Characterization and Genetic Diversity of *Rhizoctonia spp.* Associated with Rice Sheath Diseases in India

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ABSTRACT

Isolates of *Rhizoctonia spp.* were obtained from rice in India. Characterization by conventional techniques and PCR showed that from 110 isolates, 99 were *R. solani* and 11 were *R. oryzae- sativae*. Of 99 isolates identified as *R. solani*, 96 were AG1-IA, 1 was AG1-IB, and 2 were AG1-IC . Amplified fragment length polymorphism (AFLP) was used to estimate genetic relationships in pathogen populations distributed in space and in time, also between two species of *Rhizoctonia* infecting rice in India. Cluster analysis based on the AFLP data separated isolates belonged to the 3 different intraspecific groups of *R. solani* AG1 and differentiate *R. solani* from *R. oryzae- sativae*. Clones (i.e., cases where two or more isolates shared the same AFLP genotype) were identified in the *R. solani* AG1-IA and *R. oryzae-sativae* populations. Eighteen clones from AG1-IA isolates represented 51% of the AG1-IA population. *R. solani* AG1-IA appears to exhibit an epidemic population structure, whereby novel genotypes apparently have been introduced into a region over time.

INTRODUCTION

The *Rhizoctonia* sheath diseases of rice, comprising *Rhizoctonia solani*, R. *oryzae*, and *R. oryzae-sativae* cause significant yield losses in many rice growing regions of the world (5). *R. solani*, a ubiquitous pathogen, incites rice sheath blight which is one of the most serious fungal diseases of rice. Among the 14 anastomosis groups (AGs) that have been described in *R. solani* to date, isolates of AG1-IA have been associated with the rice sheath blight pathogen (1,2). *R. oryzae* and *R. oryzae-sativae* causal agents of sheath spot and aggregate sheath spot, respectively, both produce lesions on the leaf sheath very similar to those of sheath blight (5). In addition to the similarity of disease symptoms, distinguishing the species in culture is difficult due to the lack of stable morphological characters. Accuracy in distinguishing these pathogens is essential to ensure the success of the extensive disease management (5). Resistant rice cultivars have not been bred due to the low inherent level of resistance in rice to *Rhizoctonia spp.* These difficulties are compounded by the lack of adequate information on the population biology of the pathogen.

The objectives of the present study were to (i) characterize *Rhizoctonia spp.* associated with rice sheath diseases in India by morphological characterization and hyphal anastomosis in conjunction with molecular techniques such as PCR-based detection of *Rhizoctonia* species using species-specific primers and PCR-RFLP of rDNA-ITS regions to identify different ISGs of *R. solani* AG1 isolates, (ii) describe the genetic relationships among *Rhizoctonia spp.* populations from diverse rice cultivars using AFLP markers.

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MATERIALS AND METHODS

Fungal isolates. In this study a total of 110 *Rhizoctonia spp.* isolates were obtained from different rice cultivars in India during 2000 to 2003 (Table 1). Samples from each 10 rice growing areas in India were collected using transect sampling.

Morphological characterization. Fungal isolates were initially characterized as either binucleate or multinucleate. Colonies of *Rhiozoctonia* on PDA, 2-3 days old, were stained to determine nuclear number and were examined for the presence of a septal pore (dolipore) apparatus. The nuclear condition

TABLE 1. Origin and designation of the isolates used in this study

State	Location	Population	Year	Cultivar	Isolates
Andhra Pradesh	Mertheru	MR	2001	Swarna	MR-1.1.2, 1.2.0, 1.1.6, 1.2, 1.44, 1.43, 1.98, 2.2, 2.4, 2.1, 1.46, 1.47, 1.92, 1.73, 1.80. 1.83, 1.106, 1.26, 1.27
				Mukamala	MR-1.4, 1.35, 1.37, 1.38, 1.42, 1.50
				IR-50	MR-1.32, 1.48, 1.87
Andhra Pradesh	Nellore	NL	2001	HR-12	NL-3, 10, 11, 51, 54, 84, 97, 99, 100, 102
				IR50	NL-18, 20, 30, 42, 44, 70, 104, 105
Karnataka	Immavu	IMV	2000	Swarna	IMV-2.2, 3.0, 3.3,
Kaarnataka	Nanjangudu	NG	2000	Swarna	NG-1.4, 1.5, 1.6, 1.7
				HR-12	NG-1.1, 1.3, 1.8,
Karnataka	Kalla	KAL	2003	M. Vijaya	KAL-34, 35, 36, 65
				Sania	KAL-29, 31, 32, 33, 38
Karnataka	Mandiya	MAN	203	IR-50	MAN-38, 40,
				M. Vijaya	MAN-33, 43, 51, 79, 86, 87, 88, 89, 90
Kerala	Moncombu	MC	2000	Neeraja	MC-1.8, 1.32, 16
				Pokkali-04	MC-1.43, 3.4, 4.1
Kerala	Pattambi	PTB	2000	Pavizham	PTB-3.6, 3.7, 3.12, 5
				PTB-9	PTB-8.7, 9.1, 20, 48, 61
Tamil Nadu	Keelkudaloor	KKD	2000	IR-20	KKD-1.4, 1.5, 1.12, 1.13, 1.15, 1.23, 1.26, 1.33. 1.35
Tamil Nadu	Keelakamanakanpat	KKP	2000	IR-20	KKP-1.12, 1.15, 1.16
				IR-36	KKP-1.37, 1.38, 1.52, 1.54, 1.65
				ADT-24	KKP-1.27, 1.28

in penultimate cells of vegetative hyphae was determined by the trypan blue stain technique as described by Martin and Lucas (7).

DNA extraction. Cultures were grown at room temperature in potato dextrose broth. Mycelium was harvested after 5 days, preceding sclerotium formation. Total genomic DNA was extracted by the hexadecyl-trimethylammonium bromide (CTAB) method according to Zolan and Pukkila (12).

PCR-based distinguishing of *Rhizoctonia* species. For identification of *Rhizoctonia* species, genomic DNAs from all of isolates listed in table 1 were amplified by PCR using three species-specific primer combinations including GMRS-3/ ITS1, GMRO-3/ R635, and GMROS-6/ R635 for identification of *R. solani*, *R. oryzae*; and *R. oryzae-sativae*, respectively (5).

Anastomosis grouping. Each *R. solani* isolate was paired with tester strains of *R. solani*. Positive controls consisted of pairing between tester strains of the same anastomosis group and sub group. Hyphal anastomosis was determined at × 400 and fusion confirmed at × 1,000 magnification.

PCR-RFLP of rDNA-ITS region. RFLP of rDNA-ITS was employed to clarify whether isolates of *R. solani* AG1 obtained from rice belonged to subgroup IA, IB, or IC. Tester strains of AG1-IA, AG1-IB and AG1-IC were included for comparison. Genomic DNA of the isolates was used for PCR amplification of the ITS region using a pair of primers, RS1 and RS4 with the reaction conditions described by Guillemaut et al. (3). Restriction polymorphisms were detected using two discriminant restriction enzymes *Mse*I and *Mun*I . Restriction digests of PCR products were resolved by electrophoresis in agarose gel and viewed by UV transIumination.

AFLP analysis. Genomic DNA was subjected to AFLP analysis based on the method described by Vos et al. (11). *Eco*RI and *Mse*I primers without selective nucleotides (*Eco*RI + 0 and *Mse*I + 0) were employed for pre-amplification. For the final selective amplification, the 1:10 diluted pre-amplified DNA was amplified using a $\gamma-$ [^{33}P] -ATP 5' end labelled *Eco*RI-primer carrying two selective nucleotides (*Eco*RI + CA) in combination with a *Mse*I-primer containing two selective nucleotides (either *Mse*I + AT, *Mse*I + CG, *Mse*I + GC or *Mse*I + GT). The reproducibility of the AFLP data was measured previously by repeating the entire procedure using one isolate from each ISG of *R. solani* AG1 (-IA, IB, IC) and one isolate of *R. oryzae-sativae*.

Genetic relationships among *Rhizoctonia spp.* isolates. For each isolate, the DNA fingerprints were scored by visual inspection for presence (1) or absence (0) of specific AFLP bands. All dendrograms were created with the TREE program of NTSYS-pc or the DRAW option of Treecon. The 'goodness of fit' of the clustering to the data matrix was determined by calculating the cophenetic correlation coefficient between the similarity matrix and the cophenetic matrix derived from the dendrogram, using the COPH and MXCOMP procedures of the NTSYS-pc. Reliability of the dendrogram was tested by bootstrap analysis with 1,000 replications using Treecon.

RESULTS

Morphological characterization. Of the 110 isolates recovered from rice sheath lesions, 99 were multinucleate and 11 binucleate. Of the 99 multinucleate *Rhizoctonia* isolates, 96 isolates fitted with *R. solani* AG1 type 1 (AG1-IA), 1 isolate fitted with AG1 type 2 (AG1-IB), and 2 isolates fitted with AG1 type 3 (AG1-IC) in colony morphology and sclerotial type described by Sherwood (10).

PCR-based distinguishing of *Rhizoctonia* species. The rDNA-ITS region for 110 *Rhizoctonia* isolates was amplified using 3 primer combinations. GMRS-3 / ITS1 primer pair which is specific for amplification of the rDNA-ITS region of *R. solani*, gave a single product of 550 bp only for 99 multinucleate isolates and primer pair GMROS-6 / R635, which is specific for identification of *R. oryzae-sativae*, gave a single product of 1,200 bp only for 11 binucleate *Rhizoctonia* isolates. Primer combination GMRO-3 / R635 (specific for characterization of *R. oryzae*) did not give any band for any *Rhizoctonia* isolate.

Anastomosis grouping. All of 99 *R. solani* isolates anastomosed with the AG1-IA tester isolate and with the other ISGs of AG1, including IB and IC.

PCR-RFLP of rDNA-ITS region. The rDNA-ITS region for 99 isolates of *R. solani* and the tester isolates of AG1-IA, AG1-IB, and AG1-IC was amplified and digested using two discriminating restriction enzymes (*Mse*I and *Mun*I). The amplified fragment of AG1-IA showed no restriction site variation among AG1-IA isolates. However, the three ISGs of *R. solani* AG1 (IA, IB, and IC) differed from each other in restriction sites generated with *Mse*I and *Mun*I.

AFLP analysis. When bands from all of the isolates were considered, the four primer combinations used revealed a total of 443 bands, ranging in length from 50 to 500 base pairs. From these fragments, 292 unambiguous bands were scored. Only 37 bands were monomorphic across the complete set of isolates, resulting in 87.4% of the scored bands being polymorphic. Out of the 278 markers scored, 43 were shared among all 96 *R. solani* AG1-IA isolates. As a result, the band polymorphism level within the AG1-IA isolates was 85.3%.

Genetic relationships among Rhizoctonia spp. isolates. The AFLP data were used to make pairwise comparisons of the genotypes based on both shared and unique amplification products to generate a similarity matrix. Comparing all the dendrograms generated, and selecting the cut off points depending on the similarity coefficient and the clustering method employed, the two species (*R. solani* and R. *oryzae-sativae*) and three ISGs of *R. solani* AG1 (-IA, IB, and IC) clearly clustered separately and the same four main lineages within the AG1-IA population and two main lineages within *R. oryzae-sativae* isolates could be identified. The dendrogram generated using Treecon with the Nei and Li similarity coefficient based on 292 AFLP markers revealed the presence of 2 predominant lineages, joined by a single node at the 60% similarity level and supported with bootstrap values of 100% **(Fig. 1).** Lineage 1 contained 99 *R. solani* isolates, clearly separated from lineage 2 containing 11 *R. oryzae-sativae* isolates. Within the lineage 1, three ISGs of *R.solani* AG1 isolates were distinguishable. Lineage 1A contains 96 AG1-IA isolates, separated from lineage 1B containing the single isolate of AG1-IB and also clearly separated from lineage 1C containing two isolates of AG1-IC. At the 92% similarity level, four lineages could be identified in *R. solani* AG1-IA and 2 lineages in *R. oryzae-sativae* populations **(Fig. 1)**. Lineage 1Aa contains isolates collected from Andhra-pradesh in 2001. The isolates collected from Karnataka in 2003 were separated from all other isolates in the lineage 1Ab. Lineage 1Ac consists of the isolates collected from Kerala in 2000 and also two isolates from Tamil-Nadu. The isolates of Karnataka collected in

2000 and the rest of *R. solani* AG1-IA isolates of Tamil-Nadu and one of the isolates of Kerala were grouped in lineage 1Ad. In the *R. oryzae-sativae* population, both lineages 2A and 2B contain isolates collected from Tamil-Nadu in 2000. Lineage 1Aa was also a heterogeneous group, containing 8 unique clones (i.e. cases in which two or more isolates shared the same AFLP genotype) among 46 total isolates.

Sixty-eight AFLP genotypes were identified among isolates of *R. solani* and 10 genotypes among isolates of *R. oryzae-sativae*. There were no common AFLP patterns shared between isolates of *R. oryzae-sativae* and *R. solani* **(Fig. 1).**

DISCUSSION

Binucleate *R. oryzae-sativae* and multinucleate *R. solani* isolates were identified from diseased leaves and sheaths of rice plants. Of the 99 multinucleate *Rhizoctonia* isolates, 96 isolates fitted with *R. solani* AG1 type 1 (AG1-IA), one isolate fitted with AG1 type 2 (AG1-IB), and 2 isolates fitted with AG1 type 3 (AG1-IC) in colony morphology and sclerotial type described by Sherwood (10), but changes in morphological characters of AG1-IA and AG1-IB isolates were observed with changing environmental factors. Therefore, morphological characters are not reliable to identify ISGs in *R. solani*. A PCR-based method using primers designed from unique regions within the rDNA region allowed rapid and accurate identification of the *Rhizoctonia* species responsible for sheath diseases in rice. PCR-RFLP was useful only in differentiating the three ISGs of AG1. However no genetic differences in the restriction pattern of ITS region were observed among AG1-IA isolates. This may be because the ITS region is highly conserved and covers relatively few markers, thus additional restriction enzymes may be needed to detect variation between isolates.

In contrast, the AFLP technique analyses multiple loci, so differences between genotypes were more easily detected. The AFLP analysis in the present study confirmed the enormous genetic differentiation between *R. solani* and *R. oryzae-sativae*, and showed a close relationship among three ISGs of *R. solani* AG1 (-IA, IB, and IC). The method allowed discrimination of *Rhizoctonia* to and below the species level. The level of genetic variability detected within the *Rhizoctonia* isolates by AFLP analysis confirmed that it is a reliable, efficient, and effective marker technology for determining genetic relationships in *Rhizoctonia spp.*

In general, isolates of the same taxonomic group with distance range from 9 km (between NG and IMV populations) to 290 km (between NG and KKP populations) were clustered together at 92% similarity level. All *R. solani* AG1-IA isolates linked at 84% similarity level and it was slightly lower than previously found in India (95%) using Rep-PCR fingerprinting (6).

The isolates from the same locations of Karnataka in 2000 and 2003 were not available in the present study. But the distance between sampling locations in Karnataka in two years of sampling (2000 and 2003) ranged from 71 to 80 km. If we consider that isolates of NG and KKP populations (which are 290 km apart from each other) were clustered together in lineage 1Ad, but isolates of MAN population (which are only 71 km apart from IMV population) were clustered separately in lineage 1Ab, we can conclude that maybe new genotypes were introduced into a region over time.

AFLP genotypes were not shared among different populations. The same genotype was only found in the isolates belonging to the same population. Therefore, there was not any evidence for long-distance dissemination of asexual propagules (sclerotia or mycelia). In two clones from NL (consist of isolates: NL-44, NL-54, and NL-20) and MAN (isolates MAN-51, and MAN-89) populations, the same genotype was found to be shared in the isolates separated by 4 km and 3km, respectively. But in all other clones, the same genotype was found to be shared in the isolates separated by 5m (in the case of isolates KAL-32 and KAL-33), to 500 m (in the case of KAL-65 and other isolates of the same clone; KAL-34, KAL-35, and KAL-36). However, small sample sizes was a possibility in minimizing the chances of finding shared clones among different populations. A similar case previously reported by Linde et al. (6) in 15 *R. solani* AG1-IA populations from rice in India who found the same genotypes, separated by 30 m to 280 m using Rep-PCR. In another study in Texas, clones were identified in populations separated by up to 280 km using RFLP fingerprint probe (9). These findings could indicate

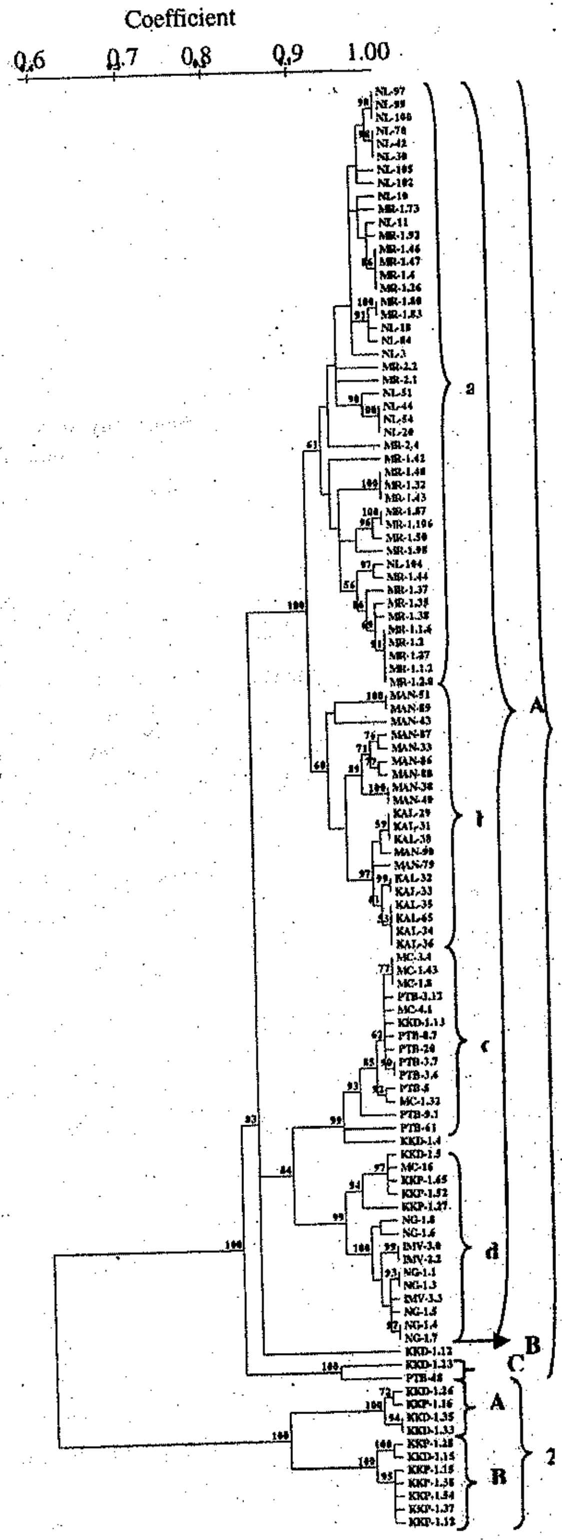

Fig. 1. Unweighted pair-group method, arithmetic mean dendrogram produced from AFLP fingerprint patterns observed for 110 *Rhizoctonia spp.* isolates. Scale bar indicated the horizontal distance corresponding to genetic similarity as measured by the Nei and Li similarity coefficient. Bootstrap values greater than 50 are indicated adjacent to the nodes and are based on 1,000 replications. Lineages with significant bootstrap support are indicated.

that the AFLP and Rep-PCR fingerprint techniques are more effective for distinguishing clones than RFLP fingerprint probe. Another possible reason is that man-aided dispersal of asexual propagules is more common in Texas than in India (6). The results of the present study indicate that gene flow among populations in India was not achieved by asexual propagule dispersal; as we did not find any clones shared among different geographic populations of each pathogen. This can be attributed to the relatively small sample sizes analyzed. Also, sexual reproduction in each pathogen can be involved in this case. The first major effect of sexual reproduction at the population level is the production of recombinant genotypes, which maintain genotype diversity (8). There was a clear link established among morphology, PCR-RFLP, and AFLP genotypes. Our results indicate that not only *R. solani* AG1-IA, but also AG1-IB and AG1-IC can cause rice sheath blight. Also, *R. solani* AG1-IA was the established, and perhaps the predominant, AG1 subgroup in the warm rice growing areas of India.

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