGTGAACTGTCGTTATTTGTTGTGTGTGTGCGCGTTGC 59
P. sylvaticum           CCACACCTAAAAAA-CTTTCCACGTGAACTGTCGTTATTTGTTGTGTGTCTGCGCGTTGC 59
P. spinosum             CCACACCTAAAAAA-CTTTCCACGTGAACTGTCATTATTTGTTGTGTGTCTGCGCGTTGT 59
                        ************** ****************** *************** *********

P. cylindrosporum       TAGCATGC--GCGTTTGCTTACGCTTCGGTGTTTGCGAGTG--CGCGTGCTGGCGGTGCG 116
P. regulare             TAGCATGC--GCGTTTGCTTACGCTTCGGTGTTTGCGAGTG--CGCGTGCTGGCGGTGCG 116
P. mamillatum           TGGCGTGCGTGCGTTTGCTTACGCTTCGGTGTTTGCGAGTGTGCGCGTGCTGGCGGTGCG 119
P. spiculum             TGGCGTGC----GTTTGCTTACGCTTCGGTGTTTGCGAGTGCGCGCGTGCTGGCGGTGCG 115
P. paroecandrum         TGGCGTGC----GTTTGCTTACGCTTCGGTGTTTGCGAGTG----CGTGCTGTCGGTGCG 111
P. sylvaticum           TGGCGTGC----GTTTGCTTACGCTTCGGTGTTTGCGAGTG----CGTGCTGGCGGTGCG 111
P. spinosum             TGGCATGC----ATTTGCTTACACTTTGGTGTTTGCGAGTG----CGTGTTGGCAGTGTG 111
                        * ** ***     ********* *** **************    **** ** * *** *

P. cylindrosporum       CAGACTGAACGAAGGTCGTGTGTTTGCTGTGT--GCCTGCTGCACCGCTGACTTTTGCAT 174
P. regulare             CAGACTGAACGAAGGTCGTGTGTTTGCTGTGT--GCCTGCTGCACCGCTGACTTTTGCAT 174
P. mamillatum           CGGACTGAACGAAGGTCGTGTGTT-GCTGTGTGTGCCTGCTGCACCGCTGACTTT-GCAT 177
P. spiculum             CAGACTGAACGAAGGTCGTGTGTTTGCTGTGTGTGCCTGCTGCACCGCTGACTTT-GCAT 174
P. paroecandrum         CGGACTGAACGAAGGTCGTGTGTTTGCTGTGT--GCCTGCTGCACCGCTGACTTT-GCAT 168
P. sylvaticum           CGGACTGAACGAAGGTCGTGT---TGCTGTGT--GCCTGCTGCACTGCTGACTTT-GCAT 165
P. spinosum             CGGACTGAACGAAGGTTGTGTGT-TGTTATGT--GTCTGCTGCACTGCTGACTTT-GCAT 167
                        * ************** ****    * * ***  * ********* ********* ****

P. cylindrosporum       TGATTTGCATGATGTTGGCGGAGCGGCGGGTG--CTGTGCGTGCGCGCGGCTGACCTATT 232
P. regulare             TGATTTGCATGATGTTGGCGGAGCGGCGGGTG--CTGTGCGTGCGCGCGGCTGACCTATT 232
P. mamillatum           TGATTTGCATGGTGTTGGCGGAGCGGCGGGTGTGCTGTGCGTGCGCGCGGCTGACTTA-T 236
P. spiculum             TGATTTGCATGGTGTTGGCGGAGCGGCGGGTGTGCTATGCGTGCGCGCGGCTGACTTA-T 233
P. paroecandrum         TCATTTGCATGGTCTTGGCGGAGCGGCGGGTG--CTGTGCGTGCGCG--GCTGACTTATC 224
P. sylvaticum           TGATTTGCATGGTCTTGGCGGAGCGGCGGGTG--CTGTGCGTGCGCG--GCTGACTTA-C 220
P. spinosum             TCATTTGTATGGTCTTGGCGGAGTGGCGGGTA--CTGTGCATGCACA--GCTGACTTA-T 222
                        * ***** *** * ********* *******   ** *** *** *   ****** **

P. cylindrosporum       TTTTTCAAACCCCATACCTAAATGACTGATTATACTGTGAGAACGAAAGTTCTTGCTTTA 292
P. regulare             TTTTTCAAACCCCATACCTAAATGACTGATTATACTGTGAGAACGAAAGTTCTTGCTTTA 292
P. mamillatum           TTTTTCAAACCCGATACCTAAATGACTGATTATACTGTGAGAACGAAAGTTCTTGCTTTA 296
P. spiculum             TTTTTCAAACCCGATACCTAAATGACTGATTATACTGTGAGAACGAAAGTTCTTGCTTTA 293
P. paroecandrum         TTTTTCAAACCCCATACCTAAATGACTGATTATACTGTGAGAACGAAAGTTCTTGCTTTA 284
P. sylvaticum           TTTTTCAAACCCCATACCTAACTTACTGATTATACTGTGAGAACGAAAGTTCTTGCTTTT 280
P. spinosum             TTTTTCAAACCCCATACCTAAATGACTGATTATACTGTGAGAACGAAAGTTCTTGCTTTA 282
                        ************ ******** * ***********************************

P. cylindrosporum       AACTAGATAA 302
P. regulare             AACTAGATAA 302
P. mamillatum           AACTAGATAA 306
P. spiculum             AACTAGATAA 303
P. paroecandrum         AACTAGATAA 294
P. sylvaticum           AACTAGATAA 290
P. spinosum             AACTAGATAA 292
                        **********
```

Fig. 3. CLUSTAL W (1.81) multiple sequence alignment of the internal transcribed spacer-1 sequences of *Pythium spiculum* (DQ 205094) with those of *Pythium cylindrosporum* (Genbank accession AY 598643), *Pythium regulare* (AF 492018), *Pythium paroecandrum* (AJ233453), *Pythium sylvaticum* (AY 598645), *Pythium mamillatum* (AY 598703) and *Pythium spinosum* (AF 492017).

all the pythiaceous isolates, it was observed that the identification was wrong. Not only the sporulation, but also some morphological features and the ITS sequences are different which are outlined in Table 2 and Fig. 3. Oogonia and oospores, are often cylindrical or dumbbell-shaped in the former instead of spherical as in the latter. Antheridia are strictly monoclinous in this case and the growth patterns of the two oomycetes are also different on solid media.

Molecular analyses of the newly erected taxon, supports the morphological observations, and a Blast search with the sequence of the ITS region of the rRNA genes of *P. spiculum* gives close resemblance with *P. mamillatum* (Genbank accession AY598703, 98.5% identity), *P. regulare* (AF492018, 94.8%), *P. paroecandrum* (AJ233453, 94.6%), *P. cylindrosporum* (AY598643, 93.5%), *P. irregulare* (AB108000, 93.5%), *P. sylvaticum* (AY598645, 92.3%), and *P. spinosum*

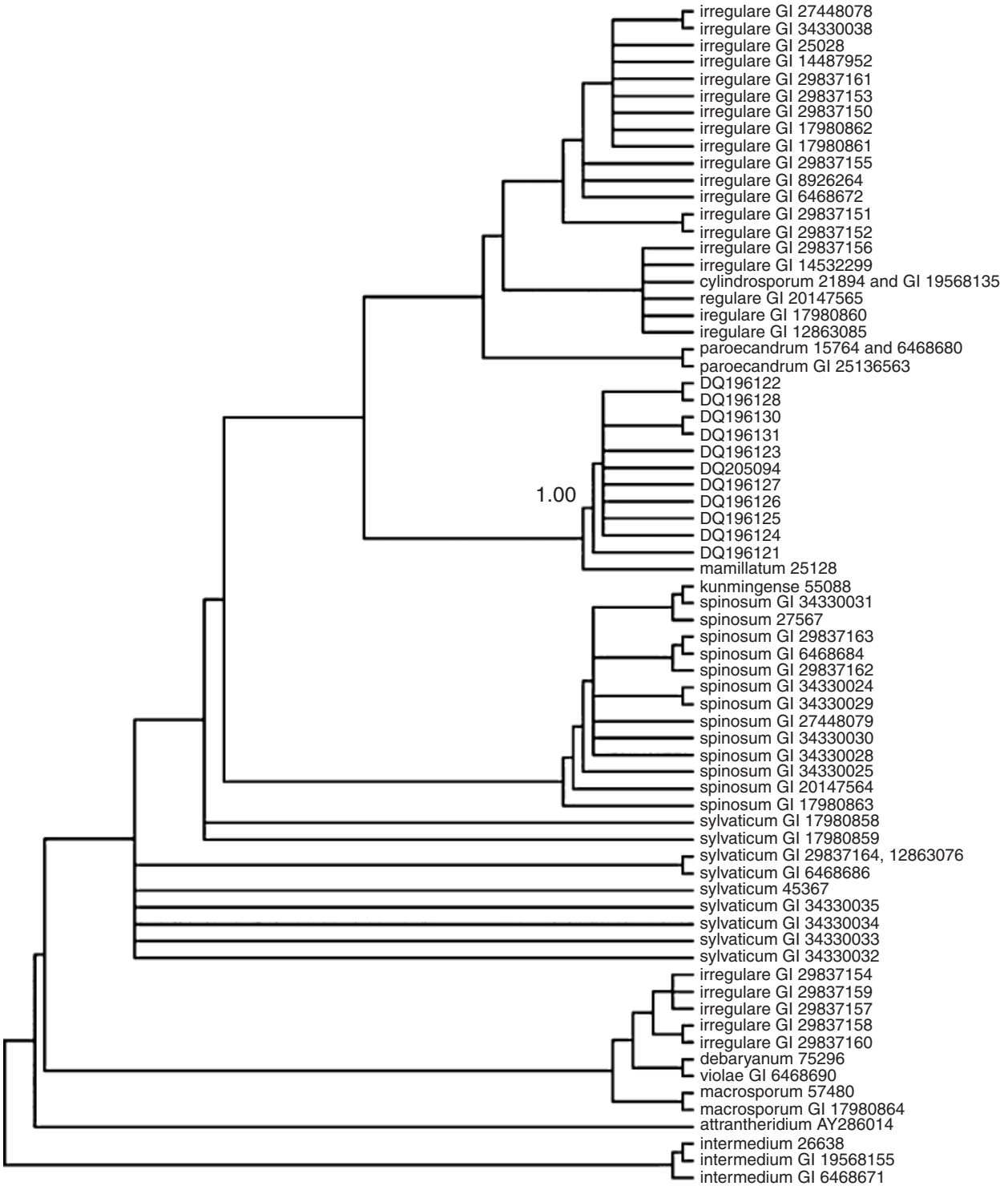

Fig. 4. Phylogenetic position of DQ196121, DQ196122, DQ196123, DQ196124, DQ196125, DQ196126, DQ196127, DQ196128, DQ196130, DQ196131 and DQ205094 derived from internal transcribed spacer sequences using Bayesian inferences method. The number at node *Pythium mamillatum–Pythium spiculum* represents posterior probabilities.

Table 2. Comparison of morphological characters of *Pythium mamillatum* and *Pythium spiculum*

Characters	*P. mamillatum*	*P. spiculum*
Growth on PCA	25 mm day^{-1} vague rosette pattern	27 mm day^{-1} broad chrysanthemum pattern
Sporangia	Intercalary or lateral, 17–25 μm zoospores produced	Both terminal and intercalary, 15–33 μm zoospores not produced
Oogonia	Mostly spherical	Spherical, cylindrical to peanut shaped
Antheridia	Monoclinous and diclinous, one to two in number	Only monoclinous, one to three in number
Oospores	Globose, plerotic with wall 0.8–1.4 μm in diameter Double oospores not present	Globose, cylindrical to peanut shaped, plerotic and aplerotic, double oospores present, oospore wall 0.5–1 μm in diameter

PCA, potato carrot agar.

(AF492017, 91.2%). According to this analysis, the closest ally of *P. spiculum* is *P. mamillatum*. The new species fits well in this group of oomycetes with the exception that it has lost its sporangia forming abilities during evolution and adaptation to the terrestrial habitat. The close resemblance of the ITS sequences in the genus *Pythium* is also well known, and it has been reported that species may vary by only one or two base pairs (Timothy *et al*., 2003).

Phylogenetic analysis of the ITS region consistently indicates that *Pythium* isolates described in this study share a common ancestor with all *Pythium* sequences used here. In all analyses, the sequences of *P. spiculum* form a monophyletic group. This is supported by high values of BS and PP confirming the species status of these isolates. Thus molecular evidence proves that *Pythium* isolates described in this article represents a new species.

References

Cavalier-Smith TA (1998) A revised six-kingdom system of life. *Biol Rev Cambridge Philos Soc* **73**: 203–266.

Cooke DEL, Drenth A, Duncan JM, Wagels G & Brasier CM (2000) A molecular phylogeny of *Phytophthora* and related oomycetes. *Fung Gen Biol* **30**: 17–32.

Galtier N, Gouy M & Gautier C (1996) SEAVIEW and PHYLO_WIN, two graphic tools for sequence alignment and molecular phylogeny. *Comput Appl Biosci* **12**: 543–548.

Guindon S & Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol* **52**: 696–704.

Hasegawa M, Kishino H & Yano TA (1985) Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *J Mol Evol* **22**: 160–174.

Huelsenbeck JP & Ronquist F (2001) MRBAYES: Bayesian inference of phylogeny. *Bioinformatics* **17**: 754–755.

Lanave C, Preparata G, Saccone C & Serio G (1984) A new method for calculating evolutionary substitution rates. *J Mol Evol* **20**: 86–93.

Lévesque CA & de Cock AWAM (2004) Molecular phylogeny and taxonomy of the genus *Pythium*. *Mycol Res* **108**: 1363–1383.

Martin F (1995) Meiotic instability of *Pythium sylvaticum* as demonstrated by inheritance of the nuclear markers and karyotype analysis. *Genetics* **139**: 1233–1246.

Paul B (1987) A new species of *Pythium* with ornamented oogonia from Algeria. *Mycologia* **79**: 797–802.

Paul B (1992) *Pythium radiosum*, a new species from the Bank of Lake Zurich. *Mycol Helv* **5**: 1–8.

Paul B (1999) *Pythium ornacarpum*: a new species with ornamented oogonia isolated from soil in France. *FEMS Microbiol Lett* **180**: 337–344.

Paul B (2002) *Pythium segnitium* sp. nov., isolated from the Canary islands, its taxonomy, ITS region of rDNA, and its comparison with related species. *FEMS Microbiol Lett* **217**: 207–212.

Paul B (2003) *Pythium carbonicum*, a new species isolated from a spoil heap in northern France, the ITS region, taxonomy and comparison with related species. *FEMS Microbiol Lett* **219**: 269–274.

Paul B, Galland D, Bhatnagar T & Dulieu H (1998) A new species of *Pythium* isolated from the Burgundy region of France. *FEMS Microbiol Lett* **158**: 207–213.

Paul B, Galland D & Masih I (1999) *Pythium prolatum*, isolated from soil in the Burgundy region: a new record for Europe. *FEMS Microbiol Lett* **173**: 69–75.

Plaats-Niterink AJ (1981) Monograph of the genus *Pythium*. *Stud Mycol Baarn* **21**: 1–242.

Rodriguez F, Oliver JF, Martin A & Medina JR (1990) The general stochastic model of nucleotide substitution. *J Theor Biol* **142**: 485–501.

Timothy C, Paulitz TZ, Adams K & Mazzola M (2003) *Pythium abappressorium* – a new species from eastern Washington. *Mycologia* **95**: 80–86.

White TJ, Burns T, Lee S & Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols: A Guide to Methods and Applications* (Innis MA, Gelfand DH, Sinsky JJ & White TJ, eds), pp. 315–322. Academic Press, San Diego, CA.