Isozyme Variation and Genetic Relatedness in Binucleate *Rhizoctonia* Species

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ABSTRACT

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Fifty isolates of binucleate *Rhizoctonia* representing 12 Japanese and five North American anastomosis groups (AGs) were characterized by isozyme electrophoretic patterns. Of the 23 enzyme systems screened, eight enzymes with a total of 63 phenotypes were applied to study the genetic relationship among the AGs. Hexokinase and malate dehydrogenase displayed the most polymorphic banding patterns. Cluster analysis of isozyme bands generated four distinct groups (I, II, III, and IV). Group I was represented by all AG-G isolates. Group II was subdivided into two subgroups (IIA and IIB). Subgroup IIA was made up of the cross-anastomosing groups AG-D/CAG-1 and AG-E/CAG-3, and subgroup IIB was made up of groups AG-F/CAG-4, AG-I, and CAG-5. Group III included isolates belonging to AG-K and CAG-2 with one isolate each of AG-A and AG-B(o). Group IV consisted mainly of AG-Bb isolates. AG-A, AG-Ba, AG-C, and AG-O isolates were placed in different groups and were considered genetically diverse. Isozyme groups of binucleate *Rhizoctonia* were consistent with prior groupings determined by hyphal anastomosis and by DNA restriction pattern analysis. The present study provides evidence that isozyme phenotypes are good indicators of genetic diversity among anastomosis groups of binucleate *Rhizoctonia* species.

Additional keywords: *Ceratobasidium* species, molecular differentiation, multivariate analysis, phylogeny.

Plant pathogenic species of binucleate *Rhizoctonia* (teleomorph: *Ceratobasidium*) occur worldwide, causing seedling damping-off, seed decay, foliage blights, and root, crown, and stem rotting of several important crops (13,15). They constitute a complex of isolate groups characterized by the binucleate state of their hyphal cells and share with *Rhizoctonia solani* Kühn common morphological features, such as nonsporulating, vegetative, and septate mycelia with a dolipore apparatus and monilioid cells that aggregate to form sclerotia (33). Earlier attempts to group the binucleate isolates according to their morphology (36,42), host range (30), and pathogenicity (9,20,31) have not been successful due to the great variations observed in these characters. Most investigators have resorted to classifying their isolates into distinct anastomosis groups (AGs) based on their affinities for hyphal fusion. To date, 17 Japanese AGs, designated as AG-A through AG-Q (27–30), and seven North American AGs, CAG-1 through CAG-7 (2), have been identified. Five out of the seven North American AGs have isolates that cross-anastomose with those of a corresponding Japanese group (CAG-1/AG-D, CAG-2/AG-A, CAG-3/AG-E, CAG-4/AG-F, and CAG-6/AG-E) (27–29). Although useful, anastomosis grouping provided little insight into the genetic diversity of these fungi.

Techniques in molecular biology like DNA/DNA hybridization and restriction fragment length polymorphism (RFLP) have proved useful in understanding the genetic relatedness among isolates of *Rhizoctonia solani* (14,46) and binucleate *Rhizoctonia* (5,45). Groups formed by DNA/DNA hybridization and by RFLP analysis of ribosomal DNA (rDNA) corresponded well with previous anastomosis groupings. These results strongly indicate that anastomosis is genetically based.

Electrophoretic separation of enzymes, which exploits the polymorphism of detected isozyme forms, is another molecular technique that has been widely used to generate a large number of markers for the assessment of genetic diversity in fungi (4,32,48). In *Rhizoctonia solani*, isozyme electrophoretic profiles provided a good indication of the genetic diversity among selected AGs and reconfirmed the genetic basis of the AG concept (16,18). In the case of binucleate *Rhizoctonia*, work with pectic enzyme patterns defined subgroups within selected binucleate *Rhizoctonia* whose teleomorphic stage belong to *Ceratobasidium cornigerum* (Bourd.) D.P. Rogers (25,41). To date, however, no research with isozyme patterns has clearly defined groups within binucleate *Rhizoctonia*. The aims of this study were to use gel electrophoresis of several metabolic enzymes to determine the genetic relatedness among and within 17 AGs of binucleate *Rhizoctonia* and to examine whether the electrophoretic groups are congruent with those determined by anastomosis. A preliminary report of this work has been published (6).

MATERIALS AND METHODS

Isolate culture. Fifty isolates of binucleate *Rhizoctonia* representing 12 Japanese and five North American AGs from different geographical regions were selected for this study (Table 1). Representative isolates of AG-J, AG-L, AG-M, AG-N, AG-P, AG-Q, CAG-6, and CAG-7 were not available for isozyme analysis at the time the study was conducted. All isolates were grown on Difco potato-dextrose agar (PDA; Difco Laboratories, Detroit, MI) in darkness at 20 ± 3 C.

Enzyme extraction. Fresh mycelium was obtained by growing individual isolates on PDA at room temperature for 5 days in darkness. Two mycelial disks (7 mm in diameter) per isolate were transferred to 100 ml of Difco potato-dextrose broth in 250-ml Erlenmeyer flasks and were incubated without shaking for 7 days under the same conditions. The mycelial mats were collected by vacuum-filtration and ground into a fine powder with liquid nitrogen. Enzyme extraction was carried out according to Laroche et al (16). Frozen mycelial powder (1.5 mg) was homogenized by vortexing in 500 ml of extraction buffer and was centrifuged at 13,000 *g* for 15 min in a benchtop microfuge. The collected supernatant was submitted to a second centrifugation at 100,000 *g* for 10 min in a Beckman airfuge ultracentrifuge (Beckman,

Fullerton, CA) and was stored at −80 C for electrophoretic analysis. Enzyme manipulations were performed at 4 C.

Electrophoresis and enzyme detection. Enzyme supernatants were subjected to discontinuous native polyacrylamide gel electrophoresis (PAGE) as described by Davis, using a Tris-glycine (pH 8.3) buffer system (8). To separate enzymes, electrophoresis was run in either 6, 7.5, or 12% (w/v) polyacrylamide (pH 8.9) at 4 C with a constant current of 25 mA. Bromophenol blue was inserted on either side of the samples and served as a boundary front marker. For each enzyme system, reference isolates showing the maximum variation in their electrophoretic patterns were used as internal standards. Relative mobilities (Rm) of isozyme bands were recorded from the gel origin to the anode. Three electrophoretic runs were performed for all isolates.

After completion of electrophoresis, gels were stained for each of the 23 enzymes by selective protocols (Table 2). Stained gels were fixed in 5% methanol/10% acetic acid (v/v) solution and dried between two cellulose membranes of BioGel Wrap (Bio-Design Inc. of New York, Carmel).

Genetic nomenclature and inference. The genetic nomenclature of May et al (21) and Yeh and Layton (49) was employed. Abbreviations with all capital letters referred to enzymes;

TABLE 1. Anastomosis groups, hosts, and references of binucleate *Rhizoctonia* isolates used for isozyme analysis

Anastomosis group	Isolate collection no.	Host or habitat	Isolate reference[a]
AG-A	SN-1	Soil	Ogoshi
AG-A	C-662	Soil	Ogoshi
AG-Ba	C-460	*Oryza sativa*	Ogoshi
AG-Bb	C-157	*O. sativa*	Ogoshi
AG-Bb	C-348	Unknown	Ogoshi
AG-B(o)	POER-2	Unknown	Ogoshi
AG-B(o)	C-302	Unknown	Ogoshi
AG-C	70B	Soil	Ogoshi
AG-C	A001C	Soil	Ogoshi
AG-D	10B7	Unknown	Ogoshi
AG-D	7-4-2	*Triticum aestivum*	Cook
AG-D	2-3s-3	*T. aestivum*/soil	Cook
AG-D	C-60	Unknown	Ogoshi
AG-E	RH-155	Unknown	Ogoshi
AG-E	1-1-6	*T. aestivum*	Cook
AG-F	SIR-1	*Ipomoea batatas*	Ogoshi
AG-G	AHC-9	Unknown	Ogoshi
AG-G	Fa-479	Unknown	Ogoshi
AG-G	232-CG	*Azalea* sp. Pine bark medium	Echandi
AG-G	JF-3N1-1	*Fragaria* × *ananassa*	Echandi
AG-G	JF-3S4-3	*F.* × *ananassa*	Echandi
AG-G	R-95	*F.* × *ananassa*	Echandi
AG-G	BN-160	*Festuca rubra*	Echandi
AG-I	Cre5-3a	Unknown	Ogoshi
AG-I	Pt-5	*Beta vulgaris*	Ogoshi
AG-I	AV-2	*Artemisia vulgaris* var. *indica*	Ogoshi (ATCC 76143)
AG-K	1-2w1-2	*Hordeum vulgaris*	Cook
AG-K	AC-1	*Allium cepa*	Ogoshi (ATCC 76145)
AG-K	55-D-33	Unknown	Ogoshi
AG-K	2-5-6	*Medicago sativa*	Cook
AG-O	FKo-2-10	Soil	Ogoshi
AG-O	FKo-6-2	Soil	Ogoshi (ATCC 76149)
CAG-1	BN-163	*T. aestivum*	Burpee
CAG-1	BN-162	*Juncus* sp.	Burpee (ATCC 62063)
CAG-1	BN-185	*T. aestivum*	Burpee
CAG-1	BN-160	*Agrostis* sp.	Burpee
CAG-1	BN-171	*H. vulgaris*	Burpee
CAG-2	539-I4 SDF	Soil	Sumner
CAG-2	BN-1	*Cucumis sativus*	Sumner
CAG-2	BN-4	*Arachis hypogaea*	Sumner
CAG-2	547	*Arabis candensis*	Sumner
CAG-2	BN-4-T	Soil	Burpee (ATCC 34969)
CAG-3	594-CIII1	*Arachis hypogaea*	Sumner
CAG-3	371-300-2C	*Phaseolus vulgaris*	Sumner
CAG-3	471-332	Soil	Sumner
CAG-4	511-45-PLT-2	*Cuphea petiolata*	Sumner
CAG-4	539-I13-SDF	Soil	Sumner
CAG-4	BN-38	*Glycine max*	Burpee
CAG-5	449-I7E-B	*P. vulgaris*	Sumner
CAG-5	580-III DTR1-SDF	Soil	Sumner

[a]ATCC = American Type Culture Collection, 12301 Parklawn, Rockville, MD; L. L. Burpee, Department of Plant Pathology, University of Georgia, Griffin; R. J. Cook, United States Department of Agriculture, Root Disease & Biological Control Research Unit, 367 Johnson Hall, Pullman, WA; E. Echandi, Department of Plant Pathology, North Carolina State University, Raleigh; A. Ogoshi, Faculty of Agriculture, Hokkaido University, Kita-Ku, Sapporo 060, Japan; and D. R. Sumner, Coastal Plain Experiment Station, The University of Georgia College of Agriculture, Tifton.

abbreviations with only the first letter capitalized indicated the putative genetic locus coding for the enzyme (Fig. 1). Presumed loci were numbered in decreasing order of anodal mobility (e.g., *Mdh*-1 represents the most cathodal locus; Fig. 1B). The most common allele at each locus was arbitrarily designated 100, with additional alleles given numerical values according to their relative migration to the 100-allele. Inference of the genetic basis of electrophoretic banding patterns was made in accordance with known subunit compositions of the enzymes (7,11) and in relation to observations in other fungi in which crossing experiments have been conducted (22).

Data analysis. Genetic relatedness among isolates was assessed by two multivariate statistical analyses: 1) cluster analysis and 2) principal component analysis (PCA). For the cluster analysis, a matrix of Jacquard similarity coefficients was generated from a binary code based on the presence or absence of isozyme bands. The unweighted pair-group method with arithmetic averages (UPGMA) (40) was performed on this matrix by the R software (44). For the PCA analysis, electrophoretic bands of individual isolates were translated into coded vectors according to their presumed genotypes with the algorithm of Smouse and Neel (39) and Yeh and Layton (49). The coded vectors were submitted to PCA by SAS based on a variance-covariance matrix (37). The percent contribution of each locus was calculated according to the method of Bousquet et al (1).

RESULTS

Nine out of 23 enzyme systems showed well-resolved electrophoretic banding patterns and strong staining activity (Table 2). They were used as phenotypic markers for the analysis of the 50 isolates (Table 1). Crosses between monokaryotic isolates were not attempted due to the difficulty involved in inducing the teleomorph state of binucleate *Rhizoctonia* species. The genetic basis of the isozyme phenotypes was inferred directly from the observed banding patterns. Based on the Rm values of their isozyme bands, phenotypes were genetically interpreted in terms of putative alleles present at specific loci coding for the isozymes. The locus coding for the enzyme leucine amino-peptidase was not considered because the displayed banding patterns did not provide a clear indication of the enzyme subunit structure.

Enzyme banding patterns. A total of 63 electrophoretic phenotypes showed consistency between replications, with the number of phenotypes varying from four to 17 per enzyme (Fig. 2). All enzymes behaved as monomers, except for MDH and HK, which produced complex banding patterns and were interpreted as each being coded by two loci (Fig. 2). Presumed null alleles were proposed when no isozyme bands were detected for a particular enzyme (Table 3).

Glucose 6-phosphate dehydrogenase (G6PD). All isolates were characterized by a single G6PD banding pattern (Fig. 2; Table 3). Four electrophoretic phenotypes were detected, with phenotype 1 specific to isolate 594-CIII1 (CAG-3). Identical banding patterns were observed among isolates of CAG-2 (phenotype 2), AG-Bb and AG-G (phenotype 3), and AG-Ba, AG-C, and AG-F (phenotype 4). Phenotypic intragroup variation was noted in the AG-A, AG-K, CAG-5, and AG-B(o) subgroup. No G6PD activity was detected in AG-E and AG-I nor in some isolates of AG-D, AG-O, CAG-1, CAG-3, and CAG-4 (Table 3).

Malate dehydrogenase (MDH). Two separate zones of MDH activity were scored among eight of the 17 AGs (Fig. 2; Table 3). They were interpreted as the products of two different loci (*Mdh*-1 and *Mdh*-2) that shared the double-banded phenotypes 2–6 (Fig. 2). Isolates of the remaining AGs expressed only one of the two loci. Phenotypic variation was noted among isolates of AG-C, AG-D, AG-K, AG-O, CAG-1, CAG-2, and CAG-5 (Table 3). Phenotypes 1–3, 8, 9, 12, and 13 were detected in all isolates of AG-Ba, AG-A and AG-Bb, AG-G, AG-E, AG-B(o), CAG-4, and AG-I, respectively. Except for AG-A/CAG-2, phenotypes 7 and 12 were shared between the cross-anastomosing

TABLE 2. List of enzymes screened and their identification

Class and recommended name[a]	Abbreviation[a]	EC no.[a]	Subunit no.[b]	Staining activity[c] (reference)
Oxidoreductases				
Alcohol dehydrogenase	ADH	1.1.1.1	4	± (43)
Shikimic acid dehydrogenase	SDH	1.1.1.25	-[d]	− (3)
Malate dehydrogenase[e]	MDH	1.1.1.37	2	+ (3)
Isocitrate dehydrogenase	IDH	1.1.1.42	2	± (3)
Glucose dehydrogenase	GLUDH	1.1.1.47	-	± (3)
Glucose 6-phosphate dehydrogenase[e]	G6PD	1.1.1.49	2	+ (3)
Glutamate dehydrogenase	GDH	1.4.1.3	-	− (3)
Diaphorase	DIA	1.6.4.3	1	± (35)
Menadione reductase	MR	1.6.99.2	1	± (3)
Nicotinamide adenine dinucleotide dehydrogenase	NADHDH	1.6.99.3	1	± (3)
Peroxidase	PER	1.11.1.7	1	− (9)
Transferases				
Hexokinase[e]	HK	2.7.1.1	2	+ (3)
Phosphoglucomutase	PGM	2.7.5.1	-	± (3)
Hydrolases				
α-Esterase	α-EST	3.1.1.1	1	± (38)
β-Esterase	β-EST	3.1.1.1	1	± (34)
Alkaline phosphatase	ALP	3.1.3.1	2	± (3)
α-Acid phosphatase[e]	α-ACP	3.1.3.2	2	+ (17)[f]
β-Acid phosphatase[e]	β-ACP	3.1.3.2	2	+ (17)[f]
Leucine amino-peptidase[e]	LAP	3.4.11.1	1	+ (3)
Lyases				
Aldolase[e]	ALD	4.1.2.13	2	+ (3)
Fumarase[e]	FUM	4.2.1.2	4	+ (3)
Aconitase	ACO	4.2.1.3	1	± (3)
Isomerases				
Phosphoglucose isomerase[e]	PGI	5.3.1.9	2	+ (38)

[a] Based on the Nomenclature Committee of the International Union of Biochemistry (47).
[b] Based on Darnall and Klotz (7) and Harris and Hopkinson (11).
[c] + = strong staining activity; ± = weak activity; − = no detectable activity.
[d] Subunit number not reported.
[e] Enzymes are those selected for isozyme analysis of binucleate *Rhizoctonia*.
[f] A modification of Lilly and Charvat (17) protocol was used with fast blue RR diazonium salt stain replaced by fast garnet GBC.

groups AG-D/CAG-1 and AG-F/CAG-4, respectively.

Hexokinase (HK). Two putative loci with 17 electrophoretic phenotypes coded for this dimeric enzyme (Fig. 2). The number of HK bands per phenotype varied between one (for phenotypes 1, 3, 9, 13, and 15–17), two (for phenotypes 2, 5, 7, 8, 10, 11, and 14), three (for phenotype 4), and four (for phenotypes 6 and 12). Significant phenotypic variation was noted among isolates of 12 AGs (Table 3). Identical banding patterns were observed, however, among most isolates of AG-G (phenotype 1), CAG-4 (phenotype 3), and CAG-2 (phenotype 4).

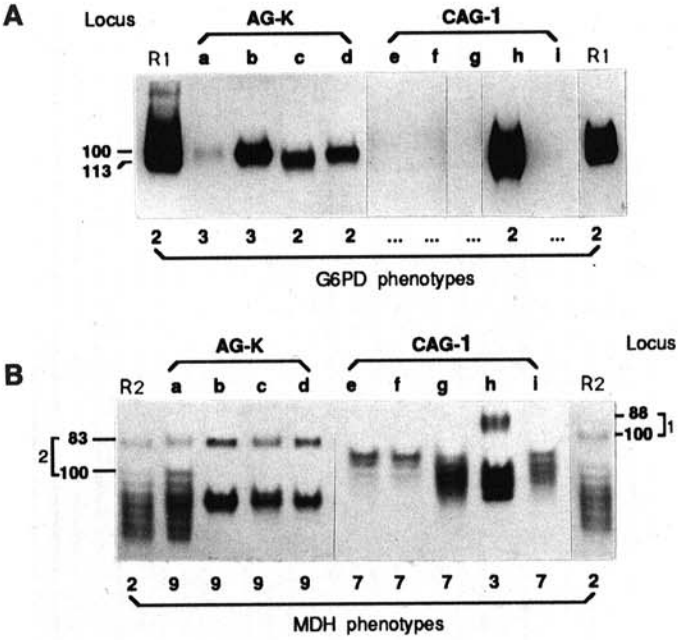

Fig. 1. Example for scoring isozyme gels for **A,** a monomeric, G6PD, and **B,** a dimeric, MDH, enzyme. Isolate designations are: a, 1-2w1-1; b, AC-1; c, 55-D-33, d, 2-5-6; e, BN-163; f, BN-162; g, BN-185; h, BN-160; and i, BN-171, with reference isolates designated as R1 and R2. Numbers at either side represent the putative allelic designations.

α-Acid phosphatase (α-ACP). Seven electrophoretic phenotypes were identified in this enzyme. Phenotypes 1, 2, 4, and 7 were characterized by a single α-ACP banding pattern (Fig. 2). The double-banded phenotypes 3, 5, and 6 were interpreted as presumed heterozygous genotypes 91/100, 76/91, and 59/91, respectively. These genotypes were only expressed in isolates 70B (AG-C) and Cre5-3a (AG-I), isolates A001C (AG-C) and 10B7 (AG-D), and isolate C-302 (AG-B(o)), respectively (Table 3). Most isolates of AG-Ba, AG-D, AG-I, AG-G, and CAG-3 displayed similar banding patterns (phenotype 2). Phenotype 7 was present in all isolates of CAG-5 and in the majority of CAG-4 isolates. Phenotypic variation was detected in AG-C, AG-K, AG-O, CAG-1, and AG-B subgroups. AG-E isolates showed no detectable activity at this locus and were presumed to express a null allele.

β-Acid phosphatase (β-ACP). A total of seven electrophoretic phenotypes were observed (Fig. 2). No isozyme bands were detected for β-ACP in AG-Ba nor in some isolates of AG-E and CAG-1 (Table 3). Phenotypes 3, 5, and 7 were characterized by a double-banded pattern (Fig. 2) and were detected in only four AGs (Table 3). Phenotypes 1, 2, 4, and 6 displayed a single-banded pattern and were distributed among isolates of the remaining AGs (Table 3). Heterozygosity was noted only in AG-C (isolate 70B, phenotype 5) and CAG-4 (isolate 539-I13-SDF, phenotype 7) with putative alleles 77/92 and 77/82, respectively. Isolates of AG-B, AG-C, AG-E, AG-O, CAG-1, and CAG-4 expressed more than one phenotype (Table 3). Most AG-K and CAG-3 isolates shared phenotype 1, whereas phenotype 2 was detected in AG-I and CAG-5 and phenotype 4 in AG-A, AG-D, AG-F, and CAG-2.

Aldolase (ALD). Six different electrophoretic phenotypes were detected in 13 out of 17 AGs (Table 3). Phenotype 2 (presumed genotype 89/117) was characterized by a double-banded pattern and was unique to CAG-3 (Fig. 2). Phenotype 6 was specific to CAG-5 isolates. The most common phenotype, 3, was expressed in all isolates of AG-Ba and AG-K and among the majority of CAG-2 isolates. Only one phenotype was detected among all isolates of AG-D and AG-I (phenotype 5), AG-K (phenotype 3), and CAG-1 (phenotype 4). More than one phenotype was expressed in the remaining AGs (Table 3).

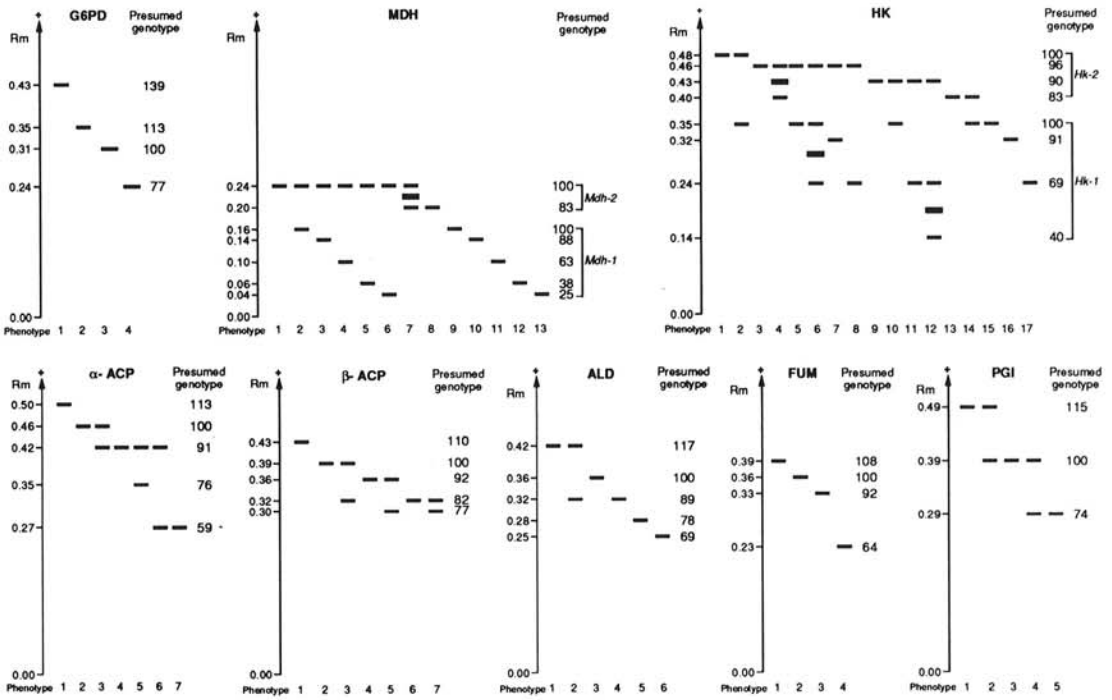

Fig. 2. Electrophoretic phenotypes of eight enzymes with interpretable genetic basis for 10 presumed loci. Phenotypic groups are indicated by numbers from 1 to 17. Numbers at right represent presumed loci and alleles. Alleles are represented by relative distance from the protein coded by the most common allele (designated 100). Direction of migration is toward the anode (+).

TABLE 3. Distribution of phenotypes of 50 binucleate *Rhizoctonia* isolates

Anastomosis group and isolate collection no.	Phenotype no.[a]							
	G6PD[b]	MDH	HK	α-ACP	β-ACP	ALD	FUM	PGI
AG-A								
SN-1	3	2	9	4	4	3	2	-[c]
C-662	2	2	10	4	4	4	-	3
AG-Ba								
C-460	4	1	13	2	-	3	3	2
AG-Bb								
C-157	3	2	10	4	3	-	2	3
C-348	3	2	15	4	6	4	4	2
AG-B(o)								
POER-2	2	9	10	2	2	3	2	3
C-302	4	9	14	6	6	-	2	3
AG-C								
70B	4	5	1	3	5	5	-	2
A001C	4	6	17	5	6	-	-	4
AG-D								
10B7	3	6	12	5	6	5	2	2
7-4-2	-	7	8	2	4	5	4	3
2-3s-3	-	7	6	2	4	5	4	3
C-60	3	13	-	2	4	5	-	-
AG-E								
RH-155	-	8	1	-	-	1	2	3
1-1-6	-	8	15	-	2	3	-	3
AG-F								
SIR-1	4	12	16	4	4	-	2	3
AG-G								
AHC-9	3	3	9	2	3	3	2	2
Fa-479	3	3	1	2	3	1	2	3
232-CG	3	3	1	-	2	-	2	1
JF-3N1-1	3	3	2	2	6	1	2	2
JF-3S4-3	3	3	1	2	2	1	2	3
R-95	3	3	1	2	2	1	2	1
BN-160	3	3	1	2	2	1	2	3
AG-I								
Cre5-3a	-	13	-	3	4	5	-	1
Pt-5	-	13	11	2	2	5	-	1
AV-2	-	13	16	2	2	5	-	1
AG-K								
1-2w1-2	3	2	10	4	2	3	2	1
AC-1	3	9	4	2	1	3	3	1
55-D-33	2	9	9	1	1	3	3	1
2-5-6	2	9	9	2	1	3	3	3
AG-O								
FKo-2-10	-	10	8	-	2	1	-	4
FKo-6-2	3	12	13	7	4	3	-	4
CAG-1								
BN-163	-	7	7	4	4	4	-	1
BN-162	-	7	6	4	4	4	-	1
BN-185	-	7	5	2	2	4	-	1
BN-160	2	3	1	1	1	-	-	2
BN-171	-	7	8	1	-	-	-	3
CAG-2								
539-I4SDF	2	10	13	4	4	5	3	1
BN-1	2	-	4	2	2	3	2	5
BN-4	2	12	4	4	4	3	2	5
547	2	10	13	4	4	3	2	1
BN-4-T	2	9	4	4	4	3	2	3
CAG-3								
594-CIII1	1	-	-	-	2	3	1	1
371-300-2C	-	-	1	2	1	2	2	1
471-332	-	-	13	2	1	2	2	1
CAG-4								
511-45PLT2	-	12	-	4	2	-	-	-
539-I13-SDF	4	12	3	7	7	5	3	1
BN-38	-	12	3	7	4	-	3	1
CAG-5								
449-I7E-B	2	11	16	7	2	-	-	2
580-IIIDTR1-SDF	4	4	15	7	2	6	-	-
Total	4	13	17	7	7	6	4	5

[a] Phenotype numbers as indicated in Figure 2.
[b] Enzyme abbreviations as given in Table 2.
[c] No detectable enzymatic activity.

Fumarase (FUM). Isolates belonging to AG-C, AG-I, AG-O, CAG-1, and CAG-5 showed no activity for FUM. Four phenotypes with single-banded patterns were identified in the other AGs (Fig. 2; Table 3). Intragroup phenotypic variation was noted only within AG-A, AG-D, AG-E, and AG-B (Table 3). All isolates belonging to AG-B(o), AG-F, and AG-G, and the majority of those belonging to CAG-2 and CAG-3 displayed phenotype 2. Phenotype 3 was detected only in AG-Ba, AG-K, and CAG-4. Phenotype 1 was specific to isolate 594-CIII1 (CAG-3), whereas phenotype 4 was specific to AG-Bb and AG-D.

Phosphoglucose isomerase (PGI). Five electrophoretic phenotypes were observed, with phenotypes 2 and 4 showing a double-banded pattern. They were interpreted as putative heterozygous for alleles 100/115 and 74/100, respectively (Fig. 2). Phenotypic variation was noted among isolates belonging to AG-A, AG-B, AG-C, AG-D, AG-G, CAG-1, and CAG-2 (Table 3). Phenotype 1 was produced by all isolates of AG-I, CAG-3, and CAG-4 and by the majority of AG-K isolates. All isolates of AG-B(o), AG-E, and AG-F expressed phenotype 3, whereas those of AG-O displayed phenotype 4. Phenotype 5, representing the most cathodal putative allele, 74, was specific to only two CAG-2 isolates (BN-1 and BN-4).

Cluster analysis. The dendrogram presented in Figure 3 was generated with a cophenetic correlation of 0.65. Four major groups, designated I, II, III, and IV, were delineated at 69% similarity. Within most groups, isolates belonging to a particular AG were grouped at a high-similarity level before they joined those from other AGs. Group I is made up of all AG-G isolates clustering at 84% similarity. Group II could be split into two subgroups, IIA and IIB, linked to each other at 73% similarity. Subgroup IIA is formed at 76% similarity and is made up of AG-D, AG-E, CAG-1, and CAG-3 isolates. Subgroup IIB is formed at 74% similarity and is made up of AG-Ba, AG-F, AG-I, CAG-4, CAG-5, and one isolate each of AG-B(o), AG-D, and AG-O. Group III represents all isolates of AG-K and CAG-2 and one isolate each of AG-A and AG-B(o), clustering at 74% similarity before joining groups I and II at 70% similarity. Group IV is formed at 73% similarity and is made up of AG-Bb isolates and one isolate each of AG-A, AG-C, AG-D, and AG-K. It is linked to the remaining groups at 69% similarity. Isolates FKo-2-10 (AG-O) and 70B (AG-C), the most dissimilar, joined group I at 74 and 73% similarity, respectively.

PCA. Further demonstration of genetic variation was evaluated with PCA of 10 putative polymorphic loci (Fig. 4). A total of 25.1% of the overall variance was attributed to the first two principal components. Component I represented 14.7% of the variation and was dominated by alleles *Ald*, *Fum*, and *G6pd*, accounting for 11.5, 13.2, and 16.3% of the contribution, respectively. It differentiated AG-G, AG-K, CAG-2, and CAG-3 from the other AGs (Fig. 4). Component II accounted for 10.3% of the total variation and was heavily weighted by alleles *β-Acp* (11.4%), *Hk*-2 (12.6%), and *Pgi* (30%). This component separated AG-D and CAG-4 isolates from the others. The remaining AGs were not clearly delineated by either component (Fig. 4).

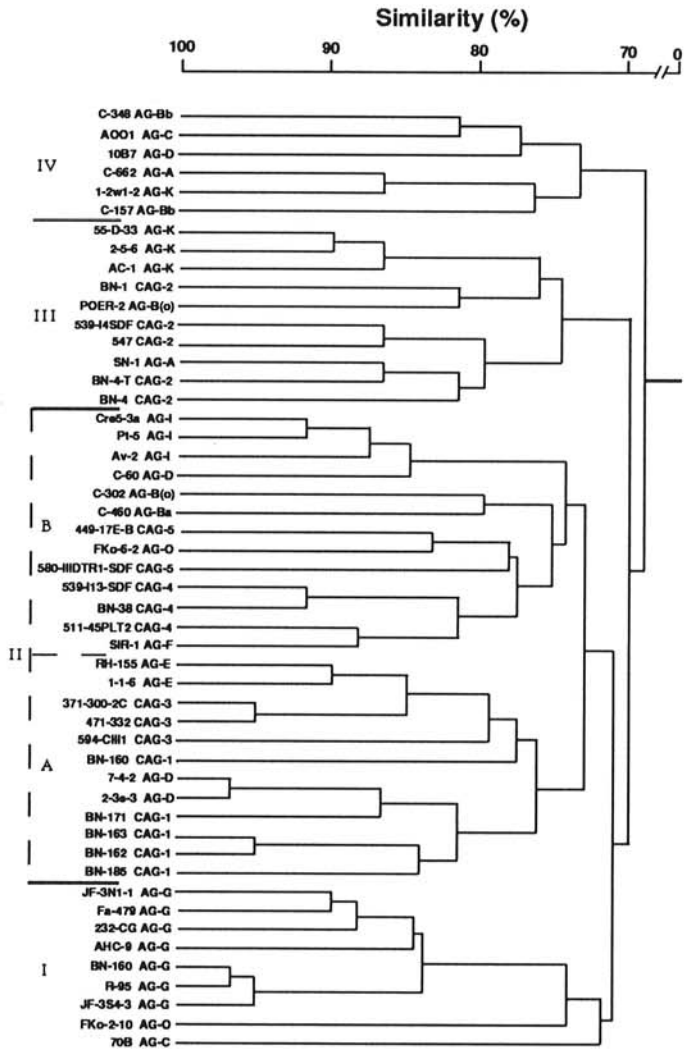

Fig. 3. Dendrogram based on isozyme analysis of 50 binucleate *Rhizoctonia* isolates. The dendrogram was constructed by the unweighted pair-group method with arithmetic averaging with Jacquard's similarity values.

DISCUSSION

Electrophoresis of enzymes was applied to differentiate phenotypes among 17 AGs of binucleate *Rhizoctonia* species. The eight enzyme systems were selected based on the high resolution and reproducibility of their banding patterns on native PAGE. Although we assumed that the lack of enzymatic activity in some isolates is due to the expression of putative null alleles, genetic crosses are essential to verify whether null alleles or other mechanisms are involved (23,24). The presence of multiple bands for MDH and HK was interpreted as the expression of alleles at different loci coding for the same enzyme, because two zones of activity were detected at separate regions on the gel.

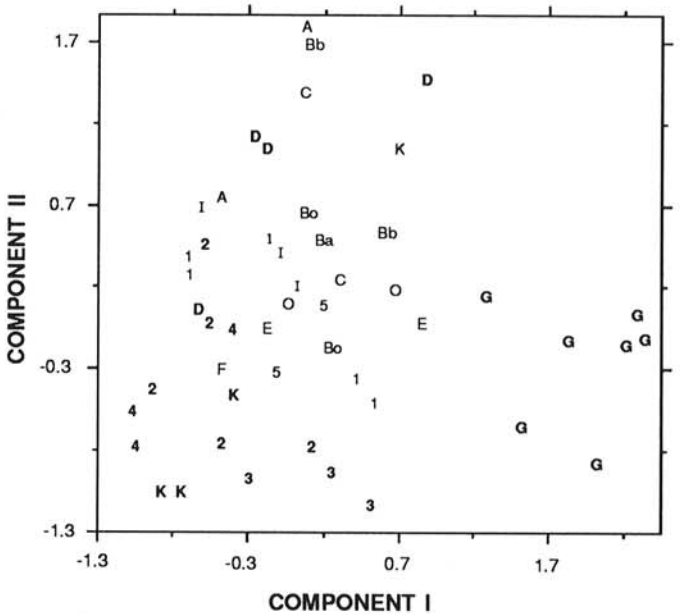

Fig. 4. Principal component analysis scatterplot of scores of 50 binucleate *Rhizoctonia* isolates belonging to 12 Japanese (A = AG-A; Ba = AG-Ba; Bb = AG-Bb; Bo = AG-B(o); C = AG-C; D = AG-D; E = AG-E; F = AG-F; G = AG-G; I = AG-I; K = AG-K; and O = AG-O) and five North American anastomosis groups (1 = CAG-1; 2 = CAG-2; 3 = CAG-3; 4 = CAG-4; and 5 = CAG-5). Scale of axes is proportional to the eigenvalues of the first two principal components.

In this study, analysis of isozyme electrophoretic phenotypes demonstrated that four genetically distinct groups (I, II, III, and IV) of binucleate *Rhizoctonia* were formed and that within each group, isolates described as possessing the same hyphal anastomosis reaction (2,27–30) were clustered at a high-similarity level before joining isolates of a different AG. In addition, these groups are congruent with five out of seven RFLP groups (I, II, IV, V, and VII) of binucleate *Rhizoctonia* species identified by rDNA RFLP patterns (5). Similar correlations between isozyme analysis and RFLPs of certain AGs of *R. solani* have also been reported (14,16,19).

Isolates of AG-G, designated as *Rhizoctonia fragariae* with *Ceratobasidium* species teleomorphs (13), formed a genetically distinct group (group I) based on cluster analysis and phenotype similarity. Interestingly, in a recent study conducted by Cubeta et al (5), the same AG-G isolates were assigned to RFLP group IV based on their unique and identical rDNA restriction patterns and DNA fragment size. It appears that AG-G isolates share a conserved genetic region that evolved from a common ancestor.

Isolates of AG-D and CAG-1, producing the same teleomorph (*C. graminearum*) (30) and whose hyphae are known to cross-anastomose, clustered at a high-similarity level before joining other AGs. The same trend was found with AG-A/CAG-2 (teleomorph: *C. cornigerum*), AG-F/CAG-4 (teleomorph: *Ceratobasidium* species), and AG-E/CAG-3, all of which are known to cross-anastomose. Genetic similarity between each of these cross-anastomosing groups also has been demonstrated by RFLP patterns (5). These results corroborate the findings of Ogoshi et al (27,29) on hyphal anastomosis, concerning the relatedness of the Japanese and North American strains.

Ogoshi (26) considered AG-B a heterogeneous group and subdivided it into three intraspecific groups (AG-Ba, AG-Bb, and AG-B(o)) based on hyphal anastomosis, whereas Gunnell and Webster (10) found that these groups correspond to different teleomorphs: *C. setariae* for AG-Ba, *C. oryzae-sativae* for AG-Bb, and *C. cornigerum* for AG-B(0). More recently, Herr (12) reported on the inability of the tester isolates of these subgroups to cross-anastomose with each other. The cluster analysis derived from the electrophoretic data demonstrated that the AG-B subgroups formed genetically distant groups. Further evidence of this genetic variability is supported by RFLP analysis (5).

In this study, the great phenotypic intragroup variation detected in AG-C and AG-O resulted in the placement of isolates into separate and genetically distant groups in the dendrogram. These findings are not consistent with the conclusions of Ogoshi et al (29) based on anastomosis tests. It is likely that the testing of additional isolates may clarify the genetic relationship within AG-C and AG-O. Isolates 70B (AG-C) and FKo-2-10 (AG-O) gave consistently unique phenotype patterns with the majority of isozymes tested and are considered genetically less related to other isolates belonging to the same AG. We are not sure why these particular isolates are grouped at lower levels of similarity. In the case of FKo-2-10, presumed null alleles for more than one locus were detected, and the expression of such null alleles may artificially lower the degree of relatedness with other isolates.

PCA provided more information about the contribution of each presumed locus to the total genetic variability observed among isolates. Certain loci of some enzymes were responsible for the inter- rather than the intragroup genetic variation. On the first principal component, *G6pd* showed the highest percent contribution and accounted for the greatest similarity among AG-G isolates and for the dissimilarity between AG-G and the remaining AGs. Other loci of highly polymorphic enzymes like *Hk*-2 contributed mostly to the intragroup variation on the second principal component.

The binucleate *Rhizoctonia* species are a complex group of organisms, some of which are important plant pathogens (1,30). Their identification as well as their characterization can be a challenge to plant pathologists and mycologists. We have shown that gel electrophoresis of enzymes was able to consistently differentiate the seventeen anastomosis groups previously identified by Burpee et al (2) and Ogoshi et al (28,29). Furthermore, in a recent study conducted on multinucleate *R. solani* isolates, we were able to distinctly differentiate by enzyme electrophoresis AGs 3 and 9 (16). Thus, this technique offers researchers a simple, reliable, and economical method that can be used alone or in conjunction with anastomosis.

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