Pythium brassicum sp. nov.: A Novel Plant Family-Specific Root Pathogen

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Abstract

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Roots of stunted broccoli plants (*Brassica oleracea*) from the Palo Verde Valley, CA, were observed at various stages of decay. A species of *Pythium* with large spiny oogonia was microscopically observed and consistently isolated from decayed roots. Isolates produced spherical, intercalary sporangia (average 34.5 μ m in diameter) and aplerotic oospores (average 37.3 μ m in diameter) in oogonia (average 47.4 μ m in diameter) bearing numerous conical spines (average 8.5 μ m in height and 5.5 μ m basal width) with blunt apices. A representative broccoli isolate (P1) had a 99% internal transcribed spacer (ITS) sequence similarity with *Pythium jasmonium* (nom. inval., GenBank AF216654.1), a species which has not been formally described. Three other accessions in GenBank also carry the specific epithet of *P. jasmonium* and were originally isolated from diseased plants in the family Brassicaceae. In our study, these isolates were pathogenic on broccoli and morpho-

logically similar to P1. P1 was pathogenic to 10 cultivated and 12 wild plants in the family Brassicaceae but not to 18 species of cultivated plants belonging to nine other plant families. Mycelial growth of our isolates occurred between <10 and 35°C, with an optimum of 25°C (maximum growth rate 25 mm/day). Our broccoli isolates are related to other species in subclade B of clade J. Members of subclade B include *P. mastophorum*, *P. uncinatum*, *P. buismaniae*, *P. polymastum*, and *P. megalacanthum*. However, the broccoli isolates, in addition to all those in GenBank that carry the specific epithet of *P. jasmonium*, possess unique ITS, β -tubulin, and *cox1* sequences that are sufficiently different from other species in subclade B to justify status as a new species. We propose that isolates previously designated as *P. jasmonium* (nom. inval.) be renamed as *P. brassicum* sp. nov. based on their apparent plant family-specific pathogenicity.

Pythium spp. are members of the kingdom Stramenopila: phylum Oomycota and many are plant pathogens of economic importance in agriculture. Commonly, they cause damping-off diseases of emerging seedlings in greenhouses and in agricultural fields but some species can also cause root diseases of older plants. *Pythium* spp. tend to be nonspecific in their host range and many species can infect a large number of hosts (e.g., *Pythium ultimum* with 719 host records), whereas some species such as *P. graminicola* and *P. arrhenomanes* are primarily pathogens of graminaceous hosts (8,10,12).

In late October 2011, severely stunted broccoli plants (*Brassica oleracea* var. *italica*), were submitted for diagnosis from the Palo Verde Valley of California. This desert valley is a vegetable crop-growing region and part of the lower Colorado River Basin along the eastern border of southern California adjacent to Arizona. The 30-ha furrow-irrigated crop had been planted in early September and, after 6 to 7 weeks, over 50% of the plants were declining in the field and showing root rot symptoms.

A *Pythium* sp. was isolated and was initially thought to be *P. polymastum* based on morphological features. *P. polymastum* has never been associated with root rot of any field-grown crop in California; however, the pathogen was reported as the cause of damping-off of broccoli and cauliflower seedlings in greenhouses in California in 1998 (1). At first, it was thought that the pathogen could have been introduced into the field on infected nursery stock; however, the broccoli crop had been direct seeded into the field.

The unusual field occurrence and the association of this particular species of *Pythium* with a major crop in California prompted this investigation.

Phylogenetic analysis of internal transcribed spacer (ITS) sequence data divided known *Pythium* spp. into 11 major clades (5,9). Clade J contains two subclades, with *P. nunn*, *P. orthogonon*, *P. acanthophoron*, *P. perplexum*, *P. nodosum*, and *P. cystogenes* in one subclade (herein referred to as subclade A) and *P. buismaniae*, *P. polymastum*, *P. uncinatum*, *P. mastophorum*, *P. megalacanthum*, and *P. jasmonium* in the other subclade (herein referred to as subclade B) (5,9). The species in subclade A do not have many common morphological characteristics, whereas species in subclade B are morphologically distinct, with large oogonia ornamented with spines and thick-walled oospores. Oogonia and oospores of the *Pythium* spp. from our broccoli isolations have the morphological characteristics of species in subclade B of clade J.

The objectives of this study were to clarify the identity and characterize the *Pythium* spp. from broccoli using morphological and molecular methods, as well as determine mycelial growth over a range of temperatures and determine the pathogenicity and host range of the isolates from broccoli.

Materials and Methods

Isolations from root samples and culturing of *Pythium* spp.

Four stunted broccoli plants from three widely separated locations (North, West, and South) within a 30-ha commercial broccoli field were collected. Ten necrotic roots were excised from each stunted plant, washed in running tap water for 10 min, blotted dry, plated onto 1.5% water agar, and incubated at 25°C. Hyphae emerging from the roots were transferred to 10% clarified V8 juice (V8C) agar medium for identification.

Three isolates from broccoli (P1, P2, and P3) and five reference isolates identified as *P. jasmonium* based on ITS sequence information in GenBank, their reference numbers, host, and year of isolation are listed in Table 1. Six isolates (i.e., P1, CBS 101876,

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PPRI8402, PPRI8404, MAFF725020, and MAFF725021) were employed for most of the studies reported herein and will be collectively referred to hereafter as Pj6. Additionally, a reference culture of *P. polymastum* (CBS 811.7) was used in some studies. Stock cultures of all isolates were maintained on 10% V8C agar.

Morphological characteristics of *Pythium* isolates. For these studies, all isolates were grown in 10% V8C broth for 14 days. In total, 50 measurements were made of morphological features of sexual spores of all Pj6 isolates. Asexual structures (i.e., hyphal swellings and sporangia) were not produced by any of the isolates in 10% V8C broth. However, asexual structures were occasionally and sparingly formed in water agar adjacent to broccoli roots colonized by isolates P1 and PPRI8404 in the pathogenicity studies described below.

Growth-temperature studies. A 5-mm-diameter disk of each Pj6 isolate from a 4-day-old culture was placed at the perimeter of three replicate petri plates containing 10% V8C agar. Plates were incubated at 10, 15, 20, 25, 30, or 35°C in the dark. At 24-h intervals, the radial growth of the developing colony was measured and the mean of three consecutive measurements per plate was used to estimate the daily growth rate at the various temperatures. Additionally, colony morphology and hyphal widths were recorded. The experiment was repeated once.

Pathogenicity and host range studies. In a greenhouse pathogenicity test, 2-week-old broccoli seedlings reared in Rockwool starter cubes were individually transplanted into 10-cm-diameter pots containing steam-pasteurized University of California (UC) mix amended with 2 g of P1-colonized millet seed. Control plants were transplanted into pots containing UC mix amended with 2 g of autoclaved millet seed. There were four replications for each treatment. Fresh shoot weights were recorded 21 (first experiment) or 28 (second experiment) days after inoculation. Roots from inoculated and noninoculated plants were randomly collected, examined at $\times 400$ magnification for oospores, and plated onto water agar medium.

In laboratory pathogenicity tests, 10 to 12 seeds of selected plant genera and species (Table 2) were sown on 1.5% water agar in 9-cm-diameter petri dishes. Upon radicle emergence, a 7-mm-diameter plug of a 48-h-old 10% V8C agar culture of selected isolates of the *Pythium* spp. mentioned above was placed in the center of the plate. This method provided the pathogen with a food base sufficient to support vegetative growth across the plate within 48 h at 24°C. Data on postemergence root rot and seedling death or stunting were recorded after 10 to 14 days of incubation in a growth chamber at 24°C with a 12-h photoperiod. Shoot and root length and tissue fresh weights were measured. Disease rating of each seedling was recorded as follows: 0 = no evidence of root rot or shoot stunting, 1 = slight tap root discoloration, 2 = severe stunting or necrosis of tap root and stunting of shoot, and 3 = plant dead. Three replicate petri dishes were used for each isolate and plant species and the experiment was repeated once.

DNA extraction, amplification, and sequencing. Pj6 isolates and two additional isolates from broccoli (P2 and P3) were cultured in 10 ml of V8C broth for 7 days at 25°C. DNA was extracted (17) and 30 ng was used as a template for amplification of the nuclear ribosomal internal transcribed spacer ITS (ITS1, 5.8 S rRNA, ITS2) region (1,000 to 1,100 bp in size), and partial β -tubu-

lin (658 bp in size) and mitochondrial cytochrome c oxidase subunit I (*coxI*) genes (727 bp in size). The ITS universal primers were ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCC TCCGCTTATTGATATGC-3') (9,16). The β -tubulin gene primer pair was forward BT5 (5'-GTATCATGTGCACGTACTCGG-3') and reverse BT6 (5'-CAAGAAAGCCTTACG ACGGA-3') (12). The *coxI* primer pair was forward (OomCoxI-Levup (5'-TCA WCWMGATGGCTTTTTC AAC-3') and reverse (FM85mod 5'-RRHWACKTGACTDATRATA CCAA-3') (10).

Table 2. Pathogenicity of broccoli isolate P1 on cultivated and non-cultivated plants

Plants	Common name	Disease rating ^a
In the family Brassicaceae		
Cultivated		
<i>Brassica oleracea</i> var. <i>acephala</i>	Kale	3
<i>B. oleracea</i> var. <i>botrytis</i>	Cauliflower	3
<i>B. oleracea</i> var. <i>capitata</i>	Cabbage	3
<i>B. oleracea</i> var. <i>gemmifera</i>	Brussel spouts	3
<i>B. oleracea</i> var. <i>italica</i>	Broccoli	3
<i>B. rapa</i> var. <i>rapa</i>	Turnip	3
<i>B. rapa</i> var. <i>ruvo</i>	Rapini	3
<i>Lepidium sativum</i>	Cress	2.4
<i>Lobularia maritima</i>	Sweet alyssum	2.8
<i>Raphanus sativus</i>	Radish	3
Noncultivated		
<i>Arabidopsis thaliana</i> (C24)	Thale cress	3
<i>B. nigra</i>	Black mustard	3
<i>B. rapa</i>	Field mustard	2.6
<i>B. tournefortii</i>	Sahara mustard	3
<i>Cardaria draba</i>	Hoary cress	3
<i>C. pubescens</i>	Hairy whitetop	2.3
<i>Hirschfeldia incana</i>	Shortpod mustard	3
<i>Sinapis arvensis</i>	Charlock mustard	3
<i>Sisymbrium erysimoides</i>	Mediterranean rocket	3
<i>S. irio</i>	London rocket	3
<i>S. officinale</i>	Hedge mustard	3
<i>S. orientale</i>	Oriental hedge mustard	3
In other families		
<i>Bellis perennis</i>	Daisy	0.3
<i>Bouteloua gracilis</i>	Blue grama grass	0
<i>Capsicum annuum</i>	Pepper	0
<i>Cucumis melo</i>	Cantaloupe	0
<i>C. sativus</i>	Cucumber	0
<i>Daucus carota</i>	Carrot	0
<i>Gypsophila paniculata</i>	Baby's breath	0
<i>Gossypium hirsutum</i>	Cotton	0
<i>Lactuca sativa</i>	Lettuce	0
<i>Linum usitatissimum</i>	Fax	0
<i>Lupinus perennis</i>	Lupine	0
<i>Medicago sativa</i>	Alfalfa	0
<i>Nicotiana tabacum</i>	Tobacco	0.2
<i>Ocimum basilicum</i>	Sweet basil	0
<i>Solanum lycopersicum</i>	Tomato	0
<i>Triticum aestivum</i>	Wheat	0
<i>Vinca minor</i>	Dwarf periwinkle	0
<i>Zea mays</i>	Sweet corn	0

^a Disease rating was recorded for each seedling as follows: 0 = no evidence of root rot or shoot stunting, 1 = slight tap root discoloration, 2 = severe stunting or necrosis of tap root and stunting of shoot, and 3 = plant dead.

Table 1. Isolates of *Pythium* spp. used in cultural, temperature, and pathogenicity studies

Isolate	Morphological spp.	Host	Year	Origin	GenBank accession number		
					ITS	β -Tubulin	<i>coxI</i>
CBS101876	<i>Pythium jasmonium</i>	<i>Arabidopsis thaliana</i>	1998	Washington, United States	AF216654	KJ424409	KJ424404
P1	<i>P. brassicum</i>	<i>Brassica oleracea</i> (broccoli)	2011	California, United States	KJ183088	KJ183097	KJ183091
P2	<i>P. brassicum</i>	<i>B. oleracea</i> (broccoli)	2011	California, United States	KJ183089	KJ183098	KJ183092
P3	<i>P. brassicum</i>	<i>B. oleracea</i> (broccoli)	2011	California, United States	KJ183090	KJ183099	KJ183093
PPRI8402	<i>P. jasmonium</i>	<i>B. oleracea</i>	1994	South Africa	FJ415959	KJ424412	KJ424407
PPRI8404	<i>P. jasmonium</i>	<i>B. napus</i> (canola)	1994	South Africa	KJ424403	KJ424413	KJ424408
MAFF725020	<i>P. megalacanthum</i>	<i>B. oleraceae</i> (cabbage)	2004	Japan	AB512739	KJ424410	KJ424405
MAFF725021	<i>P. megalacanthum</i>	<i>B. oleraceae</i> (cabbage)	2004	Japan	AB512782	KJ424411	KJ424406

The polymerase chain reaction (PCR) reaction mixture in 25 μ l consisted of DNA template (30 ng), 10 $\times$ buffer containing 15 mM MgCl₂ (Apex; Genesee Scientific), 2.5 mM each dNTP, 2.5 μ g of bovine serum albumen, 10 μ M each forward and reverse primers, and 1 U of Apex Taq polymerase (Genesee Scientific). PCR amplification conditions using a PTC-100 thermal cycler (MJ Research) consisted of initial denaturation at 95°C for 4 min; followed by 40 cycles of denaturation at 94°C for 1 min, primer annealing for 1 min at 57°C for ITS, 52°C for *coxI*, and 63°C for β -tubulin, and extension at 72°C for 2 min; with a final extension at 72°C for 7 min (15). PCR products were visualized in 2% (wt/vol) agarose gels stained with ethidium bromide. Amplification products were purified by ethanol-sodium acetate precipitation. Sequencing of PCR amplicons was carried out using the same forward and reverse primers as described above in a standard Sanger dideoxy sequencing reaction in a 3730 XL DNA analyzer (ABI, Institute for Integrative Genome Biology, UC Riverside).

Sequence analysis. A BLAST search in GenBank for the three gene sequences (ITS, *coxI*, and β -tubulin) was done for isolates CBS101876, P1, P2, P3, PPRI8402, PPRI8404, MAFF725020, and MAFF725021 (Table 1). Nucleotide sequences representing various isolates within phylogenetic clade J of *Pythium* (5,9) with the highest homology to P1 were selected (Table 3) and used in constructing phylogenetic trees using MEGA version 5 (11). Multiple sequence alignment was performed using ClustalW. Phylogenetic relationships among *Pythium* isolates, based on gene sequences for ITS, *coxI*, and β -tubulin, were inferred by maximum parsimony (MP) using the Subtree-Pruning-Regrafting search method of MEGA. Bootstrap 50% majority-rule consensus trees were generated using 1,000 replications. Phylogenetic trees were constructed for ITS, β -tubulin, and *coxI* individually and for the combined dataset of ITS and *coxI*. *Phytophthora megasperma* isolate P1278 was used as outgroup for β -tubulin analysis.

Results

Isolations from root samples. Diseased broccoli plants were stunted (Fig. 1A) and roots were at various stages of decay. Light microscopy of symptomatic root tissues revealed the presence of

numerous large spiny oogonia in cortical tissues (Fig. 1B and C). A *Pythium* sp. with large spiny oogonia was consistently isolated from necrotic roots of all broccoli plants sampled. One isolate from a plant collected in the North, South, and West portions of the 30-ha commercial field (i.e., P1, P2, and P3, respectively) was randomly selected and placed in our culture collection. All isolates were morphologically indistinguishable. Therefore, P1 was selected as representative of the three isolates for most studies.

Morphological characteristics of *Pythium* isolates. On V8C agar medium, colonies of all isolates from broccoli and the remaining Pj6 isolates grew with little aerial mycelium and without any special growth pattern, with the exception of isolate CBS101876 that produced clumps of aerial hyphae. All isolates produced spiny oogonia in V8C broth and had coenocytic hyphae 5 to 7.5 μ m in diameter. Morphometrics of oogonia, oospores, as well as spine height, width, and shape of the Pj6 isolates are presented in Table 4 and illustrated in Figure 2A and B. All isolates were morphologically similar but dimensions of all structures of isolate CBS101876 from *Arabidopsis thaliana* were smaller. Additionally, spines on oogonia of the latter isolate were less numerous and of varied shapes compared with the other five isolates. Antheridia were bulbous and broadly attached to oogonia and ranged in size from 12.2 by 17.5 to 11.25 by 18.75 μ m. The number of antheridia ranged from 1 to 4 per oogonium for each isolate (Fig. 2A).

None of the isolates produced asexual spores in V8C broth. However, intercalary and terminal sporangia, measuring 25 to 40 μ m in diameter, were occasionally observed in water agar adjacent to broccoli roots colonized by P1 and PPRI8404 but rarely by others. When flooded with sterile distilled water and incubated at 25°C, most sporangia germinated directly within 1 h with a germ tube (Fig. 2C inset). A few sporangia (fewer than 5%) germinated indirectly, with a discharge tube culminating in vesicle and zoospore formation (Fig. 2C). Zoospores were 10 to 12.5 μ m in diameter.

Growth temperature studies. All Pj6 isolates exhibited a similar mycelial growth response for the temperatures evaluated, with an optimum of 25°C and a maximum of 35°C (Fig. 3). However, there were differences in the mycelial growth rates among the iso-

Table 3. Reference isolates of *Pythium* spp. with internal transcribed spacer (ITS), β -tubulin, and cytochrome c oxidase subunit I (*coxI*) sequence accession numbers obtained from GenBank and used for constructing phylogenetic trees

Species	Isolate	Isolation host	Origin (year)	Sequence accession number		
				ITS	β -Tubulin	<i>coxI</i>
<i>Pythium buismaniae</i>	CBS28831	<i>Chrysanthemum</i> sp.	Netherlands (1999)	AY598659	–	HQ708737
<i>P. jasmonium</i>	CBS101876	<i>Arabidopsis</i> mutant	Washington, United States (1998)	AF216654	–	–
<i>P. jasmonium</i>	DAOM229150	<i>Arabidopsis</i> mutant	Washington, United States (2010)	–	–	EU350525
<i>P. jasmonium</i>	DAOM239123	<i>Arabidopsis</i> mutant	Japan (2007)	–	–	EU350524
<i>P. jasmonium</i>	PPRI8402	<i>Brassica oleracea</i>	South Africa (2008)	FJ415959	–	–
<i>P. mastophorum</i>	CBS37572	<i>Apium graveolens</i>	England (2010)	–	–	EU350523
<i>P. megalacanthum</i>	DSMZ62964	<i>Chrysanthemum</i> sp.	Germany (1999)	AF216660	–	–
<i>P. megalacanthum</i>	DAOM229154	Not listed	Germany (2010)	HQ643693	–	HQ708737
<i>P. nodosum</i>	MAFF305905	Soil	Japan	DQ071346	–	–
<i>P. nunn</i>	ATCC20693	Soil	Colorado, United States	–	DQ071325	–
<i>P. orthogonon</i>	DS2-6-9D	<i>Zoysia</i> sp.	Japan	–	DQ071329	–
<i>P. polymastum</i>	ADC9817	Not listed	Netherlands (2010)	–	–	HQ708476
<i>P. polymastum</i>	CBS81170	<i>Lactuca sativa</i>	Netherlands (2013)	AY598660	–	–
<i>P. uncinulatum</i>	CBS51877	<i>L. sativa</i>	Netherlands (2013)	AY598712	–	AY598712
<i>Pythium</i> sp.	CAL-2011a	–	Washington, United States (2010)	–	–	HQ708819

Table 4. Morphometrics of sexual spores of isolates of *Pythium* spp. evaluated in this study^a

Isolate	Oogonium diameter (μ m)	Oospore diameter (μ m)	Spine height (μ m)	Spine base width (μ m)
CBS101876	39.2 (30.0–46.8)	32.6 (26.2–38.2)	6.0 (3.7–11.2)	5.1 (2.5–7.0)
P1	47.4 (37.5–61.6)	37.3 (30.0–50.0)	8.5 (4.7–16.2)	5.5 (1.6–10)
PPRI8402	42.2 (35.1–52.5)	33.1 (26.5–40.0)	7.7 (4.7–15.6)	5.3 (3.0–8.6)
PPRI8404	44.6 (35.9–57.5)	36.2 (28.1–47.5)	6.2 (3.9–10.0)	5.2 (3.1–7.8)
MAFF725020	42.1 (34.3–47.5)	35.1 (25.1–42.5)	6.5 (3.1–9.5)	5.5 (3.1–7.8)
MAFF725021	42.6 (32.7–50.0)	34.6 (27.3–45.0)	6.1 (3.1–11.2)	5.3 (3.1–7.8)

^a Values represent means of 50 measurements from four independent experiments. Numbers in parentheses are the ranges of lowest and highest measurements. Isolates were cultured in 10% clarified V8 juice broth.

lates. CBS101876 and PPRI8402 had the slowest mycelial growth rates at the lower temperatures evaluated.

Pathogenicity tests. In greenhouse tests, broccoli isolate P1 caused stunting and reduction in fresh weight relative to noninoculated controls. Fresh weight of plants was significantly reduced from 16.4 and 23.7 g in the controls to 6.9 and 12.1 g in the inoculated treatments after 3 and 4 weeks for experiments 1 and 2, respectively (Fig. 4). Oospores of the pathogen were microscopically observed in necrotic roots and the pathogen was reisolated, thus completing Koch's postulates. Additionally, laboratory tests confirmed the pathogenicity of P1 to broccoli as well as 9 cultivated and 12 noncultivated plant species in the family Brassicaceae but not to 18 species of plants belonging to 11 other plant families (Table 2). Roots of all seedlings in the family Brassicaceae became stunted and necrotic within 7 days and most seedlings died within 10 days after inoculation (Fig. 5). Numerous spiny oögonia were observed in necrotic roots and in agar adjacent to necrotic roots.

Fig. 1. Broccoli (*Brassica oleracea* var. *italica*) plants collected 55 days after seeding in a commercial field in Blythe, California. **A**, Infected (left) and healthy (right) plants. Bar = 4 cm; **B**, Oospores of *Pythium brassicum* inside a necrotic root. Bar = 260 μ m; and **C**, a large spiny oögonium (diameter = 48 μ m) and aplerotic oospore of *Pythium brassicum* inside a necrotic root. Bar = 25 μ m.

Additionally, sporangia were also observed in agar adjacent to infected roots. Similar to P1, the other five Pj6 isolates and P2 and P3 were pathogenic (disease rating of 3) to broccoli, with significant reductions in stem and root lengths and plant fresh weights (Fig. 6), but not to lettuce or flax. Only an isolate of *Pythium polymastum*, CBS811.7, was pathogenic (disease rating of 3) to lettuce but not (disease rating of 0) to broccoli. All pathogenicity studies were repeated with the same results.

Phylogenetic analysis. Sequences for ITS, *coxI*, and β -tubulin genes for all Pj6 isolates were deposited in GenBank with accession numbers listed in Table 1. Using the MP method on the ITS dataset, *Pythium* isolates from Japan (*P. megalacanthum* MAFF725020 and MAFF725021), South Africa (*P. jasmonium* PPRI8402 and PPRI8404), and the United States (*P. jasmonium* CBS101876 and our broccoli isolates P1, P2, and P3) grouped

Fig. 2. Sexual and asexual reproductive structures of *Pythium brassicum*. **A**, Spiny oögonium with four antheridia (a) attached; **B**, Spiny oögonium with a mature thick-walled aplerotic oospore. Bars = 30 μ m. **C**, Intercalary and terminal sporangia (s). The terminal sporangium has germinated indirectly with an evacuation tube (et) and a vesicle (v) with zoospores. Inset: Sporangium germinating directly with a germ tube (gt). Bars = 25 μ m.

Fig. 3. Growth-temperature relationships of *Pythium brassicum* isolates. Means of six isolates are from two experiments.

closely together and formed a distinct cluster separate from other species in subclade B with 100% bootstrap support (Fig. 7A). The latter species included *P. mastophorum* CBS37572, *P. uncinulatum* CBS51877, *P. megalacanthum* DAOM229154 and DSMZ62964, *P. polymastum* CBS81170 and ADC9817, and *P. buismaniae* CBS28831. The consistency index (CI) was 0.958084 and the retention index (RI) was 0.987784.

Analysis of β -tubulin sequences using MP showed the same clustering of all *Pythium* isolates from Japan, South Africa, and the United States, with P1, P2, and P3 slightly distant from the rest of the other isolates. The CI and RI values were 0.902913 and 0.782609, respectively. As an outgroup, *P. orthogonon* DS-2-6-9D,

Fig. 4. Fresh shoot weights of broccoli plants 3 and 4 weeks after inoculation with *Pythium brassicum* (isolate P1). There were four replications of each treatment. Bars within each experiment marked with a different letter differ significantly and *P* values indicate levels of significance within each experiment.

Fig. 5. Pathogenicity tests with *Pythium brassicum* (isolate P1) on garden cress (left), broccoli (center), and rapini (right) on water agar 7 days after inoculation. Inoculated plants in foreground and respective controls in background.

Fig. 6. Pathogenicity tests with Pj6 isolates on broccoli seedlings. All isolates significantly reduced stem and root length, and decreased tissue fresh weight 7 days after inoculation compared with the noninoculated control.

P. nodosum MAFF305905, and *P. nunn* ATCC20694 formed a separate cluster (subgroup A), with 83% bootstrap support and considerable diversity (Fig. 7B).

Phylogenetic analysis for *cox1* showed clustering of the Pj6 isolates, *P. jasmonium* DAOM229150 and DAOM239123, as well as *Pythium* sp. CAL2011a (CBS101876), with 100% bootstrap support. There were slight genetic distances among them. All four Japanese and South African isolates (*P. megalacanthum* MAFF725020 and MAFF725021 and *P. jasmonium* PPR18402 and PPR18404) together with the *P. jasmonium* isolate from Washington (DOAM 229150), formed a subgroup (77% bootstrap support)

Fig. 7. Phylogenetic analyses of *Pythium* isolates based on: **A**, internal transcribed spacer (ITS); **B**, β -tubulin; **C**, *cox1*; and **D**, combined ITS and *cox1* gene sequences using maximum parsimony. Numbers on the branches represent bootstrap values greater than 50 obtained from 1,000 replications. Bars = numbers of substitutions per site.

distinct from that of P1, P2, and P3 isolates (89% bootstrap support). The CI and RI values for this MP analysis were 0.904255 and 0.938776, respectively. *P. megalacanthum* DAOM229154 and *P. polymastum* ADC9817 had identical *coxI* sequences and grouped distantly with *P. uncinulatum* CBS51877 and *P. mastophorum* CBS37572 (Fig. 7C).

The topology of combined ITS and *coxI* gene sequences revealed that the Pj6 isolates, as well as P1, P2, and P3; *P. jasmonium* isolates DAOM 229150 and DAOM239123; and *Pythium* sp. CAL-2011a (CBS101876) constitute a cluster with 100% bootstrap support within subclade B of clade J, which is distinct from the second cluster of isolates in subclade B represented by *P. mastophorum*, *P. buismaniae*, *P. megalacanthum*, and *P. polymastum* (Fig. 7D). Genetic differences among *Pythium* isolates in the first cluster were small, whereas there was much more diversity among isolates in the second cluster. The analysis for combined ITS and *coxI* sequence data had a CI = 0.941176 and RI = 0.970954.

Discussion

Isolations from diseased roots of stunted broccoli plants yielded a *Pythium* spp. with distinct morphological characteristics and could not be easily assigned to any known species infecting this crop. The broccoli isolates (e.g., P1, P2, and P3) had 99% ITS sequence similarity with *P. jasmonium* (4) and 100% with two isolates of *P. megalacanthum* (MAFF725020 and MAFF725021). *P. jasmonium* CBS101876 was originally identified as *P. mastophorum* when first recovered from necrotic roots of a jasmonate-suppressed *A. thaliana* mutant in 1998 (14). Because it had a distinct ITS sequence, it was later designated a new species, *P. jasmonium* (4,7). However, this species has not been formally described and, thus, is an invalid name. A search by us in GenBank indicated that three other accessions carry the specific epithet of *P. jasmonium* (DAOM229150, DAOM239123, and PPRI8402) but were previously identified as either *P. megalacanthum* or *P. polymastum* based upon similar morphological characteristics. Other isolates labeled *P. megalacanthum* (DAOM229154) or *P. polymastum* (ADC9817) grouped into the second cluster of subclade B of clade J in our molecular analysis (Fig. 7A, C, and D).

Our data support the naming of a new species for all Pj6 isolates, DAOM229150 and DAOM239123 isolates, and additional isolates from broccoli in California (e.g., P2 and P3) because these isolates all grouped closely together in the phylogenetic analyses of multilocus sequence data. Additionally, these isolates originated from diseased roots of plants in the family Brassicaceae and, as we demonstrated for several of them, are apparently pathogenic only to species in this plant family. The specific epithet “*jasmonium*”, however, is not appropriate because none of the susceptible plants in our study were jasmonate suppressed and, second, the epithet “*jasmonium*” is an invalid name. Additionally, although two isolates assigned to *P. megalacanthum* are found in the phylogenetic cluster together with the broccoli isolates, this species epithet is also not suitable because another isolate of *P. megalacanthum* (DAOM229154) grouped with the second cluster of species in subclade B of clade J. Thus, we suggest placing *P. jasmonium* in synonymy with a new species, *P. brassicum*, as described below.

Morphological description: *Pythium brassicum* Stanghellini, Mohammadi, Förster, & Adaskaveg sp. nov. CBS137315, Holotype, P1, isolated from roots of broccoli, California, USA.

Colonies on clarified V8 juice agar submerged without a special pattern. Main hyphae up to 7.5 µm wide. Sporangia globose to irregularly shaped, 25 to 40 µm (average 34.5 µm) in diameter, terminal or intercalary. Zoospores (10 to 12.5 µm diameter) released from vesicles at 25°C. Discharge tubes 20 to 200 µm in length and 5 to 7 µm wide arising from any part of the sporangium. Oogonia mostly terminal on side branches, globose, 37.5 to 61.6 µm (average 47.4 µm) in diameter with numerous spines. Spines mostly conical with blunt tips, 4.7 to 16.2 µm (average 8.5 µm) in height, 2 µm in diameter at spine tip, and 5.5 µm diameter at spine base. Antheridia 1 to 4, mostly 1 per oogonium, primarily decli-

nous, bulbous, 12.5 to 18.7 µm in diameter. Oospores aplerotic, 30 to 50 µm (average 37.3 µm) in diameter, wall up to 2.5 µm thick. Isolate P1 of *P. brassicum* is herein designated the holotype and has been deposited at the CBS-KNAW Fungal Biodiversity Center, The Netherlands.

In addition to possession of unique sequences (ITS, β-tubulin, and *coxI*) that are sufficient to justify status as a new species, spines on oogonia of *P. brassicum* are primarily conical with blunt tips. This is in contrast to spine shapes reported for other species in subclade B (2,3,8,12). Spines of *P. mastophorum* and *P. polymastum* are primarily mammiform with acute tips. Spines of *P. uncinulatum* are curved (resembling rose thorns) with acute tips, and spines of *P. buismaniae* are conical with irregular terminal projections. Spines of *P. megalacanthum* have been reported as conical with acute tips or mammiform.

Host specificity, coupled with distinct molecular characteristics, has been used in naming of a new species of *Phytophthora* (i.e., *Phytophthora brassicae*) (6). The concept of a Brassicaceae-family-specific species of *Pythium* is not new. In 1974, Vanterpool reported on the host-specificity of a “*Brassica* strain of *P. polymastum*” (13). The morphological characteristics and host range of his isolates closely resemble those we recovered from broccoli. Unfortunately, a culture of his isolate that was deposited in the culture collection of CMI (IMI173501) is no longer available.

Whereas crop rotation is generally not a recommended management strategy for *Pythium* spp. because these pathogens generally have wide host ranges, the existence of host specificity (i.e., *Pythium brassicum*) indicates that rotation with non-Brassicaceae plants and management of weeds belonging to this plant family could be efficacious in disease management.

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