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FULL PAPER

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Pythium apinafurcum: its morphology, molecular phylogeny, and infectivity for plants

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Abstract During a survey of *Pythium* species in soils of Japan, *Pythium* isolates growing at high temperatures were obtained from an uncultivated field soil in Wakayama Prefecture. All six isolates showed similar morphology to each other and had complexly branched secondary hyphae, globose nonproliferating sporangia, and smooth-surfaced oogonia that have one or two oospores per oogonium. The combination of these characteristics differentiated these isolates from other *Pythium* species reported. Phylogenetic analyses based on sequences of the ribosomal DNA ITS and D1/D2 region of the large subunit showed that all *Pythium* isolates were clustered in a single clade that was distantly related to other known clades of the genus. We described these isolates as a new *Pythium* species, *Pythium apinafurcum*, based on morphology and molecular phylogeny. The *P. apinafurcum* isolates nonsymptomatically infected the roots of seedlings of bermudagrass, cabbage, and cucumber in a pot inoculation test.

Key words D1/D2 · Intraspecific · New species · rDNA · Taxonomy

Introduction

The genus *Pythium* is widely distributed throughout the world, and approximately 150 species are reported (Dick 2001; Kirk et al. 2008). Members of the genus *Pythium* are ubiquitous and occupy several ecological niches (van der Plaats-Niterink 1981). *Pythium* species are known to inhabit uncultivated field soils as saprophytes or potential plant pathogens (Schmitternner 1972; van der Plaats-Niterink 1981; Watanabe 1994). Recently, many new *Pythium* species have been described based on the strains isolated from uncultivated fields or seminatural environments in several countries (Allain-Boulé et al. 2004; Nechwatal et al. 2005; Nechwatal and Mendgen 2006; Paul 2006; Belbahri et al. 2008; Moralejo et al. 2008; Paul and Bala 2008). These facts suggest that more undescribed *Pythium* species exist in the soils of uncultivated fields and thus a survey of *Pythium* species is required.

Identification of *Pythium* is mainly based on morphological features such as size and shape of sporangia or oogonia (van der Plaats-Niterink 1981). However, members of *Pythium* are considered to be a very difficult group for identification of species because these characteristics are often very similar among different species and sometimes do not form on agar medium plates (Hendrix and Papa 1974). Therefore, morphological taxonomy of the genus *Pythium* is increasingly being supplemented by the molecular characteristics of a given species (Paul 2003). The internal transcribed spacer (ITS) region of rDNA has become a useful tool for the separation, identification, and determination of *Pythium* species (Matsumoto et al. 1999; Paul 2002; Lévesque and de Cock 2004). Sequence variations among *Pythium* species were also recognized in D1/D2 region of the large subunit (LSU) rDNA, and the region has been used for phylogenetic studies (Riethmüller et al. 2002; Lévesque and de Cock 2004). These reports suggested that the sequence data of the D1/D2 region are also useful for clarifying the identity and the phylogenetic position of unknown *Pythium* species.

During a survey from 2003 to 2007 of *Pythium* species in soils of 18 prefectures of Japan, 554 isolates of *Pythium* species were obtained. Among them, 6 isolates from Wakayama Prefecture produced both asexual and sexual structures and showed morphological characteristics different from *Pythium* species previously reported. Their rDNA ITS region and LSU D1/D2 region sequences were unique and quite different from those of known *Pythium* species. We described these isolates as a new species based on morphology and molecular phylogeny. Inoculation tests

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Table 1. Dimensions of sexual and asexual structures and accession numbers of internal transcribed spacer (ITS) and D1/D2 gene sequences of *Pythium apinafurcum* isolates

Strain no. (intra specific group)	International no.		Specimen no. TNSF	Primary hyphae width (μm)	Sporangium diameter (μm)	Oogonium diameter (μm)	Oospore diameter (μm)	Oospore wall thickness (μm)	Oogonia with two oospores (%)[a]	Accession no.	
	MAFF	NBRC								ITS	D1/D2
UZ298 (GII)	241065	–	24281	2.4–5.1	8.3–28.9	11.4–24.3	10.5–22.0	0.4–2.2		AB458649	AB458656
UZ299 (GI)	241058	105193	24282	2.5–5.9	13.3–26.1	12.6–29.8	10.9–28.1	0.2–1.5	11.5	AB458650	AB458659
UZ300 (GI)	241059	105194	19008	2.0–5.0	10.3–24.4	11.3–21.4	10.4–19.8	0.3–1.8	11.2	AB458651	AB458660
UZ301 (GII)	241060	105195	24283	2.3–5.7	9.0–27.1	11.4–27.8	10.4–21.9	0.4–2.4	8.3	AB498652	AB458657
UZ302 (GII)	241061	105196	24284	2.1–5.0	12.8–26.9	11.7–24.5	11.2–19.8	0.6–1.5	13	AB498653	AB458658
UZ303 (GI)	241066	–	24285	2.4–5.2	11.4–27.3	12.9–22.5	11.7–22.3	0.3–1.8	8.8	AB498654	AB458661

[a] Proportion of oogonia with two oospores to the total number of oogonia

conducted for the new species on several plants revealed its nonsymptomatic infection of the plants.

Materials and methods

Isolation

All six isolates of *Pythium* were obtained from a soil sample collected in a field naturally growing gramineous weeds at Susami Town (33°30′ N, 135°35′ E, 5 m alt.), Wakayama Prefecture. Isolations from the soil sample were performed with the baiting technique (Watanabe 1981) using seeds of green pepper and cucumber and potato tuber cubes (0.125 cm^3) as baiting substrates. Substrates embedded in the soil sample in Petri dishes were incubated at 20°–30°C for 24–48 h. The substrates were then removed from the soil, washed under running tap water, dried, and placed on water agar (WA). After incubation on WA at 25°C, a single hyphal tip of colonies developed from the substrates was transferred onto WA. *Pythium* isolates obtained were maintained on corn meal agar (CMA; Becton Dickinson, Sparks, MD, USA) until used for analyses. All isolates were deposited in Microbiological Genebank, National Institute of Agrobiological Sciences (MAFF), Japan, and Biological Resource Center, National Institute of Technology and Evaluation (NBRC), Japan (Table 1).

Examination of morphology and hyphal growth

Morphology of the six isolates of *Pythium* was examined in grass-leaf water culture (van der Plaats-Niterink 1981). Forty sporangia, oogonia, and oospores were measured for each isolate after incubation for 1–10 days at about 25°C. Colony patterns were examined on potato dextrose agar (PDA; Becton Dickinson) and potato carrot agar (PCA) prepared according to van der Plaats-Niterink (1981) in Petri dishes after incubation for 6 days at 25°C. To determine hyphal growth rates, the isolates were incubated on PCA at 4°–43°C at intervals of 3°C for 7 days and measured for colony diameter. The experiment was repeated three times with one plate per repetition. Analysis of variance was conducted for the growth rate using JMP software (version 5.1.1; SAS Institute, Cary, NC, USA). Means of the data were compared by least significant difference based on the Tukey–Kramer honestly significant different (HSD) test ($P < 0.05$).

DNA extraction and amplification

DNA extractions and polymerase chain reaction (PCR) for the six isolates of *Pythium* were prepared by the method described previously (Uzuhashi et al. 2008). In the PCR reactions, primer pairs of ITS5 (5′-GGAAGTAAAAGTC GTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATT GATATGC-3′) as described by White et al. (1990) and NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′)

and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) described by O'Donnell (1993) were used for ITS region, including the 5.8S rDNA and the D1/D2 region of the LSU rDNA, respectively. All PCR products were purified with the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) following the manufacturer's instructions and then used for sequence analysis.

DNA sequencing and phylogenetic analysis

Sequence reactions for the six isolates of *Pythium* were performed as previously described (Uzuhashi et al. 2008). All sequences determined were deposited in GenBank (see Table 1). The sequences were aligned with relevant *Pythium* sequences obtained from the GenBank database using the ClustalX program (Thompson et al. 1997) and manually using MacClade 4.0 (Maddison and Maddison 2000). At least one species of *Pythium* was chosen from each clade of "A" to "K" as described by Lévesque and de Cock (2004), using their sequence data. The complete alignments were deposited in TreeBASE as SN4223 (ITS) and SN4220 (D1/D2). The ITS and D1/D2 multiple sequence alignments were analyzed with the RAxML software (Stamatakis 2006) under the maximum-likelihood (ML) criterion. Nodal support was evaluated with 100 nonparametric bootstrapping pseudoreplications using the GTRMIX algorithm for the analysis. A ML tree constructed by the RAxML program was visualized by TREEVIEW program version 1.6.6 (Page 1996). Bootstrapping for the ITS and D1/D2 analyses were also conducted based on heuristic search under the unweighted maximum parsimony (MP) criterion (Fitch 1971) with PAUP* (Swofford 2003). Parsimony bootstrap analysis with 500 replicates was performed by subsequent tree-bisection-reconnection (TBR) branch swapping and random addition sequence.

Pathogenicity test

Two isolates of *Pythium*, UZ300 and UZ301, were tested with seedlings of bermudagrass (*Cynodon dactylon* (L.) Pers., cv. Sundevil II), cabbage (*Brassica oleracea* L. capitata group, cv. Okina), and cucumber (*Cucumis sativus* L., cv. Jibai). These plants were chosen because they were commonly found around the field where all the isolates of the new species were obtained. A broad host range plant pathogen, *Pythium ultimum* Trow var. *ultimum* isolate OPU774 (Kida et al. 2007), was used for comparison. Inoculum was prepared by the method described previously (Uzuhashi et al. 2008). Infested soil was prepared by mixing 30 g inoculum in 1 l nursery soil (Aisai-1; Katakura Chikkarin, Tokyo, Japan). Each geminated seed of the plants was placed in a well of a 24-well plastic plate (BD Falcon, Franklin Lakes, NJ, USA) containing 200 ml infested soil per plate. Each plate was enclosed in a double-layer polyethylene bag and kept at 28°C under continuous light (73 μmol m^{-2} s^{-1}, measured at plant levels) in a growth chamber. The soil in the plates was watered each day to keep the moisture near –10 kPa. Uninfected soil was used as a control. Six days after inoculation, the mortality of seedlings was recorded as the number of seedlings showing pre- and postemerging damping off. Recovery of the inoculated *Pythium* isolates from infected roots was checked by using the *Pythium* selective NARM medium (Morita and Tojo 2007). Colonization of the isolates was confirmed by optical microscopic observation (400×). The experiments were repeated four times with one plate per repetition. Analysis of variance was conducted for the mortality and the recovery of different *Pythium* isolates using JMP software (version 5.1.1; SAS Institute). Means of the data were compared by least significant difference based on the Tukey–Kramer HSD test ($P < 0.05$).

Taxonomy

Pythium apinafurcum Uzuhashi & Tojo, sp. nov.

Figs. 1, 2

Coloniae in PCA submersae, cum ordinationibus rosularibus; crescens inter 10° et 40°C, optime ad 34°C, cum incremento radianum quotidianus 12 mm ad 12 mm. Hyphae principales hyalinae, non-septatae, 2.0–5.0 μm latae. Hyphae secundariae tenues et pluriramosae. Appressoria raro formata, falciformes. Sporangia intercalaria, raro terminalia, globosa, 10.3–24.4 μm diametro. Zoosporae raro formatae inter 15° et 25°C. Culturae homothallicae. Oogonia intercalaria, terminalia, globosa, laevia, 11.3–21.4 μm diametro. Antheridia monoclina vel diclina, 1–2 in quoque oogonio, sessilia, raro breviter stipitata; stipites antheridiorum sine ramis. Oosporae pleroticae vel apleroticae, 10.4–19.8 μm diametro; paries 1.8 μm crassus, 1–3 in quoque oogonio. Incrementum diurnum in PCA 25°C, 12 mm.

Holotypus: On gramineous weeds, Susami Town, Wakayama Prefecture, Japan (33°30′ N, 135°35′ E, 5 m alt.), collected by Shihomi Uzuhashi, TNS-F-19008 (dried culture); ex-type cultures: MAFF 241059 and NBRC 105194.

Etymology: *apinafurcum* refers to the branches frequently observed on tips of secondary hyphae.

Colonies on PCA submerged with a rosette pattern. Daily growth at 25°C on PCA 12 mm. Minimum temperatures for hyphal growth on PCA 10°C, optimum 34°C, and maximum 40°C. Main hyphae hyaline, aseptate, 2.0–5.0 μm wide, with narrow wide secondary hyphae, frequently branched complexes with various lengths. Appressoria sickle shape. Sporangia intercalary, occasionally terminal, globose, 10.3–24.4 μm (mean, 18.9 μm) in diameter. Zoospores occasionally formed at 15° to 25°C. Oogonia produced in single culture, globose, smooth, intercalary or terminal, 11.3–21.4 μm (mean, 17.1 μm) in diameter. Antheridia, usually one to three per oogonium, diclinous or monoclinous, occasionally on short unbranched stalk. Oospores plerotic or aplerotic, one or two per oogonium, 10.4–19.8 μm (mean, 15.4 μm) in diameter, wall up to 1.8 μm thickness.

Specimen examined: See holotypus.

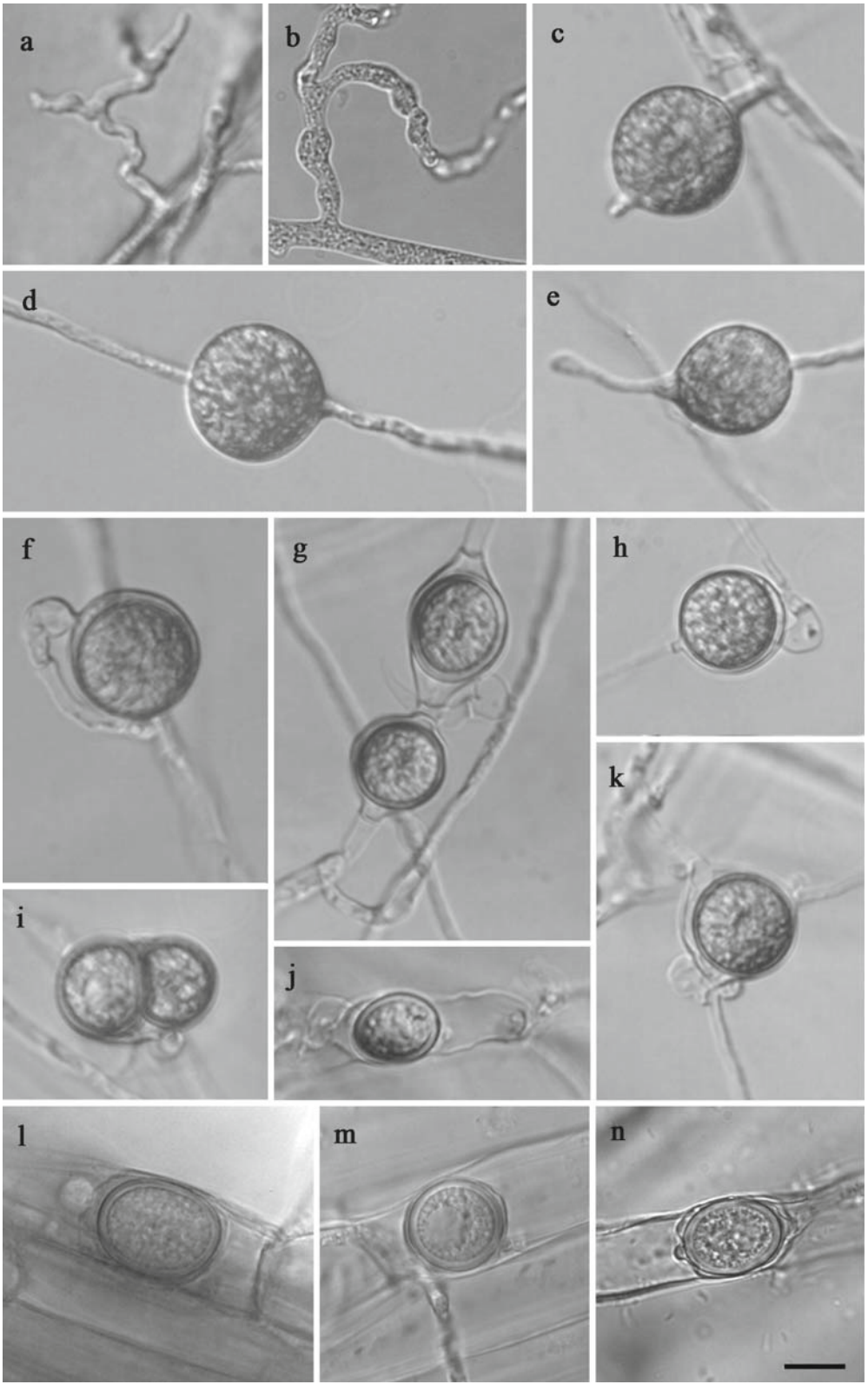

Fig. 1. Morphology of *Pythium apinafurcum* (UZ300, ex-type strain). **a, b** Primary hyphae formed thin secondary hyphae frequently branched and formed complexes with various lengths. **c, e** Terminal globose sporangia. **d** Intercalary globose sporangium. **f** Oogonium with monoclinous antheridium. **g** Internal oogonium. **h** Terminal oogonium with diclinous antheridium. **i** Oogonium with two oospores. **j** Elongated oogonium with aplerotic oospore. **k** Oogonium with some antheridia. **l–n** Oospores formed in root cells [bermudagrass (**l**); cucumber (**m**); cabbage (**n**)]. *Bar* 10 μm

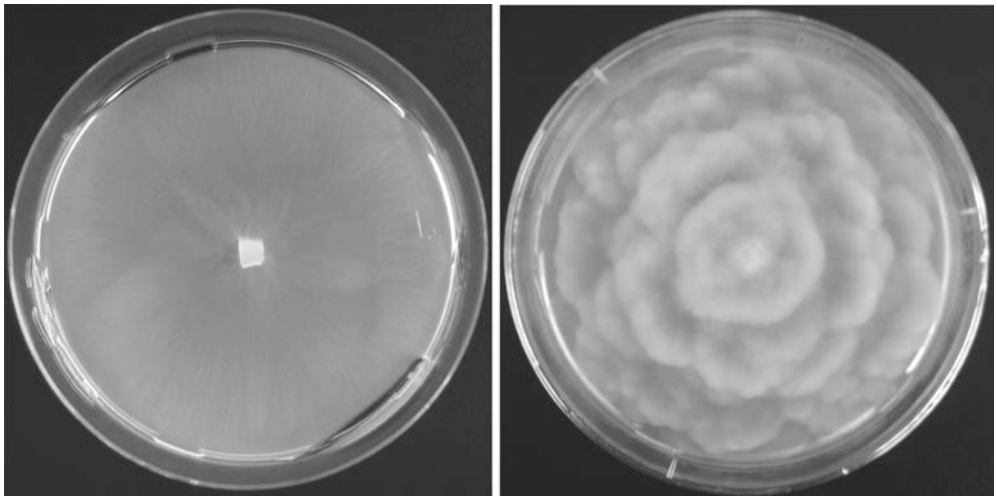

Fig. 2. Colony patterns of *Pythium apinafurcum* (UZ300, ex-type strain) on potato carrot agar (PCA, *left*) and potato dextrose agar (PDA, *right*) at 25°C for 6 days

Results and discussion

Morphological characteristics

All six isolates of *P. apinafurcum* had globose, nonproliferated sporangia, smooth-surfaced oogonia, and complexly branched secondary hyphae (Fig. 1). The morphological features, such as globose, nonproliferated sporangia and smooth-surfaced oogonia, of *P. apinafurcum* resembled those of *P. irregulare* Buisman, *P. ultimum* var. *ultimum*, and *P. paroecandrum* Drechsler. *Pythium apinafurcum* differed from them by the complexly branched secondary hyphae, consistent production of two oospores in one oogonium, and variable size of sporangia and oogonia. All isolates of *P. apinafurcum* showed chrysanthemum or fine radiate patterns of mycelia without aerial hyphae on PCA and a rosette pattern with abundant aerial hyphae on PDA (Fig. 2). All the isolates grew at a range of 10°–40°C and showed the most rapid growth at 34°C (Fig. 3). They grew constantly at 40°C. The isolates did not grow at 4° or 7°C but showed regrowth after incubation at 25°C. Regrowth was not observed after incubation at 43°C in all isolates. Constant growth at 40°C has been known in some species of *Pythium* including *P. acanthicum* Drechsler, *P. aphanidermatum* (Edson) Fitzp., *P. arrhenomanes* Drechsler, *P. echinulatum* V. D. Matthews, *P. helicoides* Drechsler, *P. hypogynum* Middleton, *P. myriotylum* Drechsler, *P. oedichilum* Drechsler, *P. periilum* Drechsler, and *P. periplocum* Drechsler (Middleton 1943). The temperature response of *P. apinafurcum* is similar to that of these high-temperature-growing *Pythium*.

All six isolates of *P. apinafurcum* had the same morphological characteristics (see Table 1, Fig. 1). However, the isolates were divided into two groups by the amount of reproductive organs and the temperature response for growth (see Fig. 3). The first group included the isolates UZ299, UZ300, and UZ303, and the second group included the isolates UZ298, UZ301, and UZ302. Both groups were isolated from a single soil sample with the same isolation frequency; i.e., three isolates of each group were obtained.

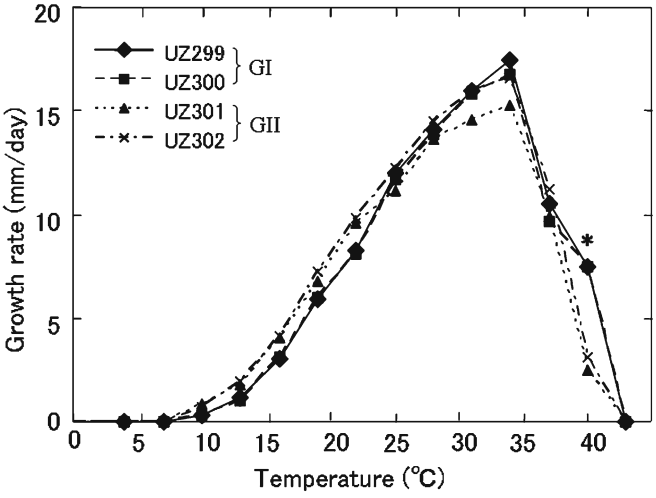

Fig. 3. Comparison between the intraspecific group I (*GI*) and the intraspecific group II (*GII*) of *Pythium apinafurcum* isolates on hyphal growth on potato carrot agar (PCA) at different temperatures. The isolates UZ299 and UZ300 of GI and UZ301 and UZ303 of GII were used. Significance of the difference between the GI and the GII isolates in the Tukey–Kramer honestly significant different (HSD) test is indicated (*$P < 0.05$)

Because morphology was equivalent between these two groups (Table 1), their cultural differences were thought to be an intraspecific variation. We defined the first group as intraspecies group I (GI) and the second group as intraspecific group II (GII). The GI isolates produced relatively sparse mycelia, and many sporangia and oogonia, compared with the GII isolates. The mycelial growth rate at 40°C was significantly lower ($P < 0.05$) in the GII isolates than in the GI isolates (Fig. 3).

Phylogenetic position of *P. apinafurcum*

In the phylogenetic analyses based on the ITS sequences, ML and MP trees showed similar topology to each other. Tree topology by ML analysis is shown in Fig. 4 together with bootstrap values from MP analysis. All isolates of

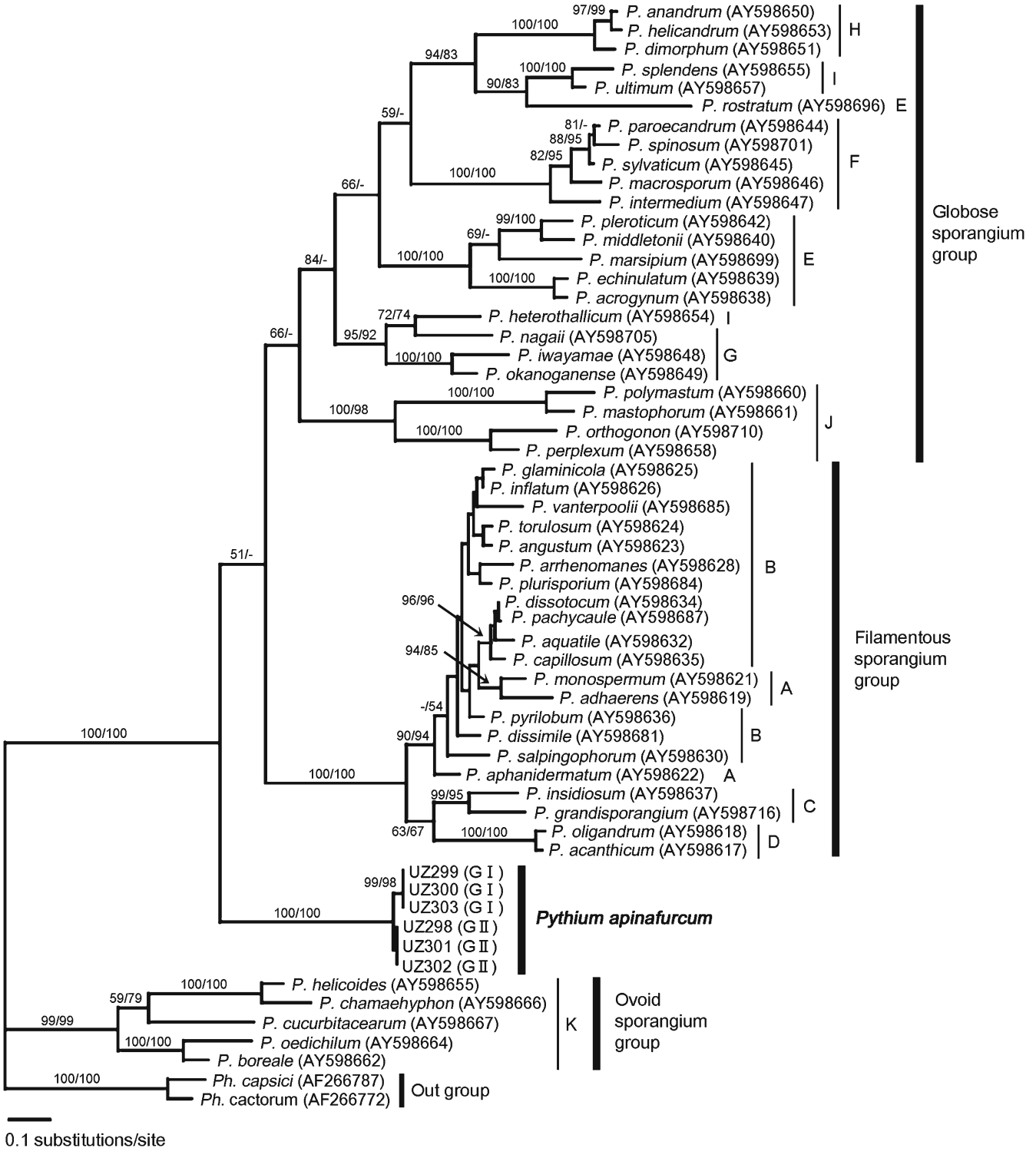

Fig. 4. Phylogenetic position of *Pythium apinafurcum* isolates among *Pythium* species based on the internal transcribed spacer (ITS) sequence of the rDNA. Branch lengths were estimated with RAxML under maximum likelihood (ML). *Numbers on the branches* represent bootstrap values greater than 50%. ML bootstrap values from 100 replicates conducted with RAxML (*left*) and MP bootstrap values from 500 replicates conducted with PAUP* (*right*) are shown. *Numbers* following the species name indicate GenBank accession numbers. Letters *A* to *K* indicate each clade described by Lévesque and de Cock (2004). Sporangial morphological groups were based on van der Plaats-Niterink (1981) and Lévesque and de Cock (2004)

P. apinafurcum appeared to be a monophyletic group that did not belong to any clades of A to K as described by Lévesque and de Cock (2004). This phylogenetic position of *P. apinafurcum* was supported by the phylogenetic trees based on the D1/D2 sequences (Fig. 5). This unique position, strongly supported by all analyses in this study, indicates that *P. apinafurcum* is phylogenetically distinct from any other *Pythium* species reported.

The entire length of the final ITS sequence alignment was 1346 bp. The log-likelihood of the best ML tree recovered by RAxML was –19220.494695. The ML tree scores were base frequencies of A = 0.217453, C = 0.193299, G =

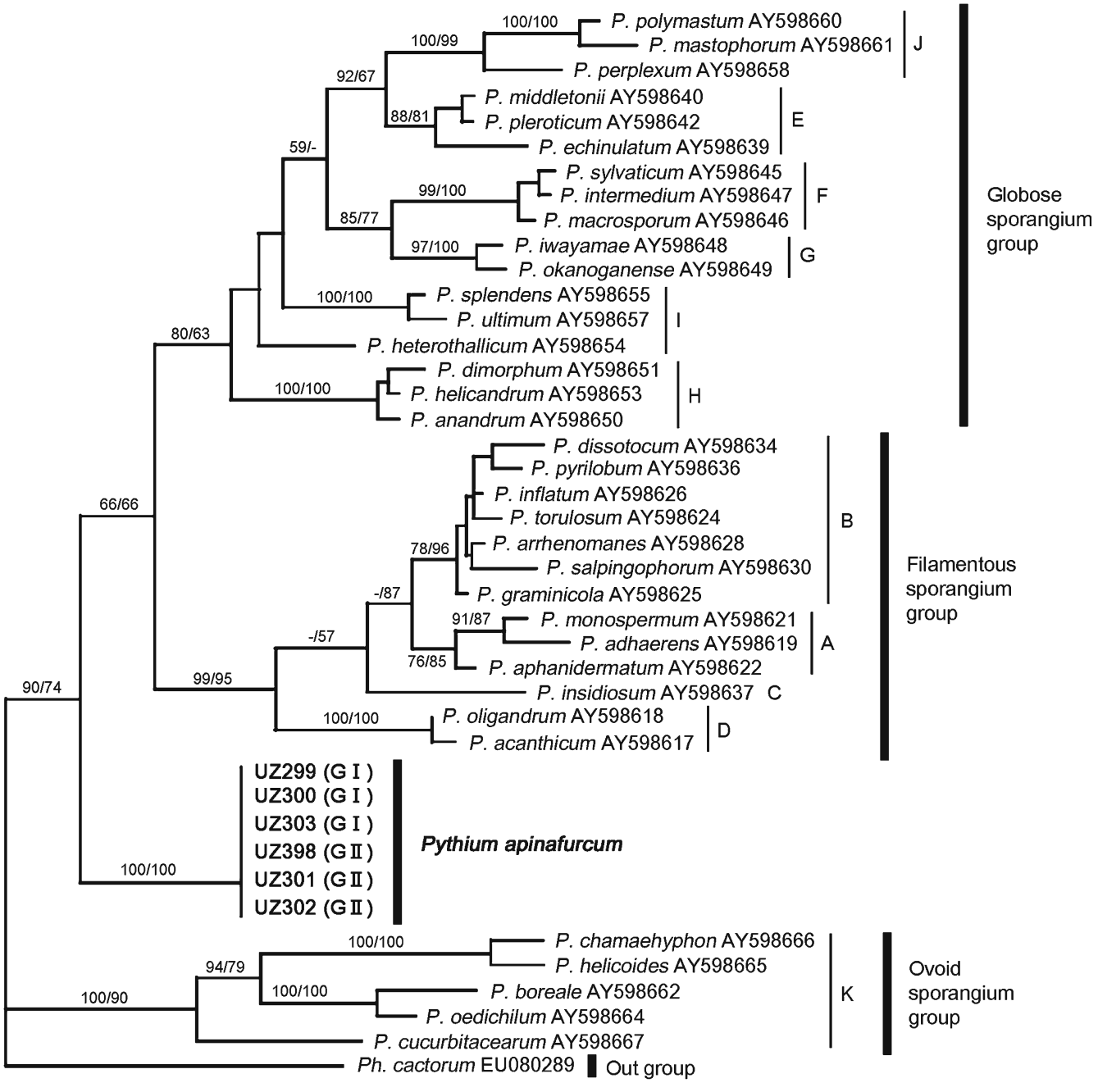

Fig. 5. Phylogenetic position of *Pythium apinafurcum* isolates among *Pythium* species based on the D1/D2 sequence of the LSU rDNA. Branch lengths were estimated with RAxML under maximum likelihood (ML). *Numbers on the branches* represent bootstrap values greater than 50%. ML bootstrap values from 100 replicates conducted with RAxML (*left*) and MP bootstrap values from 500 replicates conducted with PAUP* (*right*) are shown. *Numbers* following the species name indicate GenBank accession numbers. Letters *A* to *K* indicate each clade described by Lévesque and de Cock (2004). Sporangial morphological groups were based on van der Plaats-Niterink (1981) and Lévesque and de Cock (2004)

0.263765, and T = 0.325483. Of 1346 total characters, 921 were variable and 742 were parsimony informative, and parsimony analysis resulted in 4311 most parsimonious trees of length of 4466, consistency index (CI) of 0.414, and retention index (RI) of 0.672. The entire length of the final D1/D2 sequence alignment was 809 bp. The log-likelihood of best ML tree recovered by RAxML was –6157.245641. The ML tree scores were base frequencies of A = 0.228767, C = 0.179132, G = 0.308804, and T = 0.283298. Of 809 total characters, 280 were variable and 249 were parsimony informative; parsimony analysis resulted in 1087 most parsimonious trees of length = 1117, CI = 0.415, and RI = 0.731.

Nucleotide sequences of the D1/D2 region were identical among all isolates of *P. apinafurcum*, whereas the sequences of the ITS region varied between the two intraspecific groups defined as GI and GII. The sequence size of the GI and GII isolates was 713 and 720 bp, respectively. The similarity between the sequences of GI and GII isolates was 93.9%, in which 31 bp of indels and 14 bp of substitutions were presented (Table 2). BLAST searches of these sequences through the GenBank database revealed that the unique sequences differ enough from those of any described *Pythium* species to justify the new species status. Similar intraspecific variation of the ITS sequences relating with growth temperature has been known in *P. myriotylum* (Perneel et al. 2006). Intraspecific variations of the sequence of the ITS region has also been known in *P. irregulare* (Matsumoto et al. 2000), *P. graminicola* (Kageyama et al. 2005), *P. helicoides* (Kageyama et al. 2007), and *P. merculiale* (Belbahri et al. 2008). These reports and our present data suggest that the intraspecific variations of the ITS sequence region are common in this genus.

The main tree topology in all phylogenetic trees in this study was similar to that of the previous study by Lévesque and de Cock (2004), although some minor differences were detected in the relationship among clades A to K. The same three major monophyletic groups were detected in all trees, categorized as the globose sporangium group, the filamentous sporangium group, and the ovoid sporangium group (see Figs. 4, 5). Monophyly of the globose sporangium group was not well supported, whereas the others were strongly supported by bootstrap analyses in all phylogenetic trees. The relationship between phylogeny and sporangium morphology has been also shown by several previous studies (Matsumoto et al. 1999; Martin 2000; Villa et al. 2006). *Pythium apinafurcum* formed globose sporangia, but it was clearly phylogenetically differentiated from other *Pythium* species forming this type of sporangia. The results suggest that *P. apinafurcum* evolved independently from the other species of *Pythium* with globose sporangia.

Phylogeny of the *Pythium* has been evaluated mainly based on ITS sequence data (Matsumoto et al. 1999; Lévesque and de Cock 2004; Villa et al. 2006). However, nucleotide lengths of the ITS region varied more extensively among *Pythium* species analyzed in this study than those of the D1/D2 region; the ITS sequences ranged from

Table 2. Sequence differences between the intraspecific group I (GI) and the intraspecific group II (GII) of *Pythium apinafurcum* on rDNA ITS region

Region of rDNA	Kind of difference	Position	Intraspecific group GI	Intraspecific group GII
ITS1	Deletion	102–104, 111–112	GTT, GC	–
ITS1	Transition	55, 67, 73, 100, 108, 114	C, A, G, T, A, A	T, G, A, C, G, G
ITS1	Transversion	106	C	A
ITS1	Insertion	56–64	–	GCTTGTATT
		77–82	–	CAAAAA
ITS2	Deletion	28–29, 257–258, 287–289	TT, TT, CAT	–
ITS2	Transition	271, 301, 328	G, G, A	A, A, G
ITS2	Transversion	291–293, 358	TTC, C	CAT, A
ITS2	Insertion	335–336, 361–362	–	GA, TA

Table 3. Pathogenicity of *Pythium apinafurcum* isolates on seedlings of bermudagrass, cabbage, and cucumber and their recovery from roots of the inoculated plants

Pythium species	Isolate (intraspecific group)	Mortality (%)			Recovery of *Pythium* from roots (%)		
		Bermudagrass	Cabbage	Cucumber	Bermudagrass	Cabbage	Cucumber
P. apinafurcum	UZ300 (GI)	0	0b	0	100	94.4 ± 1.4b	100
P. apinafurcum	UZ301 (GII)	0	0b	0	100	90.2 ± 1.3b	100
P. ultimum var. *ultimum*	OPU774	100	98.6 ± 1.4a	100	100	100a	100
Uninoculated		0	0b	0	0	0b	0
LSD ($P < 0.05$)		–	2.7	–	–	4.4	–

LSD, least squares difference

Data given as means ± standard errors ($n = 3$). Values followed by the same letters in a column are not different significantly ($P < 0.05$) according to the Tukey–Kramer honestly significant different (HSD) test. Mortality is calculated from the number of seedling pre- and postemergence damping off 4 days after inoculation. Recovery of *Pythium* from roots is calculated from the number of seedlings showing recovery of *Pythium* species on NARM (Morita and Tojo 2007) from roots 4 days after inoculation

Dashes, LSD not determined because of lack of statistical variation

713 to 1050 bp, whereas the D1/D2 sequences ranged from 785 to 800 bp. Alignment of sequences with high length variation such as the ITS was very difficult because of the large number of sequence gaps. Although the effectiveness of utilization of the D1/D2 region in phylogenetic analysis of *Pythium* species should be further examined, the region is more useful than the ITS for analyses of this genus.

Pathogenicity

Pythium apinafurcum isolates did not cause any damage on seedlings of bermudagrass, cabbage, and cucumber after their inoculation, although the *P. ultimum* var. *ultimum* isolate produced severe damage on all the plants (see Table 3). *Pythium apinafurcum* isolates infected the plant roots with oospore formation (see Fig. 1, l–m) and were consistently recovered from roots (Table 3). There was no difference between the two intraspecific groups GI and GII in infectivity to plants. The results suggest that *P. apinafurcum* infects a wide range of plants without symptoms. The nonsymptomatic infection of the plants by *P. apinafurcum* indicates that this species probably survives as a plant symbiont or a saprophyte under natural conditions.

In conclusion, *P. apinafurcum* isolated from an uncultivated field soil in Wakayama Prefecture, Japan, is described as a distinct new *Pythium* species on the basis of morphological and molecular characteristics. The species is characterized by its complexly branched secondary hyphae, oogonium with one or two oospores, hyphal growth at 40°C, and nonsymptomatic infection of several plants.

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