Population Structure of *Rhizoctonia oryzae-sativae* in California Rice Fields

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ABSTRACT

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Six pairs of single-locus microsatellite primers were developed to study the population structure of *Rhizoctonia oryzae-sativae*, the cause of aggregate sheath spot disease of rice, among and within three rice-growing areas in California over a 3-year period. A high level of gene flow among growing areas was indicated by low population subdivision according to analysis of molecular variance and moderate to no population differentiation between pairs of populations based on the fixation index (F_{ST}). Gametic equilibrium of most pairs of microsatellite loci, high numbers of unique multilocus genotypes, and high genotypic diversity indicated extensive sexual recombination within growing areas. Because there was little differentiation among populations in all hierarchical levels, including among growing areas within sampling years, fields within growing areas, and corners within individual fields, a high level of gene flow was revealed in all levels. Basidiospores were likely the main vehicle of gene flow among populations, including short and long distances. Asexual inocula (sclerotia and mycelia) probably overwinter because a few clones were detected over a 2-year period within the same field. A few clones were shared among fields but were not commonly shared among growing areas.

Aggregate sheath spot of rice, caused by *Rhizoctonia oryzae-sativae*, occurs in California in the United States and in other countries, including Japan, China, Vietnam, India, Thailand, Iran, Venezuela, Uruguay, and Australia (6,14,24,25,36). The disease was observed in California in the 1960s after susceptible semidwarf, high-yielding cultivars were introduced and widely grown (15). Disease symptoms are characterized by oval lesions with green or gray centers surrounded by a distinct brown margin. Several lesions can coalesce, resulting in an aggregate of lesions. The fungus may also attack panicles, which results in sterile grain (14,15,32). Sclerotia often form inside and on the surface of infected tissue. Overwintering sclerotia or mycelia in soil or crop residue probably serve as reservoirs of inocula (14,15,27,32,33). A subsequent rice crop is infected by floating sclerotia in the rice paddy (14,15,32). Aggregate sheath spot caused yield losses of 20% in Australia (26) and 4 to 9% in Uruguay (25); however, significant yield losses have not been documented in Korea (23) and California (16).

Ceratobasidium oryzae-sativae, the teleomorph of *R. oryzae-sativae* (17), is distinct from other *Ceratobasidium* spp. because it has globose to subglobose basidia bearing two stout sterigmata. Basidiospores are typically subglobose, hyaline, and repetitive (17). The fruiting body occurs on the outside of leaf sheaths as a thin, effuse whitish layer (14).

Aggregate sheath spot is presumed to be a monocyclic disease; numbers of sclerotia, the primary inocula, in a field may directly affect disease incidence in a subsequent rice crop (32). However, Miller and Webster (32) demonstrated that populations of sclerotia in rice seedbeds in California were not always correlated with subsequence levels of disease. They suggested that other propagules, including mycelia in straw residue, may serve as an alternative inoculum source, and basidiospores may serve as secondary inoculum. Later, Lanoiselet et al. (27) discovered that *R. oryzae-sativae* could overwinter as mycelia on straw debris. Although the sexual stage was discovered in California by Gunnell and Webster in 1987 (17), the role of the teleomorph in disease epidemics has never been examined.

Microsatellites or simple-sequence repeats (SSRs) are widely dispersed in most eukaryotic and prokaryotic genomes (8,43). The number of repeat units in a particular microsatellite region generates DNA polymorphism which can be detected by specific polymerase chain reaction (PCR) primers amplifying such regions (37). Microsatellites have become powerful tools in genetic analyses of numerous organisms, including animals, plants, fungi, and humans, for the study of genomic evolution, population structure, gene flow, intraspecific phylogeny, mating systems, and genetic mapping (2,8,37). Recently, highly polymorphic microsatellite markers were developed for studying population genetics of rice-, soybean-, and maize-infecting *R. solani* AG-1 IA (3,46) and potato-infecting *R. solani* AG-3 (12).

The objectives of this research were to determine the genetic diversity within populations of *R. oryzae-sativae* and estimate the relative importance of sexual and asexual reproduction of the fungus in California rice-growing areas. Because the disease was assumed to be monocyclic with primarily asexual reproduction, a high degree of clonality was expected. We also evaluated genetic structure and inferred gene flow of *R. oryzae-sativae* in hierarchical levels, including among sampling years, growing areas within sampling years, fields within growing areas, and within individual fields. In addition, genetic differentiation of *R. oryzae-*

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sativae in the same field in consecutive years and among lesions along the same leaf sheath was determined.

MATERIALS AND METHODS

Collection of isolates. Rice stems with aggregate sheath spot symptoms were randomly collected from three rice-growing areas parallel to the Sacramento River in Sutter, southern Colusa, and northern Colusa Counties, California, in 2004 to 2006 (Fig. 1). Pieces of rice leaf sheaths of ≈4 to 9 mm^2 were cut from the margin of lesions, surface sterilized in 0.6% sodium hypochlorite (10% bleach) for 3 min, and plated on potato dextrose agar (PDA) + 0.01% gentamicin sulfate. *R. oryzae-sativae*-like cultures were transferred to PDA and incubated at room temperature (≈25°C) for 2 weeks. Agar plugs of all *R. oryzae-sativae* cultures were maintained in 1 ml of sterile distilled water at 4°C for long-term storage.

In 2004, diseased rice sheaths were collected from 7 to 14 rice fields in each growing area (Fig. 1; Table 1). One to five isolates of *R. oryzae-sativae* were collected in each field. A hierarchical sampling scheme was used in 2005 and 2006; diseased rice sheaths were collected from four rice fields in each of the three growing areas (Fig. 1; Table 1). Ten isolates were collected in each of three (2005) or two (2006) fields from each growing area. In addition, 10 isolates were cultured from stems from each of the four corners of one (2005) or two (2006) fields per growing area.

In 2007, 10 isolates were collected from each of the four corners in one field in each of the three rice-growing areas (Table

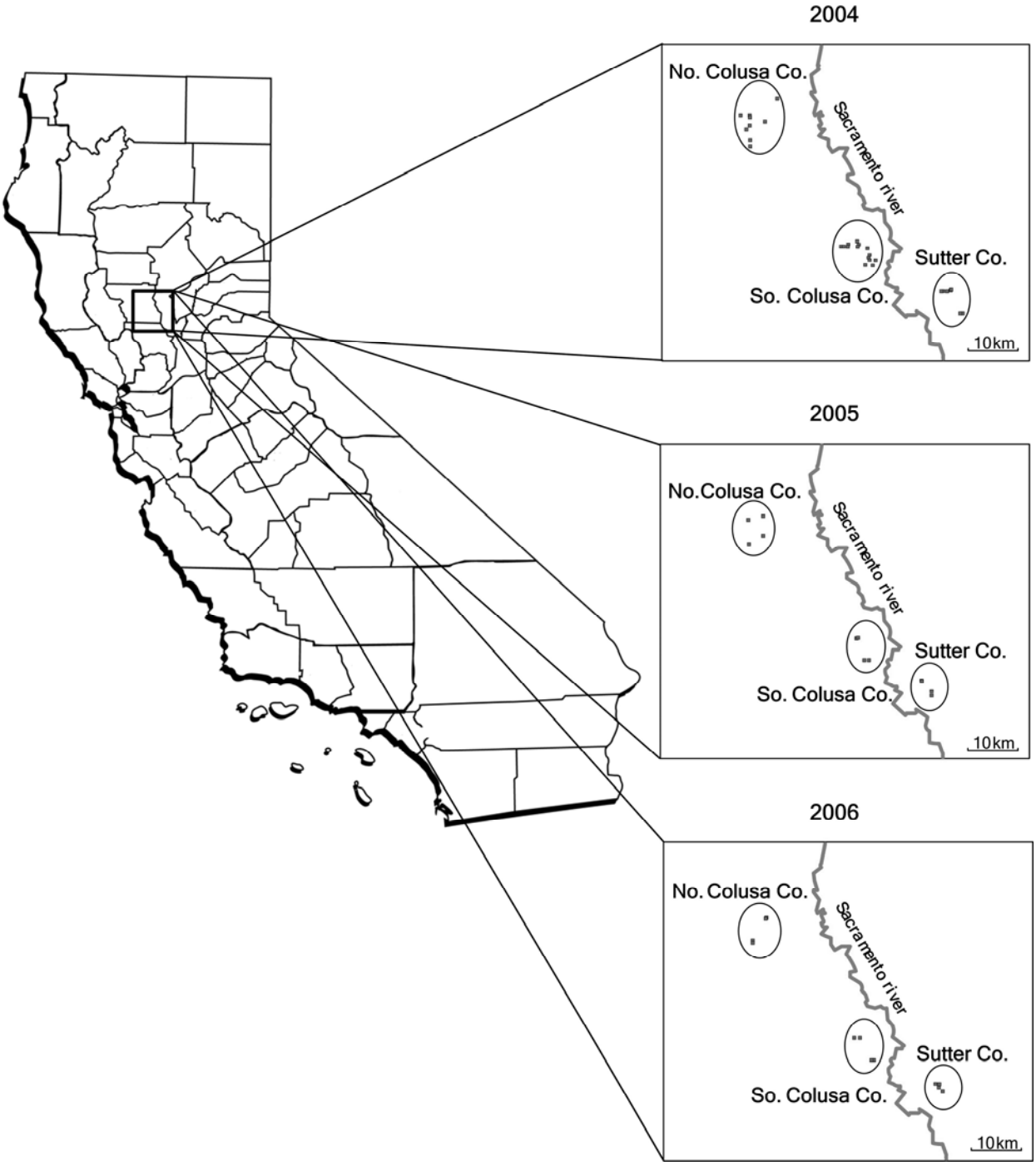

Fig. 1. Sampling locations in three rice-growing areas in Sutter, southern Colusa, and northern Colusa Counties, CA, in 2004 to 2006 (California map from California Secretary of State website).

1). The corners of a total of 12 fields were sampled in 2005 to 2007 but data from only 11 fields were analyzed due to small sample sizes in each corner of a field in southern Colusa County in 2006. In addition, the same rice field in each of the three rice-growing areas was sampled in 2005 to 2007. Ten isolates were collected from each of the four corners in each field. Because the field in Sutter County was rotated to sunflower in 2007, data from only 2005 and 2006 were included in the analysis. In order to examine whether isolates from multiple lesions on the same leaf sheath were clonal, one isolate was collected from each of four individual lesions on each of four leaf sheaths.

Identification of isolates. All isolates were identified as *R. oryzae-sativae* based on colony characteristics. The production of globose brown sclerotia on white to pale-brown mycelia on PDA after 2 weeks was diagnostic (14). The identity of 30 isolates was confirmed by the examination of morphological characteristics (the appearance of T cells and constricted hyphae) (33), binucleate condition of cells, and internal transcribed spacer (ITS) sequences. Nuclei stained with 4′-6′-diamidino-2-phenylindole (DAPI) were examined in UV light (13). ITS1, 5.8s rDNA, and ITS2 regions were amplified by primers ITS4 and ITS5 (44). The amplified products were purified with QIAquick PCR Purification kit no. 28104 (Qiagen, Valencia, CA) according to the manufacturer's instructions. The purified PCR products were sequenced with both primers using an ABI 3730 Capillary Electrophoresis DNA Analyzer (Applied Biosystem, Foster City, CA) at the College of Biological Sciences DNA Sequencing Facility, University of California (UC) Davis. DNA sequences were compared with sequences in the National Center for Biotechnology Information (NCBI) database using BLASTN with the following parameters: expect threshold = 10, word size = 28, match/mismatch scores = 1, –2, and linear gap costs.

TABLE 1. *Rhizoctonia oryzae-sativae* isolates used in this study

Year, location	No. of fields	No. of isolates	
		Total	Clone-corrected[a]
2004			
Sutter Co.	7	15	14
South Colusa Co.	14	24	22
North Colusa Co.	9	21	20
Total	30	60	56
2005			
Sutter Co.	4	70	54
South Colusa Co.	4	70	60
North Colusa Co.	4	67	58
Total	12	207	172
2006			
Sutter Co.	4	100	65
South Colusa Co.	4	78	62
North Colusa Co.	4	99	67
Total	12	277	194
2007			
Sutter Co.	1	38	24
South Colusa Co.	1	40	23
North Colusa Co.	1	40	24
Total	3	118	71

[a] Clone-corrected data included only isolates representing a different multilocus genotype in each growing area.

Culture preparation for DNA extraction. Each isolate was grown for 7 days in 125-ml Erlenmeyer flasks with 50 ml of liquid media consisting of 20.7 g of D-glucose, 1.2 g of DL-asparagine, 1.2 g of $K_2HPO_4 \cdot 3H_2O$, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of NaCl, and 0.5 g of yeast extract per liter. The unshaken flasks were incubated at room temperature. Mycelia were then strained through Miracloth (Calbiochem, Gibbstown, NJ) and lyophilized. All lyophilized cultures were sent to the College of Agricultural and Environmental Science Genomics Facility, UC Davis for DNA extraction, single-locus microsatellite primer design, microsatellite amplification, and microsatellite fragment size analyses.

Microsatellite identification and primer design. Genomic DNA was extracted from one isolate of *R. oryzae-sativae* and ≈5 μg of the purified DNA was randomly sheared with the HydroShear device (GeneMachines, San Carlos, CA). After separation of the sheared DNA on an agarose gel by electrophoresis, fragments of 2 to 4 kb were isolated and used to construct a shotgun sublibrary in a pUC18 plasmid. Bacterial colonies of the sublibrary were picked and organized in microwell plates using the Q-bot system (Genetix, Queensway, UK). DNA sequencing was performed with 1,152 picked clones using an ABI Big Dye Terminator Cycle Sequencing kit and a 3730xl DNA analyzer (Applied Biosystems). Over 700 kb of high-quality *R. oryzae-sativae* sequences were obtained after trimming out poor-quality and vector-contaminated sequences. SSR sequences were identified using a perl script and PCR primers were designed around the SSR motifs with the PRIMER3 program (Whitehead Institute for Biomedical Research, Cambridge, MA) (40). Thirty-seven sets of the designed oligonucleotides, with 6-FAM labeling on the forward primers of each set, were used to screen microsatellite markers showing polymorphism.

Of the 37 primer sets, 13 primer pairs were selected based on stability and SSR polymorphism. Gametic disequilibrium of the 13 primers with DNA from 32 *R. oryzae-sativae* isolates was analyzed by the program ARLEQUIN version 2.0 (Genetic and Biometry Laboratory, University of Geneva, Switzerland) to confirm that the assumption of independent markers was not violated. Six unlinked single-locus microsatellite markers were selected for population structure studies (Table 2). In addition,

TABLE 2. Microsatellite primers used in this study

Locus	Repeat motif	Primer sequence (5′–3′)	No. of alleles	Product size (bp)[a]	Gene diversity[b]			
					2004	2005	2006	2007
P2	TG	F: ACGTCGTCGAGGAATTCTGT	3	136–140	0.53	0.59	0.58	0.57
	…	R: ACGTGGAACCCCAGACATAG	…	…	…	…	…	…
P7	CT	F: CGCTGACTTTGGTGAGCATA	2	175–177	0.35	0.43	0.43	0.30
	…	R: AAAACAACCGATCCAGTGCT	…	…	…	…	…	…
P20	GGC	F: CACAGGGTGACTACGGCAAC	8	90–114	0.69	0.62	0.62	0.59
	…	R: GTAGCTCCCTCCATAGCCG	…	…	…	…	…	…
P33	CCGC	F: CGTGTCTCGTACGTCTGCAT	9	187–202	0.75	0.85	0.79	0.81
	…	R: GCTGACAGGGCTATCATTGG	…	…	…	…	…	…
P36	CG	F: TACCCTTTGGGTTTGAGACG	3	177–181	0.10	0.24	0.19	0.23
	…	R: TGCTGGTTATTCTCTGCACG	…	…	…	…	…	…
P37	AC	F: CAAACACCGAGAAGCAGGTT	9	165–173	0.75	0.84	0.74	0.82
	…	R: GGGTGCAATTGCTGTTCATA	…	…	…	…	…	…
All loci	…	…	34	…	…	…	…	…
Mean	…	…	5.7	…	0.53	0.59	0.56	0.55

[a] Range of amplified product size.
[b] Unbiased gene diversity was computed according to Nei (34) using FSTAT version 2.9.3.2.

species specificity of the markers was tested with *R. solani* (originally isolated from cotton seedlings from Tulare County, CA) and two binucleate *Rhizoctonia* spp., isolates 249 and 250, from wheat (collection of R. K. Webster).

DNA extraction and PCR. Genomic DNA of *R. oryzae-sativae* was extracted by using TRIzol reagent and the manufacturer's protocol (Invitrogen, Carlsbad, CA). DNA concentration was calculated by using PicoGreen double-stranded DNA quantitation reagent (Molecular Probes, Eugene, OR) and a SPECTRAFluor Plus fluorometer (Tecan, Durham, NC). After normalizing the concentration of fungal DNA, ≈5 ng of the DNA was used in each PCR, which was performed in 25-μl volumes using the QIAGEN HotStarTaq DNA polymerase with a 6-FAM-labeled primer. PCR was carried out in a Tetrad thermal cycler (MJ Research, Watertown, MA) with the following program: 15 min at 95°C; followed by 35 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C; then 10 min at 72°C as a final extension step. The amplified PCR products were separated with an ABI 3730xl DNA analyzer system and the fragment size was analyzed using GeneScan 500 LIZ size standard (Applied Biosystem).

Microsatellite data analyses. The electropherogram data were analyzed by GENEMAPPER software (version 3.0; Applied Biosystem) to determine the size of products. Multilocus genotyping data were obtained by considering different product sizes as different alleles at each locus. One or two alleles per locus were present and were scored as homozygote or heterozygote. Null alleles were treated as missing data. Isolates having the same multilocus genotypes (MGs) were assumed to be clones. There were two sets of data: the non-clone-corrected data, which included all isolates, and the clone-corrected data, which included only isolates representing each different MG in each growing area (Table 1). Clone-corrected data were used to reduce the bias of sampling the same clone within growing areas. The significance levels for multiple comparisons were adjusted by the sequential Bonferroni correction (38).

Genetic diversity within populations. The effective number of alleles was computed according to Hartl and Clark (20) in POPGENE software (version 1.32; University of Alberta and Center for International Forestry Research, Alberta, Canada) (45). The clonal fraction was defined as the proportion of asexually derived fungal isolates and was calculated as 1 − [(number of different genotypes)/(total number of isolates)] (47). Genotypic diversity, computed in MULTILOCUS software (version 1.3; Department of Biology, University College London, Silwood Park, UK), was calculated by $(n/n - 1)(1 - \Sigma p_i^2)$, where p_i is the frequency of the ith genotype and n is the number of individuals sampled. All possible pairs of isolates were sampled; the proportion of pairs that were different was then calculated. Genotypic diversity was defined as the probability that two individuals taken at random had distinct genotypes; 1 if every individual was different and 0 if every individual was the same (1). Non-clone-corrected data were used in these analyses.

Average unbiased gene diversity (34) and allelic richness using clone-corrected data were calculated in FSTAT software (version 2.9.3.2; Institute of Ecology, Lausanne, Switzerland). Allelic richness in 14 samples (the minimum number of samples) was calculated, using a rarefaction index (22) to eliminate the dependency on sample size (9). The differences in gene diversity and allelic richness among groups of populations were tested with 1,000 permutations in FSTAT version 2.9.3.2. In addition, the clonal fractions within multiple-scale hierarchical populations and percentages of clones shared among those scale populations were determined to estimate the dispersal of asexual inocula.

Reproductive mode: Hardy-Weinberg and gametic equilibrium tests. The clone-corrected data were used to test the null hypothesis that population structure conformed to random mating by Hardy-Weinberg and gametic equilibrium tests. Hardy-Weinberg equilibrium (HWE) was determined by the exact test (18) using a Markov chain (chain length: 100,000; dememorization: 1,000) in ARLEQUIN version 2.0. Inbreeding coefficients (F_{IS}) were calculated for detection of significant excesses or deficits of heterozygotes, which may be the reason for deviation from HWE. The significance of F_{IS} within populations was tested with 1,080 randomizations and the differences of F_{IS} among groups of populations were tested with 1,000 permutations in FSTAT version 2.9.3.2.

Gametic disequilibrium was assessed by determining the association between pairs of loci using the likelihood ratio test (10) with 1,023 permutations in ARLEQUIN version 2.0. Index of association (I_A), a measure of multilocus gametic disequilibrium (29), was also computed in MULTILOCUS version 1.3. The significance of I_A was tested by comparing the observed value and the expected value under a null hypothesis of random mating with 1,000 randomizations (1).

Population admixture. To detect admixture of subpopulations differing in allele frequency, which could be the cause of departure from HWE and gametic disequilibrium (21), Bayesian analysis of admixed genotypes within populations was carried out in STRUCTURE software (version 2.2; Department of Human Genetics, University of Chicago) (35). The posterior probability for each individual assigned to each population was computed by using prior information of sampling area as a predefined population (six runs; $K = 9$; 10,000 burn-in period; 10,000 Markov chain Monte Carlo steps after burn-in).

Hierarchical distribution of genetic variation and population differentiation. The clone-corrected data were used to test the null hypothesis that populations were not genetically differentiated over the multiple hierarchical spatial scales or among distinct sampling years. Analysis of molecular variance (AMOVA) estimated genetic variance components at different hierarchical levels of population structure (11), including among sampling years, among growing areas within sampling years, among fields within growing areas, and among corners within fields. In addition, the pairwise fixation index (F_{ST}) between growing areas was calculated to examine population differentiation. Both analyses were performed with 1,023 permutations in ARLEQUIN version 2.0.

RESULTS

Collection of *R. oryzae-sativae* isolates. Different sampling schemes were used to collect *R. oryzae-sativae* isolates. In 2004, only a few isolates ($n = 1$ to 5) were collected from each of several fields in each growing area (Fig. 1). In 2005 and 2006, numerous isolates were collected in each of four fields in each growing area. In addition, isolates were collected from each of the four corners (40 isolates per fields) in one or two fields in each growing area in 2005 and 2006. In total, 544 *R. oryzae-sativae* isolates were collected from the three rice-growing areas in 2004 to 2006 (Table 1). Based on cultural characteristics, all isolates were identified as *R. oryzae-sativae*. The occurrence of T cells, constricted hyphae, and binucleate cells was confirmed in 30 isolates. In all, 672 bp of ITS sequences were 99.7% similar to *R. oryzae-sativae* sequences in the NCBI database (e.g., accession no. AJ000192). All 30 isolates of *R. oryzae-sativae* shared identical ITS sequences.

Microsatellite primer development. None of the primers amplified DNA from *R. solani* or binucleate *Rhizoctonia* sp. isolate 250. Two primer pairs weakly amplified two microsatellite loci, P20 and P33, of *Rhizoctonia* sp. isolate 249. The fluorescence signal of loci P20 and P33 of binucleate *Rhizoctonia* sp. isolate 249 was 3.8 and 2.5 times less intense than the fluorescence signal of the corresponding loci of *R. oryzae-sativae*, respectively.

The allelic variation in loci P2, P7, P20, and P36 was due to differences in the number of repeat units. Allelic variation for P33 was due to differences of 1, 2, and 3 bp and repeat units; variation

in P37 was due to 1 bp and repeat units. Multiple loci of P33 were sequenced and variability in lengths of the alleles was caused by an indel or an imperfect SSR motif. Therefore, the infinite allele model (all alleles sizes are independent) was assumed in the analyses.

The number of alleles observed at each locus was 2 to 9, with an average of 5.7 alleles per locus. P7 was the least polymorphic locus and P33 and P37 were the most polymorphic loci (Table 2). The average gene diversity was high in every year of the study, ranging from an average low of 0.53 in 2004 to an average high of 0.59 in 2005 (Table 2).

A total of 34 alleles was recovered by six microsatellite loci in 2004 to 2007 (Table 2). There were 32, 32, and 31 total alleles and 3.3, 3.2, and 2.8 effective alleles per locus in isolates collected in Sutter, southern Colusa, and northern Colusa Counties, respectively. In all, 28 alleles (82.4% of the total number of alleles) were recovered in all growing areas, 5 alleles were present in two growing areas, and only 1 private allele of P20 was restricted to northern Colusa County.

Genetic diversity within populations. Diversity of MGs and genotypic diversity within each of the three growing areas in 2004 to 2006 were high. The frequency of MGs was 65.0 to 95.2% of the total number of isolates, genotypic diversity was 0.981 to 0.996, and gene diversity was 0.48 to 0.63 (Table 3). The lowest allelic richness within populations was 3.4 per locus and the highest allelic richness was 4.5 per locus (Table 3). Gene diversity was not significantly different among years ($P = 0.423$). Allelic richness of the 2004 populations was significantly different from that of the 2005 populations ($P = 0.013$) but allelic richness of the 2006 populations was not significantly different from either 2004 populations ($P = 0.661$) or 2005 populations ($P = 0.097$). Clonal fractions within growing areas in 2004 to 2006 were 0.05 to 0.35 (Table 3). Because different sampling schemes were used, clonal fractions could not be compared among years. However, clonal fractions were proportional to number of isolates within fields (i.e., clonal fractions increased as more isolates from individual fields were included in the analysis).

Population admixture. Eleven admixed MGs (2.9% of the total MGs) were detected in five populations in 2004 to 2006 (Table 4). Four populations had no admixture. There were three admixed genotypes detected in the 2004 populations; two MGs were assigned to another growing area in the same year and one MG was assigned to another year. There were seven admixed genotypes detected in the 2005 populations; two MGs were assigned to another growing area in the same year and five MGs were assigned to another year. The admixed MG in 2006 was assigned to the population in 2005.

Reproductive mode: Hardy-Weinberg and gametic equilibrium tests. The null hypothesis that population structure conformed to random mating was tested. After removing admixed MGs, the number of loci in HWE increased and number of pairs in gametic disequilibrium and I_A also decreased in some populations (Table 4). F_{IS} in all nine populations (from each of the three growing areas in 2004 to 2006) also decreased but all were significantly positive. F_{IS} among sampling years was not significantly different ($P = 0.204$). The number of loci in HWE was 1 to 5. There was no gametic disequilibrium between any pairs of loci in six of the nine populations of *R. oryzae-sativae*. Only 1 of the 15 pairs of loci in the other three populations was in gametic disequilibrium. Significant I_A was not detected in the 2006 populations but significant I_A was observed in a few populations in 2004 to 2005 (Table 4).

Dispersal of asexual inocula. Most clones within growing areas were due to clones within fields in each growing area. Only a few MGs were shared among growing areas and some were shared among fields (Table 5). In 2005 to 2007, clonal fractions within corners of fields were 0.10 to 0.39 but there were a few clones shared among corners (Table 5).

When an individual field in each growing area was sampled in 2005 to 2007, a small percentage (3.6%) of overwintering clones in the fields in southern Colusa and northern Colusa Counties was observed (Table 5). Three MGs (1.8%) in these fields were shared in two consecutive years. No MG was found in three consecutive years in any field. However, three MGs (1.8%) were shared in individual fields between 2005 and 2007. The field in Sutter County was rotated to sunflower in 2007 and no clone was common between 2005 and 2006. When all isolates collected in 2004 to 2007 were combined, 32 MGs (7.1%) were shared among years (Table 5). In total, 24 MGs were shared in two consecutive years and 8 MGs were shared in 2005 and 2007 but no MGs were shared in three consecutive years.

Of the 448 different MGs, 318 (71.0%) were unique (no clones were detected). In each year of the study, the unique MGs accounted for 92.9, 83.6, 72.5, and 59.2% of the total MGs in 2004 to 2007, respectively. MGs of isolates from different lesions along the same leaf sheath of a single rice plant were identical in each of four replications.

Hierarchical distribution of genetic variation and population differentiation. According to AMOVA, populations were not significantly differentiated among sampling years when data from the three growing areas in 2004 to 2006 were analyzed (Table 6). However, there was a significant but low population differentiation among years when three individual fields were analyzed (Table 6). When data from these individual fields were analyzed

TABLE 3. Genetic diversity within nine *Rhizoctonia oryzae-sativae* populations from three rice-growing areas in California in 2004 to 2006

Population	No. of isolates	No. of MGs[a]	Clonal fraction	Genotypic diversity[b]	Gene diversity[c,d]	Allelic richness[d,e]
Sutter-2004	15	14	0.07	0.981	0.60	3.7
South Colusa-2004	24	22	0.08	0.993	0.48	3.5
North Colusa-2004	21	20	0.05	0.991	0.49	3.5
All areas-2004	60	56	0.07	0.996	0.53	3.6
Sutter-2005	70	54	0.23	0.989	0.63	4.5
South Colusa-2005	70	60	0.14	0.995	0.58	4.0
North Colusa-2005	67	58	0.13	0.996	0.51	3.9
All areas-2005	207	171	0.17	0.998	0.59	4.1
Sutter-2006	100	65	0.35	0.989	0.57	3.9
South Colusa-2006	78	62	0.21	0.992	0.57	3.7
North Colusa-2006	99	67	0.32	0.985	0.51	3.4
All areas-2006	277	182	0.34	0.995	0.56	3.7

[a] Multilocus genotypes (MGs).
[b] Genotypic diversity was calculated using MULTILOCUS version 1.3; diversity was defined as the probability that two individuals taken at random had distinct genotypes, where the value was 1 if every individual was different and 0 if every individual was the same (1).
[c] Unbiased gene diversity (34) was calculated using FSTAT version 2.9.3.2 and averaged across all loci.
[d] Differences of gene diversity and allelic richness among groups of populations were tested with 1,000 permutations in FSTAT version 2.9.3.2.
[e] Allelic richness in 14 samples (the minimum number of samples) was calculated according to El Mousadik and Petit (9), using rarefaction index (22) implemented by FSTAT version 2.9.3.2 and averaged across all loci.

separately, population differentiation among years was not significant in one of the three fields (data not presented). There was low population differentiation of *R. oryzae-sativae* among growing areas within sampling years. Similarly, population differentiation among fields within growing areas was also small (Table 6).

To test the null hypothesis that populations are not genetically subdivided within a paddy, isolates from four corners of fields were sampled and analyzed by AMOVA. The population differentiation of the fungus among corners within fields was low (Table 6). When the data from each of 11 fields were analyzed separately, the population differentiation was not significant ($P > 0.05$) among the corners in eight fields. In the other three fields, the differentiation of the populations among corners (F_{ST}) was 0.07, 0.012, and 0.012 (data not presented).

Based on the F_{ST} of the populations between pairs of growing areas in 2004 to 2006, there was moderate ($F_{ST} = 0.080$) to no population differentiation (Table 7). Populations of *R. oryzae-sativae* from Sutter County versus southern Colusa County were not genetically differentiated in all 3 years.

DISCUSSION

The microsatellite markers developed in this study were species specific and provided the resolution to distinguish a large number of genotypes of *R. oryzae-sativae*. At least four loci were amplified for all *R. oryzae-sativae* isolates and all six loci were amplified for the majority of them. A few *R. oryzae-sativae* isolates exhibited null alleles in two primers, possibly caused by a mutation at the 3′ end of the primer binding sites (5).

Although the sexual stage of *R. oryzae-sativae* is known (17), aggregate sheath spot is considered to be a monocyclic disease because sclerotia produced at the end of season overwinter and infect the next rice crop (14,15,32). Therefore, it was surprising

TABLE 4. Tests for random mating hypothesis in nine *Rhizoctonia oryzae-sativae* populations from three rice-growing areas in California in 2004 to 2006

Population	No. of admixed MGs[a]	No. of loci in HWE[b]	F_{IS}[c,d]	P[c]	No. of pairwise GD[e]	I_A[f]	P[f]
Sutter-2004	0	1/6	0.329	0.001*	0	0.135	0.173
South Colusa-2004	2	2/5[g]	0.389	0.001*	0	0.197	0.065
South Colusa-2004[h]	–	2/5[g]	0.366	0.001*	0	0.160	0.078
North Colusa-2004	1	4/6	0.201	0.006*	1	0.487	0.001*
North Colusa-2004[h]	–	5/6	0.145	0.025*	1	0.376	0.009*
Sutter-2005	0	2/6	0.150	0.001*	0	0.155	0.002*
South Colusa-2005	4	1/6	0.239	0.001*	1	0.184	0.006*
South Colusa-2005[h]	–	2/6	0.226	0.001*	0	0.176	0.007*
North Colusa-2005	3	1/6	0.263	0.001*	1	0.267	<0.001*
North Colusa-2005[h]	–	2/5[g]	0.224	0.001*	1	0.087	0.073
Sutter-2006	0	2/6	0.278	0.001*	0	0.011	0.409
South Colusa-2006	0	2/6	0.266	0.001*	1	0.078	0.149
North Colusa-2006	1	4/6	0.236	0.001*	0	0.014	0.399
North Colusa-2006[h]	–	4/6	0.233	0.001*	0	0.021	0.352

[a] Admixed multilocus genotypes (MGs) were determined by Bayesian statistics in the prior information model (six runs; $K = 9$; 10,000 burn-in period; 10,000 Markov chain Monte Carlo steps after burn-in) implemented in STRUCTURE version 2.2 (35).
[b] Hardy-Weinberg equilibrium (HWE) test was performed by exact test (18) using a Markov chain (chain length: 100,000; dememorization: 1,000) in ARLEQUIN version 2.0.
[c] Inbreeding coefficients (F_{IS}) and P values were calculated with 1,080 randomizations in FSTAT version 2.9.3.2.
[d] After removing admixed MGs, there was no significant difference in F_{IS} among years ($P = 0.204$) tested with 1,000 permutations in FSTAT version 2.9.3.2.
[e] Fifteen pairs of loci were tested for gametic disequilibrium (GD) using the likelihood ratio test (10) with 1,023 permutations in ARLEQUIN version 2.0; the value indicates the number of significant associated pairs of loci at $\alpha = 0.05$ with sequential Bonferroni adjusted (38).
[f] Index of association (I_A) and P values were computed in MULTILOCUS version 1.3; the significance of I_A was tested by comparing the observed value and expected value under the null hypothesis of random mating with 1,000 randomizations (1); * indicates significant at $\alpha = 0.05$.
[g] One locus was monomorphic.
[h] Admixed multilocus genotypes were removed.

TABLE 5. Dispersal of asexual inocula of *Rhizoctonia oryzae-sativae* within and among multiple-scale hierarchical populations in California in 2004 to 2007

Year	Population	Total no. of		Clonal fraction[b]	Clones shared among populations	
		Isolates	MGs[a]		No. of MGs	MGs (%)
2004[c]	Areas	60	56	0.07	1	1.8
2005	Areas	207	171	0.17	1	0.6
	Fields	207	171	0.21	2	1.2
	Corners of fields[d]	39	34	0.10	0.3	0.9
2006	Areas	277	182	0.29	12	6.6
	Fields	277	182	0.24	21	11.5
	Corners of fields[e]	40	26	0.30	0.8	3.1
2007	Fields	118	71	0.40	3	4.2
	Corners of fields[d]	39	24	0.39	0.7	2.9
2005–2007[f]	Years (Sutter Co.)[g]	79	60	0.24	0	0.0
	Years (South Colusa Co.)	120	84	0.30	3	3.6
	Years (North Colusa Co.)	117	83	0.29	3	3.6
2004–2007	Years (all areas)	662	448	0.25	32	7.1

[a] Multilocus genotypes (MGs).
[b] Mean of clonal fractions within multiple-scale hierarchical populations in three growing areas, 12 fields, four corners of a field, three sampling years (individual fields), or four sampling years (2004 to 2007).
[c] Sample size in each field was small ($n = 1$ to 5) in 2004, so only the area level was evaluated.
[d] Mean of three fields.
[e] Mean of five fields.
[f] Individual field in each of the three growing areas was sampled in 2005 to 2007.
[g] In this field, only data from 2005 and 2006 were analyzed because sunflowers were rotated in 2007.

that many MGs were unique and a high degree of genotypic diversity was discovered in this study. Moreover, only a few populations of *R. oryzae-sativae* had significant I_A, and gametic equilibrium of most pairs of microsatellite loci was revealed. Unique MGs might be the products of sexual reproduction, which creates unique individuals from meiosis (19), or an artifact due to small sample sizes. Nonrandom association of alleles at different loci should be low in sexually reproducing populations due to the decay of gametic disequilibrium by recombination over time (19,31). These results indicated recombination by sexual reproduction in *R. oryzae-sativae*, which apparently is more common in California rice-growing areas than assumed. However, some loci deviated from HWE and a significant deficit of heterozygotes was observed in all populations, indicating that *R. oryzae-sativae* populations did not mate randomly and the departure from HWE of some loci may have been due to significant inbreeding.

Asexual reproduction of *R. oryzae-sativae* may also play an important role in the production of inocula (mycelia and sclerotia) within California rice fields because most clonal genotypes were shared within fields. It is possible that sclerotia or mycelia may survive at least 2 years because a few clones were shared between 2005 and 2007. The failure to detect clones in 2006 may be due to small sample sizes or the possibility that sclerotia may be constitutively dormant for at least a year after they are formed. In the field where crop rotation with sunflower was practiced, no clone was detected between years and disease incidence was seemingly lower than disease incidence in fields under continuous rice cultivation (P. Chaijuckam, *personal observation*). Thus, crop rotation might reduce numbers of overwintering asexual inocula. Although it has been assumed that asexual reproduction plays a major mode in the epidemiology of aggregate sheath spot (14,15,32), only a few clones were shared among years and a high degree of genetic diversity was displayed. This scenario might be explained by a small population of overwintering asexual inocula or frequent sexual reproduction, which may include widespread dispersal of airborne basidiospores. This explanation may support the conclusion of Miller and Webster (32), who noted that the number of recovered sclerotia in seedbeds was not always associated with subsequent disease incidence.

Based on evidence presented here, the population structure of *R. oryzae-sativae* in California rice-growing areas may be the result of both sexual and asexual reproduction. Mixed reproductive modes were also reported for *R. solani* AG-1 IA in Louisiana using 10 microsatellite loci (3). The estimation of reproduction modes is confounded by the different distances that sexual and asexual inocula are distributed. Hence, the results may be affected by sampling spatial scales (7). In this study, different sampling schemes were used to collect *R. oryzae-sativae* isolates. In 2004, only a few isolates were collected from each of several fields in each growing area. In 2005 and 2006, numerous isolates were collected in each of four fields in each growing area. Populations in 2004 tended to be more variable than populations in 2005 and 2006. Higher clonal fractions of *R. oryzae-sativae* were observed as greater numbers of isolates from one field were sampled, highlighting the short-distance dispersal of asexual inocula within fields. A similar case was reported for *Mycosphaerella graminicola* on wheat; different sampling spatial scales were conducted between California (most isolates from same leaves) and Oregon populations (most isolates from same fields). The clonal fraction of *M. graminicola* within the California population was higher than that of the Oregon population (7,30).

On a single rice plant, isolates on the same leaf sheath were clonal, indicating that the fungus may spread from an initial infection to the other areas on the leaf sheath as described previously (14,15). The genetic variability of populations of *R. oryzae-sativae* in California was significant but low in all hierarchical levels, including among growing areas within sampling years, fields within growing areas, and corners within fields, which suggested a high level of gene flow in all hierarchical levels. More-

TABLE 7. Pairwise fixation index (F_{ST}) based on six microsatellite loci of *Rhizoctonia oryzae-sativae* populations from three rice growing areas in 2004 to 2006

	F_{ST}[a]		
Pairwise location	2004	2005	2006
Sutter Co. vs. South Colusa Co.	0.022	0.005	0.000
Sutter Co. vs. North Colusa Co.	0.080*	0.076*	0.028*
South Colusa Co. vs. North Colusa Co.	0.046*	0.080*	0.048*

[a] Pairwise fixation indices were computed between pairs of populations with 1,023 permutations in ARLEQUIN version 2.0; * indicates significant at $\alpha = 0.05$ with sequential Bonferroni adjusted (38).

TABLE 6. Analysis of molecular variance (AMOVA) based on six microsatellite loci of *Rhizoctonia oryzae-sativae* within and among multiple-scale hierarchical populations in 2004 to 2007

Source of variation	df[a]	Variance components	Variation (%)	Fixation indices	P[b]
2004–2006					
Among years	2	0.036	2.1	$F_{CT} = 0.021$	0.057
Among areas within years	6	0.068	4.0	$F_{SC} = 0.041$	0.000*
Within areas	835	1.605	93.9	…	…
Total	843	1.709	…	…	…
2005 and 2006[c]					
Among areas	5	0.046	2.7	$F_{CT} = 0.027$	0.000*
Among fields within areas	18	0.062	3.7	$F_{SC} = 0.037$	0.000*
Within fields	708	1.577	93.6	…	…
Total	731	1.685	…	…	…
2005–2007[d]					
Among fields	10	0.052	3.1	$F_{CT} = 0.031$	0.000*
Among corners within fields	33	0.087	5.2	$F_{SC} = 0.054$	0.000*
Within corners	566	1.533	91.7	…	…
Total	609	1.672	…	…	…
Same fields (2005–2007)[e]					
Among fields	2	0.041	2.4	$F_{CT} = 0.024$	0.056
Among years within fields	5	0.046	2.7	$F_{SC} = 0.028$	0.000*
Within years	460	1.609	94.83	…	…
Total	467	1.697	…	…	…

[a] Cloned-corrected data representing different multilocus genotypes were used in AMOVA (11) with 1,023 permutations implemented in ARLEQUIN version 2.0.
[b] Probability of having a larger variance component than the observed values by chance alone; * indicates significant at $\alpha = 0.05$.
[c] Data from 2005 and 2006 were combined and analyzed together.
[d] Data from 11 fields in 2005 to 2007 were combined and analyzed together.
[e] An individual field in each of the three growing areas was sampled from 2005 to 2007; data from three fields were combined and analyzed together.

over, the admixed genotypes indicated gene flow among growing areas. Furthermore, there was moderate to no differentiation based on F_{ST} between pairs of growing areas. These results pointed to high gene flow of *R. oryzae-sativae* populations among Sutter, southern Colusa, and northern Colusa Counties. Populations from Sutter and southern Colusa Counties were not significantly differentiated in all three years, indicating that the Sacramento River was not a barrier to gene flow. These growing areas are separated by a distance of only ≈18 km. A high level of gene flow among populations of *R. solani* AG-1 IA in Texas (39) and in India (28) was also documented by using seven single-copy restriction fragment length polymorphism markers. When temporal structure over a 3-year period was determined in this study, little to no population differentiation was detected among sampling years. Furthermore, average gene diversity over time was uniform and admixed genotypes were assigned to different years, suggesting gene flow among sampling years. Thus, geographical structure seemed to have more influence on population subdivisions than temporal structure. Similar results were described in a study by Taheri et al. (42), where geographic regions and not rice cultivars were the primary factor shaping population structure of *R. solani* AG-1 IA, the cause of sheath blight on rice in India.

Only a few clones were shared among growing areas in this study, suggesting that asexual inocula may play a small role in long-distance dispersal. In contrast, sexual reproduction, which occurs after the mid- to late-tillering stage (17), was apparently prevalent in California rice-growing areas. This supports the conclusion that airborne basidiospores were likely a major cause of gene flow among populations, including short and long distances. Long-distance dissemination of basidiospores may be due to dispersal-infection-dispersal sequences (4) or the spore itself may be distributed over long distances under favorable conditions. This has been reported for *R. solani* AG-2 type 2, the cause of leaf spot of flue-cured tobacco, which disperses by airborne basidiospores throughout the eastern half of North Carolina (41). Long-distance dispersal of sclerotia of *R. oryzae-sativae* may be transported by irrigation water or on soil or farm equipment. Dissemination of the fungus via infected seed is less likely because rice growers in California usually treat seed with sodium hypochlorite to control bakane disease.

Basidiospores may play a more important role in the development of aggregate sheath spot disease epidemics in California rice-growing regions than realized. If basidiospores are the primary agent involved in the surprisingly high gene flow among populations of *R. oryzae-sativae*, consideration of airborne spore dispersal may be important for disease management.

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