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Population structure of the rice sheath blight pathogen *Rhizoctonia solani* AG-1 IA from India

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Abstract

The population structure of *Rhizoctonia solani* AG-1 IA causing rice sheath blight from India was evaluated for 96 isolates using seven RFLP loci. Nineteen of the isolates did not hybridise to *R. solani* AG-1 IA RFLP probes and rDNA analyses subsequently confirmed that they were either *Ceratobasidium oryzae-sativae* isolates or another *Rhizoctonia* sp. The population structure of the remaining 77 *R. solani* AG-1 IA Indian isolates was similar to that of a previously characterized Texas population. Clonal dispersal of *R. solani* AG-1 IA in India was moderate within fields and no clones were shared among field populations. Low levels of population subdivision and small genetic distances among populations were consistent with high levels of gene flow. Frequent sexual reproduction was indicated by the fact that most populations were in Hardy–Weinberg equilibrium (HWE). The two loci (R68 and R111) that deviated significantly from HWE showed an excess of heterozygosity. Although Texas and Indian populations were geographically very distant, they exhibited only moderate population subdivision, with an F_{ST} value of 0.193.

Introduction

Rhizoctonia solani Kuhn. is an important pathogen of rice in many rice-growing regions of the world. *Rhizoctonia* belongs to the Basidiomycetes, with *R. solani* being multinucleate. *Rhizoctonia solani* species [teleomorph *Thanatephorus cucumeris* (Frank)] represent a 'collective species' (Sneh et al., 1996), which has been divided into 13 anastomosis groups (AG-1–AG-13) and AG-BI (the bridging isolate AG) (Carling, 1996; Carling et al., 2002a, b). The anastomosis group AG-1 can be further subdivided into three intraspecific groups based on disease symptoms, cultural characteristics, rDNA similarity and isozymes (Liu and Sinclair, 1993; Mohammadi et al., 2003). The intraspecific groups are AG-1 IA (sheath blight on rice), AG-1 IB (web blight) and AG-1 IC (damping off) (Sneh et al., 1991). Rice sheath blight is a particularly important component of the rice disease complex, occurring in most rice-producing areas (Dagupta and Vilgalys, 1997), including India.

Rice sheath blight has been the subject of several population studies measuring variation based on virulence (Neeraja et al., 2002), isozymes (Neeraja et al., 2002), RFLPs (Rosewich et al., 1999), or simple-sequence repeat PCR (Banniza and Rutherford, 2001). The species concept for *R. solani* has been poorly defined in the past and it has been unclear whether different molecular subgroups represent different species or populations (Cubeta et al., 1996). Some AGs and their subgroups have

been differentiated with specific rDNA markers (Salazar et al., 2000) and most of the molecular subgroups were shown to be phylogenetically distinct and comprised of different biological species (Gonzalez et al., 2001).

In a previous population genetic study of *R. solani* AG-1 IA from Texas based on RFLP markers, results were consistent with a high degree of gene flow between populations and regular outcrossing within field populations (-Rosewich et al., 1999). Prior to these findings, the perception was that *R. solani* AG-1 IA is a mostly asexual fungus, with dissemination over relatively short distances by natural movement of asexual propagules (sclerotia), and over longer distances via movement of contaminated machinery, seed, or irrigation water. The aims of this study were to determine which species of *Rhizoctonia* are associated with rice sheath blight in India, and to determine the population genetic structure of *R. solani* AG-1 IA from India, with subsequent comparisons to previously described *R. solani* AG-1 IA populations from Texas (Rosewich et al., 1999). Information on dispersal (gene and genotype flow) and mode of reproduction could influence the current control and management strategies for rice sheath blight in India.

Materials and methods

Isolates

Infected leaves were collected from rice plants showing characteristic symptoms of sheath blight. Leaf and sheath samples were plated on 2% Water agar plates and hyphal tips from subsequent *Rhizoctonia* growth was transferred to Potato dextrose agar (PDA) after 24 h to isolate *R. solani* AG-1 IA. Sclerotium production was documented after 12 days of growth on PDA. Samples from eight rice fields in southern India were collected using transect sampling. Samples were collected at locations distributed approximately every 10 m along a transect in each rice field. At and around each sampling location, 10 diseased samples were collected when available. A sample usually consisted of a single rice tiller which either had sheath blight lesions on the sheath/pseudostem, or the leaves, or both. The sampled tillers had green leaves and were in late tillering or flowering stage of the crop. One of the rice fields was collected from northern Kerala (PTB = Pattambi), three were collected from southern Kerala (MC = Moncombu, NP = Neelamperur, KID = Kidankara), two from Karnataka (NG = Nanjangudu, IMV = Immavu) and two were from Tamil Nadu (KKD = Keelgudaloor, KKP = Keelakamanakanpatti) (Figure 1). The rice fields from MC and KID were geographically closest to each other, separated by ~10 km in a large rice-growing area planted to thousands of hectares.

DNA extraction and RFLP analyses

For each isolate, scraped mycelium from 5-week-old cultures grown on PDA amended with 50 $\mu g\ l^{-1}$ kanamycin was added to 40 ml of PDB (also amended with 50 $\mu g\ l^{-1}$ kanamycin) and incubated in an Erlenmyer flask on a rotary shaker at 20 °C. Mycelium was harvested after 5 days and freeze dried. DNA was extracted from the lyophilized tissue with the DNeasy Plant Mini DNA extraction kit (Qiagen, Hilden, Germany) according to the specifications of the manufacturer. Restriction enzyme digestion of total genomic DNA with *Hind*III, Southern blotting, and RFLP analysis were performed as described previously for *R. solani* (Rosewich et al., 1999). The seven probes used to assess allele frequencies were the same ones used in that study (Rosewich et al., 1999), namely R44, R61, R68, R78, R111, R116, and R148. Clones were differentiated using rep-PCR fingerprinting with primers *ERIC2F* and *BOXA1R* (Linde et al., 2003), which provided clear, easily scored banding patterns, instead of the RFLP fingerprint probe, R18, used in the Rosewich et al. (1999) study, which had a high background hybridization signal and was difficult to interpret. Allele designations in this study followed the previous RFLP study on *R. solani* (Rosewich et al., 1999). Isolates having the same multilocus RFLP and rep-PCR patterns were considered members of the same clonal lineage.

Sequence analysis for molecular identification

The rDNA internal transcribed spacer (ITS including 5.8S rDNA) sequences were determined for the isolates where species identification was

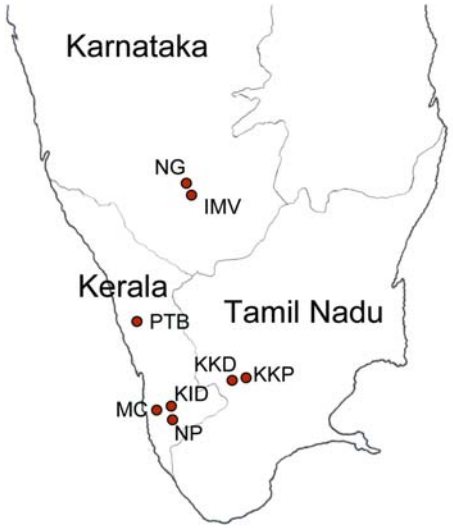

Figure 1. Geographic locations of the eight *Rhizoctonia solani* AG-1 IA field populations sampled in India.

in doubt because of the absence of hybridisation to the RFLP probes. In total, 16 isolates were sequenced using primers ITS4 and ITS5 (White et al., 1990). Seven of the sequenced isolates did not hybridise to *R. solani* AG-1 IA probes, and the other nine were chosen randomly from the Indian population that did hybridise (Table 1). Sequencing was performed on an ABI 3100 Sequencer using Taq-Cycle automated sequencing with DyeDeoxy Terminators (BigDye™ Terminator v3.0 Cycle Sequencing Ready Reaction; Applied Biosystems) with both primers to ensure reliability of the sequence data. Sequences were assembled and aligned using Sequencher™ 4.1 (Gene Codes Corporation, Ann Arbor, MI). BLAST searches (Altschul et al., 1997) were done against the NCBI/GenBank databases.

RFLP data analyses

All data were analysed using the programme FSTAT version 2.9.3 (Goudet, 2001) unless stated otherwise. Data were analysed for both clone-corrected and uncorrected (all isolates) datasets. Analyses included Nei's gene diversity (Nei, 1987) and pairwise θ (F_{ST}) among populations (Weir and Cockerham, 1984). To measure the population differentiation between the Texas (Rosewich et al., 1999) and Indian populations, the pairwise θ (F_{ST}) (Weir and Cockerham, 1984) was calculated on the clone-corrected data sets. Unbiased genetic distance (Nei, 1978) between populations was calculated in Popgene32 (Yeh et al., 1999). A phenogram derived by UPGMA (unweighted pair group method with arithmetic means) analyses based on Nei's (1972) genetic distance among the Indian and Texas populations (Rosewich et al., 1999) was calculated with Popgene32 (Yeh et al., 1999). To compare the relative contributions of asexual and sexual reproduction in each population of *R. solani* AG-1 IA we tested (a) whether each pair of loci was at genotypic equilibrium (in FSTAT), and (b) the significance of association between genotypes at loci in each population with a χ^2 test and the log-likelihood ratio G^2-statistic where probability values are obtained with a weighted randomization test (in Popgene32). For these tests, data were clone-corrected and pooled into a single data set. The excess heterozygosity within populations (F_{IS}) (Weir and Cockerham, 1984) was tested with a permutation test (1000 permutations) where alleles were permuted among individuals within the clone-corrected population.

Results

Ninety-six strains were isolated from rice sheath blight-infected leaves and sheaths. Nineteen of the isolates did not hybridise to any of the seven *R. solani* AG-1 IA probes, resulting in RFLP analyses of 77 isolates. Out of the 18 strains that produced spherical microsclerotia, 14 did not hybridise to any of the seven *R. solani* AG-1 IA probes. Sixteen of the non-hybridising isolates originated from the Tamil Nadu population, one from Karnataka, and two from north Kerala (Table 1). The hybridising and non-hybridising isolates could be distinguished by Rep-PCR analyses as they showed different amplification patterns (Figure 2).

Sequence analysis for molecular identification

rDNA-ITS analyses of the non-hybridising isolates revealed that four of them (from Tamil Nadu) showed highest similarity to an unidentified *Rhizoctonia* sp. (97% similarity, e-values ranged from 10^{-104} to 8×10^{-14}), while the other three tested isolates (two from Tamil Nadu and one

from Karnataka) were similar to *Ceratobasidium oryzae-sativae* with a BLAST search (98% similarity, e-value = 0). The nine isolates that hybridised to *R. solani* AG-1 IA probes (1 from Karnataka, 1 from South Kerala, 5 from North Kerala, 2 from Tamil Nadu) had DNA sequences that identified them as isolates belonging to *R. solani* AG-1 IA (*T. cucumeris* AG-1 IA) (similarity ranged from 98 to 100%).

RFLP analysis

The seven loci analysed were all polymorphic among the 77 isolates analysed, yielding two alleles per locus except for locus R68, which had four alleles. All isolates behaved in a diploid fashion with only one (homozygote) or two (heterozygote) hybridizing DNA fragments observed for each RFLP locus. *Rhizoctonia solani* AG-1 IA alleles found in the Indian population were identical to those found in the Texas population (Rosewich et al., 1999) except for a new allele (E) at locus R68 that was not detected in the Texas population. On the other hand, allele C at locus R68, C at locus R78, B and D from R116, and C from R148 in the Texas population were absent in the Indian population (Table 1). Thirty-three multilocus genotypes were identified with RFLPs and Rep-PCR. Rep-PCR fingerprinting mostly confirmed clones suggested by RFLP multilocus genotypes, except for members of three RFLP genotypes which could be further distinguished into six multilocus genotypes with Rep-PCR. Differences of at least two Rep-PCR bands were present between each of the three otherwise identical RFLP genotypes. In four cases, genotypes identified as clones with Rep-PCR differed at one to three loci with RFLPs. Shared multilocus genotypes (i.e., clones sharing the same RFLP genotype and Rep-PCR fingerprint) were commonly found within the same sampling sites of a rice field as well as among locations of the same field, but were not shared among rice fields.

Pairwise θ (F_{ST}) values among populations ranged between −0.01 and 0.05 for the clone-corrected data set and between 0.01 and 0.16 for the total data set (Table 2). For the pairwise analyses, samples from rice fields were grouped together according to geographic location because sample sizes for individual rice fields were too small for meaningful comparisons. Pairwise θ between India and Texas was 0.193. Nei's genetic distance (D) among populations varied between 0.002 and 0.070 for clone-corrected and between 0.008 and 0.067 for the total data set (Table 3). Genetic distances among Indian populations were smaller than those among Texas populations (Figure 3). Indian and Texas populations were genetically divergent based on the genetic distance between them (Figure 3). The average gene diversity for the seven loci in the clone-corrected data ranged from 0.17 to 0.47 (Table 4). The average gene diversity for the clone-corrected population was 0.34 (Table 4) and 0.30 for the total non-clone corrected population. Five locus pairs exhibited significant genotypic disequilibrium after 200,000 permutations at a 0.1% nominal level where $P = 0.000005$. They were R68:R44 ($P = 0.021$), R68:R111 ($P = 0.031$), R68:R111 ($P = 0.004$), R111:R44 ($P = 0.038$), and R148:R116 ($P = 0.031$). Testing log-likelihood ratios in the clone-corrected data set revealed that locus R111 deviated significantly from HWE for the combined Indian data set (Table 4). Locus R111 also showed significant linkage disequilibrium with the χ^2 test (Table 4). Significant excess heterozygosity (F_{IS}) was observed at loci R68 and R111 of the clone-corrected data set (Table 4). None of the loci showed significant excess heterozygosity in the individual clone-corrected populations (data not shown).

Discussion

Several *Rhizoctonia* isolates obtained from rice in this study proved to be another *Rhizoctonia* sp., or *C. oryzae-sativae* (anamorph *Rhizoctonia oryzae-sativae*). Mis-identification of *R. solani* AG-1 IA is uncommon. However, an earlier report from India indicated that some isolates did anastomose with an AG-1 IA tester isolate, but probably belonged to *R. oryzae-sativae* as shown with isozymes (Neeraja et al., 2002). *Rhizoctonia. oryzae-sativae* causes aggregate sheath spot of rice and might have been mistakenly isolated from sheath blight-infected leaves. These findings emphasize the advantages of molecular as opposed to phenotypic markers to distinguish and identify *Rhizoctonia* species.

We found no evidence for long-distance dispersal of asexual propagules, e.g., sclerotia, as

Table 1. Populations of *Rhizoctonia solani* AG-1 IA from India

Population	Geographic area	Sample size[a]	No. of isolates	Multilocus RFLP-rep-PCR genotype	RFLP alleles (R68/R116/ R111/R44/R78/R61/ R148)
Karnataka	Nanjangudu (NG)	8 (9)	3	1	AD/AA/AA/BB/AA/AA/AA
			5	2	AD/AA/AA/BB/AA/AA/BB
	Immavu (IMV)	2 (2)	1	3	AD/AA/AB/BB/AA/AA/BB
			1	4	AD/AA/AB/BB/AA/AA/AA
	Subtotal	10 (11)			
North Kerala	Pattambi (PTB)	40 (42)	6	5	AD/AC/AB/AB/AA/AB/AB
			5	6	DD/AA/AA/BB/AA/AA/BB
			2	7	DD/AA/AA/BB/AA/BB/BB
			1	8	DD/AA/AB/AB/AA/BB/BB
			4	9	DD/AA/AB/BB/AB/AB/BB
			2	10	AD/AA/AA/BB/AB/AA/BB
			1	11	AD/AC/AB/AB/AB/AB/AB
			1	12	AD/AA/AB/BB/AA/AA/AB
			8	13	DD/AA/AA/BB/AA/AB/BB
			1	14	AD/AC/AB/BB/AB/AB/AA
			1	15	AD/AC/AB/AB/AB/BB/AB
			1	16	AD/AA/AB/AB/AB/BB/BB
			3	17	AD/AA/AB/BB/AA/AA/AB
			1	18	DD/AA/AA/?/AA/AB/?
			1	19	DD/AA/AA/BB/AA/AA/BB
			1	20	DD/AA/AB/BB/AB/AB/BB
			1	21	AD/AA/AA/AB/AB/AB/AA
	Subtotal	40 (42)			
South Kerala	Moncombu (MC)	13(13)	4	22	DD/AA/AA/BB/AA/BB/BB
			2	23	DE/AA/AB/AB/AA/AB/BB
			2	24	AD/AA/AB/AB/AA/AA/AA
			1	25	DD/AA/AA/BB/AA/AA/AB
			4	26	DD/AA/AA/BB/AA/BB/AB
	Kidankara (KD)	4 (4)	4	27	AD/AA/AB/BB/AA/AB/AB
	Neelamperur (NP)	4 (4)	1	28	AD/AA/AB/BB/AB/AA/AB
			3	29	AD/AA/AB/AB/AA/AA/BB
	Subtotal	21(21)			
Tamil Nadu	Keelakamanakanpatti (KKP)	3 (12)	1	30	DB/AC/AB/BB/AA/AA/BB
			2	31	DD/AA/AB/BB/AA/AA/AB
	Keelakudaloor (KKD)	3 (10)	2	32	AD/AA/AA/BB/AA/AB/BB
			1	33	AD/AC/AA/BB/AA/AB/BB
	Subtotal	6 (22)			
Total		77 (96)			

[a] First number indicates the number of isolates used in the RFLP analyses. The number in brackets indicates the total number of isolates obtained in this study with the difference between the two values indicating the number of isolates that did not hybridise to any of the *R. solani* AG-1 IA probes.

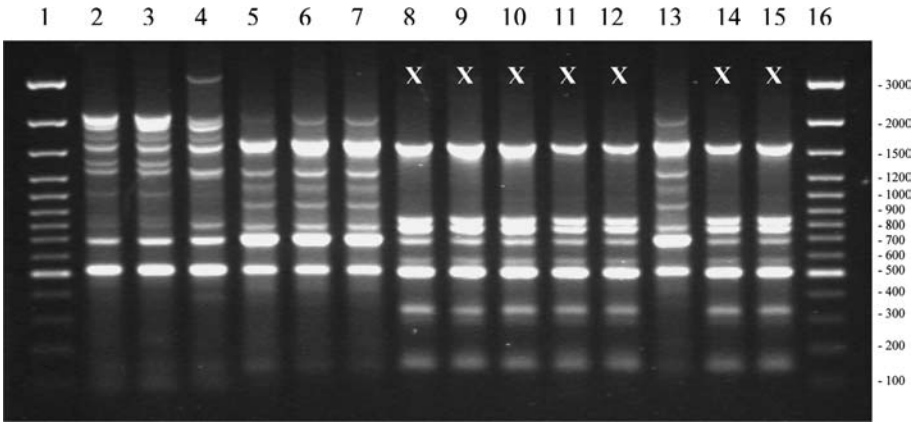

Figure 2. An example of Rep-PCR fingerprints of *Rhizoctonia* isolates in India. Lanes 1 and 16 are 100-bp Ladders Plus (PeQLab). Lanes 2–4 are *Rhizoctonia* isolates from IMV (the isolate from lane 3 was not included in the RFLP study because of low DNA concentration), lanes 5–15 are isolates from KKD. Lanes indicated with an X represent isolates that did not hybridise to *R. solani* AG-1 IA RFLP probes.

Table 2. Population pairwise θ of *Rhizoctonia solani* AG-1 IA populations from total data (above diagonal) and for clone-corrected data (below diagonal) collected from India

	Karnataka	North Kerala	South Kerala	Tamil Nadu
Karnataka	–	0.13	0.16	0.06
North Kerala	0.01	–	0.01	0.01
South Kerala	0.01	−0.01	–	0.03
Tamil Nadu	0.05	0.01	−0.01	–

Table 3. Nei's unbiased measure of genetic distance (D) for total data (above diagonal) and clone-corrected (below diagonal)

	Karnataka	North Kerala	South Kerala	Tamil Nadu
Karnataka	–	0.060	0.067	0.018
North Kerala	0.070	–	0.008	0.010
South Kerala	0.030	0.002	–	0.020
Tamil Nadu	0.038	0.023	0.020	–

genotypes were not shared among Indian populations. Population sizes were, however, small, minimizing the chances of finding shared clones. The same genotype was only found to be shared among sampling locations of the same rice field (separated by 30 m in the case of genotype 27, or 280 m in the case of genotype 7) consistent with asexual dispersal over only moderate distances. The low to moderate pairwise population subdivision of *R. solani* AG-1 IA and the low genetic distances among populations in both the clone-corrected and total data set, indicate moderate gene flow among populations. As our data suggest that gene flow among populations in India was not achieved by asexual propagule dispersal; the most likely alternative explanation is the movement of basidiospores. Hymenia have been observed on rice with sheath blight infection in Texas (Jones and Belmar, 1989), providing a source of basidiospores. But basidiospores of *T. cucumeris* are hyaline and thin-walled, and are thought to disperse only short distances. Circumstantial evidence on tobacco, however, suggest, that basidiospores can disperse over long-distances given favourable environmental conditions (Shew, 1985). Long distance dispersal of asexual propa-

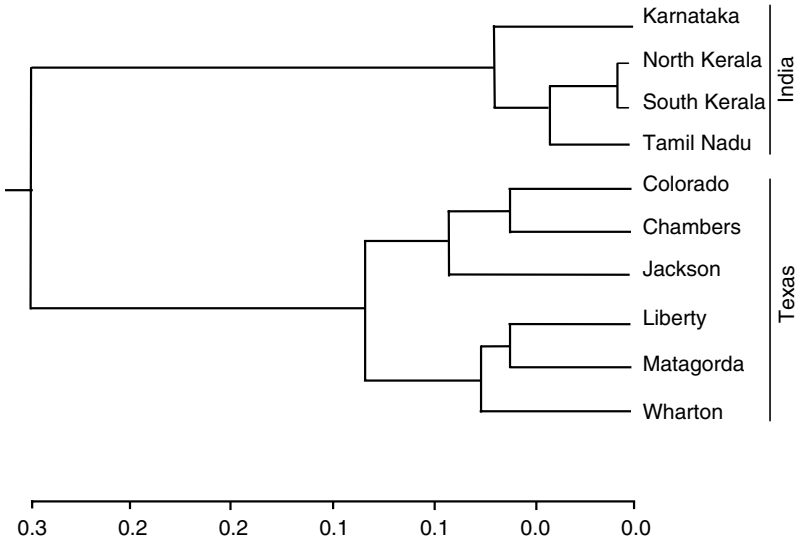

Figure 3. Phenogram derived by UPGMA (unweighted pair group method with arithmetic means) based on Nei's genetic distance (Nei, 1972) in Popgene32 (Yeh et al., 1999), between six Texas and eight Indian populations of *Rhizoctonia solani* AG-1 IA.

gules might be achieved by movement of contaminated machinery, seed, or irrigation water. Frequent dispersal of asexual propagules among populations would lead to widespread distribution of clones across populations. The fact that we did not find any clones shared among populations can be attributed to the relatively low sample sizes analyzed, and/or frequent sexual reproduction in *R. solani* populations. Frequent sexual recombination would lead to a rapid loss of specific clonal genotypes because individual gene combinations would be broken up and incorporated into a much larger pool of genetic backgrounds.

In the Texas study, clones were identified in populations separated by up to 280 km (Rosewich et al., 1999). In our study, clones were not shared among populations after clone identification using Rep-PCR fingerprinting, even though the MC and KID rice fields were only 10 m apart. If only the RFLP multilocus haplotypes were considered, then some clones were found to be shared among populations. These findings could indicate that the

Table 4. Tests for Hardy–Weinberg equilibrium for 33 multilocus RFLP-rep-PCR genotypes of *Rhizoctonia solani* AG-1 IA from India

RFLP locus	H^a	G^{2b}	P^c	Prob[d]	F^e_{IS}	P^f
R44	0.25	1.316	0.251	0.386	−0.148	0.506
R61	0.47	1.725	0.189	0.186	0.229	0.960
R68	0.45	9.723	0.137	0.341	−0.396	0.009
R78	0.24	1.269	0.260	0.396	−0.143	0.515
R111	0.41	7.500	0.006	0.025	−0.391	0.024
R116	0.17	0.501	0.479	0.602	−0.085	0.761
R148	0.44	2.591	0.108	0.101	0.287	0.976
Overall	0.34				-0.079	0.136

[a]H = Nei's (Nei, 1987) gene diversity on clone-corrected data.
[b]Log likelihood ratio test for HWE was calculated using Popgene32 (Yeh et al., 1999).
[c]Probability of G^2.
[d]Probability of χ^2 test for homogeneity of gene frequency across loci calculated in Popgene.
[e]Weir and Cockerham's (Weir and Cockerham, 1984) calculation of F_{IS} in FSTAT.
[f]P value for F_{IS} based on 1000 randomisations and indicating the proportion of randomizations that gave a larger F_{IS} value than the observed.

Rep-PCR fingerprint technique utilized in this study is more effective at distinguishing clones than the RFLP fingerprint probe used by Rosewich et al. (1999). Another possibility is that man-aided dispersal of sclerotia is more common in Texas than in India.

Although gene diversity for the seven *R. solani* loci was moderately high (0.17–0.47), it was lower than previously found in Texas (0.49–0.59) (Rosewich et al., 1999). F_{ST} (population subdivision) between the Texas and Indian populations was moderate (0.193). The genetic distance and F_{ST} between Indian and Texas populations indicated geographical subdivision consistent with little or no gene flow among continents. Population subdivision among the Indian *R. solani* populations was low to moderate including some negative values. As θ_{ST} (F_{ST}) is a covariance, negative values can occur if isolates between populations are genetically more similar than isolates within populations (Schneider et al., 1997). Due to the small population sample sizes and low to moderate population subdivision observed as well as the low genetic distance among Indian populations, we pooled all the genotypes into one data set for HWE calculations, but nevertheless did the analyses also across populations for the clone-corrected data set. In the clone-corrected combined population, significant Hardy–Weinberg disequilibrium was observed with the log-likelihood ratio test as well as the χ^2 test only for locus R111, a finding consistent with sexual reproduction. The deviation from HWE in R111 is due to an excess of heterozygotes at this locus. Our data thus indicate that the Indian *R. solani* sheath blight population is most likely outbreeding.

Negative values of F_{IS} indicate excess heterozygosity. Previously, the highest F_{IS} values in the Texas population were obtained for loci R44, R78 and R111, although F_{IS} values for all loci were negative (Rosewich et al., 1999). In the Indian population, only five of the seven loci displayed negative F_{IS} values, of which only two were significant. Moreover, values were never as high as in the Texas population (highest $F_{IS} = -0.396$ for R68 in India compared to −0.551 for locus R44 in Texas). Several reasons for excess heterozygosity were hypothesized in the earlier (Rosewich et al., 1999) paper, with the most likely explanation being different allelic frequencies in male and female parents (binomial sampling error) due to a small breeding population (Pudovkin et al., 1996), the same effect which is obtained by a predominance of a few successful clones in the breeding population. Indeed, a successful clone was observed in almost all the Indian populations sampled. These clones could not be found more than approximately 280 m apart, but for the relatively small sample sizes analysed in this paper, this would likely lead to a binomial sampling error, and thus excess heterozygosity.

Many similarities were found between the *R. solani* AG-1 IA populations from India and Texas. These include evidence for short-distance asexual propagule dispersal, possible long-distance dispersal of basidiospores (leading to high levels of gene flow regionally), sexual reproduction (few deviations from HWE), and persistence of successful clones in sub-populations (as seen by excess heterozygosity and occurrence of clones). *Rhizoctonia solani* from rice also appeared to be a genetically homogeneous group, although three species were found to be involved in the disease in India. The main difference between the Texas and Indian populations was that clones were not shared among Indian populations, while some shared clones were found among the Texas populations. The regular outcrossing and high gene flow indicated for *R. solani* AG-1 IA has significant implications for effective control and resistance breeding strategies in India (McDonald and Linde, 2002).

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