Mating Types Analysis of *Magnaporthe oryzae* Populations by Molecular Methods

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Abstract: Rice blast is a devastating disease which is caused by the heterothallic fungus *Magnaporthe oryzae.* Compatible sexual recombination which occurs between two *M. oryzae* strains of different mating types, can enhance genetic variability. Assessment of mating type alleles is used as a marker to measure population diversity. Forty six isolates of *M. oryzae* were collected from infected rice leaves from various ecosystems of coastal Odisha, India, and the mating type analysis using molecular markers was carried out. MAT1-1 mating type was dominating in all the ecosystems and MAT1-2 was found to be present in uplands as well as in irrigated fields. Both mating types could be found in the same field in irrigated ecosystem. The disease spread was very fast vertically as well as horizontally in those fields resulting in blast lesions looking as 'green islands (gi) produced in senescence leaves', and MAT1-2 was found to be associated with all gi lesions. Consequently, the management of the disease in those plots was very difficult. Interestingly, ribosomal RNA IGS region could not be amplified in MAT1-2 isolates but consistent amplification was obtained in MAT1-1 mating type isolates.

Keywords: IGS region, *m. oryzae*, mating type, *oryza sativa*, rice.

INTRODUCTION

Rice (*Oryza sativa* L.) supplies approximately 23% of the per capita energy for six billion people worldwide [1]. Rice blast, caused by Magnaporthe oryzae is a serious disease responsible for yield loss to the tune of 80% s [2, 3]. Each year, this disease is estimated to destroy enough rice that could feed more than 60 million people [4].

Magnaporthe oryzae B. Couch (anamorph *Pyricularia oryzae* Cavara; synonym *Magnaporthe grisea* (Hebert) Barr), is a heterothallic fungus, meaning the population comprises of two distinct mating types (MAT1-1 and MAT1-2) and the sexual reproduction is possible only between the two opposite mating types [5, 6]. This fungus is considered as highly variable and high degree of pathogenic variation (pathotypes) is observed in the field [8, 9]. Sexual reproduction can enhance and influence the genotypic variability of *M. oryzae* populations where the recombinant progenies may have new capability to infect different host cultivars. Both *MAT 1-1* and *MAT 1-2* are idiomorphs of a single mating type locus *Mat* [10]. Assessment of mating type alleles has been used as a marker to measure population diversity in this pathogen [7, 11].

Conventional approach to determine mating type in the pathogen population depends upon the appearance of mature perithecia in a cross between known tester and an unknown strain on culture media which is time consuming and requires high technical expertise [9, 12-14]. PCR amplification methods using *Mat* gene specific primers are a rapid method to explore the mating type population of *M. oryzae* [15-17].

In the current study 46 *M. oryzae* isolates (from coastal Odisha, India) were studied for mating type analysis by *Mat* gene specific molecular markers. In addition, we have also attempted the amplification of the intergenic spacer (IGS) region as this is one of the most rapidly evolving regions in the genome [18].

MATERIAL & METHODS

A total of 46 *M. oryzae* isolates, used in the current investigation were isolated from blast lesions produced on leaves collected from various ecosystems of coastal Odisha, India. Fourteen isolates were from rain-fed upland fields, 21 isolates were from Uniform blast nursery at CRRI, Cuttack and 11 isolates from farmer's fields with irrigation facilities (Table **1**). *M. oryzae* associated with blast lesions were isolated by a selective isolation method [19]. Lesion quality of samples was assessed [20]. Isolates were grouped numerically on the basis of lesion color and mating type by NTSYS Pc [21].

Genomic DNA Extraction

M. oryzae cultures were grown in OM broth (oatmeal broth medium; i.e. oatmeal 30 g, water one lit.). Cultures of 14 days old were used for DNA extraction. Genomic DNA of *M. oryzae* was extracted according to Liu *et.al.* [22] with following modifications. Mycelia was ground with 1ml of

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Table 1. Passport data and mating type analysis of *Magnaporthe oryzae* isolates from coastal Odisha by molecular methods.

Sl. No.	Isolate ID	Lesion Colour	Source	Mating Type	IGS Amplification
1	B2	g.i	Upland ecosystem	MAT2	No
2	B4	g.i	Upland ecosystem	MAT2	No
3	B5	bg	Upland ecosystem	MAT2	No
4	B7	g.i	Upland ecosystem	MAT2	No
5	B10	g.i	Upland ecosystem	MAT2	No
6	B11	g.i	Upland ecosystem	MAT2	No
7	B14a	g.i	Upland ecosystem	MAT2	No
8	B15a	chocolate	Upland ecosystem	MAT1	Yes
9	B15b	g.i	Upland ecosystem	MAT2	No
10	B19a	ybg	Upland ecosystem	MAT1	Yes
11	B19b	bg	Upland ecosystem	MAT1	Yes
12	B21	chocolate	Upland ecosystem	MAT1	Yes
13	B24	chocolate	Upland ecosystem	MAT1	Yes
14	B29	chocolate	Upland ecosystem	MAT1	Yes
15	B73	bg	UBN#	MAT1	Yes
16	B83a	ybg	UBN	MAT1	Yes
17	B83b	ybg	UBN	MAT1	Yes
18	B305	ybg	UBN	MAT1	Yes
19	B312a	bg	UBN	MAT1	Yes
20	B312b	ybg	UBN	MAT1	Yes
21	B313	bg	UBN	MAT1	Yes
22	B318a	w.g.m	UBN	MAT1	Yes
23	B318b	w.g.m	UBN	MAT1	Yes
24	B332	grey	UBN	MAT1	Yes
25	B339	grey	UBN	MAT1	Yes
26	B342	grey	UBN	MAT1	Yes
27	B364	ypg	UBN	MAT1	Yes
28	B366a	bg	UBN	MAT1	Yes
29	B366b	bg	UBN	MAT1	Yes
30	B371	ypg	UBN	MAT1	Yes
31	B376	pg	UBN	MAT1	Yes
32	B380a	pg	UBN	MAT1	Yes
33	B380b	pg	UBN	MAT1	Yes
34	B385a	bg	UBN	MAT1	Yes
35	B385B	bg	UBN	MAT1	Yes
36	B387	g.i	Irrigated ecosystem	MAT2	No
37	B388	bg	Irrigated ecosystem	MAT2	No

Table 1. Contd…..

Sl. No.	Isolate ID	Lesion Colour*	Source	Mating Type	IGS Amplification
38	B389	bg	Irrigated ecosystem	MAT2	No
39	B390	g.i	Irrigated ecosystem	MAT2	No
40	B391	ybg	Irrigated ecosystem	MAT1	Yes
41	B392	ybg	Irrigated ecosystem	MAT2	No
42	B393	grey	Irrigated ecosystem	MAT1	Yes
43	B394	ybg	Irrigated ecosystem	MAT1	Yes
44	B395	ybg	Irrigated ecosystem	MAT1	Yes
45	B396	ypg	Irrigated ecosystem	MAT2	No
46	B397	grey	Irrigated ecosystem	MAT1	Yes

*g.i- green island; bg-brown grey; ybg-yellow brown grey; ypg-yellow purple grey; pg- purple grey; w.g.m-whitish with grey margin; [Dhua, 1998], #UBN-Uniform Blast Nursery.

extraction buffer (400mM Tris-HCL pH8.0, 150 mM NaCl, 60 mM EDTA pH8.0, 1%SDS) and mixed with 100 µl of 3M potassium acetate (pH 4.8) and then centrifuged. To the supernatant 500 µl of chloroform: isoamyl alcohol (24:1) was mixed and centrifuged. The aqueous phage was collected and mixed with equal volume of isopropanol and centrifuged. The supernatant was discarded and DNA pellet was saved and then washed with 70% ethanol. After air drying DNA pellet was redissolved in 1X TE. To remove RNA the DNA solution was treated with RNaseA for 30 min at 37°C. RNaseA was denatured by incubating at 60°C for 1 h. Quality of DNA was checked on 0.8% agarose gel and quantity of DNA was estimated by spectrophotometer (Varian, Cary100 BioUV-Visible Spectrophotometer).

Amplification of *Mat* Gene and IGS Region

Mating type genes *mat1-1* and *mat1-2* were amplified according to [15] using following primer pairs:

(MAT1-1 F 5'TCAGCTCGCCCAAATCAACAAT3')

(MAT1-1R 5'ACTCAAGACCCGGCACGAACAT3')

(MAT1-2 F 5'GAGTTGCCTGCCCGCTTCTG3')

(MAT1-2 R 5'GGCTTGGTCGTTGGGGATTGT3')

PCR was performed in a 50 µl reaction mixture containing 60 ng of DNA, 1X PCR buffer with KCL, 1.5 mM $MgCl_2$, 10 pico moles of forward and reverse primers, 0.2 mM dNTPs, and 1U of Taq DNA polymerase, in a thermocycler PTC 100 (MJ Research). Thirty cycles of amplification were performed in a PCR program of initial denaturation at 94 °C for 5 min. Each cycle consisted of a denaturation step at 94 °C for 30 s, annealing temperature at 55 °C for 30 s and an extension step at 72 °C for 1 min, with a final extension at 72 °C for 5 min. The amplified products were fractioned on 1% agarose gel, visualized by ethidium bromide staining and documented in Syngene G Box Gel Documentation system.

The IGS region was amplified according to Kumar et.al. [23] using the primer pair LR12R F 5'GAACGCCTCTAA-GTCAGAATCC3' and InvSR1R R5' ACTGGCAGAATC-AACCAGGTA 3'.

RESULTS

Approximately 809 bp amplicon was obtained corresponding to MAT1-1 locus with about 70% of the isolates Fig. (**1**). Rest 30% tested positive for MAT1-2 locus with an amplicon size of 940 bp (Fig. **2**). About 20% isolates were from green island producing blast lesions, 24% were

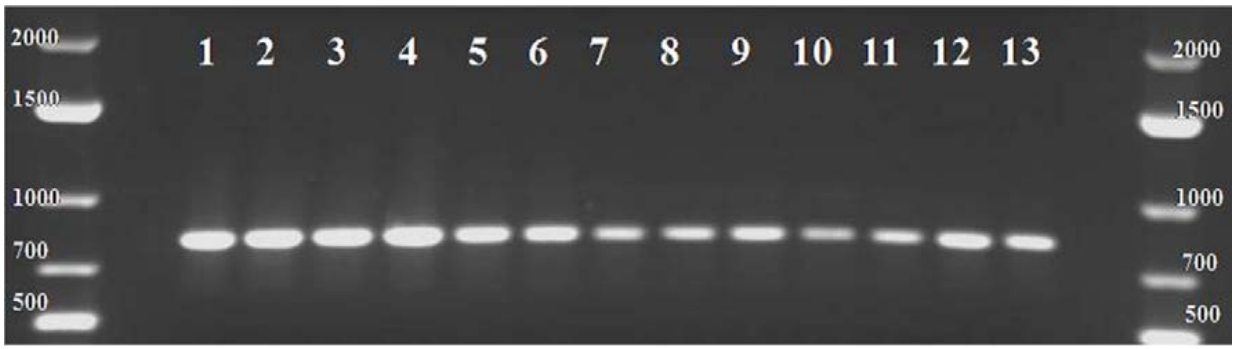

Fig. (1). Amplification of MAT I-1 mating types of *M. oryzae* population by MAT1-1F and MAT1-1R primers.
1= B-15a; 2= B-19a; 3= B-19b; 4= B-21; 5= B-24; 6= B-29; 7= B-73; 8= B-83a;
9= B-83b; 10= B-305; 11= B-312a; 12= B-312b; 13= B-313.

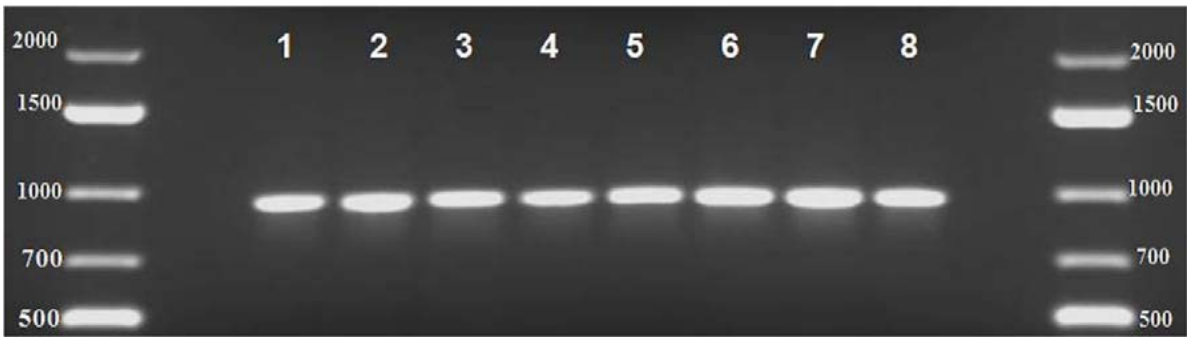

Fig. (2). Amplification of MATI-2 mating types of *M. oryzae* population by MAT1-2F and MAT1-2R primers.(1= B-2; 2= B-4; 3= B-5; 4= B-7; 5= B-10; 6= B-11; 7= B-14a; 8= B-15b).

from bg lesions, 9% from chocolate colour lesions, 11% from grey lesions, 6% from pg lesions, 4% from whitish grey lesions, 19.5% from ybg lesions and 6% from ypg lesions. This grouping on the basis of lesion colour has been clearly depicted from the dendrogram constructed by NTSYS (Figs. **3** and **4**) It was interesting to note that all the isolates from gi blast lesions were of MAT1-2. However 35% MAT1-2 isolates in present study were also from ypg /bg/ybg. MAT1-1 was not present in gi lesions.

Distribution of Mating Populations in Various Locations

(i) Upland Ecosystem

Both mating types were present in the samples from upland fields. MAT1-2 mating type was present in 57% cultures which were isolated from blast lesions where infected portions of leaf produced green islands and didn't senesce, while un-infected parts of leaf senesced [24]. The MAT1-2 was also isolated from one big broad brown grey lesion (Table **1**; Figs **3** and **4**). In blast infected samples from uplands, the MAT1-1 was present in *M. oryzae* cultures associated mostly with avirulent 'yellow brown grey' and chocolate lesions.

(ii) Uniform Blast Nursery (UBN)

The seeds supplied by co-ordinating centre were grown in UBN and all the samples collected from UBN i.e. brown grey (bg), yellow brown grey (ybg), yellow purple grey (ypg), purple grey (pg) and whitish with grey margin (w.g.m) yielded MAT1-1 mating type (Figs. **3** and **4**).

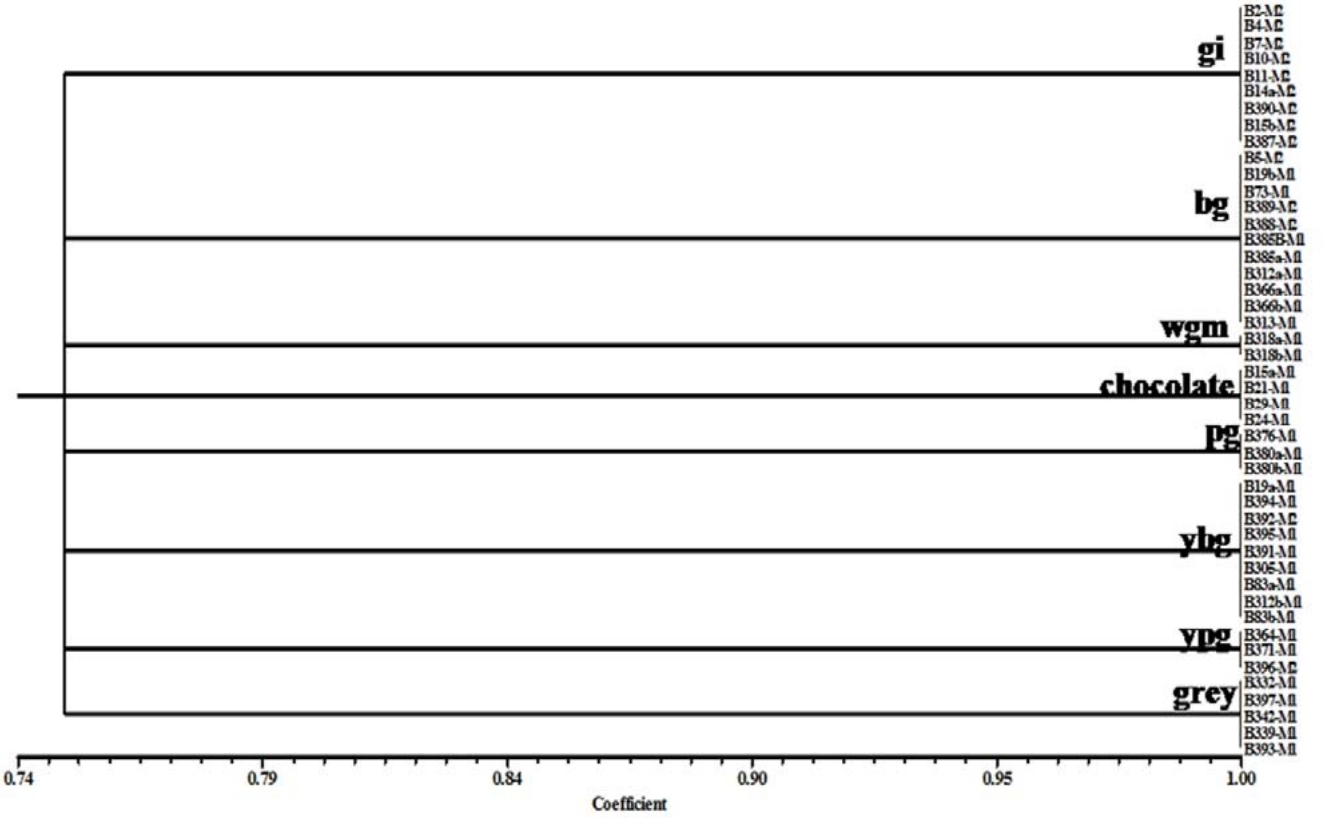

Fig. (3). Grouping of isolates on the basis of lesion colour.

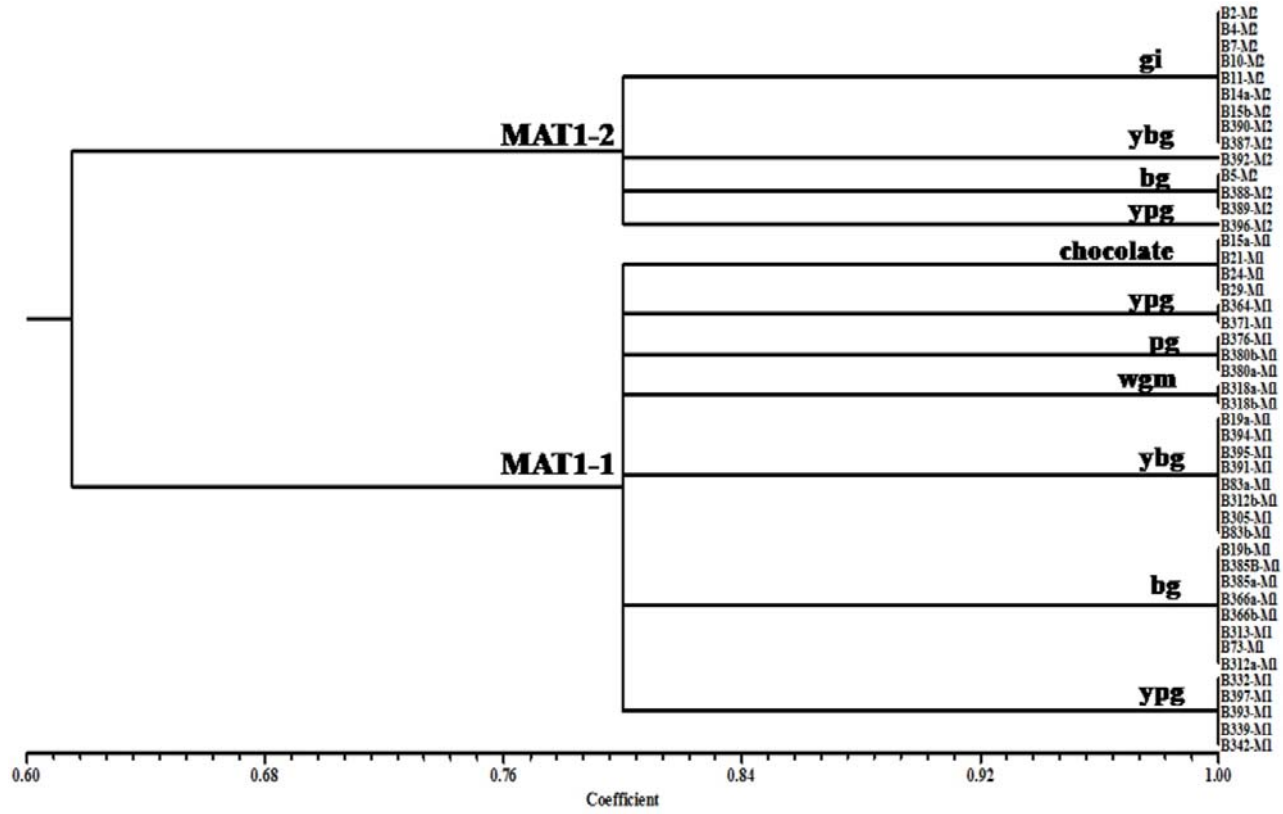

Fig. (4). Grouping of isolates on the basis of mating type.
MAT1-1 MAT1-2.

(iii) Irrigated Ecosystem

Severe blast infection was observed in fields of irrigated ecosystem included in this investigation. Chemical control measures were not effective and the disease spread rapidly in those fields. Both mating populations were observed in the same field. MAT1-2 was found in 55% samples. MAT1-1 mating type was isolated from grey, ybg and ypg blast lesions. MAT1-2 was observed from 'green islands produced in senescence leaves' or the ypg /bg / ybg lesions possessing thin margin and thick broad grey centre.

Amplification of IGS Region in Various Mating Types

It was interesting to note that all the isolates classified as MAT1-1 mating type were amplified by IGS specific primers LR12R - InvSR1R resulting in an amplicon size of approximately 3.3 kb, whereas no amplification could be obtained from MAT1-2 populations (Fig. **5**).

DISCUSSION

Analysis of mating type can provide an estimation of genetic diversity among *M. oryzae* populations from rice. Bao-Hua *et al.* [16] compared the PCR method and the standard strain GUY11 / KA3 mating type assays and there was a good consensus (95.1%). They suggested PCR based mating type detection method to be more feasible, quick and simple. Same set of primers were used in the present study.

In the samples analyses in the present investigation, MAT1-1 was the dominating mating type in all the ecosystems of coastal Odisha. Using the same primer pair, Dong-mei *et al.* [17] studied 141 isolates and it was found

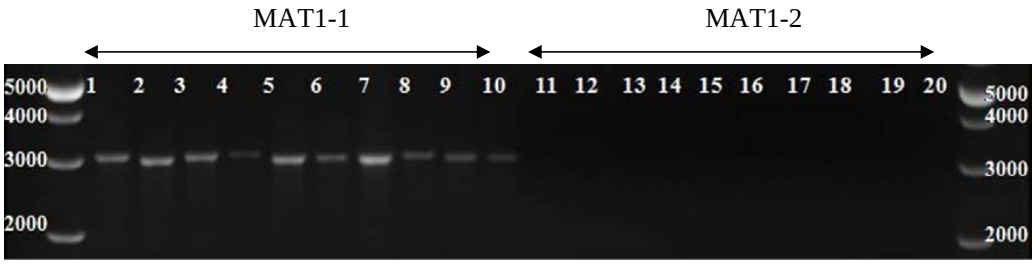

Fig. (5). Amplification of IGS region of MAT1-1 and MATI-2 population by LR12R and InvSR1R. (1=29; 2= B73; 3= B83a; 4= B83b; 5= B305; 6= B312a; 7= B312b; 8= B313; 9= B318a; 10= B318b; 11= B2; 12= B4; 13= B5; 14= B7; 15= B10; 16= B11; 17= B14a; 18= B15b; 19=B387; 20=B388).

that 19% of the samples belonged to MAT1-1 and 17% to MAT1-2. Urak *et al*. [11] found 160 of 168 isolates tested to be MAT1-1 in North Central California. In a survey of 467 *P. grisea* rice isolates from 34 countries in Europe and Africa, only mating type MAT 1-1 was found [13]. Zarrinnia *et al.* [7] found no MAT1-2 isolate among 100 Iranian populations.

MAT1-2 was found to be present in uplands as well as in irrigated fields during the present study. The presence of both mating types in same field in irrigated ecosystem was reflected in the aggressive behaviour of the pathogen. The disease spread was very rapid in those fields resulting in blast lesions as 'green islands produced in senescence leaves' and making the management of the disease challenging. These findings support the earlier observations of Kumar *et al.* [9] where the populations from the Himalayas were analyzed. They suggested a possible occurrence of sexual recombination in this region. A high MAT1-2 population (83%) was observed by Bau-Hua *et al.* [16] among 150 field isolates in rice from Fujian province of China. The size of PCR product produced by MAT1-1 / MAT1-2 in this study was similar to the bands observed by Bau-Hua *et al.* [16].

The IGS region that separates the repeated ribosomal genes appears to be the most rapidly evolving spacer region among fungi [25]. The entire IGS region can be amplified using primers anchored in the 3' end of the LSU gene e.g., LR12R and 5' end of the SSU RNA gene e.g., invSR1R [26, 27]. The ability and superiority of IGS regions as a highly variable marker for detecting compatible mating spores of mushroom species have been demonstrated in *Ferula sinkiangensis* [28], *Schizophyllum commune* [29], *Hebeloma cylindrosporum* [30] and *Pleurotus cornucopiae* [31, 32]. In present study, the IGS region did not amplify in MAT1-2 isolates but isolates possessing MAT1-1 were amplified. The correlation of lesion colour and the mating type indicates that there is a relationship between these two as evident from the dendrogram obtained using lesion colour (Fig. **3**), as well the dendrogram using both lesion colour and mating type (Fig. **4**). This observation suggests that the virulence of the pathogen could be predicted from the lesion colour, as reported earlier by Dhua [33] and while scoring the blast disease the colour of the lesion should be considered [20].

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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