Genetics

Genetic Relatedness Among Anastomosis Groups in *Rhizoctonia* as Measured by DNA/DNA Hybridization

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ABSTRACT

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Genetic relationships of 44 multinucleate and binucleate isolates of *Rhizoctonia* were investigated by the technique of heterologous DNA/DNA hybridization. Genomic DNA was isolated from representative isolates of eight different anastomosis groups (AG) in *Rhizoctonia solani*, five different anastomosis groups (CAG) of binucleate *Rhizoctonia*, and several isolates of uncertain anastomosis affinity. Results of more than 200 pairwise DNA hybridization experiments strongly support previous intraspecific groupings based on anastomosis. Isolates belonging to different AG show little DNA sequence complementarity, usually less than 25%. DNA hybridization values within each AG range considerably (30–100%) and indicate that substantial genetic variation is present within some AG. Within AG, reduced DNA hybridization values (< 60%) indicate the presence of genetically divergent subgroups, some of which had previously been recognized on the basis of colony morphology, pathogenicity, or anastomosis behavior. The lowest DNA hybridization values from this study were observed between the multinucleate *R. solani* reference isolates and isolates representing five CAG of binucleate *Rhizoctonia*. Highly reduced levels of DNA hybridization indicate that multinucleate and binucleate *Rhizoctonia* spp. are unrelated and provide genetic evidence to support the taxonomic separation of the teleomorph genera *Thanatephorus* and *Ceratobasidium*.

Additional key words: *Ceratobasidium cornigerum, Thanatephorus cucumeris.*

Isolates of *Rhizoctonia* are known to vary greatly in their morphology, pathogenicity, and physiology, making taxonomic study difficult (25). Cytologically, isolates of *R. solani* Kühn (teleomorph = *Thanatephorus cucumeris* (Frank.) Donk.) can be distinguished by their multinucleate hyphae, in contrast to isolates of *Rhizoctonia* having binucleate cells and a *Ceratobasidium*

teleomorph (25,31,32). Isolates referable to a single teleomorph show substantial intraspecific variation. Talbot (29,30) noted the apparent diversity of *R. solani* isolates grouped under *T. cucumeris* and suggested that further studies may support the recognition of more species within the genus *Thanatephorus*. Based on study of morphology and pathogenicity, several workers have recognized a number of varieties and species in the *R. solani* complex (21,29–32).

Grouping of isolates on the basis of anastomosis provides the best method to date for recognizing intraspecific groups within *Rhizoctonia* (3,21,24). At least nine major anastomosis groups (AG) have been identified in *R. solani* (3,5,7,8,19,20,21,24). Anastomosis has also been useful for delimiting intraspecific groups within other taxa possessing a *Rhizoctonia* anamorph. The discovery of numerous anastomosis groups within the teleomorph genera *Ceratobasidium* (4,22) and *Waitea* (23) now provides a useful method for recognizing intraspecific variation in these fungi as well.

Much evidence already exists to support the view that different AG represent genetically isolated groups and may in fact be good species. Sherwood (28) studied AG-1 through AG-4 and found significant physiological and morphological variation between groups. Although it was not always possible to make absolute distinctions without performing anastomosis tests, he suggested that different AG represent divergent lines of evolution and could be considered as species (28). Numerous subsequent studies have reinforced this view (reviewed in 21). Anastomosis groups of *R. solani* have been shown to differ in their serological characteristics (1), soluble protein electrophoretic profiles (26), and DNA base composition (9), suggesting that there may be major genetic differences between them.

With the recent advent of molecular biology, it is now possible to determine genetic relationships among the different AG based on direct comparison of their nucleic acids. Because DNA reassociation is highly dependent on precise base pairing between homologous nucleotide sequences, heterologous DNA hybridization provides a powerful criterion for determining taxonomic and phylogenetic relationships in many groups of fungi (6,17). Recently, Kuninaga and Yokosawa reported on a series of studies comparing different AG of *R. solani* from Japan, based on a spectrophotometric determination of DNA sequence similarity (10–16). They found high DNA-sequence similarity among isolates belonging within each AG, but only little similarity when different AG were compared to each other. Reduced DNA-sequence similarity among isolates within a single AG was observed in AG-1, AG-2, AG-4, and AG-6, suggesting that microevolutionary groups were present.

In conducting a phylogenetic study on the *Rhizoctonia* complex, I also chose to compare different AG by the method of DNA/DNA hybridization (6). The aims of the present study were to investigate: 1) genetic relationships between different AG of *R. solani*; 2) genetic heterogeneity between cosmopolitan isolates belonging to a single AG; and 3) the affinity of binucleate and multinucleate *Rhizoctonia* isolates placed in different teleomorph genera. At the time this study was undertaken, I was unaware of Kuninaga and Yokosawa's results comparing different AG. Because the isolates and techniques I employed differ from the previous workers, the results from the present study confirm and extend our understanding of genetic diversity within *Rhizoctonia*.

MATERIALS AND METHODS

Isolates. Forty-two isolates of binucleate and multinucleate *Rhizoctonia* were obtained through private and established culture collections. The majority of these isolates had been placed into anastomosis groups by previous investigators (Table 1). Several additional multinucleate isolates that did not anastomose with standard tester isolates from AG-1–AG-5 were included in the study (L. Burpee, *personal communication*).

DNA extraction. Starter cultures were established by transferring actively growing mycelial plugs into 50 ml 1.5% of malt extract, 1 mg/L of thiamine, 50 μg/L of biotin. After five to 10 days' growth at 25 C without shaking, two starter cultures per isolate were macerated for 30 sec at low speed in a sterilized 250-ml blender cup (Eberbach) and used to inoculate two 2-L Erlenmeyer flasks each containing 700 ml of either 1.5% malt extract or potato-dextrose broth (Difco). The cultures were incubated at 25 C on an orbital shaker at 150 rpm for five to 10 days. The mycelial pellets were harvested by vacuum filtration over Miracloth (Calbiochem, Inc.), washed with cold saline buffer (150 mM NaCl, 50 mM Tris, 10 mM Na_2EDTA, pH 8.0), lyophilized, and stored at −20 C until use.

DNA from *Rhizoctonia* was isolated using a modification of the procedure developed by Murray and Thompson (18). Five to 10 grams of lyophilized mycelium was ground in liquid nitrogen using a prechilled mortar and pestle and placed into a 500-ml extraction flask. Before the powder could thaw, 100 ml of hot extraction buffer (60 C) was added. The contents were swirled to avoid clumping and then placed in a 60 C water bath for 5 min with occasional swirling. Each lysate was extracted two times with 50 ml of phenol-chloroform mixture (25 parts liquid phenol, 24 parts chloroform, 1 part isoamyl alcohol, 0.1% w/v 8-hydroxyquinoline), once with chloroform-isoamyl alcohol (24:1 v/v), before precipitation of the nucleic acid. After resuspension into TE buffer (10 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0), final purification of genomic DNA was obtained by ethanol precipitation, followed by one or two series of equilibrium density gradient centrifugations using cesium chloride. High molecular-weight DNA from *Escherichia coli* (strain HB 101) was obtained by the procedure described (6). DNA yields were calculated from absorbance ratios and hyperchromicity of native DNA (6). DNA was stored at −20 C in 15 mM NaCl, 1.5 mM Na_3-citrate, pH 7.0 until use.

DNA reassociation using S1 nuclease. All DNAs were sheared before hybridization by passage through a French Press (Aminco) at 16,000 psi to a modal fragment size of 500 nucleotides (size determined by agarose electrophoresis). Representative isolates were chosen from AG-1, AG-2, AG-3, and AG-4 to serve as radioactively labeled reference isolates (Table 2). DNA from each reference isolate was chemically labeled in vitro with ^{125}I to an average specific activity of 3×10^6 cpm/μg (27). Both radiolabeled and unlabeled DNA samples were denatured by boiling for 5 min and stored at −20 C in 15 mM NaCl, 1.5 mM Na_3-citrate, pH 7.0 until use. DNA hybridization was carried out in duplicate glass microvials, each containing 30 ng of radiolabeled reference DNA and 40 μg of unlabeled DNA in a total volume of 110 μl adjusted to 1.17 M NaCl/1 mM HEPES, pH 7.0, at 65 C for 24–48 hr. The ratios of unlabeled driver DNA to labeled reference DNA in the experiments were in excess of 1,000 to minimize self-renaturation of the labeled reference DNA, and the incubations were carried out to a minimum C_0t of 100 to provide for complete reannealing under the given salt conditions. For each series of hybridizations with a given radiolabeled reference DNA, control vials in which unlabeled DNA had been omitted were also included to determine the extent of self-renaturation of the labeled reference DNA. After hybridization, each sample was treated with S-1 nuclease (Calbiochem) to hydrolyze unreacted single-stranded DNA. The remaining double-stranded DNA was precipitated and collected onto glass fiber filters for counting (6). Radioactivity was counted using an LKB Rack-Gamma counter. After subtracting to correct for self-renaturation of the labeled reference DNA, hybridization was quantitated by dividing the number of counts in each heterologous reaction by the value obtained for the homologous control vials (containing additional unlabeled reference isolate DNA).

RESULTS

DNA sequence similarity in *R. solani*. The results of more than 200 pairwise DNA hybridization experiments are summarized in Table 2. The mean standard error for all replicated homology experiments was less than 3%. Dashed lines in Table 2 indicate those isolates that show highest similarity to the different reference isolates. Most isolates belonging to a single DNA homology group had greater than 50% homology with their respective reference isolate. Groupings based on DNA hybridization in Table 2 show

good correspondence with differences in AG or their subgroups in nearly every case. DNA hybridization values between isolates from different AG were mostly below 25% and indicate that different AG are genetically unrelated (6,17).

Three isolates believed to represent different AG failed to hybridize with their respective reference isolates and instead showed highest similarity with reference DNA from AG-4. Two of these isolates, 17-RS and 25-RS (mistakenly believed at first to represent AG-3 and AG-2, respectively), were later tested for their anastomosis reactions and found to belong in AG-4, as indicated by DNA hybridization (D. Carling, *personal communication*). The third isolate, 31-RS, was also tested for anastomosis and found to belong in AG-5, as originally believed. The discrepancy between anastomosis affinity and DNA hybridization in these cases is best attributed to cross-contamination between cultures rather than inaccuracy of previous anastomosis tests.

Intragroup DNA hybridization. DNA hybridization experiments reveal considerable genetic heterogeneity within several AG. For AG-1 and AG-2, this heterogeneity appears to correspond, at least in part, with different subgroups that have been defined on the basis of pathogenicity and reduced anastomosis frequency (20). For example, DNA hybridization between AG-1-IA, the rice sheath blight pathogens (strains 1-RS, 2-RS, and 11-RA), and AG-1-IC, web blight pathogens (strains 3-RS and 13-RS) of AG-1, ranged between 18–50%. In contrast, DNA hybridization within either of these subgroups was generally much higher (mostly above 94%). Similarly, only 24% DNA hybridization was observed between isolates from different subgroups of AG-2. A higher degree of hybridization was observed between AG-2-1 isolates and their respective reference strain (>60%). All AG-3 isolates showed high hybridization (>67%) with their corresponding reference strain.

AG-4 isolates from this study belong to at least two distinct DNA hybridization groups. The two reference strains (7-RS and 25-RS) have very high hybridization (>90%) with each other and with most other AG-4 strains (17-RS, 18-RS, 30-RS, and 43-RS). Several AG-4 strains (6-RS, 44-RS, and 45-RS) showed reduced DNA hybridization with the two reference strains (30–40%). These low intra-AG hybridization values are still higher than the hybridization of the AG-4 reference strains with other AG (<30%), indicating that AG-4 isolates are still more closely related to one another than with any other AG.

Five multinucleate isolates (33-RS, 34-RS, 35-RS, 36-RS, and 47-RS) of uncertain anastomosis affinity showed little DNA hybridization with any of the labeled reference isolates, with the exception of 34-RS and 36-RS, which showed some similarity (47 and 34%, respectively) with reference DNA from AG-1-IA. Anastomosis tests later revealed that 34-RS and 36-RS belong to AG-1-IB (D. Carling, *personal cummunication*). Since another isolate of AG-1 (-IB) (12-RS) did not show significant

TABLE 1. Isolates of *Rhizoctonia* included in the study with information on their anastomosis (AG or CAG), geographic origin, host, and isolate reference

Isolate	AG/CAG	Origin	Host	Isolate reference
1-RS	AG-1-IA	Louisiana	*Glycine max*	N. O'Neill 3472
2-RS	AG-1-IA	Louisiana	*Oryza sativa*	N. O'Neill 17574
3-RS	AG-1-IC	Canada	*Pinus* sp.	Whitney R43, ATCC 44661
4-RS	AG-3		*Phaseolus* sp.	Papavizas R-1, ATCC 14006, LR 9673
5-RS	AG-3	Minnesota	*Solanum tuberosum*	C. B. Hill P42, ATCC 44660
6-RS	AG-4	California	Conifer	Parmeter C283, LR 9773, ATCC 42127
7-RS	AG-4	Minnesota	*Medicago sativa*	Anderson 140, ATCC 44662
8-RS	AG-2-1	Australia	Soil	Flentjes 48, Parmeter C289, ATCC 44658
10-RS	AG-5	Japan	*Glycine max*	Ogoshi GU-2, Adams & Butler 462
11-RS	AG-1-IA	Japan	*Oryza sativa*	Ogoshi CS-2
12-RS	AG-1-IB	Japan	*Beta vulgaris*	Ogoshi B-39
13-RS	AG-1-IC	Japan	Soil	Ogoshi F-2
14-RS	AG-2-1		*Pisum sativum*	Ogoshi PS-4
15-RS	AG-2-2	Japan	*Juncus* sp.	Ogoshi C-96
16-RS	AG-2-2	Japan	*Beta vulgaris*	Ogoshi B-62
17-RS	AG-4			
18-RS	AG-4	Japan	*Beta vulgaris*	Ogoshi RH-74
19-RS	AG-5	Japan	*Glycine max*	Ogoshi GM-10
20-RS	AG-6	Japan	Soil	Ogoshi OT-2-1
22-RS	AG-BI	Japan	Soil	Ogoshi SN-1-2
23-RS	AG-1		*Phaseolus* sp.	W. R. Cuelho 630, ATCC 52660
24-RS	AG-2		*Brassica* sp.	J. E. Kotila R-276, ATCC 10159
25-RS	AG-4			
26-RS	AG-2		*Beta vulgaris*	J. E. Kotila R-255, ATCC 10176
27-RS	AG-2		*Phaseolus vulgaris*	R. T. Sherwood W-22, ATCC 18619
28-RS	AG-3		*Solanum tuberosum*	J. E. Kotila R-72, ATCC 10182
29-RS	AG-4		*Picea glauca*	E. E. Butler 41, S. Whitney 41, ATCC 48802
30-RS	AG-4			E. E. Butler 113, Vaartaja, ATCC 48803
31-RS	AG-5		*Beta vulgaris*	S. Naito Rh 184, ATCC 46138
33-RS	AG-8[a]	Scotland	*Hordeum vulgare*	L. Burpee RH 88
34-RS	AG-1-IB[a]	Washington, DC	*Poa* sp.	L. Burpee RH 90
35-RS	AG-5[a]		*Poa* sp.	L. Burpee RH 90
36-RS	AG-1-IB[a]	Pennsylvania	*Poa* sp.	L. Burpee RH 46
37-RS	CAG1	Pennsylvania	*Agrostis* sp.	L. Burpee Bn 1/T, ATCC 44233
38-RS	CAG5	Georgia	*Cucumis* sp.	L. Burpee Bn 37/T, Rhs 53
39-RS	CAG3	Georgia	*Arachis* sp.	L. Burpee Bn 31/T, Rhs 14
40-RS	CAG2	Ohio		L. Burpee Bn 4/T, ATCC 34969
41-RS	CAG4	Georgia	*Glycine* sp.	L. Burpee Bn 38/T, Rhs 68
42-RS	AG-3		*Solanum tuberosum*	K. R. Barker 24, ATCC 14701
43-RS	AG-4	Michigan	*Medicago sativa*	J. E. Kotila R-40, ATCC 10154
44-RS	AG-4		*Beta vulgaris*	Papavizas R-5, ATCC 14007
45-RS	AG-4	Virginia	*Beta vulgaris*	J. E. Kotila R-355, ATCC 10177
46-RS	AG-1		*Brassica oleracea*	K. R. Barker 29, ATCC 14703
47-RS	?[b]		*Phaseolus* sp.	K. R. Barker 27, ATCC 14702

[a] The anastomosis affinities of these solates were not known at the outset of the study; anastomosis grouping was determined afterward.
[b] Anastomosis grouping uncertain.

hybridization with reference DNA from AG-1-IA or AG-1-IC, it is likely that AG-1-IB represents a third DNA hybridization group from AG-1.

DNA hybridization between multinucleate and binucleate isolates. Little or no DNA hybridization was evident between the multinucleate reference strains used in this study and the binucleate isolates that correspond with different CAG. DNA hybridization to an unrelated control DNA (from *E. coli* HB 101) was almost zero, providing a background hybridization level for comparing relationships between the multinucleate and binucleate groups. The relative level of DNA hybridization of binucleate versus multinucleate isolates with the six multinucleate reference strains was tested by a nonparametric rank-sum test and found to be significant (Kruskal-Wallis test, $\chi^2_{(df=1)} = 20.7$, $p = 0.0001$). Thus, DNA hybridization data suggest little genetic relationship between AG and CAG used in this study.

TABLE 2. DNA hybridization among anastomosis groups (AG) from *Rhizoctonia solani* and several binucleate *Rhizoctonia* (CAG)

Unlabeled DNA[a] from:		Isolate and AG type used as reference DNA:					
AG type	Isolate	2-RS 1-IA	3-RS 1-IB	8-RS 2-1	4-RS 3	7-RS 4	25-RS 4
1	1-RS	100	23	9	3	18	12
1	2-RS	100	21	11	5	15	13
1	11-RS	100	18	6	0	12	10
1	46-RS	57	28	14	8	19	30
1	3-RS	36	100	11	9	16	14
1	13-RS	50	94	17	18	21	21
1	12-RS	26	16	4	5	7	8
2-1	8-RS	23	16	100	12	20	17
2-1	14-RS	19	18	60	25	24	25
2	24-RS	21	13	60	43	20	20
2	26-RS	15	10	71	26	28	26
2-2	15-RS	25	14	24	23	23	24
2-2	16-RS	17	15	24	25	17	17
3	4-RS	19	24	17	100	24	22
3	5-RS	19	16	25	100	26	18
3	28-RS	20	13	2	67	22	20
3	42-RS	11	13	20	95	16	14
4	6-RS	18	24	19	18	40	39
4	7-RS	14	13	13	4	100	96
4	18-RS	18	14	15	9	100	93
4	30-RS	27	21	23	23	100	100
4	43-RS	ND[b]	17	22	ND	92	ND
4	25-RS	16	16	18	14	100	100
4	17-RS	14	ND	10	16	68	ND
4	44-RS	29	12	16	8	32	30
4	45-RS	34	19	17	18	37	37
5	31-RS	29	21	27	30	59	62
5	10-RS	20	14	17	24	19	21
5	19-RS	43	15	22	33	22	21
6	20-RS	17	9	12	7	14	12
BI	22-RS	29	17	24	23	15	18
?	33-RS	16	8	18	21	12	11
?	34-RS	47	25	6	6	12	17
?	35-RS	17	19	23	3	20	20
?	36-RS	34	24	7	11	10	10
?	47-RS	29	18	22	9	23	25
CAG1	37-RS	10	4	3	0	5	4
CAG2	40-RS	ND	5	4	ND	6	7
CAG3	39-RS	24	12	12	11	21	15
CAG4	41-RS	ND	20	17	ND	27	27
CAG5	38-RS	13	10	10	0	17	13
E. coli	HB101	0	0	2	0	4	4

[a] Reference DNA was labeled with ^{125}I and hybridized with at least 1000-fold excess of unlabeled DNA under the conditions given in the text. DNA hybridization was assayed using S1-nuclease (6). Normalized hybridization values were determined as the percentage of heterologous: homologous reassociation, corrected for background and self-renaturation of the radiolabeled reference DNA. Values represent the average of two determinations.

[b] ND = hybridization value not determined.

DISCUSSION

The results of heterologous DNA hybridization experiments strongly support current views concerning genetic and taxonomic relationships in *Rhizoctonia*. DNA hybridization groups recognized in this study correspond well with previous groupings based on anastomosis and present further evidence that anastomosis is indicative of genetic relatedness. Of five multinucleate strains that did not anastomose with any of the standard AG tester strains from AG 1 to 5, four showed little or no hybridization with any of the reference strains used in this study. Two strains subsequently identified as belonging to AG-1-IB showed limited hybridization with reference DNA from AG-1-IA, which may suggest some relationship.

DNA hybridization between isolates of different anastomosis groups was usually less than 25%. In contrast, intragroup DNA hybridization was greater (>57%) for isolates hybridized against reference strains from AG-1-IA, AG-1-IC, AG-2-1, and AG-3. The reduced amount of DNA hybridization between certain isolates within AG-1 and AG-2 corresponds well with subgroupings recognized by other researchers (14). The results shown in Table 2 also show that there are at least two DNA hybridization groups present in AG-4. Most AG-4 isolates show high DNA hybridization with either AG-4 reference strain (90–100%), in contrast to five isolates that gave considerably lower hybridization values (30–62%). This low level of hybridization is particularly surprising, since AG-4 isolates may be crossed in vitro and have been demonstrated to be completely interfertile (2,3). Further study of interfertile strains having low hybridization could provide useful information about the nature of gene flow within AG and the importance of intersterility barriers in evolution of these fungi.

A series of previous studies by Kuninaga and Yokosawa used a spectrophotometric technique to study DNA sequence hybridization within each of several AG from Japan (10–16). Those results demonstrated very high DNA hybridization between isolates belonging within AG, but only negligible similarity between different AG. Reduced levels of DNA similarity within AG-1, AG-2, AG-4, and AG-6 were also reported. Their results are in exact concordance with this study. The results from the present study, which used isotope-labeled DNAs to directly measure sequence hybridization, thus confirms previous reports about independent evolution of different AG within *R. solani*.

The range of intragroup hybridization values reported in this study is greater than in the previous reports, which found relatively high intragroup DNA hybridization, mostly above 90% (10–16). In this study, DNA hybridization groups showed hybridization levels as low as 57%. The difference in DNA hybridization ranges observed in this study and the previous ones may be attributable to the different methods used for determining sequence hybridization. The spectrophotometric assay for DNA sequence similarity used by Kuninaga and Yokosawa measures DNA similarity based on rate of reassociation, yielding an indirect measure of DNA sequence similarity. In contrast, assaying heterologous hybridization using a labeled reference DNA provides a direct measure of sequence relatedness between different genomes. Both methods have been demonstrated to give similar results (6), and the close agreement between the previous study and the present one demonstrates the consistency of a DNA-based approach for measuring genetic relationships among AG. An alternative explanation as to why I observed a higher level of intragroup variation may be the geographic diversity of cultures used in this study. Because strains used in the previous studies all originated in Japan, the cosmopolitan sample of material in this study may better represent genetic diversity within different AG. Additional studies aimed at detecting geographic variation could yield interesting information about microevolution within AG.

Representative isolates of several binucleate *Rhizoctonia* sp. anastomosis groups (CAG) were found to have significantly lower DNA hybridization with the reference strains than did the multinucleate *R. solani*. These results support taxonomic conclusions regarding phylogenetic relationships of *R. solani* with other *Rhizoctonia* isolates. Cytologically, isolates of *R. solani*

(teleomorph = *T. cucumeris*) can be distinguished by their multinucleate hyphae, in contrast to other *Rhizoctonia* isolates having binucleate cells (teleomorph = *Ceratobasidium* spp.). Results of my DNA hybridization experiments support taxonomic separation of *Thanatephorus* and *Ceratobasidium*, which had been previously based on cytology and basidial morphology (29,32).

Evidence for genetic differences within *R. solani* now provides additional evidence that different AG are discrete evolutionary units and probably merit some sort of taxonomic status. Several AG and their subgroups had been recognized as good species by numerous previous workers (21,29,30). Taxonomic revision of intraspecific groups in *R. solani* and other *Rhizoctonia* may seem more desirable as we understand more about the enormous genetic diversity present in these fungi. Further study of the extensive genetic variation within some groups may also provide information about their evolution, particularly regarding the association between anastomosis, interfertility, and genetic divergence.

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