Characterization of *Ustilago segetum* causing loose smut of wheat

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Abstract

Isolates of loose smut of wheat caused by *Ustilago segetum* (Pers.) Roussel *tritici* Jensen were collected from different cultivars of wheat grown at various locations of North-Western India (Punjab, Haryana, U.P. and Rajasthan). The genetic relationships of molecular variability and and virulence pattern among these races is unknown. In this study, a total of 24 isolates of representing six groups of *U. segetum tritici* were studied using molecular and virulence data. In total, eighteen random decamer primers and ISSR primers were used to characterize twenty four isolates of the pathogen. The RAPD and ISSR primers were used for polymorphism analysis and generated a total of 206 scorable bands collectively. All the isolates could be precisely differentiated from each other employing these primers and grouped into two distinct clusters. The classification at molecular level did not exactly collate with the geographical distribution of the isolates and virulence / pathogenicity.

Keywords: Loose smut, wheat, molecular characterization

Introduction

Loose smut of wheat (*Ustilago segetum* (Pers.) Roussel *tritici*) Jensen is distributed worldwide. It is seed-born and the earheads emerging from infected seeds get partially or wholly converted into black powdery mass. The varietal patterns keep changing which lead to change in the pathogens. Several studies have revealed the existence of pathogenic variability in this pathogen (Nielson, 1987, Rewal and Jhooty, 1986). Studying the mechanisms of pathogenicity and source of genetic variation in plant pathogens is critical for the future control of the diseases they cause (Nelson *et al*., 1994). Knowledge of the pathogen population structure can contribute to resistance breeding efforts and to the development of strategies for the deployment of resistance. The most widely used method for characterizing pathogen population is determining the virulence structure of isolates from the population by evaluating their reactions on a set of differentials. However, virulence analysis used to infer population spectrum because the genes involved in virulence represent a very small fraction of genes in the pathogen genome (Lueng *et al*., 1993). This measure Changes in pathogen are also reflected at molecular level. The objective of present investigation was to determine the genetic relationships among the races of *U. segetum tritici* collected from wheat based on molecular and virulence data.

Materials and Methods

Collection and maintenance of pathogen isolates

The present investigation was carried out in the laboratories of Seed Technology Centre, School of Agriculture Biotechnology and experimental areas of wheat section, Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana (2008-2011). Twenty four different isolates of *Ustilago segetum*, collected from various locations of north-western India (Punjab, Haryana, Uttar Pradesh, Rajasthan and Delhi) and from different types of wheat were maintained on highly susceptible variety, WL 711(Table 1). All the isolates were morphologically looking black in colour and powdery. Isolates collected in different years and places were maintained by artificially inoculating the highly susceptible variety from a single properly bagged infected earhead by standard method of inoculations using dry teliospores (Gothwal and Pathak, 1977).

Table 1. Isolates collected from different places in India and types of wheat

Isolate	Place of collection	States in India	Type of wheat
LS 1, LS 31	Mansa	Punjab	*Triticum aestivum*
LS 2, LS 30, LS 12	Gurdaspur	Punjab	*Triticum aestivum*
LS 4, LS 25, LS27	Bathinda	Punjab	*Triticum aestivum*
LS 5	Mansa	Punjab	*Triticum durum*
LS 6	Hoshiarpur	Punjab	*Triticosecale*
LS 7,	Ludhiana	Punjab	*Triticum aestivum*
LS 8	Ferozepur	Punjab	*Triticum aestivum*
LS 9	Faridkot	Punjab	*Triticum aestivum*
LS 13	Pantnagar	Uttarakhand	*Triticum aestivum*
LS 15	Pantnagar	Uttarakhand	*Triticum durum*
LS 16	Dhaulakuan	Himachal Pradesh,	*Triticum aestivum*
LS 18	Shriganganagar	Rajasthan	*Triticum aestivum*
LS 20	Hoshiarpur	Punjab	*Triticosecale*
LS 22	Hisar	Haryana	
LS 23	New Delhi	New Delhi	*Triticum durum*
LS 28	Sangrur	Punjab	*Triticum aestivum*
LS 29	Bathinda	Punjab	*Triticosecale*
LS 33	Kapurthala	Punjab	*Triticum durum*
LS 35	Ludhiana	Punjab	*Triticosecale*

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Molecular analysis

Genomic DNA extraction

The Total genomic DNA of each isolate of *Ustilago segetum* was isolated from mycelia using CTAB method as described elsewhere (Saghai-Maroof *et al.,*1984). Isolates were incubated at 25°C for 48–72 h in flasks containing 20 ml of potato dextrose broth, agitated at 100 rpm for 3 days at 25°C. Mycelia were harvested by filtration through double layers of filter paper, dried between two layers of sterilized filter paper in laminar air flow cabinet and stored at -80°C.

Seven milligrams of fungal tissue was ground in liquid nitrogen and the powder was quickly transferred to 50 ml Okridge tubes. After all the samples were ground, 15 ml of 2X CTAB extraction buffer was added to it. The powder was suspended in the buffer by inverting and rotating the tubes. The tubes were incubated at 65°C for 45 minutes in water bath. The samples were mixed occasionally while maintaining at 65°C. After incubation, 15 ml of chloroform: isoamyl alcohol (24:1) was added and tubes were swirled, till it made an emulsion. The tubes were placed on a platform shaker for 30 minutes and then centrifuged at 8000 rpm for 30 minutes at room temperature. The supernatant was transferred to a clean sterile falcon tubes. About 10 ml of chilled isopropyl alcohol was added to precipitate nucleic acids and then tubes were inverted gently several times. The DNA formed white cotton like precipitate and good quality DNA floated up. The floating DNA was hooked out using a sterile hooked pasteur pipette / by centrifugation. The hooked or pelleted DNA was transferred into clean sterile 1.5 ml microfuge tube and was rinsed with 70 % ethanol 2-3 times. The ethanol was dried completely placing the microfuge tubes inverted on a blotting paper. The DNA was finally dissolved in appropriate quantity of 1X TE buffer (Tris-EDTA buffer- 10 mM Tris HCl, 1mM EDTA and pH 8.0). The tubes were left for few hours at room temperature to allow DNA to dissolve. Then 5µl RNAse (10 mg/ml) was added and tubes were incubated at 37°C in water bath for 1 hour and stored at -20^{0}C.

PCR amplification

A set of nine ISSR and RAPD primers each were used for amplification of genomic DNA of all the isolates of the pathogen (Table 2).

ISSR analysis

In vitro amplification using Polymerase Chain Reaction was performed in a 96 well micro titer plate in a MJ Research Thermal Cycler (PTC-100) and BIORAD My Cycler. A set of nine ISSR primers (Table 2) was synthesized according to the sequences obtained from the University of British Columbia, Canada (Zietkewicz et al., 1994).

Table 2. Primers used for differentiating isolates of *Ustilago segetum tritici*

S.No.	Primer	Sequence
ISSR primers		
1	ISSR-811	GA GA GA GA GA GA GA GA C
2	ISSR-824	TC TC TC TC TC TC TC TC G
3	ISSR-826	AC AC AC AC AC AC AC AC C
4	ISSR-827	AC AC AC AC AC AC AC AC G
5	ISSR-835	AG AG AG AG AG AG AG AG AG YC
6	ISSR-848	CA CA CA CA CA CA CA CA RG
7	ISSR-849	GT GT GT GT GT GT GT GT YA
8	ISSR-855	AC AC AC AC AC AC AC AC AC AC YT
9	ISSR-856	AC AC AC AC AC AC AC AC YA
RAPD primers		
10	OPG 01	CTA CGG AGG
11	OPG 02	GGC ACT GAG G
12	OPG 03	GAG CCC TCC A
13	OPG 04	AGC GTG TCT G
14	OPG 05	CTG AGA CGG A
15	OPG 06	GTG CCT AAC C
16	OPG 07	GAA CCT GCG G
17	OPG 08	TCA CGT CCA C
18	OPG 09	CTG ACG TCA C

RAPD analysis

PCR amplification of DNA Random amplified polymorphic DNA polymerase chain reaction (RAPD–PCR) procedure described by Saghai-Maroof *et al.* (1984) was followed using a reaction mixture of 25 µl volume consisting of 2.5µl of 10X PCR buffer (20 mm Tris-HCl, pH-8.4; 50 mm KCl and 1.5 mm MgCl2; Genetix, Biotech Asia Pvt Ltd, New Delhi, India), 2.0 µl dNTPs mix (25 mM each of dATP, dTTP, dGTP, dCTP; MBI), 0.2 µl Taq DNA polymerase (5 U/ µl; Genetix, Biotech Asia Pvt Ltd, New Delhi, India), 1.0 µl of primer (10 mm; Operon Technologies Inc., CA, Alameda, USA), 2.0 µl (20 ng) genomic DNA and 17.3 µl of sterilized double-distilled water. The reaction mixture was vortexed and centrifuged for 30 s in microcentrifuge for proper mixing. Amplification was carried out in a thermal cycler (Thermal cycler (PTC-100) and BIORAD My Cycler) by using three temperature profiles, programmed for initial DNA denaturation at 94^{0}C for 5 min, followed by 40 cycles consisting of DNA denaturation for 4 min at 94^{0}C, primer annealing at 37^{0}C for 1 min and polymerization for 2 min

at 72^{0}C with a final extension period of 5 min at 72^{0}C. PCR mixture supplemented with sterilized distilled water instead of template DNA was kept as control. Amplification products were separated on 1.6% agarose gels (Bangalore Genei Pvt Ltd, Bangalore, India), stained with ethidium bromide (0.5 lg/ml) and photographs were taken by using a Gel documentation system (BIORAD gel documentation system). Each primer combination was replicated thrice to check the consistency of the banding pattern.

Results and Discussion

Cluster analysis of 24 isolates of *U. segetum tritici* indicated that out of a total of 18 primers used for the molecular analysis of the pathogen (ISSR and RAPD) only 7 primers were able to give scorable bands with some polymorphism (Fig.1). These primers gave a total of 206 bands. ISSR primers were found better than RAPD. Of nine ISSR primers, five primers (ISSR 811, ISSR 824, ISSR 826, ISSR 827 and ISSR 856) were able to produce scorable bands. Of these, primer ISSR 827 gave highest polymorphism.

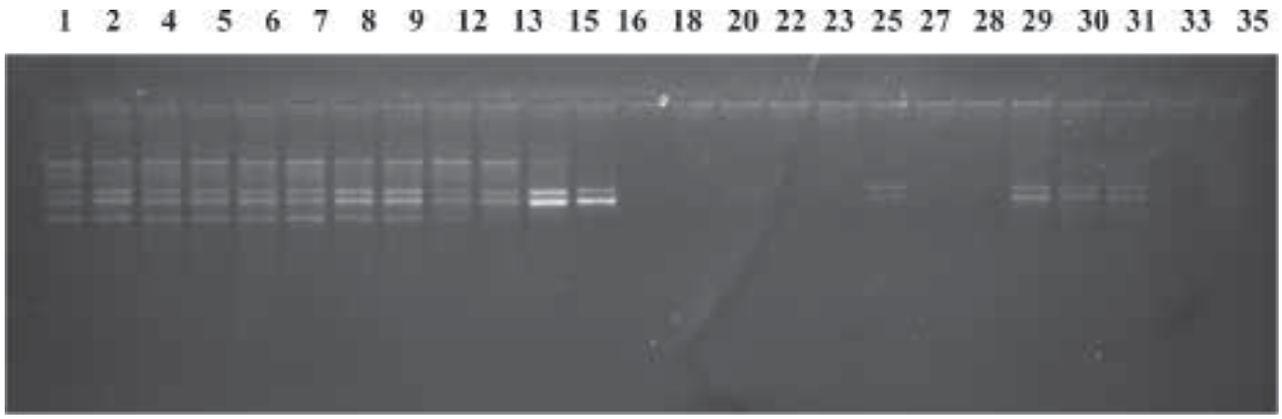

Fig. 1. A representative picture depicting ISSR based profiling of loose smut isolates with primer ISSR 827 (for lane numbers see Table 1).

Of nine RAPD primers, only two primers (OPG02 and OPG 05) were able to produce scorable bands. Of these primers, primer OPG 02 gave highest polymorphism. However, Karwasra *et al.* (2002) could observe 210 scorable bands using RAPD and ISSR primers.

Table 3. Molecular characterization based grouping

	At 16% level of similarity					
Major	Group1 At 34% similarity					Group 2
Clusters	C1	C2	C3	C4	C5	
Isolates	LS1, LS5, LS31, LS6, LS16, LS25, LS28, LS35, LS2, LS8, LS33, LS15, LS7, LS30 and LS13	LS4, LS9 and LS12	LS27	LS20 and LS29	LS18 and LS23	LS 22

Based upon molecular characterization evaluated at 16% and 34% level of similarity, *U. segetum tritici* isolates were divided into two groups as indicated in Table 3. Banding pattern obtained from polymorphic markers was subjected to cluster analysis using software package NTSYS PC version 2.02e (Rohlf, 1993). Jaccard's similarity coefficient between 24 isolates of *U. segetum tritici* varied from 16.0% to 100.0%. Out of 24 isolates, LS16 and LS25 showed maximum similarity coefficient (Fig. 2). This study was further supported by the pathogenicity test on differential set (Kaur 2010, Table 4). Phylogentic analysis divided the isolates into two major groups namely group 1 and group 2. At 36% level of similarity, group 1 gets sub divided into five clusters (Fig. 2), while group 2 contained only isolate LS22. This clustering showed that isolate LS22 was all together dissimilar (84% level of dissimilarity) from the rest isolates of *U. segetum tritici.* Isolate, LS7 and LS30 showed 87% level of similarity followed by LS8 and LS33 showed 62 % level of similarity. Cluster 4 contains only two isolates, LS20 and LS29, which showed 40% level of similarity.

Isolate, LS22 formed a separate category in molecular studies which was the only isolate from geographical region, Hissar. Isolates, LS1 and LS 31 (*T. aestivum)* and LS 5 (*T. durum*) from

geographical region, Mansa (Table 1) were clustered in one group based on molecular analysis. Similar to the present study, RAPD, ISSR and AFLP profiling were evaluated for assessing the extent of genetic variation among the isolates of *U. segetum tritici* which causes the loose smut disease of wheat (Karwasra *et al.*, 2002). A study by Randhawa *et al.* (2009) indicated that molecular diversity in *U. tritici* can be associated with the virulence patterns and most of the races were observed to be virulent only on either bread wheat or durum wheat, but race T39 could infect both the species.

We conclude that *U. segetum tritici* causing loose smut of wheat in the north-Western region of India possesses variable populations as is evident from differential inoculation and molecular analysis. The phylogenetic grouping based on DNA based markers data did not appear to be congruent with either morphological (data not shown) or virulence pattern.

Table 4. Grouping of isolates based on reaction on Canadian differential set

Group	Susceptible reaction on differentials	Isolates
1	TD4, TD5, TD7, TD13	LS1
2	TD7, TD13	LS3, LS4, LS8, LS12, LS15, LS18, LS19, LS20, LS33, LS34
3	TD5, TD7, TD13	LS2, LS5, LS10, LS13, LS14, LS16, LS21, LS24, LS25, LS26, LS27, LS28, LS30, LS31, LS32, LS35
4	TD5, TD7	LS6, LS9, LS22, LS23, LS29
5	TD5, TD13	LS11
6	Only TD7	LS7, LS17

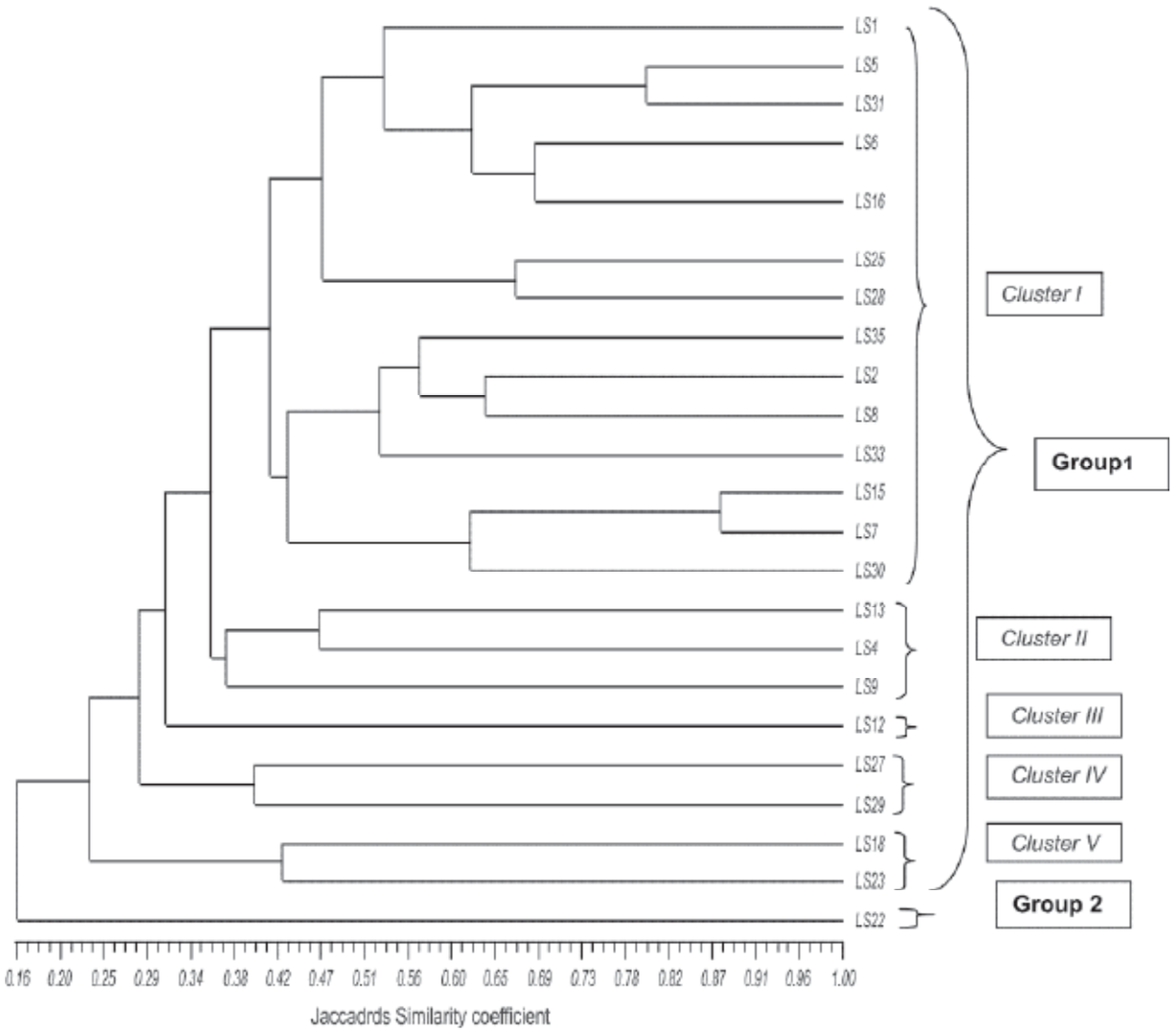

Fig 2. Dendrogram obtained using ISSR and RAPD bands of 24 isolates of *Ustilago segetum tritici* generated by unweighted pair group method arithmetic mean (UPGMA). Scale at the bottom depicts the similarity values obtained using Jaccard's similarity coefficient.

The use of a large number of random primers or use of other DNA markers may yield race-specific DNA marker for the detection of particular race.

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