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ORIGINAL ARTICLE

Molecular variability of finger millet blast pathogen of *Magnaporthe grisea* by Random Amplified Polymorphic DNA analysis

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ABSTRACT
In the present study twenty four Magnaporthe grisea infected samples were collected from major finger millet growing areas of Tamil Nadu and AICSMIP centers in India and four other blast isolates from various crops viz., rice, foxtail millet, kollukattai grass and dub grass. For genetic variability analysis of M. grisea isolates, twenty RAPD primers with 10 nucleotides long were used (OPA01, OPA02, OPA09, OPB01, OPC20, OPE04, OPF01, OPF05, OPF06, OPF07, OPF08, OPF10, OPF11, OPF12, OPF14, OPG19, OPZ13, OPZ19, C3 and R3). RAPD markers used in this investigation increased the marker density for finding out genetic relationships in blast pathogen from eleven different geographical regions in Tamil Nadu and AICSMIP centers. Similarity coefficient values were then utilized to cluster the data using UPGMA dendrogram revealed that the existence of two principal clusters (viz., A and B) with two sub clusters in A (A1, A2) and B (B1, B2). The isolates of M. grisea from finger millet blast infected samples collected from different geographical locations were distinctly different from rice, foxtail millet, kollukattai grass and dub grass isolates of M. grisea. However, there was no variation among the isolates of finger millet irrespective of geographical regions as they have been placed in one cluster. It is concluded that Indian finger millet pathogen and rice blast, foxtail millet, kollukattai grass and dub grass fungus may be genetically heterogeneous and the inter relationships amongst the different isolates can be easily, precisely and reliably done using RAPD-PCR technology.
Keywords: *Blast, PCR, RAPD, Magnaporthe grisea*

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INTRODUCTION

Finger millet (*Eleusine coracana* L.) is one of the most important millet crops belonging to the family Poaceae and sub family Chloridoidea [3]. Among the small millets, finger millet is widely grown traditional grain cereal cultivated in semi-arid areas of East and Southern Africa and South Asia as a stable food for millions of poor population. In India is cultivated in the states of Karnataka, Andhra Pradesh and Tamil Nadu and northern hills [21]. The total area under finger millet production in India is about 2.8 m.ha with an annual production of about 2.78 mt. [13]. The crop constitutes about 81 per cent of the miner millets produced in India [7]. Finger millet blast caused by *Magnaporthe grisea* (Hebert) Barr. (anomorph *Pyricularia grisea* (Cooke) Sacc. is a heterothallic, filamentous fungus, one of the major destructive disease causing excessive damage to this crop from seedling to ear head forming stages. The disease occurs during all growing seasons and on almost all finger millet varieties cultivated. *M. grisea* parasitizes over 50 grasses, including economically important crops like wheat, rice, barley and millet [14]. Yield loss due to blast can be as high as 50% when the disease occurs in epidemic proportions. Use of resistant varieties is the most economical and effective method of controlling finger millet blast mainly in resource-poor farmer's fields. Unfortunately, blast fungus is able to overcome the resistance within two to three years after these plants are cultivated widely [1]. The breakdown in resistance has been attributed to the high variability of the pathogen and there are numerous reports that this diversity may

be due to continuous generation of novel pathogenic variation. Knowledge of the genetic variation within and among populations is an important component of understanding the population biology of pathogenic fungi and infers the impact of driving force influencing the evolution of pathogen populations [10]. Therefore, information on population diversity can be used for developing strategies to increase the durability of resistance [25, 26]. Molecular markers play a major role in analyzing the genetic diversity and variation of fungal pathogens. Among the molecular markers, random amplified polymorphic DNA (RAPD) is the widely used effective marker for assess the genetic variation of fungal pathogens [11; 16]. Thus, the RAPD markers have become efficient to identify the genetic variations between the *M. grisea* isolates.

In the present study, *M. grisea* samples were collected from different geographical regions of Tamil Nadu and AICSMIP centers. The single spores of *M. grisea* were isolated and maintained in the laboratory using Oat meal agar. The genomic DNA was isolated using standard cetyl trimethyl ammonium bromide (CTAB) protocol with minor modifications 20 random primers (10 mer) were used to generate genomic finger prints of twenty four *M. grisea* isolates of different geographical regions and other blast isolates from various crops *viz.,* rice, foxtail millet, kollukattai grass and dub grass. All RAPD markers depicted high polymorphism (50 - 90%). The banding patterns generated by these markers were used to generate "squared Euclidean distances". The linkage distances were further used to construct a dendrogram using "unweighted pair group averages" and Non-metric dendro scaling analysis. This study revealed that populations of *M. grisea* in hilly geographical region of Tamil Nadu and AICSMIP centre (India), may be genetically heterogeneous and the interrelationship amongst the different isolates can be easily, precisely and reliably explained by RAPD polymerase chain reaction (PCR) technology.

MATERIAL AND METHODS

Samples of infected leaves, necks and fingers were collected from eleven location *viz.,* Krishnagiri, Dharmapuri, Coimbatore, Ranchi, Dholi, Vizianagaram, Dindori, Berhampur, Sisai, Rewa and Bangalore (Table 1). The collected samples were packed in paper bags and stored at 4°C till further use. Twenty four monoconidial isolates were obtained by directly transferring conidia (from one lesion per leaf/neck/finger of a plant) on petri plates having Oat meal agar medium. For DNA extraction, the fungus was grown in Richard's broth medium. After seven days of incubation at room temperature, fungal mycelium was harvested by filtration through Whatman filter paper and washed with distilled water. The mycelial mats were transferred to sterilized butter papers (150 x 100 mm size) and freeze dried for 16 hours at -400 ^{0}C, in a lyophilizer. Total DNA was extracted following the procedure of (Murray and Thompson, 1980) for plant DNA with modifications for mini-scale preparation as described by (Scott *et al.,* 1993). The concentration of the DNA was measured by using U. V. spectrophotometer (Thermo Spectronic - Biomate 5) and adjusted to 40ng/µL and quality was assessed by a mini-gel electrophoresis (1.0 % agarose gel).

Genomic DNA Isolation

Cetyltrimethyl ammonium bromide (CTAB) method was adopted to extract the total DNA from the mycelial mat of *M. grisea*. A nine mm hyphal plug from the edge of the actively growing mycelium was transferred and cultures were grown in 250 ml conical flasks containing 100 ml Richard's broth at room temperature (25 ± 1 °C) for 15 days. The culture filtrate was removed and the mycelia were blot dried. Mycelium (1 g) was ground in a pestle and mortar using liquid nitrogen. Powdered mycelia were mixed in pre-warmed (65 °C) extraction buffer (500µl) consisting of 100 mM Tris (pH 8.5), 250 mmol l $^{-1}$ NaCl, 0.5 mmol l^{-1} EDTA and 0.5% SDS. The samples were incubated at 65 °C for 30 minutes followed by the addition of 5 mol l^{-1} potassium acetate solution (final concentration 1.7mol l^{-1}). Then the tube was incubated at 60°C for 30 minutes. During the incubation, the tubes were vortexed for 5 seconds for complete mixing of the buffer and spores. After incubation, the tubes were spun at 10000 rpm for 3 minutes and the supernatant was collected. To this equal volume of chloroform and isoamyl alcohol (24: 1) (v/v) was added and incubated for 30 minutes in a horizontal position. Then the tubes were centrifuged at 10000 rpm for 10 minutes and the aqueous phase was transferred to new eppendorf tube and the above step was repeated to extract the entire DNA. To the pooled supernatant equal volume of isopropanol was added and incubated for 10 minutes and centrifuged the tubes at 10000 rpm for 3 minutes. The obtained DNA precipitate was washed with 70 per cent ethanol (centrifuged for 3 minutes at 10000 rpm) and the pellet was dried and finally dissolved in 50 µl of the sterile distilled water. The final concentration of DNA in the extract was tested for the amount of DNA by 1.5 per cent agarose gel electrophoresis [9].

RAPD-PCR analysis

RAPD-PCR analysis was performed to all the isolates. Twenty DNA primers, 10 nucleotides long (Table 2) were used for the experiment (OPA01, OPA02, OPA09, OPB01, OPC20, OPE04, OPF01, OPF05, OPF06, OPF07, OPF08, OPF10, OPF11, OPF12, OPF14, OPG19, OPZ13, OPZ19, C3 and R3). DNA from each fungal isolate was screened for RAPD markers generated by random decamer primers (Operon, Inc., Alameda, CA) and amplified by PCR in 25-µl reactions using the method described by Vandemark *et al*. [22]. Reactions were resolved by agarose gel (1.4 per cent w/v) electrophoresis, stained with ethidium bromide, visualized under ultraviolet light and recorded with an Alpha Imager 2000 (Alpha Innotech, San Leandro, CA). RAPD-PCR was carried out using an Agilent SureCycler 8800 gradient thermal cycler programmed for one cycle of denaturation at 94°C for five minutes followed by 40 cycles of denaturation at 94°C for one minute, annealing at 37°C for one minute, extension at 72°C for two minutes and a final extension at 72°C for five minutes. Each amplification mix (25 µl) contained template DNA (50 ng), ampli-taq polymerase (1unit), primer 20 µM, dNTPs (200 µM), PCR buffer [1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris (pH 8.0), BSA (2 mg/ml)] and sterile distilled water.

Agarose gel electrophoresis

The RAPD-PCR products were separated in 1.5 per cent (w/v) agarose gel in 1x TAE buffer (0.4 MTris, 0.2 M acetic acid, 10 mM EDTA; pH 8.4) containing 0.5 µg/ml ethidium bromide. The PCR product along with gel loading buffer (6x containing 0.25 per cent bromophenol blue, 0.25 per cent xylene, cyndol FF and 3 per cent glycerol) was loaded and electrophoresis was carried out at 90 V. Then, the gel was viewed in an UV illuminator.

Data analysis

DNA banding pattern generated by RAPD primers were scored as "1" for presence of an amplified band and "0" for its absence. All gels were scored twice manually and independently. Presence or absence of unique and shared polymorphic bands was used to generate "Suared Euclidean Distances". The non metric dendro scaling analysis is a common estimator of genetic identity or estimation of interspecific relationships. Similarity coefficients were used to construct the UPGMA (Unweigted pair group method with arithmetic means) dendrogram [5].

RESULT AND DISCUSSION

Twenty four *M. grisea* isolates from finger millet and four other blast isolates from various crops *viz.,* rice, foxtail millet, kollukattai grass and dub grass were used to study the genetic variability through RAPD analysis using random primers**.** RAPD analysis with twenty random primers amplified DNA fragments with different sizes (Plate1). Similarity coefficient values were then utilized to cluster the data using UPGMA dendrogram revealed that the existence of two principal clusters (*viz.,* A and B) and each with two sub clusters in A (A1, A2) and B (B1, B2). The finger millet blast infected samples collected from different geographical locations were distinctly related with blast infected rice, foxtail millet, kollukattai grass and dub grass samples. The sub cluster A1 comprised of the samples from JAFB20 and MANB23, while A2 had all other finger millet blast infected samples *viz.,* ANNB14, BIFB13, ODNB17, KALB24, JANB19, MALB22, JANB18, MALB21, TNLB1, TNLB2, MANB15, ODNB16, TNLB3, TNNB8, TNLB4, TNNB5, TNNB6, JALB9, BINB12, TNNB7, JANB10, BILB11. The sub cluster B1 had blast infected samples of rice and foxtail millet and the sub cluster B2 comprised of blast infected kollukattai grass and dub grass. From dendrogram it could be deducted that finger millet blast pathogen from different geographical locations have 65 to 90 per cent similarity while, 50 – 60 per cent similarity was observed within the out groups of samples *viz.,* rice, foxtail millet, kollukattai grass and dub grass (Fig. 1). RAPD marker based genetic analysis have clearly clustered the blast pathogen from finger millet and other host blast pathogen based on geographic distance clearly. Non-metric dendro scaling analysis of RAPD marker showed *M. grisea* isolates collected from finger millet are distinct group and further all the finger millet isolates were diverse from rice, foxtail millet, kollukattai grass and dub grass (Fig. 2).

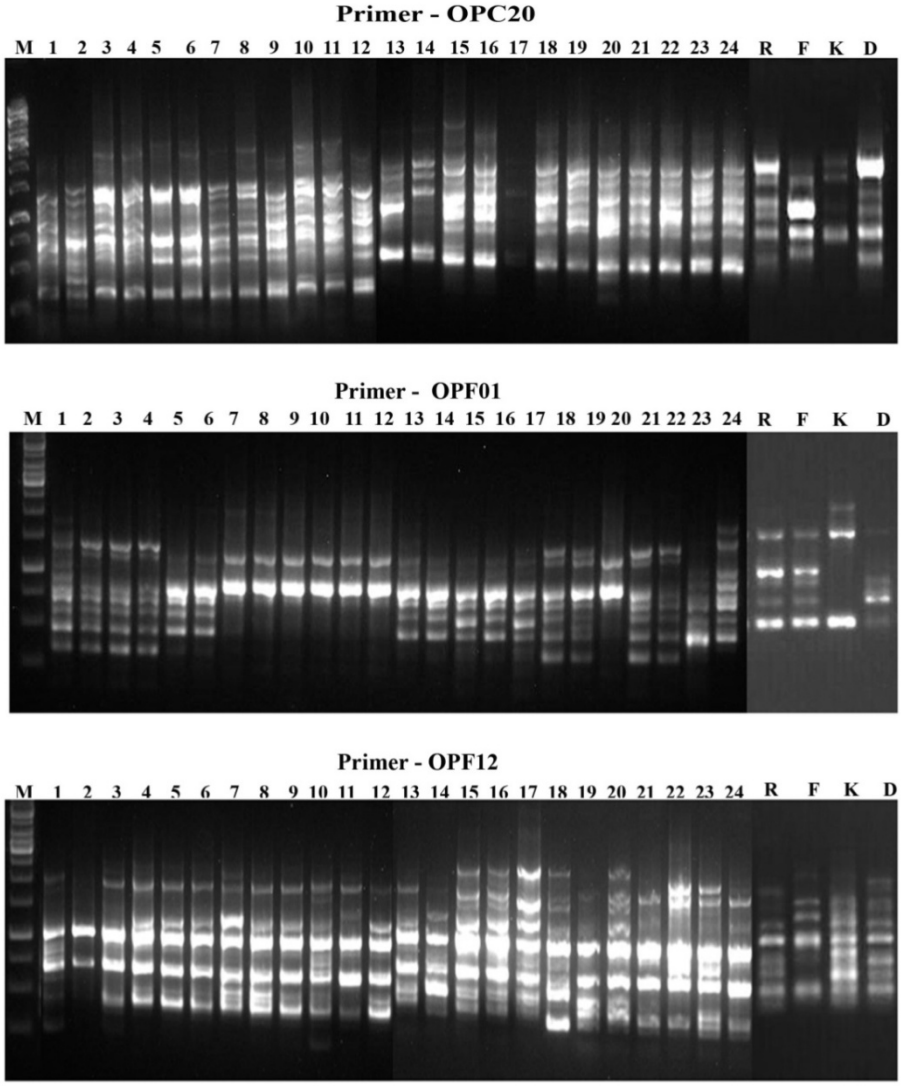
Table 1. Collection of *M. grisea* isolates from different regions of India

S. No.	Isolates	Location	States	Plant parts	Variety	Season
1	TNLB1	Krishnagiri	Tamil Nadu	Leaf	CO14	Kharif 2012
2	TNLB2	Krishnagiri	Tamil Nadu	Leaf	CO14	Kharif 2012
3	TNLB3	Dharmapuri	Tamil Nadu	Leaf	CO14	Kharif 2012
4	TNLB4	Coimbatore	Tamil Nadu	Leaf	CO14	Kharif 2012
5	TNNB5	Krishnagiri	Tamil Nadu	Neck	GPU28	Kharif 2012
6	TNNB6	Krishnagiri	Tamil Nadu	Neck	GPU28	Kharif 2012
7	TNNB7	Dharmapuri	Tamil Nadu	Neck	GPU28	Kharif 2012
8	TNNB8	Coimbatore	Tamil Nadu	Neck	KM252	Kharif 2012
9	JALB9	Ranchi	Jharkhand	Leaf	RAU8	Kharif 2012
10	JANB10	Ranchi	Jharkhand	Neck	RAU8	Kharif 2012
11	BILB11	Dholi	Bihar	Leaf	PR202	Kharif 2012
12	BINB12	Dholi	Bihar	Neck	PR202	Kharif 2012
13	BIFB13	Dholi	Bihar	Finger	PR202	Kharif 2012
14	ANNB14	Vizianagaram	Andhra Pradesh	Neck	VR 708	Kharif 2012
15	MANB15	Dindori	Madhya Pradesh	Neck	GPU28	Kharif 2012
16	ODNB16	Berhampur	Odisha	Neck	KM252	Kharif 2012
17	ODNB17	Berhampur	Odisha	Neck	KM252	Kharif 2012
18	JANB18	Sisai	Jharkhand	Neck	Local	Kharif 2012
19	JANB19	Sisai	Jharkhand	Neck	Local	Kharif 2012
20	JAFB20	Sisai	Jharkhand	Finger	Local	Kharif 2012
21	MALB21	Rewa	Madhya Pradesh	Leaf	Local	Kharif 2012
22	MALB22	Rewa	Madhya Pradesh	Leaf	Local	Kharif 2012
23	MANB23	Rewa	Madhya Pradesh	Neck	Local	Kharif 2012
24	KALB24	Bangalore	Karnataka	Leaf	GPU64	Kharif 2012

Table. 2 Amplification performance of oligonucleotide on the isolates from *Magnaporthe grisea*

S.No.	Primer code	Nucleotide sequence
1	OPA-01	5'CAGGCCCTTC3'
2	OPA 02	5' TGCCGAGCTG 3'
3	OPA-09	5'AGTCAGCCAC3'
4	OPB-01	5'GTTTCGCTCC3'
5	OPC-20	5'ACTTCGCCAC3'
6	OPE-04	5'GTGACATGCC3'
7	OPF-01	5'ACGGATCCTG3'
8	OPF-05	5'CCGAATTCCC3'
9	OPF-06	5'GGGAATTCGG3'
10	OPF- 07	5'CCGATATCCC3'
11	OPF-08	5'GGGATATCGG3'
12	OPF -10	5'GGAAGCTTGG3'
13	OPF -11	5'ACGGTACCAG3'
14	OPF -12	5'GGCTGCAGAA3'
15	OPF-14	5'TGCTGCAGGT3'
16	OPG-19	5'GTCAGGGCAA3'
17	OPZ-13	5' GACTAAGCCC 3'
18	OPZ-19	5'GTGCGAGCAA3'
19	C3	5'CGGCTTGGGT3'
20	R3	5'TGCCGAGCTG3'

Plate 1. RAPD analysis of *M. grisea* isolates with random primers



Lane:

M - 1kb ladder	8.TNNB8	16. ODNB16	24. KALB24
1. TNLB1	9. JALB9	17. ODNB17	25. R - Rice
2. TNLB2	10. JANB10	18. JANB18	26. F - Foxtail millet
3. TNLB3	11. BILB11	19. JANB19	27. K - Kollukattai grass
4. TNLB4	12. BINB12	20. JAFB20	28. D - Dub grass
5. TNNB5	13. BIFB13	21. MALB21	
6. TNNB6	14. ANNB14	22. MALB22	
7. TNNB7	15. MANB15	23. MANB23	

The analysis of RAPD polymorphism in isolates of *M. grisea* from different regions across India revealed the occurrence of high level of polymorphism, indicating a wide and diverse genetic base. A repeat sequence termed MGR586 was identified in the genome of rice infecting strains of *M. grisea* [17] This sequence has been widely used for DNA fingerprinting of *M. grisea* to investigate the epidemiology of the rice blast disease [6, 23]. Another retrotransposon, *fosbury* has also been used for genetic differentiation studies and the results indicate that isolates from Bangladesh lack both MGR586 and *fosbury*. MGR586 probe also failed to detect karyotypic changes [25]. Thus there is a need to develop different DNA fingerprinting techniques to identify various forms of *M. grisea* diversity. RAPD markers used in this investigation increased the marker density for finding out genetic relationships. The dendrogram study revealed that the geographic origin of strains does not play crucial role in lineage formation, as in each

lineage (group), there were mixed populations of the eleven geographical regions. Similar result was obtained from other fungal pathogen as well. Chadha and Gopalakrishna [2] studied the genetic diversity of the rice isolates of *M. grisea* and were found to exhibit 64 per cent polymorphism. Overall, the random amplified polymorphic DNA patterns did not show high level of polymorphism which was opined by Zhou *et al*. [27]. Singh and Kumar [19] carried out a study to generate genomic finger prints using random amplified polymorphic DNA markers as well as to find out genetic diversity in *M. grisea* isolates collected from three different geographical regions (hilly area) of Uttarakhand. A total of forty five isolates and fifteen RAPD primers were used to generate genomic fingerprint profile which depicted about 25 to 40% linkage distance and resulted in formation of two major groups. Polymorphism range shown by RAPD primers was 71.40 to 90%, while the range of total loci scored was from 07 to 10. The molecular weight of scorable loci ranged from 150 to 2500 bp which indicated the genetic diversity and virulence complexity of rice blast fungus among samples tested. Shanmugapackiam *et al*. [16] who studied the genetic variability of *M. grisea* isolates collected from different locations found significant variations between the isolates. Similar pattern of genetic variability of *M. grisea* in rice was obtained by Madhavan *et al.* [8]. RAPD profile showed that minimum and maximum per cent similarities of *P. oryzae* isolates were in the range of 35 to 80 per cent respectively. The cluster analysis by unweighted pair group method with arithmetic average (UPGMA) separated the *P.oryzae* isolates into two major groups at 0.53 of similarity coefficient [4, 20]. These results can be used for screening resistance in commercial finger millet varieties in areas where there is high risk of *M. grisea* infection. On the basis of the present study, it is concluded that the Indian population of finger millet blast like rice blast fungus may be genetically heterogeneous and the interrelationships amongst the different isolates can be easily, precisely and reliably explained by RAPD-PCR technology.

Fig.1. Dendrogram showing the molecular variability of *M. grisea* isolates

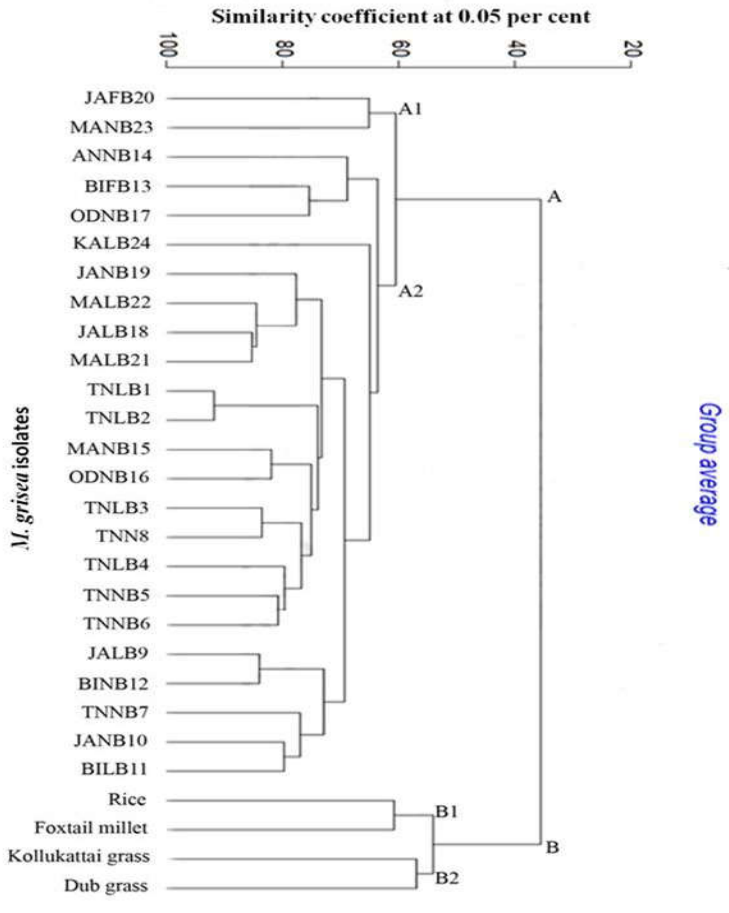
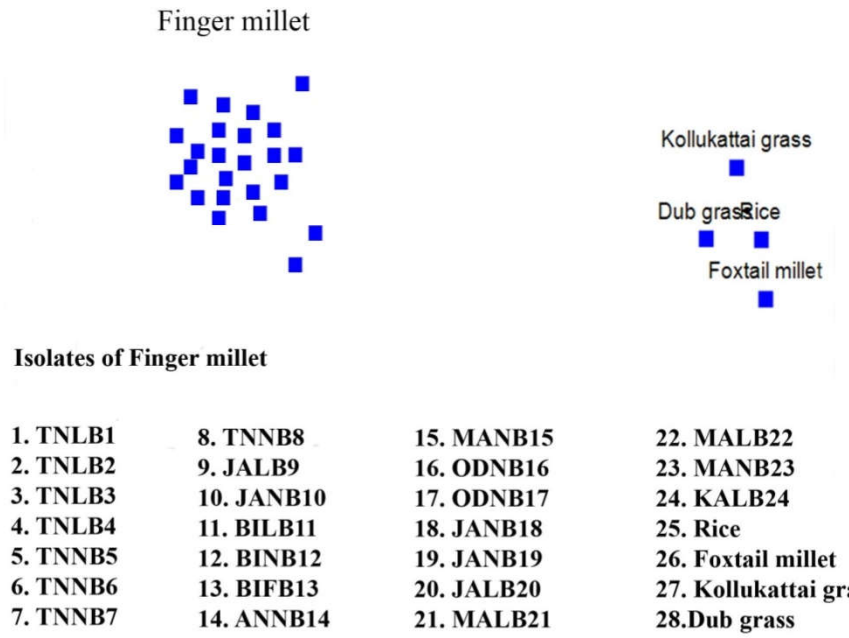


Fig. 2. Non-metric dendroscaling alaysis of host species of *M. grisea* isolates



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