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High Levels of Gene Flow and Heterozygote Excess Characterize *Rhizoctonia solani* AG-1 IA (*Thanatephorus cucumeris*) from Texas

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Rosewich, U. L., Pettway, R. E., McDonald, B. A., and Kistler, H. C. 1999. High levels of gene flow and heterozygote excess characterize *Rhizoctonia solani* AG-1 IA (*Thanatephorus cucumeris*) from Texas. *Fungal Genetics and Biology* 28, 148–159. To date, much of the genetics of the basidiomycete *Thanatephorus cucumeris* (anamorph = *Rhizoctonia solani*) remains unknown. Here, we present a population genetics study using codominant markers to augment laboratory analyses. Seven single-copy nuclear RFLP markers were used to examine 182 isolates of *Rhizoctonia solani* AG-1 IA collected from six commercial rice fields in Texas. Thirty-six multilocus RFLP genotypes were identified. Population subdivision analyses indicated a high degree of gene flow/migration between the six geographic populations. Tests for Hardy–Weinberg equilibrium (HWE) among the 36 multilocus RFLP genotypes revealed that four of the seven loci did not significantly differ from HWE. Subsequent analysis demonstrated that departures from HWE at the three remaining loci were due to an excess of heterozygotes. Data presented here suggest that *R. solani* AG-1 IA is actively outbreeding (heterothallic). Possible explanations for heterozygote excess, which was observed at all seven RFLP loci, are discussed.

***Index Descriptors:* *Rhizoctonia solani; Thanatephorus cucumeris;* sheath blight; rice; heterozygote excess; gene flow; RFLPs; Hardy–Weinberg equilibrium.**

The filamentous basidiomycete *Thanatephorus cucumeris* (Frank) Donk (anamorph = *Rhizoctonia solani* Kuehn) is a species complex composed of distinct groups of fungi with diverse life histories. Current classification within the *R. solani* complex is based largely on grouping of isolates into 14 anastomosis groups (AGs) (Carling, 1996). In addition, 5 AGs have been further divided into subgroups that differ from each other in morphology, pathogenicity, or molecular characters (Ogoshi, 1987). Using molecular approaches, it has been established that all AGs and subgroups are genetically isolated and represent highly divergent evolutionary units (Laroche *et al.,* 1992; Kuninaga and Yokosawa, 1982; Kuninaga *et al.,* 1997; Vilgalys, 1988; Vilgalys and Gonzalez, 1990).

The elucidation of the genetics of these evolutionary units has been hampered due to several attributes of the species. Hyphae are multinucleate and lack clamp connections. Therefore, homokaryons cannot be distinguished from heterokaryons. This not only makes it difficult to determine the nuclear condition of naturally occurring isolates but also hinders the detection of mating reactions (Cubeta and Vilgalyis, 1997). In addition, isolates of most AGs or subgroups do not readily sporulate *in vitro,* even though they may produce meiotic basidiospores in nature (Ogoshi, 1987). Despite these obstacles, some generalizations about the nuclear condition and mating behavior of

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fungi in the *R. solani* complex have been made. For example, it is generally assumed that the unlike nuclei of heterokaryotic isolates fuse to form a diploid nucleus, with consequent recombination during meiosis (Flentje *et al.*, 1970). Three AGs (AG-1 IC, AG-4, and AG-8) have been crossed in the laboratory and possess a heterothallic (outcrossing) mating system. In other AGs, the mating system is not known or is presumed to be homothallic (self-fertile) (Cubeta and Vilgalys, 1997). There is evidence that isolates within an AG that possesses a heterothallic mating system may also undergo recombination in the laboratory through heterokaryon–homokaryon mating (Cubeta and Vilgalys, 1997).

In general, *R. solani* is perceived as a fundamentally asexual fungus which does not reproduce sexually (Adams, 1996). The fungus survives and is disseminated primarily by the asexual production of sclerotia and/or vegetative mycelium. The presumption that *R. solani* is primarily asexual is associated with our lack of understanding of how heterokaryotization occurs in nature. Meiotic basidiospores are generally monokaryotic, in addition to being extremely fragile. Field isolates are generally assumed to be heterokaryotic (Flentje *et al.*, 1970). Therefore, heterokaryotization *via* somatic fusion of the mycelia from two (monokaryotic) basidiospores must occur prior to infection, if recombination contributes at all to population structure.

One important subgroup within the *R. solani* complex is AG-1 IA, the causal agent of sheath blight of rice. This pathogen was first described from southwestern Japan at the beginning of the century (Kozaka, 1975) and has been subsequently reported from most rice-growing regions of the world (Dagupta, 1992), including the southern United States (Lee and Rush, 1983; Marchetti, 1983).

In the southern United States, *R. solani* AG-1 IA is thought to have been an endemic pathogen (Marchetti, 1983), being present mainly on many undomesticated plant species but also found on commercial crops like soybean and rice (Ryker and Gooch, 1937; Stroube, 1954). As late as 1966, sheath blight on rice was considered to be of minor importance (Rush, 1972). The sudden increase in incidence and severity of rice sheath blight in the United States and other rice-growing countries in the early 1970's is generally linked to the rapid adoption of high-yielding semidwarf cultivars and increased nitrogen fertilization (Lee and Rush, 1983). These and other changes in agronomic practices have lead to a microclimate in the crop that is advantageous to the pathogen (Lee and Rush, 1983).

AG-1 IA is one of the subgroups of the *R. solani* complex that has been resistant to genetic analysis in the laboratory. Though basidiospore-bearing hymenia of *R. solani* AG-1 IA have been observed in rice fields (Jones and Belmar, 1989), it is currently unknown whether recombination occurs in field isolates and whether *R. solani* AG-1 IA is homothallic or heterothallic.

The objectives of this study were to develop RFLP markers for determining whether recombinant genotypes exist in populations of *R. solani* AG-1 IA from rice in Texas. We were also interested in the distribution of genotypes within and among populations and in the approximately spatial scale over which population structure arises as a consequence of recombination and gene flow.

MATERIALS AND METHODS

Fungal cultures. In July 1994, *R. solani* AG-1 IA isolates were obtained from six commercial rice fields in Texas (Table 1). Each field was located in a different county (Fig. 1). Field collections were made using one transect across a field with sampling locations spaced at ca. 20-m intervals. Each location was designated with a letter in the order of collection, i.e., A, B, C, etc. From each sampling location, symptomatic leaves were collected from four different plants. Each leaf was assigned a different number (1–4). Fungal cultures were derived from leaf lesions and pure cultures were established by subculturing mycelium from single sclerotia on potato dextrose agar (24 g/L Difco potato dextrose broth (PDB), 15 g/L agar). Isolates were stored at room temperature as dried sclerotia in 2-ml cryogenic vials.

Cultures for DNA extraction were grown at room

TABLE 1

Locations (Indicating the Town Closest to Commercial Rice Fields), Texas Counties, and Rice Cultivars from Which *Rhizoctonia solani* AG-1 IA Was Sampled

Location	County	Cultivar	Sample size[a]
Buckeye	Matagorda	Gulfmont	39
Garwood	Colorado	Cypress	26
El Campo	Wharton	Gulfmont	21
Winnie	Chambers	Cypress	40
Ganado	Jackson	Gulfmont	27
Dayton	Liberty	Cypress	29

[a] Number of individuals electrophoresed.

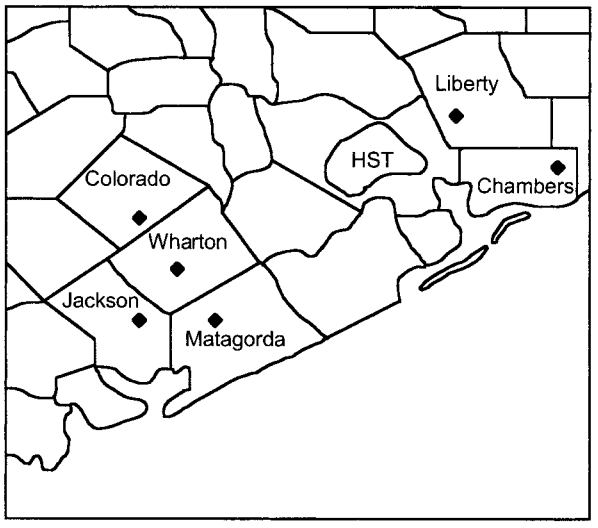

FIG. 1. Texas counties from which geographic populations of *R. solani* AG-1 IA were sampled. Black diamonds indicate the approximate field locations. The two most widely separated sampling locations (Jackson and Chambers) were separated by ca. 280 km. Houston (HST) is inserted for illustration purposes.

temperature without shaking in 125-ml Erlenmeyer flasks containing 75 ml PDB. Mycelium was harvested after 3–4 days, preceding sclerotium formation. Lyophilized tissue was pulverized and suspended in 10 ml SDS extraction buffer (100 mM EDTA (pH 8), 1% sodium dodecyl sulfate, 1 µl/ml diethylpyrocarbonate). After vortexing for 1 min, incubating for 60 min at 65°C, and centrifuging at 12,000*g* for 15 min at room temperature, 8 ml of the supernatant was transferred to a fresh tube and 800 µl of 4 M potassium acetate (pH 5.75) was added. After mixing, the tubes were kept on ice for 45 min and then centrifuged at 28,000*g* for 15 min at 4°C. The supernatant was incubated at 37°C for 1 h in the presence of pronase E at a final concentration of 0.5 µg/ml. DNA was precipitated by addition of 1 vol of isopropanol. Samples were pelleted and resuspended in 700 µl of TE (10 mM Tris, 1 mM EDTA, pH 8) and the solution was transferred to a 1.5-ml polypropylene tube. The DNA was extracted once with phenol:chloroform: isoamyl alcohol (25:24:1 vol, respectively) and once with chloroform:isoamyl alcohol (24:1) and precipitated with 2 vol of 95% ethanol and 0.5 vol of 5 M ammonium acetate. The DNA was air-dried and suspended in approximately 100–300 µl of TE, depending on the size of the nucleic acid pellet, before storage at 4°C.

Development of RFLP probes. A first library was constructed with the *Pst*I-digested DNA of a randomly chosen Texas isolate of *R. solani* AG-1 IA cloned into pUC 18 as described previously (Rosewich *et al.*, 1998). Thirty-nine clones were retained. One screening blot contained the *Eco*RI-digested DNA of 14 Texas isolates. A second blot consisted of DNA from 7 isolates from different southern states (Arkansas, Alabama, Mississippi, Texas, and Louisiana) digested with *Eco*RI, *Pst*I, *Hin*dIII, and *Xho*I. Southern blots and RFLPs were generated as described below. A second library was constructed using the *Hin*dIII-digested DNA of another randomly chosen Texas isolate. Sizes of clones resulting from the second library were determined by digestion with *Hin*dIII and separation of DNA on a 0.8% agarose gel with a 1-kb ladder as size standard. Clones selected for screening had single inserts in a size range between 1 and 1.5 kb. Of 151 clones, 23 qualified. Two replicate screening blots contained 5 µg *Hin*dIII-digested DNA each of 19 isolates originating from all six Texas field populations (2–4 isolates per population).

Scoring of RFLPs. Total DNA samples (5 µg) from all 182 individuals were digested with *Hin*dIII, size-separated by electrophoresis, and transferred to nylon membranes as previously described (Rosewich *et al.*, 1998). Hybridization, wash, and detection procedures were as previously described (Rosewich *et al.*, 1998), except that hybridization and washes were carried out at 65°C.

DNA fragments with different sizes were treated as alleles at each RFLP locus. Isolates having the same multilocus RFLP genotype (i.e., having the same alleles at each of the RFLP loci) from different populations were compared using the repetitive probe R18 to determine if these isolates were part of the same clonal lineage. Isolates having the same multilocus RFLP genotype and DNA fingerprint were assumed to be members of the same clone. Isolates with the same multilocus RFLP genotype but with a different DNA fingerprint pattern were assumed to be members of the same clonal lineage.

Data analyses. For analysis, we arranged the data into various data sets. The first set included data from all isolates sorted according to the six field populations. This data set was further hierarchically subdivided into sampling stations within the six fields. In a second "clone-corrected" data set, isolates with the same multilocus RFLP genotype within each geographic population were considered only once, to eliminate the effect of repeated sampling of the same clone on the association among loci. In a third data set, which was also clone-corrected, we pooled all multilocus RFLP genotypes into a single data set. Population genetic analysis was conducted using

ARLEQUIN ver. 1.1 (Schneider *et al.,* 1997), unless otherwise stated. Within-population analysis included average gene diversity per locus (Nei, 1987) for both uncorrected and clone-corrected data. Between-population analyses were conducted to test for the presence of population structure. Data were analyzed using POPGENE (Yeh *et al.,* 1997) to calculate F_{ST} (Hartl and Clark, 1989) and unbiased genetic distance (D) (Nei, 1978). Both tests are based on allele frequency differences between populations. In addition, we conducted analysis of molecular variance (AMOVA) (Excoffier *et al.,* 1992), which takes into account the number of differences between molecular genotypes. AMOVA produces estimates of variance components and *F* statistic analogues (Φ statistics), reflecting correlation of diversity at different levels of hierarchical subdivision (between geographic populations, between sampling stations within geographic populations). Pairwise Φ_{ST} revealed similarity between the individual geographic populations. *P* values determined their significance. All tests were conducted for both total and clone-corrected data.

Subsequent analyses tested for goodness-of-fit to Hardy–Weinberg equilibrium (HWE) and determined linkage disequilibrium between all pairs of loci. These analyses involved clone-corrected data, whereby all multilocus RFLP genotypes from all populations were pooled into a single data set. To test for HWE, we used an exact test analogous to Fisher's exact test on a two-by-two contingency table, but extended to a triangular contingency of arbitrary size, according to Guo and Thompson (1992). In addition, likelihood ratio (G^2) tests were performed using POPGENE (Yeh *et al.,* 1997). Linkage disequilibrium between all pairs of loci was assessed. For genotypic data for which the haplotypic phase is unknown, linkage disequilibrium between a pair of loci can be determined using a likelihood-ratio test (Slatkin and Excoffier, 1996). Our observation of apparent heterozygote excess prompted us to calculate F_{IS}. F_{IS} at all seven loci was computed as in Weir and Cockerham (1984) using GENEPOP ver. 1.2 (Raymond and Rousset, 1995).

RESULTS

Development of RFLP probes. Though the first genomic library yielded one repetitive probe useful for DNA fingerprinting (designated R18), single-copy probes could not be identified because of complex banding patterns. Useful RFLP probes, i.e., probes that displayed not more than two restriction fragments of similar intensity and had the potential to differentiate between homo- and heterozygotes, were developed from the second library. From this library we selected clones for small insert size (1–1.5 kb) and digested the DNA of field isolates with the same enzyme as that used to construct the genomic library (*Hin*dIII). Despite the small insert size, only 4 of 23 clones tested (17.4%) from the second library were monomorphic among 19 Texas isolates, indicating high gene diversity within *R. solani* AG-1 IA. Of 12 probes that clearly distinguished between homo- and heterozygotes, we selected 7 that were used to construct multilocus RFLP genotypes. Examples of single-copy nuclear RFLPs are shown in Fig. 2.

Gene diversity. Seven single-copy probes were utilized for RFLP analysis of 182 isolates of *R. solani* AG-1 IA from six geographic populations. The seven loci surveyed in *R. solani* AG-1 IA were polymorphic in all localities, yielding an average of 2.86 alleles per locus. Most alleles (14 of 20) were present in all six populations. Private alleles were present at low frequency in two populations (allele D at locus R68 in the population from Colorado, allele D at locus R116 in the population from Chambers). In addition, 4 alleles were not present in all populations (allele C at locus R68, allele B at locus R116, allele C at locus R78, allele C at locus R148). The average gene diversity for the seven loci was high and reached values between 0.434 and 0.519 within geographic populations if all isolates were included and between 0.495 and 0.590 for clone-corrected data.

Genotypic analysis. Among the 182 *R. solani* isolates analyzed, 36 multilocus RFLP genotypes were identified (Table 2). On average, 6.83 multilocus RFLP genotypes were found per locality (range: 3–15). In contrast to alleles, which were generally shared between populations, multilocus RFLP genotypes were mainly locality specific with some exceptions. Multilocus RFLP genotype 25 was found at high frequency in three fields (Chambers, 12/26 = 0.462; Colorado, 15/22 = 0.682; Jackson, 7/27 = 0.259). Multilocus RFLP genotype 25 representatives from each field also displayed identical patterns based on the repetitive probe R18 (Fig. 3). Other multilocus RFLP genotypes were shared among some populations: multilocus RFLP genotypes 7, 9, and 20 were identified from two fields each. Again, DNA fingerprints of representative isolates of multilocus RFLP genotypes 7 and 9 selected from both respective localities corresponded to single-locus data (data not shown). The case was different for isolates of multilocus RFLP genotype 20. Though isolates of multilo-

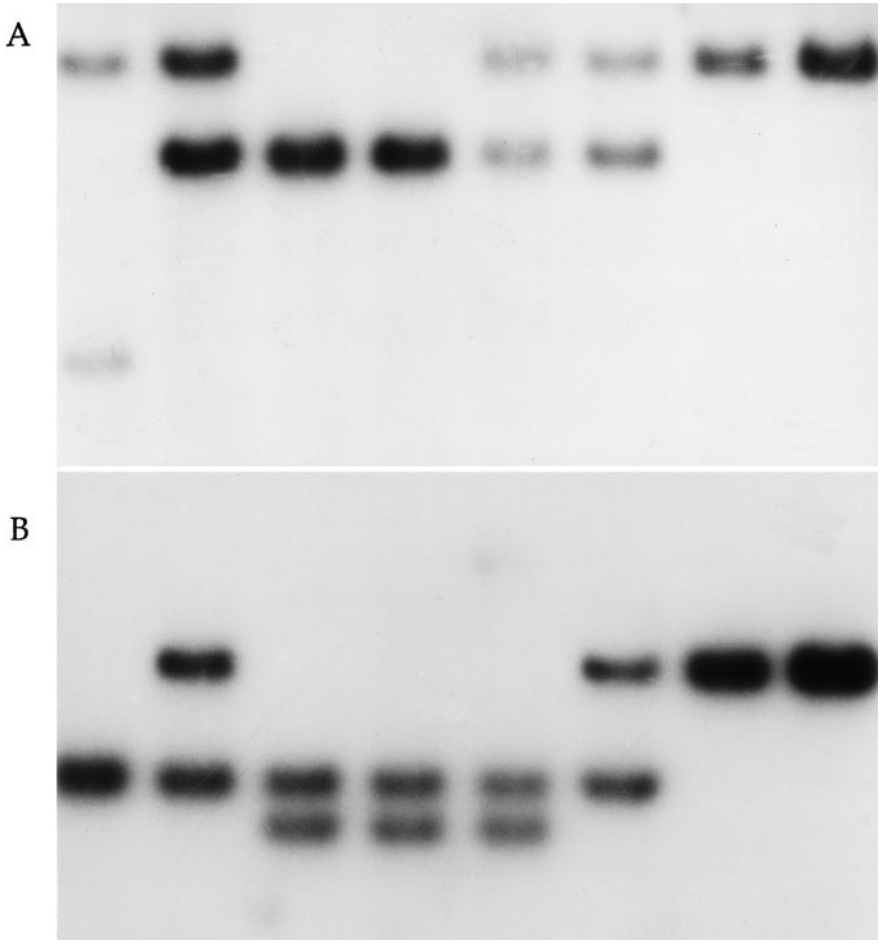

FIG. 2. Southern hybridization of *Hin*dIII-digested total DNA of *R. solani* AG-1 IA with single-copy RFLP probes R68 (A) and R116 (B), showing homo- and heterozygote individuals for both loci. Isolates are the same for both probes.

cus RFLP genotype 20 from the same field displayed the same fingerprint pattern, isolates from different fields did not (Fig. 3).

Analysis of population structure was conducted for both uncorrected and clone-corrected data. For uncorrected data, overall F_{ST} between geographic populations was determined to be 0.071; for clone-corrected data, F_{ST} was 0.046 (Table 3). Using AMOVA, overall Φ_{ST} between geographic populations was 0.070 for uncorrected data. When the uncorrected data were further hierarchically subdivided into the sampling stations within fields, subdivision was found to be slightly higher between sampling stations ($\Phi_{ST} = 0.095$) than between geographic populations.

For clone-corrected data, overall Φ_{ST} was -0.035 between the geographic populations. Table 4 compares population pairwise Φ_{ST} values and Nei's unbiased genetic distances (D) for the six geographic populations for both uncorrected and clone-corrected data. Both approaches gave equivalent results, indicating high levels of gene flow, which was more pronounced for the clone-corrected data. In addition, P values of pairwise Φ_{ST} values were not significant at the 0.05 level for any pairwise (field) comparison of the clone-corrected data.

Due to the lack of significant population structure for the nuclear genome, we pooled all multilocus RFLP genotypes data into a single (clone-corrected) data set to test (1) for goodness-of-fit to HWE and (2) for linkage disequilibrium between all pairs of loci. The results from the exact test and the likelihood ratio statistic (G^2) test for HWE are shown in Table 5. With both tests, genotypic proportions conformed to HWE at four loci. At three loci,

TABLE 2

Multilocus RFLP Genotypes Displayed by Isolates of *Rhizoctonia solani* AG-1 IA Collected from Six Commercial Rice Fields in Texas

MRG[a]	68/116/111/44/78/61/148[b]	Matagorda[c]	Chambers	Colorado	Wharton	Jackson	Liberty
1	AA/AA/AB/AA/BC/AA/AB	J (1,2,3,4)[d]					
2	AA/AA/AB/AB/AA/AB/AB	D3					
3	AA/AA/AB/AB/AB/AA/AB						B (1,4)
4	AA/AA/AB/AB/BB/AA/AB	D (1,2,4) M4					
5	AA/AA/BB/AB/BC/AB/AB	L (1,2,3)					
6	AA/AB/AA/AB/AB/AB/AB	G4					
7	AA/AB/AA/AB/AB/AB/BC			E2	A (2,3,4) B (1,3,4) G (1,2,3,4) J (1,2,3,4)		
8	AA/AC/AB/AB/AC/AB/AA				D (1,2,3,4)		
9	AA/AC/AB/AB/AB/AB/AC	C (1,2,3,4)			C3		
10	AB/AA/AA/AA/BC/AA/AC	B (1,3,4)					
11	AB/AA/AB/AB/AA/AA/AA	H (1,2)					
12	AB/AA/AB/AB/AA/AA/AB	A (1,2,3)					
13	AB/AA/AB/AB/AB/AA/AB					B (1,2,3,4) I4	
14	AB/AA/AB/AB/AB/AB/AA						H (2,3,4)
15	AB/AA/AB/AB/AB/AB/AC	F (1,2,4)					
16	AB/AA/AB/BB/AB/AB/AC	M1					
17	AB/AB/AA/AB/AB/AA/BC						A (1,2,3,4) F (1,2,3)
18	AB/AB/AA/AB/AA/AA/AC				E (1,2,3,4) F (1,3,4)		
19	AB/AB/AB/AA/AB/AB/BC				I (1,2,3,4)		
20	AB/AB/AB/AB/AA/AB/AB		B1, D1, E (3,4) F (1,2,3,4) H (1,2,3,4)		K (1,2,3) L (1,2,3)		
21	AB/AB/AB/AB/AB/AA/BB						E (1,2,3,4)
22	AB/AB/AB/AB/AB/AA/BC						G (2,4)
23	AB/AC/AA/AB/AB/AB/AB	I (1,2,3,4)					
24	AB/AC/AA/AB/AC/AB/AA						D (1,2,3,4)
25	AB/AC/AB/AA/AB/AA/AB		A (1,2,3,4) C (1,3) G (1,2,3) J (2,3,4)	A (1,2,4) B (1,2,3,4) C (2,4) D (1,4) H (1,3,4)		A (1,2,3,4) C (1,2,3,4)	
26	AB/AC/AB/AA/AC/AA/CC	G1					
27	AB/AC/AB/AB/AB/AA/AC						C (1,4)
28	AB/AC/AB/AB/AB/AB/AC						I (1,3)
29	AB/AC/AB/BB/AB/AB/AA				H (1,2,3,4)		
30	AB/AD/AA/AB/BC/AA/AB		B2, I2				
31	AC/AA/AB/AB/AB/AB/AC	K (2,3,4)					
32	AC/AC/AB/AB/AB/AB/AA						J (1,2,3)
33	BB/AC/AA/BB/AB/BB/AC					J3	
34	BB/AC/AB/AB/AA/AA/CC	M (2,3)					
35	BB/CC/AB/AB/AB/AB/AB					D (1,2,3,4) F (2,3) G (1,2) H (1,2,3) I (1,2)	
36	BD/AA/AB/AB/AA/AA/AC			F (1,2,3) G (1,2) J2			

[a] Multilocus RFLP genotype.

[b] Number and order of plasmid probes used to define alleles at RFLP loci.

[c] Texas counties in which sampled rice fields were located.

[d] Letters (A, B, C, etc.) indicate specific sampling locations across a transect. From each sampling location, symptomatic leaves were collected from four different plants. Each leaf was assigned a different number (1–4).

we detected significant departure from HWE. In addition, the likelihood ratio test of linkage disequilibrium was not significant for most pairs of loci (Table 6), with the exception of the pair R44 and R61. To test whether nonrandom mating was responsible for departure from HWE at three loci, we calculated F_{IS} (Table 7). All estimates of F_{IS} were negative, indicating heterozygote excess at all loci.

DISCUSSION

During the Second International Symposium on *Rhizoctonia,* it was anticipated that population genetics would resolve many unanswered questions concerning the genetics of the genus *Rhizoctonia,* as it would permit estimation of outbreeding, inbreeding, population structure, spatial orientation, and genetic variation (Adams, 1996). To elucidate some of these population genetic processes in *R. solani,* the necessary first step was to develop neutral genetic markers, which can unambiguously distinguish between homo- and heterozygotes. As this can be a problem with multilocus minisatellite fingerprints, RAPDs, and some allozyme markers (Baverstock and Moritz, 1996), we developed easily scorable, single-copy RFLP markers for *R. solani* AG-1 IA. To our knowledge, this is the first study in any AG of *R. solani* which used codominant markers to investigate the genetics of *R. solani* at a population level.

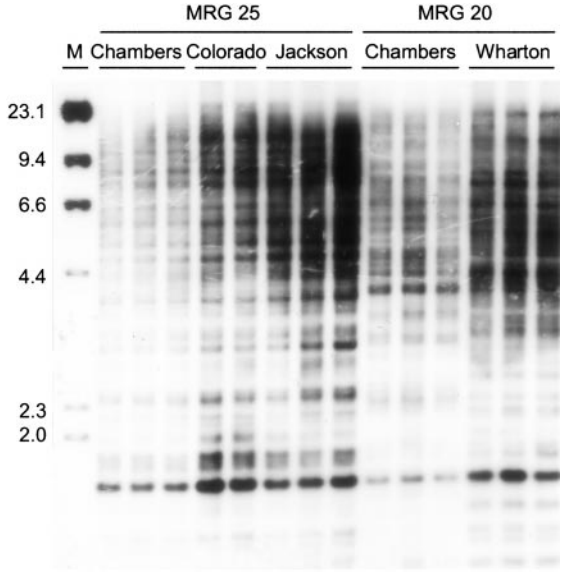

FIG. 3. Hybridization patterns of *Hin*dIII-digested total DNA of *R. solani* AG-1 IA using the repetitive probe R18. Displayed are isolates of multilocus RFLP genotype 25 and of multilocus RFLP genotype 20 from different fields. The first lane is a λ/*Hin*dIII standard and the size fragments are indicated in kilobases at the left.

TABLE 3

Population Differentiation and Gene Flow of *Rhizoctonia solani* AG-1 IA Isolates Collected from Six Commercial Rice Fields in Texas

RFLP locus	N_u [a]	F_{ST} [b]	N_m [c]	N_c [d]	F_{ST} [b]	N_m [c]
R68	364	0.113	1.964	82	0.098	2.295
R116	364	0.134	1.619	82	0.076	3.041
R111	364	0.036	6.681	82	0.005	48.007
R44	364	0.062	3.770	82	0.023	10.800
R78	364	0.037	6.584	82	0.027	9.150
R61	364	0.074	3.151	82	0.051	4.704
R148	364	0.039	6.089	82	0.042	5.738
Mean	364	0.071	3.276	82	0.046	5.209

[a] Data from all isolates were used (uncorrected data).

[b] F_{ST} was calculated according to Nei (1978) using POPGENE (Yeh *et al.*, 1997).

[c] The effective number of migrants (N_m) was estimated from $N_m = 0.25 (1 - F_{ST})/F_{ST}$.

[d] One isolate per genotype and population was retained (clone-corrected data).

We examined 182 isolates from six commercial rice fields located in six major rice-growing counties in Texas. These counties represent about 80% of the rice-growing area in the state. We first established that *R. solani* from rice is a genetically homogeneous group, which is in contrast to an earlier report (Liu and Sinclair, 1993) that suggested that the Texan population of *R. solani* AG-1 IA on rice was composed of at least two genetically distinct entities (subgroups). This conclusion was based on differences in the PCR-amplified ribosomal DNA ITS-5.8 S region of some Texas isolates. The RFLP data also allowed us to get some insight into the nuclear condition of the genome. Visual examination of the allelic information from the single-locus RFLP probes allowed an easy separation into heterozygote and homozygote classes at all seven loci. Homozygotes always generated darker bands than heterozygotes, which in turn were always characterized by two equally intense bands. In addition, isolates with three bands were never observed, which would have been indicative of a heterokaryon–homokaryon mating. We conclude that *R. solani* AG-1 IA showed characteristics of a balanced dikaryon.

We used the allelic information from seven single-locus

TABLE 4

Population Pairwise Φ_{ST} [a] and Unbiased Genetic Distance (D)[b] (in Parentheses) of *Rhizoctonia solani* AG-1 IA Isolates Collected from Six Commercial Rice Fields in Texas, for Total Data (above Diagonal) and Clone-Corrected Data (below Diagonal)

County	Matagorda	Chambers	Colorado	Wharton	Jackson	Liberty
Matagorda		0.060*	0.066*	0.059*	0.129**	0.018
		(0.070)	(0.068)	(0.068)	(0.151)	(0.025)
Chambers	−0.041		0.021	0.065*	0.054	0.027
	(0.011)		(0.027)	(0.078)	(0.061)	(0.038)
Colaroda	−0.069	−0.144		0.128**	0.067	0.052
	(−0.019)	(−0.043)		(0.145)	(0.070)	(0.053)
Wharton	−0.012	−0.056	−0.078		0.155**	0.022
	(0.014)	(0.012)	(−0.011)		(0.197)	(0.035)
Jackson	0.024	−0.087	−0.060	−0.005		0.077*
	(0.069)	(−0.003)	(0.026)	(0.056)		(0.090)
Liberty	−0.027	−0.085	−0.093	−0.038	−0.037	
	(−0.004)	(−0.025)	(−0.032)	(−0.004)	(0.013)	

[a] Population pairwise Φ_{ST} was calculated using ARLEQUIN ver. 1.1 (Schneider *et al.,* 1997). Asterisks denote significant P values at $P < 0.05$ (*) or $P < 0.01$ (**) leading to an Φ_{ST} value larger than or equal to the observed value when permuting multilocus RFLP genotypes between populations (1000 permutations).

[b] Nei's unbiased genetic distance (D) was calculated using POPGENE (Yeh *et al.,* 1997).

RFLP probes to group the 182 isolates of *R. solani* AG-1 IA into 36 multilocus RFLP genotypes. Within- and between-population analyses considered the possibility of subdivision among the six geographic populations. Sheath blight is considered a modified single-cycle disease. *R. solani* AG-1 IA grows from plant to plant; so, an initial infection can spread short distances via contact infection by mycelia (Kozaka, 1975) but usually does not spread to uninfected field regions during a single growing season. Our results agreed with these field observations. Up to four isolates, collected from different plants, were analyzed per sampling location within individual fields. In most cases, all isolates from any one sampling location displayed the same multilocus RFLP genotype, while isolates from other sampling locations were often a different multilocus RFLP genotype.

TABLE 5

Tests for Hardy–Weinberg Equilibrium for 36 Multilocus RFLP Genotypes of *Rhizoctonia solani* AG-1 IA

RFLP locus	G^2 [a]	P [b]	P_r [c]
R68	7.110	0.311	0.114
R116	6.672	0.352	0.508
R111	10.621	0.001**[d]	0.004*
R44	11.490	<0.001***	0.002***
R78	9.572	0.023*	0.031*
R61	2.253	0.133	0.231
R148	3.362	0.339	0.294

[a] Log likelihood statistic (G^2) was calculated using POPGENE (Yeh *et al.,* 1997).

[b] Probability of G^2.

[c] Exact test analogous to Fisher's exact test on a two-by-two contingency table, but extended to a triangular contingency of arbitrary size (Guo and Thompson, 1992), was calculated using ARLEQUIN 1.1 (chain length: 100,000; dememorization: 1000) (Schneider *et al.,* 1997).

[d] Asterisks denote significant P values at $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), indicating significant deviation from Hardy–Weinberg equilibrium.

TABLE 6

P Values of a Likelihood Ratio Test of Linkage Disequilibrium[a] and Their Respective Standard Deviations (below Diagonal) between Pairs of Seven RFLP Loci Based on 36 Multilocus RFLP Genotypes of *Rhizoctonia solani* AG-1 IA

RFLP locus	R68	R116	R111	R44	R78	R61	R148
R68		0.384	0.142	0.549	0.209	0.421	0.196
R116	0.003		0.176	0.854	0.161	0.389	0.090
R111	0.003	0.002		0.263	0.581	0.461	0.584
R44	0.003	0.002	0.003		0.276	0.016	0.289
R78	0.002	0.003	0.003	0.003		0.650	0.239
R61	0.003	0.003	0.003	0.001	0.004		0.143
R148	0.002	0.002	0.002	0.003	0.003	0.002	

[a] Linkage disequilibrium between pairs of loci was assessed using a likelihood-ratio test, whose empirical distribution is obtained by a permutation procedure (Slatkin and Excoffier, 1996) using ARLEQUIN ver. 1.1 (number of permutations: 20000; number of initial conditions for EM: 100) (Schneider *et al.,* 1997).

TABLE 7
Observed and Expected Number of Homo- and Heterozygotes and Resulting F_{IS} at Seven RFLP Loci of 36 Multilocus RFLP Genotypes of *R. solani* AG-1 IA

RFLP locus	Observed homozygotes	Expected homozygotes	Observed hetero-zygotes	Expected hetero-zygotes	F_{IS} [a]
R68	12	16.887	24	19.113	−0.260
R116	15	18.239	21	17.761	−0.186
R111	10	18.648	26	17.352	−0.509
R44	8	17.803	28	18.197	−0.551
R78	8	15.141	28	20.859	−0.349
R61	18	21.352	18	14.648	−0.233
R148	8	13.197	28	22.803	−0.232

[a] F_{IS} was calculated as in Weir and Cockerham (1984) using GENEPOP ver. 1.2 (Raymond and Rousset, 1995).

On the other hand, long-distance dispersal of sclerotia (or other clonally reproduced fungal material) in *R. solani* AG-1 IA was strongly suggested by the presence of the same multilocus genotype in different counties. Three multilocus RFLP genotypes, whose isolates also displayed identical DNA fingerprints, were found in more than one location. Multilocus RFLP genotype 25 was found at high frequency in three geographically separated localities (up to approx 280 km apart from each other). Two other multilocus RFLP genotypes (multilocus RFLP genotypes 7 and 9) were each identified in two fields. This is not the first time that long-distance dispersal has been detected in *R. solani.* MacNish *et al.* (1993), who used the perfect anastomosis reaction ("self-pairings") between two isolates of *R. solani* as indicator for a clonal relationship between them, identified isolates of clonal descent within AG-8 from widely separated parts of Australia.

Traditional explanations for the migration/spread of clonal structures of soilborne plant pathogenic fungi over long distances favor physical means, such as the transport of sclerotia or other fungal structures in soil or infected organic matter on machinery or other equipment (e.g., MacNish *et al.,* 1993). In our case, this is difficult to visualize. Metropolitan Houston separates the major rice-growing areas in Texas. This urban area should act as a buffer zone, preventing an exchange of contaminated soil or machinery between the rice-growing areas located southwest and northeast of Houston. Two alternative explanations for the dissemination of clones are via birds, which may ingest sclerotia and transport them over long distances or via contaminated seed.

The clonality of multilocus RFLP genotypes 7, 9, and 25 in different fields could be demonstrated by the agreement of single-copy probes and the DNA fingerprinting probe R18. The discrepancy between single-locus data and DNA fingerprinting pattern for isolates of multilocus RFLP genotype 20 collected from different fields is more difficult to explain. Based on frequencies of individual alleles of multilocus RFLP genotype 20, the probability of clonally unrelated isolates sharing the same multilocus RFLP genotype 20 type is extremely low ($P = 7.4 \times 10^{-6}$). Therefore, we propose that isolates of multilocus RFLP genotype 20 in different fields belong to the same clonal lineage, evidenced by the same multilocus RFLP genotype, but have accumulated differences in more variable regions of the genome due to substantial temporal separation.

Even though relatively few multilocus RFLP genotypes were shared among localities, gene flow was high. Analyses based on both allele frequency differences and multilocus RFLP genotype differences revealed a lack of population structure among Texas isolates of *R. solani* AG-1 IA. When data from all isolates were taking into consideration, Φ_{ST} was 0.070 between the geographic locations. When data were further hierarchically subdivided into specific sampling locations within fields, overall subdivision between sampling locations within fields was even slightly higher than the subdivision between geographic populations, with $\Phi_{ST} = 0.095$. These results once again support field observations of limited dispersal within fields during the growing season and highlight the existence of a mechanism that ensures a high level of dispersal between geographic populations of clonally reproduced fungal material.

Using clone-corrected data, Φ_{ST} among the six geographic populations was even slightly negative. As Φ_{ST} is a covariance, negative values can occur if isolates between populations are genetically more similar than isolates within populations (Schneider *et al.,* 1997). Furthermore, as all pairwise Φ_{ST} values for clone-corrected data were nonsignificant at the 5% level, we postulate that the entire rice-growing area of Texas is home to a single population of *R. solani* AG-1 IA. Therefore, we deemed it appropriate to pool all multilocus RFLP genotypes into a single data set for evaluation of HWE.

The evaluation of whether recombination is taking place in *R. solani* is important, as even a small amount of recombination may have significant effects on population structure (Milgroom, 1996). In *R. solani,* the asexual stage is traditionally viewed as predominant in the life cycle, although hymenia, consisting of basidia and basidiospores, have been observed in many AGs. In *R. solani* AG-1 IA,

hymenia have been observed not only on rice but also on alternative hosts, such as soybean, sorghum, and corn (Jones and Belmar, 1989). They are formed midseason on rice around the booting stage (Hashiba and Kobayashi, 1996). Basidiospores are formed and discharged during only the night hours (Kozaka, 1975; Naito, 1996). They are fragile and often survive for only a few hours, especially if exposed to direct sunlight or dry conditions (Kozaka, 1975; Naito, 1996). Even though it has been demonstrated that basidiospores in some AGs can initiate infection, little information is available regarding the epidemiological importance of basidiospores (Naito, 1996).

To test for HWE, we used an exact test (probability test), which is appropriate for small sample sizes (Weir, 1990), and a log-likelihood test (G^2). Both tests revealed that four loci conformed to HWE. In addition, linkage disequilibrium was detected for only one pair of loci and visual examination of genotypic data revealed that recombinants were present at all seven examined loci. Together, these data indicate that novel genotypes in *R. solani* AG-1 IA are produced by sexual recombination and that this AG is heterothallic (outbreeding).

It was evident from visual examination of data that a high proportion of genotypes at all loci were heterozygotes. To test whether nonrandom mating was responsible for departure from HWE for three loci, we calculated F_{IS}. All estimates of F_{IS} were negative, including the four loci that conformed to HWE. The three loci that significantly differed from HWE also had the highest negative values for F_{IS}, substantiating that an excess of heterozygotes was responsible for departure from HWE.

While the basis for heterozygote deficiency in populations has been theoretically and experimentally explored and has been shown to be caused by inbreeding, by positive assortative mating, or by pooling populations with different allele frequencies (the Wahlund effect), heterozygote excess in populations is not as common and therefore has not been as fully theoretically explored. Overdominant selection favoring heterozygotes (Mitton, 1989), associative overdominance (Nei, 1987), and negative assortative mating (e.g., self-incompatibility in plants; Hartl and Clark, 1989) are common textbook explanations for observed heterozygote excess and are generally used to explain heterozygote excess in natural populations (e.g., Ford *et al.*, 1998; Doligez and Joly, 1997). For our data, overdominance (heterozygote advantage) can be ruled out, as it is highly unlikely that all seven RFLP loci were not neutral. Negative assortative mating, e.g., only isolates of unlike mating type mating together, may play a small role in contributing to heterozygote excess, if *R. solani* AG-1 IA is exclusively outbreeding (as our data indicated). Conversely, an excess of heterozygotes would be expected only at loci determining or being tightly linked to mating-type genes, while other unlinked loci would be expected to be in HWE. As our results suggest that most of the seven loci were unlinked, negative assortative mating again appears to be an unsatisfactory explanation for heterozygote excess in *R. solani* AG-1 IA.

Heterozygote excess has also been previously found in two plant pathogenic fungi, *Phytophthora infestans* and *Puccinia graminis.* Goodwin (1997), who calculated fixation indices for 16 species of *Phytophthora,* calculated $F_{IS} = -0.82$ for non-Mexican isolates of *P. infestans.* Similar results were obtained by Burdon and Roelfs (1985) when examining an asexual population of *P. graminis* using isozymes. F_{IS} was negative for four of five loci examined. Both studies used data which were not clone-corrected. Therefore, heterozygote excess may be explained by high levels of heterozygosity being incidentally displayed by high-frequency clones. As our study used clone-corrected data, this also is an unsatisfactory explanation.

A recent publication (Pudovkin *et al.,* 1996) theoretically explored an alternative mechanism leading to an excess of heterozygotes. When the number of breeders producing the next generation is small, allelic frequencies in male and female parents differ due to binomial sampling error. The consequences of this difference is an excess with respect to HWE of heterozygotes in progeny (Pudovkin *et al.,* 1996). The same effect would also be expected if some successful clones monopolize breeding. In *R. solani* AG-1 IA, some clones appear to be geographically more widespread and more prevalent than others. For example, multilocus RFLP genotype 25 or other widespread clones may have a higher probability of sexually reproducing than other less prevalent clones.

This study provided initial data on the population genetics of *R. solani* AG-1 IA from rice in Texas. Though the sampling strategy and sample size was probably adequate to approximate the population parameters measured, more extensive studies are needed, especially to elucidate the extent of recombination in the population. Future sampling should try to maximize genotypic diversity to increase the power for the HWE and linkage disequilibrium calculations. Hierarchical sampling within fields should therefore be avoided.

Future work should also examine the Japanese population of *R. solani* AG-1 IA, as Japan is the assumed center of origin of *R. solani* AG-1 IA. A study by Ogoshi and Ui

(1983) used the anastomosis reaction type as marker. Many clones (determined by perfect fusion between two isolates) were found in a small rice field in Japan and, although some clones were widely distributed, others were more limited in their distribution patterns. More detailed analyses of the population genetics of *R. solani* AG-1 IA from Japan could determine similarities and differences between the Japanese and the Texas populations and therefore would help us to determine which population genetic processes are most important in shaping the population structure in this important fungal pathogen.

In summary, population genetic analysis of *R. solani* AG-1 IA revealed that several mechanisms exist which could contribute toward its continued success as a pathogen. The population of *R. solani* AG-1 IA from rice in Texas displays high genetic diversity, coupled with efficient mechanisms of migration/gene flow. In addition, *R. solani* AG-1 IA appears to exhibit an epidemic population structure (Maynard-Smith *et al.,* 1993), whereby novel genotypes apparently are generated by sexual recombination. Once favorable gene combinations are formed, selection can act upon successful individuals and increase them by asexual reproduction to high frequency.

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