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Original Article

Molecular diversity of internal transcribed spacer among the monoconidial isolates of *Magnaporthe oryzae* isolated from rice in Southern Karnataka, IndiaD. Jagadeesh<sup>a</sup>, M.K. Prasanna Kumar<sup>b</sup>, R. Chandrakanth<sup>a</sup>, N.S. Devaki<sup>a,\*</sup><sup>a</sup> Department of Molecular Biology, Yuvaraja's College, University of Mysore, Mysuru 570005, India<sup>b</sup> Department of Plant Pathology, GKVK, University of Agricultural Science, Bangalore 560065, India

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## ABSTRACT

Blast disease of rice plant is caused by *Magnaporthe oryzae* (anamorph *Pyricularia oryzae*). This disease is recognized to be one of the most serious diseases of rice crop around the world. A total of 72 monoconidial isolates of *M. oryzae* obtained from blast disease samples collected around Southern Karnataka were characterized using internal transcribed spacers of the ribosomal DNA sequences. These were analyzed by comparing with already deposited sequences in GenBank database. It helped in diagnosing the invasive pathogen in all locations. Variability of rDNA sequences was found to be highly polymorphic with 0.068962 nucleotide diversity showing 6 distinct clades. 33 haplotype groups were identified with haplotype diversity of 0.8881 and Tajima's neutrality test with a D value of  $-1.96827$  with  $P < 0.05$  showing the presence of variations among the sequences of pathogen isolates. The Tajima's D value of less than one indicates the presence of a high number of rare alleles. Our study indicates that the pathogen might have undergone recent selection pressure because of the exposure to a large number of cultivars resulting in the evolution of rare alleles. This shows the importance of characterizing internal transcribed spacer (ITS) to know pathogen diversity and its fitness which has potential to contribute to the field of breeding for blast disease resistance.

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## 1. Introduction

Blast disease of rice plant is caused by *Magnaporthe oryzae* B.C. Couch, (Anamorph: *Pyricularia oryzae* Cavara) [1]. The disease can infect paddy at all growth stages and all aerial parts of the plant. Rice blast is known to occur in epidemic proportions around different parts of the world with yield loss up to 50% [2]. Infection occurs on leaves during vegetative phase and on panicles and neck during reproductive phase of the crop. Neck blast causes higher yield loss as compared to leaf blast in the tropical lowlands of Asia [3]. This pathogen is known for its high genetic plasticity. Presence of an abundant number of transposable elements has contributed to a continued shift in its genetic makeup resulting in the breakdown of resistance of rice cultivars [4,5]. The potential of this fungus to quickly overcome resistance within a short period after the release

of a new cultivar has made breeding for resistance cultivar a constant challenge for higher productivity [2].

Molecular identification of *M. oryzae* using internal transcribed spacer (ITS) region of nuclear ribosomal DNA genes showed 99% nucleotide identity with the already deposited sequence of *M. oryzae* sequence in GenBank isolated from infected rice leaf samples of Malaysia [6]. There are scanty reports about DNA sequence divergence among the ITS regions in both plants and animals. One of the available reports in fungi is on the investigation of ITS regions using *Rhizopus* spp to clarify the taxonomic relationship among different strains [7]. Chloroplast DNA variation in Fig. cultivars (*Ficus carica* L.) in Tunisia was analyzed by using trnL-trnF intergenic spacer [8]. Results showed the confirmation of intraspecific variations in the plastid DNA of cultivars of the Tunisian *F. carica* with highest haplotype diversity shared between the two cultivar sets. Based on the sequence variation of the internal transcribed spacer region of ribosomal DNA in *Cerastoderma* Species (Bivalvia: Cardiidae) the divergence among *C. glaucum* from the Baltic and Mediterranean Seas and *C. edule* was assessed [9]. This type of study is significant due to the role of ITS in ribosome maturation.

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These regions form specific secondary structures for recognition of cleavage sites and provide the binding sites for nucleolar protein [10]. The change in the nucleotide bases of ITS can alter the formation of secondary structures which directly affects the rRNA processing [11,12].

Hence knowing the diversity of ITS regions of the genome is significant in understanding the variability of the pathogen, which is important for resistant breeding of rice cultivar against *M. oryzae*. The present study was thus undertaken to know the diversity of ITS regions of rDNA in *M. oryzae* isolates collected from different rice fields in Southern Karnataka, India.

## 2. Materials and methods

### 2.1. Sample collection and isolation of the pathogen

A total of 72 Rice blast disease samples were collected from the rice fields from 11 different districts of Karnataka (Fig. 1, Table 1). The pathogen *M. oryzae* was isolated from blast infected plant parts of 32 cultivars. The plant parts such as leaves, neck, collar, node, stem, and panicle were used for isolation of the pathogen [13]. Diseased samples were cut into small pieces around the infected area including the edge of the lesion (1–2 cm) and then it was subjected

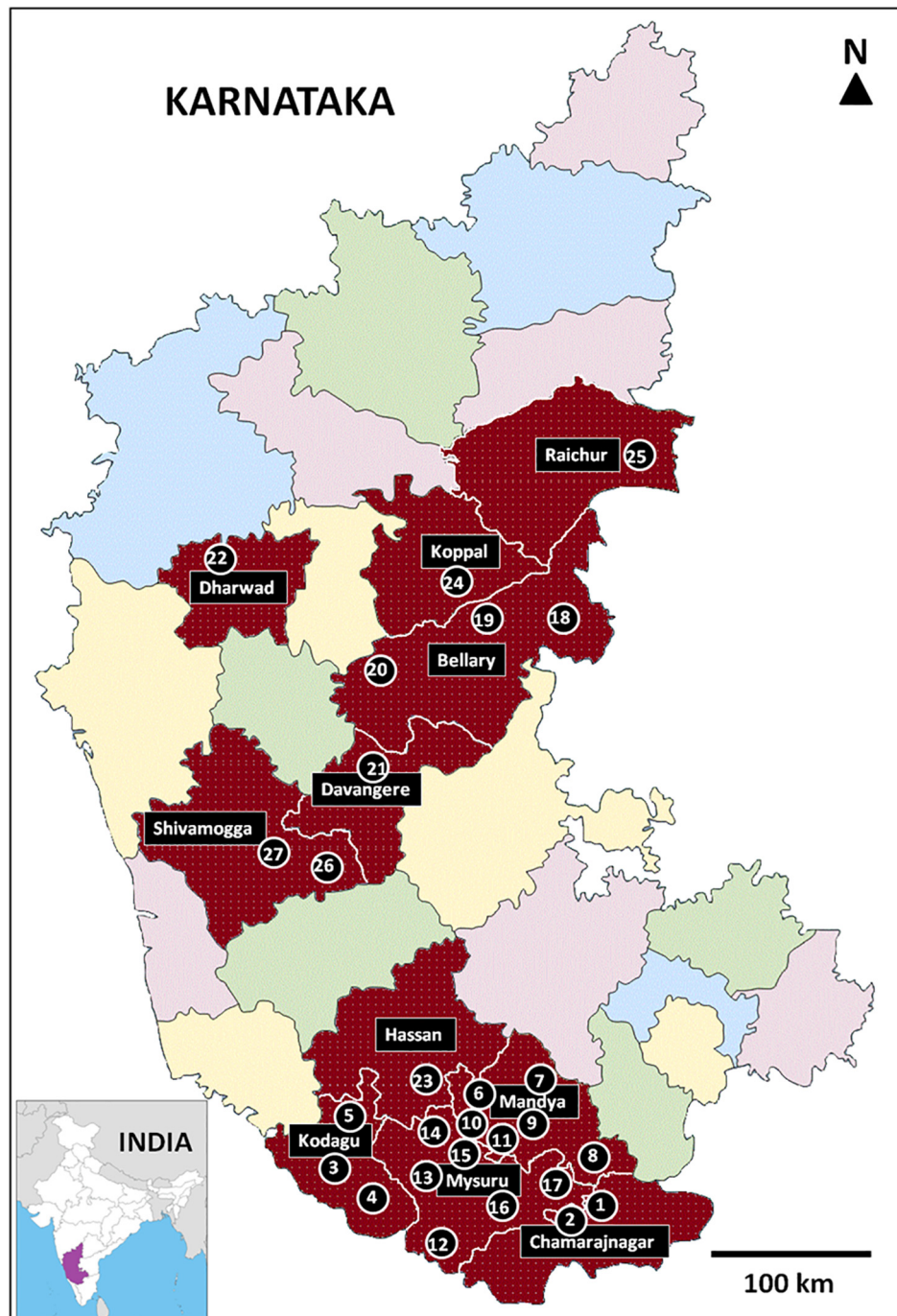


Fig. 1. Locations of rice blast disease samples collected from different Districts (dark red color) of Karnataka in India map (Inset shows the location of Karnataka in India). The number within the circle represents taluk number as given in Table 1.

to surface sterilization with 1% sodium hypochlorite. Further, these samples were kept inside moist chambers to enhance sporulation at 28 °C for 48 h.

After incubation, these infected plant pieces were examined under the stereo binocular microscope to confirm the typical elliptical or spindle-shaped *M. oryzae* spores. Single conidium was picked up into fresh oat meal agar plate with streptomycin sulfate (40 mg/L) and incubated at 28 °C for 14 Days. Isolate codes were given with first, second, third and fourth letters indicating the district, taluk, village, and place of collection respectively. Further, these four-letter codes ended in two digit numbers. The isolates are coded in alphabetical order.

## 2.2. Amplification of the ITS regions of rDNA

### 2.2.1. DNA extraction

DNA was extracted from the monoconidial cultures of *M. oryzae* isolates using DNA extraction method as described by Murray & Thompson with minor modifications [14]. The cultures were grown in Cornmeal broth for 10 to 14 days at 28 ± 1 °C in a shaker incubator at 50 rpm. Mycelial mat was filtered and ground to fine powder in liquid nitrogen and transferred to 2 ml Eppendorf tube with 1 ml of CTAB buffer. Further, these tubes were kept at 65 °C for 30 min with occasional stirring for every 10 min and centrifuged at 10,000 rpm for 10 min at room temperature and the supernatant was collected in another 2 ml Eppendorf tube. Equal volumes of supernatant and phenol: chloroform: isoamyl alcohol mixture (25:24:1) were taken in an Eppendorf tube and cen-

trifuged at 10,000 rpm for 10 min. The supernatant was again collected in another 2 ml Eppendorf tube and an equal volume of chloroform: isoamyl alcohol (24:1) was added, followed by centrifugation at 10,000 rpm for 10 min. The upper aqueous phase was pipetted out in a fresh 1.5 ml Eppendorf tube and mixed with 20 µl of sodium acetate (3M) and 1 ml of ice-cold isopropanol and kept at –20 °C for 30 min. After thawing of samples at room temperature the mixture was centrifuged again at 13,000 rpm for 15 min, the supernatant was decanted and DNA pellet was washed with 70% ethanol, dried at room temperature and dissolved in 50–100 µl TE (10 mM Tris-Cl, pH 8.0; 1 mM EDTA). Quality and quantity of DNA were checked with nanodrop as well as by running on 0.8% agarose gel.

### 2.2.2. PCR amplification of ITS and sequencing

The ITS regions of *M. oryzae* isolates were amplified using universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCC TCC GCTTATTGATATGC-3') [15] PCR reactions were performed in 20 µl mixture containing 50 ng of total DNA, 2 µl Taq DNA Polymerase (1 U/µl), 0.5 µl of both forward and reverse primer (1 µM) and 2 µl of dNTPs (10 mM). The reaction mixture was made up to 20 µl using Milli Q water. The reaction was carried out in Mastercycler Pro thermal cycler (Eppendorf, Hamburg, Germany). For amplification of the ITS regions of the rDNA, the following temperature profile was used: 5 min initial denaturation at 94 °C followed by 35 cycles of 94 °C for 45 sec, 58 °C for 1 min, 72 °C for 1 min, and a final extension step at 72 °C for 5 min. Amplification of ITS was confirmed by gel electrophoresis using

**Table 1**  
Rice blast disease samples collected from different districts and taluks of Southern Karnataka.

District	Taluk No.	Taluk Name	Isolate Code <sup>a</sup>	Accession No. <sup>b</sup>
Chamarajnanagar	1	Kollegal	CKDS01,CKHM02 CKHR03, CKHR04 CKKL05, CKML06 CKST07,CKST08 CKTR09	MF583092, MF583093 MF583094, MF583095 MF583096, MF583097 MF583098, MF583099 MF583100
	2	Yelandur	CYAG10, CYJM11 CYKT12, CYKS13 CYKK14, CYLR15	MF583101, MF583102 MF583103, MF583104 MF583105, MF583106
Kodagu	3	Madikeri	KMBT16, KMHK17 KMHD18	MF583126, MF583139 MF583154
	4	Virajpet	KVAM19, KVBL20 KVBG21, KVBL22 KVHG23, KVKM24 KVKR25, KVPN26	MF583137, MF583127 MF583128, MF583135 MF583134, MF583136 MF583138, MF5838575
Mandya	5	Somwarpet	KSAB27, KSHB28 KSKK29, KSSR30	MF583133, MF583140 MF583141, MF583142
	6	Krishnarajpete	MKKN31, MKMD32	MF583107, MF583108
	7	Maddur	MMMG33,MMNG34	MF583109, MF583110
	8	Malavalli	MMAD35,MMKO36	MF583111, MF583112
Mysore	9	Mandya	MMGV37, MMSV38 MMVF39, MMVF40	MF583113, MF583114 MF583115, MF583116
	10	Pandavapura	MPAK41,MPDY42 MPPD43	MF583117, MF583118 MF583119
	11	Srirangapatna	MSCH44, MSKM45	MF583120, MF583121
Bellary	12	Heggadadevana kote	MHHM46, MHSR47	MF583144, MF583146
	13	Hunsur	MHHN48	MF583147
	14	Krishnarajanagar	MKBD49, MKHB50,MKML51	MF583148, MF583149 MF583143
	15	Mysuru	MMKL52, MMNG53, MMSD54	MF583150, MF583151 MF583152
	16	Nanjangud	MNHJ55, MNHU56 MNHM57, MNRM58	MF583153, MF583156 MF583157, MF583158
	17	Trirumakudal Narasipur	MTHR59, MTSS60	MF583159, MF583160
Davangere	18	Bellary	BBEM61	MF583132
	19	Hospet	BHDS62	MF583161
	20	Hagaribommanahalli	BHBG63	MF583162
Dharwad	21	Davangere	DDNS64	MF583131
Hassan	22	Dharwad	DDMU65	MF583129
Koppal	23	Holenarasipura	HHCC66, HHHM67	MF583130, MF583145
Raichur	24	Gangavathi	KGGV68	MF583125
Shivamogga	25	Raichur	RRKD69	MF583124
Shivamogga	26	Bhadravati	SBGN70, SBBM71	MF583123, MF583155
	27	Shivamogga	SSHB72	MF583122

<sup>a</sup> The first, second, third and fourth letters of the isolate indicate the district, taluk, and place of sample collection respectively. These letters are followed by collection numbers.

<sup>b</sup> Accession numbers of 72 *Magnaporthe oryzae* isolates obtained from GenBank NCBI.

2% agarose. DNA ladder was obtained from APS LABS (MAGBand 100 bp DNA Ladder) Sequencing was done at Chromous Biotech Pvt. Ltd, Bangalore, India. The sequences obtained in this study were compared with those already available in the GenBank database using BLAST tool available in the National Center for Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov/BLAST/>) and these sequences were submitted to GenBank database using BankIt submission tool.

### 2.3. DNA sequence alignment and phylogenetic analysis

The ITS sequences of *M. oryzae* isolates were compared with the corresponding sequences of other *M. oryzae* isolates from GenBank database. For cluster analysis, CLUSTAL W alignment program of the MEGA 7 software was used. The generated pairwise similarity matrix was used to group isolates by the unweighted pair group method arithmetic average (UPGMA) [16]. The evolutionary

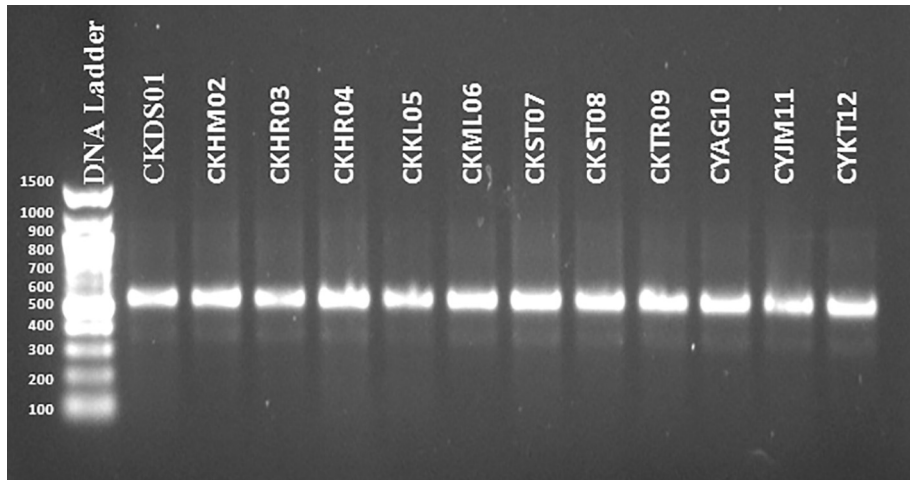


Fig. 2. Gel electrophoresis of the amplified rDNA internal transcribed spacers region of 12 *Magnaporthe oryzae* isolates using 2% of agarose. First lane from left side is 100 bp DNA ladder.

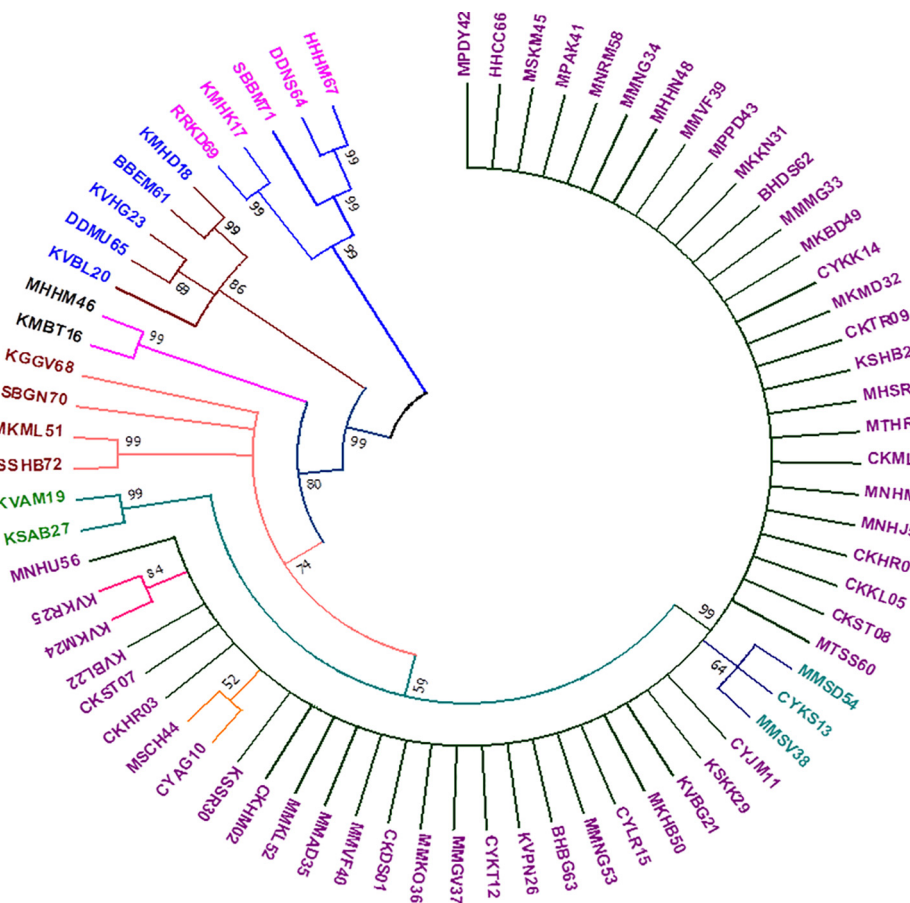


Fig. 3. A Circular Dendrogram of 72 *Magnaporthe oryzae* Internal Transcribed Spacer (ITS1/ITS4) genotypes based on UPGMA using MEGA 7.

history was inferred using the UPGMA method [17]. The bootstrap consensus tree inferred from 1000 replicates [18]. This was considered for representing the evolutionary history of the taxa analyzed [19]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The evolutionary distances were computed using the number of differences method [20] and are in the units of the number of base differences per sequence. The analysis involved 72 nucleotide sequences. All positions containing gaps and missing data were eliminated. A dendrogram was derived from the similarity matrix. A discrete Gamma distribution was used to model evolutionary rate differences among sites and also to depict nucleotide frequencies. Substitution pattern and rates were estimated as per Tamura K and Nei M model [18,19].

#### 2.4. Identification of haplotypes

Grouping haplotypes for testing genetic differentiation of populations was carried out using Chi-square test. This was based on allele frequencies in samples from different localities [21,22]. Indels Polymorphism and haplotype diversity were analyzed using DNA Sequence polymorphism software version 6.10.01 [23]. Sequences were further subjected to Tajima's Neutrality Test [24].

### 3. Results

#### 3.1. Molecular characterization of *M. Oryzae* isolates

During the present investigation, 72 monoconidial isolates of *M. oryzae* were established. The observed purity of DNA samples was  $1.8 \pm 0.3$ . Amplification of ITS1/ITS4 resulted in a single band of 550 bp in all the isolates (Fig. 2). These bands were recovered and sent to sequencing and output sequences were compared with the already deposited sequence of this fungus in NCBI GenBank database. This resulted in confirmation and matching with already submitted sequences in the database. Accession numbers obtained for all the 72 sequences of the respective isolates through BlankIt sequence submission tool [25] are given in the (Table 1).

#### 3.2. Phylogenetic analysis

All 72 sequences differed from one another and the extent of variation among the isolates is indicated in the dendrogram (Fig. 3). Dendrogram showed two major clusters sharing 99 Bootstrap values among one clade. It was further divided into two sub-clades with isolate RRKD69 and KMHK17 sharing maximum genetic similarities with the isolates DDNS64 and HHHM67. In another major clade, it was subdivided into one minor and one

**Table 2**

Haplotype distribution of 72 *Magnaporthe oryzae* isolates obtained from DNA Sequence Polymorphism software.

Haplotype (Hap)	Total number of isolates	Isolate code <sup>a</sup>
Hap_1	10	CKDS01, CYKT12, CYLR15, MMAD35, MMGV37, MMVF40, MMKL52, MMNG53, BHBG63, KVPN26
Hap_2	1	CKHM02
Hap_3	1	CKHR03
Hap_4	22	CKHR04, CKKL05, CKML06, CKTR09, CYKK14, MKKN31, MKMD32, MMMG33, MMNG34, MMVF39, MPAK41, MPDY42, MPPD43, HHCC66, KSHB28, MHSR47, MHHN48, MKBD49, MNHM57, MNRM58, MTHR59, BHDS62
Hap_5	1	CKST07
Hap_6	1	CKST08
Hap_7	1	CYAG10
Hap_8	1	CYJM11
Hap_9	3	CYKS13, MMSV38, MMSD54
Hap_10	1	MMKO36
Hap_11	1	MSC H44
Hap_12	1	MSKM45
Hap_13	2	SSHB72, MKML51
Hap_14	1	SBSG70
Hap_15	2	RRKD69, KMHK17
Hap_16	1	KGGV68
Hap_17	2	KMBT16, MHHM46
Hap_18	1	KVBL20
Hap_19	1	KVBG21
Hap_20	1	DDMU65
Hap_21	2	DDNS64, HHHM67
Hap_22	2	BBEM61, KMHD18
Hap_23	2	KSAB27, KVAM19
Hap_24	1	KVHG23
Hap_25	1	KVBL22
Hap_26	2	KVKM24, KVKR25
Hap_27	1	KSKK29
Hap_28	1	KSSR30
Hap_29	1	MKHB50
Hap_30	1	MNHJ55
Hap_31	1	SBBM71
Hap_32	1	MNHU56
Hap_33	1	MTSS60
Total	72	

<sup>a</sup> The first, second, third and fourth letters of the isolate indicate the district, taluk, and place of sample collection respectively. These letters are followed by collection numbers.

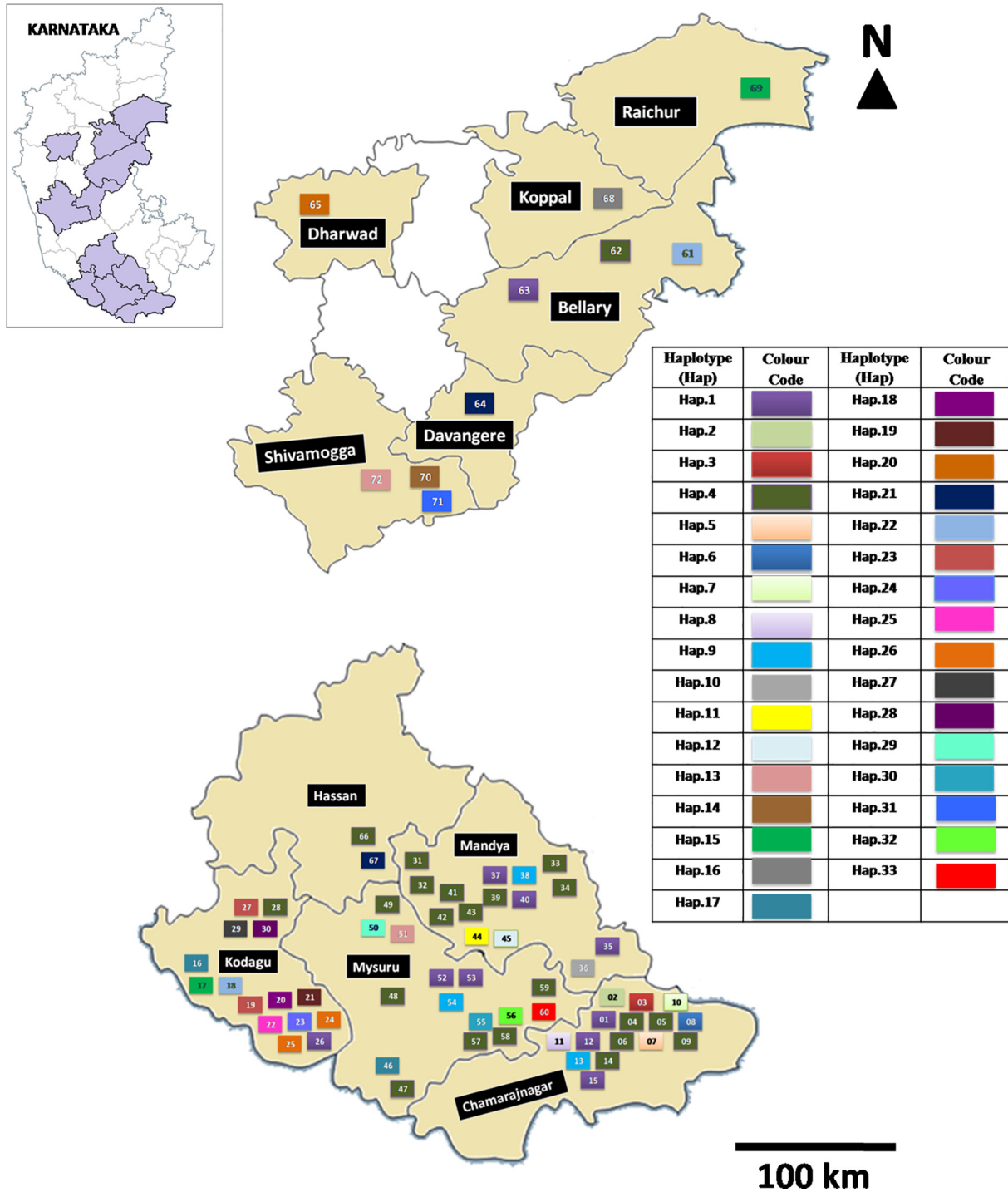


Fig. 4. Location of Haplotype distribution of 72 *Magnaporthe oryzae* isolates in 11 districts of Karnataka State (Inset shows location of different districts of sample collection in Karnataka state). Colour code represents Haplotype number and number within the colorbox indicates collection number of isolates.

major clade. The minor clade included KVBL20, KVHG23, DDMU65, BBEM61, and KMHD18 sharing a close genetic relationship. The major clade SBCN70 and KGGV68 form distinct leaves, SSHB72, MKML51 and KMBT16, MHHM46 shares equal genetic similarities between them. Again major clade is further divided into common leaves among rest of the isolates except MMSD54, CYKS13 MMSV38 which forms a separate small group with 64 bootstrap value. CYAG10, MSCH44 and KVKM24, KVKR25 showed 52 and 84 bootstrap value respectively with separate minor clades.

The estimated value of the shape parameter for the discrete Gamma Distribution is 0.8034. Mean evolutionary rates in these

categories were 0.07, 0.64, 1.20, and 2.80 substitutions per site. The nucleotide frequencies are A = 22.48%, T/U = 24.82%, C = 26.22%, and G = 26.49%. For estimating Maximum Likelihood (ML) values, a tree topology was automatically computed. The maximum Log likelihood for this computation was -2103.116.

### 3.3. Haplotype analysis

Among the *M. oryzae* ITS sequence data obtained from 72 isolates the total number of InDel sites was found to be 270 with Average InDel length event of 1.437 and Average InDel

length of 1.321. The number of InDel haplotypes was 19 with InDel haplotype diversity of 0.519. Similar work of measuring InDel length of ITS region was carried out in non-flowering seed plants [26].

Chi-square test can be directly adapted to use with the nucleotide variation by treating each distinct haplotype as an allele. In our study among the 72 *M. oryzae* ITS sequences 33 haplotype groups were formed with haplotype diversity of 0.8881 (Table 2). This was based on haplotype frequencies in the sample using the information on the extent of differences existing among the nucleotide sequences (Fig. 4). The variance of haplotype diversity was 0.00092 with a standard deviation of 0.030. A similar type of work to understand the genetic diversity where the determination of haplotypes and nucleotide diversity in the rDNA regions of the Tunisian Fig. cultivars (*Ficus carica* L.; Moraceae) was done [27].

The number of polymorphic (segregating) sites  $S$  were 5: 167 and a total number of mutations was  $\text{Eta}$ : 236. The average number of nucleotide differences,  $k$ : 21.10250, Nucleotide diversity,  $\text{Pi}$ : 0.06896.  $\text{Theta}$  (per sequence) from  $\text{Eta}$ : 48.69070;  $\text{Theta}$  (per site) from  $\text{Eta}$ : 0.15912. Tajima's  $D$  value obtained was  $-1.96827$  with a Statistical significance  $P < 0.05$ .

#### 4. Discussion

This study has revealed for the first time the utility of ITS to understand the diversity of a fungal pathogen. Earlier researchers have used rDNA for understanding speciation and classifying the taxa in some higher eukaryotes.

The six clades shown in the dendrogram in the present work reveals the extent of diversity among the 72 monoconidial isolates of *M. oryzae* isolated from infected rice samples from various locations of Southern Karnataka region in India. This was based on sequence variations observed in the ITS regions. These may be stable variations for the given pathogen as these are highly conserved segments of the genome. However, since ITS region is not as highly conserved as that of rRNA genes [28], this may facilitate the pathogen to break down the resistance in host plants.

The distance measures between nucleotide sequences were proposed by implementing gamma distribution to understand variable rates at specific sites. The substitution rate often varies from site to site within a sequence, not only with nucleotide sites but also with the type of nucleotide. This method is well fitted to both mtDNA and nuclear DNA sequence data. The rate of heterogeneity among the sites was explored under the assumption that the rate variation follows gamma distribution as reported by many researchers [29–31].

Tajima's  $D$  value of  $-1.96827$  with a Statistical significance  $P < 0.05$  indicated strong evidence against the null hypothesis. This value shows the strong significant relationship between the different ITS sequences. The Tajima's  $D$  value lesser than one indicates the presence of a high number of rare alleles [24]. The Current study has indicated that the pathogen might have undergone recent selection pressure because of the cultivation of a large number of cultivars resulting in the evolution of rare alleles.

During current investigation, 33 haplotype groups were identified among the 72 *M. oryzae* isolates. This revealed the existence of such alleles where ITS were considered for the analysis and this has shown the extent of genetic variation of *M. oryzae* population of Southern Karnataka. This study highlights use of haplotypes to understand the diversity of the given pathogen. Haplotype study was also carried out to identify speciation in *Hymenoscyphus albidus* [32]. This technique was adopted in Tunisian Fig. cultivars (*Ficus carica* L.; Moraceae) [26] to understand genetic diversity as done in our investigation. Similarly, in *Fusarium incarnatum* phylogenetic and haplotypes studies were carried out to compare the

pathogen isolates of different countries belonging to Southeast Asia, South America and North American continents [33].

The PCR amplification of ribosomal region spanning the internal spacers ITS1/ ITS4 and the 5.8S rRNA genotype showed variations distinct to one another irrespective of geographical locations from where the isolates were obtained. This may be due to the genetic variability of the 32 host cultivars used for isolation of the pathogen in Southern Karnataka region.

Isolates obtained from the same cultivars located in different geographical locations also showed significant variations. This again indicates the genome plasticity existing among the isolates of the pathogen, which may enable the fungus to overcome newer resistant host cultivars in a short period of time. The current investigation has shown the importance of characterizing ITS to know pathogen diversity and its fitness which has potential to contribute to the field of breeding for blast disease resistance.

#### 5. Conclusions

Our results demonstrated that importance of utility of ITS to understand the genetic diversity of a fungal pathogen. Variations among the DNA sequences showed the extent of genetic variation existing in the highly conserved regions of rDNA of *M. oryzae* population in Southern Karnataka. This indicates the genome plasticity existing among the *M. oryzae* isolates obtained from these regions which has enabled the pathogen to adapt to distinctly different agro climatic zones. The variations suggest that mutation might have occurred in favor of the pathogen over a period of time within its genome under various environmental conditions and they will help to develop many mechanisms to evade host plant defenses. The knowledge gained through current investigations regarding ITS diversity will be helpful in breeding blast disease resistance rice cultivars. Hence this can be used as a very effective molecular marker to understand the variability of a pathogen in a given population.

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#### Compliance with ethical standards

This article does not contain any studies with human or animal subjects.

#### Authors' contributions

Jagadeesh D performed the experiments and analyzes the data. Chandrakanth R analyzed the data. Prasanna Kumar M K and Devaki N S designed the study, analyzed the data and revise the manuscript.

#### Conflict of interest

All authors declare that they have no conflict of interest.

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