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NOTE

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First records of *Pythium aquatile* and *P. macrosporum* isolated from soils in Japan

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Abstract *Pythium aquatile* and *P. macrosporum* were isolated from the soil of a cultivated field in Gunma Prefecture and a forest in Nagano Prefecture for the first time in Japan. Their morphological characteristics are described, and their pathogenicity and taxonomy are discussed.

Key words Pathogenicity · *Pythium aquatile* · *Pythium macrosporum* · rDNA · Soil

Pythium aquatile Höhnk and *P. macrosporum* Vaartaja & Van der Plaats-Niterink, previously undescribed in Japan, were isolated from soil of a cultivated field of soybean in Gunma Prefecture and a forest of Japanese red pine in Nagano Prefecture, respectively. *Pythium aquatile* has been reported in Germany (Höhnk 1953), New Zealand (Robertson 1973a), Pakistan (Abdul-Haq and Shahzad 1998), Norway (Herrero et al. 2003), and Poland (Czeczuga et al. 2004). This species has moderate virulence to seedlings of some vegetables (Robertson 1973b). *Pythium macrosporum* has been isolated from The Netherlands (van der Plaats-Niterink 1981; van Os et al. 1999), Germany (van der Plaats-Niterink 1981), Canada (van der Plaats-Niterink 1981; Allain-Boulé et al. 2004), and the United States (Westover and Bever 2001). This species is associated with root diseases of flower bulbs (Westover and Bever 2001), grasses (van Os et al. 1999), and carrot (Allain-Boulé et al. 2004). Although *P. aquatile* and *P. macrosporum* are distributed worldwide and are known as potential plant pathogens, only a few studies have been done on their morphology (Höhnk 1953; van der Plaats-Niterink 1981) and molecular taxonomy (Allain-Boulé et al. 2004; Lévesque and de Cock 2004).

This article describes the morphology and sequences of the nuclear ribosomal DNA (rDNA) internal transcribed spacer (ITS) regions and the pathogenicity of *P. aquatile* and *P. macrosporum* isolates from Japan.

Morphology of *P. aquatile* and *P. macrosporum* was examined on grass-leaf water culture (van der Plaats-Niterink 1981). Cardinal temperature for hyphal growth was determined on potato-carrot agar (PCA). *Pythium macrosporum* is a heterothallic species, and the species isolate CBS 102343 (Tojo et al. 2000) was used for formation of sexual organs.

Pathogenicity was tested on seedlings of cabbage (*Brassica oleracea* L. capitata group cv. okina) and cucumber (*Cucumis sativus* L. cv. jibai). An aggressive plant pathogen, *P. ultimum* Trow var. *ultimum* isolate OPU774 (Kida et al. 2007), was used for comparison. Inoculum was prepared by a method described previously (Tojo et al. 1993) with slight modification. Mycelial plugs of the strains obtained from cornmeal agar cultures were transferred to 1000-ml flasks (Vidrex, Fukuoka, Japan) containing 10 g autoclaved seeds of tall fescue (*Festuca arundinacea* Schreb. cv. Davinchi), in 40 ml water. After 11 days of incubation at 22°C in darkness, colonized seeds were used as inoculum. Infested soil was prepared by mixing 30 g inoculum in 1 l nursery soil (Aisai-1; Katakura Chikkarin, Tokyo). One seed of cabbage or cucumber was placed in each well of a 24-well plastic plate (BD Falcon, Franklin Lakes, NJ, USA) containing 200 ml infested soil. Each plate was enclosed in a double layer of polyethylene bag, and kept at 22°C under continuous light (73 μmol $m^{-2}s^{-1}$ measured at plant levels) in a growth chamber. Uninfested soil was used as a control. Six days after inoculation, the mortality of seedlings was recorded as the number of seedlings showing pre- and postemergence damping-off. The experiments were repeated three times with one plate per treatment. Analysis of variance was conducted for the mortality of different *Pythium* species using JMP software (version 5.1.1; SAS Institute, Cary, NC, USA). Means of the data were compared by least

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significant difference based on a Tukey–Kramer honestly significant different (HSD) test ($P < 0.05$).

The nucleotide sequences of the ITS region including 5.8S rDNA were determined. DNA was extracted from mycelium grown on PDA. Mycelium was suspended in 20 µl extraction buffer [10 mM Tris-HCl pH. 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% sodium dodecyl sulfate (SDS), 0.01% Proteinase K]. The mixture was incubated at 37°C for 60 min, then at 95°C for 10 min. After incubation, 30 µl sterile distilled water was added to the mixture. The ITS region was amplified by polymerase chain reaction (PCR) using primers ITS5 and ITS4 (White et al. 1990). Amplification was carried out in a PCR System 9700 (Applied Biosystems, Foster City, CA, USA) according to the following amplification program: an initial denaturation at 95°C for 3 min followed by 35 cycles including denaturation at 95°C for 30 s, annealing at 55°C for 1 min, extension at 72°C for 1 min, and the final extension step at 72°C for 10 min. PCR products were purified with SUPREC-02 (Takara Bio, Otsu, Shiga, Japan) following the manufacturer's instructions and then used for sequence analysis. Sequence reaction was performed with BigDye Terminator V3.1 Cycle Sequencing Reaction Kit (Applied Biosystems) using the same primers as in the initial PCR step. Products of sequence reaction were analyzed with an ABI 3130 Genetic Analyzers (Applied Biosystems). To confirm the species identification, we compared these sequences with the ITS sequences of the same species of *Pythium*: *P. aquatile* (GenBank accession no. AY598632) and *P. macrosporum* (AY598646), respectively.

Pythium aquatile Höhnk, Veröff. Inst. Meeresforsch. Bremerh. 2:94, 1953. Figs. 1–6

Morphology: Main hyphae up to 8 µm in diameter. Filamentous and slightly inflated sporangia; zoospore formed at room temperature (25°–28°C); discharge tubes up to 200 µm long. Encysted zoospores 8.6–15.9 µm (mean, 10.5 µm) in diameter. Hyphal swellings or abortive oogonium-like structures were often observed; these were globose, 12.3–21.2 µm (mean, 16.7 µm) in diameter or pyriform, 8.8–15.1 × 10.9–26.7(–41.5) µm (mean, 12.6 × 22.8 µm). Oogonia globose, smooth, terminal or intercalary, frequently clustering in small groups, 14.3–21.8 µm (mean, 17.9 µm) in diameter. Antheridia 1–3 per oogonium, monoclinous or diclinous. Oospores aplerotic, 11.4–16.9 µm (mean, 13.8 µm) in diameter, one per oogonium. The thickness of the oospore wall was 0.3–1.6 µm (mean, 1.0 µm). Cardinal temperatures for hyphal growth on PCA were 5°C minimum, 25°–30°C optimum, and 35°C maximum, with a daily growth rate of 15 mm at 25°C. Colonies on PCA were submerged with a rosette pattern and showed no clear difference under any temperature conditions. Although hyphal swellings or abortive oognium-like structures were often found in the isolate, the other characteristics agreed with those of the monograph of *P. aquatile* (van der Plaats-Niterink 1981).

Pathogenicity: *Pythium aquatile* isolate UZ216 caused damping-off on cabbage seedlings but not on cucumber seedlings (Table 1). The disease was less severe in plants inoculated with *P. aquatile* isolate UZ216 than with *P. macrosporum* isolate UZ233 and *P. ultimum* var. *ultimum* isolate OPU774. The results were similar to those reported by Robertson (1973b), that is, damping-off caused by *P. aquatile* appeared on seedlings of tomato and *Ipomea violacea* but not on pea seedlings. As in a previous report (Robertson 1973b) and the present pathogenicity data (Table 1) for *P. aquatile*, the species is thought to be a weak plant pathogen. *Pythium aquatile* usually lives in natural soils (Höhnk 1953; Abdul-haq and Shahzad 1998; Czeczuga et al. 2004) and may cause slight to moderate diseases on crops when it is introduced into plant cultivation environments.

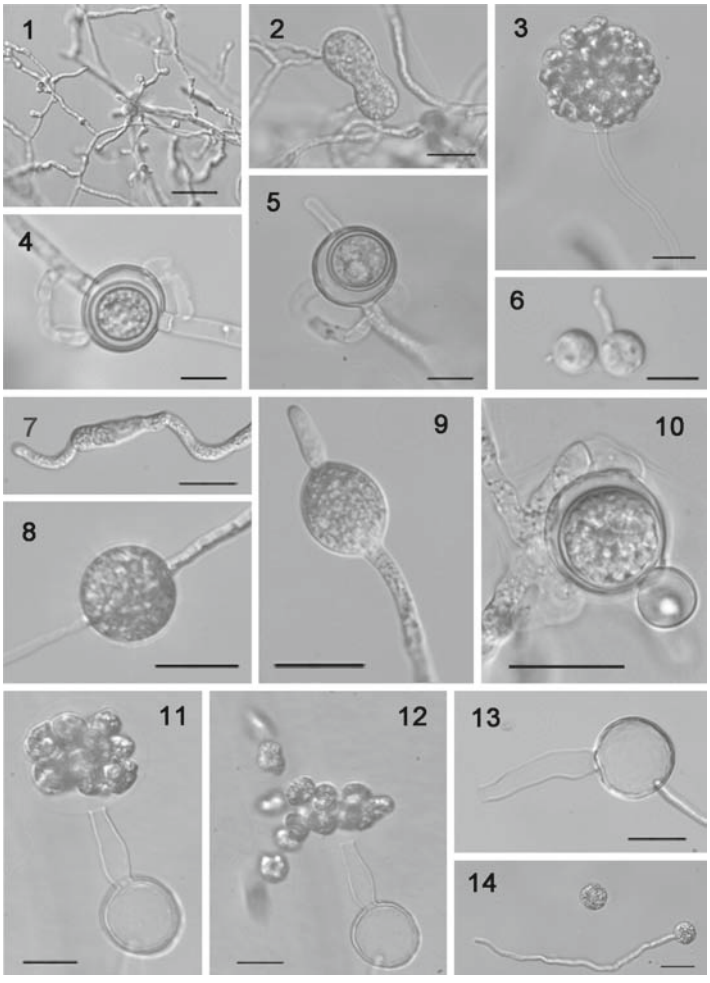
Sequence data: The ITS region (ITS1, 5.8S, ITS2) of the isolate UZ216 was 773 bp in length. The ITS1, 5.8S, and ITS2 were 174, 159, and 440 bp in length, respectively. The sequence has been deposited in Gen Bank under the accession number AB359909. The sequence was similar to that of *P. aquatile* isolate CBS 215.80 (AY598632; Lévesque and de Cock 2004) (similarity, 99.9%), which was used for the species description by van der Plaats-Niterink (1981).

Isolate examined: An isolate UZ216 was obtained from soil of a soybean cropping field, Minakami-machi, Tone-gun, Gunma Prefecture, Japan, on May 31, 2006 by a baiting technique (Watanabe 1981) using a potato tuber cube (0.125 cm^3) as a baiting substrate. The isolate was deposited in NBRC (National Institute of Technology and Evaluation, Biological Resource Center, Japan) and MAFF (Ministry of Agriculture, Forestry and Fisheries of Japan) as accession reference numbers NBRC103117 and MAFF240154, respectively.

Pythium macrosporum Vaartaja & Van der Plaats-Niterink, Stud. Mycol. 21:89, 1981. Figs. 7–14

Morphology: Main hyphae up to 7 µm in diameter. Appressoria sickle-shaped. Sporangia globose or subglobose, terminal and intercalary, 18.4–35.5 µm (mean, 26.8 µm) in diameter; zoospores formed at 15°C; discharge tubes up to 100 µm long and 5–13 µm wide. Encysted zoospores 10–18 µm (mean, 14.6 µm) in diameter. Oogonia not formed in single culture, but formed in dual culture with *P. macrosporum* isolate CBS 102343 on PCA. Oogonia globose, smooth, terminal, occasionally intercalary, (19.5–)20.5–24.5 µm (mean, 22.4 µm) in diameter. Antheridia 1–4 per oogonium, diclinous, antheridial stalks mostly simple, rarely branched, often contorted or inflated. Oospores aplerotic, 16.0–21.0 µm (mean, 18.9 µm) in diameter, 1 per oogonium. The thickness of the oospore wall was 0.5–1.7 µm. Cardinal temperatures for hyphal growth on PCA were 5°C minimum, 25°C optimum, and 30°C maximum with a daily growth rate of 26 mm at 25°C. Colonies on PCA were submerged with a rosette pattern and showed no clear difference under any temperature condition. These characteristics agreed with those of the original description of *P. macrosporum* (van der Plaats-Niterink 1981).

Pathogenicity: *Pythium macrosporum* isolate UZ233 caused damping-off on cabbage and cucumber seedlings



Figs. 1–14. Morphological characteristics of *Pythium aquatile* (**1–6**) and *P. macrosporum* (**7–14**). **1** Filamentous sporangia. **2** Pyriform hyphal swelling. **3** Vesicle with zoospores. **4** Intercalary oogonium with aplerotic oospore and two antheridia. **5** Terminal oogonium with aplerotic oospore and monoclinous anteridium. **6** Encysted zoospores. **7** Appressorium. **8** Intercalary sporangium. **9** Subspherical sporangium with apical papilla. **10** Terminal oogonium with three contorted antheridia formed in dual culture of isolate UZ233 and CBS 102343. **11** Vesicle with zoospores. **12** Dispersal of zoospores from vesicle. **13** Empty sporangium after dispersal of zoospores. **14** Germination of encysted zoospores. *Bars* **1** 40 µm; **2, 3, 7–14** 20 µm; **4–6** 10 µm

Table 1. Pathogenicity of *Pythium* species after artificial inoculation on cabbage and cucumber

Pythium species	Isolate no.	Mortality (%)[a]	
		Cabbage	Cucumber
P. aquatile	UZ216	4.6 ± 2.8a	0a
P. macrosporum	UZ233	8.8 ± 2.2a	31.9 ± 7.3b
P. ultimum var. *ultimum*	OPU774	58.3 ± 4.8b	90.7 ± 2.8c
LSD ($P < 0.05$)		15.0	19.7

Data given as means ± standard errors ($n = 3$). Values followed by the same letters in a column do not differ significantly according to a Tukey–Kramer honestly significant different (HSD) test

[a] Mortality was recorded as the number of seedlings showing pre- and postemergence damping-off 6 days after inoculation

(Table 1). The disease severity was lower in plants inoculated with *P. macrosporum* isolate UZ233 than with *P. ultimum* var. *ultimum* strain OPU774. The results suggest that *P. macrosporum* is a potential pathogen of these crops, although its impact is relatively small compared with *P. ultimum* var. *ultimum*.

Sequence data: The ITS region (ITS1, 5.8S, ITS2) of the isolate UZ233 was 903 bp in length. The ITS1, 5.8S, and ITS2 were 290, 159, and 454 bp in length, respectively. The sequence has been deposited in GenBank under the accession number AB359910. The sequence was similar to that of *P. macrosporum* isolate CBS 574.80 (AY598646; Lévesque and de Cock 2004) (similarity, 99.4%), which was used for the species description by van der Plaats-Niterink (1981).

Isolate examined: An isolate UZ233 was obtained from soil of a Japanese red pine forest, Ueda-shi, Nagano Prefecture, Japan, in June 2006 by a baiting technique (Watanabe 1981) using a potato tuber cube (0.125 cm^3) as a baiting substrate. The isolate was deposited in NBRC and MAFF as accession reference numbers NBRC103881 and MAFF240155, respectively.

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