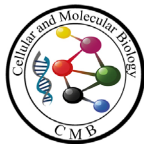

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Genetic diversity of rice sheath blight in different geographical regions of China

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ABSTRACT

Rhizoctonia solani AG1-IA (*Thandfephorus cucumeris* teleomorph) is the causative agent of rice sheath blight in Chinese paddy fields. Due to the importance of the disease and the lack of comprehensive information on the genetic structure of the fungus populations, 25 isolates collected from Hubei, Sichuan, Anhui, and Jiangsu provinces, and southern China's Yangtze River basin were examined for morphological characteristics, growth rate, and genetic diversity of this pathogen. The anastomosis group determination test results showed that all isolates belong to the AG1-IA anastomosis group. To quickly diagnose and confirm the anastomosis group of isolates, ten isolates along with AG1-IA and AGA standard isolates were examined by specific primers AG1-IA. A 256bp band was amplified in all of them. The results of the growth velocity study divided the isolates into two groups' fast growth (68% of isolates) and slow growth (32% of isolates). The genetic diversity of 25 isolates was assessed using the RAPD marker. Among the 20 primers, bands from seven primers ranging from 250 to 5,000bp were performed using the Jaccard similarity coefficient and UPGMA method by data cluster analysis of NTSYS-pc software. The cluster analysis results divided the isolates into two groups with a similarity level of 36%, which corresponded to the grouping of isolates into two groups of fast growth and slow growth. At the level of 80% similarity, the isolates were divided into 23 groups, which indicates the high genetic diversity of these isolates. The results of the molecular analysis showed that isolates belonging to a geographical area do not necessarily have a genetic affinity. This study performed rapid detection of *R. solani* AG1-IA using specific primers AG1-IA, and evaluation of genetic diversity of rice sheath blight isolates was performed by RAPD marker in this study.

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Introduction

Rhizoctonia solani is not a taxonomically distinct unit but a complex species consisting of genetically different groups. Anastomosis groups 1 to 14 have been identified and introduced in the sexual form of *Thundthephorus cucumeris* (1). *R. solani* AG1-IA is one of the pathogens of the aerial organs that cause sheath blight disease. Rice sheath blight is one of the most important fungal diseases in southern China's Yangtze River basin paddy fields (2).

Although the characteristics of anastomosis groups and subgroups have been the most successful attempts to describe the genetic diversity of Rhizoctonia based on morphological, pathogenic, and anastomosis characteristics, they caused many mistakes in recognizing the exact subspecies of this fungus because of overlapping between some anastomosis groups (3). Therefore, this method cannot estimate genetic diversity within and between anastomosis groups (4).

In addition, the lack of stable morphological and physiological characteristics in this pathogen has made it difficult and time-consuming to identify isolates of this species (2). With the introduction of biochemical and molecular methods, modern tools replaced the older methods of studying anastomosis groups and Rhizoctonia populations (4).

The use of specific primers for 28s-based anastomosis groups is one of the most appropriate methods for rapidly diagnosing *R. solani* anastomosis groups. Unlike the classical methods of diagnosing anastomosis groups, this method is not time-consuming and provides rapid subspecies diagnosis (5). This primer has been used to distinguish between different anastomosis groups of *R. solani* isolates. In studies by Matsumoto *et al*. (6), this method accurately distinguished isolates belonging to the AG1-IA, AG1-IB, AG1-IC, AG 2-1, and AG 2-2 anastomosis groups.

Molecular markers have studied various aspects of the biology of the Rhizoctonia genus (7). By reviewing 52 isolates obtained from corn and rice in India using Random Amplified Polymorphic DNA (RAPD) markers, Mishra *et al*. (8) distinguished seven groups at a 75% similarity level. By studying 41 isolates of *R. solani* belonging to 11 anastomosis groups with RAPD and Eric markers (Enterobacterial Repetitive Intergenic Consensus, ERIC), they observed high diversity within the studied anastomosis groups.

According to the Nandeesha *et al*. (9) study, the random markers were used to show differences between Australian and Japanese Inter-Specific Groups (ISGs) belonging to the same geographical area or different regions for isolates of *R. solani* AG1-IA collected from rice. They were analyzed based on the biochemical comparison, pectic zymograms marker, and rDNA-RFLP marker. According to these studies, although non-molecular markers proved the

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existence of diversity in the isolates, only the molecular markers used to study the populations of this fungus indicated the lack of genetic diversity in these isolates.

The study of the genetic diversity of this fungus seems necessary to clarify the perspective of breeding and selection of resistant plants and the effectiveness of conventional chemical control methods according to the significant damage of sheath blights caused by *R. solani* AG1-IA in rice fields in China and the restrictions on the production of plants resistant to Rhizoctonia (10). Therefore, in this study, after diagnosing the anastomosis group by classical method and confirming it using AG1-IA specific primers, the RAPD marker was used to evaluate the genetic diversity among isolates.

Materials and Methods

R. solani isolates collected from different rice cultivars of Hubei, Sichuan, Anhui and Jiangsu provinces and southern China's Yangtze River basin paddy fields were studied. Isolation and purification were performed on WA and PDA medium (200g potato extract with 15g dextrose and 20g agar in one liter of double distilled water). The morphological and molecular properties of 20 selected isolates were investigated.

Determining the anastomosis group

All isolates belonged to the AG1-IA anastomosis group based on their hyphal fusion with standard test isolator from the AG1-IA anastomosis group using the clean slide method (11, 12).

Growth rate measurement

To measure the growth rate of the isolates, a tablet was taken from the active margin of two to three-day-old cultured colonies grown at 25°C and placed in the center of the PDA culture medium in nine-cm Petri dishes. For each isolate, three replications were considered. Their growth rate was measured after 24, 48, and 72 hours of storage at 20°C. The average data were calculated in a completely randomized design. Then the isolates were grouped. It was performed using MVSP software and the Euclidean coefficient.

Preparation of mycelial mass

Mycelial mass production of pathogenic isolates was performed in a PDB liquid culture medium (200g potato extract with 15g dextrose in one liter of double distilled water). A five-millimeter tablet was separated from the margins of the two- to three-day-old colonies formed on PDA medium for each isolate and transferred to flasks containing 100 ml of sterilized PDB medium. After three to five days of mycelium growth at 20°C on a rotary shaker, the resulting mycelium is separated from the liquid medium by Whatman paper and a vacuum pump and after rinsing with distilled water in a Falcon, stored in an ultrafreezer at -70°C.

DNA extraction

To extract DNA from each isolate, 40 to 50 mg of mycelium powdered by liquid nitrogen was transferred to sterile microtubes, and 600µl of extraction form (100 mM Tris-HCl (pH 8.0), 20 mM Na EDTA, 0.5 m NaCl) was added. Then, one microliter of RNase (10 mg/ml) was added to the suspension, and after 10 minutes of storage at 65°C, it was placed on ice. Then 0.4 ml of chloroform solution and amyl alcohol in a ratio of 1:24 suspension was added and centrifuged at 7,500 rpm for 5 minutes at 4°C, and the supernatant was transferred to a new microtube. DNA was added by centrifugation of 0.7 volumes of cold isopropanol for 15 min at 10,000 rpm. After removing isopropanol and evaporation of the remaining alcohol, the samples were dissolved in 100µl of sterile deionized water (13). Isolation was used to bring the final DNA concentration of the isolates to 40ng/µl.

Identification of *R. solani* AG1-IA using specific AG1-IA primers

To confirm that the isolates belonged to the AG1-IA anastomosis group, ten isolates as species representatives were examined by the group-specific primers designed by Matsumoto *et al*. (6). In this test, AG1-IA standard isolates were used as the positive control, and AG4 standard isolates were used as the negative control. Amplification of DNA fragments in a Master Eppendorf Gradient thermocycler (Eppendorf, Germany) with initial genomic DNA denaturation was done at 95°C for five minutes. In 30 cycles, denaturation was done at 94°C for one minute. The annealing was done at 54°C for 2 minutes, and the extension was performed at 72°C for three minutes. For final extension, samples were put at 72°C for five minutes (6).

PCR products were analyzed in 0.8% agarose gel in TBE buffer (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA), constant voltage 80V, and electrophoresis for 30 minutes after staining with ethidium bromide (1 µg/ml).

RAPD Marker

RAPD-PCR technique was performed in 25µl reaction mixture containing 2.5µl of Buffer PCR (pH=9.0, 10mM Tris-HCl, and 50mM KCl) 1.5mM MgCl2, and 200µM of each dNTP (dCTP, dATP, dGTP, dTTP), 0.4pM from each of the ten nucleotide primers, 40ng of the sample DNA and 0.5 units of the Taq Polymerase enzyme in Eppendorf Gradient Thermocycler. DNA fragments were amplified by initial denaturation at 95°C for 2 minutes. Then in 30 cycles, denaturation was performed for 1 minute at 96°C. The annealing was done for 1 minute at 45.7°C, and for 2 minutes, the extension was completed at 72°C. The final extension was done for 8 minutes at 70°C. With a constant voltage of 80V, electrophoresis of PCR products was performed for 3 hours in 1% agarose gel and TBE buffer (0.09M Tris, 0.09M boric acid, 0.002M EDTA). After staining with ethidium bromide (1µg/ml), the resulting bands were observed under UV light.

Data analysis

Obtained bands were analyzed from the RAPD marker after scoring bands as zero and one (Presence or absence of band). Based on the data obtained from the RAPD marker, cluster analysis was performed by the UPGMA method (14) based on the distance matrix, Jaccard similarity coefficient (15), and NTSYS-pc Ver 2.0 software.

Results

Morphological studies

The colony of *R. solani* AG1-A was grown on the surface of the PDA medium. Thallus also grew densely on

Table 1. Isolates of *Rhizoctonia solani* AG1-IA and mean growth rates of 25 selected isolates.

Isolate Number	Collected Place	Growth Rate (mm/day)
Rs-1	Hubei Province	11.8
Rs-2	Hubei Province	21.9
Rs-3	Hubei Province	26.3
Rs-4	Hubei Province	24.7
Rs-5	Hubei Province	27.8
Rs-6	Sichuan Province	25.9
Rs-7	Sichuan Province	14.4
Rs-8	Sichuan Province	12.1
Rs-9	Sichuan Province	26.3
Rs-10	Sichuan Province	15.5
Rs-11	Anhui Province	16.2
Rs-12	Anhui Province	14.8
Rs-13	Anhui Province	12.5
Rs-14	Anhui Province	16.1
Rs-15	Anhui Province	28.1
Rs-16	Jiangsu Province	26.3
Rs-17	Jiangsu Province	28.1
Rs-18	Jiangsu Province	28.1
Rs-19	Jiangsu Province	30.9
Rs-20	Jiangsu Province	29.4
Rs-21	Yangtze River Valley	31.1
Rs-22	Yangtze River Valley	21.3
Rs-23	Yangtze River Valley	26.3
Rs-24	Yangtze River Valley	23.1
Rs-25	Yangtze River Valley	26.9

the surface and sometimes aerially. The color of the colonies changed first to white and then to cream. After that, it changed from light brown to dark brown. Measurement of growth rate and daily growth rate of isolates at 20°C was investigated. The average growth of isolates at this temperature was 21mm/day, which filled the entire 9cm container for three days (Table 1). The highest and lowest growth rates were related to Rs-21 and Rs-1 isolates.

The results of cluster grouping divided the isolates into two distinct groups: fast growth (68% isolates) and slow growth (32% isolates) (Figure 1).

Determination of anastomosis group

All isolates were tested by the clean slide method with standard test isolates from AG1-IA anastomosis groups, and based on filament fusion between them; their belonging to the AG1-IA anastomosis group was confirmed.

Anastomosis group confirmation using AG1-IA-specific primers

Ten isolates were studied as population representatives by species-specific primers to identify the isolates directly and confirm their belonging to the AG1-IA anastomosis group. These primers specifically amplified a 256bp fragment in these isolates and the AG1-IA standard isolate (Figure 2). These primers did not amplify any fragments in the AG4 isolate used as a negative control.

Investigation of genetic diversity with RAPD marker

Of the 20 primers tested, seven primers, OPA04, OPA07, OPC18, OPC19, R28, and RC09 (Table 2), showed higher reproducibility and polymorphism shown in Figure 3 of the RC09 primer gel. From the total data obtained from these seven primers, 1308 bands in the range of 200 to 5000bp were obtained for 25 isolates, which was able to

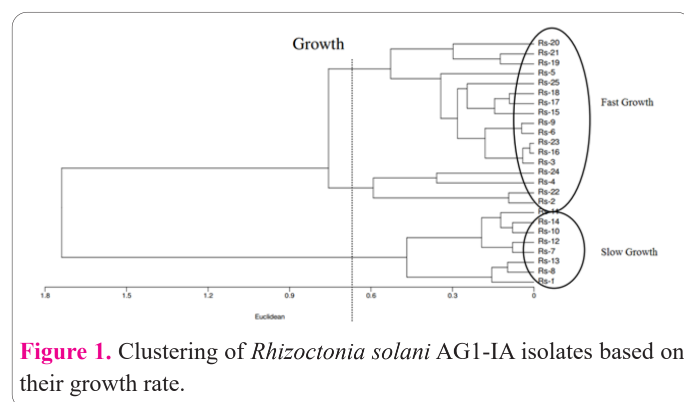

Figure 1. Clustering of *Rhizoctonia solani* AG1-IA isolates based on their growth rate.

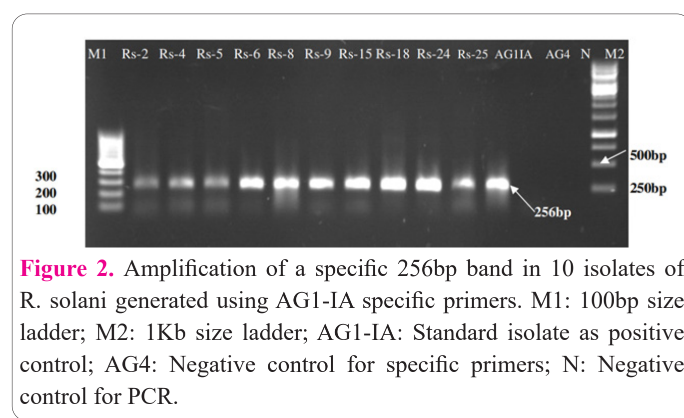

Figure 2. Amplification of a specific 256bp band in 10 isolates of R. solani generated using AG1-IA specific primers. M1: 100bp size ladder; M2: 1Kb size ladder; AG1-IA: Standard isolate as positive control; AG4: Negative control for specific primers; N: Negative control for PCR.

Table 2. The results of random primers on isolates of *Rhizoctonia solani* AG1-IA.

Primer Sequence	Number of Fragments amplified	Number of Polymorphic locus	Number of Monomorphic locus	Polymorphic Percentage
5'-GAAACGGGTG-3'	117	11	0	100%
5'-AATCGGGCTG-3'	291	22	0	100%
5'-AGCCAGCGAA-3'	140	11	2	78%
5'-TGAGTGGGTG-3'	129	14	1	93%
5'-GTTGCCAGCC-3'	162	14	1	93%
5'-ATGGATCCGC-3'	213	15	1	93%
5'-GATAACGCAC-3'	256	19	1	95%
Total	1308	107	6	94%

show a very good polymorphism. The highest number of bands was obtained by OPA04 primer and the lowest by OPC18 primer. The dendrogram of bands was divided into two groups based on the Jaccard coefficient at the 36% similarity of isolates. These primers were able to separate the isolates into fast-growing and slow-growing groups. The first group included all slow-growing isolates (32% of the total isolates) and the second group included all fast-growing isolates (68% of the total isolates). From the total RAPD band patterns, 113 loci were obtained, of which six loci (5.5%) were monomorphic, and 107 loci (94.5%) were polymorphic. The highest polymorphism was obtained by primers OPA04 and OPA07 and the lowest by primer OPC18. The highest similarity based on the Jaccard coefficient was observed between Rs-17 and Rs-18 isolates with 83 similarities, and the lowest similarity was observed between Rs-10 and Rs-17 isolates with ten similarities. At the level of similarity of 80%, the number of groups increased to 23 groups, which indicates the high diversity observed among this group of isolates. These primers were not able to detect isolates by their geographical locations.

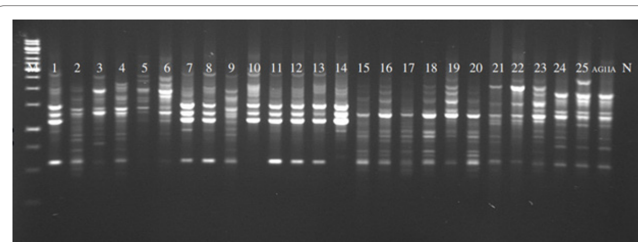

Figure 3. Fingerprint patterns of isolates of *R. solani* generated by RAPD RC09 primers; M: 1Kb size ladder; AG1-IA: Standard isolates and positive control; N: Negative control for PCR.

Discussion

Investigation of diversity based on morphological characteristics

The thallus appearance of *R. solani* in this study was following the investigations of Raj *et al*. (16) and Righini *et al*. (17). The color of the colonies varied from white to light and dark brown. In terms of growth rate, the isolates had a rapid growth of more than 21mm/day consistent with the results of Desvani *et al*. (18). Haque *et al*. (19) reported the growth rate of isolates belonging to the AG1-IA anastomosis group in 30 mm/day at 30°C, which could be due to the difference in growth temperature of the isolates. The results of cluster grouping confirmed the division of isolates into two completely separate groups in terms of growth rate.

Anastomosis group confirmation using AG1-IA specific primer

The use of ribosomal primers for rapid detection of *Rhizoctonia solani* anastomotic groups is one of the most appropriate methods, which in studies by Matsumoto et al. (6) were able to isolate AG1-IA, AG 2-1, AG 2.2, AG1-IB, AG1-IC be precisely separated from each other. In the present study, these primers examined 10 Rhizoctonia isolates isolated from rice sheaths as representative of the study population, all of which had a band of 256bp according to Matsumoto's study. These primers were unable to replicate this region in the AG4 isolate. This result was also consistent with the achievement of Matsumoto's research (6).

Evaluation of genetic diversity of isolates with RAPD marker

Studying the genetic diversity of Rhizoctonia isolates showed that the RAPD marker if used correctly, is a valuable tool for studying diversity in this fungus. These results were consistent with the findings of Chakrapani *et al*. (20). This marker is used in various fields of diagnosis and genetic diversity of fungal isolates, classification, and phylogenetic studies (21, 22).

The results of the band pattern obtained from the seven node beginnings used in this study indicate the existence of high genetic diversity within the AG1-IA anastomotic group. This result confirms the studies of previous researchers on the variety and complexity of intraspecific groups of *R. solani* (23, 24).

Results of cluster analysis

The band patterns obtained from seven RAPD primers obtained from 25 studied isolates divided the isolates into two distinct genetic groups at a level of 36% similarity. These two groups were entirely consistent with the results of growth rate measurements, but no relationship was found between the grouping of isolates and their collection areas. This finding was consistent with the results of the study of Chakrapani *et al*. (20) that there was no relationship between the geographical distribution of isolates and groups from the study of genetic diversity. They stated that the criteria of the geographical area could be examined and mentioned the role of the anastomosis group in creating more critical diversity. Although other researchers have reported a genetic relationship and geographical location (25,26), its incompatibility can be found in other factors that cause genetic diversity. In this study, at the

level of 80% similarity, the number of groups increased to 23 groups, which indicates the very high diversity observed among the isolates. This method has also been used to study genetic diversity among AG1 isolates collected from Australia and Japan, where significant variation has been observed among isolates and even among isolates belonging to a region (27).

Chakrapani *et al*. (20) also reported high diversity within the anastomotic groups studied with RAPD and ERIC markers. Sibayan *et al*. (28) also used RAPD markers to differentiate 52 AG1-IA isolates from maize and rice in the Philippines into seven groups at 75% similarity level. In the research conducted by Wang *et al*. (10), the genetic diversity results of *R. solani* AG1-IA were isolated from rice by seven shear enzymes used in molecular markers. DNA-RFLP had four cleavage enzymes with cleavage sites that could not show polymorphisms in the AG1-IA population. While the results of the genetic diversity of *R. solani* isolates by RAPD marker in our study completely refute this result and offer a high diversity among the AG1-IA subgroup. The use of a small number of shear enzymes and the lack of cleavage sites for the enzymes used in the fragment could be the inconsistent results between our studies with Wang *et al*. (10). Genetic variation at molecular, morphological, biochemical and physiological levels has been reported in many plants (29-33).

Before this study, there was little information on the genetic diversity of *R. solani* AG1-IA Chinese populations using molecular markers. In this study, for the first time, the genetic diversity of *R. solani* AG1-IA Chinese isolates was demonstrated with RAPD molecular marker after rapid molecular detection using species-specific primers. For further studies of diversity among isolates, the use of larger populations and more diverse molecular markers is essential. Also, AG1-IA-specific primers, which were optimized in this study for rapid detection of this subspecies, can be used to detect contamination in infected samples before isolation, which requires further research.

Acknowledgments
Not applicable.

Interest conflict
The authors declare that they have no conflict of interest.

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