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Genetic structure of populations of *Rhizoctonia solani* AG-3 on potato in eastern North Carolina

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Abstract: A polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was developed to identify and differentiate genotypes of *Rhizoctonia solani* anastomosis group 3 subgroup PT (AG-3 PT), a fungal pathogen of potato. Polymorphic co-dominant single-locus PCR-RFLP markers were identified after sequencing of clones from a genomic library and digestion with restriction enzymes. Multilocus genotypes were determined by a combination of PCR product and digestion with a specific restriction enzyme for each of seven loci. A sample of 104 isolates from one commercial field in each of five counties in eastern North Carolina was analyzed, and evidence for high levels of gene flow between populations was revealed. When data were clone-corrected and samples pooled into one single North Carolina population, random associations of alleles were found for all loci or pairs of loci, indicating random mating. However, when all genotypes were analyzed, the observed genotypic diversity deviated from panmixia and alleles within and between loci were not randomly associated. These findings support a model of population structure for *R. solani* AG-3 PT on potato that includes both recombination and clonality.

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INTRODUCTION

The soilborne basidiomycete *Rhizoctonia solani* Kühn [*Thanatephorus cucumeris* (Frank) Donk] anastomosis group 3 subgroup PT (AG-3 PT) is a major pathogen infecting potato (*Solanum tuberosum* L.) worldwide. Symptoms and signs of Rhizoctonia disease of potato can be found on many plant parts including roots, sprouts, stems, stolons, and tubers. As potato plants reach maturity, the fungus can infest daughter tubers, producing black sclerotia on their surface (Carling et al 1989).

The ecology and pathology of *R. solani* AG-3 PT on potato has been extensively studied (Ogoshi 1987), but little information is available concerning the role of asexual and sexual reproduction in determining population structure of this important potato pathogen. In general, *R. solani* AG-3 PT is assumed to be asexual, surviving primarily as mycelium and sclerotia in soil and on potato seed tubers (Cubeta and Vilgalys 2000). However, the sexual stage (*T. cucumeris*) is frequently observed on potato stems in commercial production fields. Although basidiospores of *T. cucumeris* are assumed to have limited dispersal and little or no importance in the disease cycle (Carling et al 1989), they are an important aspect of fungal life history that contributes to the genetic diversity and structure of field populations. The mating system of *T. cucumeris* (anamorph = *R. solani* AG-3 PT) is not known. Likewise, it is not known whether recombination associated with sexual reproduction occurs among field isolates (Vilgalys and Cubeta 1994, Cubeta and Vilgalys 1997).

Except for the studies of Rosewich et al (1999), no information about the genetic diversity and structure of field populations of *R. solani* exists. Previous studies with *R. solani* AG-3 have employed various methods to examine the genetic variation among isolates from culture collections obtained from distinct Solanaceous hosts, including eggplant, potato, tobacco and tomato (Laroche et al 1992, Liu and Sinclair 1992, Liu et al 1993, Stevens Johnk et al 1993). However, basic questions remain about the population structure of *R. solani* AG-3 PT. For example: i) How

diverse are populations of *R. solani* AG-3 PT from potato fields? and ii) Are populations of *R. solani* AG-3 PT on potato clonal or panmictic?

In this study two null hypotheses were tested: a) populations of *R. solani* AG-3 PT from potato in North Carolina (NC) have no genetic subdivision or differentiation; and b) populations of *R. solani* AG-3 PT from potato plants are randomly mating (recombining). To address these hypotheses, a strategy for rapid detection of individual genotypes of *R. solani* AG-3 PT using co-dominant polymerase chain reaction (PCR)-RFLP markers was employed. These markers were used to assess the genetic diversity and structure of population samples of *R. solani* AG-3 PT obtained from one field from each of five different counties in NC. Analysis of genetic variation (e.g., similarity) and random mating among these samples were conducted. Data were also analyzed to determine whether populations of *R. solani* AG-3 PT were subdivided.

MATERIALS AND METHODS

Population sampling and DNA isolation.—Isolates of *R. solani* AG-3 PT for this study were obtained from infected subterranean potato stems with cankers. Infected plants were obtained from one commercial field in each of 5 counties in eastern NC (Ceresini 2000). Ten plots (2 per field), each consisting of 6–8 rows of 9 m length were planted with either 240 or 320 potato seed pieces (cv. 'Atlantic'). Seed potatoes used to establish the plots were disinfested before planting with 2% formaldehyde to eliminate seedborne *R. solani* AG-3 PT (Carling et al 1989). All plants from each plot were harvested and examined for symptoms of Rhizoctonia stem canker.

Samples were assayed on alkaline water agar (AWA, pH 8.5) to isolate *R. solani* AG-3 PT (Gutierrez et al 1997). A total of 104 pure cultures of *R. solani* were established by transferring mycelium from single hyphal tips to potato dextrose agar. Isolates were stored on sterilized rice grains at −20 C. Isolates were examined for nuclear condition by the DAPI staining procedure (Martin 1987). The anastomosis grouping of each isolate of *R. solani* was determined by standard protocols (Gutierrez et al 1997, Herr and Roberts 1980). Genomic DNA was extracted by methods described previously (Rosewich et al 1999).

Development of PCR-RFLP markers.—Anonymous single-copy DNA markers were developed using a strategy outlined by Karl and Avise (1993) and Xu et al (1999). A genomic library was constructed as described previously (Rosewich et al 1999) with *Hind* III-digested DNA of a randomly chosen NC isolate of *R. solani* AG-3 PT (P-211) cloned into pUC18 (Rosewich et al 1999). Sizes of clones resulting from this library were determined by digestion with *Hind* III and separation of DNA on a 0.8% agarose gel with a 1-kb ladder. Clones with a single insert between 1 and 1.8 kb were selected (32 total). An extra clone with a 3.2 kb insert was also included in the sample. Both termini from 15 cloned DNA fragments were sequenced with forward and reverse primers for the multiple cloning site of pUC18 in an ABI Prism 377 automated sequencer (PE Applied Biosystems). Sequence chromatograms were compiled using Sequencher software (vers. 2.0, GeneCodes Corp.). Locus-specific PCR primers for 21–26 nucleotides were designed for further screening. PCR reactions were conduced in a Model 9600 DNA ThermoCycler (Perkin-Elmer) employing standard conditions described previously for *R. solani* (Vilgalys and Gonzalez 1990) with Amplitaq DNA polymerase (Perkin-Elmer). Restriction polymorphisms were detected initially by screening 21 random isolates of *R. solani* AG-3 PT from all five NC counties with restriction enzymes with 6-base recognition sites (*Bam* HI, *Eco* RI, *Eco* RV, *Hind* III, *Hinc* II, *Hae* II, *Pst* I, *Sac* I, *Sty* I, *Xho* I) or restriction enzymes with 4-base recognition sites (*Hha* I, *Hae* III, *Mbo* I, *Msp* I, *Rsa* I, *Tru* 9I). The entire sample of 104 isolates was then screened with combinations of PCR fragments and specific restriction enzymes that generated polymorphisms. Typical restriction enzyme reactions consisted of 5 μL of PCR product, 7.5 U of enzyme (Promega), 2.0 μL of 10× reaction buffer, 0.2 μL of 10× bovine serum albumin and H_2O to a total volume of 20 μL. Reactions were incubated for 5 h at 37 C (or according to manufacturer's recommendations) then stopped by incubation at 70 C for 5 min. Restriction digests of PCR products were separated by electrophoresis in 1.2 to 2.4% MetaPhor (FMC BioProducts) agarose gels in 1× TBE, containing ethidium bromide, and viewed by UV transillumination.

Data analysis.—Three data sets were constructed: (a) all isolates and retaining the five geographical populations (NC counties, uncorrected data); (b) clone-corrected data; isolates with the same multilocus PCR-RFLP genotype within each geographic population considered only once; and (c) either uncorrected or clone-corrected data of the combined isolates from all populations.

For analysis of genetic diversity within *R. solani* AG-3 PT populations, three population genetics measures were applied: (a) Stoddart's genotypic diversity (Stoddart and Taylor 1988) $G_o = 1/(\Sigma p_i^2)$, where p_i is the frequency of a particular multilocus genotype, with G_o ranging from 1 to N, where N is the sample size. The sample-corrected genotypic diversity, $G_c = G_o/N$, was used to allow comparisons between different populations. The expected overall genotypic diversity for a sample of size N was calculated using Stoddart's expected genotypic diversity (Stoddart 1983) $G_E = 1/(d + p/N)$, where $d = \Sigma g_i^2$ for all genotypes when $(g_i^* N) \geq 1$ and $p = \Sigma g_i$ for all the genotypes when $g_i^* N) < 1$; g_i is the expected frequency of the multilocus genotype calculated by Hardy-Weinberg expectations; (b) observed heterozygosity (H_O) which represents the percentage of heterozygosity at each locus (Hartl and Clark 1997). The mean observed heterozygosity (i.e., gene diversity) was calculated as the arithmetic mean of all loci sampled; and (c) unbiased estimate of expected heterozygosity ($\hat{H}_E$) or mean unbiased gene diversity, $\hat{H}_E = \Sigma_{k=1}^{r} h_k/r$, where h_k is the value of h for k^{th} locus, and r is the total number of loci studied; h is an unbiased estimate of heterozygosity for a single locus

TABLE I. Polymorphic PCR-RFLP markers for analysis of *Rhizoctonia solani* AG-3 PT from potato

PCR-RFLP locus	Primer code	Primer sequence (5′-3′)	Length	Fragment size[a]	Enzyme	Restriction site[a]
pP09	F09	TGTCAGTCGAGTTATCCGCGA	21	530	*Hha* I	320 allele 1
	R12	GATCAAGTGTATGCGCATGCG	21			250 allele 2
pP42	F12	GTTTTTGTAGTGCACGGGGGT	21	1970 allele 1	*Hind* III	1250 allele 2
	R09	ATTCAACGTCTGTCGGTACGG	21			1020 allele 3
pP45	F09	TTGTTCATCGTCCTCGAGTCTC	22	965 allele 1	*Hind* III	765 allele 2
	R03	ATCGGGTAAATTGCTACGCGA	21			
pP46	F09	ATTAGCCCTGACTGGGTCTCG	21	3050 allele 1	*Hind* III	2560 allele 2
	R01	ACGATATTGCCTAGTGCGACC	21			
pP47	F08	TTCATACCCAGATCGAGCGAC	21	1960	*Eco* RV	1460 allele 1
	R07	GCATATTTAATTTAGACAGGGACGC	25			1190 allele 2
pP83	F05	TTGTGAACCTTACAAGTACCCGA	23	1370 allele 1	*Hind* III	1000 allele 2
	R04	AAGTATTATTCTCTGCGGTTCGC	23			890 allele 3
pP89	F02	TTTGAGGAAGAACGCGTACGC	21	1200	*Rsa* I	660 allele 1
	R10	TGTCATTGAAAATACGGCCGA	21			515 allele 2

[a] Size of PCR fragments are presented in base pairs.

given by $h = 2n(1 - \Sigma x_i^2)/(2n - 1)$, where n is the number of diploid individuals, and x_i is the corresponding frequency of the i^{th} allele at a locus in a sample from the population (Levene 1949, Nei 1978, 1987). The expected heterozygosity was calculated with TFPGA version 1.3 (Miller 1997).

Between-population analyses were conducted to test for geographic structure in populations of *R. solani* AG-3 PT. Three population genetics analyses were performed: (a) analysis of molecular variance (AMOVA), which estimates variance components considering the number of differences between molecular genotypes (Weir and Cockerham 1984, Excoffier et al 1992, Weir 1996); (b) pairwise Φ statistics comparisons (F statistics equivalents produced by AMOVA), which reveals similarity between the individual geographic populations. AMOVA analyses were conducted using ARLEQUIN ver 2.000 (Schneider et al 2000); and (c) similarity among the population samples calculated with F statistics (theta P or $F_{WC(ST)}$ as in Weir and Cockerham (1984)) and Nei's unbiased genetic distance (D) (Nei 1978, 1987). These tests ($F_{WC(ST)}$ and D), which are based on allele frequency differences between population samples, were calculated with GDA version 1.0 (d15) (Lewis and Zaykin 2000).

To compare the relative contribution of clonality and recombination in each sample of *R. solani* AG-3 PT, two population genetic measures were used: (a) the Hardy-Weinberg equilibrium (HWE) test, which examines the random association of alleles within a locus by testing the observed genotypic frequencies for goodness-of-fit with Hardy-Weinberg expectations (Hartl and Clark 1997) and (b) composite genotype disequilibrium or linkage disequilibrium (Weir 1996), which estimates the allelic association between pairs of polymorphic markers by an exact test for association through permutation (Zaykin et al 1995). To test for HWE, an exact test analogous to Fisher's exact test (Guo and Thompson 1992) was calculated with ARLEQUIN 2.000 (Schneider et al 2000). In addition, the likelihood ratio statistic G^2 (recommended for genotypic data for which the haplotypic phase is unknown) was calculated with POPGENE (Yeh et al 1997). Linkage disequilibrium between all pairs of loci was calculated by an exact test analogous to Fisher's exact test (Slatkin 1994, Slatkin and Excoffier 1996). Calculations were performed with ARLEQUIN 2.000 (Schneider et al 2000).

RESULTS

Development of PCR-RFLP markers.—From the total of 32 randomly cloned *Hind* III-digested *R. solani* AG-3 PT genomic DNA fragments selected based on size, 15 were partially sequenced and used to design locus-specific PCR primers. Eight fragments were found to be monomorphic, hypervariable or otherwise problematic (e.g., failing to amplify, producing multiple PCR fragments). The remaining seven DNA fragments could be re-amplified from all isolates and possessed unique restriction polymorphisms. Each of four fragments had one polymorphic restriction site, whereas three had two polymorphic sites. Each polymorphic restriction site, which corresponded to a unique combination of primer pair, restriction enzyme, and restriction site, was defined as a locus (TABLE I). Scoring by this method is unambiguous as each locus has potentially two alleles: allele 1 represents the absence of the restriction site, and allele 2 its presence. There were two cases where a third allele was present (i.e., markers pP42-*Hind* III and pP83-*Hind* III). When the restriction enzyme *Hind* III was used for digestion, allele 1 corresponded to the original (undigested PCR product) fragment size. However, for other restriction enzymes, allele 1 or 2

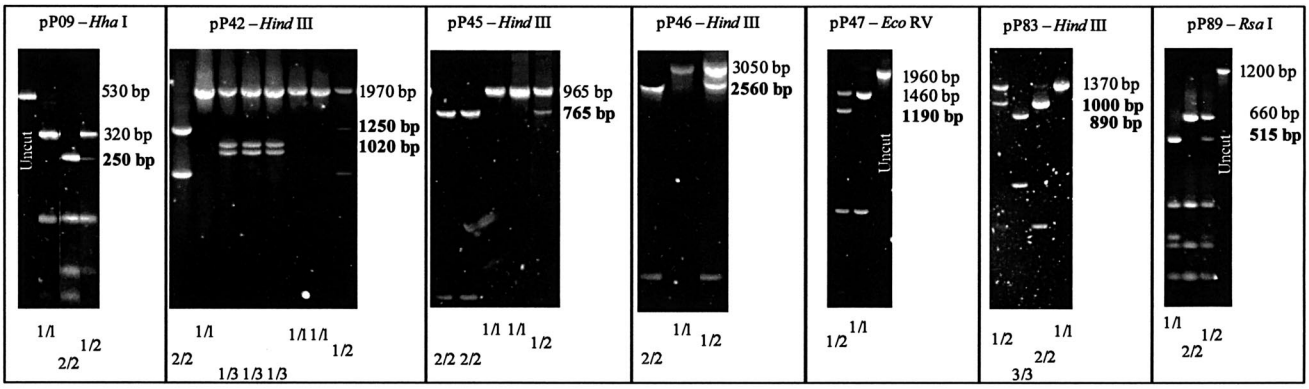

FIG. 1. PCR restriction fragment length polymorphisms (RFLPs) of *Rhizoctonia solani* AG-3 PT total DNA amplified with the locus-specific primers for pP09(F09/R12), pP42(F12/R09), pP45(F09/R03), pP46(F09/R01), pP47(F08/R07), pP83(F05/R04), and pP89(F02/R10) markers showing homozygous (1/1, 2/2, and 3/3) and heterozygous (1/2) genotypes for each locus. The approximate size of each PCR fragment is presented in base pairs (bp) and was determined by comparing each migrating fragment with a DNA standard (either 100 or 1000 bp ladder, Promega). Polymorphic restriction sites for each locus are indicated in bold.

was smaller than the original fragment. Examples of the PCR-RFLP genotyping and allele designation methods are presented in FIG. 1.

Genotypic analysis.—Genotypes and genotypic counts for each of the five samples of *Rhizoctonia solani* AG-3 PT are presented in TABLE II. Among the 104 *R. solani* isolates analyzed, 32 multilocus PCR-RFLP genotypes (MRG) were identified. On average, 8.8 MRG were found per locality (range 2–15). Most of the MRG (about 81%) were site specific (TABLES II AND III). However, isolates with the same genotype were also commonly observed between field sites. Five multilocus genotypes (MRG 3, 2, 1, 48 and 50) represented 13.5, 9.6, 8.7, 8.7, and 6.7% of the sample, respectively. Altogether, these five genotypes represented isolates, which accounted for 47.1% of the total population sample. The observed count of 14 for MRG 3, and 9 for either MRG 1 or MRG 48 was higher than the expected counts of 5.28 for both MRG 3 and MRG 1, and of 3.82 for MRG 48, which were derived under the hypothesis of panmixia (Stoddart and Taylor 1988). The observed and expected counts of genotypes for MRG 2 and 50 did not differ statistically.

As a control, a sample of the same potato seed tubers used for establishing the plots was subjected to isolation on AWA, before disinfestation with 2% formaldehyde. Six additional MRG were identified on these infested seed potatoes: one MRG 16 (1/1 1/1 1/1 1/2 1/1 1/1 2/2), two MGR 17 (1/1 1/1 1/1 1/2 1/1 1/2 2/2), one MRG 28 (1/1 1/2 1/1 1/1 1/1 1/1 2/2), one MRG 37 (1/1 1/2 1/1 2/2 1/1 2/2 2/2), one MRG 38 (1/1 1/2 1/2 1/1 1/1 1/1 2/2), and two MRG 59 (1/2 1/1 1/2 1/1 1/1 1/1 1/2). No isolates of *R. solani* AG-3 PT were recovered from potato seed tubers after disinfestation.

Stoddart's genotypic diversity (G_o) and the sample size-corrected genotypic diversity (G_c) of *R. solani* AG-3 PT are presented in TABLE III. The mean genotypic diversity (G_c) was 0.2945. Overall, there was no difference between any pairs of samples considering their G_c values. In addition, the observed overall genotypic diversity (G_o for uncorrected data = 16.64) was smaller than the expected value under panmixia ($G_E^* = 38.78$) (TABLE II).

Gene diversity, patterns of heterozygosity and deviations from HWE.—Six of the seven loci surveyed from five geographical populations of *R. solani* AG-3 PT were polymorphic at all localities, with an average of 1.97 alleles per locus. The only exception was the locus pP45-*Hind* III, which was polymorphic only at Hyde County. Most alleles (11/16) were present in all five populations. A rare allele was present at low frequencies in one population (allele 2 at locus pP45-*Hind* III in the population sample from Hyde).

The observed and expected heterozygosity of all samples from NC are presented in TABLE IV. Heterozygosity values varied greatly among loci, from 0.010 to 0.481. Two of the seven loci had heterozygosity less than 0.100 (pP45-*Hind* III and pP47-*Eco* RV). The mean heterozygosity (over loci) of the samples was between 0.186 and 0.381 when all the isolates were included in the analysis and between 0.229 and 0.357 for clone-corrected data. Comparisons of mean heterozygosity of each sample indicated no difference between expected and observed values.

Each sample of *R. solani* AG-3 PT from NC was also examined for the association of alleles within a locus

TABLE II. Frequency of multi-locus PCR-RFLP genotypes (MRG) of *Rhizoctonia solani* AG-3 PT from potato fields in North Carolina, and estimates of overall observed (G_O) and expected (G_E^*) genotypic diversity

	Genotype[b]							Frequency of MRG in each county						
MRG[a]	pP09	pP42	pP45	pP46	pP47	pP83	pP89	Washington[c]	Tyrrell	Currituck	Hyde	Camden	Observed count[d]	Expected count[d]
1	1/1	1/1	1/1	1/1	1/1	1/1	1/1			2	4	3	9	5.28
2	1/1	1/1	1/1	1/1	1/1	1/1	1/2				3	7	10	10.66
3	1/1	1/1	1/1	1/1	1/1	1/1	2/2	5			7	2	14	5.28
4	1/1	1/1	1/1	1/1	1/1	1/2	1/1					1	1	1.13
5	1/1	1/1	1/1	1/1	1/1	1/2	1/2	1				3	4	2.29
6	1/1	1/1	1/1	1/1	1/1	1/2	2/2				2		2	1.13
7	1/1	1/1	1/1	1/1	1/1	1/3	2/2				1		1	0.66
12	1/1	1/1	1/1	1/1	1/2	1/2	2/2		1				1	0.08
14	1/1	1/1	1/1	1/2	1/1	1/1	1/1				1		1	1.19
15	1/1	1/1	1/1	1/2	1/1	1/1	1/2				1		1	2.41
20	1/1	1/1	1/2	1/1	1/1	1/1	1/2				1		1	0.10
26	1/1	1/2	1/1	1/1	1/1	1/1	1/1					3	3	1.41
27	1/1	1/2	1/1	1/1	1/1	1/1	1/2	3					3	2.85
29	1/1	1/2	1/1	1/1	1/2	1/1	1/2		1				1	0.20
34	1/1	1/2	1/1	1/2	1/1	1/1	1/2				2		2	0.64
36	1/1	1/2	1/1	1/2	1/2	1/1	1/2		1				1	0.05
42	1/1	1/3	1/1	1/1	1/1	1/1	1/1				1		1	0.74
43	1/1	1/3	1/1	1/1	1/1	1/1	1/2				2		2	1.49
45	1/1	1/3	1/1	1/2	1/1	1/1	2/2	3					3	0.17
48	1/2	1/1	1/1	1/1	1/1	1/1	1/1	2	5		1	1	9	3.82
50	1/2	1/1	1/1	1/1	1/1	1/1	1/2	1	5		1		7	7.72
51	1/2	1/1	1/1	1/1	1/1	1/2	1/2					1	1	1.66
52	1/2	1/1	1/1	1/1	1/1	1/3	1/1	2				1	3	0.47
54	1/2	1/1	1/1	1/2	1/1	1/2	1/2					1	1	0.37
55	1/2	1/1	1/1	1/2	1/1	2/2	1/2				3		3	0.02
56	1/2	1/1	1/1	1/2	1/1	2/3	1/2					1	1	0.02
63	1/2	1/2	1/1	1/1	1/1	1/1	2/2					4	4	1.02
66	1/2	1/2	1/1	1/2	1/1	1/1	1/2				2		2	0.47
72	2/2	1/1	1/1	1/1	1/1	1/3	1/2					3	3	0.17
77	2/2	1/2	1/1	1/1	1/1	1/3	1/2		3				3	0.05
79	2/2	1/3	1/1	1/2	1/1	1/2	2/2	2					2	0.0046
80	2/2	2/3	1/1	1/2	1/2	1/1	1/2			4			4	0.0004
Sample size								19	16	6	32	31	104	
G_O[e]													16.64	
G_E^*[f]													38.78	

[a] Multi-locus PCR-RFLP genotype (MRG) designation. MRG designations are not in sequential numeric order.

[b] Designation and order of PCR marker used to define alleles at each locus.

[c] Counties where isolates were sampled in North Carolina.

[d] Expected count of specific MRG according to Hardy-Weinberg expectations.

[e] $G_O = 1/(\Sigma\ p_i^2)$, where p_i is the frequency of a particular multilocus genotype, with G_o ranging from 1 to N, where N is the sample size.

[f] $G_E^* = 1/(d + (p/N))$, where $d = \Sigma g_i^2$ for all genotypes where $(g_i * N) \geq 1$ and $p = \Sigma g_i$ for all the genotypes when $(g_i * N) < 1$; g_i is the expected frequency of the multilocus genotype calculated using Hardy-Weinberg expectations.

(HWE tests) for each particular locus (TABLE IV). For all samples, when data were not clone corrected, most loci (at least 5/7) had genotypic counts that did not deviate from Hardy-Weinberg expectations (HWE). Two loci (pP09-*Hha* I and pP42-*Hind* III) in the sample from Currituck, one locus (pP83-*Hind* III) in the sample from Hyde, and one locus (pP46-*Hind* III) in the sample from Camden showed significant deviation from HWE. After clone correction, all loci had genotypic counts that did not deviate from HWE.

TABLE III. Genotypic diversity of five population samples of *Rhizoctonia solani* AG-3 PT in North Carolina

Source of isolates	Sample size	Total number of genotypes	Number of site specific genotypes	Stoddart's genotypic diversity (G_o)[a]	Sample size corrected genotypic diversity (G_c)[b]
Washington	19	8	3 (8)[c]	6.3333	0.3333
Tyrrell	16	6	4 (6)	4.1290	0.2581
Currituck	6	2	1 (4)	1.8000	0.3000
Hyde	32	15	11 (16)	9.6604	0.3019
Camden	31	13	7 (14)	8.6577	0.2793
Overall	Mean			6.1108	0.2945
	Standard deviation			2.8860	0.0251

[a] $G_o = 1/(\Sigma\ p_i^2)$, where p_i is the frequency of a particular multilocus genotype, with G_o ranging from 1 to N, where N is the sample size.

[b] $G_c = G_o/N$.

[c] Number of isolates represented by these genotypes.

Population structure.—Analysis of population structure was conducted for both uncorrected and clone-corrected data. Using AMOVA (Analysis of Molecular Variance), overall Φ_{ST} between geographic populations was 0.0745 for uncorrected data. When the geographical populations were subdivided into sampling units within geographic populations (counties), subdivision was found to be even slightly higher between sampling units than between geographic populations ($\Phi_{ST} = 0.1076$). For clone-corrected data, overall Φ_{ST} was −0.0001 between geographic populations. With further hierarchical subdivision of geographical populations into sampling units, the Φ_{ST} value was again observed to be slightly higher (0.0111). In general, most of the molecular variation was detected within populations, with very little variation between geographic populations.

Population differentiation and gene flow were also analyzed by estimates of theta P (F_{ST}) as described by Weir and Cockerham (1984) (TABLE V). Overall F_{ST} between populations was 0.0741 for uncorrected data, which was significantly greater than zero. Nevertheless, this value of F_{ST} also indicates little genetic differentiation among populations. For clone-corrected data overall F_{ST} was −0.0009, which was not significantly different from zero.

Pairwise comparison between geographic populations was performed using estimates of F_{ST} equiva-

TABLE IV. Patterns of heterozygosity in different samples of *Rhizoctonia solani* AG-3 PT in North Carolina

Population	Sample type	Mean heterozygosity		No. of loci in HWE[b]	No. of loci with excess homozygotes	No. of loci with excess heterozygotes
		Expected[a]	Observed			
Washington	All genotypes[c]	0.2392	0.2105	7	0	0
	Clone-corrected[d]	0.2714	0.2500	7	0	0
Tyrell	All genotypes	0.2488	0.2946	7	0	0
	Clone-corrected	0.3333	0.3571	7	0	0
Currituck	All genotypes	0.3809	0.3809	5	2 (pP09 and pP42)	0
	Clone-corrected	0.4286	0.2857	7	0	0
Hyde	All genotypes	0.2045	0.1875	6	1 (pP83)	0
	Clone-corrected	0.2289	0.2286	7	0	0
Camden	All genotypes	0.2113	0.2073	6	0	1 (pP46)
	Clone-corrected	0.2457	0.2527	7	0	0
Total sample	All genotypes	0.2570	0.2562	6		
	Clone-corrected	0.3016	0.2748	7		

[a] Expected heterozygosity (unbiased) was computed according to Levene (1949) using TFPGA version 1.3 (Miller 1997).

[b] Hardy-Weinberg equilibrium (HWE) test was performed according to an exact test analogous to Fisher's exact test on a two-by-two contingency table, but extended to a triangular contingency or arbitrary size (Guo and Thompson 1992), using ARLEQUIN 2.000 (chain length: 100 000; dememorization: 1000) (Schneider et al 2000).

[c] Analysis performed on total data that included all 104 genotypes.

[d] Clone-corrected data comprising 32 multilocus PCR-RFLP genotypes.

TABLE V. Population differentiation and gene flow of *Rhizoctonia solani* AG-3 PT in North Carolina

		Sample Type		
	All isolates[a]		Clone corrected[b]	
PCR-RFLP locus	Nu[c]	theta P ($F_{WC(ST)}$)[d]	Nc[c]	theta P ($F_{WC(ST)}$)
pP09-*Hha* I	208	0.1420	88	−0.0003
pP42-*Hind* III	208	0.1009	88	0.0282
pP45-*Hind* III	208	−0.0116	88	−0.0352
pP46-*Hind* III	208	0.0648	88	−0.0289
pP47-*Eco* RV	208	0.2392	88	0.2355
pP83-*Hind* III	208	−0.0055	88	0.0227
pP89-*Rsa* I	208	0.0266	88	−0.0336
Overall		0.0741		−0.0009
Bootstrapping over loci	Upper value	0.1280		0.0499
	Lower value	0.0290		−0.0264

[a] Analysis performed on total data that included all 104 isolates.
[b] Clone-corrected data comprising one isolate per multilocus PCR-RFLP genotype; population was retained.
[c] N = sample size of alleles for uncorrected (Nu) and clone-corrected data (Nc).
[d] F statistics were estimated as in Weir and Cockerman (1984) using GDA version 1.0 (d15) (Lewis and Zaykin 2000).

lents (pairwise Φ_{ST} values) and Nei's unbiased genetic distances (D), for both uncorrected and clone-corrected data (TABLE VI). Pairwise Φ_{ST} values were significantly greater than zero, which would indicate population differentiation, only for uncorrected data. For example, the population from Washington was significantly different from Tyrrell and Currituck and very similar to Hyde and Camden county populations. For clone-corrected data, however, there was no indication of population differentiation considering both pairwise Φ_{ST} values and D.

HWE test for association of alleles within loci and exact test for linkage disequilibrium between pairs of loci.— Based on the lack of geographic subdivision, all MRG data were pooled into a single data set (for both uncorrected and clone-corrected samples) to test for HWE (TABLE VII) and composite linkage disequilibrium (TABLE VIII) between all pairs of loci.

The results from the likelihood ratio test (G^2) and the exact test analogous to Fisher's indicated genotypic proportions similar to HWE proportions at all seven loci (for clone-corrected data) and for six loci considering the uncorrected sample. For uncorrected data, only the locus pP09-*Hha* I showed proportions of genotypic counts that deviated from HWE. Overall, for uncorrected data, most pairs of loci were in genotypic disequilibrium (13/21). However, for clone-corrected data, the exact test for linkage disequilibrium was not significant for all pairs of loci.

DISCUSSION

This study describes a PCR-RFLP method for genotyping isolates of *R. solani* AG-3 PT. We employed a combination of two strategies for development of codominant markers as proposed by Rosewich et al (1999) and Xu et al (1999). The method established here enables unambiguous scoring of genotypes of

TABLE VI. Population pairwise Φ_{ST}[a] of *Rhizoctonia solani* AG-3 PT isolates collected from five commercial potato fields in North Carolina, for clone-corrected data (above diagonal) and total data (below diagonal)

County	Washington	Tyrrell	Currituck	Hyde	Camden
Washington	—	−0.0270	−0.0063	−0.0192	−0.0179
Tyrrell	0.0730**	—	−0.0933	0.0186	0.0035
Currituck	0.2107***	0.1232*	—	0.0656	0.0452
Hyde	0.0050	0.1057***	0.2949***	—	0.0089
Camden	0.0229	0.0320*	0.2572***	0.0146	—

[a] Population pairwise Φ_{ST} was calculated using ARLEQUIN ver 2.000 (Schneider et al 2000). Asterisks indicate significance of P values at $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) leading to values of Φ_{ST} larger than or equal to the observed value when permuting multilocus PCR-RFLP genotypes between populations (1000 permutations were performed).

TABLE VII. Test for Hardy-Weinberg equilibrium for multilocus PCR-RFLP genotypes of *Rhizoctonia solani* AG-3 PT

PCR-RFLP locus	Sample type	G^{2a}	P[b]	Prob.[c]
pP09-*Hha* I	All genotypes[d]	5.5963	0.0180*[f]	0.0220
	Clone-corrected[e]	2.6336	0.1046	0.1647
pP42-*Hind* III	All genotypes	6.6992	0.0719	0.0978
	Clone-corrected	1.6713	0.6433	1.0000
pP45-*Hind* III	All genotypes	0.0000	1.0000	1.0000
	Clone-corrected	0.0000	1.0000	1.0000
pP46-*Hind* III	All genotypes	2.2509	0.1335	0.5944
	Clone-corrected	2.0918	0.1400	0.5546
pP47-*Eco* RV	All genotypes	0.2090	0.6476	1.0000
	Clone-corrected	0.2001	0.6546	1.0000
pP83-*Hind* III	All genotypes	5.2690	0.1531	0.0738
	Clone-corrected	0.5581	0.9060	0.8412
pP89-*Rsa* I	All genotypes	0.1950	0.6588	0.6934
	Clone-corrected	3.8240	0.5363	0.7249

[a] Log likelihood statistic (G^2) 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 or arbitrary size (Guo and Thompson 1992), using ARLEQUIN 2.000 (chain length: 100 000; dememorization: 1000) (Schneider et al 2000).

[d] Analysis performed on total data that included all 104 isolates.

[e] Clone-corrected data comprising 32 multilocus PCR-RFLP genotypes.

[f] Asterisk denotes significant P value at $P < 0.05$ (*), indicating significant deviation from Hardy-Weinberg equilibrium.

isolates of *R. solani* AG-3 PT by allowing the distinction between homozygotes and heterozygotes. The PCR-RFLP method is also efficient, as only small amounts of DNA are required as starting material. Consistent with the heterokaryotic nature of *R. solani*, which has been confirmed by a similar molecular typing method (Rosewich et al 1999), every isolate had one or more heterozygous loci (TABLE II).

During the process of screening, four of the seven PCR-RFLP markers used for genotyping *R. solani* AG-3 PT (pP42, pP46, pP47 and pP89) also amplified genomic DNA from *R. solani* AG-3 TB, a pathogen of tobacco. Except for marker pP42, none of the alleles present in the sample of 20 tobacco isolates of *R. solani* AG-3 TB were shared with the potato sample (data not shown). Our findings corroborate previous information that populations of *R. solani* AG-3 are subdivided by host specialization (Ceresini et al 2001, Kuninaga et al 1997, Kuninaga et al 2000, Ogoshi 1987, Shew and Melton 1995, Stevens Johnk et al 1993). These four genetic markers could have potential applicability for phylogenetic analysis within *R.*

TABLE VIII. Exact tests for linkage disequilibrium[a] among pairs of individual PCR-RFLP loci based on 32 multilocus genotypes

PCR-RFLP locus	pP09 *Hha* I	pP42 *Hind* III	pP45 *Hind* III	pP46 *Hind* III	pP47 *Eco* RV	pP83 *Hind* III	pP89 *Rsa* I
pP09 *Hha* I	—	0.6794[b]	1.0000	0.2984	0.9692	0.0938	0.7972
pP42 *Hind* III	**0.0103**	—	0.5867	0.3298	0.0643	0.1608	0.8782
pP45 *Hind* III	0.9999	0.4509	—	1.0000	1.0000	1.0000	0.9812
pP46 *Hind* III	**0.0180**	0.00002	0.2021	—	0.5768	0.4756	0.4403
pP47 *Eco* RV	0.0662	**0.0006**	0.0689	**0.0077**	—	0.8246	0.4847
pP83 *Hind* III	**0.0001**	0.1546	0.2497	**0.0086**	0.6455	—	0.7112
pP89 *Rsa* I	0.4422	0.2082	0.7008	**0.0453**	0.2794	0.2875	—

[a] Linkage disequilibrium between pairs of loci was assessed using a exact test analogous to Fisher's exact test (Slatkin 1994, Slatkin and Excoffier 1996). The calculations were performed using ARLEQUIN 2.000 (number of permutations = 100 172; number of initial conditions for E.M. = 10) (Schneider et al 2000).

[b] P values for the exact test of association between alleles conditional to the allelic counts. Numbers above the diagonal are probabilities of association analyzed from the clone-corrected sample consisting of 32 unique multilocus PCR-RFLP genotypes. Numbers below the diagonal are probabilities of association analyzed from the total sample consisting of all 104 isolates. Significant P values are indicated in bold.

solani AG-3 as they were shown to be conserved enough to amplify genes across AG-3 PT and AG-3 TB, two genetically distinct subgroups of AG-3.

The first objective of this study was to determine the extent of genetic differentiation among populations of *R. solani* AG-3 PT and make inferences about gene flow and population subdivision. The allelic information from the seven single locus PCR-RFLP markers allowed us to group 104 isolates of *R. solani* AG-3 PT into 32 MRG. Gene flow was high, even though relatively few MRG were shared among localities. The overall Φ_{ST} between geographic populations was 0.0745 for total data. When data were subdivided into sampling locations (plots) within a field, subdivision was found to be even slightly higher between sampling units than between geographic populations (Φ_{ST} = 0.1076). However, using clone-corrected data, Φ_{ST} and F_{ST} estimates were essentially zero. In addition, for clone-corrected data, all pairwise Φ_{ST} values were not significant at 5%, and Nei's genetic distance (D) indicated no differentiation between populations.

Our results support the existence of a mechanism that ensures efficient long distance dispersal of genotypes between geographic populations. Recent analysis of the contribution of tuber-borne genotypes to the overall population of *R. solani* AG-3 PT present in potato fields of North Carolina provided evidence on how tuber-borne inoculum introduced every year affects population structure of the pathogen. High levels of genetic diversity were detected in introduced potato tubers from Canada (New Brunswick), Maine, and Wisconsin (50 different genotypes among a total sample of 58 isolates) (Ceresini 2000). Nevertheless, seven of these genotypes were among the most frequent genotypes observed in North Carolina fields (Ceresini 2000).

A similar lack of population structure was found for *R. solani* AG-1-IA from rice in Texas (Rosewich et al 1999). The authors characterized seven co-dominant RFLP loci for 182 AG-1 IA isolates in Texas rice fields and found 36 different multilocus genotypes. As there was no evidence for population subdivision, all isolates from the rice growing areas in Texas were considered to be part of one single population of *R. solani* AG-1 IA. Considering the similar observations of lack of population structure within *R. solani* AG-3 PT, we also pooled all multilocus PCR-RFLP genotypes into one single data set for further analysis of HWE and linkage disequilibrium.

The other specific question addressed in this study was: Does multilocus genetic diversity analysis support a sexual structure in local populations of *R. solani* AG-3 PT? Several studies have provided information about the structure of *R. solani* populations on wheat in Australia and rice in Texas. For example, MacNish et al (1997) found an association of anastomosis reaction type with allozymes and an overrepresentation of certain phenotypes, which was considered evidence for clonality. Evidence for clonality was also demonstrated for *R. solani* AG-1-IA through findings of repeated recovery of genotypes in the same and different rice field (Rosewich et al 1999).

To answer the question on population structure of *R. solani* AG-3 PT from potato, we considered the main effects of asexual reproduction on the pathogen population. The first major effect of asexual reproduction is the production of fewer recombinant genotypes, which decreases genotypic diversity. In this study the hypothesis of clonality was partially supported in that at least three of the population samples from potato shared the most common multilocus genotypes (MRG 1, 3 and 48) (TABLE II). In addition, observed counts for MRG 1, MRG 3, or MRG 48 were much higher than the expected counts derived under the hypothesis of panmixia (Stoddart and Taylor 1988). The higher frequency of these genotypes in potato samples may be the consequence of asexual reproduction, since all three MRGs were also found on seed potato tubers introduced from Maine into NC (Ceresini 2000). In addition, if populations of *R. solani* AG-3 PT were totally panmictic, the observed genotypic diversity would be similar to the expected value under panmixia (Stoddart 1983). Our results indicated that the overall genotypic diversity (G_o for uncorrected data = 16.64) was considerably smaller than the expected value under panmixia (G_E^* = 38.78) (TABLE II), supporting clonality. The clonal reproduction of *R. solani* AG-3 PT via mycelia or sclerotia provides a mechanism for a local collection of clones to predominate within a much larger recombining population of the pathogen on potato. However, the biological features of these asexual structures (mycelia and scletoria), especially the mode of dissemination, will influence the scale and intensity of clonal spread (Taylor et al 1999). A clonal structure for a population of *R. solani* AG-3 PT in South Australia could not be rejected because one DNA fingerprint pattern represented 30% of the isolates tested (Balali et al 1996).

The repeated occurrence of genotypes of *R. solani* AG-3 PT clearly suggests that populations are not only randomly mating (Milgroom 1996). However, the multiple occurrence of genotypes can be a confounding factor in the analyses of population structure of this potato pathogen. Therefore, the approach of analyzing the population structure of *R. solani* AG-3 PT considering only one representative of each multilocus genotype is appropriate. This approach has been recommended for making inferenc-

es about recombination in other populations of plant pathogenic fungi (Liu et al 1996, McDonald et al 1994, Rosewich et al 1999).

The second major effect of recombination on population structure is the random association of alleles both within (HWE) and between loci (linkage equilibrium). When all genotypes were included in the analysis, only one locus (pP09 *Hha* I) was not in HWE. In addition, linkage disequilibrium was detected for eight pairs of loci. However, for clone-corrected data, both tests for HWE indicated that all loci were in equilibrium and no linkage disequilibrium was detected for any pair of loci. This emphasizes the importance of using clone-corrected data to better understand aspects of reproduction that contribute to population structure (McDonald et al 1994, Rosewich et al 1999). Comparatively, analyses of the population structure of *R. solani* AG-1-IA from rice indicated that four of seven RFLP loci were in HWE and only one of all possible pairs of loci was in linkage disequilibrium, which indicated that recombination was occurring (Rosewich et al 1999).

At the extremes of models of population structure, populations may be panmictic or strictly clonal. An intermediate model was proposed by Maynard Smith et al (1993) for bacterial pathogens. This model may also be appropriate for many fungal plant pathogens that have annual sexual cycles and asexual phases. This model is characterized by frequent recombination and by the occurrence of one or a few successful individual genotypes that reproduce clonally and increase to high frequencies. Differences in fitness among genotypes expressed in the asexual phase could be responsible for the prevalence of certain genotypes. Our observations support a model of population structure for *R. solani* AG-3 PT that includes both clonality and recombination.

This study provided initial information on population genetics of *R. solani* AG-3 PT in NC. Further studies are needed to elucidate the magnitude of recombination in populations of *R. solani* AG-3 PT. In addition, increased sampling is needed to maximize genotypic diversity and enhance the power of statistical tests to infer population genetic processes. Increased resolution to infer population structure also may be achieved by the examination of additional genetic loci to ascertain that MRGs identified in this study actually represent clones.

Consideration also should be given to the elucidation of the role of other evolutionary processes that could cause linkage disequilibrium in populations of *R. solani* AG-3 PT, such as gene flow or migration. Gene flow can cause gametic disequilibrium by the immigration of individuals from populations with different allele frequencies (Milgroom 1996). Future research also should examine populations of *R. solani* AG-3 PT from the northern United States (Wisconsin and Maine) and eastern Canada, as these areas are responsible for the production of most of the seed potatoes imported into eastern North Carolina.

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