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Pathogenic and genetic variability in *Tilletia indica* monosporidial culture lines using universal rice primer-PCR

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Abstract *Tilletia indica* Mitra is the causal agent of Karnal bunt of wheat, an important disease prevalent in several countries. The disease is internationally quarantined and the pathogen due to its heterothallic nature shows high variability. In the present study, we compared the pathogenic behaviour of various isolates of *T. indica* collected from different geographical locations of India and genetically characterized monosporidial (Ms) culture lines raised from these isolates of the pathogen. Pathogenic variability revealed existence of three pathotypes based on aggressiveness on a set of differential host genotypes. Monosporidial culture lines viz., 5 each from KB1, KB2, KB4 and KB5 and three lines of KB3 were established and analyzed genetically using 12 Universal Rice Primers (URPs). Amplification showed 98.44% polymorphism and primer URP 13R produced 100% polymorphic bands. Maximum similarity (83%) was between KB1MsB and KB1MsD as calculated by Jaccard's similarity coefficient, whereas, minimum similarity was between KB1MsC and KB4MsB; KB1MsE and KB3MsA (46%). Three groups were formed among all Ms culture lines. One major group consisted of 13 lines with approximately 70% similarity, the second group consisted of 7 culture lines showing 55% similarity and the third group consisted of 3 Ms lines. URPs were able to differentiate the Ms culture lines raised from different *T. indica* isolates and the results indicated heterogeneity in the pathogen population.

Keywords DNA fingerprinting · Karnal bunt · Monosporidial cultures · URP-PCR · Variability

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Introduction

Wheat is the second most important cultivated crop in the world. Its production gained acceleration after green revolution in sixties of twentieth century in India. As two third of our population depends on agriculture, which contributes one fourth of the national GDP, its management attracts more attention. Among all the threatening diseases of wheat, Karnal bunt is an important one, as it is internationally quarantined. *T. indica* Mitra is the causal agent of the disease. Karnal bunt fungus is native to the Asian subcontinent. It was originally reported in 1931 near the city of Karnal, India from which it gets its name (Mitra 1931). Since then, it has been reported in Pakistan (Hassan 1972), Nepal (Singh et al. 1989),

Iran (Torabi and Jalalian 1996), and Iraq (CAB 1974). The disease has also been recorded in Mexico (Duran and Cromarty 1977), United States (Ykema et al. 1996) and from South Africa (Crous et al. 2000). Karnal bunt infected wheat grains have also been intercepted in seed lots received from Lebanon, Syria, Sweden and Turkey (Nath et al. 1981) but no field incidence has been reported in these countries (Singh 2005). *T. indica* is a heterothallic fungus (Fuentes-Davila and Duran 1986), belonging to the order Ustilaginales (Bonde et al. 1997). The pathogen is seed borne and adversely affects the weight and viability of seed (Bansal et al. 1984a). On germination, the diploid (2N) nucleus in teliospore undergoes meiosis followed by several mitotic divisions to produce haploid (1N) nuclei. When a teliospore germinates, a promycelium (basidium) emerges and produces as many as 180 primary sporidia (basidiospores) (Bansal et al. 1983; Fuentes-Davila and Duran 1986; Gill et al. 1993). Each primary sporidium contains a single haploid nucleus. Teliospores on the soil surface germinate and under conducive environmental conditions infect plants at flowering stage (Dhaliwal and Singh 1988). There are various conventional approaches for the management such as cultural practices, adjustment of time of irrigation and nitrogen balance in soil (Bedi et al. 1949; Goel et al. 1977; Aujla et al. 1981), mulching the soil with plastic (Singh et al. 1992) besides the use of fungicides (Singh et al. 1985). The most economical and eco-friendly management approach is cultivation of resistant varieties. Knowledge of pathogen variability based on aggressiveness is an important criterion in assessing resistance. Since the pathogen is heterothallic, i.e. it produces haploid secondary sporidia and compatible sporidia (+ & −), when these come in contact they hybridize and cause infection. Therefore, there is continuous variation in pathogen population. Most of the earlier workers have characterized pathogenic and molecular variability based on monoteliosporic populations (Bonde et al. 1996; David and Darrel 1996; Datta et al. 2000; Thirumalaisamy et al. 2006), which does not give a clear picture of genetic variability as teliospores are diploid. Since, secondary sporidia are infective in nature, in the present study molecular variability and compatibility in monosporidial culture populations were analysed. Due to limitations in virulence typing, there have been various attempts to do molecular analysis of different isolates/species of *Tilletia* using random amplified polymorphic DNA (RAPD) markers (Bonde et al. 1996; David and Darrel 1996; Shi et al. 1996; Gang and Weber 1996). Repeat sequences from Korean weedy rice, originally referred to as universal rice primer (URP) have been used for the fingerprinting of diverse genomes (Kang et al. 2002), and has been used in molecular analysis of only a very few fungi (Kang et al. 2001; Jana et al. 2005; Sharma et al. 2005; Aggarwal et al. 2008). The use of these markers to study molecular variability in Ms lines has been undertaken and the results are reported here.

Materials and methods

Cultures of *Tilletia indica*

Five cultures of *T. indica* were established from the teliospore inoculum of infected wheat seeds collected from different locations i.e., two from Haryana (KB2 and KB3); one each from Delhi (KB1), Uttar Pradesh (KB5) and Jammu and Kashmir (KB4). The cultures were raised from teliospores using technique described by Warham (1987) with some modifications. For raising Ms culture lines, when teliospores started germinating, but before formation of secondary sporidia, single teliospore with attached basidiospores (primary sporidia) were picked off with the help of sterile needle and transferred to drops of sterile water on the surface of separate agar plates. The germinating teliospore was teased over the surface to spread the basidiospores. Each basidiospore was marked on the reverse side of Petri plate, picked up with the help of dissecting needle and transferred to PDA slants and incubated at 18°C.

Pathogenic variability

Pathogenic variations among 5 different isolates was studied by inoculating monoteliosporic cultures on 18 differential host genotypes comprising of susceptible and resistant wheat, triticale and rye lines (Sharma et al. 1998) (Table 1), at physiological growth stage Z-49 (Zadocks et al. 1974). The genotypes were sown in 10 cm diameter pots filled with a mixture of loamy soil and farm yard manure (3:1, v/v) supplemented with NPK fertilizer under polyhouse conditions. After

germination, only five healthy seedlings were retained per pot. Sporidial suspension containing allantoid secondary sporidia @ 10^4/ml was prepared and five tillers of each differential host were inoculated during evening hours using hypodermic syringe (Aujla et al. 1989). High humidity was maintained by spraying the inoculated plants with water. Uninoculated checks for each genotype were also maintained. Similarly, Ms cultures derived from KB1 and KB3 isolates were also inoculated individually and in all possible combinations on highly susceptible host WL711 to study the compatibility among different Ms lines. After maturity, the inoculated ear heads were harvested, threshed and infected grains were categorized into five grades of infection on the basis of area of endosperm converted into sooty mass of fungal teliospores and coefficient of infection (CI) was calculated based on the formula

$$\text{Percent CI} = \sum \frac{X_i Y_i}{N} \times 100$$

Where, i- grade of infection (i=0 to 4), N- total numbers of grains, X- numerical value of i-th grade of infection, and Y- No. of grains of i-th grade of infection.

Based on coefficient of infection (CI) disease responses were rated as immune (I)=0.0 CI, resistant (R) = >0.1–2.0 CI, moderately resistant (MR) = >2.0–5.0 CI, moderately susceptible (MS) = >5.0–10.0 CI, susceptible (S) = >10.0–20.0 CI and highly susceptible (HS) = >20.0 CI (Singh et al. 1995). The average percent coefficient of infection was calculated and the reaction value from 0–5 was considered as resistance type and above 5 as susceptible type. Accordingly 0 and 1 were assigned for susceptible and resistance for data analysis in NTSYS-PC, version 2.00 (Rohlf 1998).

Molecular analysis of monosporidial lines

Twenty three Ms culture lines viz., 5 each from KB1, KB2, KB4 and KB5 and three lines of KB3 (KB1MsA-E from KB-1, KB2MsA-E from KB2, KB3MsA-C from KB3, KB4MsA-E from KB4 and KB5MsA-E from KB5) were established and maintained on PDA slants. The Ms cultures were seeded into potato dextrose broth in 500 ml flask and grown as static culture at 18°C for minimum 20 days. Mycelia of each isolate were harvested and processed for extraction of genomic DNA.

Extraction of genomic DNA

DNA was extracted by CTAB method (Murray and Thompson 1980). The mycelium (5 mg) was ground in liquid nitrogen and transferred to DNA extraction buffer (0.1 M Tris, 1.5 M NaCl, 0.01 M EDTA) and kept at 65°C for 1 h with occasional stirring. Equal volume of Chloroform:Isoamyl alcohol (24:1) was added to each tube followed by centrifugation. The upper aqueous phase so obtained by precipitation with 0.6 volume of ice-cold isopropanol was again centrifuged. The pellet was washed with 70% ethanol and dried at room temperature. Finally, the nucleic acid was dissolved in TE and kept at −20°C.

URP primers and polymerase chain reaction

URPs are 20-oligonucleotide primer each, originally derived from repetitive sequences of weedy rice (Kang et al. 2002). There are 12 URPs, which were synthesized by Genuine Chemical Corporation (GCC), India. PCR was performed in Temperature Gradient Thermal Cycler (BioRAD, USA). Each reaction mix consisted of 50 ng DNA, 200 μM dNTP; 0.2 μM primer, 2.5U *Taq* DNA polymerase, 1x *Taq* buffer in 25 μl of volume. The PCR amplification was performed using following thermal cycling conditions: initial denaturation for 1 min at 94°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 7 min.

The URP-PCR products were electrophoresed on 1.2% agarose gel containing ethidium bromide (0.5 μg/mL) in TBE buffer (pH 8.3) along with 1Kb DNA ladder (MBI Fermentas). The electrophoresis was carried out at a constant voltage of 60 V for 3 h and visualized under UV transilluminator and photographed in Gene Genius Gel Documentation System (Syngene Inc, Cambridge, UK).

Scoring and data analysis

Relatedness among twenty three Ms lines of five isolates of *Tilletia indica* was estimated by means of scorable DNA bands amplified from different URP markers. Each band was considered as character and was scored as either present (coded as 1) or absent (coded as 0). Cluster analysis with the unweighted pair group method with an arithmetic average

(UPGMA) algorithm was performed using NTSYS-PC (v. 2.01) (Rohlf 1998) to produce a dendrogram. Bootstrap analysis was performed using Winboot software for all the primers which amplified the Ms lines.

Results

Pathogenic variability

Five isolates when inoculated on a set of 18 differential hosts produced C.I. ranging between 0.1 by KB4 on genotype HD 30 and 25.5 by KB2 on PBW 343. Isolate KB5 from Delhi was least virulent as 8 genotypes (CMH 77.308, Shanghai 8, H 567.71, PDW 233, TL 1210, WL 6975, HD 29 and HD 30) showed immune reaction against this isolate and other 8 genotypes (HP 1531, UP 2382, W485, S486, PBW 34, WH 542, ALDAN and CPAN 3045) produced resistant reaction. This isolate induced moderately susceptible reaction on only two genotypes PBW 343 and WL 711, both highly susceptible genotypes, on which all other isolates produced 'S 'response. Isolate KB2 from Haryana (Smalkha) was highly virulent as it induced MS to S reaction on 6 genotypes (H 567.71, UP 2382, PBW 343, W485, WL 711 and WH 542) and none of the genotypes showed immune response to this isolate (Table 1). Based on disease responses three pathogenic groups were identified, KBAgI (isolates KB1 and KB3), which was highly virulent; KBAgII (isolate KB2) moderately virulent and KBAgIII (isolates KB4 and KB5) least virulent. Ms cultures when inoculated individually did not produce infection, while in combination, Ms cultures of KB1 and KB3 produced infection. The self paired Ms cultures caused no infection but cross combination resulted in successful infection in 26 combinations out of 41. This led to postulation of 3 incompatibility alleles in KB1 isolate and 2 alleles in KB3 isolate (data not shown).

Molecular variability

Out of 12 URPs used for molecular analysis only 10 primers could amplify the genomic DNA of all the Ms lines (Table 2). The amplified products of 23 Ms lines were assessed for the distribution of URP motif sequences in their genome. The size of the multiple amplified bands ranged from 100 bp to 3.0 Kb for

Table 1 Coefficient of infection and disease response of *Tilletia indica* isolates on differential hosts

Differential host	Coefficient of infection[a] (disease response) by different isolates				
	KB1	KB2	KB3	KB4	KB5
CMH77.308	1.2 (R)	2.1(MR)	1.8 (R)	1.1(R)	0.0(I)
HP 1531	2.1 (MR)	3.9(MR)	1.2 (R)	1.7(R)	0.6(R)
SHANGHAI 8	0.2 (R)	0.7(R)	3.7 (MR)	1.1(R)	0.0(I)
H 567.71	6.4 (MS)	8.2(MS)	4.6 (MR)	3.7(MR)	0.0(I)
PDW 233	0.1 (R)	0.9(R)	0.4 (R)	0.4(R)	0.0(I)
TL1210	0.6 (R)	0.4(R)	0.8 (R)	0.9(R)	0.0(I)
UP 2382	8.6 (MS)	5.3(MS)	9.4 (MS)	1.9(R)	0.9(R)
PBW 343	14.3 (S)	25.5(S)	24.0 (S)	13.8(S)	2.1(MS)
W 485	6.1 (MS)	9.2(MS)	8.2 (MS)	1.3(R)	1.2(R)
WL 711	12.4 (S)	23.1(S)	17.3 (S)	17.3(S)	8.5(MS)
S 486	9.2 (R)	8.8(R)	6.2 (R)	7.6(R)	1.5(R)
PBW 34	3.0 (MR)	4.1(MR)	3.7 (MR)	1.2(R)	0.3(R)
WH 542	8.6 (MS)	6.9(MS)	12.1(S)	1.9(R)	0.9(R)
WL 6975	0.4 (R)	2.9(MR)	6.2(MS	0.7(R)	0.1(I)
ALDAN	0.7 (R)	1.2(R)	0.7(R)	1.6(R)	1.5(R)
CAPN 3045	1.3(R)	0.2(R)	0.7(R)	2.1(MR)	0.7(R)
HD29	0.0(R)	0.8(R)	0.2(R)	0.8(R)	0.0(I)
HD 30	0.2(R)	0.6(R)	1.2(R)	0.1(R)	0.0(I)

[a] Mean of three replications

I Immune; *R* Resistant; *MR* Moderate resistant; *S* Susceptible; *MS* Moderate susceptible

Table 2 Sequences of Universal Rice Primers (URPs) and polymorphism obtained in this study

S. No	Primer	Sequence (5′–3′)	GC content (%)	Tm (°C)	Total no. of bands	Polymo-rphic bands	Monomo-rphic bands	Polymorphism (%)
1	URP6R	GGCAAGCTGGTGGGAGGTAC	50	65	15	15	–	100
2	URP4R	GGCAAGCTGGTGGGAGGTAC	50	66	15	15	–	100
3	URP30F	GGACAAGAAGAGGATGTGGA	50	65	17	12	–	100
4	URP25F	GATGTGTTCTTGGAGCCTGT	50	65	11	11	–	100
5	URP1F	ATCCAAGGTCCGAGACAACC	50	65	9	9	–	100
6	URP2F	GTGTGCGATCAGTTGCTGGG	50	67	10	10	–	100
7	URP9F	ATGTGTGCGATCAGTTGCTG	50	67	10	9	1	90
8	URP13R	TACATCGCAAGTGACACAGG	50	68	25	25	–	100
9	URP17R	AATGTGGGCAAGCTGGTGGT	50	74	9	9	–	100
10	URP38F	AAGAGGCATTCTACCACCAC	50	65	8	7	1	87.5
11	URP2R	CCCAGCAACTGATCGCACAC	50	65	–	–	–	–
12	URP32F	TACACGTCTCGATCTACAGG	50	65	–	–	–	–
Total					129	127	2	98.44%

each isolate. Out of 129 bands generated with 10 primers, 127 were polymorphic showing high range of variability (98.44% polymorphism). All URPs except URP 9F and URP 38F produced polymorphic bands showing 100% polymorphism. A single monomorphic band of 2000 bp was obtained with primer URP 9F and of 900 bp with URP 38F.

The amplification profile generated with primer URP 1F showed a prominent DNA amplicon of 850 bp in three Ms lines of KB1 (KB1MsA, KB1MsD and KB1MsE) and KB5MsD and KB4MsA (Fig. 1). Jaccard's similarity coefficient analysis and dendrogram generation showed formation of two major clusters with 55% genetic similarity between them. In first cluster, three Ms lines of KB1, viz., KB1MsA, KB1MsD and KB1MsE showed 100% similarity while KB1MsC joined the same cluster with 75% similarity. KB5MsD and KB4MsA showed 85% similarity and KB1MsB showed more than 60% similarity making a outgroup in the same cluster. The other cluster (II) is divided into two subgroups. Subgroup a consisted of KB5MsA, KB5MsC, KB5MsE, KB3MsA, KB3MsB, KB4MsB, KB4MsE and KB2MsE showing 100% similarity among them and 90% similarity with lines KB2MsB, KB2MsC, KB4MsC and KB4MsD which together clustered showing 100% similarity among them. KB5MsB also joined this cluster with 85% similarity. In subgroup b, linesKB2MsD and KB3MsC clustered together with 100% similarity along with KB2MsA in the same subgroup showing 86% similarity (Fig. 2). This data generated with URP 1F showed that the Ms lines

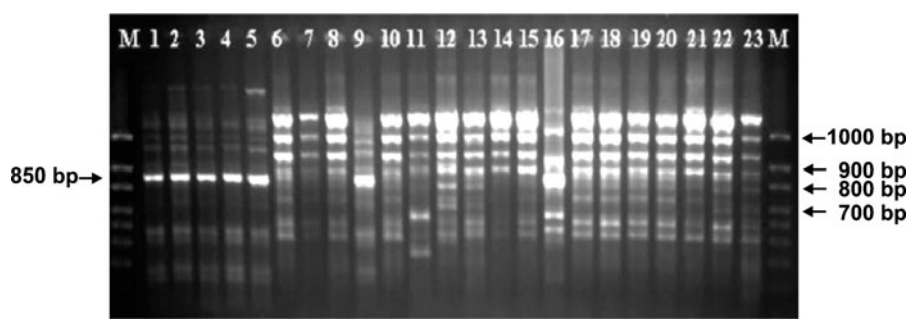

Fig. 1 DNA fingerprint profile of monosporidial culture lines of *Tilletia indica* isolates obtained with primer URP 1F. Lanes 1–5 KB1MsA-E, Lanes 6–10 KB5MsA-E, Lanes 11–15 KB2MsA-E, Lanes 16–20 KB4MsA-E, Lanes 21–23 KB3MsA-C. M—100 bp DNA ladder (MBI, Fermentas) on both sides

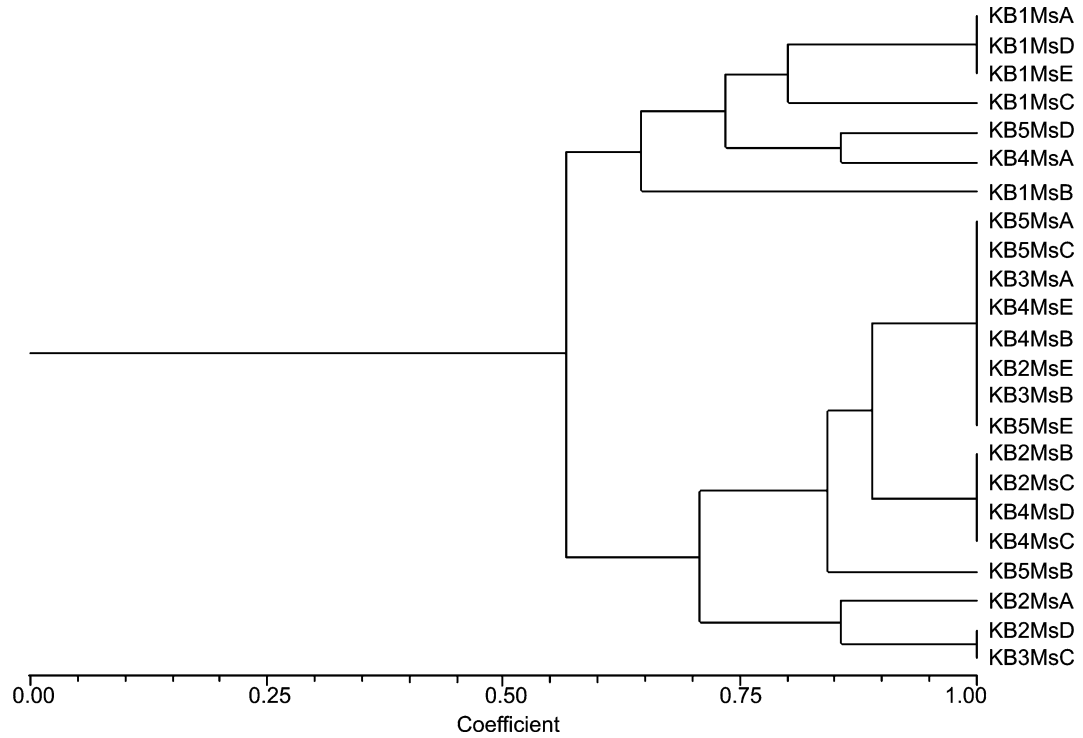

Fig. 2 Dendrogram obtained from 23 monosporidial culture lines of *Tilletia indica* with UPGMA based similarity coefficient using primer URP-1F

raised from isolates collected from different geographic locations showed high genetic similarity.

PCR amplified products of primer URP 9F showed a prominent 1000 bp band amplified in four lines of KB5 (KB5MsA-C and KB5MsE); three lines each of KB3 (KB3MsA-C) and KB4 (KB4MsB, C and E) (Fig. 3). The dendrogram generated grouped the culture lines into three clusters showing more than 45% genetic similarity (Fig. 4). The first cluster included KB1MsA and KB1MsB with 100% similarity. In the second cluster, KB2MsA, KB2MsC and KB2MsE were found to be genetically identical. Cluster 3 included KB4MsA and KB4MsD which were 100% similar on the similarity matrix. This URP 9F primer data indicated that the Ms lines raised from a particular isolate showed high genetic similarity.

The dendrogram obtained from the combined data on amplified products with all 10 primers by using bootstrap analysis showed different levels of genetic similarity among Ms lines. There were two major clusters formed among all Ms lines. The first cluster

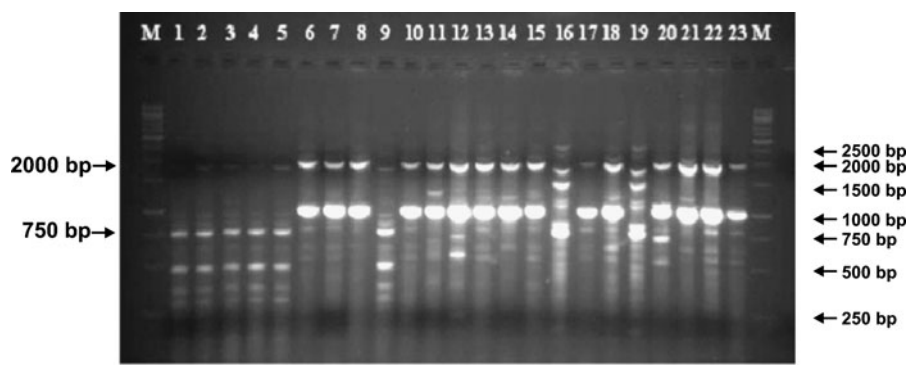

Fig. 3 DNA fingerprint profile of monosporidial culture lines of *Tilletia indica* isolates obtained with primer URP 9F. Lanes 1–5 KB1MsA-E, Lanes 6–10 KB5MsA-E, Lanes 11–15 KB2MsA-E, Lanes 16–20 KB4MsA-E, Lanes 21–23 KB3MsA-C. M is 1 Kb DNA ladder (MBI, Fermentas) on both sides

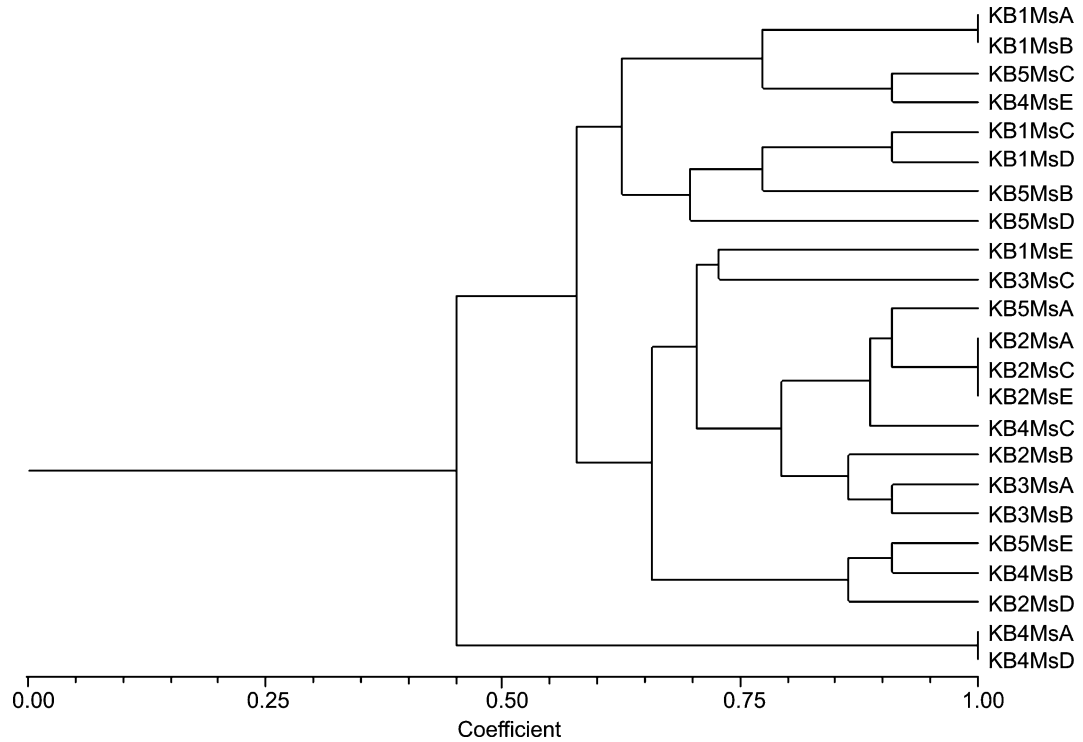

Fig. 4 Dendrogram obtained from 23 monosporidial culture lines of *Tilletia indica* with UPGMA based similarity coefficient using primer URP 9F

consisted of 7 lines showing 44–71% genetic similarity among all KB1Ms lines and one Ms line each from KB5 and KB4.The other major cluster consisted of 16 lines with 7–83% similarity among various Ms lines raised from KB2, KB3, KB4 and KB5. This cluster was further subdivided into two subgroups, with most of the Ms lines falling into one subgroup and lines KBMsA and KBMsB with bootstrap value of 37 and KBMsC at 32 bootstrap value forming a second subgroup (Fig. 5). These two subgroups showed 38% similarity between them whereas, these two groups showed 83% similarity with the other cluster. Jaccard's similarity coefficient analysis showed maximum genetic similarity (83%) between KB1MsB and KB1MsD, whereas, minimum genetic similarity was between KB1MsC and KB4MsB; and KB1MsE and KB3MsA (46%).

Thus, URP primers were able to differentiate the Ms lines raised from different *T. indica* isolates and analysis showed that in spite of being collected from different geographical locations, Ms lines of different isolates showed high genetic similarity.

Discussion

This is the first report of molecular characterization of Ms culture lines of *T. indica* using URP markers. *T. indica* is a heterothallic fungus and survives through sexual reproduction. Our results on pathogenic variability and previous results on the host pathogen interaction have indicated that there is no well-defined race concept in this pathogen based on the reaction on a set of hosts, as it exists in the wheat rusts (Bonde et al. 1996; Thirumalaisamy et al. 2006; Singh et al. 1995). However, Aujla et al. 1987 have reported the existence of races among the isolates collected from Punjab and Himachal Pradesh. In the present study, a considerable differential reaction of the isolates was observed on the host lines tested. Isolates KB1, KB2 and KB3 showed a 'MS' response on genotypes UP2382, W485 and WH 542 and were grouped together in cluster I while other isolates KB4 and KB5 caused an 'R' response on these genotypes forming a separate cluster. This indicated that there is genetic variation among *T. indica* isolates tested.

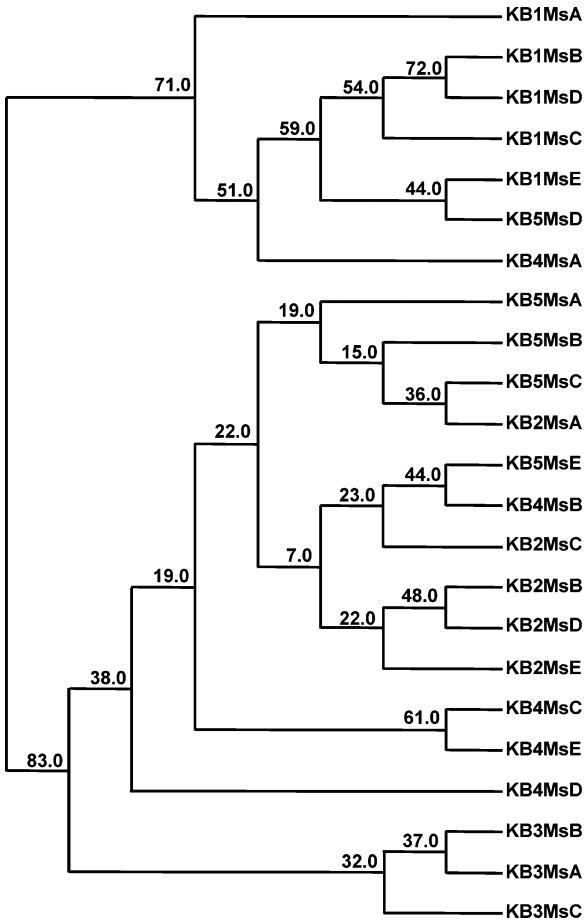

Fig. 5 Dendrogram obtained after combined bootstrap analysis. The numbers at the forks show the percentage of times the group consisting of the culture lines which are to the right of that fork occurred. KB=Karnal bunt, Ms=Monosporidial lines

Datta et al. (2000) also reported pathogenic and genetic variability in *T. indica* isolates. They suggested that the different KB resistance genes probably exist in these cultivars and there is genetic variability for pathogenicity in the isolates. Five isolates tested in present study could be grouped into 3 pathotypes based on their aggressiveness, however, a clear picture of pathotypes based on pathogenicity could not be obtained, as the disease is very much influenced by environmental conditions and reproducability is low. Identifying molecular markers is the best approach to overcome these limitations. Therefore, in this study, URP markers were used to differentiate Ms lines of the isolates. New aggressive pathotypes emerge continuously, possibly by fusion of compatible sporidia of *T. indica*, and probably for this reason some earlier commercial cultivars became susceptible to the disease (Singh et al. 1996). The genetic variability studied among monosporidial lines by URP-PCR markers showed formation of three groups. It was interesting to note that Ms lines raised from isolate KB1 and KB3 were grouped into separate clusters. The cross compatibility experiment also showed disease symptoms where compatible Ms lines of KB1 and KB3 were co-inoculated. Since, Ms lines are haploid, only compatible interactions were able to produce the disease (data not shown) indicating existence of multiple alleles. This result is supported by earliar findings of Duran and Cromarty (1977), Aujla and Sharma (1990), Fuentes-Davila (1989) and Royer and Rytter (1985). Earlier, various workers have speculated on the existence of variability based on teliospore size, shape, number of primary and secondary sporidia and differential host (Mitra 1931; Singh et al. 1995; Bansal et al. 1984b), but not many have reported on variability in Ms lines of the pathogen isolates, except for a few (Datta et al. 2000; Singh et al. 1995). DNA amplification with URP 1F produced a prominent band of 850 bp in Ms lines of KB1 (KB1MsA-E), which was present in other two lines, one of KB5 and two lines of KB4, which could be used as markers to characterize genetically similar Ms lines originating from isolates of different geographic locations. Previously, URPs were tried to determine the genetic variability of plant pathogenic fungi and they were found to be sensitive and technically simple to use (Jana et al. 2005; Sharma et al. 2005). Recently URP markers have also been used to study genetic variability among *Bipolaris sorokiniana* isolates and molecular grouping was correlated with geographical location of the isolates (Aggarwal et al. 2009). In present study also the URPs were found to be suitable for characterization of genetic variability in Ms lines of *T. indica*, which may also help in the identification of possible mating types generated during fungal life cycle.

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