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ORIGINAL ARTICLE

Multilocus sequence analysis and identification of mating-type idiomorphs distribution in *Magnaporthe oryzae* population of Karnataka state of India

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Abstract

Aims: To investigate the genetic diversity, population structure and mating-type distribution among the eco-distinct isolates of *Magnaporthe oryzae* from Karnataka, India.

Methods and Results: A set of 38 isolates of *M. oryzae* associated with leaf blast disease of rice were collected from different rice ecosystems of Karnataka, India, and analysed for their diversity at *actin*, *β-tubulin*, *calmodulin, translation elongation factor 1-α* (*TEF-1-α*), and internal transcribed spacer (ITS) genes/region. The isolates were grouped into two clusters based on the multilocus sequence diversity, the majority being in cluster-IA ($n = 37$), and only one isolate formed cluster-IB. Population structure was analysed using 123 SNP data to understand the genetic relationship. Based on $K = 2$ and ancestry threshold of >70%, blast strains were classified into two subgroups (SG1 and SG2) whereas, based on $K = 4$ and ancestry threshold of >70%, blast strains were classified into four subgroups (SG1, SG2, SG3 and SG4). We have identified 13 haplotype groups where haplotype group 2 was predominant ($n = 20$) in the population. The Tajima's and Fu's Fs neutrality tests exhibited many rare alleles. Further, the mating-type analysis was also performed using *MAT1* gene-specific primers to find the potentiality of sexual reproduction in different ecosystems. The majority of the isolates (54.5%) had *MAT1-2* idiomorph, whereas 45.5% of the isolates possessed *MAT1-1* idiomorph.

Conclusions: The present study found the genetically homogenous population of *M. oryzae* by multilocus sequence analysis. Both mating types, *MAT1-1* and *MAT1-2*, were found within the *M. oryzae* population of Karnataka.

Significance and impact of study: The study on the population structure and sexual mating behaviour of *M. oryzae* is important in developing region-specific blast-resistant rice cultivars. This is the first report of *MAT1* idiomorphs distribution in the *M. oryzae* population in any Southern state of India.

KEYWORDS

haplotype, *Magnaporthe oryzae*, *MAT1-1* and *MAT1-2*, mating type, multilocus, Rice blast, SNPs

INTRODUCTION

Rice (*Oryza sativa* L.) is a major food crop of the world, grown particularly in Asia, Africa and Latin America (Lou et al., 2012). The rice crop cultivation is affected by several biotic and abiotic stresses. Among biotic stresses, the rice blast disease caused by an *Ascomycete* fungus *Magnaporthe oryzae* B. C. Couch (syn. *Pyricularia oryzae* Cavara), is the major constraint for increasing rice production, which results in 10%–30% annual yield loss in different production zones every year (Sakulkoo et al., 2018), and up to 80%–100% loss whenever substantial epidemics occur (Prabhu et al., 2009). The *M. oryzae* infects during all stages of crop development and causes damage to all the aerial parts of the plant, but most commonly affects the leaves and panicles (Yadav et al., 2017).

Control of rice blast is the most challenging task because the pathogen can survive and multiply in harsh environmental conditions and swiftly spread to new fields (Araujo et al., 2000). However, the losses can be minimized by managing the disease using different strategies viz., modification of cultural practices, nutrition management, botanicals, biological agents, chemicals and resistant varieties (Amoghavarsha, Pramesh, Chidanandappa, et al., 2021; Amoghavarsha, Pramesh, Naik, et al., 2021; Hubert et al., 2015; Pramesh et al., 2020; Pramesh, Muniraju, et al., 2016; Pramesh, Nataraj, et al., 2016; Yang et al., 2008). Among the different strategies used, managing blast by host plant resistance is considered the most economical and best method (Sharma et al., 2021; Yadav, Aravindan, Raghu, et al., 2019). Even though effective resistance (*R*) genes are deployed, the genetic diversity of the pathogen has weakened this approach by modification of corresponding avirulence (*Avr*) genes in the pathogen (Zhong et al., 2018). The mutation in the '*Avr*' gene of the pathogen helps overcome the resistance offered by the '*R*' gene of the host. The durability of the *R* genes is not dependent on the host but on the structure of the pathogen population (McDonald & Linde, 2002). Therefore, there is a need to understand the current structure of the pathogen population and the means of diversity generation to minimize the menace caused by this disease.

Conventionally, the pathogen variability is recognized based on morphological features, ecology and mating behaviour. However, the high degree of phenotypic plasticity of morphological traits has made it very difficult to conclude the pathogen relationships (West-Eberhard, 1989). Nonetheless, the advancement of molecular studies has paved a path for the recognition of the genealogical species (i.e. species recognition based on gene trees using multiple loci), and it has surpassed the traditional grouping of organisms based on morphological data (Dettman et al., 2003; Taylor et al., 2000). Later, many molecular tools were used to understand the population diversity of *Magnaporthe* species viz., *M. grisea* Repeats (MGR), RFLP, RAPD, rep-PCR and AFLP (Amoghavarsha, Pramesh, Naik, et al., 2021; Sheoran et al., 2021).

Several molecular markers have been used for the deduction of fungal phylogeny and to classify phylogenetic species. The genome sequencing of *M. oryzae* has initiated the new era of population diversity analysis by using the genomic-loci-based markers (Choi et al., 2013; Dean et al., 2005; Kumar et al., 2017; Shirke et al., 2016). The commonly proposed DNA barcodes for the identification of fungi are the non-coding internal transcribed spacer (ITS) of the *ribosomal RNA* (rRNA) genes and coding regions like *γ-actin, β-tubulin*, *calmodulin* (CaM) and *translation elongation factor 1-α* (*TEF-1-α*) (Tekpinar & Kalmer, 2019). Due to the incompatibility and uncertainty in using a single gene marker, the best-recommended practice for evolutionary and phylogenetic analysis of fungi at the present situation is the use of different DNA barcodes in combination (Tekpinar & Kalmer, 2019).

Evolution is a continuous process, and the new subgroups of the pathogen *M. oryzae* are still evolving across the world. *Triticum* isolates arose in Brazil in the 1980s as a new pathogen of wheat crop (wheat blast fungus), which later spread to other countries in South America (Urashima et al., 1993). The new disease outbreak in the United States and Japan simultaneously was caused by another new subgroup (Lolium isolates) of the blast, *M. oryzae* (Tosa et al., 2004). The causal of blast symptoms on wheat in Kentucky, United States, differed from the wheat pathogen prevailing in South America. It is analogous to the annual ryegrass pathogen prevailing in North America, suggesting that *M. oryzae* jumped hosts from annual ryegrass to wheat (Tosa et al., 2016). The multigene-loci analysis is essential to understand the origin and evolution of M. oryzae isolates of different rice ecosystems. The past population study of rice blast isolates is restricted to the East (Yadav, Aravindan, Prabhukarthikeyan, et al., 2019) and North India (Sheoran et al., 2021). However, the data on South Indian isolates involving multi-loci is lacking.

The pathogen is known for its sexual recombination and random mating, leading to higher genetic diversity (Kotasthane et al., 2004). Asexual reproduction is more common than sexual reproduction (Saleh et al., 2012). The *M. oryzae* is heterothallic, and sexual reproduction is governed by two idiomorphs of the *MAT* locus (Kang et al., 1994). The idiomorph *MAT1-1-1* encodes an α domain, while *MAT1-2-1* encodes a DNA-binding domain of the high-mobility group (HMG) type (Debuchy & Turgeon, 2006). Isolates with either of the idiomorphs are referred to as *MAT1-1* (male fertile) or *MAT1-2* (female fertile) (Turgeon & Yoder, 2000). The previous studies on the *M. oryzae* population reported the domination of

isolates with one mating type (Onaga et al., 2015). The presence of both mating types provides an opportunity for the pathogen to reproduce sexually, thereby paving the path for variability. Apart from sexual reproduction, mutation also plays a vital role in creating variability in the pathogen population (McDonald & Linde, 2002).

The relationship between the *M. oryzae* populations of different ecosystems consisting of different cultivars has been studied using multiple gene loci sequencing (Zhong et al., 2018). Since the earliest publication of the genome sequence of *M. oryzae* strain 70–15 in 2004, multi-loci-based markers for evaluating population diversity have attained momentum (Choi et al., 2013; Feng et al., 2007). Using the nucleotide sequences of *actin*, *β-tubulin*, *calmodulin*, *TEF-1-α* and ITS regions, the genetic diversity of *M. oryzae* isolates can be analysed. Similarly, the assessment of different mating types would help understand the possibility of sexual reproduction in a geographical region. These studies are essential to understanding the existing population structure and the threat of invading new hosts.

The rice ecosystem of Karnataka, a southern state of India, has been divided into the irrigated ecosystem (Bhadra, Kavery and Tungabhadra Project command areas) and the rainfed ecosystem (Hilly ecosystem) (Muniraju et al., 2017). The disease varies from severe to moderate, from rainfed hilly to irrigated ecosystems because of the difference in soil type, cultivars grown and rainfall pattern (data not provided). Despite efforts worldwide, the understanding of *Magnaporthe* population genetic structure is still incomplete, and to understand the structure, requires genome sequences from different geographical locations (Sheoran et al., 2021).

The present investigation aimed to explore the feasibility of genetic variation and mating behaviour of the *M. oryzae* isolates from different rice ecosystems of Karnataka using the sequence data from multi-loci and mating type primers. By gathering reliable sequence data, we have analysed the population structure, evolutionary relationship, haplotype and mating type diversity within the isolates from different rice ecosystems of Karnataka, which helps develop rice blast-resistant cultivars for the region. This is the first report of the existence of both mating types in the *M. oryzae* isolates from the Southern part of India.

MATERIALS AND METHODS

Collection of diseased samples and monoconidial isolation

A total of 38 *M. oryzae* isolates used for the morphological and molecular characterization in our previous study (Amoghavarsha, Pramesh, Naik, et al., 2021) were used. All isolates were obtained from a field survey done during *Kharif* 2019–2020 in different rice ecosystems of Karnataka viz., rainfed hilly ecosystems and irrigated (Bhadra, Kaveri and Thunga-Bhadra, command areas) (Figure 1; Table 1). The infected leaf samples were collected from both cultivated and local farmer's varieties (Figure S1). The geographic coordinates for all 38 locations are provided in Table 1. The pathogen was isolated on 2% water agar (WA) using a spore-drop technique described by Dhua (1986) with some modifications. The symptomatic leaves with blast lesions were cut into small bits of 3 mm size and were surface-sterilized using sodium hypochlorite solution (1.0%) for 30 s and rinsed 3–4 times with distilled water. The bits with lesions were attached to the upper lid of a petri dish, and humidity was created using moist cotton. The upper lid was placed on the lower lid that consists of 2% water agar (WA). The set-up was placed at 25 ± 2°C for 1 day and observed for spore drop on the WA. The spores were transferred to a fresh plate containing potato dextrose agar (PDA) and allowed to grow. These single spore isolates were further maintained at 25 ± 2°C in the incubator by regular sub-culturing. The isolated cultures possessing the macroscopic and microscopic (mycelia and conidia) characteristics of *M. oryzae* were stored at −20°C for further studies as reported previously (Jagadeesh et al., 2018).

Isolation of fungal genomic DNA

With the help of a sterilized cork borer, the mycelial discs (≈5 mm) of 38 isolates were inoculated to potato dextrose broth (PDB). The 7-day-old mycelial mat was used to isolate DNA using the HiPura™ Fungal DNA Isolation kit following the manufacturer's instructions (HiMedia Laboratories Pvt Ltd). The extracted DNA was stored at −20°C for further use. The quality of the isolated genomic DNA was assessed using Qubit™ 4.0 Fluorometer (Thermo Fisher Scientific).

Primer synthesis

The oligonucleotide primers for multilocus sequence analysis were designed to amplify the *actin*, *ACT-512F* (ATGTGCAAGGCCGGTTTCGC) and *ACT-783R* (TAC GAGTCCTTCTGGCCCAT) (Carbone & Kohn, 1999), *β-tubulin, Btla* (TTCCCCCGTCTCCACTTCTTCATG), and Btlb (GACGAGATCGTTCATGTTGAACTC) (Glass & Donaldson, 1995), *Calmudulin, CAL-228F* (GAGTTCA AGGAGGCCTTCTCCC) and CAL-737R (CATCTTTCT GGCCATCATGG) (Carbone & Kohn, 1999), *TEF*

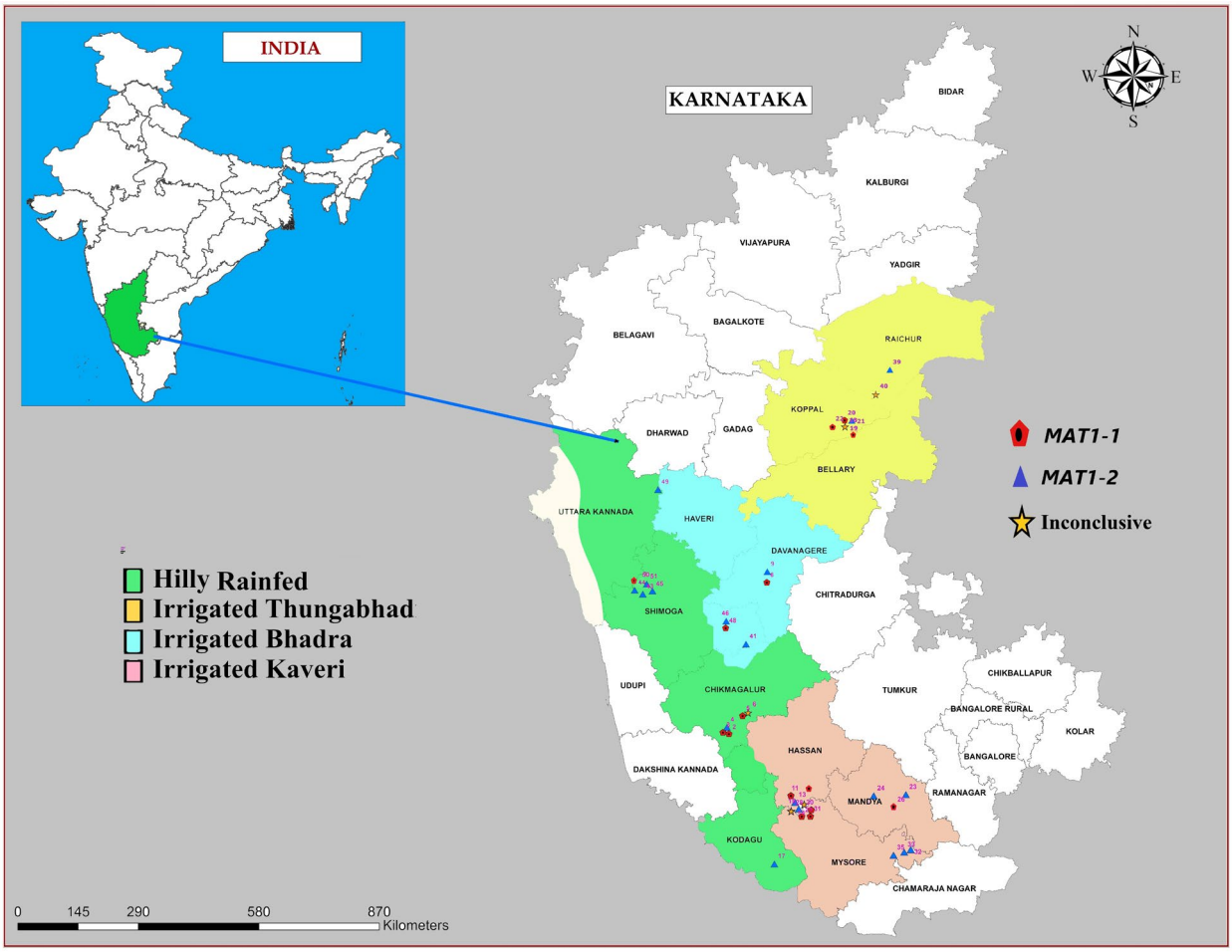

FIGURE 1 Map showing the geo-origin and mating types of *Magnaporthe oryzae* isolates from different rice ecosystems of Karnataka, India

1α, *EF1-983F* (GCY CCY GGH CAY CGT GAY TT) (Carbone & Kohn, 1999) and *EF1-2218R* (ATGACAC CRACRGCRACRGTYTGYAT) (Rehner & Buckley, 2005), ITS, *ITS1-P* (TTATATTTAGAGGAAGGAG) and *ITS4* (TCCTCCGCTTATTGATATGC) (Gardes & Bruns, 1993; White et al., 1990) were synthesized by utilizing the commercial facility (Eurofins Scientific India). Similarly, the mating-type '*MAT1*' gene primers viz., *MAT1-1, MAT1-1F* (TCAGCTCGCCCAAATCAACAAT), and *MAT1-1 R* (ACT CAAGACCCGGCACGAACAT) and, *MAT1-2, MAT1-2F* (GAGTTGCCTGCCCGCTTCTG) and *MAT1-2 R* (GGCT TGGTCGTTGGGGATTGT) were synthesized according to Zheng et al. (2008). All the primers were synthesized at a commercial facility (Eurofins Scientific India).

PCR amplification

The PCR amplification for ITS was described in our previous study (Amoghavarsha, Pramesh, Naik, et al., 2021). PCR amplification for the four selected gene loci (*actin, ß -tubulin, calmodulin* and *TEF-1-α*) was carried out in the ABS-Veriti™ 96-well thermocycler (Applied Biosystems). The PCR reaction mixture consists of 10X *Taq* buffer, 1.5 mmol L^{-1} of $MgCl_2$, 0.2 mmol L^{-1} of each dNTPs, 2 μmol L^{-1} for each forward and reverses primer and 1.26 units of *Taq* DNA polymerase. All the reagents were combined to form PCR mix (at 1 μl, except buffer at 5 μl) and distributed to PCR tubes (49 μl/ tube), to which 50 ng μl^{-1} of genomic DNA was added, and MilliQ water was used to make the final volume to 50 μl. Touchdown PCR was performed with an initial denaturation at 95°C for 5 min followed by 10 cycles of 45 s at 95°C, 45 s starting at 68°C and dropping by 1°C per cycle until a temperature of 58°C was reached and a 2 min extension at 72°C. The initial 10 cycles were then followed by 35 cycles of 45 s at 95°C, 45 s at 58°C, and 2 min at 72°C. A final extension at 72°C for 10 min completed the PCR. The amplified PCR products were separated on 1.5% agarose gel stained with ethidium bromide (0.5 μg/L) in TAE buffer (pH 8.0) along with the 1 Kb DNA ladder (MBI, Fermentas). The amplified PCR products were quantified on Qubit™ 4.0

TABLE 1 Geolocation, mating types and GenBank accession details of eco-distinct isolates of *Magnaporthe oryzae* collected for this study

Ecosystem	District	GPS coordinates		Variety	Isolates[a]	Mating type	NCBI GenBank accession no.				
		N°	E°				*Actin*	*β-Tubulin*	*CaM*[b]	*TEF-1-α*[c]	ITS[d]
Hilly region (Rainfed)	Chikkamagaluru	13.120924	75.599748	Jyothi	MoK19-02	*MAT1-1*	MT757512	MT767885	MT767924	MZ560810	MT757275
		13.125134	75.577765	Mangala	MoK19-03	*MAT1-1*	MT757513	MT767886	MT767925	MZ560811	MT757276
		13.131183	75.588651	Tunga	MoK19-04	*MAT1-2*	MT757514	MT767887	MT767926	MZ560812	MT757277
		13.257016	75.702945	Jyothi	MoK19-05	*MAT1-1*	MT757515	MT767888	MT767927	MZ560813	MT757278
		13.266959	75.713744	IET-7191	MoK19-06	NI	MT757516	MT767889	MT767928	MZ560814	MT757279
	Kodagu	12.141513	75.941336	Mysuru mallige	MoK19-17	*MAT1-2*	MT757523	MT767896	MT767935	MZ560821	MT757286
	Shivamogga	14.211397	74.922873	Hemavathi	MoK19-43	*MAT1-2*	MT757543	MT767916	MT767955	MZ560841	MT757306
		14.225189	74.905390	Jyothi	MoK19-44	*MAT1-1*	MT757544	MT767917	MT767956	MZ560842	MT757307
		14.195452	75.025073	Jyothi	MoK19-45	*MAT1-2*	MT757545	MT767918	MT767957	MZ560843	MT757308
	Uttara Kannada	14.957528	75.066326	Intan	MoK19-49	*MAT1-2*	MT757548	MT767921	MT767960	MZ560846	MT757311
		14.258470	74.921130	Mysuru Mallige	MoK19-50	*MAT1-2*	MT757549	MT767922	MT767961	MZ560847	MT757312
		14.240383	74.920195	Jyothi	MoK19-51	*MAT1-2*	MT757550	MT767923	MT767962	MZ560848	MT757313
Thunga bhadra project (TBP) (Irrigated)	Koppal	15.445302	76.489877	BPT-5204	MoK19-18	NI	MT757524	MT767897	MT767936	MZ560822	MT757287
		15.477044	76.522991	Gangavathi Sona	MoK19-19	*MAT1-1*	MT757526	MT767899	MT767937	MZ560823	MT757289
		15.451497	76.526463	RNR-15048	MoK19-20	*MAT1-1*	MT757528	MT767901	MT767938	MZ560824	MT757291
		15.456990	76.523490	BPT-5204	MoK19-21	*MAT1-2*	MT757525	MT767898	MT767939	MZ560825	MT757288
		15.433294	76.377980	Gangavathi Sona	MoK19-22	*MAT1-1*	MT757527	MT767900	MT767940	MZ560826	MT757290
	Raichur	15.859583	76.810204	BPT-5204	MoK19-39	*MAT1-2*	MT757540	MT767913	MT767952	MZ560838	MT757303
		15.672504	76.703817	Gangavathi Sona	MoK19-40	NI	MT757541	MT767914	MT767953	MZ560839	MT757304

(Continues)

TABLE 1 (Continued)

Ecosystem	District	GPS coordinates		Variety	Isolates[a]	Mating type	NCBI GenBank accession no.				
		N°	E°				*Actin*	*β-Tubulin*	*CaM*[b]	*TEF-1-α*[c]	ITS[d]
Kaveri region (Irrigated)	Hassan	12.601683	76.090019	Rajamudi	MoK19-11	*MAT1-1*	MT757519	MT767892	MT767931	MZ560817	MT757282
		12.594505	76.114303	Mangala	MoK19-13	*MAT1-1*	MT757520	MT767893	MT767932	MZ560818	MT757283
		12.582438	76.116941	Jyothi	MoK19-14	NI	MT757521	MT767894	MT767933	MZ560819	MT757284
		12.573898	76.133272	MC 13	MoK19-15	*MAT1-2*	MT757522	MT767895	MT767934	MZ560820	MT757285
	Mandya	12.663002	76.930126	BR2655	MoK19-23	*MAT1-2*	MT757529	MT767902	MT767941	MZ560827	MT757292
		12.653541	76.688456	Jyothi	MoK19-24	*MAT1-2*	MT757530	MT767903	MT767942	MZ560828	MT757293
		12.568497	76.840563	MTU 1010	MoK19-26	*MAT1-1*	MT757531	MT767904	MT767943	MZ560829	MT757294
	Mysuru	12.562384	76.150656	Jyothi	MoK19-28	NI	MT757532	MT767905	MT767944	MZ560830	MT757295
		12.553039	76.160709	Jyothi	MoK19-29	*MAT1-1*	MT757533	MT767906	MT767945	MZ560831	MT757296
		12.542335	76.184115	Intan	MoK19-30	*MAT1-1*	MT757534	MT767907	MT767946	MZ560832	MT757297
		12.537814	76.185323	Mangala	MoK19-31	*MAT1-1*	MT757535	MT767908	MT767947	MZ560833	MT757298
		12.249573	76.966535	Jyothi	MoK19-32	*MAT1-2*	MT757536	MT767909	MT767948	MZ560834	MT757299
		12.242727	76.947166	BR2655	MoK19-33	*MAT1-2*	MT757537	MT767910	MT767949	MZ560835	MT757300
		12.207643	76.837320	Thanu	MoK19-35	*MAT1-2*	MT757538	MT767911	MT767950	MZ560836	MT757301
Bhadra region (Irrigated)	Davanagere	14.260334	75.884341	BPT5204	MoK19-08	*MAT1-1*	MT757517	MT767890	MT767929	MZ560815	MT757280
		14.340148	75.887339	JLL-1758	MoK19-09	*MAT1-2*	MT757518	MT767891	MT767930	MZ560816	MT757281
	Shivamogga	13.796000	75.728003	Jyothi	MoK19-41	*MAT1-2*	MT757542	MT767915	MT767954	MZ560840	MT757305
		13.969020	75.579158	IR-20	MoK19-46	*MAT1-2*	MT757546	MT767919	MT767958	MZ560844	MT757309
		13.978198	75.578882	Sharavathi	MoK19-48	*MAT1-1*	MT757547	MT767920	MT767959	MZ560845	MT757310

NI: Not identified

[a] These isolates were obtained from our previous study (Amoghavarsha, Pramesh, Naik, et al., 2021).

[b] *CaM*, *Calmodulin*.

[c] *TEF 1α*, *Translation elongation factor-1α*.

[d] ITS, Internal transcribed spacer.

Fluorometer (Thermo Fisher Scientific). All the PCR reactions were repeated twice to cross-check the sequence data.

Sequencing and sequence analysis

The amplified PCR products of each locus (*actin, ß-tubulin, calmodulin* and *TEF-1-α*) were purified using the HiPura® PCR Product Purification Kit following the manufacturer's instructions (HiMedia™ Laboratories Pvt Ltd, Mumbai, India). The amplified PCR products of 38 isolates were sequenced at a commercial facility (AgriGenome Labs Pvt Ltd, Kochi, India). The sequences of each gene and isolates were aligned in a BioEdit software (Version 7.2.5) (Hall, 1999), and the obtained consensus sequences were deposited in the NCBI GenBank (Table 1). The consensus nucleotide sequences were employed to ascertain the level of genetic variation in the *M. oryzae* population. For all 38 isolates, the ITS fragment sequenced in our previous study (Amoghavarsha, Pramesh, Naik, et al., 2021) was also used in the present study.

Phylogenetic analysis

The phylogenetic relationship between the isolates (Table 1) was studied using sequnce sets of five loci. The consensus sequence was generated by assembling the raw sequences using BioEdit Sequence Alignment Editor (Version 7.2.5) (Hall, 1999). The sequences obtained from reverse primer were converted into reverse complement and aligned with forward sequences to obtain a consensus sequence. The partition homogeneity test (PHT) was performed between the *actin, ß -tubulin, calmodulin, TEF-1-α* and ITS gene fragment sequences with PAUP v. 4.0a169 (Swofford, 2003). The evolutionary history was inferred using the Maximum Likelihood method and Tamura-Nei model (Tamura & Nei, 1993) in the MEGA X software package (Version 10.1.7) (Kumar et al., 2018). The *Actin, ß -tubulin, calmodulin, TEF-1-α* and ITS loci were used in the same order to form concatenated sequences to assess evolutionary history.

Population structure analysis

The population structure was analysed using the SNP data using the STRUCTURE v. 2.3.4 software (Earl & VonHoldt, 2012; Pritchard et al., 2000). The number of sub-groups (ΔK) was determined using the ad-hoc statistical method, at different K values from $K = 1$ to 10, with five independent iterations per K and 200,000 burn-in lengths and 200,000 MCMC. The programme STRUCTURE HARVESTER was used to determine the peak value of ΔK according to the method described by Evanno et al. (2005).

Nucleotide polymorphism and identification of haplotypes

The five gene sequences were subjected to haplotype analysis using DNA Sequence polymorphism software (DnaSP) version 6.12.03 X 64 (Rozas et al., 2017). Haplogroups were identified based on the difference in their allelic frequencies. The tight span walker network was established using PopART (Leigh & Bryant, 2015) (http://popart.otago.ac.nz). The nucleotide sequences of the five loci of 38 isolates were subjected to nucleotide polymorphism analysis using the DnaSP program. The total number of variable sites (S), nucleotide diversity (Pi), the average number of pairwise nucleotide differences (k), and the total number of mutations (Eta) were estimated. Sequences were further subjected to the neutrality test (Fu's Fs statistic and Tajima's Test) (Jagadeesh et al., 2018).

Identification of SNP/InDel from multi-loci

The nucleotide sequences of four loci such as *actin, ß-tubulin, calmodulin* and *TEF-1-α* fragments were used for a BLAST search against the reference genome of *M. oryzae* strain 70–15 using NCBI nucleotide database (https://blast.ncbi.nlm.nih.gov) and downloaded the sequence of multi-loci of strain 70–15. The sequences of each gene were used for sequence alignment between the isolate and reference strain, 70–15 using ClustalW multiple alignment method of BioEdit software (Version 7.2.5) (Hall, 1999), and manually discovered the SNP.

Mating-type loci distribution

The mating-type of each isolate was determined using the mating-type-specific primers, *MAT1-1* and *MAT1-2*. 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 $\mu mol\,l^{-1}$ for each primer (each component at 1 µl, except buffer at 5 µl) and distributed to PCR tubes. Later, 50 $ng\,\mu l^{-1}$ of template DNA from the respective isolates was added, and MilliQ water was used to make the final volume 20 µl. The PCR was carried out in an ABS-Veriti™ 96-well thermocycler (Applied Biosystems), and the PCR program for the amplification consisted of initial denaturation at 94°C for 5 min,

followed by 30 cycles of denaturation for 30 s at 94°C, primer annealing at 55°C for 30 s; extension at 72°C for 60 s, and with a final extension at 72°C for 5 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 *MAT1* loci confirms the presence/absence of the particular loci in the isolate.

RESULTS

Multilocus sequence analysis of *Magnaporthe* isolates

The 38 leaf blast isolates were obtained from four rice ecosystems of Karnataka state following a single spore isolation technique and further used for genetic diversity analysis. The *actin*, *β-tubulin*, *calmodulin* and *TEF-1-α* genes of *M. oryzae* isolates were successfully amplified with ~250 bp, ~ 550 bp, ~500 bp, and ~900 bp long products, respectively. The BLASTn queries involving the five genes confirmed the isolates under study were identical to *M. oryzae* in the GenBank. Sequences of the *actin*, *β-tubulin*, *calmodulin* and *TEF-1-α* genes of *M. oryzae* isolates were deposited in the GenBank (Table 1). In addition to the sequence data of 38 isolates under study, four more species of *Magnaporthe*, namely, *M. grisea*, *M. salvinii*, *M. poae* and *M. rhizophila*, were used to find the evolutionary relationship between the isolates under study and other species of the *Magnaporthe*. For all 38 isolates, the ITS fragment sequenced in our previous study (Amoghavarsha, Pramesh, Naik, et al., 2021) was used for all analyses.

The partition homogeneity test of the combined data set conducted in PAUP resulted in a *p*-value of 0.038, indicating no significant incongruence between the data sets of five gene sequences. The final sequence alignment from the combined datasets consisting of 42 taxa (38 from this study and four *Magnaporthe* species obtained from GenBank) had 2709 positions in the final dataset, of which 1392 were parsimony informative, 356 were conserved, 2353 were variable and 961 were singleton sites. The data set had 1663 zero-fold degenerate sites, 74 two-fold degenerate sites and 207 four-fold degenerate sites. However, within the 38 *M. oryzae* isolates, there were 2429 constant sites, 280 variable (polymorphic) sites, 263 singleton sites and 17 were parsimony-informative characters.

Using the combined sequence data of five genes, a single most parsimonious tree (MPT) was generated (Figure 2). The tree resolved into two clades with high bootstrap values. The first clade consisted of *M. oryzae* and *M. grisea*, into two clusters. The isolates under study were clustered together into Cluster-IA except for one isolate (MoK19-06), forming the sister group from the rest of the isolates (Cluster-IB) (Figure 2). The second clade consisted of the remaining *Magnaporthe* species viz., *M. salvinii*, *M. poae* and *M. rhizophila*, in which the *M. salvinii* was clustered equidistantly from the *M. poae* and *M. rhizophila*.

Population structure analysis

Using a model-based program structure, the population structure was analysed using 123 SNP data to understand the genetic relationship among 39 isolates (including 70–15 isolate as a reference pathogen). The most probable number of subpopulations was observed at $K = 2$ and $K = 4$, indicating that the optimum number of subpopulations would be either two or four (Figure 3). Based on $K = 2$ and ancestry threshold of >70%, blast strains were classified into two subgroups (SG1 and SG2) (Table 2). The first structure group (SG1) consisted of 2 isolates such as MoK19-05 and MoK19-06. The second main cluster (SG2) consisted of 37 isolates (including 70–15 reference isolate). On the other hand, based on $K = 4$ and ancestry threshold of >70%, blast strains were classified into four subgroups (SG1, SG2, SG3 and SG4) (Table 2) along with one admixture (AD). The largest group was in SG3 with 29 isolates, followed by SG2 with five isolates, SG1 with three isolates and SG4 with one isolate. In SG2, 70–15 reference isolate along with MoK19-03, MoK19-28, MoK19-36 and MoK19-45 were included.

Haplotypes diversity

The 38 isolates with 2709 sites were grouped into 13 haplotypes with a haplotype diversity of 0.7127 (Table 3; Figure 4). Majority of the isolates ($n = 20$) were under the Hap_2 group followed by Hap_9 ($n = 5$) and Hap_1 and Hap_12 ($n = 2$), respectively. The remaining haplogroups consisted of one isolate each. The Hap_2 included most ($n = 17$) isolates from the irrigated ecosystem. The three haplogroups viz., 1, 3 and 4 comprised isolates from a rainfed hilly ecosystem, whereas six haplogroups viz., 5, 6, 7, 8, 10, 11 and 13 included isolates from an irrigated ecosystem. Three haplogroups such as 2, 9 and 12 consisted of isolates from irrigated and rainfed ecosystems. The variance of haplotype diversity was 0.00592, with a standard deviation of 0.077. The neutrality test was employed using Fu's Fs statistics and Tajima's *D* test and obtained the value of 5.598 and −2.86883, respectively, at $p < 0.001$. The total number of variable sites (S) was 280, with nucleotide diversity (Pi) of 0.00604 and an average number of pairwise nucleotide differences (k) of 16.36273. The

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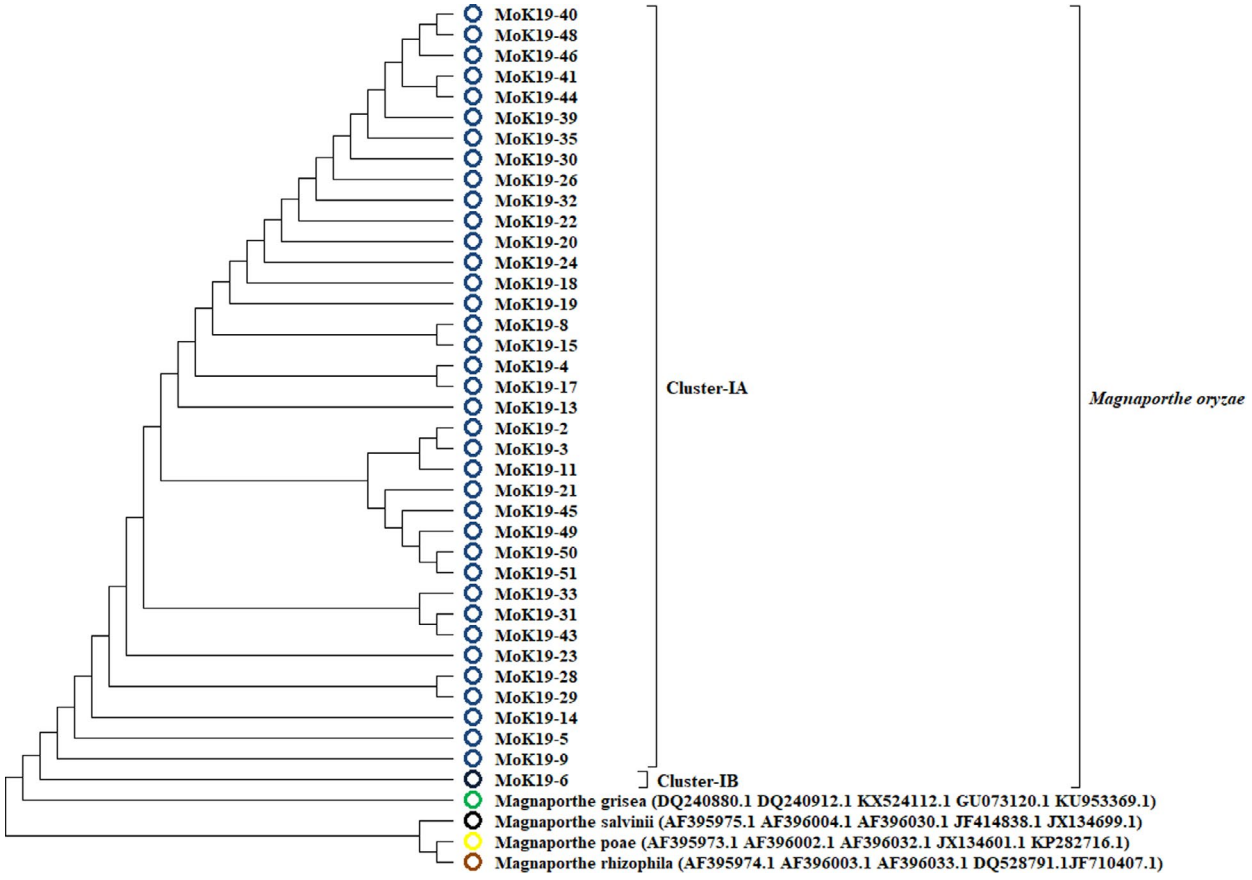

FIGURE 2 Phylogenetic tree showing the evolutionary relationship among the *Magnaporthe oryzae* isolates of Karnataka. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. Initial tree(s) for the heuristic search were obtained by Neighbour-Join and BioNJ algorithms. Evolutionary analyses were conducted in MEGA X

total number of mutations (Eta) was 291, with Theta (per sequence) from Eta and Theta (per site) from Eta being 69.25956 and 0.02557, respectively. The average pairwise differences (k) were 16.363, with observed and expected pairwise differences being 2.7223 and 0.4555 (Figure 5).

Identification of SNP/InDel from multi-loci

The SNPs were manually discovered for four genes (*actin*, *ß-tubulin, calmodulin* and *TEF-1-α*) using BioEdit software (Table 4; Figure 6). There were 18 in the *actin* gene, with an SNP frequency of 5.94%. In the case of the *ß–tubulin* gene, total SNP and InDel were 57 and 2, respectively, with a frequency of 11.56%. The first InDel was at 72nd base (−/CAGTGTACCAATGCAAG), and another InDel was at 301st base (−/G). The total SNP and InDel of the *calmodulin* sequence were 16 and 3, respectively, with 3.72%. SNP frequency. The first InDel was at 38th base (−/ACTT), the second was at 39th base (−/ACTT), and the third was at 43rd base (−/CTCT). In the *TEF-1-α* gene, the total SNP was 3 with an SNP frequency of 0.38%.

Distribution of mating type in blast population

A total of 38 monoconidial *M. oryzae* isolates from infected leaf blast samples were analysed using a PCR-based assay for mating-type distribution. The isolates produced the band of size 800 bp for *MAT1-1* (male fertile) and 950 bp for *MAT1-2* (female fertile) on 1% agarose gel (Figure S2). Among the 38 isolates, amplification for *MAT1* loci was observed in 33 isolates, whereas it was inconclusive in five isolates. Among the 33 isolates, 18 isolates were *MAT1-2* (54.5%), while 15 (45.5%) were *MAT1-1* (Table 1). Within the different rice ecosystems, the rainfed hilly ecosystem consisted of 63.64% of *MAT1*-2 and 36.36% of *MAT1-1* isolates in the population (Figure 7). In the irrigated ecosystem, *MAT1-1* (11) and *MAT1-2* (11) were equally distributed. Within irrigated ecosystems, the isolates from the Kaveri ecosystem possessed 50% of *MAT1-1* and *MAT1-2* mating types, respectively. In Thungabhadra (TBP) and Bhadra ecosystems, 60.00% and 40.00% of isolates had *MAT1-1*, whereas 40.00% and 60.00% isolates had *MAT1-2* mating types, respectively.

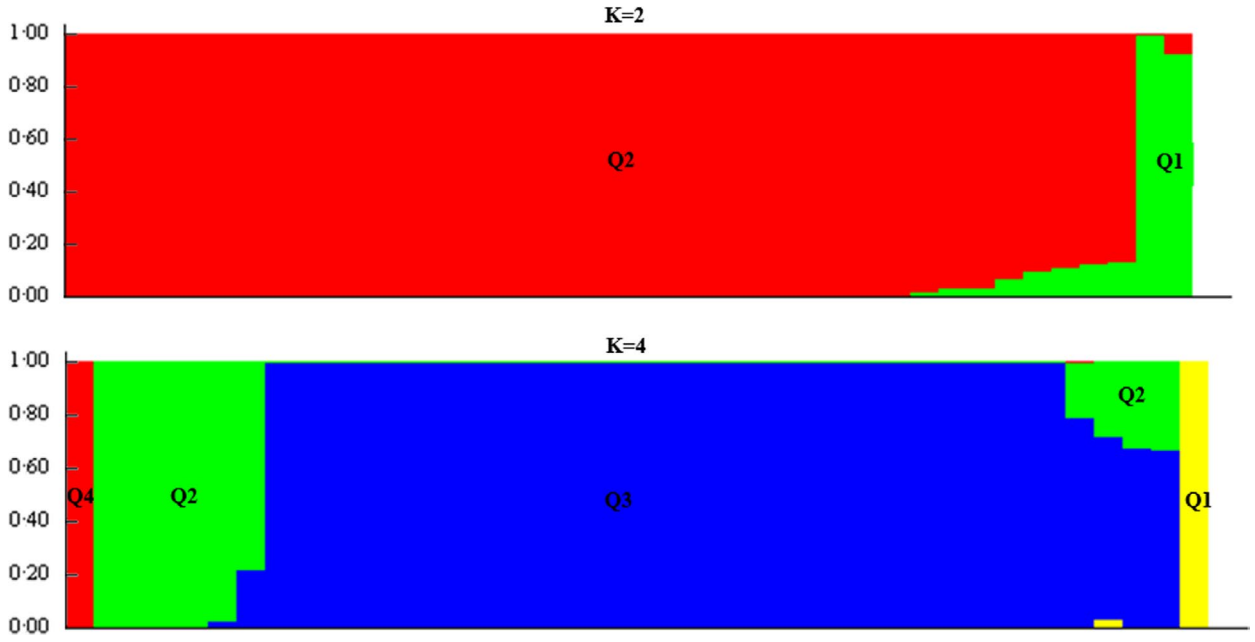

FIGURE 3 Model-based population clustering of 39 isolates at estimated membership fraction for $K = 2$ and $K = 4$, respectively. The population was classified into two clusters ($Q1$ and $Q2$) under $K = 2$ and into four clusters ($Q1$, $Q2$, $Q3$ and $Q4$) under $K = 4$. The numbers on the *y*-axis show the subgroups, and the *x*-axis shows different accessions. The distribution of accessions into the different populations is indicated by colour coding (Cluster 1, $Q1$ is green, Cluster 2, $Q2$ is red in $K = 2$, whereas, $Q1$ is yellow, $Q2$ is green, $Q3$ is blue and $Q4$ is red in $K = 4$)

DISCUSSION

One critical impediment to increasing global rice production is rice blast disease caused by *M. oryzae* (Sakulkoo et al., 2018). Several efforts have been made to breed resistant cultivars against the blast disease of rice in India (Sharma et al., 2021). However, resistant varieties are effective for a few years as the pathogen is fast-evolving and manages to break the resistance in the cultivar (Sharma et al., 2021). The heterothallic reproduction and the modification of genes by *M. oryzae* have resulted in the emergence of new virulent races (Dai et al., 2010).

The knowledge of the population genetics of the rice blast pathogen is useful in formulating the breeding strategy to slow the evolution of the pathogen (Lopez & Cumagun, 2019). The population analysis of the isolates may help to know the potential of a pathogen development into new races. In the present study, we have employed the multi-loci technique for evaluating the genetic variability and phylogenetic relations among the 38 *M. oryzae* isolates collected from the diverse rice ecosystems of a Karnataka state of India. The multi-loci technique directly measures the variations in DNA sequence in a gene set and characterizes the isolates. Mutations within the housekeeping genes are anticipated to be neutral and, consequently, likely to disclose the evolutionary relationship of the microbial strains correctly (Maiden et al., 1998).

In the present study, 38 isolates were grouped into two clusters based on concatenated sequences of five genes. Most of the isolates in our study were grouped in a single cluster, suggesting the homogenous population, which may be due to the movement of the pathogen with seed material from one region to another as suggested previously (Long et al., 2001). Among the 38 isolates, MoK19-06, obtained from a rainfed hilly ecosystem, diverged and formed a separate sub-cluster. The phylogenetic distinctiveness of this isolate reveals a separate/distinct ancestral origin and the possibility of sexual recombination. The critical reason for the divergence of MoK19-06 into a separate cluster might be the cultivation of local blast-resistant varieties by the farmers of a rainfed hilly ecosystem. This isolate has also been reported previously as a moderately virulent type (Amoghavarsha, Pramesh, Naik, et al., 2021). There is a strong relationship between the structure of the host population and the pathogen population, which highly influence each other, and the genotype of the host exerts intense selection pressure on the rice blast pathogen (Chen et al., 1995).

The presence of different resistance genes in different hosts exerts selection pressure on the rice blast population, which further results in the differential compatibility between rice blast pathogen and the cultivar (Prabhu et al., 2002). Khan et al. (2016) reported the influence of varietal differentiation between two ecosystems (rainfed and irrigated) in the differentiation of rice blast isolates into two distinct populations. In our study, most of the

TABLE 2 Population structure group of *Magnaporthe oryzae* isolates based on inferred ancestry values ($K = 2$ and $K = 4$)

		Clusters (Q) based on $K = 2$			Clusters (Q) based on $K = 4$				
Sl no	**Strains**	***Q*1**	***Q*2**	**Subgroups**	***Q*1**	***Q*2**	***Q*3**	***Q*4**	**Subgroups**
1	70–15 (Reference isolate)	0.034	0.966	SG2	0	0.996	0.003	0	SG2
2	MoK19-02	0.001	0.999	SG2	0	0.002	0.997	0	SG3
3	MoK19-03	0.072	0.928	SG2	0	0.974	0.026	0	SG2
4	MoK19-04	0.001	0.999	SG2	0	0.002	0.998	0	SG3
5	MoK19-05	0.923	0.077	SG1	0	0	0	0.999	SG4
6	MoK19-06	0.999	0.001	SG1	0.999	0	0	0	SG1
7	MoK19-08	0.003	0.997	SG2	0	0.002	0.998	0	SG3
8	MoK19-09	0.13	0.87	SG2	0	0.279	0.69	0.031	AD
9	MoK19-11	0.003	0.997	SG2	0	0.002	0.997	0	SG3
10	MoK19-13	0.003	0.997	SG2	0	0.003	0.996	0	SG3
11	MoK19-14	0.036	0.964	SG2	0.003	0.205	0.789	0.003	SG3
12	MoK19-15	0.001	0.999	SG2	0	0.002	0.998	0	SG3
13	MoK19-17	0.001	0.999	SG2	0	0.002	0.997	0	SG3
14	MoK19-18	0.001	0.999	SG2	0	0.002	0.997	0	SG3
15	MoK19-19	0.001	0.999	SG2	0	0.002	0.998	0	SG3
16	MoK19-20	0.001	0.999	SG2	0	0.002	0.998	0	SG3
17	MoK19-21	0.001	0.999	SG2	0	0.001	0.998	0	SG3
18	MoK19-22	0.001	0.999	SG2	0	0.001	0.998	0	SG3
19	MoK19-23	0.001	0.999	SG2	0	0.002	0.997	0	SG3
20	MoK19-24	0.001	0.999	SG2	0	0.003	0.997	0	SG3
21	MoK19-26	0.001	0.999	SG2	0	0.002	0.997	0	SG3
22	MoK19-28	0.094	0.906	SG2	0	0.782	0.217	0	SG2
23	MoK19-29	0.02	0.98	SG2	0	0.004	0.996	0	SG3
24	MoK19-30	0.003	0.997	SG2	0	0.002	0.998	0	SG3
25	MoK19-31	0.001	0.999	SG2	0	0.001	0.998	0	SG3
26	MoK19-32	0.001	0.999	SG2	0	0.003	0.997	0	SG3
27	MoK19-33	0.001	0.999	SG2	0	0.001	0.998	0	SG3
28	MoK19-35	0.001	0.999	SG2	0	0.003	0.997	0	SG3
29	MoK19-36	0.001	0.999	SG2	0	0.993	0.006	0	SG2
30	MoK19-39	0.003	0.997	SG2	0	0.003	0.996	0	SG3
31	MoK19-40	0.001	0.999	SG2	0	0.003	0.997	0	SG3
32	MoK19-41	0.001	0.999	SG2	0	0.002	0.997	0	SG3
33	MoK19-43	0.001	0.999	SG2	0	0.001	0.998	0	SG3
34	MoK19-44	0.001	0.999	SG2	0	0.003	0.997	0	SG3
35	MoK19-45	0.123	0.877	SG2	0	0.998	0.002	0	SG2
36	MoK19-46	0.001	0.999	SG2	0	0.001	0.998	0	SG3
37	MoK19-48	0.001	0.999	SG2	0	0.322	0.677	0	SG1
38	MoK19-49	0.108	0.892	SG2	0	0.331	0.669	0	SG1
39	MoK19-50	0.001	0.999	SG2	0	0.001	0.998	0	SG3

M. oryzae isolates exhibited nearly identical clustering profiles indicating the clonal nature of the pathogen. The combined sequence data based on multi-loci phylogenetic analysis grouped the isolates based on their host origin. All rice isolates were clustered in the *Oryza* group.

TABLE 3 Haplotype groups (Hap) of *Magnaporthe oryzae* isolates used in this study

Hap group	No. isolates	Isolates	Ecosystem
Hap_1	2	MoK19-2 and MoK19-3	Rainfed
Hap_2	20	MoK19-04, MoK19-08, MoK19-15. MoK19-17, MoK19-18, MoK19-19, MoK19-20, MoK19-22, MoK19-23, MoK19-24, MoK19-26, MoK19-30, MoK19-32, MoK19-35, MoK19-39, MoK19-40, MoK19-41, MoK19-44, MoK19-46 and MoK19-48	Rainfed and Irrigated (Both)
Hap_3	1	MoK19-05	Rainfed
Hap_4	1	MoK19-06	Rainfed
Hap_5	1	MoK19-09	Irrigated
Hap_6	1	MoK19-11	Irrigated
Hap_7	1	MoK19-13	Irrigated
Hap_8	1	MoK19-14	Irrigated
Hap_9	5	MoK19-21, MoK19-45, MoK19-49, MoK19-50 and MoK19-51	Both
Hap_10	1	MoK19-28	Irrigated
Hap_11	1	MoK19-29	Irrigated
Hap_12	2	MoK19-31 and MoK19-43	Both
Hap_13	1	MoK19-33	Irrigated

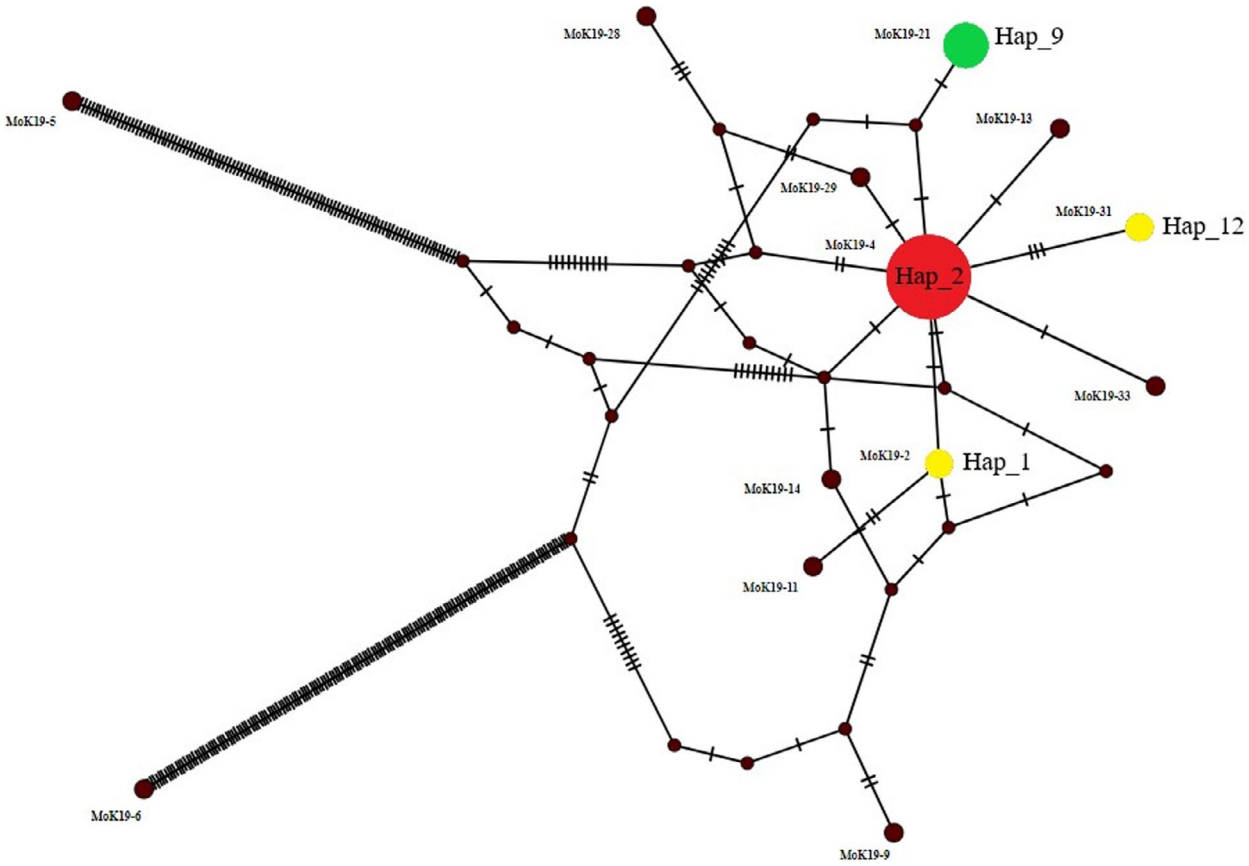

FIGURE 4 Haplotype network tree showing the distribution of isolates in different haplotypes where each node is represented by the isolate of a particular haplotype group

Population structure analysis to understand the genetic relationship among 39 isolates (including reference isolate) showed two probable subpopulations ($K = 2$ and $K = 4$), indicating the optimum number of subpopulations would be either two or four. Based on $K = 2$ and ancestry threshold of >70%, blast strains were classified into two

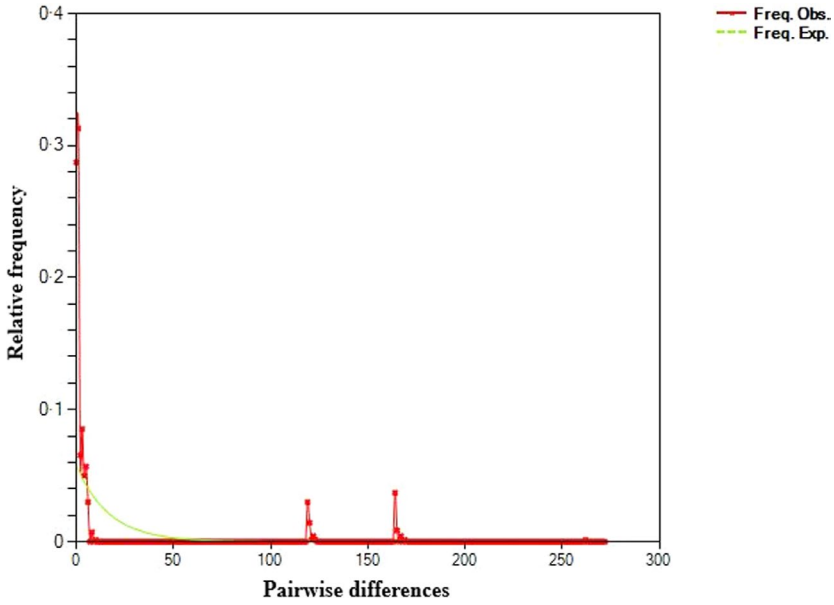

FIGURE 5 Graph showing the average number of nucleotides pairwise differences. The graph was obtained using DnaSP6 software version 6.12.03 X 64

TABLE 4 Details of SNP and InDel discovered in the four loci

Sl no.	Loci	SNP	InDel	Amplicon size (bp)	SNP frequency (%)
1	*Actin*	18	—	303	5.94%
2	*β-tubulin*	57	2	510	11.56%
3	*calmodulin*	16	3	510	3.72%
4	*TEF-1-α*	3	—	777	0.38%

subgroups (SG1 and SG2), with the first structure group (SG1) consisting of only two isolates. On the other hand, based on $K = 4$ and ancestry threshold of >70%, the whole 39 strains formed four subgroups (SG1, SG2, SG3 and SG4) along with one admixture (AD). However, these population structure studies could not separate the mating-type into different subgroups, suggesting the complexity of these populations and the requirement of additional genetic markers or whole-genome sequence of the blast pathogens. Similarly, population structure analysis could not separate the different phylogenetic groups identified for 39 isolates into different subgroups. For instance, MoK19-06 grouped separately in the phylogenetic study but formed a subgroup SG1 with MoK19-05 in population structure analysis when $K = 2$. Interestingly, these two isolates (MoK19-05 and MoK19-06), grouped as SG1 when $K = 2$, were separated into two groups when $K = 4$, MoK19-05 in SG1 and MoK19-06 in SG4. MoK19-05 with *MAT1-1* mating type was isolated from the Jyoti variety of Chikkamagaluru district (hilly ecosystem), whereas MoK19-06 was isolated from IET-7191 variety grown in the same ecosystem and were sampled within the distance of 30 km.

The haplotype analysis and neutrality test furnished the information about several polymorphic/variable sites in the DNA sequences and possible neutral mutation within the genetic makeup of the isolates (Jagadeesh et al., 2018). The haplotype analysis based on the ITS nucleotide sequences was employed for the *M. oryzae* isolates of Karnataka and found significant genetic diversity (Jagadeesh et al., 2018); however, that analysis was based on only ITS sequences of a few isolates from a limited geographical region. In the present investigation, we used nucleotide sequences of five genes and classified the 38 isolates into 13 haplogroups with a haplotype diversity of 0.7127. The nucleotide polymorphism analysis disclosed variations in the total number of variable sites (S), nucleotide diversity (Pi), the average number of pairwise nucleotide differences (k), and a total number of mutations (Eta). In the present investigation, Tajima's D test and Fu's Fs statistics supported higher rare alleles in the population. This observation might be due to the cultivation of local rice varieties with varied genetic backgrounds that might have forced the pathogen to undergo selection in different ecosystems. Interestingly, we found that the *M. oryzae* population studied is homogenous based on phylogenetic analysis, but it is quite diverse when analysed for haplotype diversity.

Based on sequence alignment of five loci, a total of 123 SNPs were discovered, of which the *β-tubulin* gene showed a maximum number of SNP with a frequency of 11.56%. The five InDel in *β-tubulin* and *calmodulin* genes/

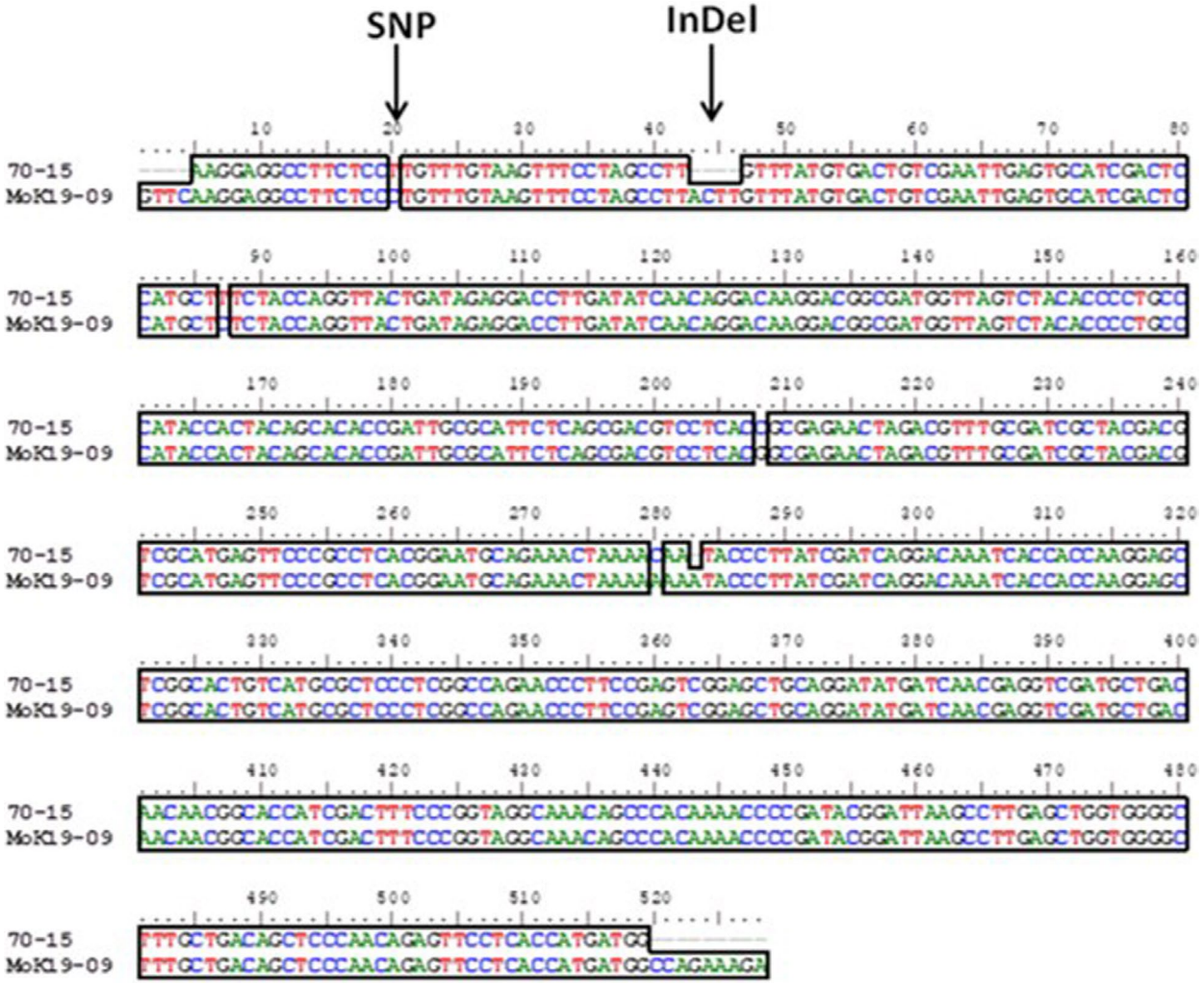

FIGURE 6 Single nucleotide polymorphisms (SNP) and insertion/deletion polymorphisms (InDel) in *Magnaporte oryzae* sequences

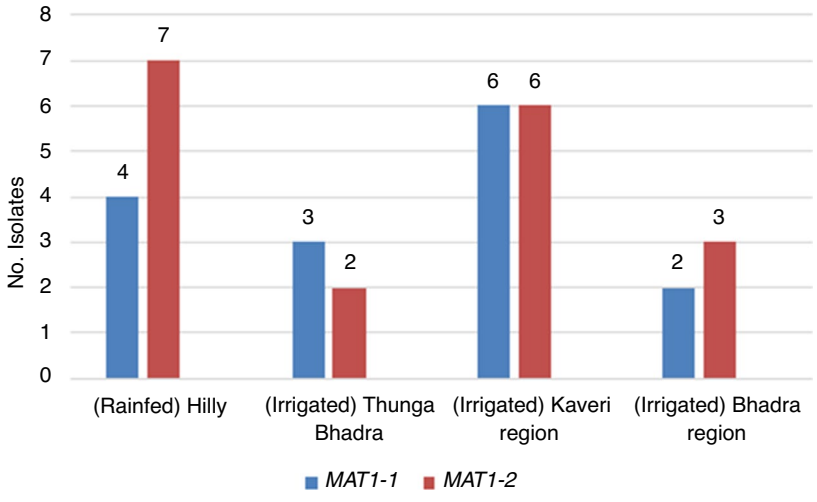

FIGURE 7 Distribution of mating types in different rice ecosystems of Karnataka. *MAT1-2* was predominant in the rainfed hilly and irrigated Bhadra ecosystem, whereas *MAT1-1* was predominant in irrigated Thungabhadra ecosystem. The Kaveri ecosystem was found to have both mating types in equal proportion

regions would be useful to develop isolate-specific PCR-based molecular markers for the differentiation of isolates.

The genetic makeup of the rice blast population is affected by selection, genetic drift, mutation, gene or genotype flow and mating systems (McDonald & Linde, 2002). However, the recombination is believed to play no role in the population structure of *M. oryzae* as sexual reproduction is absent in fields, except few regions

of Asia (Saleh et al., 2014). Nevertheless, the mating types were responsible for the major genetic groups of *M. oryzae* worldwide (Tharreau et al., 2009). In the present investigation, the mating-type present in different *M. oryzae* isolates of a Southern state of India was analysed. Out of 38 isolates, only 33 were PCR positive for either *MAT1-1* or *MAT1-2* alleles, whereas PCR result for five isolates was inconclusive (Data not shown). Among the MAT1-positive isolates, 54.5% had *MAT1-2* allele, whereas 45.5% possessed *MAT1-1* allele. The *MAT1-1* mating type was predominant in the TBP ecosystem, whereas; the *MAT1*-2 mating type was predominant in the rainfed hilly and Bhadra ecosystems. The Kaveri ecosystem had an equal proportion of isolates with *MAT1-1* and *MAT1-2*. The presence of *MAT1-2* allele in the population at a higher rate (>90%) was also reported by Tansian and Parinthawong (2018) in Thai isolates. Samanta et al. (2014) reported the occurrence of *MAT1-2* population in the uplands and irrigated ecosystems of Central India, and they also reported the occurrence of both mating types in the irrigated ecosystem. A higher *MAT1-2* mating type distribution was also reported from North-East and Eastern India (Imam et al., 2015).

For the existence of sexual reproduction at the field level, the presence of both mating types *MAT1-1* and *MAT1-2*, approximately in equal proportion, is very much essential, as the highly skewed mating-type ratio hindered the mating (Zeng et al., 2009). In the present study, the isolates exhibited the presence of *MAT1-1* and *MAT1-2* genes at 45:55 proportions, thereby indicating the possibility of sexual reproduction in the paddy fields.

The unravelling of the population structure and mating diversity of rapidly evolving pathogens like *M. oryzae* is essential. The present study efficiently deduces the phylogenetic relationship of the *M. oryzae* isolates using the multilocus technique and also found the presence of both mating types in the *M. oryzae* population of Karnataka. The study identified the nearly homogenous population of *M. oryzae* in different irrigated rice ecosystems, except the rainfed hilly ecosystem. The distribution of the mating types in the *M. oryzae* is nearly proportional and poses a threat to sexual reproduction. Hence, there is a need to plan the host plant resistance breeding programs to prevent the production of pathogen variables that could overcome the resistance of the cultivars.

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CONFLICT OF INTEREST

No conflict of interest was declared.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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