Widespread Occurrence of *Pythium arrhenomanes* Pathogenic to Rice Seedlings Around Japanese Rice Fields

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Abstract

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In Japan, rice seedlings grown in nurseries and used for transplanting are subject to a damping-off disease caused by *Pythium* spp. In this study, 148 isolates of *Pythium* spp. were obtained from rice seedlings in 39 locations of northern Japan. Among the isolates, 137 were identified as *Pythium arrhenomanes* using polymerase chain reaction (PCR) with species-specific primers, DNA sequencing analyses of the internal transcribed regions of ribosomal DNA, and the morphologies of oogonia, antheridia, oospores, and zoosporangia. Inoculation tests showed that the isolates identified as *P. arrhenomanes* were pathogenic to rice seedlings and parasitic to

southern crabgrass with only minor damage. *P. arrhenomanes* was reisolated from the roots of both rice seedlings and southern crabgrass. Poaceae weeds, hosts of *Pythium* spp., grow in and around nurseries and in ridges surrounding rice fields. To detect *Pythium* spp., 188 Poaceae weeds were collected from 37 locations in Akita Prefecture. *P. arrhenomanes* was frequently detected in 164 weed roots from all locations by PCR using species-specific primers. Thus, we determined that *P. arrhenomanes* exists in and around rice seedling nurseries and rice fields, and that it is much more widely distributed than previously recognized in northern Japan.

Transplanting rice seedlings that have grown for a month in nurseries is the main rice cropping system in most parts of eastern Asia, whereas direct seeding to rice fields is conventional in the United States and Australia. Directly seeding Japanese cultivars results in inconsistent seedling establishment (Takita 1987), although research on direct-seeding issues has long been conducted in Japan (Yamauchi and Chung 1995). Diseases of rice seedlings caused by soilborne fungi inhibit seedling establishment in the field (Chun and Schneider 1998; Cother and Gilbert 1993). Thus, the transplanting system has the merit of raising healthy rice seedlings by avoiding soilborne diseases and low establishment rates. Nevertheless, the transplanting system also has problems with soilborne pathogens. Damping off caused by *Pythium* spp. is the main problem in nursery rice seedlings. The disease occurs frequently when the night temperature is approximately 5 to 10°C for two or three consecutive days (Kato et al. 1985a). This disease has been mainly found in the cooler areas of Japan, such as eastern and northern Honshu Island.

Seven species within the genus of *Pythium* are known as rice seedling pathogens in Japan. Kato et al. (1985a,b) reported that *Pythium irregulare* Buisman, *P. spinosum* Sawada, and *P. sylvaticum* W. A. Campb. & F. F. Hendrix would attack the roots of the rice seed's budding shoots just after germination. They also described that *P. graminicola* Subraman. infected seedling roots grown for a few weeks. *P. inflatum*, *P. torulosum*, and *P. aristosporum* were also reported to be associated with rice seedling diseases (Endo and Ibaraki 1986). *P. arrhenomanes* Drechsler has been isolated from the roots of rice seedlings exhibiting symptoms of damping off and slow growth in nurseries (Toda et al. 2010). *P. arrhenomanes* has also been isolated from wheat, barley, corn, sugarcane, sorghum, sugar cane, and species of *Billbergia*, *Cajanus*, *Commelina*, *Ipomoea*, *Musa*, *Phaseolus*, and *Solanum* (Cother and Gilbert 1993; Deep and Lipps 1996; Gilbert et al. 1995; van der Plaats-Niterink 1981; Yanar et al. 1997). However, the main species causing damping off has not been clarified. Weed plants belonging to the family Poaceae are also host

plants of *Pythium* spp. (Dissanayake et al. 1997; Nechwatal et al. 2005). Rice seedlings in nurseries are usually placed in greenhouses, which are built next to the ridges surrounding the rice field. Because many weeds, including species of Poaceae, grow in and around nurseries and rice fields, *Pythium* spp. pathogenic to rice seedlings may exist where Poaceae weeds grow.

The objectives of this study were to reveal the most frequently isolated *Pythium* sp. among the seven identified species based on morphological features, molecular analyses, and the pathogenicity of *Pythium* isolates obtained from rice seedlings, and to clarify the distribution of the most frequently pathogenic *Pythium* spp. in the roots of Poaceae weeds growing in the ridges around rice fields using polymerase chain reaction (PCR) detection with species-specific primers.

Materials and Methods

Isolation of *Pythium* spp. from roots of rice seedlings. More than 20 rice seedlings that showed damping-off symptoms were obtained from 39 locations in northern Honshu Island, Japan; 3 from Aomori, 16 from Iwate, 7 from Akita, 3 from Miyagi, and 10 from Niigata. The roots of 20 to 25 seedling plants were well washed using tap water, placed on paper towels, and blotted dry using filter paper to remove water from the roots. Roots were cut into 1-cm-long segments and transferred to petri dishes containing 10 ml of streptomycin water agar medium (2% water agar and 0.06 mg of streptomycin sulfate oxide) medium or 10 ml of nystatin-ampicillin-rifampicin-miconazole medium (half-strength corn meal extract and 2% agar with ampicillin at 0.25 mg/ml, nystatin at 0.1 mg/ml, rifampicin at 0.1 mg/ml, and miconazole at 0.01 mg/ml). Root segments on each medium were incubated at 25°C for 2 days. The grown hyphae from each root segment were observed by microscope at $\times 100$ or $\times 200$ magnification. When hyphae showing no septa were confirmed microscopically, the tip of each hypha was placed into a glass tube containing 10 ml of slanted corn meal extract agar (CMA) medium. Isolates were obtained from the roots of each single rice seedling, and 20 isolates were obtained in each location.

Morphology of isolated *Pythium* spp. In total, 20 isolates of *Pythium* spp. obtained from each location were grown on slanted CMA medium, and 2 to 4 isolates were randomly selected from each group. Selected isolates were incubated on 9-cm petri plates containing 10 ml of CMA medium at 25°C for 7 to 10 days. The *Pythium* spp. isolates were identified based on the morphology of zoosporangium,

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oogonium, and oospore, and the number of antheridium adhering to the oogonium (van der Plaats-Niterink 1981).

DNA extraction. The total genomic DNA of the two to four isolates selected in the previous section were extracted using the potassium ethyl xanthogenate method (Nakahara et al. 1999). Three 5-mm² agar plugs of CMA medium with the leading mycelium edge were transferred to a petri dish (9 mm in diameter) containing 10 ml of potato dextrose broth at 25°C for 2 days in the dark. Mycelial mats were harvested by filtration, washed with sterile distilled water, blotted dry, and packed into polyethylene plastic bags for storage at -30°C until use. Frozen mycelial mats were transferred into 2-ml tubes and ground using a bubble crusher (Taitech Co. Ltd., Koshigaya, Saitama, Japan). Powdered mycelia were suspended in 300 µl of potassium ethyl xanthogenate buffer (6.25 mM potassium ethyl xanthogenate, 700 mM NaCl, 100 mM Tris-HCl [pH 7.5], and 10 mM EDTA [pH 8.0]) and then heated at 60°C for 30 min. The 160 µl of supernatant obtained after centrifugation at 15,000 rpm for 5 min at 25°C was transferred into another 1.5-ml tube, and 400 µl of 99.5% ethanol was added. The DNA precipitate was

collected by centrifugation at 15,000 rpm for 5 min at 25°C, washed with 600 µl of 70% ethanol, dried in a desiccator for 5 min, and dissolved in 100 µl of sterilized distilled water.

PCR of the ribosomal DNA internal transcribed region. PCR was conducted to amplify the ribosomal DNA internal transcribed (rDNA-ITS) region of *Pythium* spp. isolates. PCR was carried out using approximately 10 ng of DNA. The 20-µl reaction mixture consisted of 200 µM each dATP, dCTP, dGTP, and dTTP; 0.5 µM oligonucleotide primer pair; 10 mM Tris-HCl at pH 8.3; 50 mM KCl; 1.5 mM MgCl₂; and AmpliTaq Gold (Applied Biosystems, Foster City, CA) at 0.025 U/µl. The primer pair used to amplify the rDNA-ITS region was ITS1/ITS4 (White et al. 1990). For amplification, the DNA Thermal Cycler (PerkinElmer, Waltham, MA) was programmed for one cycle of 10 min at 95°C; followed by 35 cycles of 1 min at 95°C, 1 min at 55°C, and 3 min at 72°C; and 1 cycle of 10 min at 72°C. The amplification products were observed by electrophoresis on 2% agarose gel with Tris-acetic EDTA buffer. The agarose gels were stained with ethidium bromide (10 mg/ml) and visualized under ultraviolet transillumination.

Table 1. Presence or absence of oospores and zoosporangia, and polymerase chain reaction (PCR) products amplified with species-specific primers from isolates of *Pythium* spp. obtained from the roots of rice seedlings showing symptoms of damping off^x

Location number, name	Isolates	Oospores ^y	Zoosporangia ^y	AsPyF/AsRRR ^z	AsGRAF/AsPyR ^z
Aomori Prefecture					
a-1, Fujisaki-A*	A01-1, A01-2, A01-3, A01-4	+	-	-	-
a-2, Fujisaki-B	A02-1, A02-2**, A02-3, A02-4	-	+	+	-
a-3, Inakadate	A03-1**, A03-2, A03-3, A03-4	-	+	+	-
Iwate Prefecture					
b-1, Shiba-A	B01-1**, B01-2	-	+	+	-
	B01-3	NT	NT	+	-
	B01-4	+	-	+	-
b-2, Hiraizumi	B02-1**, B02-2, B02-3, B02-4	+	-	+	-
b-3, Hanamaki-A	B03-1, B03-2	-	+	+	-
	B03-3**, B03-4	NT	NT	+	-
b-4, Kanegasaki	B04-1**, B04-2, B04-4	-	+	+	-
	B04-3	+	+	+	-
	B05-1**, B05-2, B05-3, B05-4	-	+	+	-
b-6, Kitakami-A	B06-1**, B06-2	-	+	+	-
b-7, Ohshu-A	B06-3, B06-4	+	+	+	-
	B07-1, B07-2	-	+	+	-
	B07-3, B07-4	+	+	+	-
b-8, Kitakami-B	B08-1, B08-2	-	+	+	-
	B08-3, B08-4	+	+	+	-
	B09-1, B09-2, B09-3, B09-4	-	+	+	-
b-10, Ohshu-C	B10-1, B10-4	+	-	+	-
b-11, Hanamaki-B	B10-2, B10-3	-	+	+	-
	B11-1, B11-2, B11-3	-	+	+	-
	B12-1, B12-2, B12-3, B12-4	-	+	+	-
b-13, Touno-A	B13-1, B13-2, B13-3, B13-4	-	+	+	-
b-14, Touno-B	B14-1, B14-2, B14-3, B14-4	-	+	+	-
b-15, Shiba-B	B15-1, B15-2, B15-3	-	+	+	-
	B15-4	+	+	+	-
b-16, Izawa	B16-1, B16-2, B16-3	+	-	+	-
	B16-4	-	+	+	-
Akita Prefecture					
c-1, Yuri-Honjo-A***	C01-1, C01-2	+	-	-	-
	C01-3, C01-4	NT	NT	-	-
c-2, Yuri-Honjo-B***	C02-1, C02-2, C02-4	+	-	-	-
	C02-4	NT	NT	-	-
c-3, Yuri-Honjo-C	C03-1, C03-4	-	+	+	-
	C03-2, C03-3**	+	-	+	-

(continued on next page)

^x Location labels correspond to those in Figure 1. Asterisks: * indicates isolates obtained from Fujisaki-A, identified as *Pythium dissotocum*; ** indicates isolates used for pathogenicity test and sequence analysis using ribosomal DNA internal transcribed region; and *** indicates isolates obtained from Yuri-Honjo-A and -B, identified as *Pythium spinosum*.

^y Presence (+) or absence (-) of oospores or zoosporangia microscopically observed from isolates grown on corn meal extract agar in each location. NT = Not tested.

^z Presence (+) or absence (-) of oospores and sporangia, as well as PCR products amplified with specific-primers AsPyF/AsRRR for *P. arrhenomanes* or AsGRAF/AsPyR for *P. graminicola*.

PCR using species-specific primers and sequence analysis. PCR using primer pairs specific for *P. arrhenomanes* and *P. graminicola* was also conducted using *Pythium* isolates, which confirmed the amplification of the rDNA-ITS. The primer pair used for *P. arrhenomanes* was AsPyF/AsARRR (AATTCTGTACGCGTGGTC/TCCAAGAGCAATAA CCACTCTCA) and for *P. graminicola* was AsGRAF/AsPyR (TGGGCTGCATGTA TGTGTAGTCT/CTCCTTTACCCTCGA GAGGGC) (Asano et al. 2010). PCR amplification reactions for both primer pairs were carried out using approximately 10 ng of DNA. The 20- μ l reaction mixture was the same as that for the amplification of the ITS region. The DNA Thermal Cycler program for amplification of PCR products and confirmation by agarose gel were conducted as described above. For confirming the consistency of amplification with species-specific primer pairs, 15 *Pythium* spp. isolates selected randomly from each prefecture were used for the DNA sequencing analysis of the rDNA-ITS region. PCR products were purified using GFX PCR DNA and a Gel Band Purification Kit (GE Healthcare UK Limited, Buckinghamshire, UK). The rDNA-ITS regions of the 15 isolates were then sequenced using primers ITS1 and ITS4 and the Ready Reaction Cycle Sequencing Kit (Applied Biosystems). They were analyzed using an ABI 3700XL DNA sequencer (Applied Biosystems). Forward and reverse chromatograms were aligned and

sequences of the rDNA-ITS region were resolved by visual examination using GENETYX-Mac (Genetyx Science, Tokyo, Japan). Sequences reported in this article have been submitted to GenBank (accession numbers AB932589 to AB932595 and LC053666 to LC053674). Resolved sequence data were used as queries of the GenBank database using the megablast algorithm to identify similar *Pythium* spp.

Pathogenicity to rice seedlings. The six *Pythium* spp. isolates which were used in the sequence analysis of the rDNA-ITS region were also used as inocula to test for pathogenicity to rice seedlings. An isolate of *P. arrhenomanes* Py0901 obtained from Toyama Prefecture, Japan (Toda et al. 2010) was also used for comparison. Isolates of *Pythium* spp. for inocula were grown in petri dishes containing 10 ml of CMA at 25°C for 7 days in the dark. The agar containing grown *Pythium* mycelia was cut into three pieces (5 by 5 mm²) and transferred into plastic pots (7 cm in diameter and 8 cm in height) containing bentgrass seed medium. This medium is a mixture of 1 g of bentgrass seed and 5 ml of distilled water. The plastic pots containing this medium were sterilized before incubation. Then, 1 g of grown mycelia and bentgrass seed medium containing agar pieces was mixed with 600 g of soil to make the infested soil. Infested soil was put into plastic pots (17 by 12 by

Table 1. (continued from preceding page)

Location number, name	Isolates	Oospores ^y	Zoosporangia ^y	AsPyF/AsARRR ^z	AsGRAF/AsPyR ^z
c-4, Yuri-Honjo-D	C04-1, C04-2	+	–	+	–
	C04-3, C04-4	NT	NT	+	–
c-5, Yuri-Honjo-E	C05-1, C05-2	–	+	+	–
	C05-3	NT	NT	+	–
c-6, Akita-A	C05-4	+	+	+	–
	C06-1, C06-2, C06-3	–	+	+	–
c-7, Akita-B	C06-4	–	+	+	–
	C07-1	–	+	+	–
	C07-2**	+	–	+	–
	C07-3, C07-4	NT	NT	+	–
Miyagi Prefecture					
d-1, Rifu	D01-2	–	+	+	–
	D01-3, D01-4**	NT	NT	+	–
d-2, Kurihara	D02-1, D02-3	–	+	+	–
	D02-2	NT	NT	+	–
d-3, Okeya	D03-1**, D03-2	–	+	+	–
	D03-4	+	+	+	–
Niigata Prefecture					
f-1, Niigata-A	F01-1	+	–	+	–
	F01-2, F01-3	+	+	+	–
	F01-4	NT	NT	+	–
f-2, Joetsu-A	F02-1**	+	+	+	–
	F02-2	+	+	+	–
	F02-3	NT	NT	+	–
f-3, Nagaoka-A	F03-1**, F03-2	+	–	+	–
	F03-3, F03-4	–	+	+	–
f-4, Nagaoka-B	F04-1	–	+	+	–
	F04-2	+	–	+	–
	F04-3, F04-4	NT	NT	+	–
f-5, Niigata-B	F05-1**	+	–	+	–
	F05-2	–	+	+	–
	F05-3, F05-4	NT	NT	+	–
f-6, Nagaoka-C	F06-1, F06-2	+	–	+	–
	F06-3	+	+	+	–
	F06-4	–	+	+	–
f-7, Joetsu-B	F07-1, F07-2	+	–	+	–
	F07-3, F07-4	NT	NT	+	–
f-8, Joetsu-C	F08-1, F08-2	+	–	+	–
f-9, Joetsu-D	F09-1, F09-2	+	–	+	–
	F09-3, F09-4	NT	NT	+	–
f-10, Joetsu-E	F10-1	+	–	+	–
	F10-2	–	+	+	–
	F10-3, F10-4	NT	NT	+	–

5 cm) and 30 rice seeds were planted in each pot. Three replicates were prepared for each inoculum. Rice seedlings were grown in growth chambers (NK System Co, Ltd., Osaka, Japan) with light provided on a cycle of 12 h/day and 12 h/night. The temperatures for rice seedling growth were 25°C during the light and 15°C during the dark period. The pathogenicity of *Pythium* spp. to rice seedlings was estimated 3 weeks after seeding. The number of grown seedlings was counted in each plastic pot to calculate the germination

rate. Disease symptoms, including leaf wilting and death and the discoloration of roots, were observed. The shoot and root lengths of each grown seedling were measured and compared between inoculated pots and noninoculated controls for the statistical analysis using Tukey's test. *Pythium*-like hyphae showing no septa by microscope were isolated from 10 roots of randomly selected rice seedlings, with or without disease symptoms. The reisolation of *Pythium* spp. was confirmed when isolated hyphae grown on CMA showed characteristics typical of *Pythium* mycelium. The pathogenicity test was performed twice.

Pathogenicity to southern crabgrass. Isolates of *Pythium* spp. used for the inoculation of rice seedlings were also used to inoculate southern crabgrass (*Digitaria ciliaris* (Retz.) Koel). *P. arrhenomanes* mycelium and bentgrass seed medium (1 g) was mixed with 100 g of soil to make infested soil. The infested soil (100 g) was put into plastic pots (8 by 5 by 3 cm), 50 ml of water was added, and then 10 seeds were planted in each pot. Seed were incubated in the greenhouse with light provided on a cycle of 12 h/day and 12 h/night at 22 to 27°C. Pathogenicity of *Pythium* spp. to southern crabgrass was estimated 3 weeks after seeding. The germination rate, shoot length, crabgrass symptoms, and pathogen reisolation were determined based on the methodology in the previous section. The pathogenicity test was performed twice.

PCR detection and isolation of *Pythium* spp. in roots of weeds. In total, 188 Poaceae weed plants were collected from 37 locations that were less than 5 m from rice fields in Akita Prefecture, Japan. However, some weeds in location Akita-E were 50 m from a rice field. Mainly, southern crabgrass (*D. ciliaris* (Retz.) Koel), yellow foxtail (*Setaria pumila* (Poir.) Roem. et J. A. Schult.), giant foxtail (*S. faberi* Herrm), and yellow bristle grass (*S. pallide-fusca* (Schumacher.) Stapf & C. E. Hubb) were chosen for collection. The roots of southern crabgrass in Kami-Koani, Ohgata, Akita-E, Yuri-Honjo-C, Yuri-Honjo-F, and Ugo-C were used for the isolation of *Pythium* spp. to confirm the detection consistency using species-specific primers. The roots were washed using tap water, placed on paper towels, blotted dry using filter paper to remove water from inside the roots, and processed as described above.

Roots of all of the weeds were cut into segments that weighed 100 mg and were then used for DNA extraction with a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Extracted DNA from each sample was used for PCR amplification with primer pairs

Fig. 1. Locations of nurseries where *Pythium* spp. were obtained from the roots of rice seedlings in northern Japan. The code number for each location corresponds to that in Table 1.

Fig. 2. Oogonium, antheridia, oospore, and zoosporangium of *Pythium* spp. obtained from roots of rice seedlings. **A**, Oogonium and antheridia of isolate D01-4; **B**, oogonium and antheridia of isolate C07-2; **C**, zoosporangium of isolate A02-2; **D**, oospore of isolate C07-2. Bar indicates a scale of 10 μm.

AsPyF/AsRRR and AsGRAF/AsPyR based on the description in the previous section.

Results

Morphology of isolated *Pythium* spp. In total, 148 isolates of *Pythium* spp. were obtained from rice seedlings collected from 39 locations (Table 1; Fig. 1). Between 95 and 100% of the plants in each location showed *Pythium* hyphal elongation on the medium. Two to four randomly selected isolates of *Pythium* spp. from each location were used for morphological observations on CMA medium. All of the isolates that grew on the CMA medium showed white mycelia, and no septum was observed by microscope. The isolates from 36 locations in Aomori (2 areas), Akita (5 areas), Iwate (16 areas), Miyagi (3 areas), and Niigata (10 areas) had the same morphology as those of *P. arrhenomanes* described by van der Plaats-Niterink (1981). The oogonium, antheridia, oospore, and zoosporangium of representative isolates identified as *P. arrhenomanes* are shown in Figure 2. These isolates produced oogonia with diameters of 30.0 to 37.5 μm after a 5- to 7-day incubation. The number of antheridium adhering to oogonia was between one and six. The diameters of oospores in those isolates were 27.5 to 30.0 μm . Zoosporangia were observed from isolates obtained from 32 locations, and those were 5 μm in width and 20 to 30 μm in length.

PCR and sequence analysis. The *Pythium* spp. isolates obtained from rice seedlings in 39 locations gave a single PCR product when the primer pair ITS1/ITS4 was used. Then, the specificity of species-specific primers was confirmed using the extracted DNA of all of the isolates. An ITS region of approximately 850 to 1,000 bp was amplified. *Pythium* spp. isolates obtained in 36 locations showed PCR products by primer pair AsPyF/AsRRR but isolates from three locations (Fujisaki-A, Yuri-Honjo-A, and Yuri-Honjo-B) did not show PCR products by either set of primer pairs (Table 1). Sequences of 15 representative *Pythium* isolates that showed positive reactions by PCR amplification using primers AsPyF/AsRRR were analyzed to determine the species based on an analysis using the BlastN algorithm in the National Center for Biotechnology Information. These isolates were also used to test for pathogenicity to rice. ITS regions in the six isolates were 98.7 to 100% similar to those in isolates of *P. arrhenomanes* (GenBank accession numbers AB160842, AF330182, and AY598628).

Pathogenicity to rice seedlings. All isolates used for the inoculation test were pathogenic to the rice seedlings grown in both experiment I and II. Seed germination rates in infested soil were 66.7 to 96.7% in

experiment I and 65.6 to 98.9% in experiment II, whereas control pots showed a 100% seed germination rate (Table 2). All of the leaves from plants grown on infested soil were shorter than those from plants grown on noninfested soil. Leaf wilting and the death of germinated seed was also observed in seedlings inoculated with *Pythium* isolates. Several roots of these seedlings showed light brown or brownish colors on their surfaces. The seedlings grown in soil inoculated with isolates of different *Pythium* spp. had some variations in shoot and root lengths (Table 2). A statistical analysis showed that the shoots and roots of seedlings grown in control soil were significantly longer than those grown on infested soil ($P < 0.05$) in both experiments I and II (Table 2). Based on the reisolation method in the previous section, mycelia of *Pythium* spp. were

Table 3. Plant responses due to pathogenicity

Isolates	Germination (%) ^x	Shoot length (cm) ^y	Reisolation ^z
Experiment I			
A02-2	13.3 b	10.2 $\pm$ 5.3 a	+
B06-1	26.7 ab	22.9 $\pm$ 4.9 a	+
C03-3	10.0 b	7.2 $\pm$ 3.8 a	+
C07-2	20.0 ab	9.1 $\pm$ 5.6 a	+
D01-4	33.3 ab	18.5 $\pm$ 3.8 a	+
F02-1	30.0 ab	19.2 $\pm$ 5.7 a	+
Py0901	20.0 ab	13.2 $\pm$ 2.3 a	+
Control	50.0 a	19.4 $\pm$ 3.0 a	–
Experiment II			
A02-2	13.3 b	11.2 $\pm$ 6.0 a	+
B06-1	16.6 b	17.8 $\pm$ 7.5 a	+
C03-3	10.0 b	14.6 $\pm$ 1.4 a	+
C07-2	20.0 b	15.6 $\pm$ 2.1 a	+
D01-4	16.7 b	14.8 $\pm$ 1.5 a	+
F02-1	26.6 b	16.7 $\pm$ 1.8 a	+
Py0901	30.0 b	16.8 $\pm$ 3.1 a	+
Control	60.0 a	13.2 $\pm$ 1.1 a	–

^x Indicates seed germination (%) in three replicates based on (number of grown weeds/total number of seed per pot) $\times$ 100. Values followed by the same letter were not significantly different based on Tukey's test ($P < 0.05$).

^y Means of shoot lengths from three replicates. Values followed by the same letter were not significantly different based on Tukey's test ($P < 0.05$).

^z Symbols: + indicates that *Pythium* sp. was isolated from two weed roots from each pot and – indicates that *Pythium* sp. was not isolated.

Table 2. Plant responses due to pathogenicity

Isolates	Germination (%) ^x	Shoot length (cm) ^y	Root length (cm) ^y	Reisolation (%) ^z
Experiment I				
A02-2	86.7 ab	4.2 $\pm$ 2.5 c	1.2 $\pm$ 0.8 d	93.3
B06-1	76.7 bc	16.1 $\pm$ 3.1 b	13.0 $\pm$ 2.1 b	100.0
C03-3	90.0 ab	15.5 $\pm$ 2.0 b	10.8 $\pm$ 2.3 bc	100.0
C07-2	66.7 c	11.6 $\pm$ 5.0 bc	4.9 $\pm$ 1.7 d	96.7
D01-4	93.3 ab	9.4 $\pm$ 3.9 bc	5.2 $\pm$ 3.2 d	93.3
F02-1	96.7 ab	11.6 $\pm$ 4.0 bc	6.5 $\pm$ 2.9 d	96.7
Py0901	76.7 bc	11.0 $\pm$ 2.1 bc	6.5 $\pm$ 2.5 cd	96.7
Control	100.0 a	26.9 $\pm$ 5.1 a	20.4 $\pm$ 1.6 a	0.0
Experiment II				
A02-2	83.3 a	9.8 $\pm$ 1.4 c	3.4 $\pm$ 0.6 c	90.0
B06-1	95.6 a	14.2 $\pm$ 0.1 bc	6.0 $\pm$ 0.3 b	93.3
C03-3	98.9 a	10.7 $\pm$ 0.3 c	3.3 $\pm$ 0.6 c	100.0
C07-2	97.8 a	16.3 $\pm$ 1.0 b	6.7 $\pm$ 0.3 b	90.0
D01-4	65.6 a	9.9 $\pm$ 0.2 c	3.5 $\pm$ 1.4 c	96.7
F02-1	76.7 a	10.4 $\pm$ 4.4 c	3.6 $\pm$ 1.1 c	96.7
Py0901	94.4 a	12.4 $\pm$ 1.1 bc	4.9 $\pm$ 1.3 bc	93.3
Control	100.0 a	25.1 $\pm$ 2.5 a	12.2 $\pm$ 0.3 a	0.0

^x Indicates the percent germination in three replicates. Values followed by the same letter were not significantly different based on Tukey's test ($P < 0.05$).

^y Indicates the root and shoot lengths of germinated rice seedlings within three replicates. Values followed by the same letter were not significantly different based on Tukey's test ($P < 0.05$).

^z Indicates the reisolation percent obtained from 10 roots of rice seedlings in three replicates.

isolated from more than 90% of the roots of rice seedlings grown in the inoculated pots used for the pathogenicity tests in both experiments.

Pathogenicity test on southern crabgrass. The germination rate of southern crabgrass in inoculated soils was 10.0 to 33.3% in both experiments, whereas the rates in noninfested soil were 50.0 and 60.0% in experiment I and II, respectively (Table 3). Germination rates in noninfested soils were significantly higher than those in inoculated soils in experiment II, whereas only a slight difference in germination rates between noninfested and infested soils was confirmed in experiment I. Except for inhibiting seed germination, none of the isolates showed strong pathogenicity to southern crabgrass, although the soil used for this pathogenicity test contained a higher concentration of *Pythium* inoculum than the soil used for the pathogenicity test on rice seedlings. Some crabgrass leaves grew less than control leaves at a low frequency but there was no wilting or death, nor any root discoloration. Germinated crabgrass shoot lengths in the inoculated soil of experiment I were in the range of 7.2 to 22.9 cm, whereas the length in the control soil was 19.4 cm. In experiment II, germinated crabgrass shoot lengths in the inoculated soil ranged from 10.5 to 17.8 cm, and the control shoot length was 13.2 cm. There were no significant differences in shoot lengths between inoculated and control soils based on Tukey's test in both experiment I and II. Based on the reisolation method in the previous section, *Pythium* spp. were isolated from crabgrass roots in all inoculated pots, and no *Pythium* spp. were isolated from the roots grown in control pots in both experiments I and II.

Detection of *P. arrhenomanes* and *P. graminicola* from roots of weeds. In total, 188 Poaceae weeds were used for PCR with *Pythium* spp.-specific primer pairs. PCR products amplified by primer pair AsPyF/AsRRR were obtained from weed roots sampled at 37 locations in Akita Prefecture (Table 4; Fig. 3). PCR products were obtained from 121 southern crabgrass samples, representing 85.8% of collected samples from the 37 locations close to or surrounding rice fields. *Pythium* spp.-specific PCR products were also confirmed from other weeds, including 24 yellow foxtails as 88.9% of the collected samples from 14 locations, 13 giant foxtails as 100.0% from 9 locations, and 6 yellow bristle grasses as 85.7% from 4 locations. PCR products amplified by primer pair AsGRAF/AsPyR were also obtained from weeds in a total of 22 locations (Table 4; Fig. 3). Those PCR products included 31 southern crabgrasses as 21.8% of collected samples from 17 locations, 7 yellow foxtails as 25.9% from 6 locations, and 1 giant foxtail as 7.7% from 1 location (Table 4).

Isolates of *P. arrhenomanes* were successfully obtained from southern crabgrass at six locations: Kami-Koani, Ohgata, Akita-E, Yuri-Honjo-C, Yuri-Honjo-F, and Ugo-C. Isolates of both *P. arrhenomanes* and *P. graminicola* were obtained from southern crabgrass at the location Akita-E. Those isolates were used for PCR with species-specific primer pairs AsPyF/AsRRR and AsGRAF/AsPyR, respectively. Primer pair AsPyF/AsRRR amplified PCR products from isolates of weed roots obtained in Kami-Koani, Ohgata, Akita-E, Yuri-Honjo-C, Yuri-Honjo-F, and Ugo-C. Primer pair

Table 4. Tested numbers of samples polymerase chain reaction (PCR) amplified using species-specific primers

Location		Sampled weed plants	Tested ^z	Detected amplicons ^y	
Number	Name			AsPyF/AsARRR	AsGRAF/AsPyR
1	Kita-Akita	<i>Digitaria ciliaris</i>	3	2	0
2	Ohdate-A	<i>D. ciliaris</i>	5	2	0
		<i>Setaria pumila</i>	1	0	0
		<i>S. faberi</i>	2	2	0
3	Ohdate-B	<i>D. ciliaris</i>	5	2	0
4	Ohdate-C	<i>D. ciliaris</i>	5	4	2
5	Ohdate-D	<i>D. ciliaris</i>	3	2	0
6	Kami-Koani	<i>D. ciliaris</i>	9	9	4
7	Gojohme-A	<i>D. ciliaris</i>	2	2	0
8	Gojohme-B	<i>D. ciliaris</i>	2	1	1
		<i>S. pumila</i>	3	3	0
9	Katagami	<i>D. ciliaris</i>	2	1	0
		<i>S. faberi</i>	3	3	0
10	Hachirohgate	<i>D. ciliaris</i>	2	2	0
		<i>S. pumila</i>	3	3	0
11	Ohgata	<i>D. ciliaris</i>	13	11	4
12	Akita-A	<i>D. ciliaris</i>	3	2	0
		<i>S. pumila</i>	3	3	1
13	Akita-B	<i>D. ciliaris</i>	2	2	2
		<i>S. pumila</i>	3	2	0
14	Akita-C	<i>D. ciliaris</i>	2	2	0
15	Akita-D	<i>D. ciliaris</i>	1	1	0
16	Akita-E	<i>D. ciliaris</i>	27	27	5
17	Daisen	<i>D. ciliaris</i>	4	4	1
		<i>S. pallide-fusca</i>	1	1	0
18	Yuri-Honjo-A	<i>D. ciliaris</i>	3	3	0
		<i>S. pumila</i>	1	1	0
		<i>S. faberi</i>	1	1	0
19	Yuri-Honjo-B	<i>D. ciliaris</i>	3	2	0
		<i>S. pumila</i>	1	1	0
		<i>S. pallide-fusca</i>	2	2	0
20	Yuri-Honjo-C	<i>D. ciliaris</i>	12	12	3
21	Yuri-Honjo-D	<i>D. ciliaris</i>	2	2	0

(continued on next page)

^y Tested numbers of samples used for amplification; AsPyF/AsARRR and AsGRAF/AsPyR = the number of samples that yielded PCR products using specific primer pairs for *P. arrhenomanes* and *P. graminicola*, respectively.

^z Tested numbers of samples used for PCR amplification using AsPyF/AsARRR and AsGRAF/AsPyR primers.

AsGRAF/AsPyR amplified PCR products from isolates of weed roots obtained in Akita-E.

Discussion

Seven *Pythium* spp. are known as pathogens to rice seedlings in Japan (Endo and Ibaraki 1986; Kato et al. 1985a,b; Toda et al. 2010). However, the significance of each species in the actual disease incidence in commercially grown rice seedlings has never been investigated. In this study, *P. arrhenomanes* was the most frequently isolated microorganism from the diseased roots of rice seedlings in northern Japan (Table 1). In addition, the fungus was found in diseased rice seedlings collected from nurseries at 15 Toyama Prefecture locations in 2009 (Toda et al. 2010) and in 7 Nagano Prefecture locations in 2011 (Yamashita et al. 2012). Both prefectures are located in the middle of Honshu Island. These results strongly suggest that *P. arrhenomanes* is the most common species causing damping off in rice seedlings in the eastern half of Honshu Island. This is the first report that reveals the significance of *P. arrhenomanes* among the seven species reported as the causal pathogens of rice seedling blight in Japan.

As a pathogen to rice, *P. arrhenomanes* has been reported to inhibit rice seedling growth (Chun and Schneider 1998). In addition, *P. arrhenomanes* is the most pathogenic to rice seedlings among three *Pythium* spp. (*P. arrhenomanes*, *P. graminicola*, and *P. inflatum*) in the Philippines (van Buyten and Höfte 2013). Also, van Buyten et al. (2013) reported that a variation of virulence to young rice was confirmed for *P. arrhenomanes*. Isolates of *P. arrhenomanes* used in a pathogenicity test were virulent to rice seedlings and simultaneously showed variation in their inhibition to rice growth (Table 2). Isolates of *P. arrhenomanes* nonvirulent to rice seedlings were not found in either our results or those of van Buyten

Fig. 3. Sampling locations in Akita Prefecture in northern Japan where *Pythium arrhenomanes* (black squares) or *P. arrhenomanes* and *P. graminicola* (black and white squares) were detected. Numbers shown on the map correspond to locations in Table 4.

Table 4. (continued from preceding page)

Location		Sampled weed plants	Tested ^z	Detected amplicons ^y	
Number	Name			AsPyF/AsARRR	AsGRAF/AsPyR
22	Yuri-Honjo-E	<i>S. pumila</i>	2	2	1
		<i>S. faberi</i>	2	2	0
		<i>D. ciliaris</i>	3	1	1
23	Yuri-Honjo-F	<i>S. pumila</i>	3	3	2
		<i>D. ciliaris</i>	3	2	1
		<i>S. pumila</i>	1	1	1
24	Yokote-A	<i>D. ciliaris</i>	1	1	1
		<i>S. pumila</i>	1	1	1
		<i>S. pallide-fusca</i>	3	2	0
25	Yokote-B	<i>D. ciliaris</i>	1	1	1
26	Yokote-C	<i>D. ciliaris</i>	2	2	0
		<i>S. pumila</i>	1	1	0
		<i>S. pallide-fusca</i>	1	1	0
27	Yokote-D	<i>S. faberi</i>	1	1	0
		<i>D. ciliaris</i>	2	2	1
		<i>S. faberi</i>	1	1	1
28	Yokote-E	<i>D. ciliaris</i>	2	2	0
29	Yokote-F	<i>S. faberi</i>	1	1	0
		<i>D. ciliaris</i>	1	1	1
		<i>D. ciliaris</i>	1	1	1
30	Yokote-G	<i>D. ciliaris</i>	1	1	1
31	Yokote-H	<i>D. ciliaris</i>	1	1	1
32	Yokote-I	<i>D. ciliaris</i>	1	1	1
33	Yuzawa-A	<i>D. ciliaris</i>	2	2	0
		<i>S. pumila</i>	1	1	0
		<i>D. ciliaris</i>	3	2	0
34	Yuzawa-B	<i>S. pumila</i>	1	0	0
		<i>D. ciliaris</i>	2	2	0
		<i>S. pumila</i>	1	1	1
35	Ugo-A	<i>S. faberi</i>	1	1	0
		<i>D. ciliaris</i>	3	2	0
		<i>S. pumila</i>	1	1	0
36	Ugo-B	<i>S. faberi</i>	1	1	0
		<i>D. ciliaris</i>	3	3	0
		<i>D. ciliaris</i>	3	3	0

et al. (2013). Therefore, we hypothesize that *P. arrhenomanes* affects the growth of young rice even though variations in *P. arrhenomanes* virulence to rice seedlings exist.

Assumptions regarding the possible source of the pathogen and its mode of transmission to rice seedling roots include the following: (i) the soil used for seedling culture has not been properly sterilized; (ii) seedling roots penetrate the bottom of seedling boxes, contacting infested soil; (iii) irrigation water obtained from rivers is contaminated; and (iv) the pathogenic oomycetes are introduced into the nursery soil by wind, or worker operations. These hypotheses are based on pathogenic oomycetes being distributed ubiquitously as saprophytes in soil near and around the nurseries, which has not been proven.

P. arrhenomanes apparently infects and causes minor damage to southern crabgrass (Table 3), indicating that this plant is a host plant of the fungus. This oomycete has a wide host range, mainly in the Poaceae family, and was detected from many species in the family, including *D. ciliaris*, *S. pumila*, *S. faberi*, and *S. pallide-fusca* (Table 4). Weed hosts play significant roles in the epidemiology of *Pythium* root rot of sugarcane (Dissanayake et al. 1997). These weeds are assumed to be inoculum sources during the growing season and a means of persistence during the winter and fallow periods (Dissanayake et al. 1997). Likewise, wild Poaceae plants such as southern crabgrass, *S. pumila*, *S. faberi*, and *S. pallide-fusca* would also play significant roles in the epidemiology of the disease caused by *P. arrhenomanes*.

Southern crabgrass is a common wild plant in Japan (Kataoka et al. 1986) that is distributed in and at the sides of cultivated fields, wastelands, gardens, and roadsides of not just the agricultural regions but also of urban areas. The plant can be found roughly every 50 m on the roadsides of agricultural regions, at least in the northeast of Honshu, which includes Nagano and Toyama Prefectures. Approximately 90% of the southern crabgrass collected from the agricultural region of Akita Prefecture, where rice is mainly grown in paddy fields, contained oomycetes in the subterranean tissues. Therefore, it is highly probable that *P. arrhenomanes* exists wherever southern crabgrass grows, at least in Akita Prefecture, and most probably in northeastern Honshu. Southern crabgrass is not the only Poaceae plant, as mentioned above, and many other Poaceae plants could also be infected (Middleton 1943). Thus, this pathogen appears to be commonly distributed in the agricultural regions of Japan. Additionally, it was revealed that the distribution of *P. arrhenomanes* could be significantly affected by that of Poaceae plants, such as southern crabgrass and the three other weeds used in this study, and the common distribution of root-infecting oomycetes, at least, could be partly explained by their relationship. In addition to the distribution, we suggest that the population density of Poaceae plants influences the inoculum potential of *P. arrhenomanes* in each environment.

Most species of the genus *Pythium* are soil-inhabiting oomycetes that, according to the ecological classifications of Garrett (1956, 1970), can survive, grow, and reproduce on soil organic matter as saprophytes, even in the absence of a host plant (Curl and Truelove 1986; Garrett 1956, 1970). They are, however, not vigorous saprophytes (Hendrix and Campbell 1973) and, thus, the distribution of each species is rather restricted (Paulitz and Adams 2003) compared with vigorous saprophytes, such as the many species of *Penicillium*, *Aspergillus*, *Trichoderma*, *Fusarium*, and *Mucorales* (Curl and Truelove 1986). The limited distribution of each species suggests that several factors, in addition to the quality and quantity of organic materials introduced into the soil, could profoundly affect the survival of *Pythium* spp. in soil. Soil type (soil texture) (Dick and Ali-Shtayeh 1986; Paulitz and Adams 2003), precipitation (Dick and Ali-Shtayeh 1986; Larkin et al. 1995; Paulitz and Adams 2003), soil temperature (Hancock 1985; Larkin et al. 1995), crop management practices (Dick and Ali-Shtayeh 1986; Paulitz and Adams 2003), and mycoparasitic relationships (Martin and Hancock 1986; Paulitz and Adams 2003) were reported to influence the distribution of *Pythium* spp. Paulitz and Adams (2003) found that the composition and distribution of *Pythium* spp. in wheat fields could be influenced by environmental factors similar to those described above at a

mesoscale level (100 to 1,000,000 ha). Because the *Pythium* spp. are not vigorous saprophytes, the presence of host plants provides significant advantages for the oomycetes, which might profoundly affect their survival. The environmental factors mentioned above, and the presence of particular vegetation, especially host plants, could significantly influence the survival and, hence, the distribution of *Pythium* spp.

Based on the results obtained in this study, we suggest that soil collected from areas where Poaceae weeds grow should not be used as potting soil for growing rice seedlings. If such soil exists in the nurseries, then it should be covered by plastic sheets before placing the seedling containers on the ground. In addition, the removal of the Poaceae weeds surrounding the nurseries could be effective in reducing soil pathogen levels. Stanghellini (1975) suggested that, because the pathogenic activity of *Pythium* spp. is fairly specific in each environment, a reduction in the population level would have a significant effect on reducing rice seedling damage. Therefore, detailed information on the inoculum sources, geographical distribution, and dissemination routes is critical to developing management procedures for root-infecting and soil-inhabiting pathogenic microorganisms such as *Pythium* spp., whose distributions are believed to be common and widespread.

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