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Assessing the molecular variability in *Ustilaginoidea virens*, the rice false smut pathogen with ISSR markers

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Abstract

Rice false smut disease is gaining importance because of its impact on the grain yield and its toxin production ability. Fifty-eight isolates of *Ustilaginoidea virens* were collected from different rice-growing regions of India. DNA of *U. virens* was isolated by the CTAB method and fifty ISSR primers were screened for molecular variability studies. Twelve primers *viz*., UBC series 807, 808, 809, 810, 811, 812, 834, 835, 836, 840, 841, and 842 were selected to study the genetic variability. Different parameters of tested primers *viz*., heterozygosity (Hn), polymorphism information content (PIC), effective multiplex ratio (EMR), marker Index (MI), and resolving power (RP) were calculated. Primers UBC 812 and UBC 809 recorded maximum heterozygosity (Hn). The PIC values ranged from 0.10 to 0.27 and UBC 807 recorded the maximum value of 0.27. The EMR value varied from 6.75 to 24.0, Similarly, UBC 807 recorded the highest value of MI (24) and RP (8.55). A dendrogram was generated using the DARwin software (version 6.0.21A) based on the unweighted neighbor-joining cluster method. All the fifty-eight *U. virens* isolates were grouped into three major clusters. Clusters I and II had 21 and 35 *U. virens* isolates respectively. Cluster III had only two isolates. The isolates showed genetic variations and there was no specific grouping based on the geographical distance.

Keywords: Rice, False smut, Molecular variability, ISSR, Isolate

Introduction

Rice crop is affected by fungal, bacterial and viral diseases. Among the fungal diseases, the false smut disease of rice has become one of the important grain diseases. The pathogen *Ustilaginoidea virens* (Cooke) (Takahashi) causes up to 50% yield loss under favorable environmental conditions. It infects the pollen and stigma and converts the rice grain into a ball-like structure that contains mycelia and chlamydospores. Initially, young smut balls are covered with a thin layer of a white membrane which later bursts, and upon maturity, the smut ball turns into yellow and green color. The exact mode of infection of the pathogen was less understood and management of the disease mainly depends on the application of fungicides. The pathogen produces both sexual (sclerotia) and asexual (chlamydospores) resting structures. The pathogen infects the pollen and produces the typical smut balls (Tang *et al*., 2013). Ustiloxins and Ustilaginoidins are the toxins produced by *U. virens* (Wang *et al*., 2019). A study on the genetic diversity of pathogen will give an idea about the survival strategy of the pathogen. The present study was carried out to know the existence of variability in the isolates of *U. virens* collected from different rice-growing regions of India using Inter Simple Sequence Repeats (ISSR) primers.

Materials and Methods

Isolation of *U. virens* and pathogenicity

Smut ball samples were collected from different rice-growing states of India. Care was taken to collect the samples from different fields and different varieties.

Yellow-coloured smut balls were surface sterilized with 1% sodium hypochlorite for two minutes and washed three times with sterile water. By using a sterile bacterial inoculation needle, the chlamydospores were streaked onto Petri dishes containing potato sucrose agar (PSA) medium amended with 100 ppm streptomycin sulfate (Ladhalakshmi *et al*., 2012). The plates were incubated at 27°C for seven days. Typical white fungal colonies of *U. virens* were transferred onto fresh PSA slants for purification and stored under 4°C. The injection method of inoculation (Ladhalakshmi *et al*., 2019) using a hypodermic syringe was adopted in the booting stage of the plants for proving the pathogenicity of the pathogen. Around 4 to 5 tillers were inoculated per plant. The identity of all the collected isolates was confirmed by using specific ITS primers (Zhou *et al*., 2003; Ladhalakshmi *et al*., 2012).

Genetic variability

DNA Isolation

Total fungal DNA was isolated by following Cetyl trimethyl ammonium bromide (CTAB) method (Zhou *et al*., 2003). *U. virens* mycelium was harvested by filtration and washed with sterile distilled water repeatedly. The mycelial mat was frozen in liquid nitrogen and ground into fine powder and to the powdered mycelia (50-60 mg), 750 µl of CTAB buffer was added and incubated at 65°C for 45 min. During the incubation period, the tubes were vortexed and spun at 10,000 rpm for 15 min and equal volume of chloroform and isoamyl alcohol (24: 1) (v/v) was added to the collected supernatant and again centrifuged at 13,000 rpm for 15 min. Supernatant was pooled, and an equal volume of chilled iso-propanol was added and incubated for an hour at −20°C. The DNA was precipitated by centrifugation at 10,000 rpm for 15 min and finally, the DNA pellet was washed with 70% ethanol, air dried and finally dissolved in 50 µl of the sterile distilled water.

PCR conditions and gel electrophoresis

Initially, 50 ISSR primers (UBC series from 801 to 850) were screened with a subset of samples. Among them, twelve primers that gave a scorable banding pattern were selected for the study. ISSR-PCR was performed in a total volume of 20 µl contained 2 µl of genomic DNA (50 ng), 1 µl of primer of 5 µM primer solution, 2 µl of 10x buffer (0.1 M *Tris* pH 8.3; 0.5 M KCl; 7.5 mM $MgCl_2$; 0.1% gelatin), 1 µl of 2.5 mM dNTPs and 1.0 unit of Taq polymerase. PCR amplifications were performed in a thermal cycler (Applied Biosystems, USA) with the following conditions: initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturing at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 7 min. The amplified products were resolved in 2% agarose gel in 1X TBE buffer under room temperature at a constant voltage of 90 V. The molecular weight markers, 100 bp and 1 Kbp ladder (Banglore Genei Private Limited, India) were used for band sizing.

Data Analysis

Each amplified product/band was scored as 1 and 0 respectively based on the presence (1) and absence (0). Based on the scored data, various parameters *viz*., number of loci, number of polymorphic loci, polymorphism (%), polymorphism information content (PIC), and primer resolving power (Rp) were calculated. The PIC value for each primer was calculated. $PIC = 1 – (f^{2} + (1 - f)^{2})$, where *Pi* is the frequency of *i* th allele, *n* is the number of bands. Effective multiplex ratio (EMR) is calculated as a total number of polymorphic loci per primer multiplied by the rate of polymorphic loci from their total number. The Rp of each primer was calculated; $Rp = \Sigma Ib$, where $Ib = 1 – [2 \times (0.5 – Pi)]$, where *Pi* is the proportion of accessions containing band *i* (Chesnokov and Artemyeva, 2015). A distance-based unweighted neighbour-joining cluster tree was constructed based on the dissimilarity index and a dendrogram was constructed using the DARwin software version 6.0.21.

Results and Discussion

A total of 58 isolates of *U. virens* from 18 different rice-growing states of India were isolated and maintained (**Table 1**). The pathogen produced white coloured colony which later turned into yellow and green colour. All the collected isolates were confirmed by specific ITS primers. Pathogenicity of the false smut was proved and typical yellow-colored smut balls (up to 10 smut balls per panicle) were observed in the inoculated panicles 20 days after inoculation.

Fifty ISSR markers (801 to 850) were screened and among them, twelve markers were selected as potential makers *viz*., 807, 808, 809, 810, 811, 812, 834, 835, 836, 840, 841, and 842 based on their amplification banding pattern and reproducibility. Across the markers, the number of amplified loci varied from 9 to 24 (**Figure 1**). Twelve ISSR primers amplified a total of 6503 bands with an average number of 541 bands for 58 isolates of *U. virens*. The maximum heterozygosity (Hn) was shown by UBC 812 and UBC 809. The value of the polymorphism information content (PIC) indicates the ability of a marker to find the polymorphism in the population (Chesnokov and Artemyeva, 2015). In the present study, the polymorphism of the primers varied from 75% to 100%. The polymorphism information content (PIC) values ranged from 0.10 to 0.27. UBC 807 recorded the maximum PIC value of 0.27. Among the tested ISSR primers, the Effective multiplex ratio (EMR) varied from 6.75 to 24.0, and the maximum value was recorded with UBC 807 (24.0) and it can be considered an efficient marker. To estimate the utility of the markers, the Marker Index (MI) is used and the values ranged from 1.0 to 5.9 and the primer UBC 807 recorded the maximum value of 5.9. The parameter resolving power (RP) determines the ability of the primer to differentiate the tested isolates and tested primers recorded a range of values from 2.14 to 8.55 and UBC 807 recorded the highest value of 8.55 (**Table 2**).

Table 1. Details on the *Ustilaginoidea virens* isolates collected from different rice-growing states of India

S. No.	Isolate No.	Place of collection	Variety from which smut balls were collected
1	WB-1	RRS, Chinsurah, West Bengal	Not Known
2	WB-2	Chinsurah, West Bengal	Not Known
3	MH-1	Sakoli-1, Maharastra	RPN
4	MH-2	Sakoli-2, Maharastra	PKV Khamang
5	MH-3	Sakoli-3, Maharastra	Swarna
6	MH-4	Sakoli-4, Maharastra	Jai Sriram
7	MH-5	Sakoli-5, Maharastra	HMT
8	GJ-1	Nawagam, Gujarat	Not Known
9	HP	Malan, Himachal Pradesh	Not Known
10	PU-1	PAU-1, Ludhiana, Punjab	IRRI line
11	PU-2	PAU-2, Ludhiana, Punjab	Signet 5050
12	PU-3	PAU-3, Ludhiana, Punjab	NK6704
13	PU-4	PAU-4, Ludhiana, Punjab	HRI107
14	PU-5	PAU-5, Ludhiana, Punjab	Not Known
15	PU-6	PAU-6, Ludhiana, Punjab	PR-120
16	PU-7	PAU-7, Ludhiana, Punjab	GSK-37
17	PU-8	PAU-8, Ludhiana, Punjab	Not Known
18	PU-9	Kapurthala, Punjab	Not Known
19	HR-1	Kaul-1, Haryana	PR106
20	HR-2	Kaul-2, Haryana	HKR47
21	HR-3	Kaul-3, Haryana	Haryana Shankar Dhan
22	HR-4	Kaul-4, Haryana	HKR126

S. No.	Isolate No.	Place of collection	Variety from which smut balls were collected
23	HR-5	Gunthala	PAU201
24	HR-6	Kaul-5, Haryana	Not Known
25	HR-7	Kaul-6, Haryana	Not Known
26	HR-8	Kaul-7, Haryana	Not Known
27	HR-9	Karnal-1, Haryana	CSR-5
28	HR-10	Karnal-2, Haryana	PET-TR-2000-003
29	HR-11	Karnal-3, Haryana	IRSSTN-3
30	HR-12	Karnal-4, Haryana	Bulk
31	HR-13	Uchani, Haryana	HKR581
32	UK-1	Pantnagar, Uttarakhand	Not Known
33	UK-2	Kasipur, Uttarakhand	Not Known
34	UP-1	Nagina, Uttar Pradesh	Not Known
35	MP-1	Jabalpur, Madhya Pradesh	Not Known
36	OD-1	CRRI-1, Odisha	Not Known
37	OD-2	CRRI-2, Odisha	Not Known
38	TN-1	Aduthurai-1, Tamil Nadu	AD0812
39	TN-2	Aduthurai-2, Tamil Nadu	MDU4
40	TN-3	Aduthurai-3, Tamil Nadu	Not Known
41	TN-4	Aduthurai-4, Tamil Nadu	Not Known
42	TN-5	Aduthurai-5, Tamil Nadu	Not Known
43	TN-6	Ramanathapuram-1, Tamil Nadu	BPT 5204
44	TN-7	Ramanathapuram-2, Tamil Nadu	BPT 5205
45	TN-8	Thirurmangalam-1, Tamil Nadu	BPT 5206
46	TN-9	Thirurmangalam-2, Tamil Nadu	BPT 5207
47	KA-1	Karnataka	Not Known
48	KA-2	Mugad, Karnataka	Not Known
49	KL-1	Palakkad, Kerala	Not Known
50	KL-2	Pattambi, Kerala	Not Known
51	KL-3	Moncompu, Kerala	Not Known
52	TS-1	Medak, Telangana	Hybrid
53	TS-2	IIRR, Telangana	PAU1400
54	TS-3	Warangal, Telangana	BPT5204
55	AS	Titabar, Assam	Not Known
56	ML	Meghalaya,	Not Known
57	MN-1	Wangbal, Manipur	Not Known
58	MN-2	Imphal, Manipur	Not Known

Twelve potential ISSR markers were used to generate the unweighted Neighbor-joining dendrogram

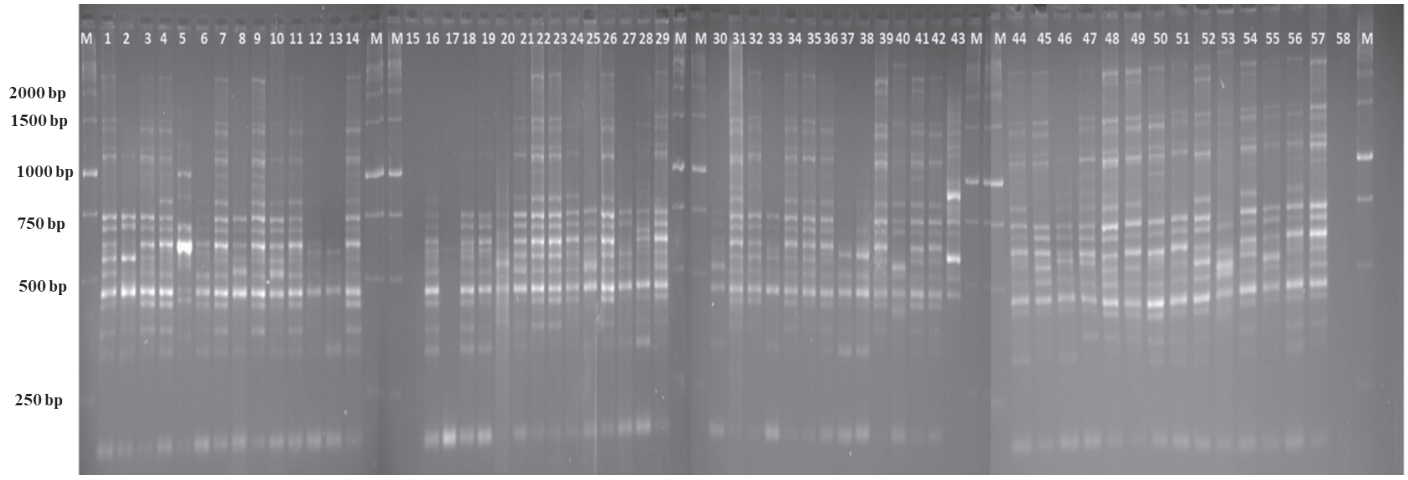

Figure 1: DNA amplified fragments profile of *U. virens* isolates using ISSR primer UBC 807; M – DNA Ladder; 1-58 – *U. virens* isolates

Table 2. Primer names with their sequences and different parameters of ISSR primers parameters

Sl. No	Primer	Primer Sequence	No of Loci	No of Polymorphic Loci	Polymorphism (%)	Hertero-zyosity	PIC	EMR	MI	RP Values
1	807	AGAGAGAGAGAGAGAGT	22	22	100	0.47	0.27	22.00	5.9	8.55
2	808	AGAGAGAGAGAGAGAGC	24	24	100	0.49	0.15	24.00	3.7	4.38
3	809	AGAGAGAGAGAGAGAGG	23	23	100	0.66	0.14	23.00	3.1	4.07
4	810	GAGAGAGAGAGAGAGAT	13	13	100	0.57	0.14	13.00	1.9	2.24
5	811	GAGAGAGAGAGAGAGAC	17	17	100	0.49	0.17	17.00	2.8	3.45
6	812	GAGAGAGAGAGAGAGAA	17	17	100	0.67	0.10	17.00	1.6	2.14
7	834	AGAGAGAGAGAGAGAGYT	15	15	100	0.34	0.21	15.00	3.1	3.93
8	835	AGAGAGAGAGAGAGAGYC	12	9	75	0.39	0.15	6.75	1.0	2.28
9	836	AGAGAGAGAGAGAGAGYA	23	22	95.65	0.55	0.24	21.04	5.0	7.83
10	840	GAGAGAGAGAGAGAGAYT	14	14	100	0.53	0.20	14.00	2.7	3.76
11	841	GAGAGAGAGAGAGAGAYC	20	20	100	0.36	0.24	20.00	4.8	6.97
12	842	GAGAGAGAGAGAGAGAYG	21	20	95.23	0.35	0.20	19.05	3.9	5.66

and the dendrogram grouped the 58 *U. virens* different isolates into three major clusters. Cluster I consisted of 21 *U. virens* isolates and it had many subclusters and the majority of the isolates belonged to Haryana and Punjab. Cluster II had 35 *U. virens* isolates were further divided into four subclusters. In this cluster, the majority of the isolates belong to Haryana and Tamil Nadu. Cluster III had only two *U. virens* isolates *viz*., TS-3 and TN-3. The cluster graph (**Figure 2**) revealed that that the variation among the isolates is minimum, except for few isolates. For example, the isolates collected from Punjab grouped together in cluster I whereas two of the Punjab isolates PU5 and 9 grouped in the cluster II. All the clusters had isolates collected from different geographical regions. Hence, the results revealed that no specific pattern of clustering was observed with respect to geographic region. ISSR markers were used

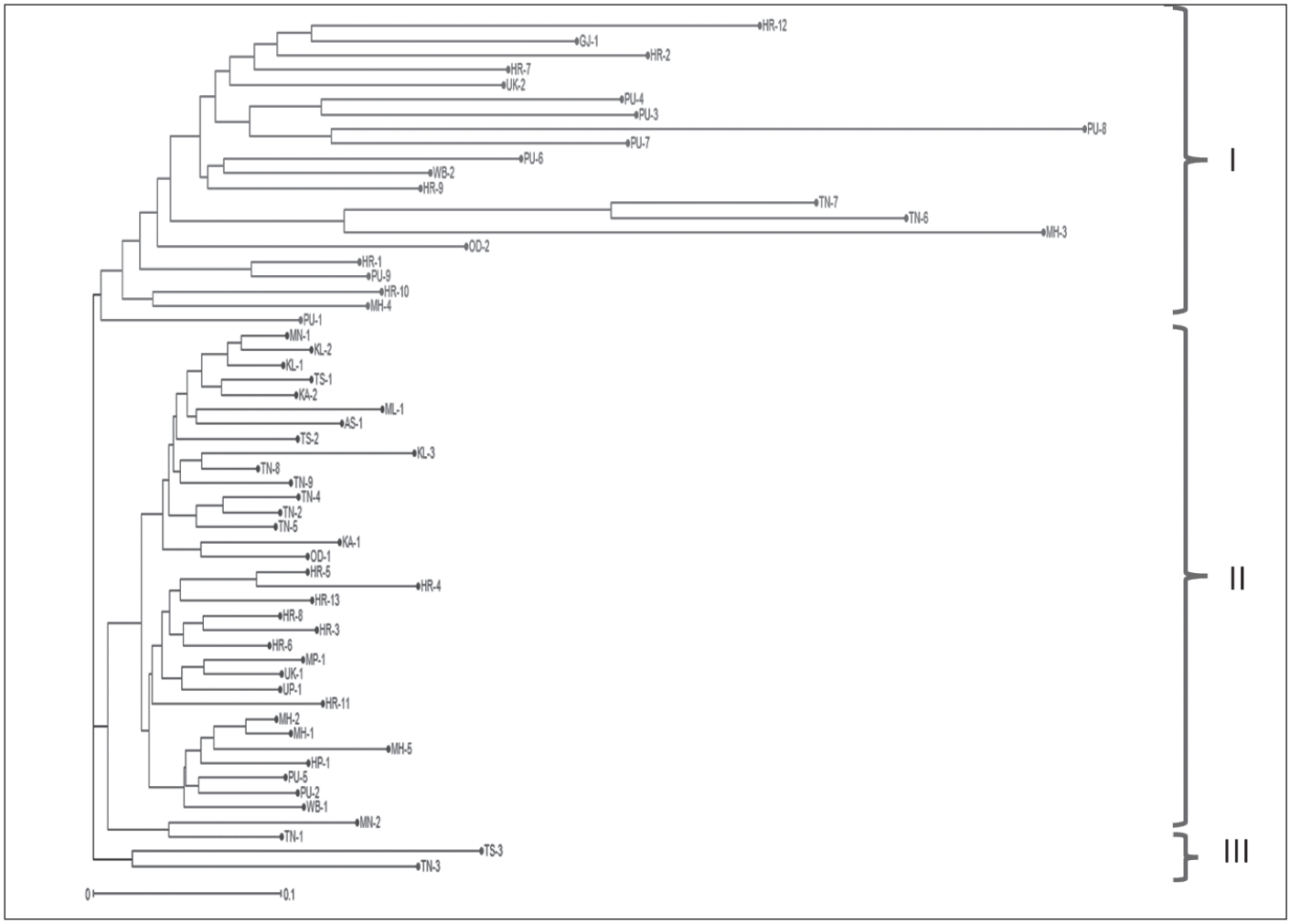

Figure 2: Dendrogram was generated using Jaccard's coefficient and UPGMA cluster analysis in DARwin for 58 *Ustilaginoidea virens* isolates based on 12 ISSR primers.

by many authors to study the molecular variability of the pathogens. Bag *et al*. (2021) reported the use of SSR markers to study the genetic variability of *U. virens* isolates collected from eastern and north-eastern India and stated that more genetic variation within populations and less among populations. Yugander *et al*. (2015) used ISSR primers to study the pathogenic and genetic variability and found that different isolates grouped based on their geographical regions. Kandan *et al*. (2015) reported that the combined use of universal rice primers (URPs), inter-simple sequence repeat (ISSR), and random amplified polymorphic DNA (RAPD) marker systems were more suitable to study the genetic variability in the rice brown spot pathogen *Bipolaris oryzae*. Anand *et al.* (2018) used ISSR markers and studied the population structure and virulence of *Alternaria carthami*, a causal agent of Alternaria leaf spot.

Conclusions

Understanding the molecular variability of plant pathogens will help to understand the nature of the existence of pathogens. With respect to false smut pathogen, twelve ISSR primers were used to understand the genetic variability. All tested primers are able to differentiate the variability and among them, UBC 807, 836 and 841 can be employed to study the genetic variability in *U. virens* isolates because of their high PIC, RP, and MI values. With respect to variability, clusters had isolates collected from different regions and there was no strong correlation based on geographical origin of

the isolates. The use of different pathogen genome-specific markers can reveal more depth in the genetic variation of the *U. virens* population.

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