Ribosomal DNA Restriction Fragment Length Polymorphisms in *Rhizoctonia solani*

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ABSTRACT

Vilgalys, R., and Gonzalez, D. 1990. Ribosomal DNA restriction fragment length polymorphisms in *Rhizoctonia solani*. Phytopathology 80:151-158.

Restriction fragment length polymorphisms (RFLPs) in the nuclear encoded ribosomal DNA repeat of the fungal pathogen *Rhizoctonia solani* revealed considerable molecular variation among and within intraspecific groups that have been recognized previously on the basis of anastomosis, morphology, and pathogenicity. Genomic DNAs from 87 isolates of *R. solani* were surveyed for RFLPs by digestion with the restriction enzymes *Eco*RI and *Bam*HI and analyzed by Southern blotting against a ribosomal DNA probe originating from anastomosis group 4. Each of the 15 intraspecific groups of *R. solani* are characterized by one or more unique RFLPs. Levels of intragroup RFLP variation were within the ranges of RFLP variation of the different anastomosis groups (AGs) and their subgroups. Isolates from anastomosis groups 3, 4, 7, 8, and BI all possess a single, invariant RFLP unique to each intraspecific group. A relatively low level of RFLP variation also was characteristic of isolates within AG-1-IB, AG-1-IC, AG-2-2, and AG-9. In contrast, a relatively high level of RFLP diversity was observed among isolates within AG-1-IA, AG-2-1, AG-5, and AG-6. We did not observe any obvious relationship between levels of RFLP within intraspecific groups and variation of other biological factors (host range, morphological variation, etc.). Instead, we propose that the pattern of RFLP variation observed is most likely the result of genetic divergence within and among intraspecific groups.

Additional keywords: genetic variation, *Thanatephorus cucumeris*.

The ubiquitous plant pathogen *Rhizoctonia solani* Kühn (teleomorph = *Thanatephorus cucumeris* (Frank) Donk) is noted for its high diversity and represents a paradigm for studies of intraspecific variation in phytopathogenic fungi. Early investigators recognized intraspecific groups within *R. solani* based primarily upon cultural characters, pathogenic behavior, and ecological criteria (25,29). Later, Parmeter et al (19) reported that isolates of *R. solani* could be divided into at least four groups based on anastomosis behavior. Within anastomosis groups (AGs), hyphal attraction and fusion occur between confronted isolates grown in culture. In contrast, isolates belonging to different AGs do not exhibit anastomosis behavior. At least nine AGs are known to occur within *R. solani* (18).

More recent studies measuring heterologous DNA/DNA hybridization have clearly demonstrated that extensive variation is present within many AGs. DNA hybridization experiments by Kuninaga and Yokosawa (11-13) revealed the existence of intraspecific groups in AG-4 and AG-6. They introduced the term "homogeneous group" for differentiating isolates of these AGs based on their DNA similarity. However, this terminology has not resolved the problem of intraspecific taxonomy in the complex of *R. solani*. Vilgalys (27) also found extensive genetic divergence among representative isolates from seven AGs and suggested that additional intraspecific groups are likely to be present in *R. solani*. The low degree of sequence similarity observed among DNA from different intraspecific groups is comparable to the divergence levels observed between different species in other fungi (14). Unfortunately, these high levels of genetic dissimilarity also make it difficult to use DNA hybridization data for determining phylogenetic and systematic relationships of different intraspecific groups.

One direct approach for assessing genetic variation in fungi is by comparative study of molecular markers (16). In particular, evolutionarily conserved markers are desirable for the group of *R. solani* because DNA hybridization studies already have demonstrated low-sequence complementarity among most intraspecific groups (27). For this purpose, the genes coding for ribosomal RNA appear to be especially well suited as molecular markers because they evolve more slowly than many other parts of the genome yet are large enough for evolutionary comparisons at many taxonomic levels (2,7). In basidiomycetes, the ribosomal RNA genes (rDNA) are present as a multigene family consisting of up to several hundred tandemly repeated units (4–6). Each rDNA repeat contains coding regions for all of the major ribosomal subunit RNAs (5S, 5.8S, 17S, and 25S RNAs), as well as spacer segments which may or may not be transcribed. Within most species, rDNA repeats from different individuals tend to have a highly conserved structure and coevolve in a concerted fashion (3). In contrast, evolutionarily isolated groups (for example, species) would be expected to have divergent rDNA genes due to independent accumulation of base substitutions, length mutations, and rearrangements. Data on the structure and organization of rDNA therefore may provide useful information for understanding evolutionary and pathogenic diversity within the complex of *R. solani* as well.

The objective of this study was to survey different intraspecific groups within the complex of *R. solani* for restriction fragment length polymorphisms (RFLPs) within rDNA genes. An abstract reporting our preliminary results has been published (28).

MATERIALS AND METHODS

Isolates and culture methods. A total of 87 isolates of *R. solani* were surveyed in this study (Table 1). They represent 15 intraspecific groups from all known AGs (AG-1–AG-9, AG-BI). A single isolate (32-RS), the AG specificity of which is unknown, was included in the present study because it meets the morphological criteria necessary to be considered as *R. solani* (1,18,19). Thirty-six of the isolates were from a previous study of DNA/DNA hybridization among AGs (27). Cultures for DNA extraction were grown in 50 ml of potato-dextrose broth (Difco Lab-

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TABLE 1. List of isolates of *Rhizoctonia solani* used in this study

Isolate, ISG[a]	Origin[b]	Host	Alternative accession number
1Rs AG-1-IA	Louisiana	*Glycine max*	LR 3472, ATCC 66159
2Rs AG-1-IA	Louisiana	*Oryza sativa*	LR 17574, ATCC 66158
11Rs AG-1-IA	Japan	*Oryza sativa*	Ogoshi CS-2, ATCC 66157
48Rs AG-1-IA	Louisiana	*Oryza sativa*	LR 6172
49Rs AG-1-IA	Louisiana	*Oryza sativa*	LR 6372
51Rs AG-1-IA	Louisiana	*Oryza sativa*	LR 174
52Rs AG-1-IA	Louisiana	*Oryza sativa*	LR 6072
54Rs AG-1-IA	Louisiana	*Glycine max*	LR 6173
55Rs AG-1-IA			Carling 7317
12Rs AG-1-IB	Japan	*Beta vulgaris*	Ogoshi B-39, ATCC 66156
34Rs AG-1-IB	District of Columbia	*Poa* sp.	L. Burpee Rh 90, ATCC 66151
36Rs AG-1-IB	Pennsylvania	*Poa* sp.	L. Burpee Rh 46, ATCC 66150
3Rs AG-1-IC	Canada	*Pinus resinosa*	ATCC 44661
13Rs AG-1-IC	Japan	soil	Ogoshi F-2, ATCC 66155
46Rs AG-1		*Brassica* sp.	ATCC 14703
81Rs AG-1	North Carolina	soil	L.T. Lucas Rs 128
8Rs AG-2-1	Australia	soil	ATCC 44658
14Rs AG-2-1	Japan	*Pisum sativum*	Ogoshi PS-4, ATCC 66154
56Rs AG-2-1	Alaska		ATCC 62805
93Rs AG-2-1	Alaska		Carling BS-63
94Rs AG-2-1	Alaska		Carling M-66
95Rs AG-2-1	Alaska		Carling DP-256
96Rs AG-2-1	Alaska		Carling F-6-M
97Rs AG-2-1	Alaska		Carling L-40
121Rs AG-2-1	Alaska		Carling S-74
98Rs AG-2-1	Alaska		Carling KHP-33
99Rs AG-2-1	Alaska		Carling C-6
100Rs AG-2-1	Alaska		Carling BS-15
9Rs AG-2-2	Minnesota	*Daucus carota*	ATCC 44659
15Rs AG-2-2	Japan	*Juncus* sp.	Ogoshi C-96, ATCC 66153
16Rs AG-2-2	Japan	*Beta vulgaris*	Ogoshi B-62
58Rs AG-2-2	Japan		Carling RI-64
27Rs AG-2		*Beta vulgaris*	ATCC 18619
4Rs AG-3		*Phaseolus vulgaris*	ATCC 14006
5Rs AG-3	Minnesota	*Solanum tuberosum*	ATCC 44660
17Rs AG-3			
42Rs AG-3		*Solanum tuberosum*	ATCC 14701
59Rs AG-3	Alaska		Carling W14L, ATCC 62803
101Rs AG-3	Alaska		Carling LG-7
102Rs AG-3	Alaska		Carling F-1-1
103Rs AG-3	Alaska		Carling Scl-8
104Rs AG-3	Alaska		Carling BS-29
105Rs AG-3	Alaska		Carling KHP-30
106Rs AG-3	Alaska		Carling M-8
107Rs AG-3	Alaska		Carling DP-216
108Rs AG-3	Alaska		Carling Scl-39
109Rs AG-3	Alaska		Carling B-37
110Rs AG-3	Alaska		Carling M-33
7Rs AG-4 HG-I	Minnesota		ATCC 44662
18Rs AG-4 HG-I	Japan		Ogoshi RH-74
30Rs AG-4 HG-I	Michigan		ATCC 10154
6Rs AG-4 HG-II	California		ATCC 42127
44Rs AG-4 HG-II			ATCC 14007
45Rs AG-4 HG-II	Virginia		ATCC 10177
29Rs AG-4	Canada		ATCC 48802
60Rs AG-4	Japan		Carling SN-1
10Rs AG-5	Japan	*Glycine max*	Adams & Butler 462, Ogoshi GU-2
61Rs AG-5	Japan		Carling ST-6-1
67Rs AG-5	Japan		Ogoshi C41
35Rs AG-5		*Poa* sp.	L. Burpee Rh 94
31Rs AG-5	Japan	*Beta vulgaris*	ATCC 46138
20Rs AG-6	Japan	soil	Ogoshi GM-10
62Rs AG-6	Japan		Carling NTA-3-1
70Rs AG-6-HG-I	Japan		Ogoshi OHT-1-1
72Rs AG-6-HG-I	Japan		Ogoshi IS-1-1
74Rs AG-6-G-V	Japan		Ogoshi ISH-1-1
75Rs AG-6-G-V	Japan		Ogoshi SO-2-1
21Rs AG-7	Japan	soil	Ogoshi 1535
63Rs AG-7	Japan		Carling 1529
76Rs AG-7	Japan		Ogoshi 1556
33Rs AG-8	Scotland	*Hordeum* sp.	L. Burpee Rh 88
64Rs AG-8	Washington		Carling C1WA
65Rs AG-9	Alaska		Carling S-21, ATCC 62802

Continued on next page

TABLE 1. (*continued from preceding page*)

111Rs AG-9	Alaska		Carling KHP-26
112Rs AG-9	Alaska		Carling M-6
113Rs AG-9	Alaska		Carling BS-35
114Rs AG-9	Alaska		Carling S9-R1
115Rs AG-9	Alaska		Carling F57-M
116Rs AG-9	Alaska		Carling 86-1
117Rs AG-9	Alaska		Carling BS-24
118Rs AG-9	Alaska		Carling V12-M
119Rs AG-9	Alaska		Carling KHP-15
22Rs AG-BI	Japan	soil	Ogoshi SN-1-2, ATCC 66152
66Rs AG-BI	Japan		Carling HTA
78Rs AG-BI	Japan		Ogoshi CA-2-1S
80Rs AG-BI	Japan		Ogoshi TS-2-4S
32Rs AG-?	Ohio	*Zea Mays*	L. Burpee Rh 83

[a] Intraspecific group (ISG) nomenclature after Ogoshi (18) denotes anastomosis group (AG) along with subgroup where known.
[b] State or country of origin.

oratories, Detroit, MI) for 5–10 days at 25 C without shaking, harvested by filtration, lyophilized, and stored at −20 C until use.

DNA extraction and plasmid preparation. Genomic DNA for restriction analysis was prepared by a "miniprep" method (21) modified as follows: After suspension in extraction buffer (0.15 M NaCl, 50 mM Tris, pH 8.0, 10 mM Na_2 ethylenediaminetetraacetic acid [EDTA], 1% sodium dodecyl sulfate [SDS]), samples were extracted once with phenol-chloroform-isoamyl alcohol (25:24:1 parts, respectively) and once with chloroform-isoamyl alcohol (24:1). After treatment with RNAse A (50 μg/ml for 30 min at 37 C), the DNA was reextracted once with chloroform-isoamyl alcohol, adjusted to 2.5 M NH_4 acetate, and precipitated with 2 v of 95% ethanol. The genomic DNA pellets were washed once with 80% EtOH, dried under vacuum, dissolved in 50 μl of 10 mM Tris, pH 8.0, 1 mM Na EDTA, and stored at −20 C until use.

The plasmid pTP1, which contains an entire 8.8-kilobase (kb) rDNA repeat from AG-4 (isolate 7-RS), was obtained by screening a genomic library containing large *Bam*HI fragments from AG-4 of *R. solani* (isolate 7-RS) (28). Plasmid DNA was prepared by the SDS-alkaline lysis method (15).

Restriction analysis. Genomic DNA samples (0.1 μg) were digested simultaneously with *Eco*RI and *Bam*HI (2–3 units each) for 2–3 hr under buffer conditions suggested by the manufacturers (Life Technologies, Inc., Gaithersburg, MD; Promega Biotech, Madison, WI). Samples were electrophoresed into 0.8% agarose gels in TBE buffer (0.89 M Tris, pH 8.0, 0.89 M boric acid, 2 mM Na_2 EDTA, 0.5 μg/ml of ethidium bromide) at 2.5 V/cm for 18 hr. *Hind*III-digested DNA from bacteriophage lambda was included on each gel as a size standard. DNA fragments were transferred onto nylon membranes (GeneScreenPlus, Du Pont Company, Boston, MA) according to manufacturer's instructions. Prehybridization, posthybridization, and hybridization conditions have been described (22). Probe DNA (from pTP1 and bacteriophage lambda) was labeled with ^{32}P-α-2′-deoxyadenosine 5′-triphosphate (400 Ci/mM, Amersham Corp., Arlington Heights, IL) with a nick-translation kit (Life Technologies, Inc.), denatured by boiling, and used for hybridization (2–10 ng/ml). Freshly hybridized membranes were wrapped in plastic film to prevent drying and exposed to Kodak AR-5 film for 6–18 hr at −70 C in the presence of an intensifying screen (Cronex Lightning Plus, Du Pont, Wilmington, DE).

Analysis of RFLP data. DNA fragments detected by autoradiography were sized by comparison with bacteriophage lambda *Hind*III restriction fragments. Within-group variability among restriction phenotypes was estimated by the equitability measure (J), based on the Shannon information measure of diversity (20):

$$J = \frac{\sum_{i=1}^{i=r} p_i \log p_i}{\log r}$$

where p = number of fragments of size i divided by the total number of fragments (a), and r = total number of fragment sizes present. Because the total number of fragment sizes (r) for an entire anastomosis group can never be known with certainty, J probably represents an overestimate of the equitability (evenness) among restriction phenotypes within a sample of isolates. The use of J as an equitability measure in this instance is therefore primarily for descriptive purposes because its statistical properties are not completely understood.

RESULTS

RFLPs in the ribosomal DNA of *R. solani*. Southern blots of DNA from 87 isolates digested with *Eco*RI-*Bam*HI and probed with pTP1 revealed 55 unique restriction patterns (Table 2, Figs. 1–3). Based on length estimates, the number of fragments detected within a single isolate ranged from 4 to 11, with a total of 32 unique fragments distinguished among all isolates. Some isolates contained weakly hybridizing bands that produced much fainter autoradiograph signals than the other bands from the same isolate (indicated by parentheses in Table 2). These weakly hybridizing bands were detected consistently in replicate experiments. We occasionally also noted spurious bands which did not always appear in all replicate experiments (underlined in Table 2).

Two fragments of 1.1 and 0.6 kb in length were present in all isolates examined. Restriction mapping data indicate that these 1.1- and 0.6-kb fragments are located within cistronic portions of the rDNA repeat and therefore would be less likely to vary than other regions (Vilgalys and Gonzalez, *unpublished data*). Many of the larger fragments detected correspond to nontranscribed portions of the rDNA repeat, particularly the large nontranscribed spacer located between tandem repeat units.

Variation between intraspecific groups. Based on the presence or absence of particular restriction fragments, each intraspecific group may be characterized by one or more unique restriction patterns not found in any other intraspecific group. For example, all of the AG-4 isolates produce a unique RFLP characterized by four *Eco*RI-*Bam*HI restriction fragments with lengths of 5.0, 2.1, 1.1, and 0.6 kb (Fig. 1). Other invariant RFLPs also were noted within AG-3 (11 restriction fragments), AG-7 (six fragments, Fig. 2), AG 8 (seven fragments) (Fig. 2), and AG-BI (eight fragments).

Varying numbers of RFLPs were observed in the remaining intraspecific groups. For some intraspecific groups, most RFLPs could be differentiated by the presence or absence of only several restriction fragments. For example, RFLPs from all AG-9 isolates produced five common restriction fragments, whereas three additional fragments were detected in some isolates but not in others (Fig. 2). In a similar manner, most isolates from AG-2-1 could be characterized by the presence of restriction fragments of 7.0, 3.7, and 2.5 kb.

In contrast to those intraspecific groups characterized by a limited number of RFLPs, AG-1 and AG-2 produced a variety of different restriction patterns within each subgroup (Fig. 3). One isolate, which does not belong to any of the currently rec-

TABLE 2. Ribosomal DNA restriction fragment sizes (in kilobases) within anastomosis groups (AGs), produced by simultaneous digestion with *Eco*RI and *Bam*HI

AG 1															
1Rs[a]	2Rs	11Rs	48Rs	49Rs	51Rs	52Rs	54Rs	55Rs	12Rs	34Rs	36Rs	3Rs	13Rs	46Rs	81Rs
												14.0[b]			14.0
		9.0													
7.0	(7.0)[c]						(7.0)					7.0	(7.0)	7.0	
	5.8		5.8	5.8							(5.8)	5.8			
5.4		5.4			5.4		5.4	5.4	(5.4)				5.4	5.4	
															4.4
3.7										(3.7)		3.7		3.7	
3.3		3.3													
2.9	2.9		2.9	2.9	2.9	(2.9)	2.9		2.9	(2.9)	2.9			(2.9)	
2.5	2.5			(2.5)			2.5			2.5	2.5	2.5	2.5	2.5	
		2.3						2.3							
		2.3						2.3							
									(2.1)	2.1	2.1			2.1	
						2.0		2.0							2.0
1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1
0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6

AG 2																
8Rs	14Rs	56Rs	93Rs	94Rs	95Rs	96Rs	97Rs	121Rs	98Rs	99Rs	100Rs	9Rs	15Rs	16Rs	58Rs	27Rs
7.0		7.0		7.0	7.0		7.0		7.0	7.0	7.0	7.0			7.0	7.0
		(5.4)	(5.4)		5.0				(5.4)		5.4	(5.4)		5.4		
	4.8					4.8										
4.4	4.4	4.4	4.4				4.4	4.4	4.4		4.4	4.4			4.4	4.4
3.7		3.7	(3.7)	3.7	(3.7)		3.7		3.7			3.7				
3.3	3.3		3.3		3.3			3.3			3.3	3.3	3.3	(3.3)	3.3	
		2.9			2.9	2.9	2.9	2.9	2.9	2.9	2.9	2.9	2.9	2.9	2.9	2.9
				2.7												
2.5	2.5		2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	(2.5)			2.5	2.5
		2.1						(2.1)	2.1		(2.1)					
					(1.7)				(1.7)			(1.7)	(1.7)	(1.7)	(1.7)	
1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1
0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6

AG 3	AG 4	AG 5					AG 6						AG 7	AG 8	AG BI	AG ?
(all)	(all)	10Rs	61Rs	67Rs	35Rs	31Rs	20Rs	62Rs	70Rs	72Rs	74Rs	75Rs	(all)	(all)	(all)	32Rs
9.0					(8.0)	8.0	8.0		8.0	(8.0)	8.0	(8.0)		8.0	8.0	
			7.0	7.0		7.0										
5.8										5.4	5.4	(5.4)	6.2		6.0	
5.2	5.0		5.0		5.0	(5.0)	5.2		5.2				5.2		5.4	
4.4			4.6	4.6	4.6	4.6				4.8	4.8	(4.8)		4.8	4.0	4.0
4.0								4.4	4.4	4.4						
3.7		2.9	(2.9)		2.9	2.9		3.7		3.7				2.9	2.9	2.9
2.9		2.5	2.5	2.5					2.9		2.9			2.5		
	2.1			2.1		2.1	2.5	2.5		2.5				2.1		
1.7				1.7					1.7	(1.7)		(1.7)			1.7	1.9
			(1.4)										1.3			
1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1		1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1
0.7													0.9			0.8
0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6

AG 9									
65Rs	111Rs	112Rs	113Rs	114Rs	115Rs	116Rs	117Rs	118Rs	119Rs
8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
			6.0		6.0	6.0		6.0	
5.4	5.4	5.4	5.4	5.4	5.4	5.4	5.4	5.4	5.4
		(3.7)			(3.7)	(3.7)	3.7	(3.7)	3.7
2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
			2.1				2.1	2.1	
1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1
0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6

[a]Isolate number from Table 1.
[b]Lines indicate fragments that were not detected in all replicate Southern blot experiments.
[c]Parentheses indicate faint bands that appeared in replicated experiments.

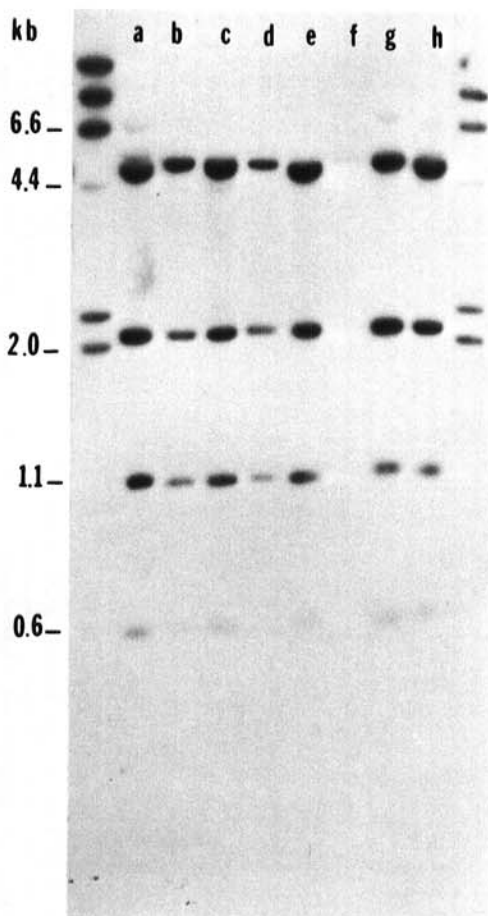

Fig. 1. Southern blot analysis of eight anastomosis group (AG)-4 isolates. Genomic DNA from each isolate was digested simultaneously with *Eco*RI and *Bam*HI, electrophoresed into an 0.8% agarose gel, blotted onto a nylon membrane, and hybridized against a ^{32}P-labeled plasmid rDNA probe. The plasmid probe pTP1 contains a single rDNA repeat cloned from isolate 7Rs (lane b) of AG-4. All of the AG-4 isolates produce the same restriction fragment polymorphism, characterized by four restriction fragments (differences in band intensity are due to varying amounts of target DNA for some isolates). Isolates: a, 6Rs; b, 7Rs; c, 18Rs; d, 29Rs; e, 30Rs; f, 44Rs; g, 45Rs; h, 60Rs. Unmarked lanes contain *Hind*III digests of DNA from phage lambda.

ognized AGs, had a unique RFLP unlike any of the other isolates in our sample. Previous tests with 32-RS indicate that it will not anastomose to any of the standard AG test strains (D. Carling, *personal communication*). Two restriction fragments, 0.8 and 1.9 kb, not found in any other isolate characterize this strain.

Variation within intraspecific groups. The number of unique RFLPs discovered within each intraspecific group varied from one to 12. To compare the amount of intragroup variation among intraspecific groups, we estimated restriction phenotype equitability (J) within each intraspecific group and AG (Table 3). Several intraspecific groups, including AG-3, AG-4, AG-7, AG-8, and AG-BI, have J values of 1.0 because they each produce a single diagnostic RFLP. Most intraspecific groups with values of $J < 1$ could be classified into two groups based on the relative within-group uniformity of their restriction phenotypes. Intraspecific groups AG-1-IA and AG-2-1 showed the greatest amount of restriction pattern variation among their isolates, reflected by lower equitability values of 0.908 and 0.890, respectively. In contrast, increasingly higher J values reflecting lower within-group heterogeneity were observed in AG-1-IB ($J = 0.952$), AG-5 ($J = 0.955$), AG-9 ($J = 0.964$), AG-6 ($J = 0.966$), AG-1-IC ($J = 0.973$), and AG-2-2 ($J = 0.983$).

DISCUSSION

RFLPs and the anastomosis groups of *R. solani*. RFLPs in the ribosomal RNA-coding DNA from 87 isolates of *R. solani* reveal a pattern of variation that is consistent with current intraspecific classifications based on anastomosis. Each of the nine anastomosis groups of *R. solani* and their subgroups is differentiated by one or more RFLPs using only two restriction endonucleases: *Eco*RI and *Bam*HI. Because none of the RFLPs that we detected was shared by isolates from different intraspecific groups, we conclude that restriction polymorphisms of rDNA in each group are unique.

Based on RFLPs, AG-1 isolates appear to be composed of several genetically heterogeneous groups (Fig. 3). Because of its variability in anastomosis behavior, pathogenicity, and cultural morphology, AG-1 has been divided by other workers into three subgroups (17,18). Our results indicate that all three subgroups in AG-1 are highly variable with respect to their rDNA restriction patterns. RFLPs within AG-1 varied considerably from isolate to isolate (Fig. 3). This variability is reflected in the relatively lower estimate of equitability for AG-1 as a whole ($J = 0.910$). Measureable amounts of polymorphism within all three subgroups

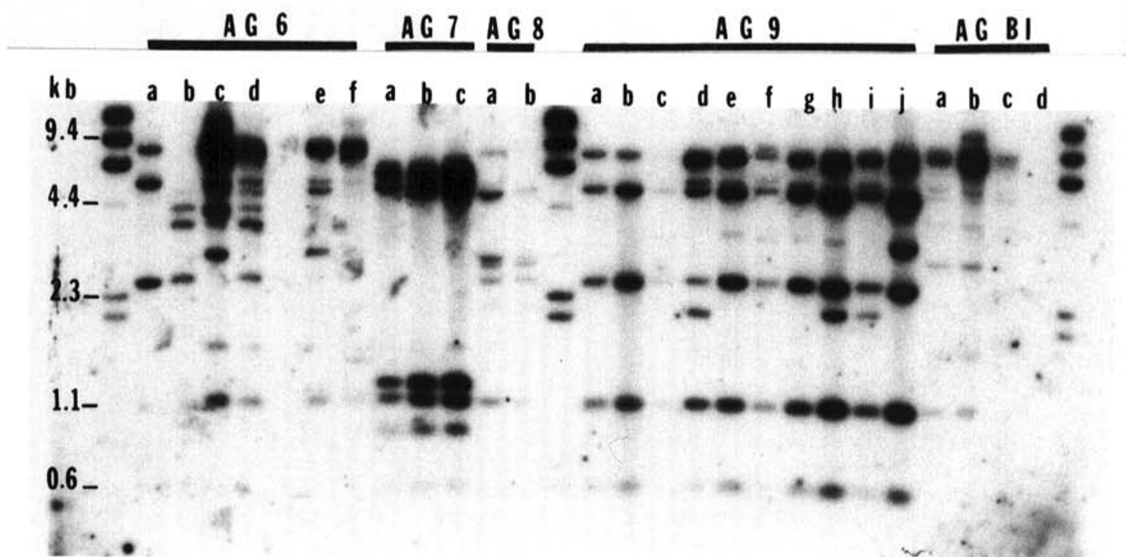

Fig. 2. Restriction fragment length polymorphisms (RFLPs) vary among anastomosis groups (AGs) in *Rhizoctonia solani*. Genomic DNA from each isolate was digested with *Eco*RI/*Bam*HI, and RFLPs were detected by Southern blot analysis as described in the caption for Figure 1. Isolates belong to five anastomosis groups: AG-6: a, 20Rs; b, 62Rs; c, 70Rs; d, 72Rs; e, 74Rs; and f, 75Rs; AG-7: a, 21Rs; b, 63Rs; and c, 76Rs; AG-8: a, 33Rs; and b, 64Rs; AG-9: a, 65Rs; b, 111Rs; c, 112Rs; d, 113Rs; e, 114Rs; f, 115Rs; g, 116Rs; h, 117Rs; i, 118Rs; and j, 119Rs; and AG-BI: a, 22Rs; b, 66Rs; c, 78Rs; and d, 80Rs. Unmarked lanes contain *Hind*III digests of DNA from phage lambda.

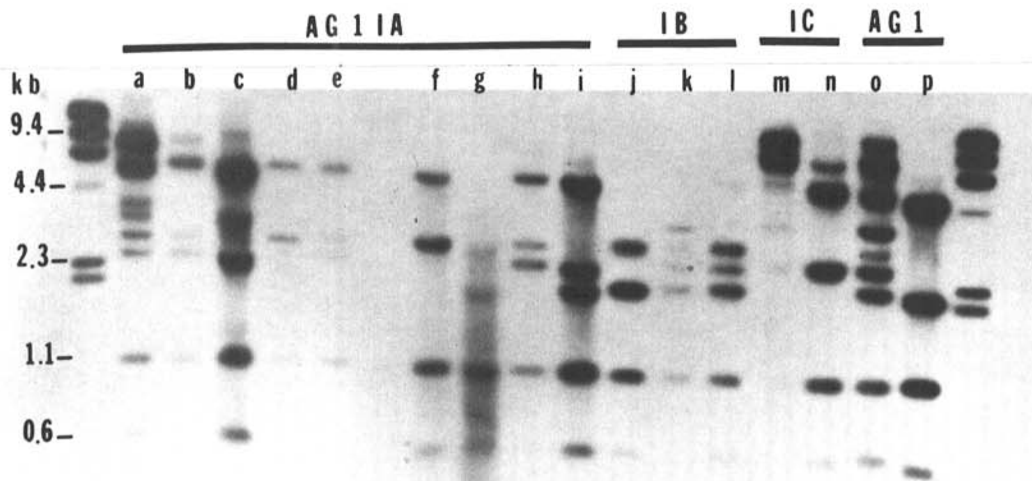

Fig. 3. Restriction fragment length polymorphisms (RFLPs) within anastomosis group (AG)-1 of *Rhizoctonia solani*. Genomic DNA from each isolate was digested with *Eco*RI/*Bam*HI, and RFLPs were detected by Southern blot analysis as described in the caption for Figure 1. Isolates from AG-1 are distinguished by unique RFLPs. Isolates: AG-1-IA: a, 1Rs; b, 2Rs; c, 11Rs; d, 48Rs; e, 49Rs; f, 51Rs; g, 52Rs; h, 54Rs; and i, 55Rs; AG-1-IB: j, 12Rs; k, 34Rs; and l, 36Rs; AG-1-IC: m, 3Rs; and n, 13Rs; and AG-1—subgroup unknown: o, 46Rs; and p, 81Rs). Unmarked lanes contain *Hind*III digests of DNA from phage lambda.

of AG-1 also suggest that genetic differences exist within intraspecific groups as well ($J = 0.908$, 0.952, and 0.973 for AG-1-IA, AG-1-IB, and AG-1-IC, respectively).

A high level of restriction pattern variation also was observed within AG-2. Ogoshi (17) initially divided AG-2 into AG-2-1 and AG-2-2 based on the frequency of anastomosis among isolates. Isolates in AG-2-2 are further subdivided into cultural types which also differ in their requirement for thiamine (17). Our results, based on RFLPs, also demonstrate considerable genetic diversity within different subgroups of AG-2. Although we could not discern a unique RFLP that distinguished either subgroup AG-2-1 or AG-2-2, the subgroups' relatively higher intraspecific group identities ($J = 0.890$ and 0.983, respectively) suggest that they are less heterogeneous than AG-2 as a whole ($J = 0.837$).

Anastomosis groups 3, 7, and 8 each have unique RFLPs that distinguish them from all the other intraspecific groups. These three AGs have not presented any problems in their classification previously, based on either anastomosis behavior or culture morphology (18). The presence of a uniform, diagnostic RFLP within AG-3,- 7, and -8 supports the view that they are genetically homogeneous (18).

AG-4 isolates also are characterized by a single invariant RFLP (Fig. 1). The apparent lack of rDNA variation within AG-4, however, contrasts with what is known about its variable genetics and pathology. AG-4 isolates are pathogenic on a wide variety of host plants worldwide. At least two subgroups have been recognized within AG-4: HG-I and HG-II, which differ in their DNA similarity, cultural appearance, and electrophoretic pattern of their proteins (10,12,27). Further studies of rDNA variation using additional restriction enzymes may reveal additional differences between the two AG-4 subgroups. Although both subgroups show reduced DNA sequence similarity, they each share the typical RFLP characteristic of AG-4. The presence of a common RFLP between subgroups HG-I and HG-II is consistent with DNA/DNA hybridization data which indicate that they are more closely related to one another than to any other AG.

Although AG-5 isolates have not presented problems previously in their classification based on anastomosis, our results showed variation in RFLPs, demonstrated by a lower equitability among AG-5 restriction phenotypes ($J = 0.955$). We often encountered difficulty performing restriction digests using DNA from AG-5 isolates, even after extended efforts to purify the DNA (for example, cesium chloride centrifugation). As a result, the RFLP experiments with each isolate were repeated a minimum of three times to insure their reproducibility. The poor ability of restriction enzymes to digest AG-5 DNA, but not that of other AGs, suggests that the DNA of this group has unique modifications (for example, methylation) not present in other intraspecific groups.

TABLE 3. Equitability of restriction fragment length polymorphisms (RFLPs) within different intraspecific groups of *Rhizoctonia solani*

Intraspecific group[a]	Number of: Isolates[b]	RFLPs detected	Restriction fragments detected (a)	Fragment types (r)	Equitability (J)
AG-1-IA	9	9	48	12	0.908
AG-1-IB	3	3	17	8	0.952
AG-1-IC	2	2	12	8	0.973
AG-1 (all)[c]	16	16	90	14	0.910
AG-2-1	12	12	91	13	0.890
AG-2-2	4	4	33	8	0.983
AG-2 (all)[c]	17	17	124	13	0.837
AG-3	15	1	135	9	1.000
AG-4	8	1	32	14	1.000
AG-5	5	5	33	11	0.955
AG-6	6	6	38	10	0.966
AG-7	3	1	18	6	1.000
AG-8	2	1	14	7	1.000
AG-9	10	6	63	8	0.964
AG-BI	4	1	32	8	1.000

[a]Intraspecific groups identified by Ogoshi (18). AG = anastomosis group.
[b]Number of individual isolates sampled.
[c]Average for entire anastomosis group (all isolates).

AG-6 isolates, like those of AG-4, have been divided into two genetically dissimilar subgroups, HG-I and HG-V, on the basis of genomic DNA hybridization (11). Our results demonstrate variation among the six AG-6 isolates that we examined ($J = 0.966$). Because of our relatively small sample of isolates, however, it is not certain whether the two subgroups could be differentiated on the basis of their RFLPs using the two restriction enzymes from this study.

Carling et al (5) reported on a new anastomosis group from Alaska, AG-9, whose isolates varied in their requirement for thiamine. Although our data show that there is variation in the RFLPs of AG-9 (Fig. 2), most isolates only differ by the presence or absence of one or two restriction fragments.

AG-BI has been reported to be a "bridging isolate" (13) because isolates from this AG have the property to fuse frequently with AG-2-2 and occasionally with AG-2-1, AG-3, and AG-6. Our results indicate that AG-BI isolates have RFLPs that are not like the RFLPS of any of the other AGs, and that the only similarity between AG-BI and AG-2-1, -3, -6, and -2-2 is the presence of a fragment of 1.7 kb. Based on the available evidence, therefore, the present RFLP data alone do not support a specific relationship between AG-BI and any other AG.

One isolate included in the present study (32-RS) does not anastomose with tester isolates from any of the other known AGs. The RFLP produced by this lone isolate was unlike any of the other isolates of *R. solani* and contained two unique restriction fragments. These results suggest again, as in a previous study (27), that 32-RS is distinct from all other known intraspecific groups within *R. solani*.

rDNA variation and evolutionary relationships among intraspecific groups. Evidence for rDNA variation among intraspecific groups suggests that additional study of ribosomal RNA genes should prove useful for determining evolutionary relationships (phylogeny) within the group of *R. solani*. The highly similar restriction patterns observed within several intraspecific groups are strong evidence for genetic homogeneity within those groups. In contrast, the complexity of restriction phenotypes observed in several intraspecific groups will require additional study before it is possible to make conclusions regarding their genetic relationships. The assignment of relatedness based solely on banding patterns, without any knowledge of their mutational basis, could yield erroneous estimates of genetic similarity. Difficulties in interpreting fragment homology often occur when dealing with multiple-fragment phenotypes without any knowledge of their molecular basis, as is the case here. A second limitation of our data set is that only two restriction enzymes were used, both of which may not sample a sufficient number of restriction sites necessary for phylogenetic analysis. For these reasons, we are reluctant to infer relationships among isolates based only on similarity of RFLPs without further mapping studies.

Significance of molecular variation within rDNA. The present study detected considerable differences in restriction patterns of the rDNA within the complex of *R. solani*. These differences were observed among AGs, among different isolates, and even within an individual isolate (based on the results from different experiments).

The complexity of the restriction patterns observed in our survey was unexpected because similar studies of rDNA variation in other fungi usually have detected less variability (8,9,23). Differences in numbers of restriction fragments can be attributed to several causes, including genetic factors (loss and gain of restriction sites, length mutations) and factors that affect restriction enzyme specificity (methylation) and digestability. Partial methylation within the rDNA repeat would be expected to yield a larger number of fragment sizes after digestion with methylation-sensitive enzymes. Experiments with methylation-sensitive and -insensitive restriction enzyme isoschizomers indicate that the rDNA in different intraspecific groups is indeed methylated to varying degrees (Vilgalys and Gonzalez, *unpublished observations*) and that multiple-band phenotypes of many intraspecific groups are probably the result of this difference. Although weak or spurious bands also could be attributed to the inability of some DNA samples to digest to completion, we suspect that, in some cases, restriction pattern differences also may be the result of minor length variation among rDNA repeats. Differences in methylation patterns and length mutations among strains belonging to the same species are commonly observed in many groups of fungi (4,8,9,24,26,30).

The current intraspecific group classification of *R. solani* is based mainly on anastomosis, host specificity, morphology, and nutrition. These classifications have been useful, but they also could be misleading. For example, estimates of restriction pattern equitability within intraspecific groups do not correspond necessarily with host range or pathogenicity. This is demonstrated best by the limited rDNA variation ($J = 1.0$) observed among the widely pathogenic isolates known from AG-4, which is known to contain at least two genetically distinct subgroups. In contrast, AG-1-IA, which is usually specific on a single host (rice), is characterized by a relatively high degree of restriction fragment length polymorphism among the isolates in our sample ($J = 0.908$).

The high degree of rDNA polymorphism that we observe in *R. solani* contrasts with the general pattern often seen in other fungi, where individuals tend to share a single, conserved rDNA restriction pattern (8,9). One possible explanation for intragroup variation in RFLPs is that they may represent the result of genetic divergence within anastomosis groups or their subgroups. We propose that the restriction polymorphism observed is due primarily to evolutionary divergence among AGs, many of which are already known to behave as biological species (1,18). The nonhomogeneity of RFLPs among anastomosis groups of *R. solani* thus supports the common belief that they represent independent evolutionary units (1,18). Higher levels of variation among RFLPs within some intraspecific groups also might suggest that additional genetically divergent groups may be present.

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