Genetic Diversity of *Rhizoctonia solani* Anastomosis Group 2

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ABSTRACT

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Genetic diversity among 70 isolates of *Rhizoctonia solani* anastomosis group (AG) 2 was studied by isozyme polymorphism and DNA restriction analyses. Five genetically distinct intraspecific groups (ISGs) (ISG 2A [9 isolates], 2B [13 isolates], 2C [22 isolates], 2D [14 isolates], and 2E [12 isolates]) were distinguished within AG 2 based on binary characters of isozyme alleles and restriction sites by using numerical cladistic analysis with isolate ISG 1C19 (AG 1-IC type) as an outgroup. Isozyme phenotypes were developed for these groups. Some isozyme alleles and loci were identified as useful molecular markers for population studies. DNA restriction mapping showed that these groups shared the same gene of mitochondrial small subunit rDNA and a high level of similarity in nuclear rDNA for internal transcribed spacers (ITS), including the 5.8S ribosomal RNA gene. The polymerase chain reaction (PCR) amplified DNA fragments varied for the five groups. In the ITS region, groups 2A and 2E had the same lengths (0.69 kb) but differed at one *Eco*RI site; groups 2B, 2C, and 2D had the same lengths (0.74 kb) but differed from one another by at least one restriction site, *Msp*I or *Taq*I. ISG 2A corresponded to AG 2-1; 2B to AG 2-2 IIIB; 2C to AG 2-2 IV; 2D to a newly defined group related to AG 2-2; and 2E to a newly defined group, previously identified as AG 2-2, related to AG 2-1. When evaluated from a single source and developed at different levels, ISGs 2A and 2E were closely related, and ISGs 2B–D were closely related. The merits of using isozyme and DNA analyses for the study of *R. solani* populations or ISGs are discussed.

Additional keywords: molecular differentiation, phylogeny, *Thanatephorus cucumeris*.

Rhizoctonia solani Kühn (*Thanatephorus cucumeris* (Frank) Donk) is a soilborne plant pathogen with a wide host range (15). As a collective species or a species complex, it is made up of divergent populations (1,29,42). The lack of understanding of the relationships among populations within the species has hampered other studies on the fungus, including studies of disease control methods. A method based on anastomosis group (AG) has been used for identification and classification of isolates of *R. solani* (3,39,43,45,47,48). Eleven AGs (AG 1–10 and AG BI) have been recognized, including some nonpathogenic groups (12,16,24,36, 39,40,50). Although the AG system does not fully describe characteristics of various populations, it has been useful in identifying the various groups that cause *Rhizoctonia* diseases and the related saprophytic soil populations.

R. solani AG 2, consisting of economically important plant pathogens, has been divided into two subgroups, AG 2-1 and AG 2-2, on the basis of the frequency of anastomosis among its isolates (39). Isolates in AG 2-1 are autotrophic, and those in AG 2-2 are auxotrophic for thiamine. Two ecological types within AG 2-2, AG 2-2 IIIB (rush type) and IV (root rot type), have been recognized on the basis of their pathogenicity on mat rush (*Juncus effusus* L. var. *decipiens*) and sugarbeet (*Beta vulgaris* L.), respectively (39). Separation of these subgroups was supported by DNA guanine-cytosine content, DNA-DNA reassociation, and isozyme polymorphism studies (23,25,28) but not by serological, total protein, or single enzyme studies (2,32,44). These types also have been placed in separate intraspecific groups (ISGs) (39). However, the use of ISGs is uncertain because of the lack of genetic evidence (50). Restriction fragment length polymorphism (RFLP) of ribosomal DNA and RNA-RNA hybridization studies indicated divergent variations among isolates of *R. solani*, and no subgroup relationships within AG 2 were recognized (5,19,56).

Anastomosis grouping is a convenient but not an ideal method for classification of isolates of *R. solani*. Misidentification occurs because of the varied frequency of hyphal fusion. For example, within AG 2 it is often difficult to distinguish between isolates of AG 2-1 and AG 2-2 by hyphal fusion, and many populations are left unclassified (50). In addition, hyphae of isolates in AG 2 fuse with isolates in AG 8 or AG BI (50). The current AG system needs genetic verification and adjustment.

An isozyme is a direct expression of genotype and can be used as an indicator of genetic relationships within related populations (4,8,33,34,38). Genes coded for ribosomal RNA are conserved elements containing component sequences with different evolutionary rates, which are phylogenetically and taxonomically informative (7,20,26). Polymorphism of rDNA has been used to study genetic relationships of populations of other fungi (21,37). Oligonucleotides can be extended in the presence of polymerase and, thus, synthesize DNA. The technology of polymerase chain reaction (PCR) provides a convenient means of obtaining sufficient amounts of DNA for molecular studies. The method has been demonstrated as an effective and reliable approach for genetic studies of higher plants and animals as well as fungi (17).

Using starch gel electrophoresis and PCR technology, we investigated polymorphism of isozyme and DNA restriction fragments of populations of *R. solani* from divergent geographic and host origins. The objectives of this study were to examine the genetic diversity among populations of *R. solani* AG 2 and to evaluate the current classifications of the subgroups within AG 2. A preliminary report has been published (29).

MATERIALS AND METHODS

Fungal isolates. Seventy isolates of *R. solani* AG 2 from a range of geographic areas and hosts and isolate 1C19 (AG 1 IC type), which served as an outgroup for data analysis, were studied (Table 1). All isolates were maintained on potato-dextrose agar (PDA) (Difco Laboratories, Detroit, MI) culture plates or test tube slants at 20 or 4 C, respectively. Cultures were transferred every 6 mo. To ensure culture purity, each isolate was initially recovered from 9-cm PDA culture plates at 28 C and transferred

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TABLE 1. Identification number (ID), anastomosis group (AG), original host of isolation, origin, and source of isolates of *Rhizoctonia solani*

ID number	AG and type	Host	Origin	Original isolate designation	Source
2A01	2-1	Unknown	Alaska	F56L	D. C. Carling
2A02	2-1	Strawberry	Japan	FC-2	A. Ogoshi
2A03	2-1	Barley	Japan	HV-1	A. Ogoshi
2A04	2-1	Tulip	Japan	TG-1	A. Ogoshi
2A05	2-1	Unknown	Australia	48	N. A. Anderson
2A06	2-1	Soil	Japan	HB618-28	A. Ogoshi
2A08	2-1	Soil	Japan	SH-20	A. Ogoshi
2A09	2-1	Pea	Japan	PS-4s	A. Ogoshi
2A10	2-1	Anemone	Japan	AN-1	A. Ogoshi
2B03	2-2 IIIB	Maize	Georgia	Rhs36	D. R. Sumner
2B09	2-2 IIIB	Mat rush	Japan	C330	A. Ogoshi
2B11	2-2 IIIB	Soybean	Illinois	61D-3	Z. L. Liu
2B12	2-2 IIIB	Soybean	Illinois	65L-2	Z. L. Liu
2B81	2-2 IIIB	Sugarbeet	Japan	BV-43	A. Ogoshi
2B82	2-2 IIIB	Monochoria	Japan	C-61	A. Ogoshi
2B84	2-2 IIIB	Locust	Japan	C-104	A. Ogoshi
2B85	2-2 IIIB	Soil	Japan	C-109	A. Ogoshi
2B87	2-2 IIIB	Rice	Japan	C-116	A. Ogoshi
2B88	2-2 IIIB	Rice	Japan	C-148	A. Ogoshi
2B89	2-2 IIIB	Rice	Japan	C-354	A. Ogoshi
2B90	2-2 IIIB	Rice	Japan	C-355	A. Ogoshi
2B91	2-2 IIIB	Unkown	Japan	C-116S	A. Ogoshi
2C01	2-2	Carrot	USA	H3-77	N. A. Anderson
2C02	2-2	Sugarbeet	Ohio	H500	L. J. Herr
2C04	2-2	Sugarbeet	California	454	D. E. Carling
2C05	2-2	Sugarbeet	Minnesota	133-A-4	C. E. Windels
2C06	2-2	Sugarbeet	Minnesota	86-42-4	C. E. Windels
2C07	2-2	Sugarbeet	Minnesota	86-62-1	C. E. Windels
2C08	2-2	Sugarbeet	Minnesota	86-72-7	C. E. Windels
2C10	2-2 IV	Sugarbeet	Japan	RI-64	A. Ogoshi
2C21	2-2	Sugarbeet	N. Dakota	01-S-25	C. E. Windels
2C22	2-2	Sugarbeet	Minnesota	12-T-1	C. E. Windels
2C23	2-2	Sugarbeet	Minnesota	12-T-9	C. E. Windels
2C24	2-2	Sugarbeet	Minnesota	12-T-10	C. E. Windels
2C25	2-2	Sugarbeet	Minnesota	13-T-8	C. E. Windels
2C26	2-2	Sugarbeet	Minnesota	18-S-22	C. E. Windels
2C27	2-2	Sugarbeet	Minnesota	18-T-2	C. E. Windels
2C28	2-2	Sugarbeet	Minnesota	18-T-9	C. E. Windels
2C29	2-2	Sugarbeet	Minnesota	85-34-A3	C. E. Windels
2C30	2-2	Sugarbeet	Minnesota	86-12-7	C. E. Windels
2C31	2-2	Sugarbeet	Minnesota	86-49-11	C. E. Windels
2C32	2-2	Sugarbeet	Minnesota	86-59-3	C. E. Windels
2C33	2-2	Sugarbeet	N. Dakota	86-76-1	C. E. Windels
2C34	2-2	Sugarbeet	Minnesota	88-32-4	C. E. Windels
2D61	2-2	Turfgrasses	Texas	PFC 1	P. F. Colbaugh
2D62	2-2	Turfgrasses	Texas	PFC 2	P. F. Colbaugh
2D63	2-2	Turfgrasses	Texas	PFC 13	P. F. Colbaugh
2D64	2-2	Turfgrasses	Texas	PFC 16	P. F. Colbaugh
2D65	2-2	Turfgrasses	Texas	PFC 17	P. F. Colbaugh
2D66	2-2	Turfgrasses	Texas	PFC 21	P. F. Colbaugh
2D67	2-2	Turfgrasses	Texas	PFC 33	P. F. Colbaugh
2D68	2-2	Turfgrasses	Texas	PFC 14	P. F. Colbaugh
2D69	2-2	Turfgrasses	Texas	PFC 3	P. F. Colbaugh
2D70	2-2	Turfgrasses	Texas	PFC 9	P. F. Colbaugh
2D71	2-2	Turfgrasses	Texas	PFC 20	P. F. Colbaugh
2D72	2-2	Turfgrasses	Texas	PFC 11	P. F. Colbaugh
2D73	2-2	Turfgrasses	Texas	2-23-017	R. K. Jones
2D74	2-2	Turfgrasses	Texas	PFC 10	P. F. Colbaugh
2E41	2-2	Tobacco	N. Carolina	Sampson	H. D. Shew
2E42	2-2	Tobacco	N. Carolina	Craven	H. D. Shew
2E43	2-2	Tobacco	N. Carolina	Columbus	H. D. Shdw
2E44	2-2	Tobacco	N. Carolina	Wilson	H. D. Shew
2E45	2-2	Tobacco	N. Carolina	Edgecombe	H. D. Shew
2E46	2-2	Tobacco	N. Carolina	Carteret	H. D. Shew
2E47	2-2	Tobacco	N. Carolina	Guilford	H. D. Shew
2E48	2-2	Tobacco	N. Carolina	Granville	H. D. Shew
2E49	2-2	Tobacco	N. Carolina	Halifax	H. D. Shew
2E50	2-2	Tobacco	N. Carolina	1602	H. D. Shew
2E51	2-2	Tobacco	N. Carolina	1606	H. D. Shew
2E52	2-2	Tobacco	N. Carolina	1609	H. D. Shew

by two sequential hyphal tip transfers at 48-h intervals. No cultures with sectoring were used.

Extraction of enzymes. Three 5-mm-diameter PDA plugs from a culture of each isolate were placed separately in 50 ml of autoclaved potato-dextrose broth (PDB) (Difco) in 250-ml Erlenmeyer flasks and incubated on a rotary shaker for 6 days at 120 rpm at 25 ± 1 C. Mycelia of each isolate were harvested by filtration through cheesecloth and pressed dry on paper towels. A 150-mg dry mycelial sample was rinsed and suspended in 1.5 ml of an extraction buffer (0.1 M Tris, 10.0 mM KCl, 0.1 mM $MgCl_2$, 1.0 mM ethylene diaminetetraasorbic acid, 14.0 mM β-mercaptoethanol, 0.1 mM asorbic acid, and polyvinylpyrrolidone [0.2 g/ml of buffer], pH 7.0) (29). The mycelial suspension was then homogenized with a polytron (Ultrasonics, Germany) at high speed for 1 min and then sonicated (Branson 450 sonifier; Branson Ultrasonics Corp., Danbury, CT) twice for 10 s with a 10-s interval. The sample was processed in a minivial or a microcentrifuge submerged in ice water and kept on ice. The preparation was then centrifuged for 30 min at 13,000 *g* at 4 C. The supernatant was stored at −80 C and used within 1 mo.

Starch gel electrophoresis. We studied 10 enzymes with 13% starch gels (Sigma Chemical Co., St. Louis, MO) by using standard procedures (Table 2) (13,28,34,46,51). Isolate 2C10 showed a common band with most of the enzymes studied and was used as a reference marker at both margin lanes of each gel. The relative mobility of isolate 2C10 to the gel anode was designated 100. This isolate showed two bands for the three enzymes, acid phosphatase, esterase, and 6-phosphoglucomutate dehydrogenase. The band with slower mobility was designated 100. Each single band was considered a discrete character for each enzyme (10) and was measured for relative mobility to the gel anode with the marker. Five electrophoretic runs were completed for two sets of sample extractions. Repeatable banding patterns were obtained. A handmade comb of gel-slab-backing filter paper was used to load the enzymes. We pressed the extraction-soaked sample comb between paper towels to remove excess enzyme and loaded the sample on freshly prepared and prechilled starch gels by using a corresponding buffer (Table 2).

TABLE 2. Enzyme systems with enzyme commission (EC) number used for evaluation of the relationships among isolates of *Rhizoctonia solani* anastomosis group 2

Enzyme	Abbreviation	EC	Buffer[a]
Acid phosphatase	ACP	3.1.3.2	1
Aconitase	ACO	4.7.1.3	2
Esterase	EST	3.1.1.1	3
Hexose kinase	HXK	2.7.1.1	1
Isocitric dehydrogenase	IDH	1.1.1.42	1
Leucine amino peptidase	LAP	3.4.11.1	3
Malate dehydrogenase	MDH	1.1.1.37	2
Phosphoglucomutase	PGM	2.7.5.1	3
Phosphoglucoisomerase	PGI	5.3.1.9	3
6-Phosphogluconate dehydrogenase	6-PGD	1.1.1.44	1

[a] 1 = Tris-citrate (0.223 M Trisma base, 0.086 M citric acid, adjusted with 1.0 N NaOH, pH 7.5) (51); 2 = citrate-morpholine (0.04 M citric acid [anhydrous], adjusted to pH 6.5 with N-[3-aminopropyl]-morpholine for electrode buffer, pH 6.0 for gel buffer) (13,28); 3 = Tris-borate-EDTA (0.5 M Tris, 0.65 M boric acid, 0.02 M NaEDTA, adjusted to pH 8.1 with 1.0 N NaOH) (46).

Isolation of DNA. Four isolates of *R. solani* from sugarbeet, 2C02, 2C05, 2C06, and 2C08, were lost during the DNA studies, and 66 isolates were investigated for DNA polymorphism. We ground separately fresh dried mycelia of each isolate to a fine powder in liquid N in a prechilled mortar by using a pestle. A powder sample of 150 mg was suspended in 700 μl of lysis buffer (50 mM Tris-HCl [pH 7.2], 50 mM EDTA, 3% sodium dodecyl sulfate, 1% 2-mercaptoethanol) and homogenized with a Vortex mixer. Isolation of genomic DNA was done by the procedure of Lee and Taylor (27). The contents of each tube were incubated for 1 h at 65 C and shaken by hand at 20-min intervals. We added a chloroform/phenol (1:1) mixture of 700 μl to precipitate cellular debris and proteins. After centrifugation for 10 min at 12,000 *g*, the upper aqueous phase (containing DNA) was transferred into a clean tube. The DNA was precipitated by the addition of 0.54 vol of isopropanol in the presence of sodium acetate, rinsed with 70% ethanol, dried on a paper towel, and redissolved in 100 μl of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA).

Polymerase chain reaction (PCR). Six primers were used for amplification of the nuclear rDNA region of internal transcribed spacers (ITS), including the 5.8S gene and mitochondrial small subunit rDNA for populations of *R. solani* (Table 3) (57). The primers were synthesized by Operon Technologies Inc. (Alameda, CA). The PCR was done in 500-μl microcentrifuge tubes containing 60 μM each of dATP, dCTP, dGTP, and dTTP, 0.8 μM each of a pair of primers, and 2.5 units of *Taq* DNA polymerase in 1× amplification buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.01% gelatin) (17,18). Filtered pipet tips (USA/Scientific Plastics, Ocala, FL) were used to avoid cross contamination. A DNA template negative control with all reaction reagents was included in each set of PCR reactions. One drop of light mineral oil (Sigma Chemical Co.) was layered on top of each reaction mixture to prevent evaporation. Various temperature profiles were used (Table 3). We controlled the temperature cycles by using a DNA thermal cycler controller (MJR Research Inc., Watertown, MA). The effectiveness of DNA amplification was monitored by running standard agarose gel electrophoresis, and the amplified DNA was visualized under UV light.

The amplified nuclear ribosomal DNA, including 5.8S rDNA and the two regions of internal transcribed spacers (ITS) on both sides, and a small portion of the 18S and 28S subunit rDNA (mostly the primer region) were referred to as ITS and were amplified with primers ITS1 and ITS4. The spacer connected with 18S rDNA, amplified with primers ITS1 and ITS2, was referred to as ITSa, and the spacer between 5.8S and 28S, including most of 5.8S rDNA, amplified with primers ITS3 and ITS4, was referred to as ITSb.

Restriction endonuclease analysis. Approximately 0.25 μg of PCR-amplified DNA of each isolate was digested separately with 11 restriction enzymes, *Bam*HI, *Eco*RI, *Rco*RV, *Hae*III, *Hin*fI, *Kpn*I, *Mbo*I, *Msp*I, *Pst*I, *Taq*I, and *Xho*I, under conditions recommended by the manufacturer (Bethesda Research Laboratories, [BRL] Inc., Gaithersburg, MD). The DNA restriction fragments were separated by horizontal agarose gel electrophoresis (composite gel containing 1% SeaKem LE agarose and 1%

TABLE 3. Oligonucleotide primers and their sequences used for amplification of portions of nuclear and mitochondrial RNA genes by polymerase chain reaction for *Rhizoctonia solani*

Primer	Sequence	T_m C[a]	Annealing temperature (C)[b]
ITS1 (19 mer)	5′-TCCGTAGGTGAACCTGCGG-3′	62	57
ITS2 (20 mer)	5′-GCTGCGTTCTTCATCGATGC-3′	62	57
ITS3 (20 mer)	5′-GCATCGATGAAGAACGCAGC-3′	62	57
ITS4 (20 mer)	5′-TCCTCCGCTTATTGATATGC-3′	58	53
MS1 (25 mer)	5′-CAGCAGTCAAGAATATTAGTCAATG-3′	68	63
MS2 (22 mer)	5′-GCGGATTATCGAATTAAATAAC-3′	58	53

[a] Calculated according to Thein and Wallace (18).
[b] Calculated according to Innis and Gelfand (18).

NuSieve GTG agarose; FMC BioProducts, Rockland, MN) prepared with Tris-borate-EDTA buffer (89 mM Tris-HCl, 89 mM boric acid, and 20 mM EDTA), stained with ethidium bromide (0.5 μg/ml), and visualized under UV transillumination (31). A DNA ladder of 123 bp (BRL) was used as a molecular size reference in each gel. The target DNA of 66 isolates was amplified and digested by 11 enzymes. Five isolates, 2A08, 2B12, 2C10, 2D61, and 2E45, each representing a distinct restriction banding pattern for the 66 isolates, were used for DNA restriction mapping. Isolate 1C02, a representative of ISG 1C (AG 1 IC), was used for comparison with the AG 2 populations. We determined the order of restriction sites by using information on the restriction patterns and double-enzyme digestions.

For the PCR-amplified ITS region of rDNA, nine isolates of group 2A, 13 isolates of 2B, 18 isolates of 2C, 14 isolates of 2D, and 12 isolates of 2E showed the same length and restriction digest patterns for 11 enzymes in each group, respectively (data not shown). On the basis of these results, one isolate from each group was selected for restriction mapping. Isolate 2A08 was representative of 2A; 2B12 of 2B; 2C10 of 2C; 2D61 of 2D; and 2E45 of 2E. Isolate 1C02, representative of group 1C (AG 1 IC), was also compared for mapping and served as an outgroup for data analysis.

Data analyses. Electrophoretic phenotypes of 10 enzymes for 70 isolates were developed. For all isolates of *R. solani* tested, each phenotype was given an individual number under the specific enzyme. Therefore, phenotypes were not necessarily complete for all AG 2 isolates because of the lack of certain patterns in this group. Although some isozyme alleles or loci could be interpreted for genetic background, because of the indirect evidence and lack of cross verification of the putative alleles and loci, a more conserved approach was taken. No effort was made to define the genetic composition from the isozyme data. When no band was detected for an enzyme, no null allele was proposed for that

Fig. 1. Summary of electrophoretic phenotypes (EP) of 10 enzymes for populations of *Rhizoctonia solani* anastomosis group 2; relative mobilities are indicated (alleles for the second putative locus indicated in parentheses). Phenotypes of EST for groups 2A and 2E were not detected and were treated as unsolved rather than as null alleles for loci in this study. Enzyme abbreviations are ACP, acid phosphatase; ACO, aconitase; EST, esterase; HXK, hexose kinase; IDH, isocitric dehydrogenase; LAP, leucine amino peptidase; MDH, malate dehydrogenase; PGM, phosphoglucomutase; PGI, phosphoglucoisomerase; 6-PGD, 6-phosphogluconate dehydrogenase.

enzyme (although it could be a set of null alleles), instead, it was treated as unsolved. Such a case was marked by 9 for future reference. The terms relative mobility and allele have been used for describing isozyme bands, depending on genetic interpretation. Each independent band was considered a binary character and was recorded by its relative mobility to the anode; isolate 2C10 was used as reference. A matrix of the presence and absence of isozyme bands was compiled from the electrophoretic data for each isolate. A defined band was expressed by 1, the absence of a band by 0, and unsolved by 9, which was treated as missing data. We used the principle of independent character of putative isozyme alleles and performed a numerical cladistic analysis to infer relationships of populations among AG 2. No data transfer was made in the pursuit of a significant evolutionary variable (10). Data were analyzed by PAUP, which seeks the shortest trees that are optimal under maximum parsimony (53). We performed a heuristic search using 1C19 (a member of the isozyme monophyletic group) as an outgroup, and we generated a 50% majority-rule consensus tree with the 100 shortest multiple, equal-parsimonious trees obtained.

For DNA data, the restriction site and the distinct size of the amplified target fragment were treated as a character. To avoid violation of independence of character, we did not use banding patterns as data entry (6,54). Also, deletions and insertions of a short piece of DNA were not entered, although they are important factors and are discussed below. We performed cladistic analysis with PAUP using an exhaustive search based on restriction site information to infer phylogenetic relationships of different groups of AG 2. Group ISG 1C, previously identified as AG 1 IC, was used as an outgroup.

RESULTS

Multiple isozyme banding patterns were resolved for 10 enzymes of 70 isolates of AG 2; 58 isozyme electrophoretic phenotypes were identified (Fig. 1). The outgroup used for analysis, isolate 1C19, represented a monophyletic group of 14 isolates (AG 1-IC type) from Canada, Japan, and the United States and showed a unique monomorphic banding pattern distinct from those of the AG 2 isolates (Fig. 1). Although the isolates of group 2E

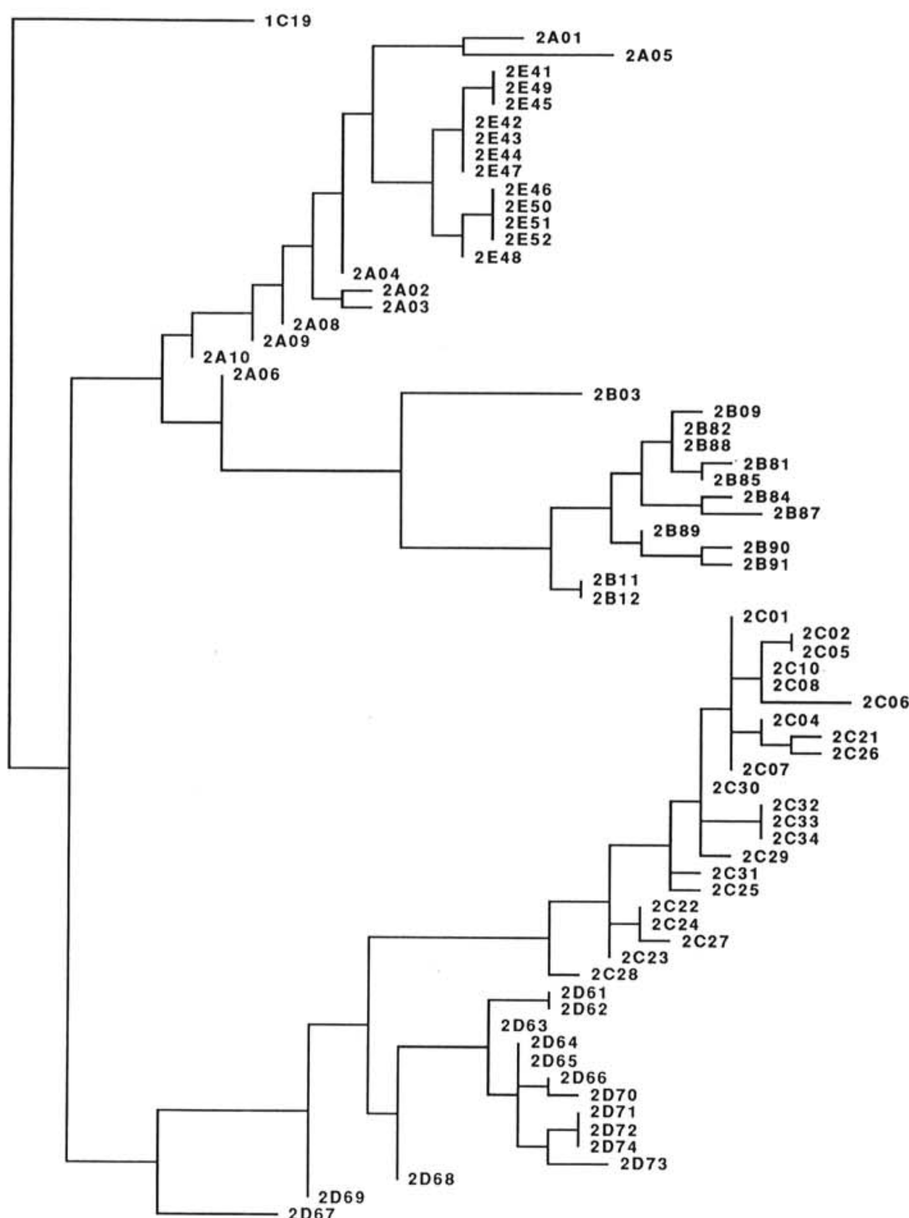

Fig. 2. A consensus tree of 50% majority-rule (length = 132; normalized consensus fork index = 0.841; Ruhlf's consistency index = 0.940) derived from isozyme data shows relationships of divergent populations of *Rhizoctonia solani* anastomosis group (AG) 2; isolate 1C19 (AG 1 IC) was used as an outgroup. The topology was supported 100% at all nodes by the 100 shortest trees (length = 132; consistency index = 0.311 for each tree) by a heuristic search with PAUP (phylogenetic analysis using parsimony) (53).

showed simpler band patterns (except for MDH), none could be used to differentiate them from the other groups. Most of the enzymes were encoded for at least two loci except for the monomeric enzyme, IDH, which was coded by a single allele *idh*. The alleles of the second putative locus (more for MDH) are indicated in parenthesis next to the column showing relative mobility (Fig. 1). Among the five groups, shared banding patterns were common for many enzymes: phenotypes 1, 4, 5 for ACP; 1, 5, 6 for ACO; 7 for EST; 9, 11 for HXK; 3 for IDH; 2, 3 for LAP; 4, 5, 11, 12, 13 for MDH; 1, 2 for PGI; 5, 8 for PGM; and 2, 3, 6 for 6-PGD. However, a few phenotypes were distinct for certain groups: phenotypes 5 of EST and 1 of IDH for group 2C, and phenotypes 2 of IDH and 6 of PGM for 2D (Fig. 1). Phenotypes not presented for all AG 2 isolates were present in other AGs.

Among the populations investigated, no single band or banding pattern defined the current groups within AG 2. Isozyme banding patterns of *R. solani* AG 2 isolates showed that each band was distinct and represented an independent allele for various loci (Fig. 1). For example, for the dimeric enzyme ACP, two loci, *acp-1* and *acp-2*, could be distinguished. In phenotype 1, ACP was encoded at allele 80 for locus *acp-1*; phenotype 4 at alleles 80 and 100 for *acp-1*; phenotype 3 at allele 100 for locus *acp-2*; phenotype 5 at two 100 alleles for the two loci; and phenotype 6 at all three alleles for the two loci. Allele 100 for phenotype 6 was not a heterozygous form but was an independent allele of locus *acp-1*, because it was presented in both phenotypes 4 and 5 as a distinct allele. Similar independent alleles were found for all other enzymes studied except for MDH. The dimeric enzyme MDH showed a complicated banding pattern, which could be attributable to either gene duplication of a single allele or multiple loci. Nonetheless, bands at 70 and 160 (relative mobility) were independent alleles that existed as alleles for phenotypes 8 and 5, respectively.

A data base of isozyme profiles was developed on the basis of relative mobilities to the anode; 2C10 was used as reference (data not shown). A consensus tree of 50% majority-rule (132 steps, Ruhlf's consistency index = 0.940; normalized consensus fork index = 0.841) was generated on the basis of the 100 shortest trees (132 steps and consistency index = 0.311 for each); isolate 1C19 was used as an outgroup (Fig. 2). The topology was supported 100% at all nodes obtained through the heuristic search with PAUP. In the consensus tree, all isolates were separated into five groups, 2A–E, with the exception of isolates 2A01 and 2A05, which outbranched at the top of group 2E.

Nine isolates of group 2A containing populations from Alaska, Australia, and Japan from various hosts or soil were previously identified as AG 2-1 and were closely related to the 12 isolates of group 2E, which included the tobacco pathogen from various counties in North Carolina belonging to AG 2-2. The 22 isolates of group 2C, which represented populations from sugarbeet from Japan and the United States, were related to the 14 isolates of the 2D group from turfgrasses from Texas. Isolates of both groups were previously identified as members of AG 2-2. The 13 isolates of group 2B contained populations from locust (*Robinia* spy.), maize (*Zea mays* L.), mat rush, monochoria (*Monochoria vaginalis* L.), rice (*Oryza sativa* L.), soybean (*Glycine max* (L.)

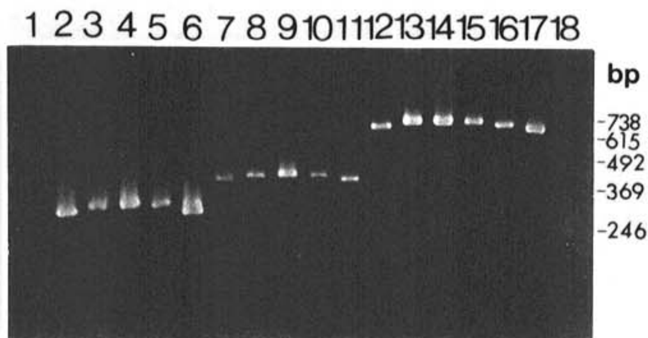

Fig. 3. Agarose gel shows polymerase chain reaction (PCR) amplified portions of nuclear ribosomal RNA genes of internal transcribed spacers (ITS), including 5.8S rDNA for intraspecific groups of *Rhizoctonia solani*. Lanes 1 and 18, DNA size marker of 123-bp ladder; 2–6, DNA region between 18S and 5.8S rDNA amplified with primers ITS1 and ITS2, referred to as ITSa; 7–11, DNA region between 5.8S and 28S, including most of 5.8S rDNA, amplified with ITS3 and ITS4, referred to as ITSb; and 12–17, DNA region between 18S and 28S, including 5.8S rDNA, amplified with ITS1 and ITS4, referred to as ITS. PCR amplification profiles by the following: initial denaturation for 2 min at 94 C, then 30 cycles of denaturation for 1 min at 94 C, annealing for 1 min at 57 C (55 C for ITSb), extension for 1 min at 72 C, and a final extension for 10 min at 72 C. Lanes 1 and 18, DNA size marker of 123-bp ladder; 2, 2A08; 3, 2B12; 4, 2C10; 5, 2D61; 6, 2E45; 7, 2A08; 8, 2B12; 9, 2C10; 10, 2D61; 11, 2E45; 12, 2A08; 13, 2B12; 14, 2C10; 15, 2D61; 16, 2E45; and 17, 1C02.

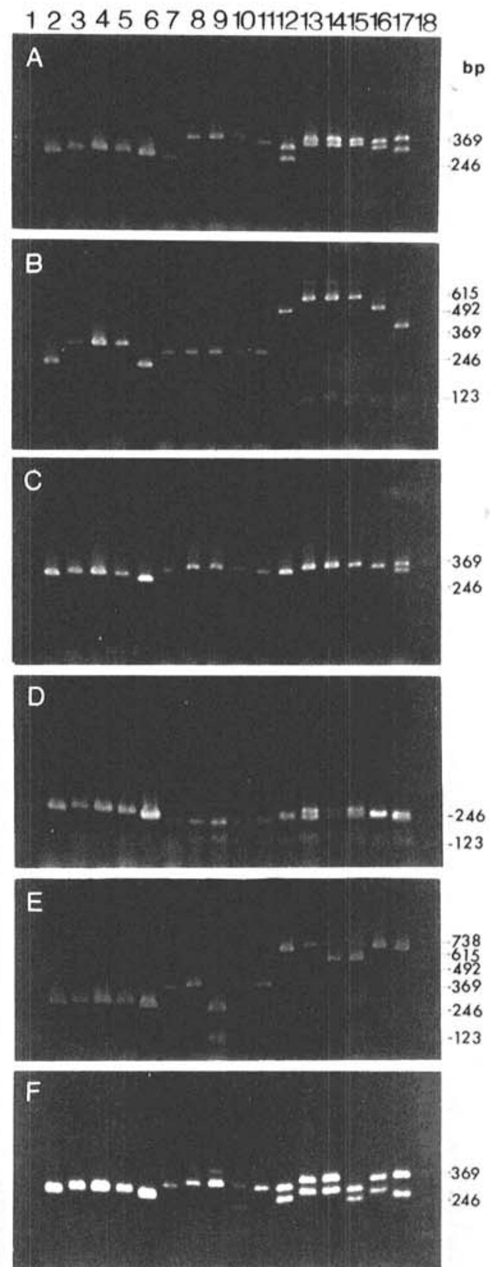

Fig. 4. Agarose gels show DNA restriction digest patterns of six intraspecific groups of *Rhizoctonia solani* anastomosis group 2 for DNA regions ITSa (lanes 2–6), ITSb (7–11), and ITS (12–17) by endonuclease **A,** *Eco*RI; **B,** *Hae*III; **C,** *Hin*fI; **D,** *Mbo*I; **E,** *Msp*I; and **F,** *Taq*I. Lanes 1 and 18, DNA size marker of 123-bp ladder; 2, 2A08; 3, 2B12; 4, 2C10; 5, 2D61; 6, 2E45; 7, 2A08; 8, 2B12; 9, 2C10; 10, 2D61; 11, 2E45; 12, 2A08; 13, 2B12; 14, 2C10; 15, 2D61; 16, 2E45; and 17, 1C02.

Merr.), sugarbeet, or soil from Japan and the United States. Members of this group were previously identified as AG 2-2 IIIB, except for isolate 2B03, which was considered an unspecialized member of AG 2-2. Group 2B was linked with group 2A and was located between the groups 2A and 2E and 2C and 2D.

The entire ITS regions of rDNA for groups 2A and 2E had the same length of approximately 0.69 kb, and those of groups 2B–D had the same length of 0.74 kb (Fig. 3). The ITSa regions for groups 2A and 2E were estimated at approximately 0.29 kb; those for groups 2B–D were estimated at 0.31 kb. The estimation for the ITSb regions of groups 2A and 2E was 0.40 kb; that for groups 2B–D was 0.43 kb. Group 1C showed a more distinct ITS length and restriction digest patterns for the six enzymes compared with the other five AG 2 groups (Figs. 3,4A–F). The entire ITS region of AG 2 did not have restriction cutting sites for enzymes *Bam*HI, *Eco*RV, *Kpn*I, *Pst*I, and *Xhn*I. For *Eco*RI, there were two restriction sites for group 2A and one for each group 2B–E, all located in the ITSb region (Fig. 4A). Two restriction sites for *Hae*III, located each at ITSa and ITSb, distinguished groups 2A and 2E from 2B–D, which had one *Hae*III site at ITSb (Fig. 4B). One *Hin*fI site with two fragments of similar size (Fig. 4C) was recorded for each of the five groups. Four fragments at three *Mbo*I cutting sites were observed for the five AG 2 groups; one site was located at ITSa and two at ITSb. The two larger fragments overlapped for groups 2A and 2E, whereas those for groups 2B–D were separated (Fig. 4D). No *Msp*I restriction site was observed for groups 2A, 2B, and 2E, and one site was located at ITSb for groups 2C and 2D (Fig. 4E). Group 2D, which had an extra *Taq*I restriction site at ITSb, with a total of three sites, differed from the remaining groups by two *Taq*I sites (Fig. 4F).

We constructed restriction maps for the ITS region of ribosomal RNA genes with additional double digestions to locate each restriction site of the five groups (Fig. 5). Most restriction sites of *Eco*RI, *Hae*III, *Hin*fI, *Mbo*I, and *Taq*I were common for this region and could be aligned with the five groups. Groups 2A and 2E had the same ITS length, but differed at one *Eco*RI site. Groups 2C and 2D shared an *Msp*I site, separating them from 2A, 2B, and 2E, but they differed by one additional *Taq*I site for 2D in the ITSb region. The five groups were distinguished from one another by at least one or more restriction sites in addition to the different lengths. However, all of the five groups had a common DNA of 0.55 kb for the mitochondrial small subunit of the ribosomal RNA gene (Fig. 6). In this region, one *Hae*III site, one *Hin*fI site, and no differentiation were found among the five groups.

Using the informative restriction site data with group 1C as an outgroup, we obtained the shortest phylogenetic tree, which consisted of seven steps (consistency index = 1.0) by an exhaustive search with PAUP (Fig. 7). According to relationships derived from DNA restriction fragment analysis, groups 2A and 2E were closely related, and groups 2B–D were closely related (Table 4; Fig. 7). The five groups were from a single source and separated at an early branch. Group 2B was located between groups 2A and 2E and 2C and 2D. However, group 2B was not linked with 2A as indicated in the consensus tree derived from isozyme polymorphism but was connected with a branch of groups 2C and 2D.

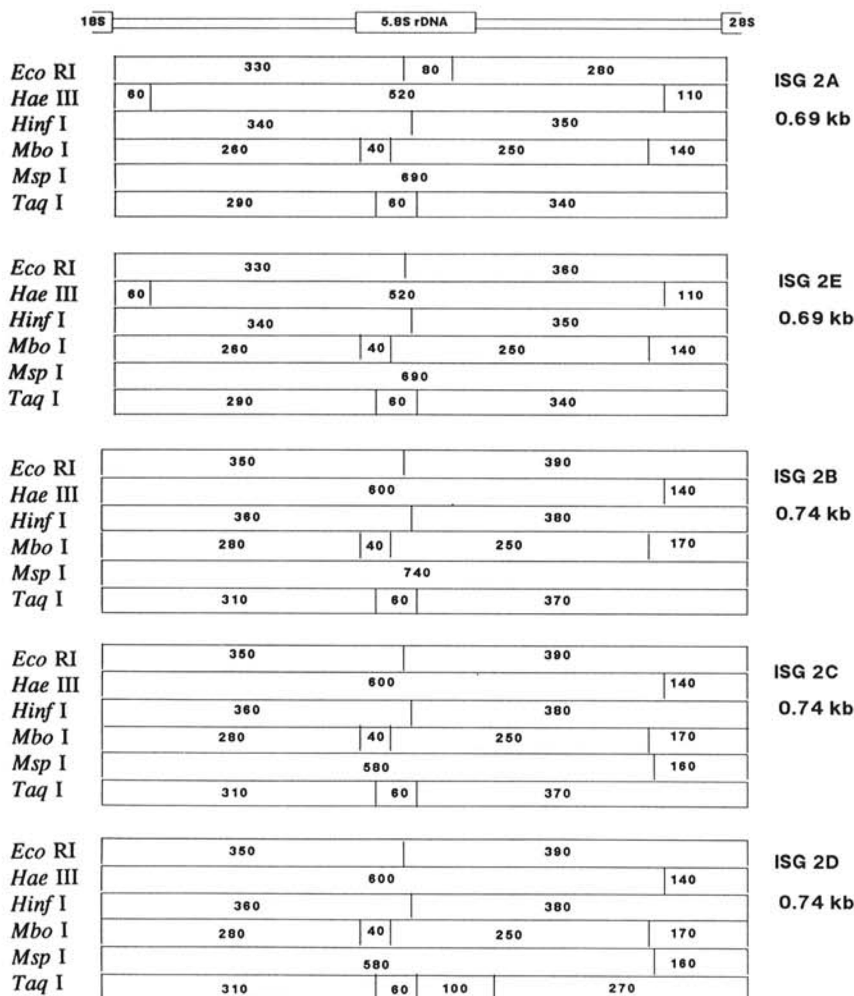

Fig. 5. Restriction maps of rDNA internal transcribed spacers, including the 5.8S ribosomal RNA gene for five intraspecific groups (ISGs) of *Rhizoctonia solani* anastomosis group 2; six enzymes with the approximate locations of 18S, 5.8S, and 28S rDNA coding regions are indicated at the top, and estimated sizes of DNA restriction fragments are indicated in base pairs.

DISCUSSION

Isolates of *R. solani* are highly variable, and groups of similar isolates can be identified by anastomosis grouping. The use of AGs might indicate genetic relatedness among populations of this fungus (39,50). The results from the present study demonstrated that populations of *R. solani* within AG 2 could be differentiated into five genetically distinct subgroups on the basis of isozyme polymorphism and DNA restriction fragment analyses. This was the first time that genetic variation within an AG was confirmed by using different molecular evidence.

Because each isozyme band was an independent character, cladistic analysis with maximum parsimony could be used. The use of alleles as characters for isozyme analysis is considered a significant way of measuring evolutionary trends, although the use of locus status is debated (10,35). With the uncertainty of the effect of environmental conditions on selection, acquisition of an allele may be more significant than subsequent modification of allelic frequency for cladistic analysis.

The 50% majority-rule consensus tree, which used isolate 1C19 as an outgroup from our isozyme data, distinguished five major groups among the AG 2 isolates. In the middle major branch, isolates of group 2B separated independently from all others. Isolates of group 2B were first reported from the United States and were closely related to AG 2-2 IIIB type C330 (2B09) according to isozyme analysis (28). They were thiamine-auxotrophic and pathogenic to a wide range of hosts (30). Isolates 2B11 and 2B12 anastomosed with isolates of IIIB and IV, but were distinct

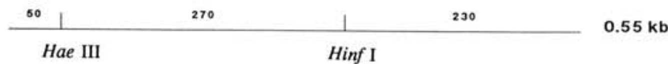

Fig. 6. Restriction map of the mitochondrial small subunit of the ribosomal RNA gene containing *Hae*III and *Hin*fI sites shared by five intraspecific groups of *Rhizoctonia solani* anastomosis group 2; estimated sizes of DNA restriction fragments are indicated in base pairs.

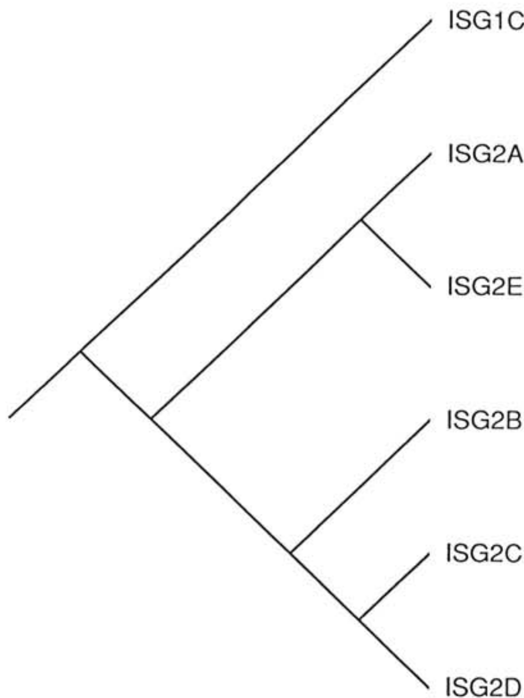

Fig. 7. Phylogenetic hypothesis for relationships of five intraspecific groups (ISGs) of *Rhizoctonia solani* anastomosis group 2 based on a numerical cladistic analysis of restriction sites of ribosomal DNA internal transcribed spacers, including the 5.8S ribosomal RNA gene. The shortest tree of seven steps with a consistency index of 1.0 was obtained by an exhaustive search with PAUP (phylogenetic analysis using parsimony) (53); ISG 1C was used as an outgroup.

in allelic composition to those of AG 2-2 VI at loci *aco*, *est-1*, *est-2*, and *pgm*. Cultures of 2B11 and 2B12 were dark brown; optimum temperature for growth was 30 C. The isozyme profile of isolate 2B03 was similar to, but not the same as, the profiles of isolates 2B11 and 2B12, which differed from isolates of AG 2-2 IV. Isolate 2B03, a maize pathogen from Georgia (52), was related to AG 2-2 IIIB by isozyme study (28) and was classified as an unspecialized member of AG 2-2 by anastomosis grouping (50). Using more isolates of AG 2-2 IIIB from different sources, we confirmed the relationships among the populations within this group and to other groups. Isolates of AG 2-2 IIIB from Japan were of high temperature types, similar to the U.S. isolates. They were all rated at 2.5 (growth rate range from 1 to 4) at 25 C. These cultural characteristics provided additional evidence that group 2B was genetically independent.

Isolates of group 2C from sugarbeet were closely related, although they came from a wide range of geographic locations. The relationship among group 2C isolates was supported by a previous study (28). In contrast, isolates of group 2D from Texas showed variations within the population; isolates 2D67 and 2D69 separated from the other isolates in the group. Isolates of groups 2C and 2D were thiamine-auxotrophic and had a low optimum temperature for growth. At 25 C, isolates of group 2D had a growth rate of 1 compared to a rate of 2 for group 2C. The two groups shared many isozyme phenotypes, but were distinguishable from phenotypes for enzymes EST, IDH, and PGM. Separation of group 2C from 2D was based on isozyme analysis and DNA restriction mapping. Isolates of *R. solani* from turfgrasses have been identified as unspecialized members of AG 2-2 (9). The relationship of those isolates with isolates in group 2D reported in this study was not known. The isolates in group 2A from various sources showed a more distant relationship than those of group 2E, which came from tobacco. The isolates in group 2E, previously identified as unspecialized members of AG 2-2 (49,50), were related to group 2A. They were separated by one restriction site, *Eco*RI. Isolates in group 2E had a growth rate of 4 and a brighter yellow appearance than those of group 2A, which had a growth rate of 2 at 25 C.

We have observed that some members of group 2E were thiamine-auxotrophic, a characteristic shared with isolates of AG 2-1. The separation of isolates AG 2-1 from AG 2-2 was based on anastomosis frequency (41). However, the AG test is not always precise, and results are difficult to interpret when a low frequency of anastomosis occurs, which could lead to misidentification. A thiamine requirement is a useful indication for separating subgroups in AG 2 (41). Auxotrophy for thiamine has also been observed for isolates in other AGs (39,50) and may be a group characteristic rather than an isolate characteristic (39). However, the significance of the limitation of the vitamin requirement at the subgroup level is not clear.

Because of the lack of cross examination, we do not propose a genotype for each of the five groups. However, many isozyme alleles and loci, such as those for ACO, EST, and PGM, were useful for distinguishing isolates in groups 2B and 2C (IIIB and IV types) (30). They could be used as molecular markers for monitoring various populations. For example, a new virulent form of *R. solani* AG 2, first reported from Illinois, was also observed in France from fields under a maize-soybean rotation (14). Isolates in this population were pathogenic to both crops, which posed a potential threat to the practice of rotation for disease control. Our study provided isozyme markers that could be used to monitor

TABLE 4. Mean pairwise distances between five intraspecific groups (ISGs) of *Rhizoctonia solani* according to DNA restriction analysis

	ISG 2A	ISG 2B	ISG 2C	ISG 2D	ISG 2E
ISG 2A	...				
ISG 2B	0.40	...			
ISG 2C	0.50	0.10	...		
ISG 2D	0.60	0.20	0.10	...	
ISG 2E	0.10	0.30	0.40	0.50	...

such populations and thus aid in effective disease management. Similarly, other alleles and loci identified in this paper could be used to study various populations. Our isozyme phenotypes could be used in an isozyme profile base; additional phenotypes could be added in the future. However, many enzymes are genetically polymorphic, and shared bands are common within and among related populations. Therefore, a single allele or locus should not be used for diagnostic purposes. For population studies of *R. solani*, isozyme analysis has special merits, particularly for those unspecialized AG 2-2 populations, or when an anastomosis test is difficult and the isolates are closely related. It provides information for individual isolates. A large population is required for determining the frequency of distribution of the particular allele and locus. Isozyme analysis provided information about individual isolates with limited variations within a population, whereas DNA analysis provided characteristics at a group level because of its more conserved nature.

The results from the DNA restriction mapping confirmed the differentiation of the five groups in AG 2. These groups had the same gene composition in the mitochondrial small subunit rDNA and a high level of similarity in nuclear rDNA of the ITS region; this indicates that they were closely related. Isolates from different origins in each of the five groups showed a unique restriction digest pattern and shared the same restriction sites and length for 11 tested enzymes, indicating the genetic stability within each group. It also indicated that DNA restriction analysis of ITS is suitable for studies of genetic diversity at the intraspecific group level for *R. solani*.

Many restriction sites were common in the ITS region and were aligned among the five ISGs with different lengths, which suggested a high level of DNA base similarity among the five groups. For those with the same ITS length, individual groups could be separated by at least one restriction site, such as between groups 2A and 2E, and groups 2B–D. These results suggest that site mutations or point mutations at the ITS region were involved in the evolution of the five groups. The variation in length of restriction fragments among the groups within a specific region suggested that insertion or deletion events of the short piece of DNA base pairs were also involved in the evolution of these groups. DNA length and site mutations have been observed among populations of other fungi (22,55). For *R. solani*, it seemed that site mutations, insertions, and/or deletions were common among these closely related subgroups.

Using DNA restriction analysis data, we hypothesized that groups 2A and 2E were closely related, and groups 2B–D were closely related. Similar results were obtained by isozyme analysis, except for with the single linkage of group 2B. However, both methods distinguished the same five congruent groups, three of which corresponded to previously identified ecological types. Each group was represented by a varied number of isolates from different geographic origins and found to be genetically independent entities. The separation of three subgroups of AG 2 (AG 2-1, AG 2-2 IIIB, and AG 2-2 IV) was supported by DNA guanine-cytosine content, DNA-DNA reassociation, and isozyme analysis studies (23,24,28), but not by esterase zymogram, serology, and total soluble protein studies (2,32,44). RFLP analysis of *R. solani* with probes of rDNA or random recombinant clones showed extensive variations and inconsistent results (19,56). Because of this variation, no subgroups were distinguished between AG 2-1 and AG 2-2, or between IIIB and IV (19,56). These results could be explained by the use of different DNA probes and too few restriction enzymes, which would provide insufficient information. We found that different gene-coding regions have an unequal rate of evolution for different groups of *R. solani*. For example, mitochondrial small subunit rDNA showed more conserved DNA homology for the five groups in AG 2 and many other AGs as well, but the large subunit rDNA had various degrees of variation for the different groups (*unpublished*). The nuclear small subunit DNA was conserved for most AGs but variable for others, including those of AG 2 (*unpublished*). Therefore, suitable conserved molecular markers are needed for genetic diversity and phylogenetic studies. The ITS regions used in this study contained informative DNA that could be used. However, before a more comprehensive phylogenetic statement can be made, more such molecular markers need to be defined and studied.

The term intraspecific group (ISG) has been used to indicate ecological pathogenetic types of AG 1 and AG 2 and was extended to include subgroups of AG 4, 6, and 9 recognized by DNA-DNA reassociation studies (11,39). However, no clear definition has been made for ISG, and because of the lack of genetic evidence to support the idea, it was withdrawn by Ogoshi et al (50). Most ISGs have been used to describe pathogenetic populations of *R. solani* in Japan, except for some isolates of AG 1 and AG 9 from the United States. ISG is a proper term that can be used to describe the genetic diversity of populations in AG 2. To avoid confusion and to simplify terminology, we define these groups as five individual ISGs with DNA restriction maps provided for each. The four isolates of group 2C from sugarbeet were not examined for DNA information, but fit in group 2C by isozyme analysis. Therefore, we treated them as members of 2C. The term ISG is used to indicate members of a genetically distinct group within *R. solani*; the number after ISG indicates anastomosis behavior, and the capital letter indicates the subgroup.

Accordingly, we propose the use of ISG 2A (9 isolates), which corresponds to AG 2-1; ISG 2B (13 isolates), which corresponds to AG 2-2 IIIB; ISG 2C (22 isolates), which corresponds to AG 2-2 IV; ISG 2D (14 isolates), a newly defined group related to AG 2-2; and 2E (12 isolates), a newly defined group previously identified as AG 2-2, but related to AG 2-1.

The five distinguished ISGs of *R. solani* were genetically related. The separation may also be related to previously reported pathogenic types. However, our separation of these ISGs was not based on host range or intended to define the host range of any group. Further studies on the host range of the various ISGs in AG 2 are required. The establishment of these genetically independent entities provides background on the genetic relatedness of these variable populations; this is essential for further studies of this fungus in biology and pathology.

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