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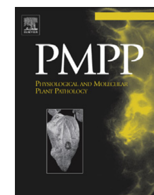

Evaluation of genetic diversity in *Magnaporthe grisea* populations adapted to finger millet using simple sequence repeats (SSRs) markers

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ABSTRACT

Finger millet blast caused by *Magnaporthe grisea* (anamorph: *Pyricularia grisea*) is a great threat to finger millet production worldwide. Genetic diversity and population structure of 72 *M. grisea* isolates collected from finger millet (56), foxtail millet (6), pearl millet (7) and rice (3) from major crop growing areas in India was studied using 24 SSR markers. None of the SSRs detected polymorphism in the *M. grisea* isolates from pearl millet. Seventeen SSR markers were polymorphic in the 65 non pearl millet isolates and detected 105 alleles, of which one was rare, 83 common, 9 frequent and 12 most frequent. A model-based population structure analysis of the genomic data identified two distinct populations with varying levels of ancestral admixtures among the 65 *M. grisea* isolates. Analysis of molecular variance (AMOVA) indicated that 52% of the total variation among the isolates used in this study was due to differences between the pathogen populations adapted to different hosts, 42% was due to differences in the isolates from the same host, and the remaining 6% due to heterozygosity within isolates. High genetic variability present in *M. grisea* isolates calls for the continuous monitoring of *M. grisea* populations anticipating blast resistance breakdown in finger millet cultivars grown in India.

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1. Introduction

Finger millet (*Eleusine coracana* L. Gaertn) is a widely grown grain cereal in the semi-arid areas of East and southern Africa and South Asia under varied agro-climatic conditions [1]. Finger millet is being increasingly recognized as a promising source of micro-nutrients and protein [2] for weak and immune-compromised people [3]. Besides energy, it contributes to alleviating micro-nutrient and protein malnutrition also called 'hidden hunger' affecting half of the world's population, especially women and pre-school children in most countries of Africa and South-east Asia [4]. Malnutrition due to protein deficiency is also found at alarming rates in the Indian subcontinent [5]. Although finger millet is tolerant to many biotic and abiotic stresses, the crop is severely affected by blast disease caused by an ascomycete fungus *Magnaporthe grisea* (Hebert) Barr. (anamorph: *Pyricularia grisea* (Cooke) Sacc.), which is very prominent among the constraints that affect yield, utilization and trade of finger millet within East Africa and South Asia [6,7]. Many of the widely grown landraces and high yielding varieties are susceptible to blast with yield losses of 10–50% being common [3] and losses can be as high as 80–90% in the endemic areas [8]. The disease affects the crop at all growth stages from seedling to grain formation, with panicle blast being the most destructive form of the disease [9,10]. *M. grisea* is pathogenic to more than 50 graminaceous hosts including food security crops such as rice, wheat, finger millet, pearl millet and foxtail millet [11,12]. Despite the wide host range of the pathogen, *M. grisea* populations mainly exist as host-specific (adapted) forms, capable of infecting a single host [13,14]. While some researchers have demonstrated successful infection of a host by an isolate from a different host under experimental conditions [15,16], others failed to confirm the results [13].

In the case of finger millet, blast management through host resistance is very economical and relevant for the resource-poor and marginal farmers who cannot afford other methods of disease control such as use of expensive chemical fungicides. However, resistance breakdown is a great challenge while breeding for blast resistance in finger millet because of pathogenic variation in

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M. grisea. It is important not only to develop cultivars with durable resistance, but also to monitor virulence change in the pathogen populations to anticipate resistance breakdown in existing finger millet cultivars, and to design strategies to sustain cultivation of high yielding, farmer and consumer preferred cultivars [17]. Lack of knowledge on the pathogen adapted to finger millet in India has hindered efforts towards identification and development of resistant cultivars adapted to local agro-ecological conditions. Consequently, research efforts have focused on understanding the *M. grisea* population structure by combining modern molecular-biotechnological approaches with traditional pathological assays. Substantial work has been done in the rice-blast pathosystem, whereas such studies are very limited for the finger millet-blast pathosystem [3,7,14]. In order to measure genetic variability more precisely, molecular markers that provide an unbiased estimate of total genomic variation and have the potential to minimize errors due to sampling variance have been developed [18]. Furthermore, determination of fungal genetic diversity based on molecular markers is reliable as it is independent of culture conditions. DNA fingerprinting techniques have created new tools for the molecular analysis of *Magnaporthe oryzae* populations [19] and this is equally applicable to *M. grisea* populations adapted to finger millet.

Assessment of genetic diversity in *M. grisea* from different crops has mostly relied on use of clones of the transposon MGR as a probe to detect restriction fragment length polymorphism (RFLP), which is an expensive and time-consuming approach. Simple sequence repeats (SSRs) or microsatellites are PCR-based molecular markers, which may be more desirable for population genetic analysis because this approach makes it simpler to obtain accurate polymorphic data due to co dominance. Besides, these markers are highly reproducible, locus-specific, multi-allelic and abundant in animal, plant and microbial genomes [20]. Although generation of SSR markers is a time-consuming, labor-intensive and expensive task, several SSR markers have already been developed for *M. grisea* infecting rice [21–24]. However, SSRs have not been used to investigate pathogen populations adapted to finger millet. Prior few studies have examined genetic diversity in finger millet-infecting populations of *M. grisea* using MGR-RFLP [14], AFLP [3] and RAPD markers [7]. Here, we analyzed finger millet infecting populations of *M. grisea*, collected from Andhra Pradesh, Bihar and Karnataka, India along with *M. grisea* isolates from pearl millet, foxtail millet and rice using SSR markers to (i) assess extent of genetic diversity in finger millet-infecting populations of *M. grisea* (ii) investigate genetic relatedness among *M. grisea* populations adapted to finger millet, foxtail millet, pearl millet and rice.

2. Material and methods

2.1. Pathogen isolates

Blast infected (leaf, neck and finger) samples of finger millet, foxtail millet and rice were collected from Vizianagaram, Patancheru, and Nandyal in Andhra Pradesh, Mandya and Naganahalli in Karnataka, and Dholi in Bihar, India during 2008–10 rainy seasons (Table 1). In addition, seven *M. grisea* isolates from four major pearl millet growing states in India – Rajasthan, Haryana, Maharashtra and Uttar Pradesh [25] were also included in this study (Table 1). Isolations of *M. grisea* were made from the blast-infected tissue on oatmeal agar medium (rolled oats 50 g, agar 15 g, distilled water 1 L) and incubated at 25 ± 1 °C for 15 days. After incubation, a dilute spore suspension (3 × 10^3 spores/ml) was prepared in sterile double-distilled water and plated onto 4% water agar in Petri plates. Single germinating conidia were marked after 10–12 h of incubation under a microscope and transferred to test tubes containing oatmeal agar for further studies.

2.2. Isolation of genomic DNA

Isolates of *M. grisea* were grown in 2X yeast extract glucose (YEG) medium [14] in shake culture for 7–10 days at 25 °C. Mycelia were harvested by filtration through Whatman filter paper No. 1, dried on blotting papers and ground to a fine powder in liquid nitrogen with a pre-cooled pestle and mortar. Genomic DNA was extracted from 200 mg of powdered mycelium of each isolate using CTAB (cetyltrimethylammonium bromide) method as suggested by Viji et al. [14]. The quantity and quality of the extracted DNA were assessed by running the DNA on 1% agarose gel, stained with ethidium bromide and photographed under UV illumination.

2.3. SSR genotyping

Twenty-four SSR markers (*Pyrms 7-8*, *Pyrms 15-16*, *Pyrms 33-34*, *Pyrms 37-38*, *Pyrms 39-40*, *Pyrms 41-42*, *Pyrms 43-44*, *Pyrms 45-46*, *Pyrms 47-48*, *Pyrms 59-60*, *Pyrms 61-62*, *Pyrms 63-64*, *Pyrms 67-68*, *Pyrms 77-78*, *Pyrms 81-82*, *Pyrms 83-84*, *Pyrms 87-88*, *Pyrms 93-94*, *Pyrms 99-100*, *Pyrms 101-102*, *Pyrms 107-108*, *Pyrms 109-110*, *Pyrms 115-116* and *Pyrms 125-126*) [22] were used for analyzing the SSR diversity in *M. grisea* isolates (Table 2). The forward primers were synthesized by adding M13-forward primer sequence (5′CACGACG TTG TAAAACGAC3′) at the 5′end of each primer. PCR was performed in 5 μl reaction volume with final concentrations of 5 ng of DNA, 2.5 mM $MgCl_2$, 0.2 mM of dNTPs, 1× PCR buffer, 0.006 pM of M13-tailed forward primer, 0.09 pM of M13-Forward primer labeled with either 6-Fam or Vic or Ned or Pet (Applied Biosystems), 0.09 pM of reverse primers and 0.1 U of *Taq* DNA polymerase (SibEnzyme Ltd., Russia) in a GeneAmp®PCR System 9700 thermal cycler (Applied Biosystems, USA) with the following cyclic conditions: initial denaturation at 94 °C for 3 min then 10 cycles of denaturation at 94 °C for 1 min, annealing at 61 °C for 1 min (temperature reduced by 1 °C for each cycle) and extension at 72 °C for 1 min. This was followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min and extension at 72 °C for 1 min with the final extension of 10 min at 72 °C. The PCR products were tested for amplification on 1.2% agarose.

Based on their expected amplicon size and/or dye, PCR products were pooled together along with internal size standard (GeneScan™ 500 LIZ® from Applied Biosystems) and capillary electrophoresis was carried out using ABI 3730*xl* Genetic Analyzer (Applied Biosystems, USA). Raw data produced from the ABI 3730*xl* Genetic Analyser was analyzed using Genemapper software (Applied Biosystems, USA) and fragment size was scored in base pairs (bp) based on the relative migration of the internal size standard.

2.4. Determination of allele frequency and diversity analysis

The alleles for each SSR locus across the samples were scored in terms of fragment length of the PCR amplified product in base pairs and used to calculate the basic statistics such as polymorphic information content (PIC), allelic richness as determined by a total number of the detected alleles, major allele frequency (M_{AF}), number of alleles per locus, gene diversity (GD), heterozygosity (H) and occurrence of unique, rare, common, frequent and most frequent alleles using PowerMarker version 3.25 [26]. These estimates were performed across all the *M. grisea* isolates, and separately among isolates from different hosts. Unique alleles are those that are present in one isolate or one group of isolates but absent in other isolates or group of isolates. Rare alleles are those whose frequency is ≤1% in the investigated isolates. Common alleles have >1%–20% frequency while those occurring with >20–50% and

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