A New Subgroup of *Rhizoctonia* AG-D, AG-D III, Obtained from Japanese Zoysia Grass Exhibiting Symptoms of a New Disease

Toshihiro Hayakawa, Research Institute of Riken Green Co., Ltd., 859-1 Minamideiheishinden, Fukude, Iwata, Shizuoka 437-1212, Japan; **Takeshi Toda**, Laboratory of Plant Pathology, Faculty of Applied Biological Science, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan; **Qu Ping** and **Joseph M. Mghalu**, United Graduate School of Agricultural Science, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan; **Shigeharu Yaguchi**, Research Institute of Riken Green Co., Ltd., 859-1 Minamideiheishinden, Fukude, Iwata, Shizuoka 437-1212, Japan; and **Mitsuro Hyakumachi**, Laboratory of Plant Pathology, Faculty of Applied Biological Science, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan

ABSTRACT

Hayakawa, T., Toda, T., Ping, Q., Mghalu, J. M., Yaguchi, S., and Hyakumachi, M. 2006. A new subgroup of *Rhizoctonia* AG-D, AG-D III, obtained from Japanese zoysia grass exhibiting symptoms of a new disease. Plant Dis. 90:1389-1394.

Isolates of an unidentified *Rhizoctonia* sp. (UN isolates) were obtained from Japanese zoysia grass (*Zoysia japonica* Steud) that exhibited symptoms of a new sheath rot disease. UN isolates were binucleate and showed hyphal fusion with tester isolates of *Rhizoctonia* anastomosis group (AG)-D. Those isolates were compared with isolates of subgroups I and II of *Rhizoctonia* AG-D based on cultural morphology, hyphal growth rate at different temperatures, anastomosis frequency, pathogenicity, and sequence analysis of the internal transcribed spacer (ITS) region of ribosomal DNA genes (rDNA-ITS region). The mycelial color of UN isolates was light yellow which differs from AG-D I but is similar to AG-D II. Sclerotia of UN isolates were dark brown in color and larger in size (1 to 3 mm in diameter) than those of AG-D subgroup I (1 mm in diameter), whereas isolates of AG-D II produced white mycelial clumps 4 to 5 mm in size. Hyphal growth rate of UN isolates was slower than that of two AG-D subgroups at several temperatures, especially 25°C. In pathogenicity tests on Japanese zoysia grass, UN isolates showed moderate disease severity and lower pathogenicity than isolates of AG-D subgroups I and II. Sequences of the rDNA-ITS region within UN isolates were almost homologous, but had lower homology with subgroups AG-D I or II. Phylogenetic trees constructed using ITS sequences showed that UN isolates formed an individual cluster that differed from the clusters of the two subgroups. We propose that UN isolates are a new subgroup of *Rhizoctonia* AG-D, subgroup III, and the name of the disease is “spring-rot” on Japanese zoysia grass.

A new *Rhizoctonia* disease on Japanese zoysia grass (*Zoysia japonica* Steud) was found on fairways and roughs of golf courses in Shizuoka, Japan, from mid-through late spring 2002. This disease formed irregular reddish-brown patches approximately 1 m in diameter. The affected turf showed symptoms of water-soaked sheath rot and reddish-brown foliar discoloration, but did not show any symptoms in the stolons and roots. Hyphae of *Rhizoctonia* spp. frequently were isolated from affected leaf sheaths. Unknown (UN) isolates had two nuclei per cell and anastomosed with tester isolates of binucleate *Rhizoctonia* anastomosis group (AG)-D.

Binucleate *Rhizoctonia* anastomosis group D (*Rhizoctonia cerealis* Van der Hoeven = *Ceratobasidium cereale* D. Murray and L. L. Burpee = *Rhizoctonia* AG-D) is the causal pathogen of foot rot, root rot, and damping-off disease in several crops (2,10,13). Based on anastomosis reactions, binucleate *Rhizoctonia* spp. have been assigned to AGs, of which 18 groups have been designated as AG-A through AG-S (12,16) in Japan. Also, *Ceratobasidium* anastomosis groups (CAGs) have been established as CAG 1 through 7 (3), and AG-D was clarified to be identical to CAG-1 (11). These patterns were supported using molecular techniques (5,6). Isolates of AG-D were further divided into subgroups I and II based on pathogenicity, morphology in culture, and molecular techniques (17,19). Both subgroups I and II can cause disease on Japanese zoysia grass (*Z. japonica* Steud) and Manila zoysia grass (*Z. matrella* Err) on fairways and roughs of golf courses in Japan (17,19). AG-D subgroup I (AG-D I) causes a *Rhizoctonia* patch disease that produces circular or irregular patches less than 1 m in diameter with a whitish color during

early and mid-spring. Affected leaves in the patch exhibit whitish lesions. AG-D subgroup II (AG-D II) causes elephant footprint disease, appearing as circular patches with a whitish color from summer through early autumn (19). Based on seasons of disease occurrence and molecular analysis, isolates of AG-D obtained from wheat, mat rush, and creeping bentgrass were clarified as AG-D I (19). The new disease caused by UN isolates differs from both *Rhizoctonia* patch disease and elephant footprint disease. Therefore, UN isolates were considered different from those of AG-D I and II.

The objectives of this study were to compare the UN isolates obtained from Japanese zoysia grasses with tester isolates of *Rhizoctonia* AG-D I and II, based on cultural morphology, hyphal growth rate at different temperatures, pathogenicity on Japanese zoysia grass, and sequence analysis of the internal transcribed spacer (ITS) region on ribosomal DNA (rDNA-ITS region).

MATERIALS AND METHODS

Isolates. Twenty-one isolates of an unknown *Rhizoctonia* sp. (UN isolates) collected from Japanese zoysia grass (*Z. japonica* Steud) exhibiting symptoms of sheath rot were used in this study (Table 1). Isolates were collected from three golf courses—Kagosaka Golf Club (GC), Fuji-Kogen GC, and Fuji-Koyama GC—in Shizuoka prefecture in Japan, located 4 to 5 km apart from each other. The temperatures in the area of the three golf courses ranged from approximately 11 to 22°C during the season when sheath rot symptoms were observed. Each isolate was collected from a different patch. Nine isolates of AG-D subgroup I and 13 isolates of subgroup II were used as tester isolates (Table 2). In addition, tester isolates of other binucleate *Rhizoctonia* AGs were used for anastomosis, and two isolates of *Rhizoctonia solani* AG 2-2 LP obtained from warm-season turfgrasses exhibiting symptoms of large-patch disease also were used for anastomosis and sequence analysis of the rDNA-ITS region (Table 3).

Corresponding author: T. Toda
E-mail: ttoda@ncsu.edu

Current address of T. Toda: Department of Plant Pathology, North Carolina State University, Campus Box 7251, Partners III building, Raleigh 27695.

Accepted for publication 30 April 2006.

DOI: 10.1094/PD-90-1389
© 2006 The American Phytopathological Society

Anastomosis reaction. Five UN isolates (UN-1, -2, -4, -5, and -11; Table 1), three isolates of AG-D I (I-1, -6, and -9), and three isolates of AG-D II (II-1, -3, and -9; Table 2) were tested for anastomosis reaction. All tester isolates of other binucleate *Rhizoctonia* AGs and two isolates of *R. solani* AG 2-2 LP (LP-1 and -2) were used for comparison (Table 3). The isolates were grown on 9-cm-diameter petri dishes containing 10 ml of potato dextrose agar (PDA; Becton Dickinson and Company, Sparks, MD) for 2 to 3 days at 25°C in darkness. Mycelial plugs, 3 mm in diameter, taken from the advancing margin of a pure culture of each isolate were paired 3 to 4 cm apart on a 9-cm petri dish containing 5 ml of 2% water agar (WA) at 25°C in the dark.

After 2 days, the overlapping portion of hyphae growing on WA was transferred to a glass slide, stained with 0.05% cotton blue in 50% acetic acid, and observed for hyphal fusion at ×400 magnification. The types of hyphal anastomosis reactions (4) were determined by observation at ×400 magnification. Percentage of fusion frequency was determined using the equation $(A/B) \times 100$, where *A* is the sum of fusion points and *B* is the sum of contact points in 15 microscopic fields.

Cultural morphology. All UN and AG-D isolates listed in Tables 1 and 2 were observed to characterize cultural morphology. A single 5-mm-diameter agar disk of each isolate was placed on the center of a petri dish containing 10 ml of PDA and grown at 25°C in darkness. After 60 days,

the morphology and color of mycelia and sclerotia were examined.

Growth rate. The hyphal growth rate for four UN isolates (UN-1 to -4) and two isolates from each subgroup (I-1, I-2, II-1, and II-2), grown on PDA at 10, 15, 20, 25, 30, 35, and 40°C was determined. For each test isolate, a 5-mm-diameter agar disk was placed 1 cm from the edge of a 9-cm petri dish containing 10 ml of PDA. Three petri dishes for each isolates in each temperature were prepared. The radius of each hyphal mass was measured every 24 h until the colony reached the opposite edge of the petri dish. The treatment means were compared using Fisher's least significant difference (LSD) based on the differences of hyphal growth between different temperatures and between subgroups. Growth rate tests were repeated once.

Pathogenicity on zoysia grass. Ten isolates (six UN isolates [UN-1, -2, -4, -7, -8, and -11], two isolates of AG-D I [I-3 and -9], and two isolates of AG-D II [II-12 and -13]) were tested for pathogenicity on Japanese zoysia grass.

Segments of sod of Japanese zoysia grass (*Z. japonica* Steud) grown in the nursery of Riken Green Co. Ltd in Japan were transferred into 15-cm-diameter clay pots and grown for 56 days at 25°C in a greenhouse prior to inoculation. Isolates of the UN group and the two subgroups of *Rhizoctonia* AG-D, cultured on PDA medium for 4 days, were transferred to 300 ml of medium for growing inocula (a mixture of 1 liter of liquid medium, including 1% yeast extract and 1% polypeptone, 3 liters of peat moss, 3 liters of wheat bran, 3 liters of rice bran, and 6 liters of soil; distributed as 300 ml per 500-ml flask) and grown for 10 days at 25°C in darkness. The culture on medium was homogenized and filtered using 2-mm mesh. Inoculation using 20 ml of filtered mycelia was done onto established Japanese zoysia grass at 20 to 25°C. Three pots of Japanese zoysia grass for each isolate were prepared. Control plants were treated similarly but without inocula. Disease severity was observed 20 days after inoculation, and was determined by the average of individual scores for 10 leaves, using the following rating scheme: 0% = no symptoms, 5% = lesions covering less than 10% of leaf area, 30% = lesions covering from 10 to 50% of the leaf area, 75% = lesions covering more than 50% of leaf area, and 100% = leaf completely withered. The means of disease severity were compared by Kruskal-Wallis test. Prior to statistical analysis, percent data were transformed to arcsine square root values to normalize the variance. The pathogenicity test was repeated once.

Re-isolation. Samples of Japanese zoysia grass with lesions were collected 20 days after inoculation. Leaf pieces, 5 mm long, were cut from infected leaves, washed three times with distilled water, blotted dry on a sterile paper towel, and

Table 1. List of isolates obtained from Japanese zoysia grass exhibiting symptom of the new disease used in this study

Working no.	Isolate name	Origin ²
UN-1	KAG9R	Kagosaka GC, Shizuoka, Japan
UN-2	KAG10R	Kagosaka GC, Shizuoka, Japan
UN-3	KAG6R1	Kagosaka GC, Shizuoka, Japan
UN-4	KAG6R2	Kagosaka GC, Shizuoka, Japan
UN-5	KAG-12FW	Kagosaka GC, Shizuoka, Japan
UN-6	KOU04-AP	Fuji-Kogen GC, Shizuoka, Japan
UN-7	KOU04-12FW	Fuji-Kogen GC, Shizuoka, Japan
UN-8	KOU04-1FW	Fuji-Kogen GC, Shizuoka, Japan
UN-9	KOU04-18FW1	Fuji-Kogen GC, Shizuoka, Japan
UN-10	KOU04-18FW2	Fuji-Kogen GC, Shizuoka, Japan
UN-11	OYA-7FW	Fuji-Koyama GC, Shizuoka, Japan
UN-12	KAG21R	Kagosaka GC, Shizuoka, Japan
UN-13	KAG22R	Kagosaka GC, Shizuoka, Japan
UN-14	KAG7R1	Kagosaka GC, Shizuoka, Japan
UN-15	KAG7R2	Kagosaka GC, Shizuoka, Japan
UN-16	KAG9R10	Kagosaka GC, Shizuoka, Japan
UN-17	KOU17-1FW	Fuji-Kogen GC, Shizuoka, Japan
UN-18	KOU17-2FW	Fuji-Kogen GC, Shizuoka, Japan
UN-19	KOU18-FWL	Fuji-Kogen GC, Shizuoka, Japan
UN-20	KOU18-FWS	Fuji-Kogen GC, Shizuoka, Japan
UN-21	OYA-FWL	Fuji-Koyama GC, Shizuoka, Japan

²Isolates were collected from Kagosaka Golf Club (GC), Fuji-Kogen GC, or Fuji-Koyama GC in Shizuoka, Japan. The host was *Zoysia japonica* Steud.

Table 2. List of isolates of *Rhizoctonia* anastomosis group (AG)-D subgroups I and II used in this study

Working no.	Isolate	Subgroup	Host	Origin
I-1	W-12 (ATCC: 76318)	AG-D I	<i>Triticum aestivum</i> L.	Hokkaido, Japan
I-2	ST-SDS-1	AG-D I	<i>Zoysia japonica</i> Steud	Mie, Japan
I-3	YC-SDS-1	AG-D I	<i>Zoysia japonica</i> Steud	Mie, Japan
I-4	IZ-SDS-1	AG-D I	<i>Zoysia matrella</i> Err	Shimane, Japan
I-5	SD-SDS-1	AG-D I	<i>Zoysia japonica</i> Steud	Yamaguchi, Japan
I-6	TG-M-2	AG-D I	<i>Zoysia japonica</i> Steud	Hiroshima, Japan
I-7	HR-12-1	AG-D I	<i>Zoysia matrella</i> Err	Shiga, Japan
I-8	Ayu-WP-1	AG-D I	<i>Agrostis palustris</i> Huds.	Kagawa, Japan
I-9	BrG-WP-1	AG-D I	<i>Agrostis palustris</i> Huds.	Yamaguchi, Japan
II-1	YG-EF-1	AG-D II	<i>Zoysia matrella</i> Err	Yamagata, Japan
II-2	MW-EF-2	AG-D II	<i>Zoysia matrella</i> Err	Yamaguchi, Japan
II-3	WK-EF-1	AG-D II	<i>Zoysia japonica</i> Steud	Yamaguchi, Japan
II-4	ST-EF-2	AG-D II	<i>Zoysia japonica</i> Steud	Kagawa, Japan
II-5	YC-EF-1	AG-D II	<i>Zoysia japonica</i> Steud	Mie, Japan
II-6	SB-EF-1	AG-D II	<i>Zoysia matrella</i> Err	Chiba, Japan
II-7	CH-EF-1	AG-D II	<i>Zoysia matrella</i> Err	Shiga, Japan
II-8	HR-EF-1	AG-D II	<i>Zoysia matrella</i> Err	Shiga, Japan
II-9	Oak-EF-1	AG-D II	<i>Zoysia japonica</i> Steud	Okayama, Japan
II-10	MW-EF-1	AG-D II	<i>Zoysia matrella</i> Err	Yamaguchi, Japan
II-11	SD-EF-1	AG-D II	<i>Zoysia matrella</i> Err	Kagawa, Japan
II-12	KAR-18R	AG-D II	<i>Zoysia matrella</i> Err	Shizuoka, Japan
II-13	TAK-14KT	AG-D II	<i>Zoysia matrella</i> Err	Shizuoka, Japan

plated on WA. Hyphae resembling *Rhizoctonia* AG-D were transferred as hyphal tips onto PDA after 5 days of incubation at 25°C. Re-isolation frequency was scored using the equation $(A/B) \times 100$, where *A* was the number of samples that showed growth of mycelium that were identical to the original isolates used to prepare the inocula, and *B* was the total number of samples (*n* = 20) used for re-isolation.

DNA extraction. In all, 30 isolates (10 UN isolates [UN-1 to -10], 8 isolates of AG-D I [I-1 to -6, -8, and -9], 10 isolates of AG-D II [II-1, -3, -4, -5, -7 to -10, -12, and -13] and 2 isolates of AG 2-2 LP [LP-1 and -2]) for DNA extraction were incubated in potato dextrose broth at 25°C. After 3 to 4 days of incubation, the mycelial mat was harvested by filtration and stored at -80°C until use. Total genomic DNA was extracted as described by Toda et al. (19).

Sequence analysis of rDNA-ITS. Polymerase chain reaction (PCR) with primers ITS 1 and ITS 4 (Table 4) was used to amplify rDNA-ITS regions of 24 isolates (9 UN isolates [UN-1 to -4 and -6 to -10], 8 isolates of AG-D I [I-1 to -6, -8, and -9; Table 1], and 7 isolates of AG-D II [II-1, -4, -7, -9, -10, -12, and -13]). PCR amplification reactions of rDNA-ITS regions were carried out using 50 ng of DNA from each of the 22 isolates. The 50-μl reaction mix-

ture consisted of 200 μM each dATP, dCTP, dGTP, and dTTP, 50 pmol of oligonucleotide primer pair ITS 1 and ITS 4, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, and 1.25 units of *Taq* DNA polymerase (Takara Shuzo, Kyoto, Japan). The DNA Thermal Cycler (GeneAmp PCR system 2700; Applied Biosystems, Foster City, CA) was programmed for 1 cycle of 5 min at 94°C; followed by 25 cycles of 30 s at 94°C, 30 s at 59°C, and 30 s at 72°C; and 1 cycle of 7 min at 72°C. The amplification products were observed by electrophoresis on 1.5% agarose gel (LO3; Takara Shuzo, Kyoto, Japan) with Tris-acetic EDTA (TAE) buffer. The agarose gels were stained with ethidium bromide and visualized under UV transillumination. After amplification, 50 μl of the PCR mixture was mixed with 30 μl of 30% polyethylene glycol 6000 (Nakarai Tesque, Kyoto, Japan) and 1.6 M NaCl and centrifuged at 13,000 rpm at room temperature. The amplified ITS region was precipitated, rinsed with 1 ml of 70% ethanol, and dissolved in 20 μl of TE buffer (10 mM Tris-HCl, pH 7.5, and 1 mM EDTA). The ITS region then was sequenced using primers ITS 1, ITS 2, ITS 3, and ITS 4 (Table 4) with a Ready Reaction Cycle Sequencing Kit (Applied Biosystems) and analyzed by an ABI 3100 DNA sequencer (Applied Biosystems). The rDNA-ITS sequences of UN

isolates (I-1 to -4 and -6 to -10) *Rhizoctonia* AG-D I (I-1 to -6, -8, and -9) and II (II-1, -4, -7, -9, -10, -12, and -13) have been deposited in the GenBank database (accession nos. AB198709, AB198694, AB198710, AB198711, AB198712, AB198713, AB198695, AB198714, AB198715, AB198697, AB198698, AB198699, AB198700, AB198701, AB198702, AB198703, AB198693, AB198704, AB198705, AB198706, AB198707, AB198708, AB214366, and AB214367, respectively). ITS sequence data of LP-1 and -2 already had been deposited in the GenBank database (accession nos. AJ238163 and AJ238165, respectively) by Sarazar et al. (14). ITS sequences of UN isolates were aligned with those of *Rhizoctonia* AG-D I and II and ITS sequence data of *R. solani* AG 2-2 LP. ITS sequences of UN isolates were aligned with those of *Rhizoctonia* AG-D I and II and the ITS sequence data of *R. solani* AG 2-2 LP using GENETYX-WIN (version 4.0.1; Software Kaihatsu Co, Ltd., Tokyo) to evaluate sequence similarities among all isolates and ClustalX version 1.8 (18) to construct a phylogenetic tree.

RESULTS

Anastomosis reaction. Hyphae of UN isolates fused with tester isolates of both AG-D I and II but failed to fuse with hyphae of other tester isolates of *Rhizoctonia* AGs and *R. solani* AG 2-2 LP. Anastomosis frequency among five UN isolates was 25.0 to 44.4%. Within subgroups, hyphal anastomosis frequencies were 37.4 to 44.3% for three AG-D I isolates and 28.6 to 32.8% for three AG-D II isolates. Anastomosis frequency of five UN isolates with three isolates of AG-D I and II were 21.3 to 38.5% and 27.4 to 39.7%, respectively. Anastomosis frequency between three isolates of AG-D I and three isolates of AG-D II was 32.5 to 46.0%.

Cultural morphology. Mycelium of all isolates of UN and two AG-D subgroups was light yellow for 30 days. A colony of UN isolates still retained a light yellow color after 60 days. Sclerotia of UN isolates were dark brown and 1 to 3 mm in diameter. Isolates of AG-D I had brown to dark-brown colonies and 1-mm-diameter dark-brown sclerotia. Isolates of AG-D II had light-yellow colonies with large whitish hyphal clamps. Neither UN isolates nor isolates of AG-D I produced hyphal clamps, and isolates of AG-D II did not produce sclerotia.

Table 3. List of tester isolates of binucleate *Rhizoctonia* anastomosis groups (AGs) and *Rhizoctonia solani* AG 2-2 LP used in this study

AG (subgroup) or working no.	Isolate (MAFF no.) ^a	Host	Origin
A	C-538 (MAFF305271)	<i>Solanum tuberosum</i>	Japan
	AH-1 (MAFF305267)	<i>Arachis hypogaea</i>	Japan
B (Ba)	C-460 (MAFF305277)	<i>Oryza sativa</i>	Japan
	C-485 (MAFF305279)	<i>Oryza sativa</i>	Japan
B (Ba)	C-455 (MAFF305284)	<i>Oryza sativa</i>	Japan
	C-350 (MAFF305283)	<i>Oryza sativa</i>	Japan
C	Ka-1-1 (MAFF305289)	Soil	Japan
	N462	<i>Dactylis glomerata</i>	Japan
E	Lu-1 (MAFF305295)	<i>Linum usitatissimum</i>	Japan
	Oc-1 (MAFF305299)	<i>Oxalis corniculata</i>	Japan
F	PS-17 (MAFF305303)	<i>Pisum sativum</i>	Japan
	SIR-1 (MAFF305304)	<i>Ipomoea batatas</i>	Japan
G	Su-1 (MAFF305307)	<i>Arachis hypogaea</i>	Japan
	C-593 (MAFF305308)	<i>Arachis hypogaea</i>	Japan
H	STC-10 (MAFF305310)	Soil	Japan
	STC-11 (MAFF305311)	Soil	Japan
I	FKO-1-17 (MAFF305312)	Soil	Japan
	FKO-6-23 (MAFF305314)	Soil	Japan
J	STC-23 (MAFF305317)	Soil	Japan
K	AC-1	<i>Allium cepa</i>	Japan
	SN-6 (MAFF305318)	Soil	Japan
L	FKO-2-11 (MAFF305321)	Soil	Japan
	FKO-2-26 (MAFF305324)	Soil	Japan
N	STC-43 (MAFF305325)	Soil	Japan
O	FKO-6-2 (MAFF305328)	Soil	Japan
P	C-579 (MAFF305336)	Soil	Japan
	C-582 (MAFF305337)	Soil	Japan
R	BN-37	<i>Cucumis sativum</i>	United States
S	S5	Soil	Japan
	AG 2-2 LP		
<i>Rhizoctonia solani</i>			
LP-1	G4	<i>Zoysia matrella</i> Err	Gifu, Japan
LP-2	L-9-1S	<i>Zoysia matrella</i> Err	Gifu, Japan

^a Isolates of AGs-J and -M were clarified to be not *Rhizoctonia* sp. and isolates of AG-Q have been clarified as AG-D I. MAFF: Ministry of Agriculture, Forestry, and Fisheries Japan.

Table 4. Primers used in this study

Primer	Sequence
ITS1	5'-TCCGTAAGGTGAACCTGCGG-3'
ITS2	5'-GCTGCGTTCTTCATCGATGC-3'
ITS3	5'-GCATCGATGAAGAACGCAGC-3'
ITS4	5'-TCCTCCGCTTATTGATATGC-3'

Growth rate. The average mycelial growth rate of each UN and AG-D isolate at 5 to 40°C is shown in Figure 1. The mycelial growth rate of UN isolates

gradually increased from 5 to 20°C and rapidly decreased from 20 to 35°C (Fig. 1). Optimum growth temperature of UN isolates was 20°C which was statistically

($P = 0.05$) supported (Table 5). The mycelial growth rate of both AG-D I and II isolates increased from 5 to 20°C and rapidly decreased from 25 to 35°C. The optimum growth temperatures of AG-D I and II isolates were statistically ($P = 0.05$) estimated from 20 to 25°C. UN isolates grew significantly more slowly than isolates of subgroups I and II at 20 and 25°C (Table 6).

Pathogenicity on zoysia grass. The experiment was performed twice with the same trend of results, and results of both experiments 1 and 2 are shown in Table 7. Japanese zoysia grass inoculated with UN isolates showed water-soaked sheath rot and foliar discoloration with a light red-dish-brown color. Disease severities on Japanese zoysia grass inoculated with UN isolates in experiment 1 and 2 were 48.8 and 52.5%, respectively. Japanese zoysia grass inoculated with isolates of both AG-D subgroups I and II showed water-soaked foliar blight with a whitish color. Disease severities for both AG-D I and II were significantly ($P = 0.05$) higher than those of UN isolates in both experiments: 97.5 and 87.5% for AG-D I and 98.8 and 100% for AG-D II. These symptoms were similar to those of zoysia grass on Japanese golf courses. Isolates of UN and AG-D I and II were re-isolated from lesions with a frequency of 77.5, 77.5, and 77.5% in experiment 1 and 83.3, 85.0, and 92.5% in experiment 2, respectively.

Sequence analysis. PCR gave a single ITS product of approximately 620 bp from all *Rhizoctonia* AG-D I and II and UN isolates. Sequence similarity among isolates of UN and AG-D I was 94.9 to 99.7 and 96.0 to 99.5%, respectively, whereas similarity among isolates of AG-D II was 93.5 to 94.7% (Table 8). Sequence similarity between isolates of UN and the two subgroups was lower (82.4 to 92.6%) than that within each subgroup. Sequence similarity of *R. solani* AG 2-2 LP with isolates of UN and two subgroups of AG-D was 71.4 to 75.8%. A single neighbor-joining tree was obtained based on ClustalX using the sequence data of UN, AG-D I and II, and *R. solani* AG 2-2 LP isolates (Fig. 2). Isolates were separated clearly into three clusters and one outgroup with support of high bootstrap values. UN isolates formed a cluster separate from those of two AG-D subgroups. Isolates of each subgroup (AG-D I and II) were clustered individually. Phylogenetic distance showed that the cluster of UN isolates is nearer to that of AG-D I than that of AG-D II. Two isolates of *R. solani* AG 2-2 LP were not part of any clusters.

DISCUSSION

From the results of this study, UN isolates were assigned as a subgroup within *Rhizoctonia* AG-D. Therefore, we propose that the UN isolates form a new subgroup of *Rhizoctonia* AG-D, subgroup III, and

Fig. 1. Mycelial growth rate of unknown (UN) isolates and isolates of two subgroups of *Rhizoctonia* anastomosis group (AG)-D. Bars indicate standard deviation. Four UN isolates (◆, dotted line), two isolates of AG-D I (■, solid line), and two isolates of AG-D II (▲, dashed line) were represented by each line.

Table 5. Comparison of mycelial growth rate of isolates of unknown (UN) and *Rhizoctonia* anastomosis group (AG)-D subgroups I and II based on different temperatures (Temp)^z

Temp (°C)	Experiment 1			Experiment 2		
	UN	AG-D I	AG-D II	UN	AG-D I	AG-D II
5	0.6 c	1.2 d	0.8 c	0.6 c	2.3 d	1.0 c
10	2.9 b	4.0 c	3.6 b	2.9 b	4.0 c	3.6 b
15	4.1 b	6.4 b	4.1 b	4.1 b	6.6 b	6.2 b
20	6.8 a	9.0 a	9.8 a	6.7 a	8.9 a	9.8 a
25	2.9 b	8.9 a	10.8 a	2.9 b	8.8 a	10.4 a
30	2.2 bc	4.9 b	5.8 b	2.1 bc	5.1 b	5.9 b
35	0 c	0 d	0 c	0 c	0 d	0 c
40	0 c	0 d	0 c	0 c	0 d	0 c

^z UN = isolates obtained from sheath rot symptoms; AG-D I = isolates of *Rhizoctonia* AG-D subgroup I and AG-D II = isolates of *Rhizoctonia* AG-DII. Four UN isolates, two isolates of AG-D I, and two isolates of AG-D II were represented. Values followed by the same letter do not differ significantly ($P = 0.05$) according to least significant difference.

Table 6. Comparison of mycelial growth rate among unknown (UN) isolates and isolates of *Rhizoctonia* anastomosis group (AG)-D in each temperature

Subgroups ^z	Temperature (°C) ^y							
	5	10	15	20	25	30	35	40
Experiment 1								
UN	0.6 a	2.9 a	4.1 a	6.8 a	2.9 a	2.2 a	0 a	0 a
AG-D I	1.2 a	4.0 a	6.4 a	9.0 b	8.9 b	4.9 ab	0 a	0 a
AG-D II	0.8 a	3.6 a	4.1 a	9.8 b	10.8 b	5.8 b	0 a	0 a
Experiment 2								
UN	0.6 a	2.9 a	4.1 a	6.7 a	2.9 a	2.1 a	0 a	0 a
AG-D I	2.3 a	4.0 a	6.6 a	8.9 b	8.8 b	5.1 ab	0 a	0 a
AG-D II	1.0 a	3.6 a	6.2 a	9.8 b	10.4 b	5.9 b	0 a	0 a

^y Values followed by the same letter do not differ significantly ($P = 0.05$) according to least significant difference.

^z UN = isolates obtained from sheath rot symptoms; AG-D I = isolates of *Rhizoctonia* AG-D subgroup I and AG-D II = isolates of *Rhizoctonia* AG-DII. Four UN isolates, two isolates of AG-D I, and two isolates of AG-D II were represented.

that the name of a new disease caused by AG-D subgroup III is "spring rot" disease on Japanese zoysia grass.

The UN isolates were categorized as members of *Rhizoctonia* AG-D because they anastomosed with tester isolates of AG-D but not with other binucleate isolates. Anastomosis frequencies ranged from 30 to 50% without differences between subgroups. Therefore, AG-D subgroups including UN isolates could not be distinguished based on anastomosis frequencies.

Cultural characteristics on PDA and the sequence of rDNA-ITS provided unique characteristics that place UN isolates in a separate subgroup, AG-D III. The standard cultural morphology of AG-D III was defined briefly by comparison with other subgroups of AG-D. Occasionally, several AG-D III isolates did not form sclerotia. On the other hand, colonies of some AG-D I isolates remained light yellow in color (*data not shown*). Therefore, we have to be cautious when determining subgroups based on morphological characterizations alone. For conventional identification of AG-D III, comparison of hyphal growth rate among AG-D subgroups seems to be easier than observation of cultural morphology, especially at 25°C (Fig. 1).

Sequence data of the rDNA-ITS region are more efficient for identifying each AG-D subgroup. Sequence homology within each AG-D subgroup was very high, but significantly lower between subgroups (Table 7). Moreover, phylogenetic analysis using the sequence data separated into three clearly defined clusters corresponding to the three AG-D subgroups (Fig. 2).

Table 7. Pathogenicity on Japanese zoysia grass 20 days after inoculation^w

Isolates ^x	Disease severity (%) ^y	Reisolation (%) ^z
Experiment 1		
UN	48.8 b	77.5
AG-D I	97.5 c	77.5
AG-D II	98.8 c	77.5
Control	0.0 a	0.0
Experiment 2		
UN	52.5 b	83.3
AG-D I	87.5 c	85.0
AG-D II	100.0 c	92.5
Control	0.0 a	0.0

^wValues followed by the same letter do not differ significantly ($P = 0.05$) according to Kruskal-Wallis test.

^xUN = unknown isolates obtained from sheath rot symptoms; anastomosis group (AG)-D I = isolates of *Rhizoctonia* AG-D subgroup I and AG-D II = isolates of *Rhizoctonia* AG-DII. Six UN isolates, two isolates of AG-D I, and two isolates of AG-D II were represented.

^yDisease severity scale: 0 = no symptoms, 5 = lesions covering less than 10% of leaf area, 30 = lesions covering from 10 to 50% of the leaf area, 75 = lesions covering more than 50% of leaf area, and 100 = leaf completely withered.

^zReisolation (%) = binucleate *Rhizoctonia* appearance/20 plants × 100.

Analysis of the rDNA-ITS region has been recognized as a useful method for identification or grouping of many fungal species. In *R. solani*, many reports demonstrated that analysis of ITS sequences was useful for classification of intragroups within AGs (1,9,14,20). In the case of binucleate *Rhizoctonia* spp., analysis of rDNA-ITS region may not be sufficient for AG identification, because several isolates assigned

to the same AG or CAG were not clustered in the same clade of a phylogenetic tree (7,8). However, in the case of AG-D, the sequence similarities of the ITS regions can be defined as the basis of differentiation among these three subgroups.

The spring rot disease on Japanese zoysia grass caused by AG-D subgroup III initially was considered to be *Rhizoctonia* patch disease caused by AG-D subgroup I.

Table 8. Percentage of sequence similarity among isolates of unknown (UN), binucleate *Rhizoctonia* anastomosis group (AG)-D I and II, and *Rhizoctonia solani* AG 2-2 LP^z

Isolate	UN	DI	DII	LP
UN	94.9–99.7	89.5–92.6	82.4–87.8	71.4–74.7
DI	...	96.0–99.5	84.2–88.7	72.8–74.4
DII	...	...	93.5–94.7	75.2–75.8
LP	...	...	...	100

^zDI = *Rhizoctonia* AG-D I, DII = *Rhizoctonia* AG-D II, and LP = *Rhizoctonia solani* AG 2-2 LP.

Fig. 2. Phylogenetic distance tree, constructed by the neighbor-joining method, to compare the nearly complete internal transcribed spacer (ITS) region of ribosomal DNA genes sequence of unknown (UN) isolates with *Rhizoctonia* anastomosis group (AG)-D I and II. Two isolates of *Rhizoctonia solani* AG 2-2 LP were used as an outgroup. The numbers on the branches are confidence values obtained for 1,000 bootstrap replicates (only values above 70% are shown). AGs and subgroups of each isolate are shown in right column. Bar represents a phylogenetic distance of 2%.

The major diseases on zoysia grasses in spring are gray snow mold caused by *Typhula* spp. (15) and pink snow mold caused by *Microdochium nivale* (15). In turfgrass, the symptoms of spring rot are clearly different from those of gray and pink snow molds but similar to those of *Rhizoctonia* patch. When we carefully observed the symptoms of spring rot, several differences were found; discoloration was reddish brown, the shape of the patch was more irregular than *Rhizoctonia* patch disease, and severe symptoms persisted later into the season. Based on results of the greenhouse experiment, the symptoms induced by AG-D III isolates were easily distinguishable from those induced by AG-D I and II. AG-D III isolates were significantly less virulent than those of other AG-D subgroups. Therefore, the “sheath rot” disease caused by AG-D III could be recognized as a new disease on Japanese zoysia grass. However, because spring rot and *Rhizoctonia* patch diseases can occur in the same season, we should diagnose the disease symptoms carefully. Under natural conditions, it is very difficult to distinguish between the diseases based on symptoms alone. Therefore, rapid and accurate methods such as molecular techniques are required for diagnosis of these turfgrass pathogens.

All AG-D III isolates were obtained from three golf courses in Shizuoka prefecture that are 4 to 5 km apart from each other. Sheath rot disease occurs from mid-through late spring and there is no relationship with humidity and rain. The temperature of all three golf courses ranges from approximately 11 to 20°C during this season. Even though the reported occurrence of sheath rot diseases caused by AG-D III is limited to golf courses in close

proximity, many golf courses all over the world share a similar climate. Therefore the sheath rot disease caused by AG-D III may not be a local disease and may occur on other golf courses as well.

LITERATURE CITED

- Boysen, M., Borja, M., Delmont, C., Salazar, O., and Rubio, V. 1996. Identification at strain level *Rhizoctonia solani* AG 4 isolates by different sequence of asymmetric PCR products of the regions. *Curr. Genet.* 29:174-181.
- Burpee, L. 1980. *Rhizoctonia cerealis* causes yellow patch of turfgrass. *Plant Dis.* 64:1114-1116.
- Burpee, L., Sanders, P. L., Cole, H., and Sherwood, R. T. 1980. Anastomosis groups among isolates of *Ceratobasidium cornigerum* and related fungi. *Mycologia* 72:689-701.
- Carling, D. E., and Leiner, R. H. 1987. Categorization of anastomosis interactions that occur between isolates of *Rhizoctonia solani*. (Abstr.) *Phytopathology* 77:1777.
- Cubeta, M. A., Echandi, E., Abemathy, T., and Vilgalys, R. 1991. Characterization of anastomosis groups of binucleate *Rhizoctonia* species using restriction analysis of amplified ribosomal RNA genes. *Mol. Plant Pathol.* 81:1395-1400.
- Damaj, M., Jabaji-Hare S., and Charest, P. M. 1993. Isozyme variation and genetic relatedness in binucleate *Rhizoctonia* species. *Phytopathology* 83:864-867.
- Gonzalez, D., Carling, D. E., Kuninaga, S., Vilgalys, R., and Cubeta, M. A. 2001. Ribosomal DNA systematics of *Ceratobasidium* and *Thanatephorus* with *Rhizoctonia* anamorphs. *Mycologia* 93:1138-1150.
- Hyakumachi, M., Priyatomojo, A., Kubota, M., and Fukui, H. 2005. New anastomosis groups, AG-T and AG-U, of binucleate *Rhizoctonia* spp. causing root and stem rot of cut-flower and miniature roses. *Phytopathology* 95:784-792.
- Justesen, A. F., Yohalem, D., Bay, A., and Nicolaisen, M. 2003. Genetic diversity in potato field populations of *Thanatephorus cucumeris* AG-3, revealed by ITS polymorphism and RAPD markers. *Mycol. Res.* 107:1323-1331.
- Lipps, R. E., and Herr, L. J. 1982. Etiology of *Rhizoctonia cerealis* in sharp eyespot of wheat. *Phytopathology* 72:1574-1577.
- Ogoshi, A., Oniki, M., Araki, T., and Ui, T. 1983. Anastomosis groups of binucleate *Rhizoctonia* in Japan and North America and their perfect states. *Trans. Mycol. Soc. Jpn.* 24:79-87.
- Ogoshi, A., Oniki, M., Sakai, R., and Ui, T. 1979. Anastomosis grouping among isolates of binucleate *Rhizoctonia*. *Trans. Mycol. Soc. Jpn.* 20:33-39.
- Oniki, M., Ogoshi, A., and Araki, T. 1986. *Ceratobasidium strarie*, *C. cornigerum*, and *C. gramineum*, the teleomorph of the pathogenic binucleate *Rhizoctonia* fungi from gramineous plants. *Trans. Mycol. Soc. Jpn.* 27:147-158.
- Salazar, O., Julian, M. C., Hyakumachi, M., and Rubio, V. 2000. Phylogenetic grouping of cultural types of *Rhizoctonia solani* AG 2-2 based on specific rDNA-ITS sequences. *Mycologia* 92:505-509.
- Smith, J. D., Jackson, N., and Woolhouse, A. R. 1989. *Fungal Diseases of Amenity Turf Grasses*. E. & F. N. Spon, New York.
- Sneh, B., Burpee, B., and Ogoshi, A. 1991. Identification of *Rhizoctonia* Species. American Phytopathological Society Press Inc., St Paul, MN.
- Tanaka, A., Kitabayashi, H., Tani, T., and Ogoshi, A. 1994. A pathogen causing patch so-called ‘elephant footprint’ on zoysia grasses. (Abstr.) *Ann. Phytopathol. Soc. Jpn.* 60:344. (In Japanese)
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 24:4876-4882.
- Toda, T., Hyakumachi, M., Suga, H., Kageyama, K., Tanaka, A., and Tani, T. 1999. Differentiation of *Rhizoctonia* AG-D isolates from turfgrass into subgroups I and II based on rDNA and RAPD analysis. *Eur. J. Plant Pathol.* 105:835-846.
- Toda, T., Mghalu, J. M., Priyatomojo, A., and Hyakumachi, M. 2004. Comparison of sequences for the internal transcribed spacer region in *Rhizoctonia solani* AG 1-ID and other subgroups of AG 1. *J. Gen. Plant Pathol.* 70:270-272.