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Genetic diversity and population structure of *Fusarium fujikuroi* causing Bakanae, an emerging disease of rice in India

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Bakanae caused by *Fusarium fujikuroi* (Nirenberg), is emerging as a serious threat for rice (*Oryza sativa.* L.) cultivation in India. In this study, 63 isolates of *Fusarium fujikuroi* isolated from symptomatic diseased plants were characterized for their morphology, pathogenicity and molecular variability using universal rice primers (URP). Of the 12 URPs used in the study, 6 primers could produce polymorphic fragments in all the isolates. The URP 17R primer was highly polymorphic (100%), whereas, the URP 1F primer produced 75% polymorphic fragments. A dendrogram obtained from the combined analysis of 6 URP primers categorized the isolates into four clusters, where most of the isolates from Punjab and Haryana were clustered separately. Mating type of the population was identified based on MAT-1 and MAT-2 region universal primers for *Gibberella fujikuroi*. Among the 63 isolates, 18 (28.57%) were identified as MAT-1 and 45 (71.42%) as MAT-2. The effective population number for mating type was 89% of the total population. Since the distribution frequencies of both mating types were not equal in the Indian population of *F. fujikuroi*, it could conclude that majority of the multiplication of isolates under field conditions was through asexual reproduction. However, the presence of both mating types in *F. fujikuroi* indicates that the population is also capable of sexual reproduction. Therefore, it is important to develop cultivars with inbuilt resistance to bakanae disease, taking into consideration the factors such as environmental conditions and variability of the pathogen in the area of intended cultivation.

Keywords: Conidia, *Gibberella fujikuroi*, *Oryza sativa*, Rice, URP primers

Bakanae is caused by *Fusarium fujikuroi* (Nirenberg), [telomorph: *Gibberella fujikuroi* (Sawada) Ito] and is emerging as a serious disease affecting rice (*Oryza sativa*. L.) in India, Japan, Taiwan, Thailand, China, Pakistan, Bangladesh and Nepal[1-6]. The symptoms of bakanae disease are characterized by plants that are several inches taller as well as thinner and yellowish compared to normal plants in the nursery and field. Infected seedlings also show dieback at later stages and if the plants survive, they are partially filled with sterile or chaffy grains at maturity. In India disease incidence of up to 40% was reported in the Kapurthala, Ropar, Patiala, Ludhiana, Amritsar, Gurdaspur, and Hoshiarpur districts of Punjab[6]. Recent changes in climate and cropping patterns have aggravated the disease, and the incidence of bakanae disease has increased steadily, particularly in Basmati rice cultivars in north-western India, including Punjab, Haryana, and western parts of Uttar Pradesh[6]. The disease causes both qualitative and quantitative yield losses to rice crops under field conditions.

Different molecular markers have been used for identification of various *Fusarium* species (*Gibberella fujikuroi* species complex) associated with rice seed responsible for bakanae disease[7-9]. URP primers that were derived from repetitive DNA sequence of rice weedy rice have been applied for producing PCR polymorphisms in different fungal species. URP-PCR is applicable for 33 genus and 142 species of the fungi[10]. However, only *F. fujikuroi* was predominant in symptomatic diseased plants under field conditions[6]. *Fusarium fujikuroi* produces micro- and macro-conidiophores bearing micro- and macro-conidia, respectively. The microconidia contain 1-2 cells and are fusiform oval, agglutinated in chains, and remain joined or cut off in false heads. The macroconidia are delicate, slightly sickle-shaped, or

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nearly straight. They are narrow at both the ends and are occasionally bent into a hook-like structure at the apex and distinctly or slightly foot-celled at the base[11]. An understanding of the pathogen's population structure, its aggressiveness, and its genetic diversity is essential for the development and implementation of effective management strategies. However, there are no systematic studies on the characterization of *F. fujikuroi* populations affecting rice in India.

Further, presence of two mating types i.e.MAT-1 and MAT-2 is required for sexual reproduction in *F. fujikuroi*, and the distribution frequency of these types affects the stability of the population[12]. Cumagun reported different mating types in *F. verticillioides* from the Philippines[13]. However, distribution of mating types in the Indian population of *F. fujikuroi* is unknown. Therefore, in the present study, we determined the morphological, pathological, and molecular diversity, as well as the mating types of the *F. fujikuroi* population isolated from symptomatic bakanae-diseased rice plants in India.

Material and Methods

Diseased samples from bakanae infected rice fields were collected from farmer's fields from different states in India as described by Bashyal *et al.*[14]. Further, the field studies did not involve endangered or protected species.

Fungal isolates and maintenance

Sixty-three different *Fusarium*-infected rice samples were collected from rice fields of different states (Fig. 1) of India (Punjab, Haryana, Uttarakhand, Jammu and Kashmir, western Uttar Pradesh, and Bihar). The geographical coordinates, place of collection of isolates and other details are presented in Supplementary Table 1. Pathogens were isolated from the stems and roots of symptomatic rice plants. Samples were cut into 1-cm^2 pieces, surface-sterilized by immersion in 70% ethanol for 1 min, transferred to a 1% aqueous solution of NaOCl for 3 min, rinsed twice with distilled water, and dried by laminar air flow on Petri-dishes containing sterile filter paper. Next, the samples were placed on Potato Dextrose Agar (PDA) medium. The plates were incubated at 25°C for 5-7 days. A single mycelial tip taken from the growing edge of the fungus was transferred to PDA slants and incubated at 25°C for 10-15 days until full mycelium growth and thereafter maintained at 4°C until further analysis.

Cultural and morphological variability

To study cultural and morphological variability, all 63 isolates of *F. fujikuroi* were grown on PDA and carnation leaf agar (CLA) medium. Radial growth was measured on PDA medium in 9 cm Petri dishes (15 mL/plate) with 3 replications per isolate, as

Fig. 1 — Geographical distribution of *Fusarium fujikuroi* isolates taken for the study

Table 1 — Details of Universal Rice Primers (URP) used to amplify DNA of *Fusarium fujikuroi* isolates

Primer	Primer sequence (5' to 3')	Total no. of bands	Polymorphic bands	Monomorphic bands	Polymorphism (%)	Band size (kb)
URP 1F	ATCCAAGGTCCGAGACAACC	8	6	2	75	0.25–5
URP 9F	ATGTGTGCGATCAGTTGCTG	12	11	1	91.67	0.25–5
URP 32F	TACACGTCTCGATCTACAGG	14	14	0	100	0.5–5.0
URP 6R	GGCAAGCTGGTGGGAGGTAC	15	15	0	100	0.5–7
URP 13R	TACATCGCAAGTGACACAGG	9	9	0	100	0.5–5
URP 17R	AATGTGGGCAAGCTGGTGGT	16	16	0	100	0.25–7
	Total	74	71	3	94.44	

described by Lilly & Barnett[15]. Inoculated plates were incubated at 25°C in a biochemical oxygen demand incubator for 5 days. The presence of macroconidia was observed by growing the isolates on CLA. All isolates were tested in a single test. Experiment was designed in complete randomized design with 5 replications per isolate and data was statistically analyzed using SPSS software (SPSS, Inc., Chicago, IL, USA).

Pathogenic variability

Pathogenic variability in the *F. fujikuroi* isolates was studied based on their ability to induce bakanae on rice seedlings of the susceptible cultivar, Pusa Basmati 1509. Isolates were allowed to grow on the PDA for 15 days and fully grown Petri plates were flooded with water and the mycelia were scraped with a sterile slide to form a suspension. The suspension was filtered through sterile muslin cloth and the final concentration was prepared to 10^6 spores/mL. Seeds of rice genotype Pusa Basmati 1509 were surface disinfected with 1% sodium hypochlorite (seeds were dipped in sodium hypochlorite solution for 3 min and rinsed with sterile water), air dried and inoculated with conidial suspension. Hundred rice seed inoculated with one isolate were sown in pots containing sand and soil in the ratio of 3:1. The experiment was conducted in the glasshouse and temperature was maintained as 28-30°C during the day and 20-25°C at night. Observations for the bakanae disease were taken after 15 and 30 days of inoculation as disease severity which was calculated as no. of plants infected over total no. of plants observed[14]. The experiment was conducted in the randomized block design with 20 seed per replication and 5 replications per isolate.

Molecular variability

Genomic DNA extraction

The isolates of *F. fujikuroi* were grown in potato dextrose broth for 5 days at 25±1°C in a biochemical oxygen demand incubator. Mycelia were filtered through Whatman No.1 filter paper and genomic DNA was extracted following the CTAB method[16]. The mycelium was ground in liquid nitrogen, transferred into DNA extraction buffer (0.1 M Tris, 1.5 M NaCl, 0.01 M EDTA), and stored at 65°C for 1 h with occasional stirring. A similar volume of chloroform: isoamyl alcohol (24:1) was added to each tube and the tubes were centrifuged at 12857 ×g (eppendorf 5804 R, Germany). The upper aqueous layer was carefully removed and precipitated with 0.6 volume of ice-cold isopropanol. The precipitate was again centrifuged at 10,000 rpm at 4°C and the pellet obtained was washed with 70% ethanol. Excessive ethanol was removed by drying at room temperature (25°C), and the pellet was dissolved in TE buffer and stored at –20°C until further use.

Universal rice primers and polymerase chain reaction

The universal rice primers (URPs) include 12 primers, 20-oligonucleotides each, originally derived from repetitive sequences of weedy rice[10]. Primers were synthesized by GCC Biotek Ltd. West Bengal, India. The polymerase chain reaction (PCR) was optimized by varying the concentration of template DNA (25, 50, and 100 ng), Taq DNA polymerase (0.5 and 1.0 U), and $MgCl_2$ (1.5, 2.5, and 3.5 mM). PCR was performed in a temperature gradient thermal cycler (Bio-Rad, Hercules, CA, USA). Primers showing reproducible and scorable amplification products were used for molecular characterization of all isolates. The URP-PCR products were then resolved on a 1.2% agarose gel containing ethidium bromide (0.5 µg/mL) in TAE buffer (pH 8.0) along with 1-kb DNA ladder (MBI Fermentas, Vilnius, Lithuania). Electrophoresis was carried out at a constant voltage of 60 V for 1.5 h, visualized under a UV transilluminator, and photographed using a Gene Genius Gel Documentation System (Syngene Inc., Cambridge, UK).

Mating type evaluation

Mating types of the isolates were evaluated using the PCR primers GFmat1a (5′-GTTCATCAAAG GGCAAGCG-3′) and GFmat1b (5′-TAAGCGCCCTCT TAACGCCTTC-3′) for the MAT-1 region and the primer pairs Gfmat2c (5′-AGCGTCATTATT CGATCAAG-3′) and (5′-CTACGTTGAGAGCTG TACAG-3′) were used for the MAT-2 region as described by Steenkamp *et al.*[12]. The effective population number for mating type [Ne (mt)] was determined using the equation

$$Ne\ (mt) = \frac{4\ (N1*N2)}{(N1+N2)}$$

where N1 is the number of strains of the "1" mating type and N2 is the number of strains of the "2" mating type[17].

Statistical analysis

The relationship among the 63 isolates of *F. fujikuroi* was assessed using scorable DNA

fragments amplified from different URP markers where each band was considered to be a character and was scored as either present (1) or absent (0). Similarity between isolates was assessed by calculating the Jaccard similarity coefficient and cluster analysis was conducted using the Unweighed Pair Group Method with Arithmetical Averages (UPGMA) of NTSYS pc (v.2.02) software[18]. Correlations among the genetic distances and geographical distances were examined by the Mantel test. To calculate geographical distance values, the longitudinal-latitudinal coordinates of the place of collection were converted in kilometers and genetic distances were calculated based on the Jacquard similarity coefficient.

Results

Morphological characterization

A total of sixty three isolates of *Fusarium fujikuroi* collected from different states of the India *viz.* (i) Punjab: 32 (F203, F204, F206, F206a, F216a, F218a, F219, F220, F224, F228, F229, F231, F234, F259, F267, F268, F269, F273, F276, F278a, F338, F301, F301a, F343, F303, F304, F306, F314, F237a, F338a, F343a, F255; (ii) Haryana:15 (F308, F249, F250, F252, F252a, F253, F256, F282, F319, F320, F322, F327, F328, F337, F337a); Uttar Pradesh: 8 (F302, F210, F232, F284, F284a, F309, F310); Uttrakhand: 3 (F341, F255a, F299); Bihar: 4 (F347, F348, F350, F351); and Jammu & Kashmir: 1 (F352**)** were characterized (Fig. 1).

Radial growth

Radial growth of the isolates varied from 2.62 cm (F338) to 7.32 cm (F249). Based on radial growth isolates were categorized in three groups as less (2.0-4.0 mm); moderate (4.1-6.0 mm); Fast (6.1-8.0 mm) growing. Isolates F203, F204, F234, F276, F210, F252a, F320, and F255a were fast-growing and showed radial growth >6 cm, whereas the isolates F350, F219, F220, F224, F229, F349, F301a, F304, F310, F350, and F352 were slow-growing with radial growth of only 3-4 cm (Suppl. Table 1. *All supplementary data are available only online along with the respective paper at NOPR repository at http://nopr.res.in*). Other isolates showed moderate growing patterns (4.1-5.9 cm).

Presence of macroconidia

The presence of Macroconidia was observed in 46 isolates (73% of isolates) while it was absent in 17 isolates namely F204, F218a, F216, F338, F301, F301a, F210, F232, F284a, F309, F310, F250, F252, F252a, F319, F327 and F350 (Suppl. Table 1).

Pathogenicity of different isolates of F. fujikuroi

All the isolates studied were pathogenic and produced distinct symptoms of bakanae disease in rice variety Pusa Basmati 1509. Based on disease severity isolates were categorized into four groups, which were less virulent (0-25%); moderately virulent (26-50%); highly virulent (51-75%); extremely virulent (76-100%). For isolates collected from Punjab, the virulence (%) varied 14.28-100%, whereas it was 2-100% for isolates from Haryana; 20-90% for isolates from Uttar Pradesh; 2-30% for isolates from Uttarakhand, and 20-100% for isolates from Bihar. Isolates F269 (Punjab), F327 (Haryana), and F348 (Bihar) were found to be highly virulent, where as isolates F341 (Uttarakhand) and F252a (Haryana) were the least virulent (Suppl. Table 1).

Molecular characterization

Among the 12 URP primers used in this study, only 6 primers showed reproducible amplicons which could produce clear cut resolvable bands, whereas the remaining 6 primers did not show amplification. Genomic DNA amplification of all 63 isolates of *F. fujikuroi* produced 74 bands, of which 71 showed 94.44% polymorphism (Table 1). The fragments amplified by the primers varied in size from 250 to 7000 base pairs in all isolates. The maximum number of bands (16) was obtained using the URP 17R primer (Fig. 2).

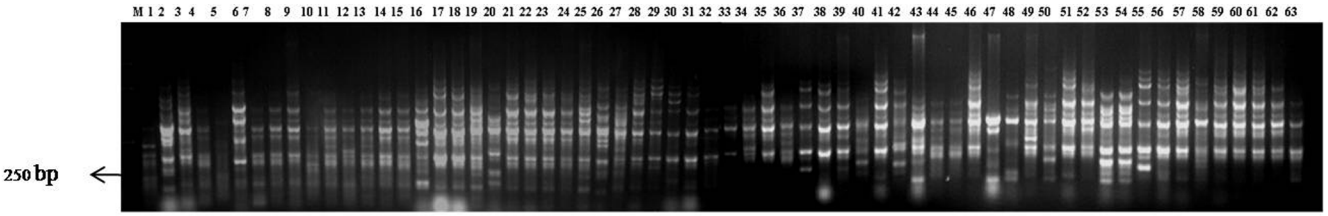

Fig. 2 — DNA finger print profile of *Fusarium fujikuroi* isolates obtained with primer URP-17R. [From left to right- Mamolecular marker lkb; *Fusarium* isolates code 1 to 63-F203, 204, 206, 206a, 218a, 219, 220, 224, 228, 229, 231, 234, 259, 267, 268, 269, 341, 273, 276, 278a, 338, 301, 301a, 302, 303, 304, 306, 314, 237a, 338a, 343a, 210, 210a, 232, 284, 284a, 309, 310, 308, 249, 250, 252, 252a, 253, 256, 282, 319, 320, 322, 327, 328, 337, 337a, 343, 255, 255a, 299, 347, 348, 350, 351 and F352]

Based on the amplified products with all 6 primers, the isolates were grouped into four major clusters. Cluster one was further divided into the three subclusters, a, b and c. 'Subcluster a' comprised of 27 isolates from Punjab belonging to rice genotypes, Pusa Basmati 1121 (PB 1121) and Pusa Basmati 6 (PB 6), five isolates from Uttar Pradesh isolated from rice genotypes PB1121, PB 6, and PB 1509, and one isolate from Uttarakhand isolated from genotype Pakistani Basmati. 'Subcluster b' comprised of isolates predominantly from Haryana belonging to rice variety PB 1121, in addition to the two isolates from Punjab collected from soil sample of rice variety PB 1121, two isolates F284a and F310 from Uttar Pradesh, and two other isolates from Uttarakhand. 'Subcluster c' included 3 isolates from Haryana. Cluster II included isolates collected from Bihar, Jammu and Kashmir. Two isolates namely F204 and F303 from Punjab grouped as clusters III and IV, respectively (Fig. 3).

Based on primer URP 17R, isolates of *F. fujikuroi* were grouped in two major clusters. Cluster I was further divided into three subclusters, a, b and c. The

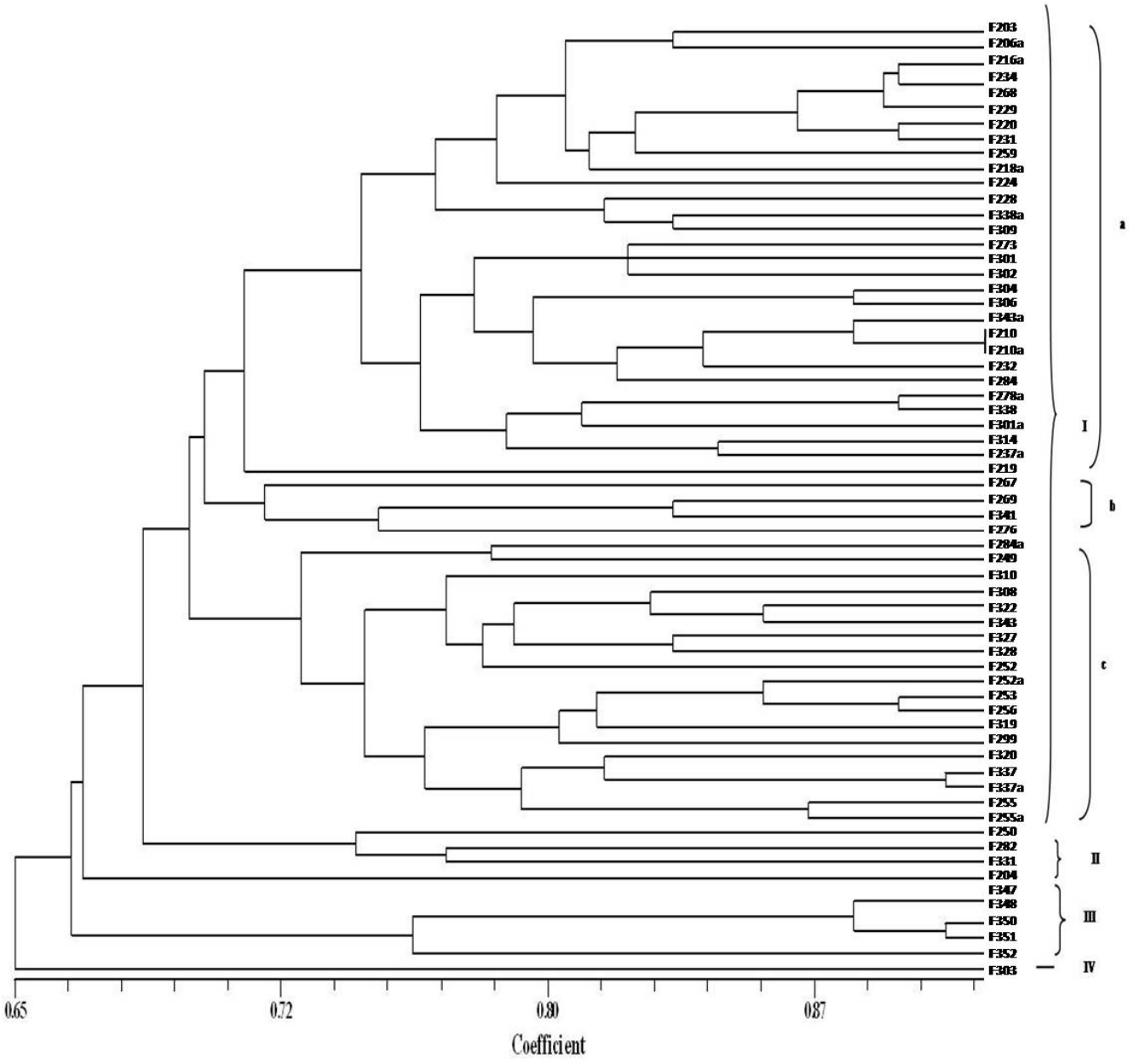

Fig. 3 — Dendrogram obtained after combined analysis of *Fusarium fujikuroi* isolates collected from different states of India using UPGMA based similarity coefficient.

subclusters "a and b" included isolates collected from Punjab, Haryana, Uttar Pradesh, Uttarakhand, Bihar, and Kashmir. Isolates of genotype PB 1509 (Uttar Pradesh, Bihar, and Jammu & Kashmir were grouped together in Subcluster a. Five isolates from Punjab and two isolates from Uttar Pradesh collected from rice variety PB 1121 were grouped together in Subcluster c. Cluster II included one isolate from Punjab and two isolates from the Karnal district of Haryana.

Based on primer URP 1F, isolates were grouped into 9 clusters. Cluster I consisted of 17 isolates from Punjab belonging to rice genotype PB 1121 and PB 6, and one isolate from Uttar Pradesh isolated from genotype PB 6. Cluster II was further divided into three Subclusters a, b, and c. Cluster a consisted of two isolates, F269 and F237a, from Punjab (PB 1121) and 1 isolate F341 (Pakistani Basmati) from Uttarakhand. 'Subcluster b' included 7 isolates from Punjab (PB 1121) and one isolate from Uttar Pradesh (PB 1509). 'Subcluster c' consisted of isolates from Punjab, Haryana, Uttar Pradesh, Bihar, Uttarakhand, Jammu, and Kashmir. Isolates F267, F219, F303, and F310 grouped separately (Suppl. Fig. 1).

Based on primer URP 32F, isolates of *F. fujikuroi* were grouped in two main clusters. Cluster I further divided in three Subclusters a, b, and c. Subcluster a mainly included isolates from Punjab, except 301 (Haryana) and F303 (Uttarakhand). Subcluster 2 consisted of isolates from all states studied. Subcluster 3 included isolates from Punjab, except F338a (Uttar Pradesh, PB 1509). Cluster II included isolates from Punjab (F228a, F304), Haryana (F252), and Uttar Pradesh (F232, F284a, and F310) (Suppl. Fig. 2).

The genetic distances of the isolates ranged from 0.5–1.0. Based on the Mantel test, isolates collected within the geographical distances of 300–600 km were found to be more variable compared to isolates collected from larger distances (Fig. 4).

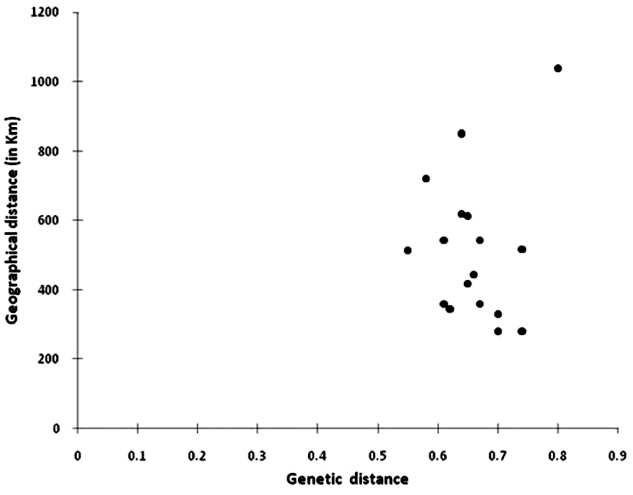

Fig. 4 — Plot of genetic distances and geographical distances derived using the Mantel test in *F. fujikuroi* isolates from six states of India (geographical distances means distances based on longitudinal-latitudinal coordinates).

Mating type

The analysis for the mating population showed that both MAT-1 and MAT-2 mating types were present in India (Fig. 5). Among the 63 isolates evaluated, 18 (28.57%) were identified as MAT-1 and 45 (71.42%) as MAT-2. The distribution of the MAT-2 population was highest in Haryana (86.67%), followed by Punjab (71.0%) and Uttar Pradesh (50%). The effective population number $N_{e(mt)}$ for mating type was 89% of the total population.

Discussion

The morphological, cultural and pathological characterization of sixty-three isolates of *F. fujikuroi* collected from different rice growing areas in northern India revealed that the pathogen is highly variable in nature. Isolates varied in radial growth, the presence of macroconidia, and virulence. However, a clear correlation could not be established between different characteristics. Bashyal & Aggarwal[8] also reported variation in the morphology and pathogenicity of *F. fujikuroi* isolates collected from rice seed. Further, Niehaus *et al*.[19] could identify two distinct pathotypes based on the variation in secondary metabolites profiles and Bashyal *et al*.[20] could identify large no. of secretary proteins in the genome of *F. fujikuroi*, which may be contributing for the variation in pathogen.

In the present study, most isolates belonging to the same geographical origin were found to be genetically

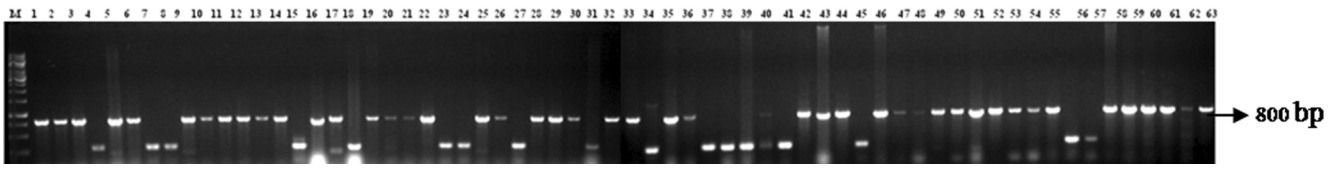

Fig. 5 — Amplification of MAT-1 and MAT-2 locus of the *Fusarium fujikuroi* isolates collected from different states of India.

distant from each other, as they grouped in different clusters. Similarity coefficients varied from 0.51–0.91 among the collected isolates. Further, the Mantel test also suggested that isolates collected from larger distances were less genetically variable compared to the isolates collected from Punjab and Haryana. Amoah *et al.*[21] collected isolates of *F. moniliforme* infecting maize, rice, and elephant grass from different geographical areas; based on molecular analysis, they concluded that the population diversity of this pathogen was not related to geographical differences. Puyam *et al*.[22] also observed dissimilarity index of 25-75% in *Fusarium moniliforme* isolated from rice. In our study, the combined analysis grouped most of the isolates from Punjab into one cluster. These results suggest that *F. fujikuroi* in India may not be spread over long distances. The lower genetic divergence of *F. fujikuroi* in different isolates from some areas in Punjab suggests that sexual reproduction in this region may be less active than in other regions. Many factors such as mutation, random genetic drift, population size, gene flow, reproduction/mating system, and selection play a vital role in the genetic variation in fungi[23]. Interactions between these factors results in either high or low variability between and within populations.

Although the pathogen mainly uses asexual reproduction, sexual reproduction can also occur through the micro- and macro-conidia due to extreme conditions prevailing in the field. Both mating types (MAT-1 and MAT-2) of *F. fujikuroi* were observed in Punjab, Haryana and Uttar Pradesh. Therefore, a mixed reproduction or mating system may also contribute to the diversity of *F. fujikuroi*. For aiding sexual reproduction in a population, the MAT-1 and MAT-2 ratio should be close to 1:1, and the frequency of the MAT-2 idiomorph should be higher[23]. Although, the MAT-2 population of *F. fujikuroi* is pre-dominant in India, the MAT-1 population is also present, which does not rule out the possibility of sexual reproduction in these populations. Therefore, sexual reproduction of the fungus may occur in India under favourable conditions. Sunder and Satyavir[24] also reported 10 VCG groups in a population of *F. moniliforme* of rice compared to 6 groups in California[25], supporting the occurrence of sexual reproduction in the Indian population of *F. fujikuroi*. Since the mixed system involves recombination, the creation of new virulent strains with high fitness and maintenance in selection pressure through the clone, spatial and temporal distribution, a higher risk of evolution exists[26].

The primary source of the inoculums for bakanae disease is infested or infected seed[27-29]. However, seed is not the only source of inoculum, as the fungus can survive in infected crop residues, soil[30-31], and weeds, which may act as reservoirs for the persistence and inoculum multiplication in the field. Our results also indicate the presence of different races/pathotypes of *F. fujikuroi* in the same area.

Conclusion

The present study revealed significant variability for morphological, pathogenic, and molecular characteristics in the Indian population of *Fusarium. fujikuroi*, which can be utilized in developing strategies for management of bakanae disease. Further, present study confirmed the presence of both mating types of *F. fujikuroi* in India, thereby indicating the possibility of sexual reproduction in this species.

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Conflict of interest

Authors declare no conflict of interest.

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