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# Molecular and morphological characterization of Indian isolates of *Ustilago tritici*, causal agent of wheat loose smut

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Morphology of teliospores of *Ustilago tritici* was studied by using light and electron microscopy (TEM & SEM). The data on teliospore length and width subjected to ANOVA showed significant difference at 1% level among the isolates. UPGMA cluster analysis divided the isolates into two major clusters. The ultrastructure of the isolates was found to be substantially similar. The isolates were also analysed by random amplified polymorphic DNA analysis (RAPD) using 25 decamer primers and showed high degree of variability among themselves. Ut12, which got separated from others by it's teliospore size, was also separated from all other isolates by UPGMA cluster analysis using RAPD analysis. The clustering based on RAPD analysis does not bear correlation with the geographical distribution of isolates as well as their groupings based on teliospore morphology. As teliospore size is a variable character, it is imperative to use approaches involving DNA polymorphism for searching variability among isolates.

**Key words :** Teliospore morphology, *Ustilago tritici*, wheat, RAPD

## INTRODUCTION

Loose smut of wheat is of worldwide occurrence and causes low to moderate annual losses. It is more common in regions with a cool, moist climate during flowering of the host. Yet, even in dry warm climates, economic losses occur. Since percentage infection equals loss in yield and since most of the monetary return from any one field goes into cost of production, even 1-2% infection can reduce profit to the farmer by 5-20% (Nielsen and Thomas, 1996).

Morphological characters of the teliospores such as teliospore measurements and teliospore surface ornamentation are used to differentiate smut species (Vanky, 1991).

Recent developments in DNA- based technology including random- amplified polymorphic DNAs (RAPD) have provided suitable tools for rapid and detailed genetic analysis of higher organisms (Rafalski *et al.*, 1991; Williams *et al.*, 1990). The genetic diversity is assessed by amplification at low stringency with a single short primer of arbitrary sequence. The RAPD technique has been used to detect genetic variations among strains or isolates within a species (Cooke *et al.*, 1996 ; Hseu *et al.*, 1996 ; Body & Carris, 1997 ; Jeng *et al.*, 1997 ; Maurer *et al.*, 1997 ; Pie *et al.*, 1997 ; Jungechulsing & Tueznski, 1997).

In the present investigation attempts have been made to use light and electron microscopical studies to see the differences in the teliospore morphology. Moreover, often there is little variation in teliospore morphology, molecular characteristics are being included in current studies in order to elucidate the existence of variability among isolates of

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*U. tritici* prevalent in India.

## MATERIALS AND METHODS

### *Collection of isolates*

The teliospores of twenty samples of *U. tritici* were collected from various parts of Northern India viz., Punjab, Haryana, Himachal Pradesh, Uttar Pradesh, Delhi and it's vicinity during February and May, 1999 (Table 1). The teliospores of these samples were taken from infected heads and subjected to microscopic examination.

**Table 1** : Isolates of *Ustilago tritici* collected from Northern India

| Isolate No. | Location | Date of Collection |
|---|---|---|
| Ut1 | Gurgaon, Haryana | March 99 |
| Ut2 | Bathinda, Haryana | March 99 |
| Ut3 | HAU, Hissar, Haryana | March 99 |
| Ut4 | Thaska, Hissar Haryana | March 99 |
| Ut5 | Kaimri, Hissar, Haryana | March 99 |
| Ut6 | Karnal, Haryana | March 99 |
| Ut7 | HPKV, Palampur, U.P. | May 99 |
| Ut8 | Palampur, H.P. | May 99 |
| Ut9 | Shimla, H.P. | March 99 |
| Ut10 | IARI, Reg. St., Shimla, H.P. | May 99 |
| Ut11 | Kumargaj, Faizabad, U.P. | March 99 |
| Ut12 | Kanpur, U.P. | March 99 |
| Ut13 | Varanasi, U.P. | March 99 |
| Ut14 | Pusa, Bihar | March 99 |
| Ut15 | Pusa, Bihar | March 99 |
| Ut16 | RAU, Bihar | March 99 |
| Ut17 | Patna, Bihar | March 99 |
| Ut18 | PAU, Ludhiana, Punjab | March 99 |
| Ut19 | PAU, Ludhiana, Punjab | March 99 |
| Ut20 | IARI, New Delhi | February 99 |

### *Microscopical Studies*

*Light microscopy* : One hundred dried telisopores of each sample were rehydrated in Shear's mounting fluid (Chupp, 1940) and observed at 2000X magnification with an Olympus BX50 microscope. Measurements for length and width of the teliospores were taken with the help of ocular micrometer.

*Scanning Electron Microscopy* : Air dried teliospores were dusted on small prices of double sided adhesive tape, mounted on specimen stub, sputter-coated with gold-palladium under vacuum, ca. 20 nm for 4.5 min, 7.5 mA. The specimens were then observed and photographed in a LEO 435 VP SEM, operated at 15kv following the procedure of Vanky (1997).

*Transmission Electron Microscopy* : The fixing of the teliospores for TEM was done as given by Vanky (1997). Teliospores were fixed with 2% glutaraldehyde in 0.1 M Na-cacodylate buffer at pH 7.2 for one week. After six continuous transfers in 0.1 M Na-cacodylate buffer, the teliospores were post fixed in 1% osmium tetraoxide in the same buffer for 1 h in the dark then washed in distilled water, and stained in 1% aqueous uranyl acetate for 1 h in the dark. After five washings in distilled water, the material was dehydrated in acetone series embedded in Spurr's plastic and sectioned the handy microtome. Semi thin sections were stained with new fuchsin and crystal violet, mounted in entellan and studied under light microscpoe. Ultra thin sections were taken by ultramicrotome with glass knives and were mounted on copper slot grids post-stained with lead citrate for 5 min and examined under a CM10 PHILIPS electron microscope at 60 kv.

### *Cultural conditions*

Monokaryotic haploid hyphae of these isolates were obtained as per method of Sharifnabi *et al.* (2000). Teliospores were surface sterilized and resuspended in sterile distille water and 150-250 ml of sterilized teliospore suspension was evenly spread on petridishes containing 1.5-2 mm thick, 1.5% water agar and DL - aspartic acid (0.147 mg/ml water). The petridishes were incubated at 20°C for about 30 h and subsequently 1 square cm blocks of medium from these plates were transferred to plates with 1.5-2 mm thick of 1/5 of normal nutrient concentration of PDA (Potato Dextrose Agar) and incubated in refrigerator overnight. The squares were then transferred to another 1.5-2 mm thick layer of 1/5 PDA pre-warmed to 25°C and kept for 4-6 h. Monokaryotic haploid hyphae were obtained and isolated by microsurgery with very thin pasture pipettes and transferred to a thick layer of 1/5 PDA and kept at 20°C for two weeks. The mycelial mass production of the mycelium of haploid hyphae obtained by incubation in PSB (Potato Sucrose Broth) in shaker incubator at 130 rpm at 20°C for 14-20 days and subsequentely mycelial growth in PSB were harvested by filtration through Whatman

filter paper No. 41. These cultures were rinsed with sterile distilled water and frozen at 20°C. The frozen samples were used for DNA extraction.

*DNA Extraction*

Because of the slow growth habit of *U. tritici*, a modified procedure of the cetyltrimethylammonium bromide (CTAB) method of Shi *et al.*, (1996) was followed. The frozen mycelium of each isolate was grounded in sterile morter and pestle using liquid nitrozen. The fine frozen powder was transferred to a sterile centrifuge tube. Pre-warmed at 65°C DNA extraction buffer [100mM Tris-HCI (pH 8.0, 50 mM EDTA (pH 8.0), 1.4M NaCI and 2% CTAB ] was added to this and incubated in a waterbath at 65°C for 1 h. After incubation an equal volume of chloroform/isoamyl alcohol (24:1) was added and mixed gently for centrifugation at 10000 rpm. 25°C for 10 min. The aqueous phase was transferred to a new sterile tube and the DNA was precipitated with 0.6 volume of cold isopropanol and 0.1 volume of sodium acetate (3M, pH 5.2) for 25 min at room temperature. DNA was pelleted by centrifugation at 10000 rpm for 10 min at 25°C. The pellet was washed with cold 70% ethanol and resuspended in sterile water.

*Purification of DNA*

A stock solution of RNase-A was prepared @ 10 mg/ml in 10mM Tris-HCI pH 8.0 and 15mM sodium chloride. The solution was boiled for 10 min to destroy DNase. From the stock, 2 µl of RNase-A was added to the crude DNA sample and incubated at 37°C for 1 h. The DNA concentration of the samples and its purity was determined by taking UV absorbance at 260 nm and 280 nm in a spectrophotometer.

*Random Amplification of Polymorphic DNA (RAPD) conditions*

Thirty-five 10 mer oligonucleotides from sets P, S, M and N (Operon Techonologies Inc, USA) were used as single primers for the amplification of sequences. The PCR reaction was performed in a Gene-Cycler (Bio-RAD, USA), in a 25 µl volume containing 4 µl of 1X reaction buffer (10 mM Tris-HCl, pH 8.3 and 50 mM KCl), 5 mM $MgCl_2$, 0.5 unit of Taq DNA polymerase, 100 µM each of dATP, dCTP, dGTP and dTTP (GENEI, Bangalore, India), 1 µM of mer primer and 25 ng of template DNA. The amplification conditions were : initial step of denaturation at 94°C for 2 min followed by 40 cycles of denaturation at 92°C for 1 min, primer annealing at 37°C for 1 min and extension at 72°C for 2 min, followed by an extended elongation step at 72°C for 5 min. Samples of 25 µl PCR products were mixed with 3 µl of 10X loading dye (0.25% bromophenol blue, 0.25% xylene cyanol and 40% sucrose, W/V) and spun briefly in a microfuge before loading (Sambrook *et al.*, 1989). The amplification products were analysed on 1.2% agarose gel containing 0.2 µg/ml of ethidium bromide in 1X TAE buffer running at 60 volts for 3 h and visualized under UV light and photographed on Polaroid 667 film under UV light. The DNA size marker used was λDNA double digested with *Eco*RI and *Hin*dIII.

***Data analysis***

*Morphological data analysis*

The data obtained from morphological studies was analysed by two methods viz., (i) Analysis of Variance : The analysis of Variance (ANOVA) was performed by using GLM procedure of PC SAS (SAS Institute, 1989). The mean comparison of the studied characters of the isolates was separately done using Duncan's Multiple Range Test. (ii) Hierarchical Cluster Analysis : The dissimilarity matrix was calculated from standardized morphological characters data using Euclidean measure of distance. Unweighted pair group method using arithmetic average (UPGMA) was selected to generate (Sneath & Sokal, 1973).

*RAPD data analysis*

Each amplification product was considered as RAPD marker. Gels were scored on the basis of the presence (1) or absence (0) of each band for all isolates. All amplifications were repeated at least twice and only reproducible bands were considered for analyse. Jaccard's similarity coefficient values for each pairwise comparison between isolates were calculated (Jaccard, 1908) and a similarity coefficient matrix was constructed. This matrix was sub-

jected to unweighted pair-group method for arithmetic average analysis (UPGMA) to generate a dendrogram using average linkage procedure. All the numerical taxonomic analysis were conducted using software NTSYS-pc, version 1.80 (Rohlf, 1988).

## RESULTS

The isolates of *U. tritici* were subjected to purification by obtaining monokaryotic haploid hyphae. The hyphae were then multiplied on potato sucrose broth to raise mycelial mass for DNA extraction. However, only 12 isolates of *U. tritici* (Ut2, Ut3, Ut4, Ut6, Ut7, Ut8, Ut10, Ut11, Ut12, Ut15, Ut17 and Ut20) yielded sufficient mycelium for DNA extraction and were further taken up for RAPD analysis.

The length of the teliospores varied from 5 µm to 12 µm and width 4 µm to 9 µm. The average mean length and width of teliospores was 6.50 µm and 5.71 µm respectively. Isolate Ut6, collected from Karnal, Haryana has the smallest teliospores having mean length (5.54 µm) and width 5.13 µm, whereas the maximum mean length of 8.41 µm was observed in Ut12 (Table 2).

**Table 2 :** Comparison of the mean length and width of different isolates of *Ustilago tritici* Using Duncan's multiple Range Test (DMRT)

| Isolate | Length (µm) | | Width (µm) | |
|---|---|---|---|---|
| | Mean | Rank | Mean | Rank |
| Ut1 | 5.940 | I | 5.270 | JK |
| Ut2 | 6.050 | HI | 5.275 | JK |
| Ut3 | 6.350 | FG | 5.670 | EFGH |
| Ut4 | 6.690 | CD | 5.800 | DE |
| Ut5 | 6.365 | FG | 5.775 | DEF |
| Ut6 | 5.540 | J | 5.135 | K |
| Ut7 | 6.470 | DEF | 5.940 | D |
| Ut8 | 5.935 | I | 5.350 | IJ |
| Ut9 | 7.710 | C | 6.170 | C |
| Ut10 | 7.140 | B | 6.480 | B |
| Ut11 | 6.420 | EFG | 5.575 | GH |
| Ut12 | 8.410 | A | 7.215 | A |
| Ut13 | 6.710 | C | 5.720 | EFG |
| Ut14 | 6.730 | C | 5.700 | EFG |
| Ut15 | 6.620 | CDE | 5.490 | HI |
| Ut16 | 6.656 | CDEF | 5.635 | EFGH |
| Ut17 | 6.200 | GH | 5.330 | IJ |
| Ut18 | 6.375 | FG | 5.590 | FGH |
| Ut19 | 6.435 | EF | 5.615 | EFGH |
| Ut20 | 6.530 | CDEF | 5.590 | FGH |

**Table 3 :** ANOVA of isolates of *Ustilago tritici* for the studied characters of teliospores.

| Source of Variance (S. V.) | Degree of Freedom (df) | Mean Square (MS)* | |
|---|---|---|---|
| | | Teliospore Length | Teliospore Width |
| Isolates | 19 | 32.461 | 22.365 |
| Error | 1980 | 0.526 | 0.337 |
| Coefficient of Variation (%) | — | 11.14 | 10.16 |

* All sources of variance are significant at 1% level.

ANOVA showed significant differences at 1% level among the isolates for both length and width of *U. tritici* (Table 3).

Based on DMRT data, results showed that there were 10 classes in case of *U. tritici* with respect to length and 11 in *U. tritici* with respect to width of teliospores. Ut12 is separated from all other isolates by DMRT. The length and width data of the teliospores of various isolate of *U. tritici was* subjected to cluster analysis using UPGMA on distance matrix based on Euclidean measure. The cluster analysis separated isolates Ut10 and Ut12 from all other isolates (Fig. 1). The remaining isolates were subdivided into 2 groups, one comprising of Ut1, Ut8, Ut2, Ut17 and Ut6 and the other comprising of the rest.

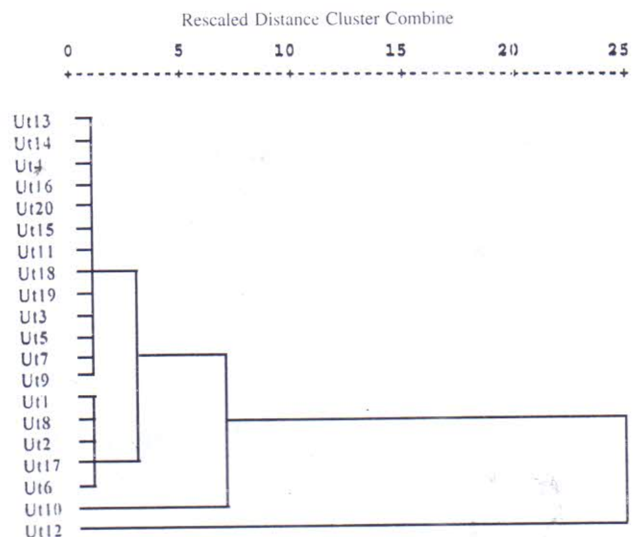


**Fig. 1 :** Dendrogram of *Ustilago tritici* isolates resulting from UPGMA based on different characteristics of teliospores.

Teliospores of *U. tritici* and dark brown, lighter coloured on one side, globose to subglobose, to

ovoid. Under SEM they are minutely echinulate. Echinulation was observed to be more on the lighter side of the teliospores. The size varying from 5-12 μm (6.50 ± 0.91) × 4-9 μm (5.71 ± 0.73). SEM showed that teliospores are minutely echinulate with sparse spines.

The cell wall has two distinct layers. The outer layer is electron dense and dark and the inner layer exhibits uniform electron density, which was corky texture with some sort of cellular organisation. The spines which are highly electron dense, are appended on the surface of the outer layer, protoplasm membrane is distinct from the cytoplasm which is granular in nature. From the results obtained it is concluded that the ultrastructure of all the isolates were found to be substantially similar.

### *Optimisation of RAPD markers*

Good quality DNA of concentration 100 ng/μl was obtained from approximately 200 mg of frozen mycelial mass. Standardization of the PCR protocol was done by changing the variables like templage DNA, Taq DNA polymerase and magnesium chloride. The optimum amplification was obtained by using the reaction mix having 25 ng templage DNA, 0.5 unit Taq polymerase, 5 mM $MgCl_2$, 1 μM primer and 100 μM dNTP in reaction volume of 25 μl.

**Table 4 :** Primers used and the number of total and polymorphic bands produced in *Ustilago tritici* isolates.

| Prime | Sequence (3'–5') | Total bands | Ploy-morphic bands | Ploy-morphic bands | Mono-morphic bands |
|---|---|---|---|---|---|
| OPM06 | CTGGGCAACT | 15 | 15 | 100 | 0 |
| OPM07 | CCGTGATCA | 20 | 20 | 100 | 0 |
| OPM13 | GGTGGTCAAG | 16 | 16 | 100 | 0 |
| OPN02 | ACCAGGGGCA | 7 | 7 | 100 | 0 |
| OPN05 | ACTGAACGCC | 15 | 15 | 100 | 0 |
| OPN17 | CATTGGGGAG | 12 | 12 | 100 | 0 |
| OPP01 | GTAGCACTCC | 11 | 11 | 100 | 0 |
| OPP02 | TCGGCACGCA | 14 | 13 | 92.85 | 1 |
| OPP03 | CTGATAGGCC | 12 | 12 | 100 | 0 |
| OPP04 | GTGTCTCAGG | 13 | 13 | 100 | 0 |
| OPP05 | CCCCGGTAAC | 6 | 6 | 100 | 0 |
| OPP06 | GTGGGCTGAC | 12 | 12 | 100 | 0 |
| OPP08 | ACATCGCCCA | 17 | 17 | 100 | 0 |
| OPP09 | GTGGTCCGCA | 13 | 13 | 100 | 0 |
| OPP10 | TCCCGCCTAC | 11 | 11 | 100 | 0 |
| OPP16 | CCAAGCTGCC | 17 | 17 | 100 | 0 |
| OPP19 | GGGAAGGACA | 13 | 12 | 92.30 | 1 |
| OPP20 | GACCCTAGTC | 12 | 12 | 100 | 0 |
| OPS01 | CTACTGCGCT | 10 | 10 | 100 | 0 |
| OPS02 | CCTCTGACTG | 11 | 11 | 100 | 0 |
| OPS03 | CAGAGGTCCC | 20 | 19 | 95.00 | 1 |
| OPS05 | TTTGGGGCCT | 26 | 26 | 100 | 0 |
| OPS06 | GATACCTCGG | 12 | 12 | 100 | 0 |
| OPS08 | TTCAGGGTGG | 18 | 18 | 100 | 0 |
| OPS09 | TCCTGGTCCC | 12 | 12 | 100 | 0 |
| | | 345 | 342 | — | 3 |

Thirty five random 10 mer Operon primer from set of OPP, OPS, OPM and OPN were screened and out of them twenty five were selected for doing RAPD analysis on the basis of reproducible and scoreable polymorphic bands obtained (Table 4).

The total number of bands obtained were 345 out of which 342 were polymorphic. Maximum numbers (26) of bands were obtained with primer OPS5 and all of them were polymorphic. The size fragments obtained varied from 0.5 to 4 kb.

### *UPGMA Cluster analysis*

The data were obtained from RAPD analysis of 12 isolates of *U. tritici* with 25 primers was subjected to UPGMA analysis. A dendrogram was prepared using the similarity coefficient of RAPD marker (Fig. 2). The dendrogram revealed the existence of variability among isolates. Among the isolates Ut12, Ut11 and Ut3 were found to form seperate groups, and the rest of the isolates were divided into two major cluster with Ut15, Ut17 and Ut20 belonging to one and Ut10, Ut8, Ut7, Ut6, Ut4, and Ut2 to the other cluster, these clusters were further divided into smaller clusters. The dendrogram revealed the existence of variability among isolates of *U. tritici.*

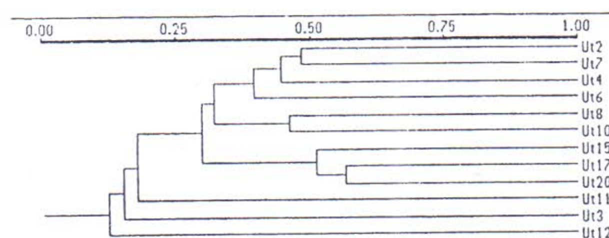


**Fig. 2 :** Dendrogram from RAPD profiles of *Ustilago tritici* isolates. Scale is distance by UPGMA method as implemented in NTSYS – pc.

## DISCUSSION

Different isolates of *U. tritici* subjected to RAPD analysis using 25 random decamer primer gave in-

teresting information regarding variability among diferent isolates. The isolates among themselves showed a high degree of variability. Ut12 isolate which got separated from others by it's teliospore size, gave a distinct pattern and was separated from all other isolates by UPGMA cluster analysis, however, Ut11 and Ut3 which got separated from others by UPGMA cluster analysis could not be separated by teliospore size. The clustering of isolates based on RAPD analysis does not bear correlation with the geographical distribution of the isolates as well as the grouping based on teliospore morphology.

Studies undertaken by us on the teliospore morphology could differentinate isolates of *U. tritici*, but for more precise grouping we did use investigation based on the molecular fingerprinting techniques in order to detect variability amongst *U. tritici.*

The present study highlights the fact that RAPD technique is a powerful tools for analyzing different population structure and need to be studied in complementation with other characters. The following conclusion can be effectively be drawn from the present studies. RAPD analysis is an effective tool, which can be used to study variability in smut fungi. This will provide us with a better understanding of the pathogen in order to differentiate species.

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