Characterization of Anastomosis Groups of Binucleate *Rhizoctonia* Species Using Restriction Analysis of an Amplified Ribosomal RNA Gene

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ABSTRACT

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Seven U.S. and 16 Japanese binucleate *Rhizoctonia* anastomosis tester isolates, representing 21 different anastomosis groups, were characterized by restriction analysis of a ribosomal RNA (rRNA) gene. Genomic DNA was extracted from each isolate and a region of DNA coding for a portion of the 25S rRNA (rDNA) was amplified using the polymerase chain reaction. Five tester isolates (CAG1, AGF, AGI, AGJ, and AGK) produced either two or three bands ranging from 1.4 to 1.8 kilobases (kb), whereas five other tester isolates (CAG5, AGBa, AGC, AGD, and AGH) produced a single, 1.8-kb fragment. The remaining 13 tester isolates produced a single, 1.4-kb fragment. Amplified rDNA from 64 additional binucleate isolates produced either a single 1.4- or 1.8-kb fragment or a combination of both fragments. Amplified rDNA was isolated and digested with eight restriction enzymes to determine restriction fragment length polymorphisms (RFLPs). RFLPs of CAG2, AGA, and AGBo were identical with all enzymes tested. Also, RFLPs of CAG6, CAG7, and AGE were identical or nearly identical with all enzymes tested. The RFLP patterns of CAG1 and AGD were also similar with all enzymes tested, and were distinct from all other anastomosis groups. Restriction analysis of nuclear encoded rDNA with four enzymes (*Hha*I, *Hpa*II, *Sau*3AI and *Taq*I), allowed the separation of 13 of 21 anastomosis groups of binucleate *Rhizoctonia* spp. into distinct groups and were found to be consistent with prior groupings based on hyphal anastomosis.

Binucleate *Rhizoctonia* spp. represent a diverse group of organisms that have been isolated from soils and plants throughout the world. These fungi can exist saprophytically in soil and plant debris or may establish parasitic relationships with plants.

More than 20 species of binucleate *Rhizoctonia* have been described (4) and have been previously classified according to their ecology (11), enzymology (8,19), host range specificity (9), morphology, and hyphal anastomosis (1,12). Because the morphological characteristics of the anamorph and teleomorph of binucleate *Rhizoctonia* spp. are very similar, reliable identification is often difficult. Because many of these fungi often exist as undifferentiated mycelium, hyphal anastomosis has been an important criterion for characterizing and identifying binucleate *Rhizoctonia* spp. At least 24 anastomosis groups (AGs) have been described for binucleate *Rhizoctonia* spp. by Burpee et al (CAG1–CAG7) and Ogoshi et al (AGA–AGQ) (1,11,12).

In another similar species, *Rhizoctonia solani*, molecular studies have demonstrated major genetic differences that are present among isolates belonging to different AGs. In *R. solani*, isolates within a single AG (i.e., intraspecific group or subgroup) share a high degree of DNA base sequence complementarity among their genomes (7,20). Isolates belonging to different AGs of *R. solani* have DNA sequence complementarity values that are less than 30%, indicating genetic nonidentity. Additional evidence based on ribosomal DNA (rDNA) restriction fragment length polymorphisms (RFLPs) also indicate that different AGs in *R. solani* are genetically divergent (21).

Although hyphal anastomosis is very useful in characterizing and identifying binucleate *Rhizoctonia* spp., some isolates do not anastomose with any of the tester strains and therefore cannot be assigned to a specific anastomosis group. Also, organisms isolated from soil provide no host information that could reduce the potential number of tester strains needed for identification. In such instances, the need for extensive anastomosis testing may require considerable time and effort.

Because of these difficulties, we sought to develop a more direct method for genetic identification and characterization of binucleate *Rhizoctonia* spp. Our approach, described in this paper, is based on the use of the polymerase chain reaction (PCR) for amplification, followed by restriction analysis of nuclear-encoded rDNA from different isolates of binucleate *Rhizoctonia* spp. Patterns of rDNA RFLPs revealed by this approach provide a convenient method for grouping isolates of binucleate *Rhizoctonia* spp., which is also consistent with prior grouping based on hyphal anastomosis. A preliminary report of this work has been published (2).

MATERIALS AND METHODS

Isolate selection and maintenance. Eighty-seven isolates of binucleate *Rhizoctonia* spp., including 23 tester isolates representing 21 different AGs, were used in this study (Table 1). Isolates were previously assigned to a specific AG by pairing with the tester strain from each AG following the procedure of Kronland and Stanghellini (6). Isolates were purified by transferring a hyphal tip to potato-dextrose agar (PDA) amended with 50 μg/ml each of streptomycin sulfate and tetracyline and were stored in the dark at 4 C on dried oat kernels and on PDA slants covered with glycerol. A 5-mm diameter plug from the edge of a 48-h-old culture of each isolate was excised, placed in 25 ml of sterile potato-dextrose broth (Difco, Detroit, MI) and incubated 4 days in the dark at 25 C. Mycelium was harvested by filtration, lyophilized, and prepared for DNA extraction.

DNA extraction and purification. Genomic DNA was extracted from each isolate following a modification of the procedure described by Vilgalys and Hester (22). Briefly, the extraction procedure involved suspending each sample (ground, lyophilized mycelium) in extraction buffer, extraction twice with phenol-chloroform-isoamyl alcohol (25:24:1, v/v), treatment with RNAase (1mg/ml, DNase-free, Sigma, St. Louis, MO), extraction twice in 250 μl of chloroform/isoamyl alcohol (24:1), resuspension in 3 M sodium acetate (pH 5.0), and precipitation for 24 h in 2 vol of 95% EtOH at −20 C. The genomic DNA pellet was washed once with 80% EtOH, dried under vacuum, and dissolved in TE buffer (10 mM Tris-HCl, 1 mM Na_2EDTA, pH 8.0).

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The extracted DNA was purified from each sample by electrophoresis in a 0.8% low melting agarose gel (Sea Plaque, FMC Corp., Chicago, IL) in TBE buffer (100 mM Tris-HCl, 20 mM Na_2EDTA, 100 mM Boric acid, pH 8.3). Agarose gels were stained with ethidium bromide and examined with UV (254 nm). The band representing the high molecular weight, genomic DNA (~100 ng) was excised, trimmed with a scalpel to remove excess agarose, and stored in sterile microfuge tubes at −20 C until further use.

DNA amplification with the PCR. Each sample was diluted to a concentration of 0.1 ng of DNA per microliter with sterile distilled water and incubated at 65 C for 30 min to dissolve the agarose. Two oligonucleotide primers LR0R (5′-ACCCGCTGAACTTAAGC-3′) and LR7 (5′-TACTACCACCAAGATCT-3′) were synthesized (22) with standard methods (Applied Biosystems 380B synthesizer, Foster City, CA) and used to amplify a portion of the rDNA repeat homologous to positions 17–1,448 within the *Saccharomyces cerevisiae* 25S rRNA. Both of these primers are complementary to highly conserved regions within the rDNA repeat and have been successfully used for amplification of rDNA from fungi, plants, and animals with PCR (22).

PCR reactions were set up using Amplitaq DNA polymerase (U.S. Biochemicals, Cleveland, OH) in 50 μl vol using buffer conditions recommended by the manufacturer. Thirty PCR cycles were conducted on an automated thermocycler (Perkin-Elmer-Cetus, Norwalk, CT). The following parameters were used: 1 min denaturation step at 94 C, annealing at 50 C for 45 sec, 50–72 C gradual increase for 1 min, and primer extension at 72 C for 1 min. To avoid possible contamination, PCR experiments were conducted in accordance with the stringent procedures described by White et al (23,24). Control tubes without DNA template were included in each experiment (negative control).

After amplification, a 3-μl aliquot from each sample was subjected to electrophoresis. Bacteriophage (λ) DNA digested with *Eco*RI/*Hin*dIII was used to determine the size of PCR products. Gels were stained with ethidium bromide and photographed over a UV transilluminator to record results.

Restriction digestion of PCR products. Amplified PCR products were extracted in chloroform/isoamyl alcohol (24:1), precipitated in 3 M Na acetate and 95% EtOH at −20 C for 24 h, washed with 80% EtOH, dried under vacuum, and resuspended in TE buffer. For restriction analysis, each PCR product was divided into equal aliquots and digested with either *Ava*II,

TABLE 1. Anastomosis group, origin and source of isolates of binucleate *Rhizoctonia* spp.

Isolate[a]	Anastomosis group	Origin	Source[b]	Isolate[a]	Anastomosis group	Origin	Source[b]
SN-2	AGA	Soil	10	*FA52909	AGK	Unknown	9
*C-662	AGA	Soil	9	BN2-2	AGK	*Glycine max*	8
R-13	AGA	*Fragaria × ananassa*	7	BN2-3	AGK	*G. max*	8
R-214	AGA	*F. × ananassa*	7	BN4	AGK	*G. max*	8
R-190	AGA	*F. × ananassa*	7	BN26A-4	AGK	*G. max*	8
S-18	AGA	*F. × ananassa*	7	BN12T28	AGK	*G. max*	8
JF-1N2-4	AGA	*F. × ananassa*	7	*FK02-1	AGL	Soil	9
S-24	AGA	*F. × ananassa*	7	*FK06-2	AGO	Soil	9
SG-1	AGA	*F. × ananassa*	7	*C-620	AGQ	Soil	4
*C-460	AGBa	*Oryza sativa*	9	*BN1	CAG1	*Agrostis* sp.	2
*C-455	AGBb	*O. sativa*	9	BN501	CAG1	*Agrostis* sp.	2
C-157	AGBb	*O. sativa*	9	BN502	CAG1	*Agrostis* sp.	2
ROS-1	AGBb	*O. sativa*	3	BN503	CAG1	*Agrostis* sp.	2
SIR-2	AGBo	*Ipomoea batatas*	9	BN504	CAG1	*Agrostis* sp.	2
*70B	AGC	Soil	9	BN505	CAG1	*Triticum aestivum*	2
AOO1C	AGC	Soil	9	BN519	CAG1	*Festuca* sp.	2
C-57	AGD	*Juncus effusus*	9	BN520	CAG1	*Festuca* sp.	2
C-73	AGD	Unknown	9	BN521	CAG1	*Festuca* sp.	2
*C-610	AGD	Unknown	10	BN523	CAG1	*Festuca* sp.	2
RH155	AGE	Unknown	9	*BN4	CAG2	Soil ATCC34969	2
*TM1-1	AGE	Soil	9	53914	CAG2	Soil	4
F-18	AGE	*Linum flavum*	9	BN4	CAG2	*Arachis hypogaea*	4
SOYCUR3	AGE	*Glycine max*	11	BN4-T	CAG2	Soil	4
*SIR-1	AGF	*Ipomoea batatas*	9	Q-III	CAG2	*Rhododendron* sp.	5
AHC9	AGG	Unknown	9	*BN31	CAG3	*Arachis hypogaea*	2
*C-653	AGG	Unknown	9	BNE-5	CAG3	*Festuca rubra*	6
BN-160	AGG	*Festuca rubra*	11	BN6	CAG3	*Ilex crenata*	1
R-95	AGG	*F. × ananassa*	7	WS905	CAG3	*Fragaria* spp.	11
232-CG	AGG	Pine bark medium	5	*BN38	CAG4	*Glycine max*	2
JF-3N1-1	AGG	*F. × ananassa*	7	511-45	CAG4	Soil	4
JF-3S4-3	AGG	*F. × ananassa*	7	539113	CAG4	Soil	4
JF-1S1-5	AGG	*F. × ananassa*	7	53112	CAG4	*Phaseolus vulgaris*	4
R-151	AGG	*F. × ananassa*	7	*BN37	CAG5	*Cucumis sativum*	2
JF-3N2-4	AGG	*F. × ananassa*	7	149IEB	CAG5	*Phaseolus vulgaris*	4
NCSTR1	AGG	*F. × ananassa*	11	580-111	CAG5	Soil	4
S-39	AGG	*F. × ananassa*	7	589	CAG5	*Ipomoea batatis*	4
*STC-9	AGH	Soil	9	BN74	CAG6	*Erigeron* sp. ATCC13247	2
*AV-2	AGI	*Artemesia* sp.	9	*BN22	CAG7	*Pittosporum* sp. FL FTCC585	2
JF-3S4-4	AGI	*F. × ananassa*	7	BN21A	CAG7	*Rhaphiolepsis* sp.	5
JF-1S5-1	AGI	*F. × ananassa*	7	BN21F	CAG7	*Rhaphiolepsis* sp.	5
JF-1S3-3	AGI	*F. × ananassa*	7	BN-29	CAG7	*Begonia* sp.	5
JF-1N4-3	AGI	*F. × ananassa*	7	BN8	CAG7	*Rhododendron* sp.	1
*184	AGJ	Unknown	8	BN-22	CAG7	*Juniperus chinensis*	5
AC1	AGK	*Allium cepa*	9				

[a]Asterisks represent seven U.S. (Burpee et al [1]) and 16 Japanese (Ogoshi et al [11,12]) anastomosis tester isolates of binucleate *Rhizoctonia* spp.
[b]Suppliers of isolates of binucleate *Rhizoctonia* spp. are designated by numbers; 1 = D. M. Benson; 2 = L. L. Burpee, University of Georgia, Griffin, GA; 3 = R. D. Cartwright, University of California, Davis, CA; 4 = S. J. Hare, University of Laval, Quebec, Canada; 5 = T. A. Law; 6 = L. T. Lucas; 7 = R. B. Martin, Clemson University, Florence, SC; 8 = B. D. Nelson, North Dakota State University; Fargo, ND; 9 = A. Ogoshi, Hokkaido University, Sapporo, Japan; and 10 = K. Yokoyama, Obihira University, Hokkaido, Japan; and 11 = authors of this paper. The suppliers lacking affiliations (1, 5 and 6) are at North Carolina State University, Raleigh.

*Hae*III, *Hha*I, *Hin*fI, *Hpa*II, *Sau*3AI, *Sau*96I, or *Taq*I following the manufacturer's directions (Promega, Madison, WI). After digestion, samples were subjected to electrophoresis in a 4% agarose gel (NuSieve, FMC Bioproducts). Bacteriophage (ϕ-X174) DNA digested with *Hae*III was used as a molecular weight standard to determine size of restriction fragments. Gels were stained with ethidium bromide and photographed over a UV transilluminator to record results. All experiments were repeated at least three times.

Analysis of RFLP data. The variability among restriction phenotypes of each anastomosis tester isolate was assessed by ordination with nonmetric multi-dimensional scaling (MDS) using the NTSYS-PC computer package (16). A pairwise phenotypic distance measurement based on the Dice coefficient (18) was calculated using the equation $1 - (2N_{ab}/[N_a + N_b])$ in which N_{ab} equals the number of common restriction fragments shared between two isolates, and $(N_a + N_b)$ equals the total number of fragments present in each isolate A and B. The resulting matrix was used to generate a two-dimensional plot of the 23 anastomosis tester isolates with NTSYS. MDS provides a graphic representation of phenotypic relationships among anastomosis tester isolates of binucleate *Rhizoctonia* spp. and was selected over other ordination analysis procedures (e.g., principal component analysis) because it provides a truer representation of pairwise differences among the binucleate isolates. Also, MDS provides a better linear relationship of plotted data points and the actual distance matrix (18).

RESULTS

Amplification of rDNA. Two sizes of rDNA products were obtained following amplification with PCR (Fig. 1). Five tester isolates (CAG1, AGF, AGI, AGJ, and AGK) each produced two bands corresponding to 1.4 and 1.8 kilobases (kb). Five other tester isolates (CAG5, AGBa, AGC, AGD, and AGH) each produced one single 1.8-kb fragment, whereas the remaining 13 tester isolates produced a 1.4-kb fragment. Of the 64 other binucleate *Rhizoctonia* spp. isolates tested, 15 were characterized by producing both a 1.4- and 1.8-kb fragment, 10 by producing a single 1.8-kb fragment and 39 by producing a single 1.4-kb fragment. Occasionally, isolates that produced the 1.4- and 1.8-kb fragments, also produced an additional fragment of 1.6 kb (Fig. 1, isolates AGF and AGI). No PCR products were obtained in any of the control reactions omitting template DNA.

Restriction analysis of amplified rDNA. RFLPs obtained after digestion of amplified rDNA differed depending on the restriction enzyme used. The enzymes, *Hha*I, *Hpa*II, *Sau*3AI, and *Taq*I were the most useful in separating the different anastomosis groups of binucleate *Rhizoctonia* spp. After digestion of amplified rDNA with *Hpa*II, all tester isolates produced a common 290-base pair (bp) fragment (Fig. 2). All tester isolates except CAG5, AGBa, AGC, and AGH also had a common 500-bp fragment. The AGC anastomosis tester isolate had two unique restriction fragments (fragments not visible in Fig. 2) of 110 and 310 bp. The tester isolates, CAG2, AGA, AGBo, AGK, AGL, and AGO had identical restriction patterns with four fragments each, while the CAG3, CAG6, CAG7, and AGE tester isolates also were identical to each other (Table 2). Also, the AGF, AGI, and AGJ tester isolates produced identical restriction patterns. Although infrequently observed, some partial digestion of rDNA is evident in Figure 2, but the resulting restriction patterns are clearly visible and were not changed.

All anastomosis tester isolates produced common 260- and 550-bp fragments after digestion of rDNA with *Taq*I, except for CAG5 and isolate C-73 (AGD), which lacked the 550-bp fragment (Fig. 3). The CAG1, AGD, and C-73 isolates produced a unique, but not identical, restriction pattern and had a unique 80-bp fragment. The AGG anastomosis tester isolate produced a unique restriction pattern not possessed by any other tester isolates (Table 2). The restriction patterns for additional isolates of binucleate *Rhizoctonia* spp. belonging to specific anastomosis groups were similar to their concomitant tester isolates. For example, the BNE-5 isolate, previously identified as belonging to CAG3, produced an identical restriction pattern to the CAG3 tester isolate (Fig. 3). The isolates BN-160, 232-CG, JF-3N1-1, JF-3S4-3, and R-95 also produced identical restriction patterns to their corresponding AGG tester isolates (Fig. 3). The isolates R-13 and Q-III previously identified as belonging to AGA or CAG2, respectively, produced a similar restriction pattern to their corresponding anastomosis tester isolates, but were not identical (Fig. 3). The restriction patterns for the SOYCUR3 (previously identified as belonging to AGE), CAG4, CAG6, CAG7, AGE, and AGO were identical to the CAG2 and AGA tester isolates (Fig. 3).

After digestion of amplified rDNA with *Sau*3AI, all anastomosis tester isolates produced a common 110-bp fragment (Table 2). The AGI and AGJ anastomosis tester isolates each had two unique fragments of 140 and 280 bp, whereas the AGH and AGBa anastomosis tester isolates had unique 400- and 900-bp fragments, respectively (Table 2).

After digestion of amplified rDNA with *Hha*I, the AGC anastomosis tester isolate produced a unique 130-bp fragment. All anastomosis tester isolates produced common 160- and 230-bp fragments (Table 2).

The restriction enzymes *Ava*II, *Hae*III, *Hin*fI, and *Sau*96AI were not useful in separating the different AGs of binucleate *Rhizoctonia* spp. However, the AGH tester isolate produced a unique 350-bp fragment after digestion of rDNA with *Hin*fI, and the CAG5 anastomosis tester isolate produced a unique 800-bp fragment after digestion of rDNA with *Hae*III (Table 2). All anastomosis tester isolates of binucleate *Rhizoctonia* spp. produced a common 220-bp fragment after digestion with *Hae*III, whereas all isolates produced common 130-, 170-, 200-, and 280-bp fragments after digestion with *Hin*fI (Table 2).

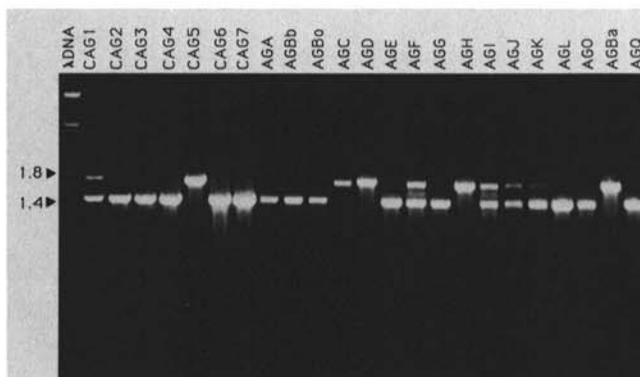

Fig. 1. Amplified rDNA of seven U.S. and 16 Japanese anastomosis tester isolates of binucleate *Rhizoctonia* spp. after electrophoresis in 0.8% Sea Plaque agarose (FMC, Chicago, IL) at 100 V for 1 h and staining with ethidium bromide. Size markers (kb) are indicated on the left.

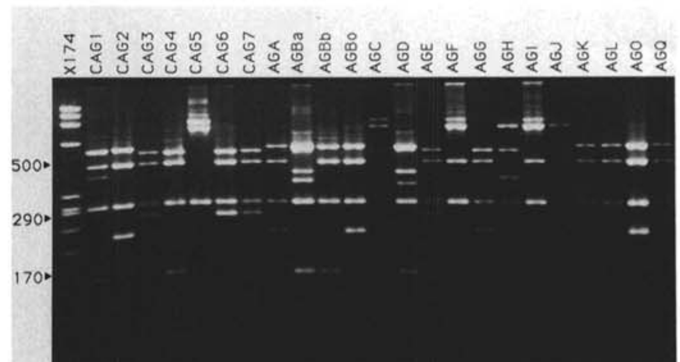

Fig. 2. Amplified rDNA of seven U.S. and 16 Japanese anastomosis tester isolates of binucleate *Rhizoctonia* spp. digested with *Hpa*II after electrophoresis in 4.0% NuSieve agarose (FMC, Chicago, IL) at 100 V for 4 h and staining with ethidium bromide. Size markers (bp) are indicated on the left.

The phenotypic relationships among the binucleate *Rhizoctonia* spp. anastomosis tester isolates based on results from the MDS analysis are presented in Table 3. Two major groups were resolved based on the size of the amplified 25S rDNA products obtained after amplification with PCR. Binucleate *Rhizoctonia* spp. anastomosis tester isolates with either a 1.8-kb or both a 1.8- and 1.4-kb fragment were all grouped together on the right hand portion of the plot, whereas isolates that produced a single 1.4-kb fragment were grouped on the left hand portion of the plot (Fig. 4). Isolates that produced identical or near identical rDNA RFLPs with all enzymes are indicated by their distinct position on the MDS plot (Fig. 4). For example, CAG1 and AGD; AGK, CAG2, AGA, and AGBo (the last three with identical RFLPs); AGE, CAG6, and CAG7 (the last two with identical RFLPs) are indicated by the proximity within the MDS plot. In addition, several anastomosis tester isolates were found to be similar or intermediate among the previously described binucleate *Rhizoctonia* spp. anastomosis tester isolates representing specific groups.

Based on combined evidence of RFLP variation, seven putative groups of binucleate *Rhizoctonia* spp. could be identified based on their rDNA RFLPs (Table 3). In the MDS plot (Fig. 4) some of these groups, such as AGBa, AGBb, AGG, and AGQ, are clearly distinguished from other AGs based on their highly dissimilar RFLPs. Groups such as AGC and AGF share more similar RFLPs with each other (55%) than other anastomosis tester isolates in Group I in Table 3 (CAG5, AGH, AGI, and AGJ) but are separated by approximately 0.9 and 0.3 units in the second dimension and first dimension, respectively, in Figure 4. Although this discrepancy is evident in the MDS plot, AGC and AGF still have consistent differences in their RFLPs that allow them to be identified.

Two other nonbinucleate *Rhizoctonia* spp. isolates (*R. solani* AG-4 and *R. zeae* WAG-Z) were also examined. Both isolates produced a single 1.4-kb fragment after amplification with PCR,

TABLE 2. Restriction fragment sizes (bp) of anastomosis tester and other isolates of binucleate *Rhizoctonia* spp. belonging to a specific anastomosis group (AG), after digestion of rDNA with *Hpa*II, *Taq*I, *Hha*I, *Sau*3AI, *Hae*III and *Hin*fI

AG[a]	Number of isolates	Restriction enzyme					
		*Hpa*II[b]	*Taq*I	*Hha*I[c]	*Sau*3AI[d]	*Hae*III[e]	*Hin*fI[f]
AGA	9	220,500, 550	260,550,690	180,340, 480	550,800	150,450, 550	100,150, 380
AGBa	1	75,170,330, 440,550	240,260,380,450, 610,800	180,480, 800	800,900	450,550, 700	250,310, 600
AGBb	3	75,170,500, 550	240,260,380,550	180,340, 480	550,800	450,550	310,380
AGBo	1	220,500,550	260,550,690	180,340, 480	550,800	150,450, 550	100,150, 380
AGC	2	110*,310*, 550,700	60,260,280,310, 800	130,180, 680,800	550,990	150,550 700	100,310, 380,600
AGD	3	75,170,330, 400,550	80,200,240,280, 450,550,690	180,680, 800	200,550, 990	250,330, 550,700	100,310, 380,600
AGE	4	260,500,550	260,550,690	340,480	550,800	180,450 550	100,310, 380
AGF	1	500,700,900	260,550,690,800	340,480, 800	250,550, 800	450,550, 700	100,310, 380,600
AGG	12	220,500,550	260,280,310,550	340,480	200,550, 600	150,450, 550	100,310, 380
AGH	1	350,550,700	110,150,280,310, 800	480,680	200,400, 600,650	250,700, 800	100,310, 350,380
AGI	5	500,700,900	260,280,310,550, 690	340,480, 680	140,280, 330,550,	450,700, 800	100,310, 380,600
AGJ	1	500,700,900	260,280,310,550, 690	340,480, 680	140,280, 550,650	450,550 700	310,380, 600
AGK	7	220,500,550	260,550,690	180,340, 480,990	550,800	150,450 550	380,510
AGL	1	220,500,550	260,550,690	340,680	550,680	150,450 550	100,250, 310
AGO	1	220,500,550	260,550,690	340,680	330,550 800	150,450, 550	100,250 310
AGQ	1	500,700	220,260,420,550	180,340 480	550,680	NT	NT
CAG1	10	75,170,400 500,550	80,200,240,260, 280,310,550	180,340 480,990	200,550, 600,990	450,550, 700	100,310, 380,600
CAG2	5	220,500,550	260,550,690	180,340 480	550,800	150,450, 550	100,380
CAG3	4	260,500,550	240,260,380,550	340,680	550,800	180,450, 550	100,250, 310
CAG4	4	90*,170,500, 550	260,550,690	340,680	550,800	250,450, 550	100,310, 380
CAG5	4	700,900	260,690,800	680,800	250,750 800	700,800	310,380, 600
CAG6	1	260,500,550	260,550,690	340,680	550,800	180,450, 550	100,310, 380
CAG7	6	260,500,550,	260,550,690	340,680	550,800	180,450, 550	100,310, 380

[a]Represent seven U.S. (Burpee [1]) and 16 Japanese (Ogoshi et al [11,12]) anastomosis tester isolates of binucleate *Rhizoctonia* spp. NT = not tested.

[b]All isolates produced a common 290-bp fragment after digestion with *Hpa*II. Fragments represented by an asterisk are not visible in Figure 2.

[c]All isolates produced common 160- and 230-bp fragments after digestion with *Hha*I.

[d]All isolates produced a common 110-bp fragment after digestion with *Sau*3AI.

[e]All isolates produced a common 220-bp fragment after digestion with *Hae*III.

[f]All isolates produced common 130-, 170-, 200- and 280-bp fragments after digestion with *Hin*fI.

and *R. zeae* produced a unique RFLP pattern after digestion with all enzymes tested and was not shared with any other binucleate *Rhizoctonia* anastomosis tester isolate (data not shown). However, the *R. solani* isolate produced a similar RFLP pattern with certain binucleate *Rhizoctonia* spp. anastomosis tester isolates after digestion with *Hha*I, *Hpa*II, and *Taq*I (data not shown).

DISCUSSION

To date, 26 anastomosis tester isolates of binucleate *Rhizoctonia* spp. have been identified (seven American and 19 Japanese), representing 24 different groups. The AGM, AGN, and AGP anastomosis tester isolates (11,12) were not available to us and therefore were not assayed in this study.

Binucleate *Rhizoctonia* spp. isolates CAG2, AGA, and AGBo produced identical restriction patterns with all enzymes tested; as did CAG6 and CAG7. The restriction phenotypes for AGE

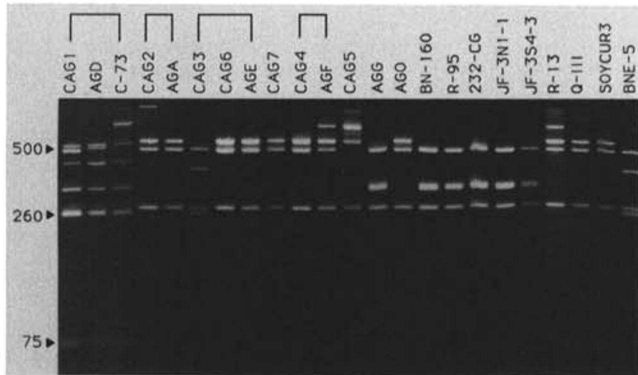

Fig. 3. Amplified rDNA of selected anastomosis tester and other binucleate *Rhizoctonia* spp. isolates digested with *Taq*I after electrophoresis in 4.0% NuSieve agarose (FMC, Chicago, IL) at 100 V for 4 h and staining with ethidium bromide. Size markers (bp) are indicated on the left. Brackets represent tester isolates capable of hyphal anastomosis according to Ogoshi et al (11,12).

TABLE 3. Groups of binucleate *Rhizoctonia* spp. based on rDNA restriction fragment length polymorphisms

Group[a]	Fragment size[b] (kb)	Anastomosis group represented[c]	Teleomorph[d]
I	1.8/1.4	CAG5, AGC, AGF AGH, AGI[e], AGJ	*Ceratobasidium sp.*
II	1.8/1.4	CAG1, AGD	*C. cerealis*
III	1.8	AGBa	*C. setariae*
IV	1.4	AGG	*Ceratobasidium sp.*
V	1.4	AGBb	*C. oryzae-sativae*
VI	1.4	AGQ	
VII	1.4	CAG2, AGA, AGBo AGL, AGO, AGK AGE, CAG6, CAG7 CAG3, CAG4	*C. cornigerum*[f]

[a]Groups are based on combined data for the four restriction enzymes *Hha*, *Hpa*II, *Sau*3AI, and *Taq*I. A similarity coefficient (Dice coefficient [18]) was used to estimate pairwise relatedness among different binucleate *Rhizoctonia* spp. anastomosis tester isolates. Isolates in group II and VII had >85% similiarity, while isolates in group I were heterogeneous with similarity values ranging from 40 to 95%.

[b]Fragment size after amplification of a portion of rDNA located within the 5′ - end of 25S rRNA coding region with polymerase chain reaction.

[c]Anastomosis tester isolates of Burpee et al (1) and Ogoshi et al (11,12).

[d]Proposed teleomorph (if known).

[e]AGI isolates have not formed a teleomorph.

[f]*C. cornigerum* is the proposed teleomorph for CAG2, AGA, and AGBo. All other isolates in this group, except AGK, have a *Ceratobasidium* teleomorph without species designation. AGK isolates have not formed a teleomorph and also produced an additional 1.8-kb fragment after polymerase chain reaction amplification.

and AGK were nearly identical to CAG6/CAG7 and CAG2/AGA, respectively, with all enzymes tested. The restriction phenotypes for CAG1 and AGD were very similar but not identical. These results support conclusions of Ogoshi et al (11,12) based on hyphal anastomosis concerning the relatedness of Japanese and American tester strains. However, this study revealed differences not found by Ogoshi et al (12). We found similar, but not identical, restriction patterns for CAG4 and AGF, a finding that differs from the results of Ogoshi et al (12), who found that CAG4 corresponded to AGF based on hyphal anastomosis behavior. Also, the restriction patterns for CAG3 and AGE were very similar, but were not identical.

After analysis of the different AGs, other isolates belonging to specific groups were tested to determine the consistency and repeatability of observed restriction patterns. Isolates belonging to AGG produced identical restriction patterns regardless of host or origin, whereas AGA, CAG2, and AGBo, which produced a common pattern, had a small amount of variability (usually one fragment). Although CAG1 and AGD isolates produced a unique and common pattern, there was considerable variability (more than one fragment) within the specific restriction pattern. This was also observed for AGI isolates.

After amplification of rDNA, two distinct size classes were observed for the anastomosis tester isolates. These bands may represent different length variants of the rRNA gene as have been found in *R. solani* (21). Occasionally after amplification of rDNA, we observed an intermediate band corresponding to 1.6 kb that may be the result of heteroduplex formation between DNA strands of different lengths. Further studies are necessary to determine the molecular basis of this phenomenon. We have never observed additional larger fragments within groups characterized by a single 1.4-kb fragment. Although we cannot explain the similarity of restriction patterns from isolates that produce different length variants of rDNA (e.g., AGD and CAG1), restriction patterns are consistent and repeatable.

Analysis of the organization and structure of genes that code for rRNA has also been used to determine genetic relatedness of other plant pathogenic fungi (5,21); however, the genetic relatedness of binucleate *Rhizoctonia* spp. has not been studied. On examination of different regions of the rRNA genes (5.8S, 17S, and 25S) of binucleate *Rhizoctonia* spp., we determined that the LR0R–LR7 portion of the 25S region provided enough varability to separate many of the anastomosis tester isolates.

Only limited inferences on genetic relationships can be made from this study in the absence of other data (e.g., base sequence analysis), but several genetically distinct groups representing different species are apparent (Table 3). For example, CAG1 and AGD, which have been designated as *Rhizoctonia cerealis*, produce a *Ceratobasidium cereale* (syn. *C. graminearum*) teleomorph (9,13) and represent a distinct group. The B group, predominately

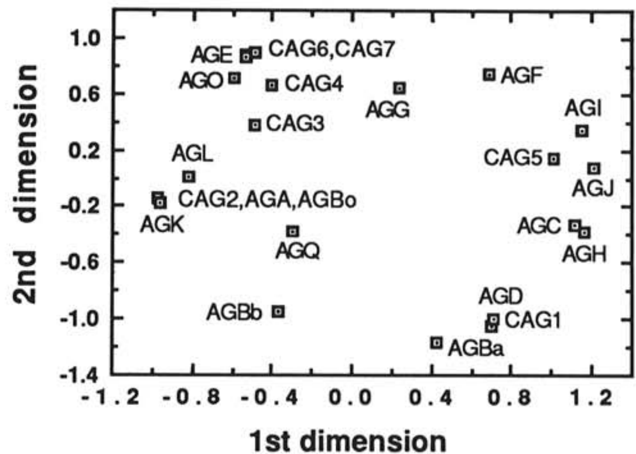

Fig. 4. Plot showing results of nonmetric multi-dimensional scaling analysis of the anastomosis tester isolates of binucleate *Rhizoctonia* spp. based on rDNA RFLPs.

rice pathogens, has been divided into three subgroups by Ogoshi et al (11,12). The AGBa, AGBb, and AGBo groups correspond to *Rhizoctonia fumigata* (teleomorph *C. setariae*), *Rhizoctonia oryzae-sativae* (teleomorph *C. oryzae-sativae*) and *C. cornigerum*, respectively (3). *C. setariae* and *C. oryzae-sativae* are morphologically similar and frequently anastomose. In our study, we found that the AGBa anastomosis tester isolate produced a 1.8-kb fragment after amplification of the rDNA, whereas the AGBb and AGBo isolates produced a 1.4-kb fragment. Although these three tester isolates had some common fragments after digestion of the rDNA, they were all distinct. The restriction pattern of AGBo was identical to AGA and CAG2, which also produce a *C. cornigerum* teleomorph (11). The testing of additional AGBo isolates may confirm this relationship; however, we have been unable to anastomose the AGBo tester isolate with either AGA or CAG2.

The phenetic relationships indicated in Figure 4 were based on the MDS analysis and probably represent an oversimplification of diversity in binucleate *Rhizoctonia* spp. Isolates in Group I (CAG5, AGC, AGF, AGH, AGI, and AGJ) in Table 3 produced either a single 1.8-kb or both a 1.4- and 1.8-kb fragment after amplification of rDNA and had similarity values ranging from 40 to 95%. The placement of these heterogeneous isolates into a single group also may be a conservative oversimplification. Further testing with additional enzymes may provide even greater resolution of the groups represented in Table 3. However, other binucleate *Rhizoctonia* isolates, as well as other *Rhizoctonia* spp. and their respective subgroups, should be assayed to determine the utility of this procedure for routine identification.

Because of difficulty inducing the teleomorph of binucleate *Rhizoctonia* spp., hyphal anastomosis is a useful and valuable tool for characterizing and identifying these isolates. Since its inception in the early 1930s for *R. solani* (17), the premise of the anastomosis concept was that isolates belonging to the same anastomosis group can recognize and fuse only with each other (14). Reports of isolates anastomosing with more than one anastomosis tester isolate and also losing their ability to anastomose (self and nonself) complicates characterization and identification of binucleate *Rhizoctonia* spp. (10,15). Some isolates may never have had the ability to anastomose, whereas others do not anastomose because of genetic mutation or imposed environmental or nutrient conditions. Perhaps the nuclear condition (either homokaryotic, dikaryotic, or heterokaryotic) influences the resulting hyphal interaction and concomitant anastomosis reaction.

Binucleate *Rhizoctonia* spp. represent a diverse group of organisms that are composed of many different species (1,3,9,11,13). Some of these species are important plant pathogens, whereas others are saprophytic species or species involved in mycorrhizal associations with orchids and other plants (4). Because of the complexity in identifying and characterizing isolates of binucleate *Rhizoctonia* spp., we have developed a method for classifying isolates into distinct groups using PCR for amplification of nuclear-encoded rDNA and analysis with four restriction enzymes. With this procedure, we can consistently differentiate 13 of the 21 anastomosis groups previously identified by Burpee et al (1) and Ogoshi et al (11,12). This method does not involve the use of radioactivity and can be used in conjunction with anastomosis and other specific characteristics to identify and characterize isolates of binucleate *Rhizoctonia* spp.

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