1 **Analysis of genetic diversity and population structure of *Magnaporthe grisea*, the causal**

2 **agent of foxtail millet blast using microsatellites**

3 Manimozhi Dhivya[a], Govindasamy Senthilraja[b,*], Nagendran Tharmalingam[c, *],

4 Sankarasubramanian Harish[b], Kalaiselvan Saravanakumari[b], Theerthagiri Anand[b],

5 Sundararajan Thiruvudainambi[a]

6

7 [a]Department of Plant Pathology, Agricultural College and Research Institute, Tamil Nadu

8 Agricultural University, Madurai 625104, Tamil Nadu, India,

9 [b]Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore 641003, Tamil

10 Nadu, India,

11 [c]Infectious Disease Division, Department of Medicine, The Miriam Hospital/The Rhode Island

12 Hospital, Alpert Medical School of Brown University, Providence. RI- 02903. The USA.

13

14 *Corresponding author: senthilraja@tnau.ac.in, ntharmalingam@houstonmethodist.org

15

16

17

18

19

20

21

22

23

Abstract

Foxtail millet blast caused by *Magnaporthe grisea* is becoming a severe problem of in foxtail millet growing regions of India. The genetic diversity and population structure of foxtail millet infecting *M. grisea* are is crucial for developing effective management strategies, such as breeding blast-resistant cultivars. We analyzed thirty-two *M. grisea* isolates from ten foxtail millet growing districts in Tamil Nadu, India for genetic diversity using twenty-nine microsatellite or Simple Sequence Repeat (SSR) markers in this study. A total of 103 alleles were identified, with a mean of 3.55 alleles/locus. Gene diversity ranged from 0.170 to 0.717, while major allelic frequencies ranged from 0.344 to 0.906. The polymorphism information content (PIC) ranged from 0.155 to 0.680, with an mean value of 0.465. Population structure analysis of genomic data sets revealed two major populations (SP1 and SP2) with different levels of ancestral admixture among the 32 blast isolates. Phylogenetic analysis classified the isolates into three major clusters. Analysis of molecular variance (AMOVA) showed high genetic variation among individuals and less among populations. Principal coordinate analysis (PCoA) showed a 27.16% genetic variation among populations. The present study provides the first report on the genetic diversity and population structure of the foxtail millet infecting *M. grisea* population in Tamil Nadu, which could be useful for the development of blast blast-resistant foxtail millet cultivars.

Keywords: Foxtail millet, diversity, blast, population structure, microsatellites, phylogenetics

1. INTRODUCTION

Foxtail millet (*Setaria italica* (L.) P. Beauv.) is one of the most important climate-resistant nutri-cereal crops that comprise a vast array of nutritional properties such as protein, starch, fiber, minerals, and antioxidants. It is widely cultivated mostly in tropical and sub-tropical countries (Sharma & Niranjan, 2018). The cultivation and consumption of small millet are increasing in Inida. The agro-industry is now focusing on increasing the small millet productivity by implementing appropriate management strategies against yield-limiting factors (Sharma & Niranjan, 2018).

The ascomycetous fungus *Magnaporthe grisea* (T.T. Hebert) M.E. Barr [anamorph: *Pyricularia grisea* (Cooke) Sacc.] causes blast disease in various cereals and millet worldwide (Noman et al., 2022; Aravind et al., 2022). The disease has become a significant obstacle in cultivating foxtail millet, particularly in the states of Karnataka, Tamil Nadu, Andhra Pradesh, Rajasthan, Madhya Pradesh, and Chhattisgarh in India (Sharma et al., 2014). The pathogen has a hemibiotrophic and heterothallic lifestyle (Koeck et al., 2011), which affects different parts of the plant, including leaves, leaf sheaths, nodes, necks, heads, or panicles at all stages of plant growth (Wilson & Talbot, 2009). Initially, the disease manifests itself as a small water-soaked lesion, after which the spots turn dark green with a central light green to greyish area. Fully developed spots have a brown to dark brown margin with a white to grey center. During the life cycle, the pathogen produces thin-walled, three-celled, pear-shaped conidia with a protruding hilum at the tips of the conidiophores, which cause and spread disease from one plant to another (Klaubauf et al., 2014).

Blast pathogens develop resistance to fungicides (Suzuki et al., 2010). Therefore, the best way to control this disease is to breed resistant cultivars. However, the pathogen can rapidly evolve new pathogenic races which that overcome the plant resistance genes in the field (Sharma et al.,

3

67 2014; Kim et al., 2019). Therefore, it is necessary to analyze the genetic diversity among the

68 *Magnaporthe* populations in a particular region to understand the mechanism of the breakdown of

69 resistance in blast-resistant cultivars (Rieux et al. 2011). Variation within a species can be

70 quantified using changes in the base sequence of DNA (SNP) or the amino acid sequence of

71 proteins, as well as a set of host differentials.

72 Previous studies have demonstrated genetic diversity among blast pathogens in rice (Aravind

73 et al., 2022), wheat (Noman et al., 2022), finger millet (Takan et al., 2012), and pearl millet

74 (Sharma et al., 2021). However, no studies have been conducted on the genetic structure of foxtail

75 millet infecting *Magnaporthe* populations in India. Although the pathogen is variable, it is very

76 host-specific to foxtail millet (Sharma et al., 2014). Improving our understanding of the genetic

77 diversity within and among populations of blast pathogens at the field level is critical to

78 formulating effective management strategies, including the development of blast-resistant

79 cultivars of foxtail millet. To determine the genetic diversity of plant pathogens, microsatellites or

80 simple sequence repeats (SSRs) are widely used marker systems (Rieux et al., 2011; Li et al.,

81 2021). The microsatellites or SSRs are tandemly repeated DNA sequences present throughout the

82 eukaryotic genome. Compared to other marker systems, SSRs are inexpensive (Schoebel et al.,

83 2013), highly polymorphic, multi-allelic, reproducible, and user-friendly (Nowicki et al., 2022).

84 Several SSR markers (Kaye et al., 2003) and minisatellite markers (Li et al., 2007) are developed

85 already for *M. grisea*. The present study aimed to analyze the extent of genetic variability and

86 population structure in field populations of foxtail millet infecting *M. grisea* in Tamil Nadu using

87 SSR markers.

2. MATERIALS AND METHODS

2.1. Survey, collection, and isolation of *M. grisea*

A detailed roving survey was conducted in major foxtail millet growing areas (hills and plains) in Tamil Nadu, India during the rainy season between 2017 and 2018. Blast severity was measured on a progressive 1-9 scale, where 1 = no lesions to small brown specks of pinhead size (0.1-1.0 mm), less than 1% leaf area affected; 2 = typical blast lesions covering 1-5% leaf area covered with lesions; 3 = 6-10%, 4 = 11-20%, 5 = 21-30%, 6 = 31-40%, 7 = 41-50%, 8 = 51-75% and many leaves dead; and 9 = typical blast lesions covering >75% leaf area or all the leaves dead (Babu et al., 2013; Sharma et al., 2014) and expressed as a Percent Disease Index (PDI) using the formula PDI = (sum of individual ratings/no. of leaves assessed x maximum disease grade value) x 100 (Wheeler, 1969; Amoghavarsha et al., 2022). Plant samples (leaves and leaf sheaths) infected with the blast were collected from the farmers' fields. From each cultivar, 25 numbers of leaves were collected. Collected samples were air-dried, bagged, labeled, and stored under refrigerated conditions at 4°C for the isolation of pathogens. The pathogen was isolated using the standard tissue isolation procedure (Tuite, 1969) with Potato Dextrose Agar (PDA) medium under aseptic conditions at 25±2°C. Mycelium growing from infected plant tissue was identified based on its morphological and cultural characteristics. The fungus was then purified by a single spore isolation technique (Ricker & Ricker, 1936) and the purified isolates were stored on PDA slants for further study.

2.2. DNA extraction

Each isolate was inoculated into a 100 ml Erlenmeyer flask containing 20 ml of potato dextrose broth and incubated at 25 ± 2°C. After 7 days of incubation, the mycelia were harvested and immediately ground in liquid nitrogen to a fine powder for DNA extraction with CTAB

5

111 (Cetyltrimethylammonium bromide) buffer, following the protocol described by Murray &

112 Thompson (1980) with minor modifications (Jagadeesh et al., 2018) using equal volumes of

113 supernatant and a mixture of phenol-chloroform-isoamyl alcohol to precipitate and isolate the

114 DNA from other impurities. The DNA was verified quantitatively and qualitatively using a

115 NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and then

116 diluted to a working concentration of 50 ng/µl and stored at -20˚C for later use.

2.3. PCR assays

2.3.1. ITS rDNA amplification

119 All the isolates were subjected to PCR amplification using a pair of universal primers, ITS1

120 and ITS4 (White et al., 1990). Primer sequences were synthesized by Eurofins Genomics India

121 (Bangalore, India). The PCR was performed in 20 µl reaction volume with 2.0 µl template DNA

122 (50 ng/µl), 10.0 µl master mix, 2.0 µl forward primer, 2.0 µl reverse primer, and 4.0 µl sterile

123 double-distilled water. The reaction mixture was centrifuged briefly to thoroughly mix the

124 components of the cocktail. Amplification was performed in a thermal cycler (C1000 Touch^{TM}

125 Thermal Cycler, Bio-Rad Laboratories, Inc., Singapore). Thermal cycling conditions were

126 achieved consisting of 35 cycles, initial denaturation at 94˚C for 5 min, denaturation at 94˚C for

127 1min, annealing at 55˚C for 1 min, extension at 72˚C for 2 min, and final extension at 72˚C for 5

128 min. The PCR products were subjected to agarose (1%) gel electrophoresis in 0.5X Tris-Borate-

129 EDTA buffer at 110V. A 100 bp DNA molecular ladder (MEDOX Biotech, Chennai, India) was

130 used to estimate the size of the amplicon. After electrophoretic separation, the gel was read under

131 the Gel Doc 2000 Bio-Rad system (Bio-Rad Laboratories, Hercules, California, USA) for a more

132 detailed analysis.

2.3.2. Microsatellite genotyping

6

134   Twenty-nine microsatellite loci (SSRs) distributed across seven *M. grisea* chromosomes

135   were used (Table S1) to analyze genetic diversity and population structure as described by Kaye

136   et al. (2003) and Adreit et al. (2007). These primer sequences were synthesized at Eurofins

137   Genomics India (Bangalore, India). PCR amplification was performed in 20 µl of reaction mix in

138   a 0.5 microcentrifuge tube using 10 ng of template DNA, 0.2 mM of dNTPs, 0.4 µM of primers,

139   1.5 mM $MgCl2$, 1X Taq buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3) and 1U Taq DNA

140   polymerase (DreamTaq, Thermo Scientific, USA). Conditions for PCR were initial denaturation

141   at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at

142   55°C for 45 s, and extension at 72°C for 45 s, with the final extension for 10 min at 72°C. For

143   marker scoring, the PCR products were separated by electrophoresis on a 2.5% agarose gel stained

144   with ethidium bromide. After electrophoretic separation, the gels were analyzed under the Gel Doc

145   2000 Bio-Rad system (Bio-Rad Laboratories, Hercules, California, USA). All PCR reactions for

146   each primer were repeated at least twice to confirm the data scored.

147   **2.4. Bioinformatics and computational and statistical analyses**

148   The percent disease index of foxtail millet blast and observations on the conidial morphology

149   of *M. grisea* were subjected to analyses of variance (ANOVA). The averages were compared by

150   the Tukey test ($p < 0.05$) in the Statistical Package for the Social Sciences (SPSS), version 21.0.

151   The amplified PCR products were sequenced at M/S Eurofins Genomics Bangalore, India

152   for double-pass DNA sequencing using the universal primers (ITS1 and ITS4) mentioned above.

153   The DNA sequences were subjected to phylogenetic analysis. A neighbor-joining tree (Saitou &

154   Nei, 1987) was constructed with MEGA v 6.1 software to study divergence patterns, and a 1000-

155   repetition bootstrap analysis was performed to support nodes in clusters (Tamura et al., 2011).

156   Basic computational and statistical analyses such as polymorphic information content (PIC),

157   gene diversity, major allele frequency, and heterozygosity were analyzed using Power Marker

158   version 3.25 (Liu & Muse, 2005). Pairwise F-statistics, Nei genetic distance, analysis of molecular

159   variance (AMOVA), and principal coordinate analysis (PCoA) were performed using the package,

160   namely GenA1Ex v. 6.502 (Peakall & Smouse, 2006). PIC values measure the significance of a

161   given DNA marker. The PIC value for each SSR locus was measured as described by Anderson et

162   al. (1993). An unweighted neighbor-joining tree was constructed based on the simple matching

163   dissimilarity matrix of SSR markers genotyped across the *M. grisea* isolates as implemented in the

164   DARwin v. 5.0.157 program (Perrier & Jacquemoud-Collet, 2006). Population structure between

165   *M. grisea* isolates was analyzed with the software package STRUCTURE (Pritchard et al., 2000)

166   in the revised version 2.3.4. This approach makes use of multilocus genotypes to infer the fraction

167   of an isolate's genetic ancestry that belongs to a population for a given number of populations (K).

168   The optimum number of populations (K) was selected after five independent runs of a burn-in of

169   50,000 iterations followed by 50,000 iterations for each value of K (testing from K = 2 to K = 10).

170   The program STRUCTURE HARVESTER was used to determine the peak value of delta K

171   according to the method described by Evanno et al. (2005).

172

3. RESULTS

3.1. Survey and assessment of blast severity in foxtail millet

The prevalence of foxtail millet leaf blast was noticed in all surveyed areas (plains and hills) in Tamil Nadu. Interestingly, the occurrence of sheath blasts was noticed only in the Kunnur village, Salem District. The severity of leaf blast disease ranged from 10.34 to 72.19 PDI. The highest incidence was observed in the cv. CO(Te)7 (72.19 PDI) in Athiyandal village, Tiruvannamalai District, followed by a local variety from a farmer's field in Kunnur village, Salem District, which had a PDI of 70.16. The minimum PDI (10.34) was observed in the cv. CO(Te)5 in Madurai. Sampling locations were marked on the map of Tamil Nadu using ARC GIS software, and the PDI or blast severity was given in three different colors according to its range. The green color represents a lesser incidence of the disease in the Salem, Madurai, and Virudhunagar districts. The yellow color indicates a moderate disease incidence in the Thoothukudi, Dindigul, Erode, Salem, Namakkal, and Dharmapuri districts. The highest incidence of the disease was recorded in Vellore, Tiruvannamalai, Dindigul, and Virudhunagar districts and is marked in red (Figure 1; Table 1). At the time of the survey, the leaf blast was observed by the type of lesion, which was characterized by a spindle-shaped lesion with a dark brown border with a grey center, and the sheath blast symptom was observed as a spindle-shaped lesion with a dark brown border with a white center on the sheath (Figure 2a,b).

3.2. Morphological characterization

In all, 32 isolates of *M. grisea* were isolated from the diseased samples (31 from leaf and 1 from sheath) and designated as TNFxM1 to TNFxM32 (Table 1). All isolates were classified based on morphological and conidial characteristics variations, namely colony color, surface appearance, size, shape, and color of the conidia. Colony color varied as greyish brown (15), slightly greyish

9

196   brown (3), creamish white (1), white (4), greyish white (4), blackish white (3), and greyish black

197   (2). Similarly, most of the isolates were smooth (19) and some were rough (13) in colony

198   appearance (Figure S1; Table 2). In all the isolates, the shape of the conidia was typically pyriform

199   with a rounded base, narrow apex, 2 septa, 3 cells, and the middle cells were broader than the

200   adjacent cells in all the isolates,. Some of them were very long and narrow, while others were quite

201   wide. The length and width of the conidia of different isolates of *M. grisea* were measured (Figure

202   S1; Table 2). The size of the conidia ranged from 20.06 μm to 37.76 μm in length and 6.88 μm to

203   11.93 μm in width. All isolates varied significantly in terms of conidial size. The blast isolates

204   TNFxM7 and TNFxM20 had the longest conidia of 37.76 μm and 33.83 μm, respectively. The

205   shortest conidial length was observed in isolates TNFxM23 (20.06 μm) and TNFxM6 (21.48 μm).

206   The highest conidial width was observed in isolates TNFxM26 and TNFxM27 at 11.93 μm and

207   10.77 μm, respectively.

208   **3.3. ITS-DNA barcoding and phylogenetic analysis**

209   ITS-based primers amplified the expected amplicon size of 560 bp (Figure S2). All the

210   sequences obtained from this study by partial gene sequencing showed more than 99% sequence

211   similarity to GenBank sequences (*M. grisea*) deposited with the NCBI. Accession numbers

212   obtained from the NCBI GenBank for each isolate are listed in Table 1.

213   The phylogenetic analysis revealed that all 32 *M. grisea* isolates were divided into two

214   groups (Group I and Group II). The two isolates (MN017168 and MN028778) from Virudhunagar

215   District were only clustered separately and formed group I. Group II consists of thirty isolates with

216   a further subdivision into subgroups I and subgroup II. Subgroup I was formed with eight isolates

217   from Tiruvanamalai (MN017167), Salem (MW534814, MW535244, MW504790, MW535295,

218   MW535961 and MW496127) and Namakkal (MW497613) districts. The remaining twenty-two

10

219   isolates from Dindigul (MT053476, MK990557, MN028779, and MN017169), Thoothukudi

220   (MN028776), Madurai (MT043762 and MT053462), Dharmapuri (MT043764), Vellore

221   (MT043805), Erode (MW497608 and MW534750), Salem (MW534813, MW534870,

222   MW535067, MW535174, MW494605, MW498280, MW504997, MW504990, and MW494316)

223   and Namakkal (MW494589 and MW535771) districts were grouped altogether and formed

224   subgroup II (Figure 3).

225   **3.4. SSR genotyping and cluster analysis**

226   All 32 isolates were genotyped with 29 SSR markers distributed throughout the

227   *M. grisea* genome. A total of 103 alleles were detected among the 29 SSR markers, ranging from

228   2 (Pyrms 67-68, Pyrms 107-108, and Pyrms 533-534) to 6 (Pyrms 607-608) alleles with an average

229   of 3.55 alleles per locus. The major allele frequency ranged from 0.344 (Pyrms 83-84) to 0.906

230   (Pyrms 533-534), with a mean of 0.605. Genetic diversity ranged from 0.170 (Pyrms 533-534) to

231   0.717 (Pyrms 657-658) with a mean value of 0.517. The polymorphism information content (PIC)

232   of the markers had an average value of 0.465 and varied from 0.155 to 0.680. The maximum value

233   of PIC was observed for marker Pyrms 37-38, while the minimum value was observed for marker

234   Pyrms 533-534. The observed mean heterozygosity was 0.007517 with a range of 0.00 to 0.094.

235   Among the markers used, only 5 (Pyrms 37-38, Pyrms 39-40, Pyrms 99-100, Pyrms 427-428, and

236   Pyrms 453-454) showed a heterozygosity value greater than zero, while 24 had zero values (Table

237   3). The unrooted unweighted neighbor Joining method was used to estimate the genetic distance

238   and dissimilarity index of 32 *M. grisea* isolates. For the unrooted tree, isolates were classified into

239   three major clusters (Figure 4). Among the three clusters, cluster ~~one~~ 1 (red color) recorded 14

240   isolates, while cluster 2 (blue color) consisted of 16 isolates, and cluster 3 (green color) had 2

241   isolates.

3.5. Population structure analysis

For genetic structure estimation, a Bayesian clustering approach was followed by taking probable subpopulations (K), and a higher delta K value using STRUCTURE 2.3.6 software. The maximum plateau of *adhoc* measurement of ΔK was K=2 with a ΔK value of 219.8 (Table S2). The population structure was analyzed for genetic relatedness among 32 *M. grisea* isolates collected from ten different districts of Tamil Nadu, India. Based on an ancestry threshold of >70%, all 32 isolates were classified into two major populations and considered pure, while <70% were considered admixture (AD). Among the 32 isolates, 13 isolates collected from Salem and Namakkal districts were classified as subpopulation 1 (SP1), and 16 isolates collected from Tiruvanamalai, Dindigul, Virudhunagar, Thoothukudi, Madurai, Dharmapuri, Vellore, Madurai, Erode and Salem districts were classified as subpopulation 2 (SP2), while 3 isolates (TNFxM6, TNFxM17, and TNFxM18) were of admixture type with a major genetic component of two subgroups (Table 4). The population shows admixture, suggesting that there is a gene flow between these populations. The fixation index (F_{st}) values of the two populations were 0.4296 for SP1 and 0.2021 for SP2. The highest divergence in allele frequency between populations was observed in SP1 and SP2 (0.1077) based on the net nucleotide distance calculated using point estimates of P. The mean distance (expected heterozygosity) between individuals in the panel population was 0.2036 and 0.2800 in response to SP1 and SP2, respectively (Figure 5a,b; Figure S3a-c).

3.6. Analysis of molecular variance (AMOVA)

The two populations, i.e., subpopulation 1 (13), and subpopulation 2 (16), along with the admixtures (3) generated from structure analysis, were analyzed to determine genetic variation among and within populations. In the AMOVA analysis, maximum variation (79%) was found

12

265 among individuals, while minimum variation (20%) was noticed between the populations and

266 within individuals (1%) (Figure 6; Table S3). The deviation from Hardy-Weinberg's prediction

267 was performed using Wright's F-statistics. The F_{is} and F_{it} values for all 29 loci were found to be

268 0.984 and 0.987 (r = <0.001), respectively, while F_{st} between populations was 0.202. The NM

269 value of assumed subpopulations was noted as 0.989. The highest pairwise genetic Nei distance

270 was observed between SP1 and AD (0.404), followed by SP1 and SP2 (0.362) and SP2 and AD

271 (0.307). Furthermore, the biplot generated from the principal coordinate analysis (PCoA) showed

272 that the first two components account for 100% of the variations (Figure 7a,b). The scatterplot was

273 developed from the PCoA analysis and displayed across isolates on the first two axes. The PCoA

274 1 biplot captured 18.03% of the variation, while PCoA 2 contributed 9.13% to the total genetic

275 variation, suggesting that the total genetic variation among the populations was 27.16% (Table

276 S4a, S4b).

277

4. DISCUSSION

Foxtail millet is grown in both the plain and hilly regions of Tamil Nadu in India. The blast disease caused by *M. grisea* (Sharma et al., 2014), caused a vast yield loss. We examined the morphological and genetic variability of 32 *M. grisea* isolates using 29 microsatellite markers (Kaye et al., 2003; Adreit et al., 2007) and universal ITS primers (ITS1 and ITS4) to characterize the genetic diversity and population structure of foxtail millet infecting *M. grisea*. We demonstrated the genetic analyses of foxtail millet blast populations, which can be used to develop blast-resistant foxtail millet cultivars.

In the present study, the severity of the blast was assessed in various foxtail millet growing areas in Tamil Nadu during the rainy season between 2017 and 2018. The highest incidence of the disease was observed in the Vellore, Tiruvannamalai, Dharmapuri, and Dindigul districts of Tamil Nadu. Similarly, Sharma et al. (2014) also noticed the occurrence of blast incidence during the rainy season in 2008 in different foxtail millet growing tracts in Telangana and Andhra Pradesh. We isolated the causative agent of foxtail millet blast from the infected leaf and sheath samples collected at surveyed fields using a PDA medium and purified by the single spore isolation technique. Other reports demonstrating that a similar single-spore isolation technique was used to isolate and purify *Magnaporthe* from rice, wheat, finger millet, and foxtail millet using different media (Sharma et al., 2014; Yadav et al., 2019) were discussed and corroborated by our study.

The present study demonstrates a variation in mycelium color and texture variation among the 32 fungal isolates collected in Tamil Nadu. This confirms previous reports by Panda et al. (2017) and Sahu et al. (2018), who classified the rice blast isolates collected from different parts of Chhattisgarh and Odisha in India into different groups based on colony color and texture. The present study shows the variation among the 32 isolates of *M. grisea* in terms of morphological

301   and conidial characteristics. However, there is no significant difference between the colony

302   morphology and conidial characters concerning the collection period and geographical distribution

303   of the isolates.

304   Sequencing of the ITS region of rDNA has become the universal barcode system for

305   identifying fungal taxonomy at the species level (Paloi et al., 2022). In the present study, all *M.*

306   *grisea* isolates were amplified using universal primers (ITS1 and ITS4) and the result showed the

307   expected 560 bp amplicon in all isolates. The isolates showed a sequence homology of >99% with

308   *M. grisea*. The results of the present study are consistent with Jagadeesh et al. (2018) who

309   examined the diversity of 72 isolates of *M. oryzae* collected in Karnataka, India using primers

310   ITS1 and ITS4. The two clades shown in the phylogenetic analysis in the present work demonstrate

311   the degree of diversity among the 32 isolates of *M. grisea* isolated from different districts of Tamil

312   Nadu in India, again reflecting the results of Jagadeesh et al. (2018), where genetic variations were

313   observed in 72 isolates of the rice blast pathogen in the ITS region. The current study identified

314   taxonomic relationships or differences between 32 isolates of foxtail millet *M. grisea*, which may

315   be useful ~~to identify~~for identifying the resistance or susceptibility to a particular genotype.

316   Phylogenetic analysis based on ITS region sequences revealed that all the ~~thirty~~ thirty-two *M.*

317   *grisea* isolates could be divided into two groups (Group I and Group II). The two isolates

318   (MN017168 and MN028778) collected from the Virudhunagar district formed separate clusters as

319   Group I and the remaining thirty isolates formed Group II. Lineages are most often split by the

320   migration of a few individuals to a newly isolated region. The two Virudhunagar isolates (where

321   we had not previously observed a millet blast) formed separate clusters as Group I, possibly due

322   to geological or climatic events. As a result of this genetic isolation, the lineages will evolve

15

323   separately and become increasingly distinct in their physiological and behavioural patterns over

324   time.

325   Microsatellites or SSRs are powerful markers used for population genetic analyses due to

326   their high specificity, polymorphism, and reproducibility (Schoebel et al., 2013; Nowicki et al.,

327   2022). We evaluated 29 SSR markers reported by Kaye et al. (2003) and Adreit et al. (2007) to

328   analyze genetic diversity in the population of foxtail millet infecting *M. grisea* from Tamil Nadu.

329   To the best of our knowledge, this is the first study to investigate the molecular diversity and

330   population structure of 32 isolates of foxtail millet infecting *M. grisea* from Tamil Nadu using

331   microsatellites. The SSR markers used in this study varied widely and the results were generally

332   consistent with previous population genetic studies conducted in India and elsewhere (Yadav et

333   al., 2019). In the present study, 103 alleles were detected using 29 microsatellite markers. The

334   number of alleles per locus ranged from 2 to 6, averaging 3.55 alleles/locus. A variation in the

335   number of alleles was observed between the markers used. Previous studies reported similar results

336   in rice (Aravind et al., 2022), wheat (Noman et al., 2022), finger millet (Takan et al., 2012), and

337   pearl millet (Sharma et al., 2021) infecting populations of *Magnaporthe* in different geographic

338   locations. Some previous studies showed that the high number of alleles per locus resulted in high

339   genetic variation. The high genetic diversity was demonstrated in the present study, confirming

340   previous results of Saleh et al. (2014), while Yadav et al. (2019) reported a low level of genetic

341   diversity among the *M. oryzae* isolates collected in North Eastern India.

342   Similarly, high levels of polymorphism were found among the 29 microsatellites used in this

343   study. The results agree with Wang et al. (2017), where a high degree of polymorphism was

344   observed when analyzing 457 rice blast isolates collected in the United States using ten SSR

345   markers. Recently, Adhikari et al. (2020) also observed high levels of polymorphism when

346   analyzing 17 pearl millet blast isolates collected in India using SSR markers. However, a low level

347   of polymorphism has been observed in the rice blast population in Brazil and Japan (Prabhu et al.,

348   2002). Analysis of genetic diversity showed a high percentage of identical alleles, indicating

349   considerable gene flow between all isolates of the *M. grisea* population in Tamil Nadu. Gene flow

350   is very essential in agroecosystems for plant pathogens since it is the process by which new genes

351   are transferred into agricultural fields far from the original mutation location. This technique is

352   likely to play a role in natural ecosystems as well in the context of changing environmental

353   conditions.

354   As most foxtail millet cultivation in Tamil Nadu is dominated by a single cultivar CO(Te)7

355   and no resistant cultivar is grown yet, there is no selection pressure on the pathogen to induce

356   change. Hence this could be why there is a high percentage of identical alleles between populations

357   of *M. grisea*. The dispersal of *M. grisea* from one location to another through airborne conidia and

358   contaminated seeds was considered an important factor in the introduction of *M. grisea* to other

359   parts of India. This is also reflected in the extent of migration between the plains and the hills. The

360   hilly region seems to have the a higher migration rate than the plains. This is probably due to the

361   different weather patterns in the hills (cold) and the plains (warm). In general, the results indicate

362   the possibility of free movement of the pathogen between the regions. The present study

363   undoubtedly shows an excellent diversity of *M. grisea* in the hilly region and that most of the

364   variability was within individuals. Our results agree with those of Wang et al. (2017), who

365   analyzed 457 rice blast isolates using the neighbor-joining method to build an unrooted tree. A

366   total of five major clusters were identified. The observed clusters were mainly related to the

367   sampling period but not to the geographic location of the isolates.

Formatted: Font: Italic

368   A Bayesian clustering approach was used to analyze the structure of the foxtail millet blast

369   isolates. The structure analysis divided the *M. grisea* isolates into two subpopulations (K=2) with

370   three admixtures. The first subpopulation (SP1) consisted of isolates from the Salem and

371   Nammakal districts of Tamil Nadu. Similarly, the second subpopulation (SP2) consisted of

372   Tiruvannamalai, Madurai, Dindigul, Erode, Virudhunagar, Tuticorin, Dharmapuri, Vellore, and

373   Salem districts in Tamil Nadu. Admixtures (AD) present in isolates can increase genetic diversity.

374   However, the structure analysis could not differentiate the blast isolates based on the geographic

375   location. Similarly, Yadav et al. (2019) identified two subpopulations of leaf and neck blast isolates

376   infecting rice crops and showed limited categorization of blast isolates based on the location.

377   Interestingly, Onaga et al. (2015) categorized the 88 rice blast isolates collected from East Africa

378   into five ancestral genetic clusters (K=5) with Structure V 2.3. Package. Wang et al. (2017)

379   analyzed 457 blast isolates using ten polymorphic microsatellites. They identified 6 genetic

380   clusters (K=6) based on collection period but not geographic location. However, a PCoA analysis

381   was performed from the results obtained from interpreting the structure. The AMOVA analysis

382   was worked out by dividing the genetic diversity within and between the populations of 32 isolates

383   of foxtail millet blast. Variation between these populations contributed 20% of the genetic

384   diversity, while 79% of the genetic variation was present between the individuals; however, one

385   percent of the variation was found within individuals. A higher genetic diversity was found among

386   the blast isolates than in the population. Identically, analysis of molecular variance values for

387   genetic diversity in the pathogen population varied from 78.66% (Wang et al., 2017) to 86.6%

388   (D'Ávila et al., 2016), 88.09% (Onaga et al., 2015) and 98% (Yadav et al., 2019). Our results are

389   consistent with previous reports in which the pathogen population was not differentiated by

390   geographic location (Onaga et al., 2015).

18

391   The genetic variation and population structure of *M. grisea* infecting foxtail millet from

392 different regions of Tamil Nadu shows significant genetic variation within populations, similar to

393 those observed in other population studies of *M. grisea* in India and elsewhere. Our results showed

394 that gene flow occurs between regions and has significant implications for foxtail millet growers

395 when virulent or fungicide-resistant strains move between regions. Preventing such a threat from

396 the foxtail millet blast requires proper prediction and forecasting systems. In addition, long-term

397 monitoring is essential to identify the population origin and evolutionary potential of foxtail millet

398 blast isolates in India.

399 **ADDITIONAL INFORMATION AND DECLARATIONS**

400
401 **Funding**

411 **Competing Interests**

412   The authors declare that they have no competing interests.

413 **Author Contributions**

414 •   M. Dhivya: Investigation, Formal analysis, Data curation, Validation. S. Harish: Methodology, Formal

415   analysis, Visualization, Writing - review and editing.

416 • G. Senthilraja: Funding acquisition, Project administration, Conceptualization, Supervision,

417 Visualization, Writing - original draft, review and editing.

418 • T. Nagendran: Data curation, Formal analysis, Writing - review and editing.

419 • S. Harish: Methodology, Formal analysis, Visualization, Writing - review & editing.

420 • K. Saravanakumari: Data curation, Formal analysis, Writing - review and editing.

421 • T. Anand: Data curation, Formal analysis, Writing - original draft, review and editing.

422 • S. Thiruvudainambi: Supervision, Methodology, Resources, Visualization, Writing – review and

423 editing.

424 **Data Availability**

425 The following information was supplied regarding data availability:

426 Raw data are available as a Supplemental File.

427 **REFERENCES**

428 Adhikari, S., Joshi, S.M., Athoni, B.K., Patil, P.V. & Jogaiah, S. (2020) Elucidation of genetic

429 relatedness of *Magnaporthe grisea*, an incitant of pearl millet blast disease by molecular

430 markers associated with virulence of host differential cultivars. *Microbial Pathogenesis*,

431 *149*, 104533.

432 Adreit, H., Santoso, Andriantsimialona, D., Utami, D.W., Notteghem, J.L., Lebrun, M.H. &

433 Tharreau, D. (2007) Microsatellite markers for population studies of the rice blast fungus,

434 *Magnaporthe grisea*. *Molecular Ecology Notes*, *7*, 667-670.

435 Amoghavarsha, C., Pramesh, D., Sridhara, S., Patil, B., Shil, S., Naik, G.R., Naik, M.K., Shokralla,

436 S., El-Sabrout, A.M., Mahmoud, E.A. & Elansary, H.O. (2022) Spatial distribution and

437 identification of potential risk regions to rice blast disease in different rice ecosystems of

438 Karnataka. *Scientific Reports*, *12*, 1-14.

439   Anderson, J.A., Churchill, G.A., Autroque, J.E., Tanksley, S.D. & Swells, M.E. (1993) Optimising

440   selection for plant linkage map. *Genome*, *36*, 181-186.

441   Aravind, K., Rajeswari, B., Babu, T.K. & Pushpavalli, S.N.C.V.L. (2022) Pathogenic and

442   molecular variability of *Magnaporthe oryzae* isolates collected from major rice growing

443   locations of Telangana State, India. *Indian Phytopathology*, 1-11.

444   Babu, T. K., Thakur, R. P., Upadhyaya, H. D., Reddy, P. N., Sharma, R., Girish, A. G., & Sarma,

445   N. D. R. K. (2013) Resistance to blast (*Magnaporthe grisea*) in a mini-core collection of

446   finger millet germplasm. *European Journal of Plant Pathology*, 135, 299-311.

447   D'Ávila, L.S., Lehner, M.S., Filippi, M.C.C., Scheuermann, K.K. & Del Ponte, E.M. (2016)

448   Genetic structure and mating type analysis of the *Pyricularia oryzae* population causing

449   widespread epidemics in southern Brazil. *Tropical Plant Pathology*, *41*, 297-305.

450   Evanno, G., Regnaut, S. & Goudet, J. (2005) Detecting the number of clusters of individuals using

451   the software STRUCTURE: a simulation study. *Molecular ecology*, *14*, 2611-2620.

452   Jagadeesh, D., Kumar, M.P., Chandrakanth, R. & Devaki, N.S. (2018) Molecular diversity of

453   internal transcribed spacer among the monoconidial isolates of *Magnaporthe oryzae*

454   isolated from rice in Southern Karnataka, India. *Journal of Genetic Engineering and*

455   *Biotechnology*, *16*, 631-638.

456   Kaye, C., Milazzo, J., Rozenfeld, S., Lebrun, M.H. & Tharreau, D. (2003) The development of

457   simple sequence repeat markers for *Magnaporthe grisea* and their integration into an

458   established genetic linkage map. *Fungal Genetics and Biology*, *40*, 207-214.

459   Kim, K.T., Ko, J., Song, H., Choi, G., Kim, H., Jeon, J., Cheong, K., Kang, S. & Lee, Y.H. (2019)

460   Evolution of the genes encoding effector candidates within multiple pathotypes of

461   *Magnaporthe oryzae*. *Frontiers in microbiology*, *10*, 2575.

462   Klaubauf, S., Tharreau, D., Fournier, E., Groenewald, J. Z., Crous, P. W., De Vries, R. P. &

463   Lebrun, M. H. (2014) Resolving the polyphyletic nature of *Pyricularia* (Pyriculariaceae).

464   *Studies in Mycology*, *79*, 85-120.

465   Koeck, M., Hardham, A. R. & Dodds, P. N. (2011) The role of effectors of biotrophic and

466   hemibiotrophic fungi in infection. *Cellular microbiology*, *13*, 1849-1857.

467   Li, C.Y., Li, J.B., Liu, L., Yang, J., Su, Y., Wang, Y.Y., Xie, Y., Ye, M. & Zhu, Y.Y. (2007)

468   Development of minisatellite markers in phytopathogenic fungus, *Magnaporthe grisea*.

469   *Molecular Ecology Notes*, *7*, 978-980.

470   Li, X., Tao, F., Fan, S., Li, H., Yang, J. & Gao, L. (2021) Genetic diversity of *Venturia inaequalis*

471   isolates (Apple scab) in China and UK determined by SSR markers. *PloS one*, *16*,

472   e0252865.

473   Liu, K. & Muse, S. V. (2005) PowerMarker: an integrated analysis environment for genetic marker

474   analysis. *Bioinformatics*, *21*, 2128-2129.

475   Murray, M. G. & Thompson, W. (1980) Rapid isolation of high molecular weight plant DNA.

476   *Nucleic Acids Research*, *8*, 4321-4326.

477   Noman, M., Al, A., Hosen, S. & Shamsi, S. (2022) Elucidating genetic diversity and population

478   structure of *Pyricularia oryzae* isolates causing wheat blast in Bangladesh. *Archives of*

479   *Microbiology*, *204*, 1-10.

480   Nowicki, M., Hadziabdic, D., Trigiano, R.N., Runge, F., Thines, M., Boggess, S.L., Ristaino, J. &

481   Spring, O. (2022) Microsatellite markers from *Peronospora tabacina*, the cause of blue

482   mold of tobacco, reveal species origin, population structure, and high gene flow.

483   *Phytopathology*, *112*, 422-434.

484   Onaga, G., Wydra, K., Koopmann, B., Séré, Y. & von Tiedemann, A. (2015) Population structure,
485   pathogenicity, and mating type distribution of *Magnaporthe oryzae* isolates from East
486   Africa. *Phytopathology*, *105*, 1137-1145.

487   Paloi, S., Luangsa-ard, J. J., Mhuantong, W., Stadler, M. & Kobmoo, N. (2022) Intragenomic
488   variation in nuclear ribosomal markers and its implication in species delimitation,
489   identification and barcoding in Fungi. *Fungal Biology Reviews*.
490   https://doi.org/10.1016/j.fbr.2022.04.002.

491   Panda, G., Sahu, C., Yadav, M.K., Aravindan, S., Umakanta, N., Raghu, S., Prabhukarthikeyan,
492   S.R., Lenka, S., Tiwari, J.K., Kar, S. & Jena, M. (2017) Morphological and molecular
493   characterization of *Magnaporthe oryzae* from Chhattisgarh. *ORYZA-An International*
494   *Journal on Rice*, *54*, 330-336.

495   Peakall, R.O.D. & Smouse, P. E. (2006) GENALEX 6: genetic analysis in Excel. Population
496   genetic software for teaching and research. *Molecular Ecology Notes*, *6*, 288-295.

497   Perrier, X. & Jacquemoud-Collet, J.P. (2006) DARwin software. http://darwin.cirad.fr/.

498   Prabhu, A.S., Filippi, M.C. & Araujo, L.G. (2002) Pathotype diversity of *Pyricularia grisea* from
499   improved upland rice cultivars in experimental plots. *Fitopatologia Brasileira*, 27, 468-
500   473.

501   Pritchard, J. K., Stephens, M. & Donnelly, P. (2000) Inference of population structure using
502   multilocus genotype data. *Genetics*, *155*, 945-959.

503   Ricker, A.J. & Ricker, R.S. (1936) Introduction to research on plant diseases. Jon. S. *Swift Co.*
504   *Inc., St. Lovis, Chicago, New York, 117*.

505 Rieux, A., Halkett, F., De Lapeyre de Bellaire, L., Zapater, M. F., Rousset, F., Ravigné, V. &
506   Carlier, J. (2011) Inferences on pathogenic fungus population structures from microsatellite
507   data: new insights from spatial genetics approaches. *Molecular Ecology*, *20*, 1661-1674.

508 Sahu, C., Yadav, M.K., Panda, G., Aravindan, S., Umakanta, N., Raghu, S., Prabhukarthikeyan,
509   S.R., Keerthana, U., Adak, T., Sharma, V. & Mohanty, M.R. (2018) Morphological and
510   molecular characterization of *Magnaporthe oryzae* causing rice blast disease in Odisha.
511   *ORYZA-An International Journal on Rice*, *55*, 467-472.

512 Saitou, N. & Nei, M. (1987) The neighbor-joining method: a new method for reconstructing
513   phylogenetic trees. *Molecular Biology and Evolution*, *4*, 406-425.

514 Saleh, D., Milazzo, J., Adreit, H., Fournier, E. & Tharreau, D. (2014) South-East Asia is the center
515   of origin, diversity and dispersion of the rice blast fungus, *Magnaporthe oryzae*. *New
516   Phytologist*, *201*, 1440-1456.

517 Schoebel, C.N., Brodbeck, S., Buehler, D., Cornejo, C., Gajurel, J., Hartikainen, H., Keller, D.,
518   Leys, M., Říčanová, Š., Segelbacher, G. & Werth, S. (2013) Lessons learned from
519   microsatellite development for nonmodel organisms using 454 pyrosequencing. *Journal of
520   Evolutionary Biology*, *26*, 600-611.

521 Sharma, N. & Niranjan, K. (2018) Foxtail millet: Properties, processing, health benefits, and uses.
522   *Food Reviews International*, *34*, 329-363.

523 Sharma, R., Girish, A.G., Upadhyaya, H.D., Humayun, P., Babu, T.K., Rao, V.P. & Thakur, R.
524   (2014) Identification of blast resistance in a core collection of foxtail millet germplasm.
525   *Plant Disease*, *98*, 519-524.

526 Sharma, R., Goud, T.Y., Prasad, Y.P., Nimmala, N., Kadvani, D.L., Mathur, A.C., Thakare, C.S.,

527   Devi, G.U. & Naik, M.K. (2021) Pathogenic variability amongst Indian isolates of

528   *Magnaporthe grisea* causing blast in pearl millet. *Crop Protection*, *139*, 105372.

529 Suzuki, F., Yamaguchi, J., Koba, A., Nakajima, T. & Arai, M. (2010) Changes in fungicide

530   resistance frequency and population structure of *Pyricularia oryzae* after discontinuance of

531   MBI-D fungicides. *Plant Disease*, *94*, 329-334.

532 Takan, J.P., Chipili, J., Muthumeenakshi, S., Talbot, N.J., Manyasa, E.O., Bandyopadhyay, R.,

533   Sere, Y., Nutsugah, S.K., Talhinhas, P., Hossain, M. & Brown, A.E. (2012) *Magnaporthe*

534   *oryzae* populations adapted to finger millet and rice exhibit distinctive patterns of genetic

535   diversity, sexuality and host interaction. *Molecular Biotechnology*, *50*, 145-158.

536 Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011) MEGA5:

537   molecular evolutionary genetics analysis using maximum likelihood, evolutionary

538   distance, and maximum parsimony methods. *Molecular Biology and Evolution*, *28*, 2731-

539   2739.

540 Tuite, J. (1969) Plant Pathological Methods. Fungi and Bacteria. *Minnesota, USA: Burgess*

541   *Publishing Company*.

542 Wang, X., Jia, Y., Wamishe, Y., Jia, M. H., & Valent, B. (2017) Dynamic changes in the rice blast

543   population in the United States over six decades. *Molecular Plant-Microbe Interactions*,

544   *30*, 803-812.

545 Wheeler, B.E.J. (1969) An introduction to plant diseases. *John Wiley and Sons Ltd., London*.

546 White, T.J., Bruns, T., Lee, S.J.W.T. & Taylor, J. (1990) Amplification and direct sequencing of

547   fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and*

548   *applications*, *18*, 315-322.

549 Wilson, R.A. & Talbot, N.J. (2009) Under pressure: investigating the biology of plant infection by

550 *Magnaporthe oryzae*. *Nature Reviews Microbiology*, *7*, 185-195.

551 Yadav, M.K., Aravindan, S., Raghu, S., Prabhukarthikeyan, S.R., Keerthana, U., Ngangkham, U.,

552 Pramesh, D., Banerjee, A., Adak, T., Kar, M.K. & Parameswaran, C. (2019) Assessment

553 of genetic diversity and population structure of *Magnaporthe oryzae* causing rice blast

554 disease using SSR markers. *Physiological and Molecular Plant Pathology*, *106*, 157-165.

555 **FIGURE LEGENDS**

556 FIGURE 1. Map showing the collection sites of *Magnaporthe grisea* isolates and foxtail millet

557 blast severity in Tamil Nadu. Sampling locations marked on the map of Tamil Nadu was

558 extracted using the ARC GIS software

559 FIGURE 2. Foxtail millet blast symptoms. (a) Leaf blast: spindle shaped lesion having dark brown

560 margin with grey colored center, (b) Sheath blast: spindle shaped lesion having dark

561 brown margin with white colored center.

562 FIGURE 3. Phylogenetic analysis. Phylogenetic tree constructed based on multiple alignments of

563 nucleotide sequences of *M. grisea* isolates. The tree was generated using the neighbor

564 joining method with 1000 bootstrap replications and the tree drawn at a cut-off value of

565 60% in MEGA 6.1.

566 FIGURE 4. Cluster analysis. Neighbor–joining cluster analysis of 32 blast isolates based on 29

567 SSR markers using DARwin software.

568 FIGURE 5. Population structure analysis. (a) Values of DK, with its modal value used to detect

569 true K of the group (K =2). For each K value, at least three independent runs were

570 considered and averaged over the replicates, (b) Population structure of 32 blast isolates

571 based on 29 markers (K = 2) and graph of estimated membership fraction for K = 2. The

572 maximum of *adhoc* measure ΔK determined by structure harvester was found to be K =

573 2, which indicated that the entire population can be grouped into two subgroups.

574 Different color within group indicates the proportion of shared ancestry with other group

575 which has the same color with the admixture.

576 FIGURE 6. AMOVA analysis of foxtail millet infecting *M. grisea* isolates.

577 FIGURE 7. Principal coordinate analysis (PCoA). (a) PCoA for Nei genetic diversity among the

578 three population, (b) PCoA of 32 blast isolates in the panel population using 29

579 molecular markers. The isolates are colored on the basis of sub-populations obtained

580 from structure analysis (SP1- yellow, SP2 - green, AD - red).

581 **SUPPORTING INFORMATION LEGENDS**

582 FIGURE S1. Colony morphology of *M. grisea* isolates. Variability in morphology and cultural

583 characters of *M. grisea* isolates on PDA. Conidial characteristics of *M. grisea*

584 isolates. Variation in conidia shapes and sizes among the isolates of *M. grisea*

585 observed under image analyzer.

586 FIGURE S2. PCR amplification of *M. grisea* using ITS Primers (ITS1 and ITS4).

587 FIGURE S3. Population structure analysis. (a) Histogram distribution of Fst1, (b) Histogram

588 distribution of Fst2, (c) Histogram distribution of alpha.

589

590

591