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IN SILICO MINING OF MICROSATELLITES IN *USTILAGO MAYDIS* AND THEIR CROSS TRANSFERABILITY TO THE KARNAL BUNT PATHOGEN *TILLETIA INDICA* FOR POLYMORPHISM

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Abstract- Simple sequence repeats (SSRs) or microsatellites are one of the valuable sources for genetic markers because of their abundance and inherent potential for determining extensive allelic variation in the genomes. A total of 1038 SSRs were detected in 12.4 Mb gene sequence data of *Ustilago maydis* in the present survey. The distribution pattern of different SSR motifs provides the evidence of greater accumulation of trinucleotide (63.1%) followed by dinucleotide (20%) and hexanucleotide (10.5%) in *U. maydis.* Among trinucleotide repeats, CAG repeats were more frequent while TC dinucleotide repeats were found to be predominant in *U. maydis.* Further, 14 simple sequence repeats (SSR) primers were designed based on genes responsible for pathogenicity in *U. maydis*. In terms of cross species transferability, a low level of transferability of SSRs was detected, and only 4 potential markers that can be used to fingerprint 18 isolates of *Tilletia indica* were identified

Keywords- *Ustilago maydis, Tilletia indica*, Simple sequence repeats, Cross transferability, Polymorphism

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Introduction

Tilletia indica Mitra [Syn *Neovossia indica* (Mitra) Mundkur], a heterothallic fungus belonging to the order *Ustilaginales* is a floral infecting organism and the causal agent of Karnal bunt disease of wheat. It has been reported in several tropical and subtropical areas of the world including India, Pakistan, Afghanistan, Iran, Iraq, South Africa, Mexico and U.S.A [1]. The Karnal bunt pathogen not only reduces the weight and impairs viability of seeds, but also causes deterioration of flour quality due to production of trimethylamine. Although yield losses are usually low, international quarantine policies against the disease may restrict the free flow of the global wheat trade [1].

T. indica has high variability in terms of cultural, virulence and genetic characteristics. The genetic variability in this pathogen is extensively studied through the use of RAPD, rDNA-ITS, ISSR, PCR-RFLP and AFLP [2,3,4,5]. *T. indica* specific primers derived from mitochondrial DNA sequence analysis and universal rice primers were also targeted for PCR [6,7]. However, in general, these methods have not been precise to detect polymorphism in *T. indica*.

Simple sequence repeats (SSRs) or microsatellites are one of the valuable sources for genetic markers because of their abundance and inherent potential for determining extensive allelic variation in the genomes. Simple-sequence repeats (SSRs), a class of repetitive DNA sequences, consist of 1-6 bp motifs repeated in tandem arrays with identical, composite or degenerate motifs [8]. SSRs have been isolated and characterized in several fungi using library enrichment method; however, fungal microsatellites indeed appeared difficult to isolate using SSR-enriched libraries [9]. In recent years, with the establishment of several sequencing projects in crop plants, animals and microorganisms resulted in a wealth of DNA sequence information. Microsatellite sequences obtained through *in silico* mining have more or less the same utility and potential comparative with those derived from a genomic library. The negligible cost of *in silico* mining and high abundance of microsatellites in different sequence resources make this approach extremely attractive for the generation of microsatellite markers. However, despite this widespread use, little is known about SSRs in fungi. In fact, there are only a limited number of studies on these seemingly important and intruding sets of sequences in fungal species. Molecular markers based on microsatellite loci have been developed from the genomes of some fungal species [10,11]. The homology of flanking regions of SSRs allows the transferability of microsatellite loci between closely related species [12]. However, there have been too few published fungal microsatellites to allow a meaningful comparison of these motifs in different fungal species [13].

Till now, there is no sequence information available for *T. Indica*. However, the complete genome of *U. maydis*, other basidiomycetes smut fungi is already sequenced by Broad Institute of MIT and Harvard, Cambridge (http://www.broad.mit.edu/annotation/fungi/ustilago_maydis/). Based on the sequence derived by Bayer Crop Science, Affymetrix DNA arrays have been designed for the parallel detection of >6200 *U. maydis* genes. This information can allow the use of *U. maydis* genome as a vehicle for the development of SSR markers that can be subsequently used for identification of different isolates of *T. indica*. Considering the importance of SSRs in pathogen identification, the work was carried outto check the cross genome transferability of SSRs from *U. maydis* to *T. Indica* for the identification of different isolates of *T. Indica* from North Western Plain Zone of India.

Materials and Methods

Source of sequence data

The genome sequences of *U. maydis* available in the public domain of Broad Institute of Harvard and MIT (http://www.broadinstitute.org/) were used for the present study.

Mining of SSRs and Primer design

The gene sequences were searched to identify SSRs using Simple Sequence Repeat Identification Tool (SSRIT), which is available at GRAMENE web site (http://www.gramene.org/db/searches/ssrtool) [14]. The program was run online and the parameters were set for detection of perfect di-, tri-, tetra-, penta-, and hexanucleotidemotifs with a minimum of 6 repeats. The data were processed and counted with Microsoft Excel 2007. The total number of repeats has been normalized in terms of relative abundance and relative density for accurate comparison of repeat types between genomes of different sizes. Relative abundance reveals the frequency of occurrence of particular repeat type in the genome, while relative density reveals the length of sequence in base pair contribute by each repeat type to total sequence analysed [15]. The relative abundance and density were calculated by following formulas: Relative abundance =Number of SSRs / Length of sequence analyzed (Mb)*;* Relative density = Length of SSR (bp) / Length of sequence analysed (Mb).

A survey was carried out to find out genes responsible for pathogenicity in *U. maydis.* Based on these genes sequences, 14 simple sequence repeats (SSR) primers were designed in *Ustilago maydis* using WebSat software [16]. WebSat was used for primer design because it allows the user to visually inspect each loci to ensure that only one SSR primer pair was designed per scaffold; to redesign or discard all primer pairs containing a mononucleotide repeat of 5 or more; and to prevent possible fragment size homoplasy before primer synthesis by discarding all primer pairs with predicted PCR products containing a mononucleotide repeat of 6 or more. Major parameters selected for primer design were a primer length of 19 to 25 bp (optimum 22 bp), a PCR product size varying between 100 and 300 bp, a GC content between 40% and 80%, and an optimum melting temperature of 55 $^{\circ}$C.

Cultures of *T. indica*

A total of 18 isolates of *T. indica* from different locations of North Western Plain Zone of India were used for the present study. Out of 18, eight cultures of *T. Indica* {KBH1 (Panipat), KBH2 (Karnal), KBH3 (Sonepat), KBH4 (Nilokheri), KBH5 (Jagadhari), KBH6 (Rohtak), KBH7 (Hansi) and KBH8 (Taraori)} were established from the teliospore inoculum of infected wheat seeds collected from different locations of Haryana during 2012-2013. The cultures were raised from *teliospores* [17]. The growing liquid cultures of *T. Indica* were harvested at desired intervals. Each mycelial mat of *T. Indica* was filtered through a Whatman Blotter Paper No.1 and washed several times with sterilized distilled water. The mycelial mats were dried between tissue paper at room temperature. Dried mycelium was stored at -20^{0}C until use. The eight isolates from various districts of Punjab were directly obtained from P.A.U Ludhiana {KBP1 (Bathinda), KBP2 (Moga), KBP3 (Hoshiarpur), KBP4 (Gurdaspur), KBP5 (Faridkot), KBP6 (Nawanshar), KBP7 (Ferozepur) and KBP8 (Kapurthala)} while two isolates (KBJ1 and KBJ2) were acquired from Jammu. All the isolates were revived, multiplied and maintained on PDA (potato dextrose agar) slants at 20°C and 80% humidity.

Isolation of DNA and PCR amplification

DNA was isolated from mycelial mats using CTAB method [18]. A total of 14SSR markers were evaluated [Table-4,5]. Polymerase chain reaction (PCR) was carried out in a final volume of 10 µL containing 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1X PCR buffer, 0.25 µM of each primer, 1 U Taq Polymerase and 30 ng extracted genomic DNA. The thermal cycler was programmed for an initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min; annealing at 52˚C to 60˚C (depending on the individual microsatellite primer) for 1 min; and extension at 72°C for 1 min followed by a 6 min final extension at 72°C. Amplification products were resolved by electrophoresis on 3% agarose gels at 4V/cm in 1XTAE buffer, visualized by ethidium bromide staining, and gel photograph taken by Geldoc system (Syngene Ltd., USA). Fragment sizes were calculated by interpolation from the migration distance of marker fragments of 100 bp DNA ladder. Using a band-based method, a matrix of binary variables (band present –1 and band absent - 0) was generated for each genotype. The clustering analysis was performed with DAR win software ver 5.0 [19,20].

Results and Discussion

Abundance and density of SSR

The SSRIT detected 1038 SSRs in 12.4 Mb gene sequence data of *U. maydis.* The distribution pattern of different SSR motifs provides the evidence of greater accumulation of trinucleotide followed by dinucleotide in genic region of *U. maydis.* triinucleotide repeats accounted 63.2% of SSR followed by dinucleotide (20.5%) and hexanucleotide (10.5%). Tetra- and pentanucleotide were the least frequent repeats accounting 3% SSR [Table-1]. The relative abundance and density of dinucleotides, trinucleotides, tetranucleotides, pentanucleotides, and hexanucleotidesin genes of *U. maydis* is given in [Table-1]. The over-representation of trinucleotides in protein-coding sequences has been previously reported in eukaryotes including fungi [15,21&22]. The relative density of trinucleotides over other SSR types has been attributed to negative selection against frame-shift mutations in the coding regions and positive selection for specific single amino-acid stretches [23].

Table-1 Relative *abundance and density of SSR in 12.4 Mb genic region U. maydis*

Motif Length	Di	Tri	Tetra	Penta	Hexa	Others	Total
SSR count	213	656	10	17	109	33	1038
SSR percentage	20.5	63.2	0.96	1.6	10.5	3.2	100
Relative abundance	17.1	52.9	0.8	1.4	8.8	2.7	83.7
Relative density	213.8	1041	34.8	38.3	397.2	133	1858.1

Most common and longest SSR

A total of ten types of dinucleotide repeats were found in the genic sequences, of which, TC (38) and CT (27) and GC (27) were the most frequent in *U. maydis* [Table-2]. The AG/GA/CT/TC dinucleotide motif was the most predominant followed by AC/CA/TG/GT motif in *U. maydis*. The AT/TA repeat motif was very rare in smut genome. The longest dinucleotide repeat motifs were found to be CA(52 bp) and TC (50bp) in genic region of *U. maydis.* Trinucleotide repeats were found in significant frequency insmut genic region. Among trinucleotide repeats, 49 different types of motifs were identified and the CAG (108) was predominant followed by GCA (62). The longest trinucleotide repeats was found to be CAA (93bp) in genes of *U. maydis.*Tri-nucleotide repeats (CAG and CAA) have been found to be a common feature in EST-derived SSRs in *F. Odum* [24]. Tetra and pentarepeats were less frequent in the rust genomes. *U. maydis* showed 10 types of tetranucleotide motifs and all of them occurred only one time in genic region. The most common tetranucleotide repeat was found to be AGCC (4) and AGTC (4). The longest tetranucleotide SSR observed was AGCA (88bp) followed by CTTT (84bp) in *U. maydis.* Among 15 pentanucleotide repeat classes, CCATC(2) and TGCTG(2) repeats are most abundant while TGCTG (40bp) was the longest repeat. Hexa nucleotide repeat types were more diverse compared to di- and trinucleotide repeats.

Table-2 *Longest and most frequent SSR motifs in coding sequences of U. maydis*

	Di	Tri	Tetra	Penta	Hexa
Longest Motif	CA (52)* TC(50)	CAA(93) CAA (84)	AGCA(88) CTTT(84)	TGCTG(40) TCGTC(35)	ACAGCA(150) AGAAGC(126)
Frequent Motif	TC(38)** CT(27)	CAG(108) GCA(62)	TTGA(1) TGGC(1)	CCATC(2) TGCTG(2)	CAGCAA(11) CAACAG(8)

*Number in parenthesis represents the number of repeats in longest SSR

**Number in parenthesis represents the occurrence of repeat motifs.

Table-3 Cross-*amplification of U. maydis SSR primers in Tilletia indica*

Locus name	Gene	Motif	Primer Sequence (5'- 3')	Expected Size (bp)	Observed Size (bp)	Polymorphism
UM03721.1	Hypothetical protein (Ste20/sid1)	(ACA)11	AGCAGCAACAACAGAAGTGGTA ACCTACAGATCCCGACCCA	143	143	Monomorphic
UM02331.1	Hypothetical protein similar to MAP kinase (Kpp2)	(GCT)12	AACCACCAAAACGAATGCAC GCAGACCTTGTACTTGCTACCC	157	300	Polymorphic
UM04258.1	Hypothetical protein similar to MAPKK kinase Kpp4 (Ubc4/Kpp4a)	(CGGCTG)6	GAAGATTGGAGCGAGAGAAGAG CAGACTTGCCATTTGTGGG	234	234	Polymorphic
UM04258.1	Hypothetical protein similar to MAPKK kinase Kpp4 (Ubc4/Kpp4b)	(CAG)5	GCGCAATCATATACCACCAAC CTGAGTGTGTGACCGGAAGA	239	239	Polymorphic
UM05734.1	Hypothetical protein similar to AF338871_1 rho GTPase; RhoA (rho)	(T)12	AGCAAAGGTCAACTCACATGG CAGCGATCAGTAAAGATGAACG	238	238	Polymorphic
UM03721.1	Hypothetical protein (Ste20/sid1)	(CAG)7	ACCCCTACTATGCGGACAATC CTTGTTGTGGTTGTGCTTGTTT	222	-	Non-specific amplification
UM03721.1	Hypothetical protein (Ste20/sid1)	(CAG)10	AAACAAGCACAACCACAACAAG CTTCTTCTAAACATGCACGCAA	184	-	Non-specific amplification
UM06328.1	Hypothetical protein (glo1)	(CAG)5	CTACAGGCAAAGAGAACCAAGC GGATGGAAGCGGTACTTGACT	211	-	No amplification
UM04556.1	Hypothetical protein (Ubc)	(CA)6	GTCTCGATCTGCACAGTCTCC CCACTAATGACCACGGATGTAA	168	-	No amplification
UM05232.1	CYAA_USTMA Adenylate cyclase (ATP pyrophosphate-lyase) (Adenylyl cyclase) (Uac1)	(GAC)5	GCCGTACAACCGGACAAG GGCAGTCTTCTTGAAAGGGTC	201	-	Non-specific amplification
UM05208.1	Hypothetical protein (Kinase)	(AGC)9	GACCCTGAGCACACAGAGACTT GAGATACGCCCATTCAACGAT	221	-	No amplification
UM02121.1	Hypothetical protein (Kinase)	(C)13	GCAGTCAGGATACACCTTTTCC AGGAGGACAGAAGCAATCAAAG	223	-	Non-specific amplification
UM02121.1	Hypothetical protein (Kinase)	(TC)25	CGTAAGTTGTTCCACACACACA TATGACAATACGCGGGAGAAG	244	-	No amplification
UM02328.1	Hypothetical protein (Kinase)	(CAG)7	CACAGCCTCTCAGGATAAAGGT ATTTGTGGCATCTGTTGTTGTC	103	-	No amplification

A total of 83 types of hexanucleotide repeats were found in *U. maydis*. CAGCAA (11) and CAACAG (8) were frequent repeats. The longest hexanucleotide repeat motifs in *U. maydis* were found to be ACAGCA (150bp) and AGAAGC (126bp), respectively. These results are similar with earlier findings, which shows that the abundance of different repeats varied extensively depending upon the species examined [25].

Amplification of target regions containing SSRs

All the 14 SSRs were evaluated using DNA from 18 isolates of *T. indica*. Five primers did not show amplification products, four primers showed non-specific amplifications, exhibiting a large number of amplification products, and 5 primers generated readable amplification bands among isolates [Table-3].

Five of the candidate primers showed a robust amplification which was selected based on their consistency of amplification in all isolates tested. Of these five primers, primer UM04258, Ubc4/Kpp4a, UM04258, Ubc4/Kpp4b, UM02331, Kpp2 and primer UM05734.rho showed polymorphic profile in *T. Indica* isolates, but primer UM03721. Ste20/sid1 was monomorphic in the 18 isolates tested [Table-3], [Fig-1]. Cluster analysis of combined data did not classify the *T.indica* isolates into clear groups [Fig-2]. However, three subgroups were identified within group. Subgroup 1 included five isolates from Haryana; KBH1 (Panipat), KBH2 ((Karnal), KBH4 (Nilokheri), KBH6 (Rohtak) and KBH7 (Hansi) along with one isolate from Punjab; KBP7 (Ferozepur). Subgroup 2 comprised 3 isolates from Haryana; KBH3 (Sonepat), KBH5 (Jagadhari), KBH8 (Taraori), two isolates from Punjab; KBP6 (Nawanshar), KBP8 (Kapurthala) and one from Jammu; KBJ1. Five isolates from Punjab; KBP1 (Bathinda), KBP2 (Moga), KBP3 (Hoshiarpur), KBP4 (Gurdaspur), KBP5 (Faridkot) were clustered together in subgroup 3. The isolate KBJ2 from Jammu was found to be most distinct from rest of the isolates [Fig-2]. To our knowledge, this is the first attempt to check the cross genome transferability of SSRs from *U. maydis* to *T. indica*. The preliminary results obtained in the present study agree with previous reports that describe a smaller fraction of cross species transfer of microsatellites within fungal genera [26].

Patterns of cross-species SSR amplification in fungi are beginning to emerge,

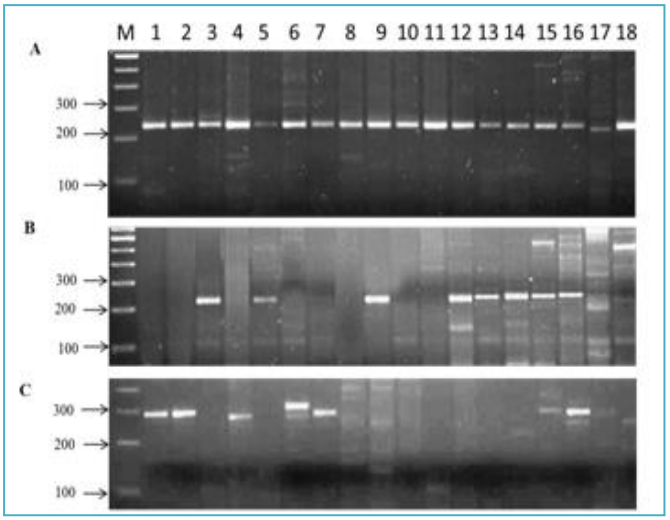

Fig.-1 Amplification of *U. maydis* SSR markers in *Tilletia indica* isolates. (A) UM04258.Ubc4/Kpp4b; (B) UM04258.Ubc4/Kpp4a; (C) UM02331.Kpp2 primers.

Lanes: 1- KBH1, 2- KBH2, 3- KBH3, 4- KBH4, 5- KBH5, 6- KBH6, 7- KBH7, 8- KBH8, 9- KBP1, 10- KBP2, 11- KBP3, 12- KBP4, 13- KBP5, 14- KBP6, 15- KBP7, 16- KBP8, 17- KBJ2, 18- KBJ1, M- 100bp ladder DNA marker

although there are still few studies that systematically explore SSR transferability beyond closely related genera [9,27]. Dracatos et al. (2006) [29] used Fifty-five PCR primers from EST-SSR of *Puccinia coronate f. sp. lolii* were used to amplify the DNA from various fungal species (*Puccinia coronate f. sp. avenae, Puccinia striiformis f.sp. tritici, Neotyphodium lolii, Blumeria graminis Aspergillus nidulans,* and *Penicillium marneffei*) and the success rate of amplification ranged from 22% to 53% [28]. Similarly, small number of markers (four out of 25) has also been described as transferable from related Uredinales species to *Hemileia vastatrix* [29]. The low rate of transferability observed in this study could

be due to mutations in the flanking regions of the SSR, the possibility of interruptions within the repeat motif or the presence of introns in the amplified region [28].

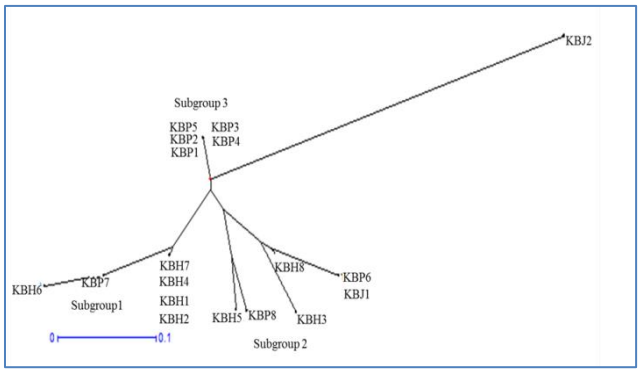

Fig.-2 Unrooted neighbor joining tree of 18 *Tilletia indica* isolates using genic SSR primers from *U. maydis*.

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