Pythium recalcitrans sp. nov. revealed by multigene phylogenetic analysis

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INTRODUCTION

Pythium is a large genus comprising more than 120 described species including many plant pathogens (Dick 1990, Lévesque and de Cock 2004). The genus is classified in the Pythiaceae within the oomycetes, a taxonomic group of predominantly filamentous diploid organisms unrelated to the true fungi (Eumycota) and currently assigned with other heterokont, biflagellate organisms to kingdom Straminipila (Dick 2001). *Pythium* species mainly cause seedling damping-off, root infections and in general rotting of soft tissues in soil and aquatic habitats (van der Plaats-Nikerink 1981). A few species have been reported as mycoparasites (van der Plaats-Nikerink 1981, Lifshitz et al 1984) and animal pathogens (de Cock et al 1987). Most *Pythium* species are homothallic with meiosis occurring in the gametangia before fertilization (Sansome 1961, Dick 1990). Only a few species have been demonstrated to be heterothallic (Campbell and Hendrix 1967, Papa et al 1967, Martin 1995) requiring the presence of an opposite mating to sexually reproduce. However low levels of homothallic behavior (selfings) have been occasionally observed in some isolates of heterothallic species (van der Plaats-Nikerink 1981, Martin 1995).

Until the modern application of molecular biology, the taxonomy of *Pythium* mainly relied on morphological and physiological aspects. The delimitation of *Pythium* taxa has been hindered by the few distinctive morphological structures (e.g. sporangia, hyphal swellings and gametangia) and often their great variability in range of dimensions that are increasingly overlapping as the number of species grows. Furthermore only a few ecological aspects are available for species discrimination because most species are cosmopolitan, nonhost-specific pathogens (i.e. generalists), which mostly lack any known niche specialization (Hendrix and Campbell 1973). Altogether these problems have resulted in many misidentifications and superfluous species epithets (Lévesque and de Cock 2004).

Phylogenetic analyses of sequence data have transformed fungal taxonomy. These analyses have been aided by the availability of large sequence databases such as GenBank. Unlike morphological characters, DNA sequence analysis is not subject to environmental interactions and hence is stable and fully

Abstract: A new species of *Pythium* collected from grapevine roots (*Vitis vinifera*) in South Africa and roots of common beet (*Beta vulgaris*) in Majorca, Spain, is described. The phylogenetic position of the new species was investigated by multigene sequence analyses of the internal transcribed spacers (ITS1 and ITS2) of the rDNA region, as well as three other nuclear and three mitochondrial coding genes. Maximum likelihood phylogenetic analyses based on ITS rDNA and concatenated β -tubulin and cytochrome c oxidase II alignment place *Pythium recalcitrans* together with *P. sylvaticum* and *P. intermedium*. *Pythium recalcitrans* sp. nov. is morphologically almost indistinguishable from other *Pythium* species that only form hyphal swellings in culture. However its species status is justified by the distinctiveness of the DNA sequences in all the genes examined. In culture *P. recalcitrans* exhibits fast radial growth, abundant spherical to subglobose hyphal swellings but produces no zoosporangia. Sexual structures are not seen in agar media but form in autoclaved grass blades floated on water. Multiple antheridia (1–7) are encountered with most of them declinuous and crook-necked. Oospores are thin-walled and either aplerotic or plerotic. *P. recalcitrans* was pathogenic to seedlings of *Beta vulgaris* and *Solanum lycopersicum*.

Key words: actin, α -tubulin, β -tubulin, *cox I* and

TABLE I. *Pythium* isolates used in the study

Isolate	Species	Host	Origin
Py26†	<i>P. recalcitrans</i>	<i>Beta vulgaris</i> (roots)	Mallorca (Spain)
STEU-6208†	<i>P. recalcitrans</i>	<i>Vitis vinifera</i> (roots)	Western Cape Province of South Africa
STEU-6209†	<i>P. recalcitrans</i>	<i>Vitis vinifera</i> (roots)	Western Cape Province of South Africa
P13245	<i>P. intermedium</i> * (+)	<i>Alnus glutinosa</i> (leaves)	Catalonia (Spain)
P17145	<i>P. intermedium</i> * (–)	<i>Rosa</i> sp. (roots)	Switzerland

* Mating types of *P. intermedium*.

† Isolates that were characterized morphologically.

reproducible. Lévesque and de Cock (2004) constructed a phylogenetic tree of 116 species of *Pythium* based on parsimony analysis of the internal transcribed spacers (ITS) sequences of the rDNA region. Their study revealed 11 clades distributed into two main clusters corresponding to *Pythium* species with filamentous (Clades A–D) and globose (Clades E–J) sporangia and a small clade including species with ovoid-papillate (Clade K) sporangia, which is out-group to the filamentous and globose clades of *Pythium*. Villa et al (2006) incorporated the cytochrome c oxidase subunit II (*cox II*) and β -tubulin genes for investigating the phylogenetic relationships of 58 isolates of *Pythium* and 17 isolates of *Phytophthora*. Their study, which included 39 *Pythium* species, identified four clades of which one clade is composed mainly of *Pythium* species with subglobose-ovoid papillate sporangia. This clade could be an evolutionary bridge between *Pythium* and *Phytophthora*.

In this paper a multigene sequence analysis approach is followed to discern the taxonomic status and phylogenetic position of a new *Pythium* species that shows morphological characters similar to those of other species, such as *P. sylvaticum* Campbell and Hendrix. A case of nonhost specificity and cosmopolitanism within the genus *Pythium* is documented.

MATERIALS AND METHODS

Isolation procedure.—Details of the origin of all the *Pythium* isolates used in the study are provided (TABLE I). A semiselective medium PARP (Jeffers and Martin 1986) was used for isolations made from necrotic roots of *Beta vulgaris* L. and *Vitis vinifera* L. Roots were washed in tap water and blotted dry on paper towels. *V. vinifera* roots were further surface sterilized by immersions in 70% ethanol for 2 s. Pieces of necrotic rootlets were plated onto PARP medium and incubated at 20 C for 2 d. Hyphal tips were transferred to carrot agar (CA, Brasier 1967) for *B. vulgaris* isolates or cornmeal agar for *V. vinifera* isolates. Stock cultures were maintained at 20 C on CA slants covered with liquid paraffin, and subcultures have been deposited at the CBS (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands).

Morphological characterization.—The main colony characters were investigated by making mycelial subcultures on CA, malt-extract agar (MEA), potato-dextrose agar (PDA) and cornmeal agar (CMA). The cultures, two replicates of each isolate, were grown at 20 C in darkness for 1 wk before the main colony characters were recorded. The relationship between radial growth rate and temperature was estimated by growing cultures, two replicates of each isolate, on CA at 5, 10, 15, 20, 25, 30, 35 and 40 C with an EchoTherm Digital Electronic Chilling/Heating plate Model IC20 (Torrey Pines Scientific, Solana Beach, California). The 90 mm diam Petri dishes were inoculated in the center with a 6 mm diam mycelial plug and left overnight at 20 C for initial growth. The margins of the colonies were marked at the bottom of the Petri dish with a grease pencil. The plates were exposed to each of the temperatures for a further 24 h using a chilling/heating plate and the radii recorded. The experiment was repeated twice.

The ability of the isolates to produce sporangia was investigated under different experimental conditions. Three blocks ca. 1 × 1 cm surface area taken from a 2 d old colony grown on CA, were transferred to 90 mm diam plates flooded with ca. 10 mL sterile distilled water or soil-water extract (Moralejo et al 2004) or with sterile distilled water plus a few autoclaved seeds of *B. vulgaris* and *Phaseolus* sp. (5–10/species). Petri dishes were incubated under continuous fluorescent white light at 20 and 25 C. The formation of sporangia and sexual structures also were investigated with either autoclaved or freshly harvested grass blades (van der Plaats-Niterink 1981) or thin autoclaved carrot slices floated on sterile distilled water (Matthews 1931). Filamentous or globose structures suspected of being capable of zoospore release were examined repeatedly with a Wild M5A binocular dissecting microscope (Leica Microsystems, Heerbrugg, Switzerland) at 50× magnification over 3 d at room temperature. The ability of these structures to release zoospores also were checked after exposing the liquid culture plates to 7 C for 45 min, followed by incubation at room temperature.

The formation of sexual structures on CA and other media in darkness was investigated for 1 mo. Because no oogonia were observed we further investigated the possibility of the new *Pythium* being heterothallic. All possible combinations of “intraspecific” pairings were tested. For this purpose mycelial plugs were placed ca. 2 cm apart on CA in 90 mm Petri dishes and incubated at 20 C in the dark for 2 wk. Interspecific pairings with both mating types of *P.*

intermedium de Bary (+/– compatible strains) also were carried out as described above. Agar blocks 1 × 1 cm in surface area from the contact line were mounted on glass slides and examined under an Olympus BX-50 compound microscope at 400× magnification. Approximately 25 sexual and asexual structures chosen at random were measured with a calibrated eyepiece at 400× magnification on CA. Digital camera images were captured with Olympus DP-12 software.

The keys to *Pythium* by Waterhouse (1967), van der Plaats-Niterink (1981) and Dick (1990) were used for identification. The morphology of the isolates was compared with the original descriptions of similar species provided by Matthews (1931), Middleton (1943) Waterhouse (1968) and van der Plaats-Niterink (1981).

Pathogenicity tests.—A soil infestation assay was performed to test the pathogenicity of isolates Py26 and STEU-6208. Inoculum was produced by first soaking 200 g of rye (*Secale cereale*) seeds overnight in an equal volume of water, followed by draining of the water and autoclaving in a 500 mL flask. The medium was inoculated with two 12 mm diam colonized agar plugs and incubated at 20 °C for 1 wk in the dark.

Two-week-old seedlings of common beet (*Beta vulgaris*), tomato (*Solanum lycopersicum* L.) and cauliflower (*Brassica oleracea* L.) were transplanted into individual pots (50 × 50 × 50 mm) containing sterile peat soil. The seedlings were left to root at 20 ± 5 °C for 2 wk in a plant growth chamber. Thereafter 200 g of the inoculum substrate were spread evenly in a tray, on top of which 12 plantlets removed from the pots were placed, and the set flooded with water for 24 h. Controls consisted of four plantlets of each species that were placed into another tray without inoculum that also was flooded with water for 24 h. The trays were incubated in a greenhouse at 20 ± 5 °C and flooded 24 h every week for 1 mo. In total 10 seedlings of each species were inoculated with each of the isolates separately. Pathogenicity on seedlings was evaluated by recording the percentage of seedlings affected by damping-off 4 wk after inoculation.

DNA isolation, amplification and sequencing.—Mycelial DNA was obtained from pure cultures of four *P. recalcitrans* isolates (TABLE II) grown in pea broth (Kroon et al 2004). DNA was extracted from frozen mycelia with a DNA-Easy Plant Minikit (QIAGEN, Basel, Switzerland) according to the manufacturer's specifications. Genomic DNA quality was checked with a NanoDrop NT-100 UV spectrophotometer (Witec AG, Switzerland). DNA amplifications were performed for three nuclear and three mitochondrial loci. Ribosomal DNA ITS amplifications were carried out using universal primers ITS4 and ITS5 that target conserved regions in the 18S and 28S rDNA genes (White et al 1990). Amplifications for the β -tubulin (β -*tub*) gene, the NADH dehydrogenase subunit I gene (*nadH1*) and the cytochrome oxidase subunit I gene (*cox I*) were performed according to Kroon et al (2004) with primers TUBUF2 and TUBUR1 for the β -*tub*, NADHF1 and NADHR1 for *nadH1* and COXF4N and COXR4N for *cox I*. The cytochrome oxidase subunit II gene (*cox II*) was amplified with primers FM75 and FM78

and PCR conditions as described by Martin and Tooley (2003). Actin and α tubulin (α -*tub*) genes were amplified with primers and PCR conditions described by Harper et al (2005). PCR products were purified with a Minelute PCR Purification Kit (QIAGEN, Switzerland) and quantity and quality were checked as described above. Amplicons were sequenced directly in both senses, and the consensus sequences were submitted to GenBank (TABLE II).

Phylogenetic analysis.—Phylogenetic analyses for ITS rDNA and concatenated β -*tub* and *cox II* alignments were performed according to Belbahri et al (2005, 2006a, b) with the ITS dataset of the study of Lévesque and de Cock (2004) and the β -*tub* and *cox II* datasets of Villa et al (2006). The ITS and concatenated β -*tub* and *cox II* datasets were analyzed separately because the much larger taxon selection for ITS precluded their combinability. The β -*tub* and *cox II* datasets in this study were compared statistically for incongruence with the nonparametric Templeton Wilcoxon signed-ranks (WS-R) test implemented in PAUP* (Sikes and Lewis 2001) with 90% bootstrap consensus trees as constraints. *Cox II* constrained to the β -*tub* consensus received a *P* value of 0.001, indicating that the two individual datasets could be combined.

Nucleotide sequences were aligned manually with Sea-view (Galtier et al 1996). The AICc criterion as implemented in Modeltest suggested GTR+I+G as an appropriate model. Metropolis-coupled Markov chain Monte Carlo analyses (MCMC; Larget and Simon 1999, Mau et al 1999) were performed with MrBayes (version 2.0; Huelsenbeck and Ronquist 2001) with the general time reversible (GTR) model allowing all rates to be different (Lanave et al 1984, Rodríguez et al 1990). To correct the among-site rate variations, the proportion of invariable sites (I) and the alpha parameter of gamma distribution (G), with eight rates categories, were estimated by the program and taken into account in all analyses. The program was run for 4 000 000 generations and sampled every 100 generations, with four simultaneous chains. A 50% majority rule consensus of the remaining trees was computed to obtain estimates for the probabilities that groups are monophyletic given the sequence data (posterior probabilities). Branch lengths were computed as mean values over the sampled trees. The trees sampled before the chains achieved stationarity were discarded. NJplot (Perrière and Gouy 1996) and Treeview (Page 1996) were used to view Bayesian trees. To confirm that the posterior probability distribution of the MCMC processes is stationary (Huelsenbeck and Ronquist 2001), the Bayesian analysis was repeated three times on a personal computer, always starting with random trees and default parameter values of the program. All these analyses were run for 4 000 000 generations.

TAXONOMY

***Pythium recalcitrans* L. Belbahri et E. Moralejo, sp. nov.** (FIGS. 1A–H)

Etymology. Because of its recalcitrance to form zoosporangia and oogonia under several treatments.

Hypae principales usque ad 10 μ m latae, hyalinae.

TABLE II. List of GenBank accession numbers for the sequences of several genes of *Pythium recalcitrans*

Isolate	ITS	Nuclear genes			Mitochondrial genes		
		Act	α -Tub	β -Tub	<i>nadh1</i>	CoxI	CoxII
Py26	DQ357833	EF434970	EF434974	EF195143	EF195144	EF426549	EF195148
STEU-6208	EF195137	EF434971	EF434975	EF195140	EF195145	EF426550	EF195149
STEU-6209	EF195138	EF434972	EF434976	EF195141	EF195146	EF426551	EF195150
STE-U 6210	EF195139	EF434973	EF434977	EF195142	EF195147	EF426552	EF195151

FIG. 1. Morphology of *Pythium recalcitrans* grown on carrot agar (CA) and grass blades floated in water. Clavate appressorium (A) formed on the plate floor. Spherical hyphal swellings produced on a colonized grass blade (B) and on CA (C). Doliiform hyphal swelling (D) formed on CA. Sexual structures developed on grass blades (E–I); many antheridia surrounding an oogonium (E–G); plerotic (H) and applerotic (I), thin-walled oospores.

Appressoria magna, simplicia, clavata, falcata vel forma irregularia formata. Temperaturae cardinales in CA: minima 4 C, optima 15–25 C et maxima 34 C. Coloniae in CA indscriptae vel modice radiantes, mycelium aerium gossypinum prope marginem. Intumescientiae hyphales in CA abundantes post 7 dies, intercalares vel in ramis secundariis terminales, globosae vel subglobosae, 25.6 μm diam, raro doliiformes vel citriformes, vel etiam cylindraceae in hyphis principalibus. Zoosporae haud visae. Oogonia solum formata abundantia ab omnibus isolatis in laminis herbarum aqua distillata submersa, levia, globosa vel subglobosa, 26.1 $\pm$ 2.9 μm diam. Antheridia diclina, hamata, vel apice angustata, multiplica, usque ad 7 in quoque oogonio. Oosporae globosae, leves, 22.7 $\pm$ 2.8 μm diam, tenuitunicatae (c. 0.6 μm), pleroticae vel apleroticae.

All measurements for each morphological structure are the average of three isolates of *P. recalcitrans* (TABLE I). Main hyphae up to 10 μm wide, hyaline, smooth, straight or slightly undulate. Hyphal branch system short and dendroid. Hyphal contents evacuate after 1 wk; many cross-septa then remain visible. *Appressoria* abundant on the plate floor, large, simple, clavate, falcate or sometimes irregularly shaped (FIG. 1A). Cardinal temperatures on CA, 4 minimum, 15–25 optimum and 34 C maximum (FIG. 2). Exposures to 35 C for 2 d were lethal for all isolates. *Colonies* on CA unpatterned to slightly radiate with a smooth outline, aerial mycelium near the margins cottony; on CMA mycelium submerged, diffuse and slightly dendroid; on PDA unpatterned with a smooth outline, and on MEA hyphae tending to be slightly spiral and dextrogyrous from above. *Hyphal swelling* abundant within 1 wk on CA, terminal on secondary branches or mostly intercalary, spherical to subglobose 25.6 (15.5–30.3) μm diam (FIG. 1B–C), a few doliiform to limoniform (FIG. 1D), or cylindrical on the main hyphae. *Zoospores* not observed under standard conditions in distilled water, soil extract or on seeds. *Sexual structures*: (On CA) not observed in agar culture, either in intraspecific or interspecific pairings with *P. intermedium* and other *Pythiums*. *Oogonia* readily and abundantly formed by all isolates on grass blades incubated on distilled water; smooth-walled, spherical to subglobose, 26.1 $\pm$ 2.9 μm diam (19.6–30.1) (FIG. 1E–I). *Antheridia* diclinous when the stalk insertion is discernible, sometimes with a broadened attachment, mostly crook-necked and multiple, with up to seven seen per oogonium (FIG. 1E–G). *Oosporae* spherical, smooth, 22.7 $\pm$ 2.8 μm diam, thin-walled (0.6 μm), both plerotic and aplerotic (FIG. 1H). Many aborted oosporae present in colonized grass blades.

Cultures examined. SPAIN, MALLORCA, Sa Pobla, 39°46'53"N, 03°02'15"E. isol. ex rhizosphaera *Beta vulgaris*, No 2001, E. Moralejo. Py26, HOLOTYPE: cultura sicca in agaro Dauci carotae in herbario Universitatis Helveticae

FIG. 2. Temperature-growth relationships of three isolates of *Pythium recalcitrans* grown on carrot agar in the darkness. RGR = radial growth rates.

Occidentalis conservatus: ex type living culture deposited at CBS 122440 culture. Leg. E. Moralejo. MYCOBANK: 510614. SOUTH AFRICA, WESTERN CAPE PROVINCE: isol. ex rhizosphaera *Vitis viniferae*, Jul 2005, CFJ Spies, cultura sicca in agaro Dauci carotae in herbario Universitatis Helveticae Occidentalis conservatus STEU6208 (CBS 122441), STEU6209, STEU6210.

RESULTS

Sequences and phylogenetic analysis.—The products of ITS amplification of the four sequenced isolates were all 821 bp long. All three South African isolates shared identical sequences and differed at a single nucleotide position in the ITS2 region from the sequence of the Spanish isolate. BLAST run through the GenBank database revealed a unique sequence pattern, distinct from those of any described species. The closest relatives were *P. irregulare* isolate 13–29 (AY907915), *P. sylvaticum* (AY907915), *P. paroecandrum* (DQ091297) and *P. spinosum* (AF492017) with minimum scores of 72–80% identity over all the ITS sequence positions. The maximum identity was 100% with *P. paroecandrum* over 79% (E value = 1e-153), 98% with *P. irregulare* over 72% (4e-154), 98% with *P. spinosum* over 71% (6e-153) and 97%, with *P. sylvaticum* over 80% (2e-152), of the rDNA ITS sequence. Phylogenetic analyses of the ITS sequence data revealed that *P. recalcitrans* falls in Clade F *sensu* Lévesque and de Cock (2004) (FIG. 3).

The sequences of each of the different nuclear and mitochondrial genes were identical within the four isolates of *P. recalcitrans* except β -tub of Py 26, which differed at a single nucleotide site and α -tub which differed at two nucleotide sites. The sequences of both genes differed substantially from those of other *Pythium* species found in GenBank. There were some minor contradictions in the clade position of some species between phylogenies based on the sequences

0.1

FIG. 3. Bayesian inference tree using rDNA ITS sequences showing phylogenetic relationships between *Pythium* species. The numbers on the nodes represent the posterior probability/percentage bootstrap support based on Bayesian analysis of the dataset. *Pythium* ITS sequences are indicated by their GenBank accession in the figure. The phylogeny was rooted with Clade K, as suggested by Villa et al (2006), who used *Phytophthora* ITS sequences as outgroups. Bar = 0.1 substitution positions.

of different genes and those based on the ITS analysis, mainly due to the lack of sequence data for all species within the genus. For example β -*tub* sequences indicated that the closest relatives of *P. recalcitrans* are *P. macrosporum* Vaartaja and Plaats-Niterink (AY944860) and *P. oedocheilum* Drechsler (DQ071326) with identity scores of 97% and 96% respectively over 658 bp. While *P. macrosporum* falls in Clade F in the phylogeny of Lvesque & de Cock (2004) based on the ITS region, *P. oedocheilum* is in Clade K forming part of a major divergent cluster within genus *Pythium*. By contrast *Cox II* gene sequences, for which there was a good species representation within the genus *Pythium*, confirmed the results obtained with ITS sequences. *Cox II* sequences showed affinities with that of *P. spinosum* Sawada (AF196616) and *P. sylvaticum* (AF196625) with identity scores of 97% over 571 bp.

Because the well known "total evidence" approach (Kluge 1989) was followed, separate analyses of the different sequenced loci were not performed. Instead analysis was conducted on a β -*tub* and *cox II* concatenated dataset. In the combined phylogenetic analysis with both genes, *P. recalcitrans* falls in Clade 2 *sensu* Villa et al (2006) together with species bearing globose to spherical zoosporangia, such as *P. irregulare* Buisman, *P. paroecandrum* Drechsler, *P. paddicum* Hirane, or those with spherical hyphal swellings (i.e. *P. sylvaticum*, *P. spinosum*, *P. intermedium*, *P. ultimum* Trow and *P. violae* Chesters and Hickman) (FIG. 4).

Pathogenicity.—Four of 10 *S. lycopersicum* seedlings inoculated with *P. recalcitrans* suffered damping-off for both Py26 and STEU-6208. The rootlets of the remaining seedlings were severely rotted compared to the controls. Seven of 10 seedlings of *B. vulgaris* inoculated with isolate Py26 and six of 10 seedlings inoculated with STEU-6208 were damped-off. In contrast none of the *Brassica oleracea* seedling showed any disease symptoms 4 wk after inoculation. All seedlings and roots in the control tests remained healthy. The pathogen could be isolated from the symptomatic plants of inoculated pots of *B. vulgaris* and *S. lycopersicum*.

DISCUSSION

The morphological examination of the new *Pythium* species barely allows discrimination from other species in the keys by Waterhouse (1967), van der Plaats-Niterink (1981) and Dick (1990). However phylogenetic analyses based on each of the sequences of the rDNA ITS region and the concatenated β -*tub* and *cox II* alignments clearly demonstrated that

Pythium recalcitrans is a new species. The same phylogenetic analyses consistently indicate that *P. recalcitrans* shares a common ancestor with all the *Pythium* species within Clade F of Lvesque and de Cock (2004) forming a monophyletic group supported by high values of posterior probabilities. On a short sequence run of the ITS region, *P. recalcitrans* displayed the highest sequence homology with *P. sylvaticum* and was positioned in the same subclade as *P. mamillatum*, *P. spinosum*, *P. paroecandrum*, *P. irregulare* and *P. intermedium* (FIG. 3).

P. recalcitrans is almost indistinguishable morphologically from other *Pythium* spp. enclosed as group HS (hyphal swellings) in the monograph of van der Plaats-Niterink (1981), except that it does produce sexual structures. The HS group is a nontaxonomic group traditionally suited for unidentified species that do not usually form zoosporangia and sexual structures under standard cultural conditions but do form hyphal swellings. As most species within this group *P. recalcitrans* is also a soil inhabitant associated with root infections of terrestrial plants. *P. recalcitrans* has been detected over a short time on two hosts having different life-forms (herbaceous plants and deciduous shrubs) in the northern and southern hemispheres and in Mediterranean climates (Balearic Islands and South Africa), always associated with horticulture. In addition *P. recalcitrans* also most likely is associated with *Rhododendron* because DNA extraction from *Rhododendron* in Poland, followed by an oomycete specific ITS-PCR, cloning and sequencing, has revealed the presence of the ITS sequences of this species (Tomasz Oszako, pers comm). These ITS sequences (UASWS0288, UASWS0209 and UASWS0291) were 100% identical to ITS sequences of the South African isolates. Therefore it seems that *P. recalcitrans* has a wide host-range, geographic distribution and ecological niche. As expected for a population that is likely not to be genetically structured by its hosts, geographical isolation and niche occupation, the isolates of *P. recalcitrans* were phenotypically similar and showed little or no polymorphism in the sequences of the six loci examined for each isolate.

P. recalcitrans conformed to the major behavioral trends described for its closer related species of Clade F in the phylogeny of Lvesque and de Cock (2004) because it exhibited fast colony growth, moderate cardinal temperatures and did not form zoospores under standard conditions tested including different temperatures. However we cannot rule out the possible release of zoospores from sporangia (=hyphal swellings) under other environmental conditions. The new species resembles *P. sylvaticum* (Campbell and Hendrix 1967) in the morphology of

FIG. 4. Phylogenetic relationships between *Pythium* and *Phytophthora* spp. derived from a matrix of concatenated alignments of *cox II* and β -Tub gene sequences. The numbers on the nodes represent the posterior probability/percentage bootstrap support based on Bayesian analysis of the dataset, as described in Materials and Methods. The sequences of β -Tub and *cox II* of *Pythium* and *Phytophthora* isolates used in this analysis are the published sequences of Villa et al (2006). The phylogeny was rooted with sequences from *Phytophthora*. Bar = 0.1 substitution positions.

its asexual structures and colony pattern but it differed in being apparently only self-breeding (homothallic) without showing any enhancement of sexual fertility when intraspecific and interspecific matings were performed, as well as in forming both aplerotic and plerotic oospores with thinner walls. Nonetheless van der Plaats-Niterink (1981) reported that some isolates of *P. sylvaticum* did form a few sexual structures in pure culture after long storage. More isolates would need to be examined before discarding the possibility of the new species being heterothallic, as occurs with its close relatives *P. intermedium* and *P. sylvaticum* (FIG. 3). When oogonia were formed *P. recalcitrans* was readily distinguishable from the other species within Clade F, such as *P. spinosum*, *P. irregulare*, *P. cylindrosporum* Paul, *P. mamillatum*, *P. kunmingense* Yu, in having smooth walled oogonia. It differed from *P. intermedium* and *P. attrantheridium* (Allain-Boulé et al 2004) by not producing terminal chains of deciduous hyphal swellings (=“conidia”). Unlike *P. paroecandrum* oogonia of *P. recalcitrans* were not catenulate and no sessile monoclinal antheridia were observed. *P. macrosporum* differed in the formation of thicker-walled oospores, usually contorted antheridial stalks and the production of zoospores. Some of the members of Clade I, *sensu* Lévesque and de Cock (2004), such as *P. ultimum*, *P. debaryanum*, *P. splendens* and *P. heterothallicum*, resembled *P. recalcitrans* morphologically. In fact some isolates identified as *P. debaryanum* Hesse by Robertson (1980) looked similar to *P. recalcitrans* and were distinguished only because the former formed sexual structures in pure culture. *P. recalcitrans* differed from *P. ultimum* in having larger oogonia, thinner-walled oospores and lack of short monoclinal antheridia; *P. splendens* was heterothallic and formed larger sporangia (=hyphal swellings) mostly in a terminal position.

ITS rDNA has been used widely in phylogenetic studies, but it does not necessarily reflect the evolutionary patterns of the entire genome (Shen 2001). However, when tree topologies based on separate genes consistently coincide in grouping together some species, there are good reasons to think of close relationships within the species. The concatenated alignment approach of the two markers β -*tub* and *cox* II undertaken in this study circumvents problems associated with rDNA or single-gene phylogenies and selection of robust phylogenetic markers. Thus genes coding for metabolic and structural proteins, such as *cox* I and II, β -*tub* and NADH dehydrogenase subunit I, are attracting attention. Two other genes tested in the genus *Pythium* in this study (α -tubulin and actin) proved to be easily sampled because their amplification was efficient

both in *Pythium* and *Phytophthora* (Belbahri unpubl data) and could be used to broaden phylogenetic markers in both genera. These genes are conserved and the alignment of their sequences is less ambiguous compared to rDNA (Bruns et al 1991).

Our results suggest that it might be possible to piece together the Peronosporales and Pythiales with concatenated alignments of these two markers (Görker et al 2007). This is of interest because we expect the number of available phylogenetic marker sequences to increase substantially. However our study also showed that certain nodes of the tree were difficult to resolve even with concatenated alignment data. Thus using new markers such as α -tubulin and actin genes could be of great value for constructing more congruent phylogenies of oomycetes.

The molecular phylogenies presented here for the genus *Pythium* support the recent studies correlating molecular clusters with subgroups defined on sporangial form (Briard et al 1995, Matsumoto et al 1999, Martin and Tooley 2000, Lévesque and de Cock 2004, Villa et al 2006). Based on these results and on our analysis it seems that the classification of oomycetes needs to be revised.

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