Brown Ring Patch: A New Disease on Bentgrass Caused by *Waitea circinata* var. *circinata*

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ABSTRACT

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Isolates of an unidentified *Rhizoctonia* sp. (NP isolates), obtained from creeping bentgrass (*Agrostis stolonifera* var. *palustris*) in Japan that exhibited symptoms of a new disease, were compared with isolates of three varieties of *Waitea circinata* var. *oryzae*, var. *zeae*, and var. *circinata*. NP isolates also were compared with isolates of *R. oryzae* obtained from creeping bentgrass exhibiting white patch-like symptoms (RW isolates). The color and size of sclerotia, color of mycelia, and pigment deposition of NP isolates was similar to that of RW isolates and *W. circinata* var. *circinata*, but distinctly different from *W. circinata* var. *oryzae* and *W. circinata* var. *zeae*. The optimal temperature for hyphal growth of NP isolates, RW isolates, and *W. circinata* var. *circinata* was 28°C, and for *W. circinata* var. *oryzae* and *W. circinata* var. *zeae* was 30°C. Pathogenicity tests on creeping bentgrass showed that the severity of disease caused by NP isolates, RW isolates, and *W. circinata* var. *circinata* was greater than with *W. circinata* var. *oryzae*, but lower than with *W. circinata* var. *zeae*. No significant differences in symptom expression were apparent among NP isolates, RW isolates, and *W. circinata* var. *circinata*. A phylogenetic tree, obtained using the results of random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR), showed that isolates of *W. circinata* var. *oryzae* and *W. circinata* var. *zeae* separated into individual clusters, while NP isolates, RW isolates, and *W. circinata* var. *circinata* clustered together. The lengths of the rDNA internal transcribed spacer (ITS) region of NP isolates, RW isolates, and *W. circinata* var. *circinata* were identical but smaller than those of *W. circinata* var. *oryzae* and *W. circinata* var. *zeae*. Restriction fragment length polymorphism (RFLP) analysis of the rDNA-ITS region, using three enzymes (*HapII*, *HinfI*, and *MboI*), also showed that NP isolates were the same as RW isolates and *W. circinata* var. *circinata*, but different from *W. circinata* var. *oryzae* and *W. circinata* var. *zeae*. Based on these results, the NP isolates causing a new disease on bentgrass are *W. circinata* var. *circinata*, and that RW isolates are also *W. circinata* var. *circinata* but not *R. oryzae*. We propose that the name of the disease on creeping bentgrass caused by *W. circinata* var. *circinata* is brown ring patch.

Additional keywords: *Rhizoctonia solani* AG 2-2 IIIB

A new disease was found in Japan on creeping bentgrass (*Agrostis stolonifera* var. *palustris* Huds.) from spring through autumn in 1994 (7,11). This disease was found on bentgrass putting greens on golf courses throughout Japan and occurred during rainy periods. Symptoms included circular or irregular small patches of tan to

yellow-brown color ranging from 10 to 50 cm in diameter. The symptoms of this disease were found on plant tissues above the soil surface. The affected turf eventually developed brownish rings, but the turf in the center of the rings recovered. In some cases, the turf recovered completely. Isolates of a *Rhizoctonia* sp. (NP isolates), obtained from plants affected by this new disease, were multinucleate and anastomosed with tester isolates of *Rhizoctonia oryzae* Ryker & Gooch and *R. zeae* Voorhees (7). However, the cultural morphology of NP isolates was different from these species.

Waitea circinata Warcup & Talbot was classified into three varieties, *W. circinata*

var. *circinata*, *W. circinata* var. *oryzae*, and *W. circinata* var. *zeae*, based on differences in the colony morphology of the vegetative state (5). Although the anamorphic name of *W. circinata* var. *circinata* has not been assigned, *R. oryzae* and *R. zeae* were represented under the anamorphic names of *W. circinata* var. *oryzae* and *W. circinata* var. *zeae*, respectively (5). *W. circinata* var. *circinata* forms orange to dark brown, globose sclerotia up to 2 mm in diameter; *W. circinata* var. *oryzae* forms orange to salmon, irregularly shaped sclerotia; and *W. circinata* var. *zeae* forms orange to brown, regularly shaped sclerotia up to 1 mm in diameter (8).

R. oryzae and *R. zeae* were assigned to *Waitea* anastomosis groups WAG-O and WAG-Z, respectively (13). Hyphal fusion was frequent among isolates belonging to each group, but was rare between the two groups (13). *R. oryzae* has been isolated from wheat, barley (9,10,12), and rice (14), while *R. zeae* has been isolated from corn (17), tall fescue (9), and rice (13). *W. circinata* var. *circinata* obtained from agricultural soil in Alaska was pathogenic to barley (8), although no diseases caused by *W. circinata* var. *circinata* have been named. Both *R. oryzae* and *R. zeae* are causal agents of turfgrass diseases in North America (3,15). Diseases of turfgrass caused by these pathogens occur most frequently during the warm and humid season, at temperatures between 28 and 36°C, inciting leaf and sheath spot (3,15). The turfgrass disease caused by *R. oryzae* in Japan was reported as a white patch disease on bentgrass in 1990 (19). However, the symptoms of white patch disease (19) are quite similar to the new disease described here. According to Tanpo et al. (19), the initial symptom of white patch disease also appears as an irregular small patch ranging from 10 to 60 cm in diameter with a light-whitish color. As with the new disease, the symptoms of white patch were found on plant tissues above the soil surface, and healthy leaves remain on several areas of turf inside the rings throughout the season. The symptoms of white

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patch disease can disappear completely at the end of the season. These characteristic symptoms of white patch disease (19) were very similar to symptoms of the new disease.

Other diseases of bentgrass caused by *Rhizoctonia* spp. are yellow patch and brown patch (2,3,21). Yellow patch, caused by *Rhizoctonia cerealis* van der Hoeven, a binucleate *Rhizoctonia* sp. in AG-D, develops yellow to straw-colored patches during cool and warm periods. Brown patch disease, caused by *R. solani* AG 2-2 IIIB, develops during the warm and humid weather in summer. Although the new disease occurs during the same season as brown patch disease, it causes less damage than brown patch and shows a yellow-brown color on creeping bentgrass leaves, while brown patch symptoms include gray-purple or gray-brown lesions on leaves.

The objectives of this study were to compare NP isolates obtained from creeping bentgrass exhibiting symptoms of the new disease with those of *W. circinata* var. *circinata*, *W. circinata* var. *oryzae*, and *W. circinata* var. *zeae* based on cultural morphology, hyphal growth rate at different temperatures, pathogenicity, and genetic relatedness using random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) analysis of ribosomal DNA internal transcribed spacer regions (rDNA-ITS). Additionally, isolates of *R. oryzae* that were reported as the causal agent of white patch disease (RW isolates) (19) on creeping bentgrass were also compared with NP isolates to confirm whether RW isolates were identical with NP isolates.

MATERIALS AND METHODS

Isolates. Eleven NP isolates were collected from creeping bentgrass exhibiting symptoms of the new disease, and five isolates of *W. circinata* var. *circinata*, six isolates of *W. circinata* var. *oryzae*, and seven isolates of *W. circinata* var. *zeae* were collected from a range of hosts and geographic areas (Table 1). Three isolates of *R. oryzae*, which were collected from creeping bentgrass exhibiting symptoms of white patch disease (RW isolates) and were provided by Tanpo et al. (19), and one isolate of *R. solani* AG 2-2 IIIB obtained from creeping bentgrass exhibiting symptoms of brown patch disease, were also used in this study.

Colony morphology. A single 5-mm agar disk of each of 30 isolates (11 NP isolates, 3 RW isolates, and 16 isolates of *W. circinata*) (Table 1) was placed onto the center of a 9-cm petri dish containing 10 ml of potato dextrose agar (PDA; Becton Dickinson and Company, Sparks, MD). These cultures were grown at 25°C in the dark. After 45 days, the color of hyphae and the shape and color of sclerotia were examined. To determine the hyphal diame-

ter and number of nuclei per cell, agar disks of each of the 30 isolates were placed onto the center of a 9-cm petri dish containing 10 ml of water agar (WA), grown at 25°C for 2 days in the dark, and observed at ×400 using bright field microscopy. The mean hyphal diameter was determined for each isolate by measuring 10 main hyphae. After this measurement, hyphae of each isolate were stained with 3% KOH and safranin O, and the number of nuclei were counted in 10 cells selected according to their location in the stain gradient so that nuclei and septa could be distinctly observed (1).

Growth rate. For each isolate, a 5-mm agar disk was placed 1 cm from the edge of a 9-cm petri dish containing 10 ml of PDA. The hyphal growth rate was determined for 19 isolates (4 NP isolates [NP-1, -4, -7, and -8], 3 RW isolates [RW-1 to -3], each of 4 isolates of *W. circinata* var. *circinata* [Wc-1 to -4], *W. circinata* var. *oryzae* [Wo-1 to -4], and *W. circinata* var. *zeae* [Wz-1 to -4] [Table 1]) grown on PDA at 15, 20, 22, 25, 28, 30, and 35°C. Each treatment was replicated three times.

The radius of each colony was measured every 24 h until the colony reached the edge of the petri dish.

Pathogenicity on creeping bentgrass. Eighteen isolates (4 NP isolates [NP-1, -4, -7, and -8], 3 RW isolates [RW-1 to -3], 3 isolates of *W. circinata* var. *circinata* [Wc-2, -3, and -4], 4 isolates of *W. circinata* var. *oryzae* [Wo-1, -3, -4, and -6], and 4 isolates of *W. circinata* var. *zeae* [Wz-1 to -4] [Table 1]) were tested for pathogenicity on creeping bentgrass.

Creeping bentgrass was grown for 24 days after seeding prior to inoculation. Seeds were sown by placing 0.14 g in 14-cm-diameter clay pots filled with 1,000 ml of sand, and then covered 2 mm deep with sand. Each pot was watered twice per day to avoid wilting. Isolates of NP, RW, and three varieties of *W. circinata* were grown on PDA medium for 4 days at 25°C in the dark. Agar disks of each isolate were transferred onto 300 ml of soil-bran medium (a mixture of 3 liters of peat moss, 3 liters of wheat bran, 3 liters of rice bran, 6 liters of turfgrass soil, and 1 liter of liquid medium containing 1% yeast extract and 1% poly-

Table 1. Isolates from creeping bentgrass with symptoms of new disease (NP), *Rhizoctonia oryzae* obtained from creeping bentgrass with symptoms of white patch disease, *Waitea circinata* var. *circinata*, *W. circinata* var. *oryzae*, and *W. circinata* var. *zeae* used in this study

Working no.	Isolate name	Host	Origin
NP isolates			
NP-1	IY-3-2	Creeping bentgrass	Ehime, Japan
NP-2	MAS-1	Creeping bentgrass	Ehime, Japan
NP-3	KT-5S-1	Creeping bentgrass	Kagawa, Japan
NP-4	HHC-13-6	Creeping bentgrass	Hyogo, Japan
NP-5	HT-1	Creeping bentgrass	Nagasaki, Japan
NP-6	KT-08-1	Creeping bentgrass	Kagawa, Japan
NP-7	Ro-1	Creeping bentgrass	Nagasaki, Japan
NP-8	HTB-A-1	Creeping bentgrass	Nagasaki, Japan
NP-9	HAT-BG	Creeping bentgrass	Aichi, Japan
NP-10	AMI-BG	Creeping bentgrass	Japan
NP-11	GEN-BG-1	Creeping bentgrass	Fukuoka, Japan
RW isolates obtained from white patch (RW) ^z			
RW-1	WP-1	Creeping bentgrass	Shiga, Japan
RW-2	R-2	Creeping bentgrass	Kagawa, Japan
RW-3	G-3	Creeping bentgrass	Kagawa, Japan
<i>W. circinata</i> var. <i>circinata</i> (Wc)			
Wc-1	Z1	Soil	Alaska, USA
Wc-2	Z4	Soil	Alaska, USA
Wc-3	Z16	Soil	Alaska, USA
Wc-4	Z41	Soil	Alaska, USA
Wc-5	Z52	Soil	Alaska, USA
<i>W. circinata</i> var. <i>oryzae</i> (Wo)			
Wo-1	Ro36	Rice	Aichi, Japan
Wo-2	Ro45	Rice	Okinawa, Japan
Wo-3	RoTTS	Rice	Toyama, Japan
Wo-4	C-301	Soil	Fukuoka, Japan
Wo-5	C-505	Soil	Yamagata, Japan
Wo-6	Ro119	Rice	Japan
<i>W. circinata</i> var. <i>zeae</i> (Wz)			
Wz-1	M003	Soil	Aichi, Japan
Wz-2	M008	Rice	Aichi, Japan
Wz-3	M009	Soil	Wakayama, Japan
Wz-4	C-504	Soil	Ishikawa, Japan
Wz-5	C-522	Rice	Fukuoka, Japan
Wz-6	OK-2TL	Creeping bentgrass	Kagawa, Japan
Wz-7	BP-Blk	Creeping bentgrass	Kagawa, Japan
<i>R. solani</i> AG 2-2 IIIB			
IIIB-1	K2-10	Creeping bentgrass	Gifu, Japan

^z Provided by Tanpo et al. (19).

peptone) in 500-ml flasks for 10 days at 25°C in the dark. Each culture was homogenized in a mixer and filtered through a 2-mm mesh screen. Twenty milliliters of filtered mycelia were then dispersed over the bentgrass in each pot. The inoculated turf was incubated at 25°C with light provided on a 14/10 h day/night cycle in the greenhouse. Each treatment was replicated 10 times. Control plants were treated similarly but without inocula. Disease severity was rated 8 days after inoculation, and was determined by averaging individual scores from 10 leaves per pot, using the following rating scale: 0% = symptomless; 1% = small lesions; 5% = lesions covering <10% of the leaf; 30% = lesions covering 10 to 50% of the leaf; 70% = lesions covering >50% of a wilted leaf; and 100% = leaf completely withered. Pathogenicity tests were done twice.

Samples of leaves with lesions were collected 9 days after inoculation. Leaf sheaths 5 mm long were cut from infected leaves, washed in three changes of distilled water, blotted dry on a sterile paper towel, and placed on WA. Hyphae resembling *Rhizoctonia* spp. were transferred as hyphal tips onto PDA at 25°C. The isolation frequency was scored among 20 samples by comparing isolates from leaves with the original isolates used to prepare inocula.

DNA extraction. Mycelia for DNA extraction were cultured in potato dextrose broth (PDB) at 25°C. After 3 to 4 days, the mycelial mat was harvested by filtration and stored at -80°C until use. Total genomic DNA was extracted as described by Hyakumachi et al. (6).

RAPD analysis. DNA samples from 25 isolates (eight NP isolates [NP-1 to -8], two RW isolates [RW-1 and -2], four isolates of *W. circinata* var. *circinata* [Wc-1 to -4], five isolates each of *W. circinata* var. *oryzae* [Wo-1 to -5] and *W. circinata* var. *zeae* [Wz-1 to -5], and one isolate of *R. solani* AG 2-2 IIIB [IIIB-1] [Table 1]) were used for RAPD-PCR. PCR amplification reactions were carried out in a 50-μl reaction mixture consisting of 200 μM each of dATP, dCTP, dGTP, and dTTP, 50 pmol of oligonucleotide primer, 10 mM Tris-HCl at pH 8.3, 50 mM KCl, 1.5 mM MgCl, and 1.25 units *Taq* DNA polymerase (Takara Shuzo Co. Ltd., Kyoto, Japan). After quantification of DNA using a DNA/RNA calculator (Amersham Bio-

tech, Piscataway, NJ), 50 ng/μl fungal DNA from each of the 25 isolates was used. RAPD analysis of *R. solani* in previous studies could distinguish the inter and intra AG subgroups and the cultural types within subgroups (4,20,21,22). Three useful random primers, R28 (5'-ATGGATCCGC-3'), RC09 (5'-GATAACGCAC-3'), and OPE04 (5'-GTGACATGCC-3'), were selected from the results of these studies. The DNA Thermal Cycler (GeneAmp PCR system 2700; Applied Biosystems, Foster City, CA) was programmed for one cycle of 3 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 35°C, and 2 min at 72°C, and one cycle of 7 min at 72°C. The RAPD-PCR products were examined by electrophoresis on 1.5% agarose gel with Tris-acetic EDTA (TAE) buffer. A 100-bp DNA ladder size marker (Invitrogen TECH-LINE, Carlsbad, CA) was also used for each electrophoresis. The agarose gels were stained with ethidium bromide and visualized under UV transillumination.

Amplification with each primer was repeated three times, and only reproducible DNA fragments were scored for each of the 25 isolates. The size of each RAPD-PCR product was estimated visually. The

length of the longest and shortest RAPD-PCR products were determined using the linear function made from GROUT, the software in Microsoft Excel, by defining X as the distance from well to each marker (mm) and Y as the length of each marker (bp). Reproducibility of DNA fragments by RAPD-PCR was tested three times. Only reproducible products were incorporated into phylogenetic analyses. The presence and absence of products were scored as 1 and 0, respectively. The data generated from three primers for each isolate were pooled, and a genetic similarity matrix was calculated based on the simple matching coefficient (16). Maximum parsimony trees were produced using the heuristic method of PAUP 4.0b1 (Sinauer Associates Inc., Sunderland, MA). Clade stability was assessed with 100 bootstrap replications.

RFLP of rDNA-ITS. The amplification reactions of the rDNA-ITS region and its post-treatment were carried out under conditions previously described (6). The entire length of the rDNA-ITS regions were compared among 32 isolates consisting of NP isolates, RW isolates, and *W. circinata*. The ITS regions of the 32 isolates were

Fig. 1. Cultural morphology of NP isolates, RW isolates, *Waitea circinata* var. *circinata*, *W. circinata* var. *oryzae*, and *W. circinata* var. *zeae* on potato dextrose agar (PDA) after 45 days incubation.

Table 2. Morphological characteristics of NP isolates, isolates of *Rhizoctonia oryzae* obtained from creeping bentgrass exhibiting the symptoms of white patch disease (RW), *Waitea circinata* var. *circinata*, *W. circinata* var. *oryzae*, and *W. circinata* var. *zeae*

Isolates/group	Nuclei per cell	Hyphal diameter (μm)	Colony color ^z	Sclerotia ^z		
				Size (mm)	Shape	Color
NP	3 to 9	6.3	Dark brown	1-3	Irregular	Dark
RW	3 to 9	6.3	Dark brown	1-3	Irregular	Dark
<i>W. circinata</i> var. <i>circinata</i>	4 to 9	6.4	Dark brown	1-3	Irregular	Brown
<i>W. circinata</i> var. <i>oryzae</i>	3 to 8	6.3	White to salmon	1-3	Irregular	Salmon
<i>W. circinata</i> var. <i>zeae</i>	3 to 10	6.4	White to orange	0.5-1	Subspheroid	Orange

^z On potato dextrose agar.

amplified by PCR and digested separately with enzymes *HapII*, *MboI*, and *HinfI* (Takara Shuzo Co. Ltd.). All digestion reactions contained 1 mM MgCl₂, 0.1 mM DTT, and 10 units of the appropriate restriction enzyme. The digestion using *HapII* was carried out in a 12-μl reaction mixture consisting of the ITS region, template DNA, and 1 mM Tris-HCl at pH 7.5. The digestion using *HinfI* was carried out in a 12-μl reaction mixture consisting of template, 10 mM NaCl, and 5 mM Tris-HCl at pH 7.5. The digestion using *MboI* was carried out in a 12-μl reaction mixture consisting of template, 10 mM KCl, and 2 mM Tris-HCl at pH 8.5. All reaction mixtures were incubated at 37°C for 4 h. Furthermore, rDNA-ITS of 19 isolates (11 NP isolates, 3 RW isolates, and 5 isolates of *W. circinata* var. *circinata*) were digested separately with enzymes *BanIII*, *EcoRI*, *HaeIII*, *HindIII*, *PstI*, *RsaI*, and *SalI*. The digestions with *BanIII*, *EcoRI*, *PstI*, *RsaI*, and *SalI* were done with the same condition as for *HinfI*. The reaction conditions for *HaeIII* and *HindIII* in a 12-μl reaction mixture consisted of template, 5 mM NaCl, and 1 mM Tris-HCl at pH 7.5. The restriction fragments were separated in a 2% NuSieve (3:1) agarose gel (FMC Bio-products, Rockl and MN, USA) with TAE buffer. The agarose gels were stained with ethidium bromide and visualized under UV transillumination. Each fragment length was determined using the linear function made from GROUT, the software in Microsoft Excel, described in the previous section.

RESULTS

Colony morphology. The colors of young colonies among NP isolates were similar and changed from white or orange to shades of dark brown with increasing age (after 30 days). The sclerotia were irregular in shape and ranged in size from 1 to 3 mm (Table 2). The color of *W. circinata* var. *circinata* colonies varied widely, from white or orange to dark brown with increasing age. The sclerotia of this variety were also light to dark brown, and their shape and size were irregular and ranged from 1 to 3 mm (Table 2). These characteristics were similar to those of RW isolates. The colony colors of *W. circinata* var. *oryzae* and *W. circinata* var. *zeae* were white to salmon pink and orange, respectively. The sclerotia of *W. circinata* var. *oryzae* were salmon pink in color and irregular in shape, and their size ranged from 1 to 3 mm. The sclerotia of *W. circinata* var. *zeae* were orange in color and globular shaped, and their size was small, ranging up to 1 mm (Table 2). The mature colonies of NP isolates, RW isolates, and the three varieties of *W. circinata* are shown in Figure 1. The diameter of the hyphae of NP isolates ranged from 4.7 to 7.8 μm (Table 2), and the number of nuclei per cell was 3 to 8 (Table 2). These charac-

teristics were almost the same as those of RW isolates, *W. circinata* var. *circinata*, *W. circinata* var. *oryzae*, and *W. circinata* var. *zeae*.

Growth rate. The mycelial growth rate of NP isolates at 15 to 35°C was not significantly different from that of RW isolates and *W. circinata* var. *circinata* (Fig. 2 and Table 3). Their mycelial growth rate gradually increased from 15 to 28°C and decreased from 28 to 35°C, and the optimum temperature for growth was 28°C (Fig. 2). The mycelial growth rate of *W. circinata* var. *oryzae* and *W. circinata* var. *zeae* increased from 15 to 30°C and decreased from 30 to 35°C; the optimum temperature for growth of both varieties was 30°C (Fig. 2). NP isolates, RW iso-

lates, and *W. circinata* var. *circinata* grew faster at 15°C but slower at 30°C than *W. circinata* var. *oryzae* and *W. circinata* var. *zeae* (Fig. 2 and Table 3). At 28°C, NP isolates and RW isolates grew more slowly than *W. circinata* var. *zeae* (Fig. 2 and Table 3).

Pathogenicity on bentgrass. The experiment was performed twice, and the results were similar in both experiments. Bentgrass inoculated with NP isolates and *W. circinata* var. *circinata* showed the same symptoms observed on bentgrass in the field; light and yellow brown on leaf sheaths in each leaf, and leaves were covered by yellow brown mycelia with sclerotia. Symptoms were not observed on the roots. Similar symptoms were observed on

Fig. 2. Mycelial growth rate of NP isolates, RW isolates, and three varieties of *Waitea circinata*. Bars indicate one standard deviation.

Table 3. Hyphal growth (mm) of NP isolates, RW isolates, and *Waitea circinata* var. *circinata*, *W. circinata* var. *oryzae*, and *W. circinata* var. *zeae*

Isolates/group	15°C	28°C	30°C
NP isolates	8.5 c ^z	19.0 a	18.1 a
RW isolates	8.6 c	19.8 a	18.9 a
<i>W. circinata</i> var. <i>circinata</i>	8.8 c	21.8 ab	20.4 a
<i>W. circinata</i> var. <i>oryzae</i>	3.3 a	18.4 a	23.7 b
<i>W. circinata</i> var. <i>zeae</i>	4.9 b	23.4 b	25.3 b

^z Values followed by the same letter do not differ significantly ($P < 0.05$) according to Duncan's multiple range test.

Table 4. Pathogenicity of NP isolates, RW isolates, *Waitea circinata* var. *circinata*, *W. circinata* var. *oryzae*, and *W. circinata* var. *zeae*

Group	Percent disease severity ^y	Reisolation frequency (%)
NP	33.4 c ^z	82.5
RW	26.0 bc	83.3
<i>W. circinata</i> var. <i>circinata</i>	21.1 b	80.0
<i>W. circinata</i> var. <i>oryzae</i>	8.4 a	83.3
<i>W. circinata</i> var. <i>zeae</i>	92.5 d	93.7

^y Disease severity scale: 0% = symptomless; 1% = small lesions; 5% = lesions covering <10% of leaf; 30% = lesions covering 10 to 50% of leaf; 70% = lesions covering >50% of a wilted leaf; and 100% = leaf completely withered. Average disease severity was estimated using NP isolates NP-1, -4, -7, and -8, *W. circinata* var. *circinata* Wc-1, -3, and -4, *W. circinata* var. *oryzae* Wo-1, -3, -4, and -6, and *W. circinata* var. *zeae* Wz-2, -3, and -4.

^z Values followed by the same letter do not differ significantly ($P < 0.01$) according to Duncan's multiple range test.

bentgrass inoculated with RW isolates. Disease severities on bentgrass inoculated with NP isolates and RW isolates did not differ (Table 4). Disease severity with *W. circinata* var. *circinata* was slightly less than with NP isolates but similar to that with RW isolates. *W. circinata* var. *oryzae* induced yellow and brown lesions, but disease severity was lower and significantly different from that induced by NP isolates, RW isolates, and *W. circinata* var. *circinata*. *W. circinata* var. *zeae* isolates induced yellow and orange lesions on

bentgrass, and leaves were covered by orange mycelia with many sclerotia. Disease severity incited by *W. circinata* var. *zeae* isolates was significantly greater than that incited by any other isolates used in this study. Isolates of NP, RW, *W. circinata* var. *circinata*, *W. circinata* var. *oryzae*, and *W. circinata* var. *zeae* were recovered from 80 to 94% of leaves with lesions (Table 4). The results shown in Table 4 are from one experiment.

RAPD analysis. All three primers amplified DNA from 25 NP isolates, RW

isolates, three varieties of *W. circinata*, and *R. solani* AG 2-2 IIIB and gave multiple products in the size range of approximately 0.4 to 4 kb (Fig. 3). The total number of bands from 25 isolates using primers R28, RC09, and OPE04 was 74. The pattern of bands produced by three primers with NP isolates was similar to the pattern with RW isolates and *W. circinata* var. *circinata* and could be distinguished from those of *W. circinata* var. *oryzae*, *W. circinata* var. *zeae*, and *R. solani* AG 2-2 IIIB (Fig. 3). In *W. circinata* var. *oryzae* and var. *zeae*, some specific fragments were observed with these three primers for each variety, although their patterns were complex. A single phylogenetic tree was obtained based on the presence or absence of fragments produced with all three primers, and showed obvious variation among NP isolates, RW isolates, the three varieties of *W. circinata*, and *R. solani* AG 2-2 IIIB (Fig. 4). Isolates were clearly separated into three clusters and one outgroup with more than 50 bootstrap values. Isolates of NP, RW, and *W. circinata* var. *circinata* were clustered together, while isolates of *W. circinata* var. *oryzae* and *W. circinata* var. *zeae* made individual clusters. The single isolate of *R. solani* AG 2-2 IIIB was not part of any cluster.

RFLP of rDNA-ITS region. The length of the rDNA-ITS region of all 11 NP isolates, three RW isolates, and five isolates of *W. circinata* var. *circinata* was approximately 645 bp (Table 5). The length of the rDNA-ITS region of both *W. circinata* var. *oryzae* and *W. circinata* var. *zeae* isolates was 660 bp. Restriction patterns of rDNA-ITS region by *HapII* showed two fragments (475 and 170 bp) for NP isolates, RW isolates, and *W. circinata* var. *circinata*, but the ITS regions of *W. circinata* var. *oryzae* and *W. circinata* var. *zeae* lacked this restriction site. RFLP analysis of rDNA-ITS with *HinfI* and *MboI* could not distinguish among NP isolates, RW isolates, and *W. circinata* var. *circinata*. RFLP analysis of rDNA-ITS with *HinfI* and *MboI* distinguished between *W. circinata* var. *oryzae* and *W. circinata* var. *zeae*. Two *MboI* sites were shared between *W. circinata* var. *zeae* and *W. circinata* var. *oryzae*, and produced three fragments of 250, 245, and 145 bp. *W. circinata* var. *zeae*, however, had one additional site within the 245-bp fragment, and this fragment was digested to form 145- and 100-bp fragments. Restriction fragments of rDNA-ITS by *HinfI* showed four fragments (200, 130, 90, and 70 bp) for *W. circinata* var. *oryzae* and three fragments (330, 90, and 80 bp) for *W. circinata* var. *zeae*, respectively. Furthermore, for *EcoRI*, *Sall*, and *BanIII*, the rDNA-ITS region of all NP isolates and *W. circinata* var. *circinata* isolates had the same restriction site, and two fragments were produced with both groups of isolates; 320 and 320 bp, 470 and 175 bp, and 375 and 270 bp for

Fig. 3. Random amplified polymorphic DNA (RAPD) banding patterns using primers R28, RC09, and OPE04 of NP isolates, RW isolates, *Waitea circinata* var. *circinata*, *W. circinata* var. *oryzae*, and *W. circinata* var. *zeae*. Lanes 1 to 4, *W. circinata* var. *circinata*; lanes 5 to 12, NP isolates; lanes 13 and 14, RW isolates; lanes 15 to 19, *W. circinata* var. *oryzae*; lanes 20 to 23, *W. circinata* var. *zeae*; lane 25, *Rhizoctonia solani* AG 2-2 IIIB. Size marker used was 100-bp DNA ladder.

these restriction enzymes, respectively. These isolates had no restriction sites for *Pst*I, *Hind*III, *Hae*III, and *Rsa*I.

DISCUSSION

A new disease on bentgrass caused by a *Rhizoctonia* sp., typified by NP isolates, was reported in Japan in 1994 (7). It was apparent that these isolates belonged to the *Waitea* anastomosis group (WAG) because they anastomosed with isolates of WAG-O (*W. circinata* var. *oryzae*) and WAG-Z (*W. circinata* var. *zeae*) (13). In this study, we clarified that these isolates were different from isolates of *W. circinata* var. *oryzae* and *W. circinata* var. *zeae*, based on cultural characteristics, including color and size of sclerotia, color of mycelia, pigment deposition of media, optimum temperature for growth, and pathogenicity. The colony color of NP isolates was similar to that of RW isolates and some isolates of *W. circinata* var. *circinata*, and the mycelial growth rate of NP isolates was the same as that of RW isolates and *W. circinata* var. *circinata*. Mycelia of NP isolates, RW isolates, and *W. circinata* var. *circinata* grew faster at temperatures below 16°C than *W. circinata* var. *oryzae* and *W. circinata* var. *zeae*, which corresponds with the report of Leiner and Carling (8). The pathogenicity test also showed that the symptoms observed on creeping bentgrass inoculated by NP isolates were similar to those caused by RW isolates and *W. circinata* var. *circinata*. The results of this study indicate that NP isolates causing a new disease of bentgrass and RW isolates causing white patch disease of bentgrass have characteristics similar to *W. circinata* var. *circinata*.

Based on RAPD-PCR analysis and RFLP of the rDNA-ITS region, NP isolates could be distinguished from *W. circinata* var. *oryzae* and *W. circinata* var. *zeae*, but could not be distinguished from RW isolates and *W. circinata* var. *circinata*. RAPD primers produced many fragments with NP isolates, RW isolates, three varieties of *R. circinata*, and *R. solani* AG 2-2 IIB, and there was a high level of variation among the isolates. On the basis of fragment patterns, a phylogenetic tree based on neighbor-joining indicated three clusters and one outgroup. Isolates of *W. circinata* var. *oryzae* and *W. circinata* var. *zeae* each made an individual cluster, while NP isolates, RW isolates, and *W. circinata* var. *circinata* made one cluster. Therefore, we can consider that NP isolates, RW isolates, and *W. circinata* var. *circinata* belong to the same RAPD group.

The length of the rDNA-ITS regions and restriction enzyme digest patterns with three enzymes could not distinguish isolates of NP, RW, and *W. circinata* var. *circinata*. However, RFLP analysis with *Hap*II of the rDNA-ITS region was useful to separate isolates of NP, RW, and *W. circinata* var. *circinata* from *W. circinata*

Fig. 4. Phylogenetic tree based on random amplified polymorphic DNA–polymerase chain reaction (RAPD-PCR) banding patterns of *Waitea circinata* isolates. An isolate of *Rhizoctonia solani* AG 2-2 IIB was used as an outgroup to root the tree. Tree length was 532 steps. Edges of length and bootstrap replication frequencies above 50% are indicated on branches. For isolate identification see Table 1.

Table 5. Estimated restriction fragment lengths (in base pairs) produced by three restriction enzymes in the ribosomal DNA internal transcribed spacer region (including 5.8S rDNA) for NP isolates, RW isolates, and three varieties of *Waitea circinata*

Restriction site	Varieties of <i>W. circinata</i>				
	NP ^y	RW	<i>circinata</i>	<i>oryzae</i>	<i>zeae</i>
<i>Hap</i> II	...	...	...	660	660
	475	475	475	...	...
	170	170	170	...	...
<i>Mbo</i> I	245	245	245	250	250
	230	230	230	...	245
	145	145	145	145	145
<i>Hin</i> fI	...	...	...	100	100
	330	330	330	...	330
	...	...	...	200	...
	...	...	...	130	...
	90	90	90	90	90
	80	80	80	70	80
Total	645	645	645	660	660

^y NP: NP isolates, RW: isolates of *Rhizoctonia oryzae* obtained from white patch disease.

^z Restriction fragments are missing.

var. *oryzae* and *W. circinata* var. *zeae*. Furthermore, restriction patterns by *Mbo*I and *Hinf*I clearly distinguished *W. circinata* var. *oryzae* from *W. circinata* var. *zeae*. The restriction sites of rDNA-ITS regions in this study did not corresponded with previously reported sequences of *R. oryzae* (10). Isolates of *W. circinata* var. *oryzae* had a high level of variation based on RAPD analysis and a different response of hyphal growth to high temperatures (8,12). There might be several subgroups in this variety. The results obtained from DNA analyses, showing that NP isolates could not be distinguished from RW isolates and *W. circinata* var. *circinata* but could be distinguished from *W. circinata* var. *oryzae* and *W. circinata* var. *zeae*, were consistent with their cultural characteristics.

Tanpo et al. (19) reported that *R. oryzae* (RW isolates) was the causal agent of white patch disease on bentgrass in Japan. The disease symptoms caused by *R. oryzae* were very similar to the new disease caused by NP isolates in this report except for whitish color. In this study, we used isolates of Tanpo et al. (19), which showed almost the same cultural characteristics and pathogenicity as NP isolates and *W. circinata* var. *circinata* but not *W. circinata* var. *oryzae*. Moreover, RW isolates were placed into the same groups as *W. circinata* var. *circinata* based on RAPD-PCR and RFLP analysis of rDNA-ITS region. Therefore, the identification of RW isolates as *R. oryzae* proposed by Tanpo et al. (19) should be reconsidered as *W. circinata* var. *circinata*.

Based on the results of this study, we recognized that the NP isolates causing the new disease and the RW isolates, which had been considered to be *R. oryzae*, cause of white patch disease, both belong to *W. circinata* var. *circinata*. However, the name "white patch" cannot be used for this turf disease, because the same name has already been used by another fungal disease

for tall fescue (*Festuca arundinacea* Schreb) caused by *Melanotus phillipsii* Singer (18). As Tanpo et al. (19) noted, the affected turf develops rings and shows a clear patch with a brownish color including yellow or white. From these symptoms, we propose that the name of the new disease is "brown ring patch".

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