Morpho-molecular characterization of Indian population of *Magnaporthe oryzae*, an incitant of blast disease in rice

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Abstract
Magnaporthe oryzae, a hemibiotrophic fungus, is one of the most destructive fungal pathogens causing potential threat to the production of rice and it can result in complete yield losses in major rice-growing areas. In the present study, seventy two M. oryzae isolates were collected from five major rice growing states of India. The blast isolates were divided into six groups based on colony characteristics viz. colony color; grayish (14), grayish white (15), grayish black (9), blackish (20), black with white cottony mass and white (9). The majority of isolates were observed as smooth (60) while few were rough (12) in appearance. M. oryzae developed characteristic spindle shaped symptoms on susceptible rice cultivar HR12 after pathogenicity assays. The virulence analysis revealed the existence of six highly virulent isolates, six moderately virulent isolates and only three mild isolates.

The sequence similarities among fifteen M. oryzae isolates ranged from 86.8 to 97.3%. The blast isolates were grouped into two groups using neighbor-joining method. Most of the Indian isolates were grouped together which represented that the Indian M. oryzae isolates are closely related. However, isolates belonged to same location were grouped in different sub-cluster which showed the existence of genetic variation among blast isolates belonging to the same location and presence of genetic similarity among M. oryzae isolates belonging to different locations. The present study would help to develop improved blast disease management through development of resistant varieties, genetic studies, and host-pathogen interaction.

Keywords: *Magnaporthe oryzae*, morphological characterization, India, virulence, cluster analysis.

Introduction

Rice (*Oryza sativa* L.) is one of the most important food crops that helps in the survival of more than 50% of the world population[1]. It played a significant role in food security of Asia, where more than 90 per cent of the rice is produced and consumed. The rice productivity is influenced by numerous biotic and abiotic factors[2]. Among biotic stresses, fungal diseases are of major concern causing significant yield losses in the rice crop throughout the world[3]. Amongst diseases, rice blast caused by *Magnaporthe oryzae* is one of the most destructive diseases in rice[4]. The blast pathogen is a heterothallic fungus and diverse phenotypic virulence develops through evolution of new races[5-7].

M. oryzae can infect a large number of plant species including rice, wheat, barley and finger millet[8]. Under blast-conducive environment, the pathogen can infect the paddy crop at all the stages of growth and development[6]. Wide geographic distribution, continuous evolution of new races, and high yield losses make this fungal disease a severe threat to rice production. It is estimated that every year rice blast is responsible to cause 10-30% yield losses in rice[8] that resulted in severe epidemics in major rice growing regions of the world.

Although, the fungicide application provided a short-term solution but it is practically uneconomical for resource poor farmers. Utilization of broad spectrum blast resistant varieties is the most successful, economical and environmental responsive approach to manage blast disease[9,10]. However, *M. oryzae* is well-known for its high genetic variation that breaks down the blast resistant varieties within 2–3 years of their release in the farmer's field[11]. The *M. oryzae* genome possessed abundant transposon, retrotransposons and repetitive segment that helps in overcoming host resistance rapidly through creation of genetic variation[12-14].

This leads to the continuous evolution of new pathogenic races that are able to infect previously reported blast resistant varieties. Disease management approach required the better understanding of the phenotypic and genetic diversity of the *M. oryzae* populations that would help in devising the strategies to manage this disease through gene stacking and gene deployment.

Understanding the genetic variation of the pathogen population not only helped in understanding the co-evolution in the plant pathosystem but also enhanced the durability of resistant cultivars[15]. However, limited information is available about the phenotypic and genetic

variation of *M. oryzae* populations from eastern India. Therefore, the present study was designed to study the variation in cultural characters, virulence analysis and genetic diversity of *M. oryzae* isolates from eastern India.

The information obtained from this study will guide to develop strategies to manage the havoc caused by blast disease in the endemic areas of the eastern India.

Material and Methods

Collection and long term storage of rice blast pathogen: Blast infected rice samples were obtained from the major rice growing regions of eastern India, particularly, Odisha (34), Jharkhand (14), Gujarat (11), Karnataka (9) and Jammu and Kashmir (4). The blast infected samples were cut into small pieces and surface sterilized by dipping into ethanol (70%) for 45 sec followed by dipping in mercuric chloride solution (0.1%) for 2–3 min, washed with autoclaved distilled water and finally leaf bits were dried with sterile filter paper and incubated for 5-7 days at 25°C. The blast fungus cultures were purified using single spore isolation and hyphal tip method. Single-conidial isolates were stored in sterile Eppendorf tubes at -20°C.

Morphological characterization and pathogenicity of *M. oryzae*: The cultural characteristics viz. texture and colony colour of all the *M. oryzae* isolates were studied by growing them on oat meal agar (OMA) medium for 7-10 days at 25±2°C. After attaining full growth, conidia were harvested and concentration was adjusted to $5x10^4$ spores/ml. The highly susceptible variety (HR12) was grown in plastic trays and inoculated at three to four leaf stage. The inoculated plants were maintained in darkness for 24 hr at 25±2°C and more than 90% relative humidity. The disease reaction was recorded 7 days after inoculation using 0-5 scale scoring system[16].

DNA isolation and quantification: Fungal mycelia (100-150 mg) was weighed and ground in liquid nitrogen using a sterilized mortar pestle. The powdered mycelium was transferred to a 2 ml Eppendorf tube containing 880 µl of extraction buffer (2% CTAB buffer, 4M NaCl, 0.5M EDTA, 1M Tris-Cl, 0.02% β-Mercaptoethanol). After incubation at 65°C for 1 hour, equal volume of phenol: chloroform: isoamylalcohol (25:24:1), was added and centrifuged @ 12000 rpm for 10 minutes.

After proper mixing, transfer the clear supernatant to an Eppendorf tube, an equal volume of chloroform: isoamylalcohol (24:1) was added followed by mixing and centrifuged @ 12000 rpm for 10 minutes.

Then, add chilled absolute alcohol to the supernatant, mix well and keep at -20°C for 2 hrs. After centrifugation, DNA pellet was washed with 70% ethanol, air dried and resuspended in 100 µl TE buffer. The gel electrophoresis and Nanodrop Spectrophotometer were used to determine the quality and quantity of fungal DNA. After quantification, the DNA samples were diluted to a concentration of 30-50 ng/µl for use in PCR reaction.

PCR amplification and gel elution: The ITS primer pairs[17] were used for molecular identification of *M. oryzae* isolate (Table 1). The PCR reaction was performed in a 25 µl reaction volume with 0.5 µM of primers, 10 mM dNTP, 1.5 mM $MgCl_2$, 50 ng of template DNA, 1X Taq buffer and 1U of DNA *Taq* polymerase. The PCR was performed as following parameters: initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 45 sec, extension at 72°C for 45 sec followed by a final extension at 72°C for 10 min.

The amplified PCR products were analyzed in gel electrophoresis and documented under UV using gel documentation system (AlphaImager, USA). The desired band was cut from the gel clean, sterile scalped blade and DNA was eluted as per standard protocol.

Table 1
List of primer pair used for PCR amplification of *M. oryzae* isolates

Primer name	Sequence (5' - > 3')
ITS – I	TCCGTAGGTGAACCTGCGG
ITS – IV	TCCTCCGCTTATTGATATGC

Sequencing and phylogenetic relationships: The PCR products obtained using the primer pairs (ITS 1 and ITS4) were purified using Wizard SV Gel and PCR Clean-Up System (Promega, USA) and T and A cloning vector kit was used to clone. The positive clones were sequenced in AB 13130 Genetic analyzer (Xcelris, India). The ClustalX software was used for version 1.81 multiple sequence alignment and pair wise alignment was made[18]. The neighbour-joining tree was constructed using Mega7 software[19].

Results and Discussion

The phytopathogenic fungus *M. oryzae* causing rice blast disease imposed a potential threat to sustainable global rice production and food security. It causes yield loss that varied from 20-100% based on susceptible cultivar and favourable environmental conditions[20]. With the emergence of new and virulent races, blast resistant varieties often break down within a few years of their release and thus imposed a continuous challenge to breeders for developing new varieties with broad and stable resistance.

The study of genetic variation in phytopathogen is required to better understand the co-evolution in the plant pathogen populations[21]. Blast infected samples were obtained from different five major rice growing regions of India (Table 2). To characterize the *M. oryzae*, it was isolated from the infected leaf samples and purified using single spore and hyphal tip method.

Table 2
Details of *M. oryzae* isolates used in the present study and their cultural characterization.

S. N.	Isolate name	Colony colour	Surface appearance	Plant parts	Location	Varieties
1.	Mo-1*	Blackish	Rough	Leaf	Odisha	Agniban
2.	Mo-2*	Grayish	Smooth	Leaf	Odisha	Sabita
3.	Mo-3*	Whitish	Smooth	Leaf	Odisha	Pooja
4.	Mo-4*	Black with white cottony mass	Smooth	Leaf	Odisha	Baunsaganthi
5.	Mo-5	Grayish	Smooth	Leaf	Odisha	Swarna
6.	Mo-6	Grayish black	Rough	Neck	Odisha	Baunsaganthi
7.	Mo-7	Whitish	Smooth	Leaf	Odisha	Haladi Ganthi
8.	Mo-8	Grayish white	Smooth	Leaf	Odisha	Tikichudi
9.	Mo-9	Grayish	Smooth	Leaf	Odisha	Swarna sub-1
10.	Mo-10	Grayish black	Smooth	Leaf	Odisha	Swarna sub-1
11.	Mo-11	Black with cottony mass	Smooth	Leaf	Odisha	Swarna
12.	Mo-12	Grayish black	Rough	Leaf	Odisha	Ranidhan
13.	Mo-13	Blackish grey	Smooth	Leaf	Odisha	Swarna
14.	Mo-14	Grayish black	Smooth	Leaf	Odisha	Pooja
15.	Mo-15	Black with white cottony mass	Smooth	Leaf	Odisha	Swarna
16.	Mo-16	Whitish grey	Rough	Leaf	Odisha	Kalajeera
17.	Mo-17	Black with white cottony mass	Smooth	Leaf	Odisha	MTU-1001
18.	Mo-18	Grayish	Smooth	Leaf	Odisha	MTU-1001
19.	Mo-19	Blackish	Smooth	Leaf	Odisha	Lalat
20.	Mo-20	Blackish	Smooth	Leaf	Odisha	Pratikshya
21.	Mo-21	Grayish	Rough	Leaf	Odisha	Pooja
22.	Mo-22	Grayish black	Smooth	Leaf	Odisha	Rajalaxmi
23.	Mo-23	White cottony mass	Smooth	Leaf	Odisha	-
24.	Mo-24	Blackish	Smooth	Leaf	Odisha	Naveen
25.	Mo-25	Grayish black	Smooth	Leaf	Odisha	Swarna
26.	Mo-26	White with cottony mass	Smooth	Leaf	Odisha	Lalat
27.	Mo-27	Grayish black	Smooth	Leaf	Odisha	Ranidhan
28.	Mo-28	Grayish white	Rough	Leaf	Odisha	Swarna sub-1
29.	Mo-29	White with cottony mass	Smooth	Leaf	Odisha	Pooja
30.	Mo-30	Grayish	Smooth	Leaf	Odisha	Naveen
31.	Mo-31	Grayish white	Rough	Leaf	Odisha	Ranidhan
32.	Mo-32	Grayish black	Smooth	Neck	Odisha	HR12
33.	Mo-33	Black with white cottony mass	Smooth	Leaf	Odisha	Karuna
34.	Mo-34	Blackish with cottony mass	Smooth	Leaf	Odisha	Karuna
35.	Mo-35*	Grayish white	Smooth	Leaf	Jharkhand	Swarna
36.	Mo-36*	Grayish white	Smooth	Leaf	Jharkhand	Swarna
37.	Mo-37*	White	Smooth	Leaf	Jharkhand	Sambha Mahsuri
38.	Mo-38	Grayish white	Smooth	Leaf	Jharkhand	Sambha Mahsuri
39.	Mo-39	Grayish	Smooth	Leaf	Jharkhand	-
40.	Mo-40	White	Rough	Leaf	Jharkhand	Vandana
41.	Mo-41	Grayish black	Rough	Leaf	Jharkhand	Vandana
42.	Mo-42	White	Smooth	Leaf	Jharkhand	Vandana
43.	Mo-43	Grayish	Smooth	Leaf	Jharkhand	Shabhagidhan
44.	Mo-44	Grayish black	Smooth	Leaf	Jharkhand	CR 40
45.	Mo-45	Grayish white	Smooth	Neck	Jharkhand	Naveen
46.	Mo-46	Blackish	Smooth	Neck	Jharkhand	Shabhagidhan
47.	Mo-47	Grayish white	Rough	Leaf	Jharkhand	Rajshri

48.	Mo-48	Grayish white	Smooth	Leaf	Jharkhand	-
49.	Mo-49*****	Blackish grey	Smooth	Neck	Gujarat	GNR-3
50.	Mo-50*****	Blackish	Smooth	Neck	Gujarat	GNR-3
51.	Mo-51*****	Grayish white	Smooth	Neck	Gujarat	Gujari
52.	Mo-52	Grayish	Rough	Neck	Gujarat	Gujari
53.	Mo-53	Blackish grey	Smooth	Neck	Gujarat	Gujari
54.	Mo-54	Blackish	Smooth	Neck	Gujarat	Gujari
55.	Mo-55	Black with cottony mass	Smooth	Leaf	Gujarat	Nathpowa
56.	Mo-56	Blackish grey	Smooth	Leaf	Gujarat	Nathpowa
57.	Mo-57	Grayish white	Smooth	Leaf	Gujarat	Gnr-4
58.	Mo-58	Grayish black	Smooth	Leaf	Gujarat	Zet-10750
59.	Mo-59	Grayish	Smooth	Leaf	Gujarat	Gujari
60.	Mo-60*****	Grayish white	Rough	Leaf	Karnataka	BPT-5204
61.	Mo-61*****	Grayish white	Smooth	Leaf	Karnataka	Gangavathi sona
62.	Mo-62*****	Grey with white cottony mass	Smooth	Leaf	Karnataka	Improved Samba Mahsuri
63.	Mo-63*****	Grayish	Smooth	Leaf	Karnataka	Jyothi
64.	Mo-64	Grayish white	Smooth	Leaf	Karnataka	BPT-5204
65.	Mo-65	Grayish	Smooth	Leaf	Karnataka	IR64
66.	Mo-66	Blackish	Rough	Leaf	Karnataka	Jyothi
67.	Mo-67	Grayish black	Smooth	Leaf	Karnataka	JGL 15098
68.	Mo-68	Grayish	Smooth	Leaf	Karnataka	Improved Samba Mahsuri
69.	Mo-69*****	Grayish	Smooth	Leaf	Jammu and Kashmir	K39
70.	Mo-70*****	Black with cottony mass	Smooth	Leaf	Jammu and Kashmir	Jhelum
71.	Mo-71	Blackish grey	Smooth	Leaf	Jammu and Kashmir	Kamad
72.	Mo-72	Blackish	Rough	Leaf	Jammu and Kashmir	Mushke budji

* Isolates used for virulence analysis and ITS sequencing

The colour of the mycelium varied from greyish to greyish black, greyish white, blackish grey and blackish with roughly circular growth. Based on conidia morphology and colony growth characteristics, the fungal pathogen was identified as *M. oryzae*. The blast isolates were stored on sterile filter paper discs and kept at -20°C for storage. Blast infected rice samples were collected from different rice growing areas of Odisha, Chhattisgarh, Tamil Nadu and purified through hyphal tip and single spore isolation technique[22-25]. Similarly, blast isolates were collected and purified from necks and nodes of infected rice plants[25].

Colony characteristics: The blast isolates (72) were collected from major rice growing regions of India. The oat meal agar media was used to study the growth characteristics of blast pathogen viz. colony color, and surface appearance. The blast isolates were characterized morphologically and categorized into six groups based on colony characteristics viz. colony color; grayish (14), grayish white (15), grayish black (9), blackish (20), black with white cottony mass and white (9). The majority of isolates was observed as smooth (60) while few were rough (12) in appearance. Significant variation in colony characteristics was observed among *M. oryzae* isolates collected from major rice growing regions of India (Table 2).

Surprisingly, most of the isolates were found to be smooth (60) and only a few were rough (12) in the colony appearance. The present study showed the presence of considerable variation among *M. oryzae* isolates originating from different rice growing regions of India for mycelial color and texture. The present study is in accordance with previous studies which showed the existence of morphological variation for the colony color and texture of the *M. oryzae* isolates.[22,27] Similarly, blast isolates collected from different regions of Odisha and Karnataka showed the morphological and texture variation among blast isolates[24,28].

Pathogenicity of rice blast isolates: The existence of the *Pyricularia oryzae* strains which varies in pathogenicity was first mooted by Sasaki[29]. In India, the existence of *M. oryzae* variation was first studied through multilocation testing of several resistant cultivars in different regions of India[30]. The pathogenic behaviour of virulent isolates was tested on the susceptible cultivar HR12. Initially, small specks symptoms developed; later enlarged into spindle, elliptical shaped

lesion with the ashy grey center. At last, spots coalesce to form large irregular patches. Based on symptom severity (lesion length and affected leaf area), 15 blast isolates from the five states of India were divided into three major groups i.e. Mo-I, Mo-II and Mo-III.

The first group, Mo-I consisted of highly virulent *M. oryzae* isolates and included six isolates (Mo-1, Mo-3, Mo-35, Mo-36, Mo-62 and Mo-69). Mo-II included six moderately virulent isolates (Mo-2, Mo-37, Mo-49, Mo-50, Mo-51, Mo-61, and Mo-63), while Mo-III included only three mild blast isolates (Mo-4 and Mo-70). Our results corroborated with previous results which showed categorization of blast isolates based on their virulence pattern and reported significant variation in their virulence characteristics[22,27].

Sequence identity and phylogeny: Identification of fungi up to the species level is of chief importance in both basic and applied research in plant pathology. The internal transcribed spacer (ITS) region of nuclear DNA has become the most commonly used molecular method for fungal species identification. In the present study, an amplicon of 520 bp was amplified using the primer pairs, ITS1 and ITS4 in selected fifteen isolates. Sequence similarities among fifteen blast isolates varied from 86.8 to 97.3%. The maximum sequence identity was recorded between Mo-36 and Mo-69, while minimum sequence identity was recorded between Mo-49 and Mo-51 (Table 3).

Our results are in agreement with previous results where the blast isolates collected from Chhattisgarh recorded sequence similarities between 78-95.6% whereas blast isolates from Odisha India showed sequence identity between 80.5 to 95.1%[22,24].

Based on ITS sequence of fifteen (present study) and eleven (retrieved through NCBI) (Table 4) blast isolates, they were grouped using the neighbour joining tree method using Mega7 software. The phylogenetic analysis divided the blast isolates originated from different rice growing regions into two clusters (Figure 1). Major cluster I included sixteen blast isolates. It further divided into two subgroups IA and IB. Sub-cluster IA included ten blast isolates dominated by Odisha isolates. Interestingly, this sub-cluster included isolates from all the five States. Surprisingly, sub-cluster IB consisted of only Indian isolates dominated by Jharkhand and Gujarat isolates. Major cluster II included ten isolates, of which, one isolate was each from Jharkhand, Odisha and Karnataka respectively.

Interestingly, major cluster I is dominated by Indian isolates whereas cluster II is dominated by isolates from different rice growing region of the world. Similarly, the blast isolates from the same geographical location did not belong to the same sub-cluster while genetically similar isolates from different geographical location were grouped together. Our study grouped most of Indian isolates in one group whereas blast isolates from other parts of the world in other group might be due to presence of distinct strain in India than rest of the world. Similarly, previous studies also reported divergence of Indian isolates from other parts of the world[22,24].

Table 3
Sequence identity matrix among *M. oryzae* isolates collected from major rice growing regions of India.

Isolates /isolates	Mo-1	Mo-2	Mo-3	Mo-4	Mo-35	Mo-36	Mo-37	Mo-49	Mo-50	Mo-51	Mo-61	Mo-62	Mo-63	Mo-69	Mo-70
Mo-1	0														
Mo-2	95.9	0													
Mo-3	91.6	90.8	0												
Mo-4	91.6	91	96.1	0											
Mo-35	91.5	91.5	87.6	87.6	0										
Mo-36	92.4	91.8	94.2	94.6	89.6	0									
Mo-37	93.5	93.1	95.5	95.5	89.8	96.3	0								
Mo-49	90.9	91.5	87.8	88	91.7	90.4	90.4	0							
Mo-50	92.8	92.8	92.1	92.1	91.7	93.5	93.2	91.5	0						
Mo-51	90.7	90.2	95.9	94.4	87	93.4	94.8	86.8	90.5	0					
Mo-61	91.6	92	87.9	87.7	92.4	89.9	90.1	93.2	91.1	87.1	0				
Mo-62	93.9	941	91.1	91.1	94.1	93.2	93.5	93.2	96.7	90.3	92.6	0			
Mo-63	91.9	91.6	91.6	91.2	91	95.1	93.6	92.5	94.1	90.7	89.3	94.1	0		
Mo-69	91.8	91.5	93.3	93.7	90.7	97.3	95.4	90.6	93.4	92.6	89.4	93.9	94.9	0	
Mo-70	92.3	92.2	95.7	96.3	89.1	95.2	96.7	89.1	93.2	94.1	89.2	92.6	92.6	94.8	0

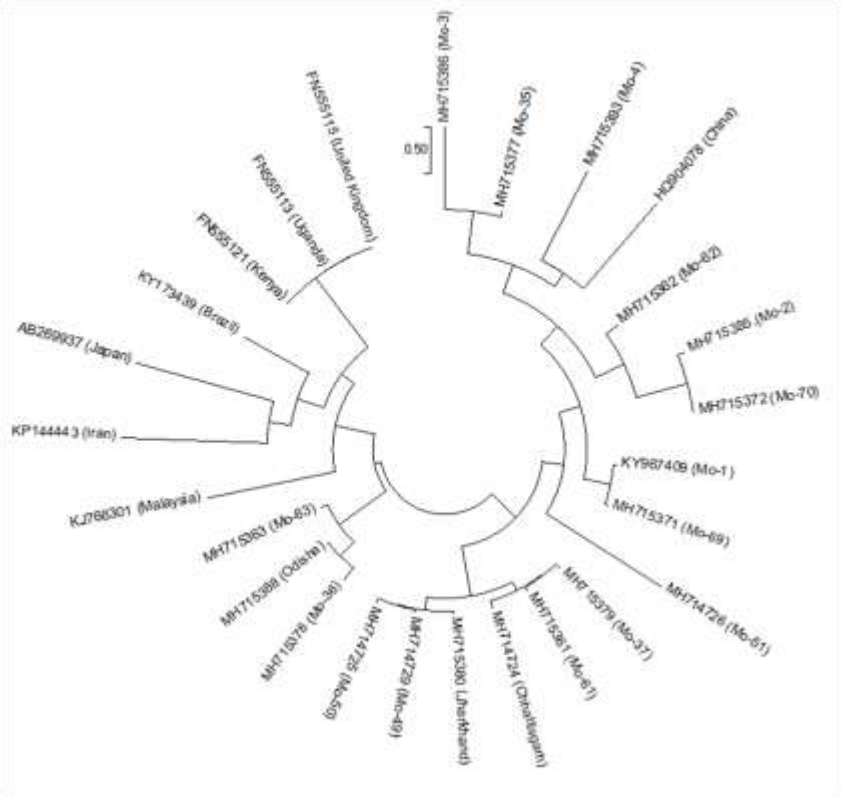

Figure 1: Phylogenetic relationship among *Magnaporthe oryzae* isolates of India with other blast isolates

Table 4
Details of *M. oryzae* accessions used for analysis in the present study.

S.N.	Isolate name	Accession No.	Location
1.	Mo-1	KY967409	Odisha
2.	Mo-2	MH715385	Odisha
3.	Mo-3	MH715386	Odisha
4.	Mo-4	MH715393	Odisha
5.	Mo-35	MH715377	Jharkhand
6.	Mo-36	MH715378	Jharkhand
7.	Mo-37	MH715379	Jharkhand
8.	Mo-49	MH714729	Gujarat
9.	Mo-50	MH714725	Gujarat
10.	Mo-51	MH714726	Gujarat
11.	Mo-61	MH715361	Karnataka
12.	Mo-62	MH715362	Karnataka
13.	Mo-63	MH715363	Karnataka
14.	Mo-69	MH715371	Kerala
15.	Mo-70	MH715372	Kerala
16.	Seth	KP144443	Iran
17.	CPC 29421	KY173439	Brazil
18.	OMZJ	HQ904078	China
19.	MG1-1	KJ766301	Malaysia
20.	D2-S26	FN555113	Uganda
21.	G22	FN555115	United Kingdom
22.	K33-184	FN555121	Kenya
23.	MAFF 306679	AB269937	Japan
24.	MG-4	MH714724	Chhattisgarh
25.	MG-70	MH715388	Odisha
26.	MG-110	MH715380	Jharkhand

Similarly, few isolates clustered into two clusters which showed the existence of high genetic variation among blast isolates originating from different rice growing region of India. Our study is in agreement with previous workers who also reported the existence of high genetic variation among rice blast isolates collected from different regions of Chhattisgarh Odisha and Andhra Pradesh[22,24,31].

Conclusion

The blast pathogen represented significant morphological and genetic variation among isolates collected from different rice growing regions. Indian isolates showed considerable genetic variation from other world isolates and the Indian isolates are related to each other at molecular level. The *M. oryzae* isolates belonged to same location clustered in different sub-groups whereas isolates from different location grouped together in the same sub-cluster. It showed existence of substantial genetic variation among isolates belonged to different location. The observed genetic similarity among Indian isolates might be due to its seed-borne nature that helps its movement in different rice growing regions.

Acknowledgement

The authors are extremely grateful to the Director, ICAR-National Rice Research Institute, Cuttack, India for his continuous support and facilitation for carrying out the research work successfully.

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(Received 12th October 2019, accepted 11th December 2019)