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ORIGINAL ARTICLE

Morpho-molecular diversity and avirulence genes distribution among the diverse isolates of *Magnaporthe oryzae* from Southern India

Chittaragi Amoghavarsha[1,2] | **Devanna Pramesh**[2] | **Ganesh R. Naik**[1] | **Manjunath K. Naik**[1] | **Manoj K. Yadav**[3] | **Umakanta Ngangkham**[4] | **Eranna Chidanandappa**[2] | **Adke Raghunandana**[2] | **Huded Sharanabasav**[2] | **Siddepalli E. Manjunatha**[2]

[1]Department of Plant Pathology, University of Agricultural and Horticultural Sciences, Shivamogga, Karnataka, India

[2]Rice Pathology Laboratory, All India Coordinated Rice Improvement Programme, University of Agricultural Sciences, Raichur, Karnataka, India

[3]ICAR-National Rice Research Institute, Cuttack, India

[4]ICAR-Research Complex for North-Eastern Hill Region, Manipur center, Imphal, Manipur, India

Correspondence
Devanna Pramesh, Rice Pathology Laboratory, All India Coordinated Rice Improvement Programme, University of Agricultural Sciences, Raichur, India.
Email: pramesh84@uasraichur.edu.in

Funding information
University of Agricultural Sciences, Raichur, India

Abstract

Aims: To investigate the diversity of eco-distinct isolates of *Magnaporthe oryzae* for their morphological, virulence and molecular diversity and relative distribution of five *Avr* genes.

Methods and Results: Fifty-two *M. oryzae* isolates were collected from different rice ecosystems of southern India. A majority of them ($n = 28$) formed a circular colony on culture media. Based on the disease reaction on susceptible cultivar (cv. HR-12), all 52 isolates were classified in to highly virulent ($n = 28$), moderately virulent ($n = 11$) and less-virulent (13) types. Among the 52 isolates, 38 were selected for deducing internal transcribed spacer (ITS) sequence diversity. For deducing phylogeny, another set of 36 isolates from other parts of the world was included, which yielded two distinct phylogenetic clusters. We identified eight haplotype groups and 91 variable sites within the ITS sequences, and haplotype-group-2 (Hap_2) was predominant ($n = 24$). The Tajima's and Fu's *F*s neutrality tests exhibited many rare alleles. Furthermore, PCR analysis for detecting the presence of five *Avr* genes in the different *M. oryzae* isolates using *Avr* gene-specific primers in PCR revealed that *Avr-Piz-t*, *Avr-Pik*, *Avr-Pia* and *Avr-Pita* were present in 73.68%, 73.68%, 63.16% and 47.37% of the isolates studied, respectively; whereas, *Avr-Pii* was identified only in 13.16% of the isolates.

Conclusions: Morpho-molecular and virulence studies revealed the significant diversity among eco-distinct isolates. PCR detection of *Avr* genes among the *M. oryzae* population revealed the presence of five *Avr* genes. Among them, *Avr-Piz-t*, *Avr-Pik* and *Avr-Pia* were more predominant.

Significance and Impact of the Study: The study documented the morphological and genetic variability of eco-distinct *M. oryzae* isolates. This is the first study demonstrating the distribution of the *Avr* genes among the eco-distinct population of *M. oryzae* from southern India. The information generated will help plant breeders to select appropriate resistant gene/s combinations to develop blast disease-resistant rice cultivars.

KEYWORDS

Avr genes, blast disease, haplotype analysis, internal transcribed spacer, *Magnaporthe oryzae*, rice

INTRODUCTION

One of the considerable constraints for increasing rice production is the rice blast disease caused by an *Ascomycota* fungus *Magnaporthe oryzae* B. C. Couch. The disease results in 10%–30% of annual yield loss (Sakulkoo et al., 2018). The pathogen infects all the crop developmental stages and damages all the aerial parts, most commonly leaves and panicles (Ou, 1985). Globally, rice is grown in different ecosystems, such as upland, rain-fed lowland, irrigated lowland and flood-prone (Khush, 1984). Karnataka, a southern state of India, is one of the major producers of rice in India. In Karnataka, rice is being cultivated in different ecosystems such as irrigated lowland ecosystems (Bhadra, Kavery, Tunga-Bhadra Project, Upper-Krishna Project and Varada command areas) and rain-fed upland ecosystem (Coastal and Hilly ecosystem) (Muniraju et al., 2017). Each ecosystem is unique concerning the availability of water, soil type, cultivars grown and has always suffered significant yield losses due to rice blast disease.

The rice blast disease continued to be the most destructive rice disease (Sharma et al., 2012). The rice blast population's genetic makeup is affected by selection, genetic drift, mutation, gene or genotype flow and mating systems (McDonald & Linde, 2002). The heterothallic reproduction and the modification of avirulence (*Avr*) genes in *M. oryzae* have resulted in new virulent races (Dai et al., 2010). The population analysis of isolates may help to know the potential of a pathogen development into new races. Previously, different markers such as amplified fragment length polymorphism (Vos et al., 1995), random amplification of polymorphic DNA (Prashanthi et al., 2010) and simple sequence repeats (Yadav, Aravindan, Raghu, et al., 2019) were used for deducing genetic diversity. The internal transcribed spacer (ITS) is still a valuable marker for taxonomic identification and deducing diversity in fungi (Schoch et al., 2012; Seifert, 2009). The haplotype analysis also helps to understand the diversity of the pathogen population (Arora et al., 2015; Cox et al., 2016).

The rice–*M. oryzae* interaction is well studied for understanding the mechanism of pathogenesis (Lopez & Cumagun, 2019; Valent, 1990). The interaction between the host resistant (*R*) genes and pathogen avirulent (*Avr*) genes follows the classical gene for gene hypothesis (Liu et al., 2005). The products of *Avr* genes are recognized by the products of corresponding *R* genes of the resistant hosts followed by the activation of hypersensitive response (Rouxel & Balesdent, 2010). To date, several *Avr* genes, such as *Avr-Pita* (Orbach et al., 2000), *Avr-Piz-t* (Li et al., 2009), *Avr- Pik/km/kp* and *Avr-Pii* (Yoshida et al., 2009), *Avr-Pia* (Miki et al., 2009; Yoshida et al., 2009), *PWL* (Kang et al., 1995; Sweigard et al., 1990), *Avr-Pi9* (Wu et al., 2015), *ACE1* (Bohnert et al., 2004) and *Avr1-CO39* (Ribot et al., 2013) have been cloned from *M. oryzae*. The evolution of *Avr* genes follows an arms race to overcome the resistance offered by *R* genes through loss or recovery or modification of its *Avr* genes (Chuma et al., 2011; Kanzaki et al., 2012; Sone et al., 2012). A previous study revealed that *Avr* gene instability is a common mechanism towards gaining virulence (Van de Wouw et al., 2010). Therefore, understanding the distribution of major *Avr* genes is essential for breeding stable resistant rice cultivars.

The present study was designed to study the morphological and molecular variability among the eco-distinct isolates of *M. oryzae* and find the distribution of selected *Avr* genes in the *M. oryzae* population. The information generated would help develop rice blast disease management strategies and enhance the resistance spectrum of cultivars grown in Southern India.

MATERIALS AND METHODS

Collection of diseased sample and pathogen isolation

The *M. oryzae* infected leaves showing characteristics symptoms of the blast disease were collected from different rice ecosystems of Karnataka viz. irrigated (Bhadra, Kaveri, Thunga-Bhadra, Upper-Krishna and Varada command) and rain-fed hilly ecosystems during *Kharif* 2019–2020 (Figure S1 and Table 1). A total of 52 isolates were recovered using the spore-drop technique on 2% water agar and subsequently purified on potato dextrose agar (PDA) (Dhua, 1986). The isolates were identified based on the characteristics of the colony, hyphae, conidiophores and conidia.

Morphological characterization

The morphological variability among the 52 isolates was characterized by studying the colony characters such as colony colour, form, elevation and margin. After purification, the actively growing mycelial tip (along with media) (≈5 mm) was transferred to a fresh PDA and incubated at 25 ± 2°C for 10 days. The colony colour, form, elevation and margins produced by each isolate were recorded. Based on

TABLE 1 List of eco-distinct isolates of *Magnaporthe oryzae* collected for this study

Ecosystem	District	GPS coordinates		Variety	Designation of isolates	Accession No.[a]
		N°	E°			
Hilly region (Rain-fed)	Chikkamagaluru	13.125579	75.642811	Mangala	MoK19-01	NS
		13.120924	75.599748	Jyothi	MoK19-02	MT757275
		13.125134	75.577765	Mangala	MoK19-03	MT757276
		13.131183	75.588651	Tunga	MoK19-04	MT757277
		13.257016	75.702945	Jyothi	MoK19-05	MT757278
		13.266959	75.713744	IET-7191	MoK19-06	MT757279
	Dharwad	15.446973	74.901461	MTU 1010	MoK19-10	NS
	Kodagu	12.141513	75.941336	Mysuru mallige	MoK19-17	MT757286
	Shivamogga	14.211397	74.922873	Hemavathi	MoK19-43	MT757306
		14.225189	74.905390	Jyothi	MoK19-44	MT757307
		14.195452	75.025073	Jyothi	MoK19-45	MT757308
	Uttara Kannada	14.957528	75.066326	Intan	MoK19-49	MT757311
		14.258470	74.921130	Mysuru mallige	MoK19-50	MT757312
		14.240383	74.920195	Jyothi	MoK19-51	MT757313
		14.874831	74.800096	Super sona	MoK19-52	NS
Thunga Bhadraproject (TBP) (Irrigated)	Koppal	15.445302	76.489877	BPT-5204	MoK19-18	MT757287
		15.477044	76.522991	Gangavathi Sona	MoK19-19	MT757289
		15.451497	76.526463	RNR-15048	MoK19-20	MT757291
		15.456990	76.523490	BPT-5204	MoK19-21	MT757288
		15.433294	76.377980	Gangavathi Sona	MoK19-22	MT757290
	Raichur	15.859583	76.810204	BPT-5204	MoK19-39	MT757303
		15.672504	76.703817	Gangavathi Sona	MoK19-40	MT757304
Kaveri region (Irrigated)	Hassan	12.601683	76.090019	Rajamudi	MoK19-11	MT757282
		12.597291	76.090376	IR-64	MoK19-12	NS
		12.594505	76.114303	Mangala	MoK19-13	MT757283
		12.582438	76.116941	Jyothi	MoK19-14	MT757284
		12.573898	76.133272	MC 13	MoK19-15	MT757285
	Mandya	12.663002	76.930126	BR2655	MoK19-23	MT757292
		12.653541	76.688456	Jyothi	MoK19-24	MT757293
		12.638914	76.705906	Mandya Sona	MoK19-25	NS
		12.568497	76.840563	MTU 1010	MoK19-26	MT757294
		12.665454	76.929560	Thanu	MoK19-27	NS
	Mysuru	12.562384	76.150656	Jyothi	MoK19-28	MT757295
		12.553039	76.160709	Jyothi	MoK19-29	MT757296
		12.542335	76.184115	Intan	MoK19-30	MT757297
		12.537814	76.185323	Mangala	MoK19-31	MT757298
		12.249573	76.966535	Jyothi	MoK19-32	MT757299
		12.242727	76.947166	BR2655	MoK19-33	MT757300
		12.229318	76.943791	MTU 1010	MoK19-34	NS
		12.207643	76.837320	Thanu	MoK19-35	MT757301
		12.234323	76.867950	Thanu	MoK19-36	NS
		12.218757	76.926333	Jaya	MoK19-37	NS
		12.218689	76.926260	Intan	MoK19-38	NS

(Continues)

TABLE 1 (Continued)

Ecosystem	District	GPS coordinates N°	GPS coordinates E°	Variety	Designation of isolates	Accession No.[a]
Bhadra region (Irrigated)	Davanagere	14.430535	75.961219	BPT5204	MoK19-07	NS
		14.260334	75.884341	BPT5204	MoK19-08	MT757280
		14.340148	75.887339	JLL-1758	MoK19-09	MT757281
	Shivamogga	13.796000	75.728003	Jyothi	MoK19-41	MT757305
		13.845125	75.676716	Jyothi	MoK19-42	NS
		13.969020	75.579158	IR-20	MoK19-46	MT757309
		14.005998	75.658183	Mangala	MoK19-47	NS
		13.978198	75.578882	Sharavathi	MoK19-48	MT757310
Varada region (Irrigated)	Haveri	14.952064	75.149498	MTU 1010	MoK19-16	NS

NS, not sequenced.

[a]Accession numbers of *M. oryzae* isolates obtained from GenBank NCBI.

the morphological studies, the isolates from diverse ecosystems were grouped.

Characterization of virulence on the susceptible cultivar

All 52 isolates were multiplied on oatmeal agar plates at 25°C for 8–10 days and regularly observed for sporulation. The spores were harvested by scraping the mycelial mass, and the spore concentration was adjusted to 2–3 × 10^5 spores per ml. About 40 ml of the conidial suspension containing Tween-20 (0.2%) was sprayed onto 14 days old rice cv. HR-12 (susceptible cultivar) using a hand atomizer. The inoculated plants were maintained in a walk-in growth chamber (Isotech Technology Pvt. Ltd) under dark at 25 ± 2°C with more than 90% relative humidity (RH) for 24 h. Later, the plants were transferred to a glasshouse and maintained at high RH (>90%) using overhead micro-sprinklers. After 7 days, the host reaction was recorded using a 0–9 scale scoring system (IRRI, 2013). The isolates with lesion type 0–3 are considered less virulent, 4–7 as moderately virulent, and more than 7 as highly virulent.

Isolation of genomic DNA

For the molecular study, 38 isolates exhibiting moderate to high virulence were selected for molecular diversity analysis. The conical flask containing potato dextrose broth was inoculated with mycelial discs (≈5 mm) with the help of a sterilized cork borer. The 7-day-old mycelial mat was used for the DNA isolation using HiPura™ Fungal DNA Isolation Kit (HiMedia Laboratories Pvt Ltd) following the manufacturer's instructions. The isolated DNA was stored at −20°C for further use. The quality and quantity of the isolated genomic DNA were assessed using both the agarose electrophoresis method (0.8%) and using Qubit™ 4.0 Fluorometer (ThermoFisher Scientific).

Primer synthesis

Oligonucleotide primers ITS-1F (Gardes & Bruns, 1993) and ITS-4 (White et al., 1990), reported previously, were synthesized and used to amplify the ITS. The *Avr*-gene-specific oligonucleotide primers of five *Avr* genes such as *Avr-Pii*, *Avr-Piz-t*, *Avr-Pik*, *Avr-Pia* and *Avr-Pita* published previously (Li et al., 2009; Miki et al., 2009; Orbach et al., 2000; Yoshida et al., 2009) were synthesized at a commercial facility (Eurofins Scientific India). Nucleotide sequences of all the primers are given in Table 2.

PCR amplification of internal transcribed spacer region

The PCR amplification for the ITS region was carried out in the ABS-Veriti™ 96-well thermocycler (Applied Biosystems). The PCR reaction mixture consists of 0.2 mmol l^{-1} of each dNTPs, 1.26 units of Taq DNA polymerase, 10X Taq buffer, 1.5 mmol l^{-1} of $MgCl_2$, 2 μmol l^{-1} for each forward and reverse primer. The PCR mix was prepared by combining all reagents (at 1 μl, except buffer at 5 μl) and distributed to PCR tubes (49 μl/tube), later 50 ng μl^{-1} of genomic DNA was added and MilliQ water was used to make the final volume to 50 μl. The PCR amplification was performed as follows: initial denaturation

TABLE 2 Oligonucleotide primers and their sequences used in the study

Gene/region	Primer name	Sequences 5→3	Annealing Temperature (°C)	Product size (bp)
ITS	ITS-1	TTATATTTAGAGGAAGGAG	55	550
	ITS-4	TCCTCCGCTTATTGATATGC		
Avr-Pita	YL169b-F	CGACCCTTTCCGCC	58	1200
	YL169b-R	TGACCGCGATTCCCTCCATT		
Avr-Piz-t	Z21-22F	AATCCCGTCACTTTCATTCTCCA	56	600
	Z21-22R	GTCGCAAGCCTCGTACTACCTTT		
Avr-Pik	Z23-24F	TCCAATTTATTCAACTGCCACTC	57	550
	Z23-24R	GTAAACCTCGTCAAACCTCCCTA		
Avr-Pia	Z27-28-F	CCCATTATCTTACCAGTCGCTTGA	56	1100
	Z27-28-R	ATTCCTCCCTAAACAGTAAACC		
Avr-Pii	Z41-42-F	TGCAGGCCCAAATCCGTAGGAA	58	450
	Z41-42-R	ACTGTCCGCTCGTTTGG		

at 94°C for 5 min; followed by 35 cycles of denaturation for 35 s at 94°C; primer annealing at 55°C for 30 s, and extension at 72°C for 40 s, and with the final extension at 72°C for 10 min. The amplified PCR product was quantified on Qubit™ 4.0 Fluorometer (ThermoFisher Scientific). The amplified product was sequenced after purification using a commercial facility (AgriGenome labs pvt ltd).

PCR amplification of *Avr* genes

The PCR master mix was prepared by combining 10X Taq buffer, 0.2 mmol l^{-1} of each dNTPs, 1.26 unit of Taq DNA polymerase, 1.5 mmol l^{-1} of $MgCl_2$, 2 μmol l^{-1} for each primer (each component at 1 μl, except buffer at 5 μl) and distributed to PCR tubes. Later 50 ng μl^{-1} of template DNA from the respective isolates was added. The PCR program for the amplification of *Avr* genes consisted of initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation for 35 s at 94°C, primer annealing (temperature as per the Table 2) for 30 s; extension at 72°C for 40 s, and with a final extension at 72°C for 10 min. The amplified PCR product was detected on 1% agarose gel electrophoresis stained with ethidium bromide and observed under a gel documentation system (UV Tech, Essential V6). The amplicon visibility for *Avr* genes confirms the presence/absence of the particular gene in the isolate.

Sequencing and sequence analysis

The PCR-amplified product of the ITS region was purified using HiPura® PCR Product Purification Kit (HiMedia™ Laboratories Pvt Ltd) following the manufacturer's instructions. The sequencing was carried out using a commercial facility (AgriGenome labs pvt ltd). The sequences of each isolate were aligned in a BioEdit (version 7.2.5) (Hall, 1999), and the consensus sequences obtained were deposited in the NCBI GenBank (Table 1).

Phylogenetic analysis

The evolutionary relationship between the different isolates of Southern India (Table 1) and other isolates was studied using their ITS sequences retrieved from the NCBI GenBank database. Phylogenetic analysis was performed in MEGA X (Kumar et al., 2018). The evolutionary history was inferred using the Neighbour-Joining method. Sequences were assembled to generate the consensus sequence for the sequence identity matrix using BioEdit Sequence Alignment Editor (version 7.2.5) (Hall, 1999).

Identification of haplotypes

The ITS sequences were subjected to haplotype analysis using DNA Sequence polymorphism software (DnaSP6) version 6.12.03 X 64 (Rozas et al., 2017). Based on the difference in the allelic frequencies, haplogroups were identified. The genetic differentiation of the population was carried out using the chi-square test. Sequences were further subjected to the neutrality test (Fu's *F*s statistic and Tajima's test) as reported previously (Jagadeesh et al., 2018).

RESULTS

Morphological characterization

Following the single-spore isolation method, 52 *M. oryzae* isolates were obtained, and the isolates were designated as MoK19-01 to MoK19-52 (Table 1). The morphological characteristics of 52 isolates showed diversity for their colony colour, appearance and colony forms. The morphological and cultural characteristics of the 52 rice blast isolates are summarized in Table 3. On PDA, isolates formed different colony colours such as greyish ($n = 13$), greyish-black ($n = 5$), greyish-cottony ($n = 7$), greyish-white ($n = 20$) and whitish cottony ($n = 7$) (Table 3). The isolates also exhibited different colony forms such as circular ($n = 28$) and irregular ($n = 24$) with raised ($n = 22$), flat ($n = 22$) and crateriform ($n = 8$) elevation with entire ($n = 9$), filiform ($n = 38$) and undulating ($n = 5$) margins (Figure 1).

TABLE 3 Grouping of *Magnaporthe oryzae* isolates based on morphological variability

(a) Based on the colour		
1	Greyish ($n = 13$)	MoK19-07, MoK19-09, MoK19-13, MoK19-14, MoK19-15, MoK19-16, MoK19-21, MoK19-25, MoK19-29, MoK19-44, MoK19-45, and MoK19-50
2	Greyish-black ($n = 5$)	MoK19-12, MoK19-28, MoK19-30, MoK19-33, and MoK19-37
3	Greyish-cottony ($n = 7$)	MoK19-06, MoK19-11, MoK19-23, MoK19-26, MoK19-31, MoK19-32, and MoK19-52
4	Greyish-white ($n = 20$)	MoK19-01, MoK19-02, MoK19-03, MoK19-04, MoK19-05, MoK19-10, MoK19-17, MoK19-18, MoK19-20, MoK19-22, MoK19-27, MoK19-34, MoK19-35, MoK19-38, MoK19-39, MoK19-42, MoK19-43, MoK19-46, MoK19-48, and MoK19-49
5	Whitish-cottony ($n = 7$)	MoK19-8, MoK19-19, MoK19-36, MoK19-40, MoK19-41, MoK19-47, and MoK19-51
(b) Based on the form		
1	Circular ($n = 28$)	MoK19-01, MoK19-03, MoK19-04, MoK19-05, MoK19-06, MoK19-07, MoK19-08, MoK19-09, MoK19-10, MoK19-14, MoK19-16, MoK19-17, MoK19-18, MoK19-19, MoK19-20, MoK19-21, MoK19-23, MoK19-24, MoK19-26, MoK19-29, MoK19-30, MoK19-31, MoK19-36, MoK19-37, MoK19-40, MoK19-47, MoK19-48, and MoK19-52
2	Irregular ($n = 24$)	MoK19-02, MoK19-11, MoK19-12, MoK19-13, MoK19-15, MoK19-22, MoK19-25, MoK19-27, MoK19-28, MoK19-32, MoK19-33, MoK19-34, MoK19-35, MoK19-38, MoK19-39, MoK19-41, MoK19-42, MoK19-43, MoK19-44, MoK19-45, MoK19-46, MoK19-49, MoK19-50, and MoK19-51
(c) Based on the elevation		
1	Flat ($n = 22$)	MoK19-01, MoK19-02, MoK19-04, MoK19-07, MoK19-09, MoK19-12, MoK19-13, MoK19-15, MoK19-16, MoK19-25, MoK19-28, MoK19-29, MoK19-30, MoK19-33, MoK19-37, MoK19-39, MoK19-42, MoK19-44, MoK19-45, MoK19-46, MoK19-49 and MoK19-50
2	Raised ($n = 22$)	MoK19-05, MoK19-06, MoK19-08, MoK19-10, MoK19-14, MoK19-17, MoK19-19, MoK19-20, MoK19-21, MoK19-22, MoK19-23, MoK19-24, MoK19-26, MoK19-31, MoK19-36, MoK19-38, MoK19-40, MoK19-41, MoK19-47, MoK19-48, MoK19-51 and MoK19-52
3	Crateriform ($n = 8$)	MoK19-03, MoK19-11, MoK19-18, MoK19-27, MoK19-32, MoK19-34, MoK19-35 and MoK19-43
(d) Based on the margin		
1	Filiform ($n = 38$)	MoK19-02, MoK19-03, MoK19-06, MoK19-07, MoK19-08, MoK19-09, MoK19-11, MoK19-12, MoK19-13, MoK19-14, MoK19-15, MoK19-16, MoK19-18, MoK19-20, MoK19-21, MoK19-22, MoK19-23, MoK19-24, MoK19-25, MoK19-26, MoK19-28, MoK19-29, MoK19-30, MoK19-31, MoK19-32, MoK19-34, MoK19-35, MoK19-37, MoK19-38, MoK19-39, MoK19-40, MoK19-42, MoK19-46, MoK19-47, MoK19-48, MoK19-49, MoK19-50 and MoK19-52
2	Entire ($n = 9$)	MoK19-01, MoK19-04, MoK19-05, MoK19-10, MoK19-17, MoK19-19, MoK19-27, MoK19-36 and MoK19-43
3	Undulating ($n = 5$)	MoK19-33, MoK19-41, MoK19-44, MoK19-45 and MoK19-51

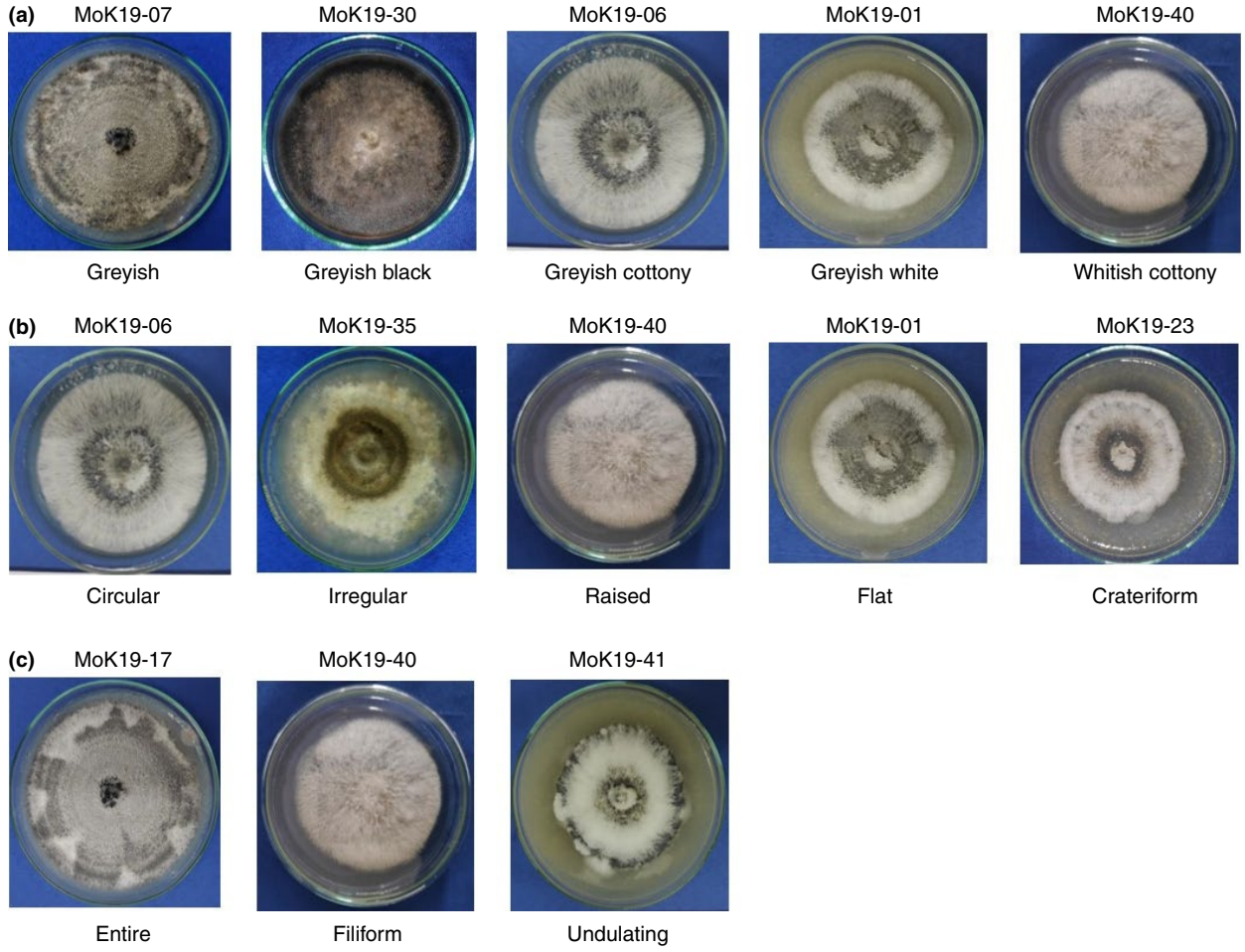

FIGURE 1 Grouping of isolates based on morphological characteristics. (a) Colour; (b) form and elevation; (c) margin

A phenotypic correlation was computed between four morphological characters of 52 *M. oryzae* isolates. A significant positive association was observed between colony colour and margin ($r = 0.41$) (Figure S2). Furthermore, colony form showed a significant positive association with colony margin ($r = 0.36$). A significant but negative correlation was observed for colony form with elevation ($r = -0.42$). The phenotypic diversity analyses of four morphometric characters of 52 *M. oryzae* isolates with heat map were depicted in Figure 2. Based on the hierarchal dendrogram, all isolates were clustered into two clades depending on the isolates similarities. The heat map indicated that colony margin and colour are closely related in comparison to other characters studied.

Virulence on susceptible cultivar HR-12

Based on disease reaction on HR-12, the isolates ($n = 52$) were classified into three types, such as highly virulent (Group Mo-I) ($n = 28$), moderately virulent (Group Mo-II) ($n = 11$) and less-virulent (Group Mo-III) ($n = 13$) (Figure 3). The highly virulent isolates were majorly from the irrigated ecosystem ($n = 21$), consisting of 11 from Kaveri, 6 from TBP and 4 from the Bhadra ecosystem (Table 4). In contrast, seven isolates from the rain-fed-hilly ecosystem are highly virulent. Among the highly virulent isolates ($n = 28$), the majority formed greyish-cottony ($n = 8$), followed by greyish-white ($n = 7$) and greyish ($n = 7$) coloured colonies. Only three highly virulent isolates were formed with whitish-cottony and greyish-black colonies on PDA. The isolates collected from different cultivars (e.g. Jyothi and MTU-1010) (Table 4) showed no correlation between virulence patterns and specific cultivar. About 85.71% (6/7) of TBP ecosystem isolates belonged to the highly virulent group, whereas 46.66% (7/15) isolates from the hilly region, 47.61% (10/21) of the Kaveri region and 50% (4/8) isolates of the Bhadra region were found to be highly virulent. Interestingly, the Varada region isolate showed a less virulent character.

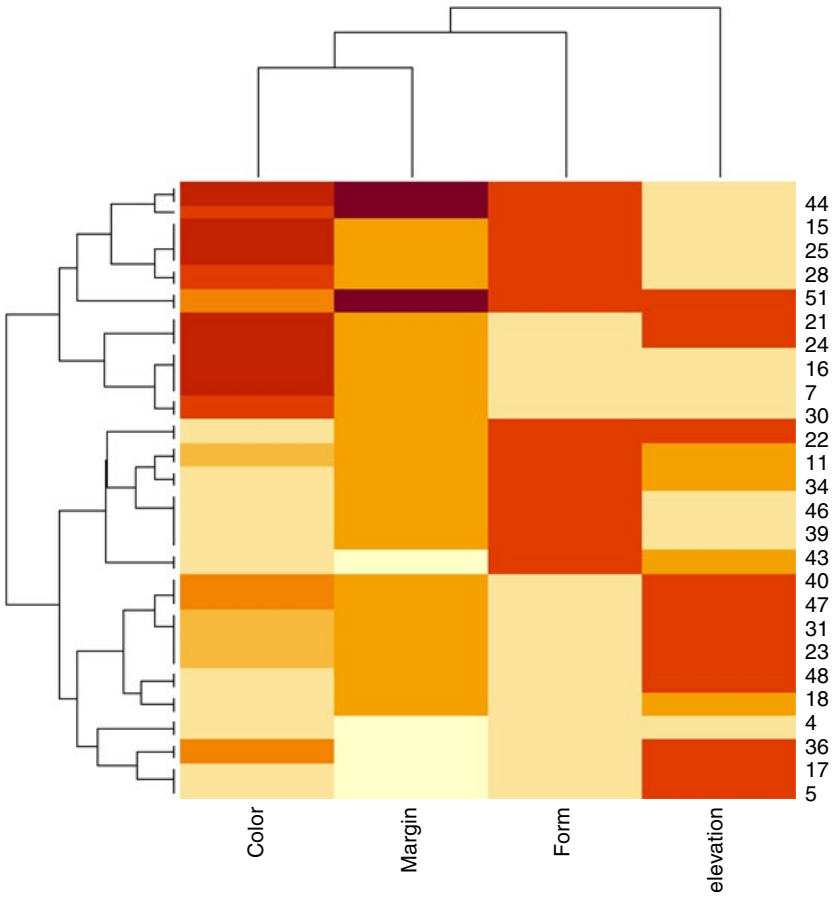

FIGURE 2 Hierarchal dendrogram of morphometric characters with a heat map

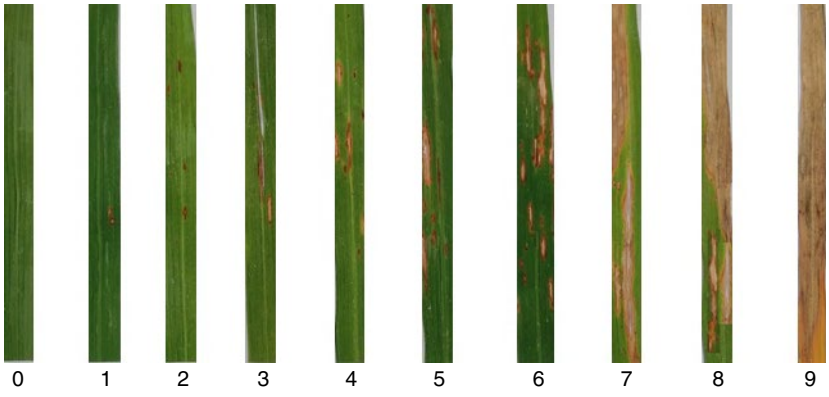

FIGURE 3 Disease rating scale used for virulence determination of isolates. 0–3 = less virulent; 3–7 = moderately virulent; >7 = highly virulent

Molecular characterization and phylogenetic analysis

Among the 52 isolates, 38 isolates that include 27 highly virulent and 11 moderately virulent, were selected. ITS-specific primer pairs amplified the ~550 bp fragment, which was confirmed on 0.8% Agarose gel. The purified PCR products were Sanger sequenced from both ends, and the consensus sequences obtained after trimming the primer sequences were submitted to the NCBI GenBank. The accession numbers were listed in Table 1.

The phylogenetic analysis of 38 isolates of this study with 38 isolates from other parts of the world (Table 5) revealed the two distinct clusters, such as cluster-I and cluster-II (Figure 4). Most isolates ($n = 74$) clustered together in cluster I, whereas only two isolates, MoK19-45 and MoK19-49, were diverged separately into cluster II. Within cluster-I, the highly virulent isolates MoK19-05, MoK19-21, MoK19-28 and moderately virulent strain MoK19-06 were equally distant from the other isolates ($n = 72$). The isolates of Tamil Nadu (South India), viz. MF678836, MF669475, MF668695 and MF668692 were clustered with the highly

TABLE 4 Virulence grouping of isolates based on the reaction on cv. HR-12

Sl. no	Ecosystem	Virulence type		
		Highly	Moderately	Less
1	(Rain-fed) Hilly[a] ($n = 15$)	MoK19-02, MoK19-05, MoK19-17, MoK19-44, MoK19-45, MoK19-50, and MoK19-51	MoK19-03, MoK19-04, MoK19-06, MoK19-43, and MoK19-49	MoK19-01, MoK19-10, and MoK19-52
2	Thunga Bhadra (TBP) (Irrigated) ($n = 7$)	MoK19-18, MoK19-20, MoK19-21, MoK19-22, MoK19-39, and MoK19-40	MoK19-19	—
3	(Irrigated) Kaveri ($n = 21$)	MoK19-11, MoK19-14, MoK19-23, MoK19-26, MoK19-28, MoK19-29, MoK19-30, MoK19-31, MoK19-32, and MoK19-33, MoK19-36	MoK19-13, MoK19-15, MoK19-24, and MoK19-35	MoK19-12, MoK19-25, MoK19-27, MoK19-34, MoK19-37, and MoK19-38
4	(Irrigated) Bhadra ($n = 8$)	MoK19-08, MoK19-09, MoK19-46, and MoK19-48	MoK19-41	MoK19-07, MoK19-42, and MoK19-47
5	(Irrigated) Varada ($n = 1$)	—	—	MoK19-16

[a]Mo = *Magnaporthe oryzae*; K = Karnataka; n = number of isolates.

virulent MoK19-11 isolate from the Kaveri ecosystem of Karnataka. At the same time, MH443357 of Thailand was clustered with highly virulent MoK19-20, MoK19-26, MoK19-30, MoK19-31, MoK19-33, MoK19-40, MoK19-44, MoK19-48, MoK19-50 and MoK19-51 and moderately virulent MoK19-41, MoK19-43 isolates. The highly virulent isolate MoK19-14 of the Kaveri ecosystem is closely related to the isolates LC552959, LC520097, LC519316, LC484289, MN999951 and FN555115 of Bangladesh, MN203028 of United Kingdom, and KY070277 of United States. Among these isolates, the MoK19-14 is related closest to the MN203028 of the United Kingdom and equally distant from LC552959, and FN555115 of Bangladesh, MH857082 of the Netherland, AY265323 of Thailand and FN555121 of the United Kingdom. Some of the isolates of this study, viz. MoK19-19, MoK19-29 and MoK19-32, are clustered together with the MH859782 of Denmark, KT693184 of Malaysia, MW042176, MW042177 and MW042178 of China, respectively. The moderately virulent MoK19-04 isolate from the hilly ecosystem of this study was equally distant from *M. grisea* isolates AB269937 of Japan, JQ911754 of Australia, GU327633 of United States and *M. oryzae* isolates KM816796, KM816797, KM816801 and KM816799 of South Korea. A similar equidistant relationship was found between highly virulent MoK19-46 isolate from Bhadra ecosystem of Karnataka and JX469384 of NE India, MT176496 of Sri Lanka, MT588875 of China, MF716846 (*M. grisea*) of Sri Lanka, MH590369 of Thailand, MF946553 of the United Kingdom, MT103090, and MT103091 of Iran, The MG642970.1 isolate from China grouped along with the Karnataka isolates viz. MoK-03, MoK-09, MoK-13, MoK-15, MoK-17, MoK-18, MoK-22, MoK-23 and MoK-24.

As limited information was observed on homology among the virulent isolates, the relationship among virulent isolates distributed in all phylogenetic groups based on sequence identity matrix was performed. The minimum and maximum similarities were varied from 68.3–100%. The maximum similarity was observed between TBP and Kaveri ecosystem isolates (MoK19-18 and MoK19-13) and Kaveri and Hilly ecosystem, respectively (MoK19-39 and MoK19-48). Similarly, the Hilly and Kaveri regions (MoK19-28 and MoK19-45) (Figure S3).

Distribution of *Avr* genes in the *M. oryzae* population

The distribution of five selected *Avr* genes in the *M. oryzae* population was studied using *Avr* gene-specific primers in PCR. The PCR analysis of *Avr* genes resulted in either amplification with expected size or no amplification. Among the *Avr* genes, the *Avr-Piz-t* (73.68%) and *Avr-Pik* (73.68%) were widely distributed in the *M. oryzae* population, followed by *Avr-Pia* (63.16%) and *Avr-Pita* (47.37%). The least frequency was observed with *Avr-Pii* (13.16%).

The isolates of irrigated ecosystems contain the highest frequency of *Avr-Pik* (84.61%), *Avr-Pit-z* (76.92%) and *Avr-Pia* (65.38%), whereas, same genes were present at 50.0%, 75.0% and 58.33% of the rain-fed ecosystem isolates, respectively. The distribution of *Avr-Pita* is found at 42.30% in irrigated and 50% in rain-fed ecosystems. Interestingly, none of the isolates of rain-fed ecosystems consisted of *Avr-Pii*, whereas 19.23% of the irrigated ecosystem isolates possess the *Avr-Pii* gene (Table 6). The highest number of isolates were found to possess three *Avr* genes ($n = 13$) followed by four ($n = 11$), two ($n = 6$) and one gene ($n = 8$) out of five genes under study (Figure 5).

TABLE 5 Details of *Magnaporthe oryzae* isolates from different parts of the world used for comparing evolutionary relationship with the isolates of present study

Sl. no.	NCBI accession no.	Strains	Origin
1	MH590369.1	*M. oryzae* isolate PO1	Thailand
2	MH443357.1	*M. oryzae* strain ERL15-9	
3	AY265323.1	*Pyricularia* sp. MC1	
4	MH857082.1	*Pyricularia oryzae* strain CBS 365.52	The Netherlands
5	MH859782.1	*P. oryzae* strain CBS 433.70	Denmark
6	MT176496.1	*P. oryzae* isolate Northern Province	Sri Lanka
7	MF716846.1	*M. oryzae* isolate AMA UOC-01	
8	KT693184.1	*M. oryzae* strain UPM-PO	Malaysia
9	KM249937.1	*M. oryzae* strain PO-FA01	
10	MW042178.1	*P. oryzae* strain Pos-3	China
11	MW042177.1	*P. oryzae* strain Pos-2	
12	MT588875.1	*P. grisea* isolate DW-2016-2	
13	MW042176.1	*P. oryzae* strain Pos-1	
14	MG642970.1	*M. e grisea*	
15	MF946553.1	*Pyricularia* sp. strain F2	United Kingdom
16	MN203028.1	*P. oryzae* isolate T49	
17	FN555121.1	*M. oryzae*	
18	JX469384.1	*M. oryzae* culture-collection NFCCI:2605	India (NE)
19	MF678836.1	*M. oryzae* isolate Gudalur	India (TN)
20	MF669475.1	*M. oryzae* isolate Nagaranai	
21	MF668695.1	*M. oryzae* isolate Aduthurai	
22	MF668692.1	*M. oryzae* isolate Maduranthakam	
23	KM816799.1	*M. oryzae* strain w11-65 18S	Korea
24	KM816801.1	*M. oryzae* strain w11-110	
25	KM816797.1	*M. oryzae* strain w11-58	
26	KM816796.1	*M. oryzae* strain w11-23	
27	AB269937.1	*M. grisea* genes	Japan
28	MT103091.1	*P. oryzae* isolate Mf3	Iran
29	MT103090.1	*P. oryzae* isolate Mb3	
30	LC552959.1	*P. oryzae* Meh17012B32	Bangladesh
31	MN999951.1	*P. oryzae* clone FH12D21_FH2A9	
32	LC520097.1	*P. oryzae* 19M7	
33	LC519316.1	*P. oryzae* 19M5	
34	FN555115.1	*M. oryzae*	
35	LC484289.1	*P. grisea* FmBWMRI-72	
36	GU327633.1	*M. grisea* strain ATCC MYA-4617	
37	KY070277.1	*M. oryzae* strain FPH2015-44	USA
38	JQ911754.1	*M. grisea* strain WAC13466 18S	Australia

Haplotype analysis and neutrality test

The 38 isolates of this study were grouped into eight haplogroups with a haplotype diversity of 0.6019 (Table 7). Majority of the isolates ($n = 24$) were under the Hap_2 group followed by Hap_6 ($n = 5$) and Hap_1 ($n = 3$), respectively. The remaining haplogroups consist of one isolate each. The Hap_2 consists of most ($n = 21$) isolate from the irrigated ecosystem. The isolates from the rainfed ecosystem were distributed under all the haplogroups

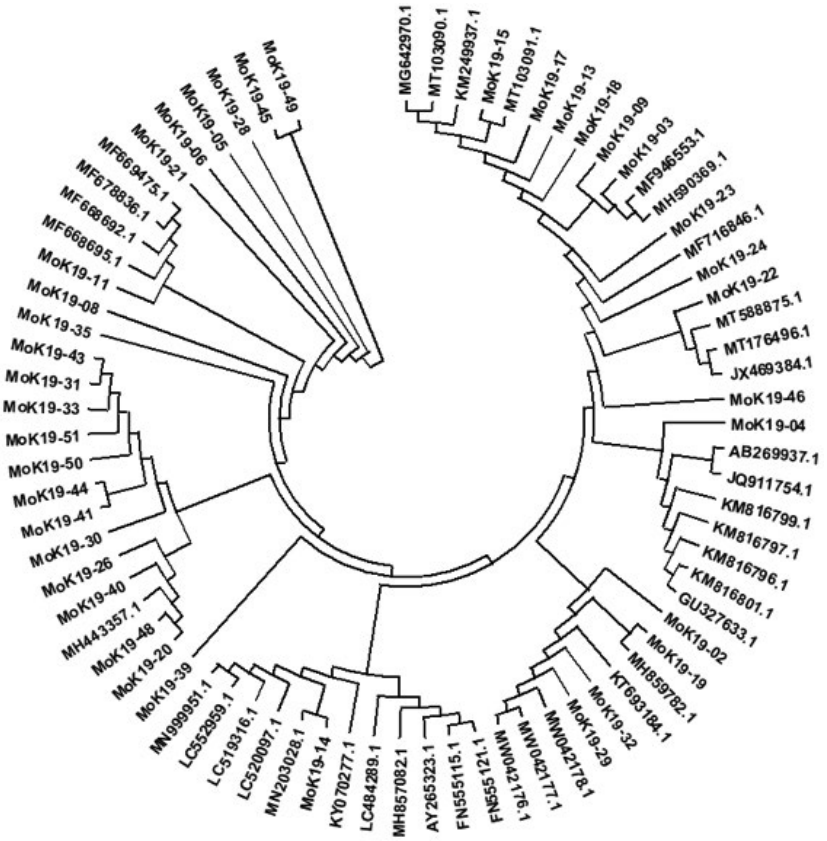

FIGURE 4 Phylogenetic tree based on internal transcribed spacer sequences. The evolutionary history was inferred using the Neighbour-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 76 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding

except Hap_5 and Hap_8. The variance of haplotype diversity was found to be 0.00695, with a standard deviation of 0.083. The neutrality test was also performed by using Fu's *F*s statistics and Tajima's *D* test and obtained the value of 3.494 and −2.75853, respectively, at $p < 0.001$. The total number of variable sites (S) was 91, with an average number of nucleotide differences of 5.70175. The total number of mutations (Eta) was 97, with Theta (per sequence) from Eta and Theta (per site) from Eta being 22.94282 and 0.04018, respectively.

DISCUSSION

Rice blast disease has been reported in more than 85 countries (Wang et al., 2014), causing significant yield losses annually (Li et al., 2011). The casual organism *M. oryzae* is still evolving; therefore, new subgroups are still emerging worldwide (Yoshida et al., 2016). In this study, significant morphological variability was observed among eco-distinct isolates in their colony colour, appearance, and forms. The colour of the colony was varied from grey to white, and similar colony features were reported previously from India (Panda et al., 2017; Singh et al., 2018; Yadav, Aravindan, Prabhukarthikeyan, et al., 2019). However, we could not find any correlation between the morphological types with the ecosystem origin of isolates. This could be due to the free movement strains between the ecosystems due to its seed transmission nature, or these isolates may have a common ancestral origin. Virulence analysis indicated three virulence types, and the majority of the isolates (53.8%) were highly virulent. Interestingly, most virulent isolates formed greyish colonies, similar to the previous report from India (Panda et al., 2017).

Internal transcribed spacer is widely used for taxonomic identification and characterization of fungal species. In the present study, we have used ITS sequence comparison to measure the genetic diversity of the *M. oryzae* population. The phylogenetic analysis using the ITS sequences of isolates from different parts of the world revealed close clustering of most isolates ($n = 74$) in cluster-I. In contrast, only two isolates (MoK19-45 and MoK19-49) that originated from the hilly ecosystem of Karnataka were diverged to form a separate cluster. However, within cluster-I, most of the isolates of this study ($n = 12$, 31%) were clustered with the Thailand isolates. Previous reports suggest that the genetic diversity of *M. oryzae* was highest in the East, South and Southeast Asia than in other regions of the world (Zeigler, 1998). The isolates of this study formed several sub-clusters with other isolates of the world indicated higher genetic variability among them. The possible reason for the divergence of MoK19-45 and MoK19-49 from the main

TABLE 6 Selected *Avr* genes distribution in the *Magnaporthe oryzae* isolates

		***Avr* genes**				
Sl. No	**Isolates**	***Avr-Pita***	***Avr-Piz-t***	***Avr-Pik***	***Avr-Pia***	***Avr-Pii***
1	MoK-19-02	−	+	+	+	−
2	MoK-19-03	+	+	+	+	−
3	MoK-19-04	+	+	−	+	−
4	MoK-19-05	+	+	+	-	−
5	MoK-19-06	−	−	−	+	−
6	MoK-19-08	−	+	+	−	−
7	MoK-19-09	+	−	−	+	−
8	MoK-19-11	+	−	+	+	−
9	MoK-19-13	−	+	+	+	−
10	MoK-19-14	−	+	+	+	+
11	MoK-19-15	−	+	+	−	−
12	MoK-19-17	−	+	+	+	−
13	MoK-19-18	−	+	+	+	−
14	MoK-19-21	+	+	+	+	−
15	MoK-19-19	−	+	+	+	−
16	MoK-19-22	−	+	+	+	+
17	MoK-19-20	+	+	−	−	−
18	MoK-19-23	−	+	+	+	+
19	MoK-19-24	+	+	+	+	−
20	MoK-19-26	+	+	+	−	−
21	MoK-19-28	−	−	+	+	−
22	MoK-19-29	−	−	+	+	−
23	MoK-19-30	−	+	−	−	−
24	MoK-19-31	−	+	+	+	−
25	MoK-19-32	−	−	+	−	−
26	MoK-19-33	+	+	+	−	−
27	MoK-19-35	−	−	+	−	−
28	MoK-19-39	+	+	+	+	−
29	MoK-19-40	−	+	+	+	+
30	MoK-19-41	+	+	+	+	−
31	MoK-19-43	+	+	+	+	−
32	MoK-19-44	+	+	+	+	−
33	MoK-19-45	+	−	−	−	−
34	MoK-19-46	+	+	+	−	−
35	MoK-19-48	+	−	−	+	+
36	MoK-19-49	−	+	−	−	−
37	MoK-19-50	+	−	−	−	−
38	MoK-19–51	−	+	−	−	−

Sign +/− indicates the amplification or non-amplification of gene.

cluster might be due to the unique evolutionary processes driven by the host (still local blast-resistant landraces are predominately cultivated by the farmers of a hilly ecosystem).

The *M. oryzae* predominately reproduces asexually. However, it has a high gene flow potential and spreads globally through infected seeds (Tharreau et al., 2009). The haplotype analysis and neutrality test provide

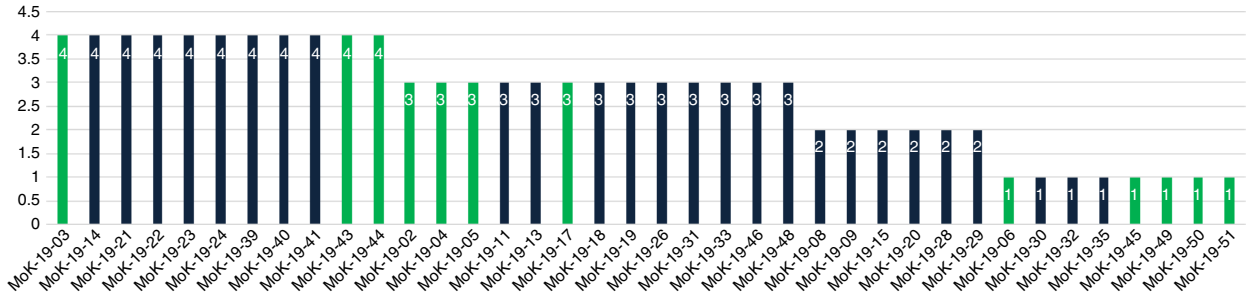

FIGURE 5 Frequency distribution of *Avr* genes in 38 *Magnaporthe oryzae* isolates of southern India. The isolates from rain-fed (green) and irrigated (black) ecosystems were presented

TABLE 7 Haplogroups of *Magnaporthe oryzae* isolates collected from diverse rice ecosystems of Karnataka

Haplotype (Hap) group	Number of isolates	Isolate code
Hap_1	3	MoK19-02, MoK19-03, MoK19-09
Hap_2	24	MoK19-04, MoK19-08, MoK19-13, MoK19-14, MoK19-15, MoK19-17, MoK19-18, MoK19-19, MoK19-20, MoK19-22, MoK19-23, MoK19-24, MoK19-26, MoK19-28, MoK19-29, MoK19-30, MoK19-32, MoK19-35, MoK19-39, MoK19-40, MoK19-41, MoK19-44, MoK19-46, MoK19-48
Hap_3	1	MoK19-05
Hap_4	1	MoK19-06
Hap_5	1	MoK19-11
Hap_6	5	MoK19-21, MoK19-45, MoK19-49, MoK19-50, MoK19-51
Hap_7	2	MoK19-31, MoK19-43
Hap_8	1	MoK19-33

information regarding the number of variable/ polymorphic sites in the DNA sequences and the neutral mutation within the genetic makeup of the population (Jagadeesh et al., 2018). The haplotype analysis was previously used for the *M. oryzae* isolates of Karnataka and found significant variability (Jagadeesh et al., 2018); however, the isolates studied were from a limited geographical region of Karnataka. During our investigation, the isolates were classified into eight haplogroups with haplotype diversity 0.6019. The haplotype diversity is controlled by many factors like recombination, mutation, demography and marker ascertainment (Stumpf, 2004). In our investigation, Fu's *Fs* statistics and Tajima's *D* supported higher rare alleles in the population. The possible cause may be the cultivation of diverse rice varieties in the different ecosystems that might have forced the pathogen to undergo selection.

Currently, the blast disease is managed by using various fungicides (Amoghavarsha et al., 2021; Pramesh, Muniraju, et al., 2016; Pramesh, Nataraj, et al., 2016); however, the most efficient strategy is the cultivation of rice varieties expressing an *R* gene corresponding to the *Avr* gene of *M. oryzae* (Li et al., 2012; Sharma et al., 2021). However, the pathogen overcomes the resistance offered by these *R* genes due to plasticity in their *Avr* genes (Chuma et al., 2011; Valent & Khang, 2010). In the present study, we screened *M. oryzae* isolates from different ecosystems for five *Avr* genes and identified all five *Avr* genes *viz*., *Avr-Piz-t*, *Avr-Pik*, *Avr-Pia, Avr-Pii* and *Avr-Pita* in the *M. oryzae* population. This result suggested the use of corresponding *R* genes in the breeding program for developing blast-resistant varieties. Interestingly, *Avr-Pii* was found most diminutive in the *M. oryzae* population, making it less preferable in the breeding program. However, a previous study reports the absence of *Avr-Pii* in any of the isolates of Karnataka (Gowda et al., 2015), which could be due to a smaller number of isolates studied (only 9). Thus, this is the first comprehensive study reporting the distribution of five *Avr* genes in the *M. oryzae* population of Southern India. Similarly, a previous study reported the distribution of nine *Avr* genes from Eastern India (Imam et al., 2015). The *Avr* genes are closely associated with transposable elements and diverse repeated sequences, leading to polymorphism and translocations, which help *M. oryzae* develop the new form rapidly (Huang et al., 2014). Therefore, continuous monitoring of *Avr* genes distribution at regular intervals is essential to update the effective *R* genes used in the resistance breeding program.

In conclusion, our study reported the significant diversity in morpho-molecular and virulence characters of *M.*

oryzae isolates collected from diverse rice ecosystems of Southern India. We also identified a high frequency of *Avr-Piz-t*, *Avr-Pik*, *Avr-Pia* and *Avr-Pita* genes in the *M. oryzae* population. This study added more information to the existing knowledge of morpho-molecular diversity and the *Avr* genes distribution in the eco-distinct population of *M. oryzae*. The information generated through the present study would help better understand blast pathogen dynamics that aids in the development of durable blast-resistant varieties required for sustainable agriculture.

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CONFLICT OF INTEREST

No conflict of interest has been declared.

AUTHOR CONTRIBUTION

AC and DP conducted the experiment. DP, GNR and MKN designed the study and arranged the funding. AC, DP, CE, RA, MKY, UN, SH and MSE collected isolates and performed the analysis. AC and DP wrote the manuscript with additional support from MKY and UN.

DATA AVAILABILITY STATEMENT

The accession numbers of all the sequences used in the study are listed in Table 1.

ORCID

Chittaragi Amoghavarsha https://orcid.org/0000-0002-5941-9661
Devanna Pramesh https://orcid.org/0000-0002-5229-443X

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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