Original Article

Genomic characterization of *Puccinia triticina* using molecular marker technology

Caracterização genômica de *Puccinia triticina* usando tecnologia de marcadores moleculares

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Abstract

Leaf rust, caused by *Puccinia triticina*, is the most common rust disease of wheat. The fungus is an obligate parasite capable of producing infectious urediniospores. To study the genetic structure of the leaf rust population 20 RAPD primers were evaluated on 15 isolates samples collected in Pakistan. A total of 105 RAPD fragments were amplified with an average of 7 fragments per primer. The number of amplified fragments varied from 1 to 12. GL Decamer L-07 and GL Decamer L-01 amplified the highest number of bands (twelve) and primer GL Decamer A-03 amplified the lowest number of bands i.e one. Results showed that almost all investigated isolates were genetically different that confirms high genetic diversity within the leaf rust population. Rust spores can follow the migration pattern in short and long distances to neighbor areas. Results indicated that the greatest variability was revealed by 74.9% of genetic differentiation within leaf rust populations. These results suggested that each population was not completely identical and high gene flow has occurred among the leaf rust population of different areas. The highest differentiation and genetic distance among the Pakistani leaf rust populations were detected between the leaf rust population in NARC isolate (NARC-4) and AARI-11 and the highest similarity was observed between NARC isolates (NARC-4) and (NARC-5). The present study showed the leaf rust population in Pakistan is highly dynamic and variable.

Keywords: leaf rust, diversity, RAPD marker, polymorphism, wheat.

Resumo

A ferrugem da folha, causada por *Puccinia triticina*, é a ferrugem mais comum do trigo. O fungo é um parasita obrigatório, capaz de produzir urediniósporos infecciosos. Para estudar a estrutura genética da população de ferrugem da folha, 20 primers RAPD foram avaliados em 15 amostras de isolados coletadas no Paquistão. Um total de 105 fragmentos RAPD foram amplificados com uma média de 7 fragmentos por primer. O número de fragmentos amplificados variou de 1 a 12. GL Decamer L-07 e GL Decamer L-01 amplificaram o maior número de bandas (doze), e o primer GL Decamer A-03 amplificou o menor número de bandas, ou seja, um. Os resultados mostraram que quase todos os isolados investigados eram geneticamente diferentes, o que confirma a alta diversidade genética na população de ferrugem da folha. Os esporos de ferrugem podem seguir o padrão de migração em distâncias curtas e longas para áreas vizinhas. Os resultados indicaram que a maior variabilidade foi revelada por 74,9% da diferenciação genética nas populações de ferrugem. Esses resultados sugeriram que cada população não era completamente idêntica e um alto fluxo gênico ocorreu entre a população de ferrugem da folha de diferentes áreas. A maior diferenciação e distância genética entre as populações de ferrugem da folha do Paquistão foram detectadas entre a população de ferrugem da folha no isolado NARC (NARC-4) e AARI-11 e a maior similaridade foi observada entre os isolados NARC (NARC-4) e (NARC-5). O presente estudo mostrou que a população de ferrugem da folha no Paquistão é altamente dinâmica e variável.

Palavras-chave: ferrugem da folha, diversidade, marcador RAPD, polimorfismo, trigo.

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1. Introduction

Wheat is a staple food of around 40% of the world population (FAO, 2007). Wheat production has been increased tremendously since the 1960s green revolution through the introduction of rust resistant and semi-dwarf wheat varieties. This helped in overcoming the problem of hunger especially in developing countries, where green revolution technologies were adopted in a better way (Heisey et al., 2002). However, the interminably increasing population and the appearance of new devastating disease races have threatened global food security. Therefore, there is a dire need to improve wheat productivity by developing high yielding disease-resistant wheat varieties.

Among wheat diseases, wheat rusts (leaf, stripe, and stem rust)), diseases have been thought the main biotic factor for production restraints in Asia and the rest of the world (Singh and Rajaran, 1991). Leaf rust caused by *Puccinia tritici* is one of the most important and destructive diseases of wheat in the world. Puccinia is an obligate parasite, which can infect the wheat during its growing period. Leaf rust damages are lesser than stem and yellow rust, but its harmful effects cause more annual damages, due to its prevalent occurrence (Huerta-Espino et al., 2011). It alone can cause 30-50% yield losses under favorable environmental conditions and by growing the susceptible cultivars. The importance of this disease is historically recognized (Borlaug, 1968; Brennan et al., 1994) as in 1978, leaf rust disease in Pakistan causes 10% yield loss (Hassan et al., 1979). There are many approaches to control this disease, one of them is genetic resistance that is cheap, environmentally friendly, and is adopted by almost all the developing countries (Rehman et al., 2013). About 60 genes of leaf rust have been reported in wheat (McIntosh et al., 2003). Wheat varieties are often relying on race-specific resistance however, these varieties readily lose their effectiveness within years. Wheat leaf rust races evolution through mutation and selection is also a major concern of this era. It is a very significant feature of uredospores that these can travel to miles through air current, wind source, even these spores can travel over the continents from the infected plant source. It is essential to regularly evaluate and categorize rust races of pathogens population. To incorporate genetic resistance, assessment of the genetic diversity and population structure of fungi within the pathogen species is a pre-requisite. Such factors are very significant to explain genetic diversity and discuss the happening of new virulence in the region. DNA based molecular markers including Amplified Fragment Length Polymorphism (AFLP) (Dadrezaie et al., 2013), Random Amplified Polymorphic DNA (RAPD) (Dinu et al., 2016), and Simple Sequence Repeat (SSR) (Ordonez and Kolmer, 2007) are widely used to study these population. RAPD sare the most simple and efficient molecular markers for genetic screening and characterization of different plants and organisms. RAPD is a multilocus marker and only a single decamer is usually used. These are short random nucleotide sequence primers that are used for the detection of polymorphism among DNA products of different species (Williams et al., 1990). RAPD technique is widely used due to its simplicity, inexpensive nature, ease of performance, and requirement of a minute quantity of template DNA (Micheli et al., 1994). Optimization of RAPDs (PCR) conditions improves the PCR product resolution and RAPDs repeatability. RAPDs markers are considered important for the characterization of variation between regionally based groups of *Puccinia triticina* virulence phenotypes but a few reports till now detected the variation between nearly linked virulence phenotypes in among or within regions. Previously RAPDs markers were applied by Chen et al. (1995) to study genetic diversity among the *P. triticina* isolates.

Our objective was to study the genetic diversity of *P. triticina* isolates collected from the different parts of Pakistan using RAPD markers which will help to identify the strains in relation to its virulence and severity in the region.

2. Materials and Methods

Fifteen samples of leaf rust-infected leaves and spores were collected from different locations of Pakistan in the early spring. Out of these, twelve samples were collected in the form of infected leaves (diseased leaf rust samples) from the National Agricultural Research Centre (NARC) Islamabad, belongs to different locations of rust affected wheat areas in Pakistan discussed in Table 1. The spores of rust were removed from leaves and dried in the open air and then kept in the refrigerator at 4 °C. Three samples were collected from wheat varieties AARI-11, Faisalabad-85, and Morocco from the experimental area of Wheat Research Institute (WRI), Ayub Agricultural Research Institute (ARRI), Faisalabad.

2.1. DNA extraction

DNA was extracted from either collected, dried urediniospores (1 to 20 mg) or from diced, infected leaf tissue with visible uredinia (20 to 30 mg). Spores were added to Eppendorf tubes and crushed mechanically using autoclaved micro pestles. DNA was extracted using the following modifications of the cetyltrimethylammonium bromide (CTAB) method (Kolmer and Liu, 2000) following incubation at 65 °C for 1.5h in 600μl of extraction buffer (0.165 M Tris-HCl, pH 8.0; 66 mM EDTA, pH 8.0; 1.54 M NaCl; 1.1% CTAB; and Proteinase K at 50 μg/mL) and 66 μl of 20% sodium dodecyl sulfate (SDS), DNA was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, vol/vol/vol), followed by extraction with an equal volume of chloroform:isoamyl alcohol (24:1, vol/vol). The aqueous phase was removed, and nucleic acids were precipitated with 0.6 volume of isopropanol and finally resuspended in 100μl of Tris-EDTA (TE) buffer (10mM Tris-HCl, pH 8.0; 0.1mM EDTA, pH 8.0). RNA was digested by incubation with 3.5μl of RNase A (10 mg mL⁻¹) at 37 °C for 1h. To precipitate DNA, 0.1 volume of 3.0 M NaOAc and 2.0 volumes of cold 95% ethanol were added to samples, mixed by inversion, and incubated at –20 °C for 1h. After centrifugation, the pellet then was washed twice with cold 70% ethanol, air-dried, and resuspended in 100μl of TE buffer. Quantification of DNA was determined by NanoDrop

ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, Delaware).

2.2. Molecular analysis with RAPD

15 RAPD primers (Table 2) was used in current study. RAPD polymerase chain reaction conditions were optimized and used for amplification as 15 ng/µl DNA was used in 25 µl reaction volume using AmpliTaq mixture (*Applied Biosystems*, USA) with 2.5 µl primer (10 µM). The thermocycler was programmed at 94 °C for 3 min; followed by 40 cycles of 1 min at 94 °C, 2 min at 36 °C, and 2 min at 72 °C; then 10 min at 72 °C for amplification of DNA with RAPD primers. The amplified product was visualized on an agarose gel, scoring was done as 0 and 1 based on the presence and absence of the band.

Table 1. List of Samples of rust isolates along with the location.

No.	Sample Name	Sample Type	Location
1.	15LPPK-336	Leaves	Jehlum, Punjab
2.	15LSPK-41	Leaves	Tandojaam, Sindh, Sehar
3.	Faisalabad-85	Leaves	Ayub Agricultural Research Centre, Faisalabad from Variety Faisalabad 85
4.	AARI-11	Leaves	Ayub Agricultural Research Institute, Faisalabad from variety AARI 11
5.	15LSPK-18	Leaves	Jhol, Sanghar, Sindh, Sehar
6.	15LPPK-322	Leaves	Syed Kaswan, Rawalpindi, Punjab
7.	15LSPK-86	Leaves	Kanbala, Punjab, Sehar
8.	SSS	Spores	Sanghar, Sindh
9.	NARC 1 (A)	Leaves	NARC, Islamabad
10	DPS	Spores	Dunyapur, Punjab
11	NARC 2 (B)	Leaves	NARC, Islamabad
12	NARC 3 (C)	Leaves	NARC, Islamabad
13	NARC 4 (D)	Leaves	NARC, Islamabad
14	NARC 5 (E)	Leaves	NARC, Islamabad
15	Morocco	Leaves	Ayub Agricultural Research Centre, Faisalabad from variety Morocco

Table 2. The total number and polymorphic produced by primers.

Sr. #	Primer Name	Sequence (5′-3′)	TNB*	TNL*	NPB*	Polymorphism %
1.	GL DecamerA-01	CAGGCCCTTC	32	4	3	75%
2.	GL DecamerA-02	TGCCGAGCTG	60	7	7	100%
3.	GL DecamerA-03	AGTCAGCCAC	15	1	0	0%
4.	GL DecamerA-04	AATCGGGCTG	50	4	2	50%
5.	GL DecamerA-07	GAAACGGGTG	48	10	10	100%
6.	GL DecamerA-08	GTGACGTAGG	37	4	3	75%
7.	GL Decamer L-01	GGCATGACCT	90	12	12	100%
8.	GL Decamer L-02	TGGGCGTCAA	44	8	7	87.5%
9.	GL Decamer L-03	CCAGCAGCTT	69	7	5	71%
10	GL Decamer L-04	GACTGCACAC	59	8	6	75%
11	GL Decamer L-05	ACGCAGGCAC	78	6	4	66.6%
12	GL Decamer L-06	GAGGGAAGAG	61	9	9	100%
13	GL Decamer L-07	AGGCGGGAAC	129	12	8	66.6%
14	GL Decamer L-08	AGCAGGTGGA	93	10	9	90%
15.	GL Decamer L-10	TGGGAGATGG	18	3	2	66.6%
Total			**883**	**105**	**87**	**74.9%**

TNB: Total No of Bands. TNL: Total No of Loci. NPB: Number of Polymorphic Bands.

2.3. Genotypic data analysis

Genetic relatedness and distance were measured using Nei's similarity indices with Popgen32 software (version 1.32). Dendrogram by Nei's Unweighted Paired Group of Arithmetic Means Average (UPGMA) method using Popgen32 software (Yeh et al., 1999) and displayed using FigTree v1.4.4.

3. Results

3.1. Analysis of band patterns

PCR amplified products were analyzed for the band patterns. All clear and complete resolution bands as depicted in Figure 1 were scored for data analysis. Results from the proliferation of bands that amplified from 15 RAPD primers on an agarose gel, revealed in total 105 clear bands out of which 87 were polymorphic bands (Table 2). The highest polymorphic percentage revealed by three primers (GL-A02, GL-L01, and GL-L06). Total 74.9% polymorphism was depicted by all primers.

3.2. Genetic similarities among Puccinia triticina

Genetic similarities among leaf rust causal agent (*Puccinia triticina*) were assessed using Nei's similarity indices. Genetic similarity ranged from 90.91% to 45.45% (Table 3). The maximum genetic similarity was found between NARC isolates (A) and 15LPPK-322 while the minimum genetic similarity was found between NARC isolate (C) and AARI-11 (AARI isolate). The narrow genetic base was found among all the *P. triticina* isolates used in this study.

3.3. Genetic relationship among Puccinia triticina

Genetic relationship among leaf rust causal agent isolates were identified through cluster analysis by Nei's Unweighted Paired Group of Arithmetic Means Average (UPGMA). All the 15 isolates of *P. triticina* were clustered into three main groups. The first cluster consisted of 5 members including four isolates of NARC (B, C, D, and E) and Morocco (AARI isolates) (Figure 2). The second group is linked consist of six members that are subdivided into 3, 2, and 1 members, the isolates within group-A1 are 15LPPK-86, Faisalabad-85, 15LSPK-41, in group-A2 are
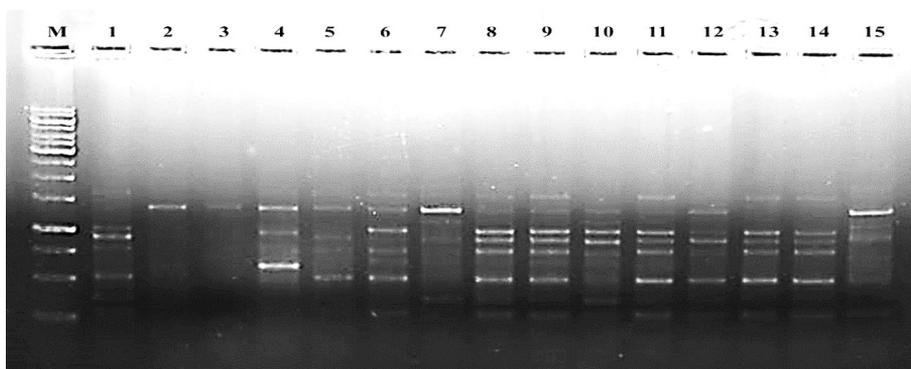

Figure 1. Amplification product of RAPD decamer primer GL Decamer L-01. M is a ladder and Labelled 1-15 are *Puccinia triticina* isolates.
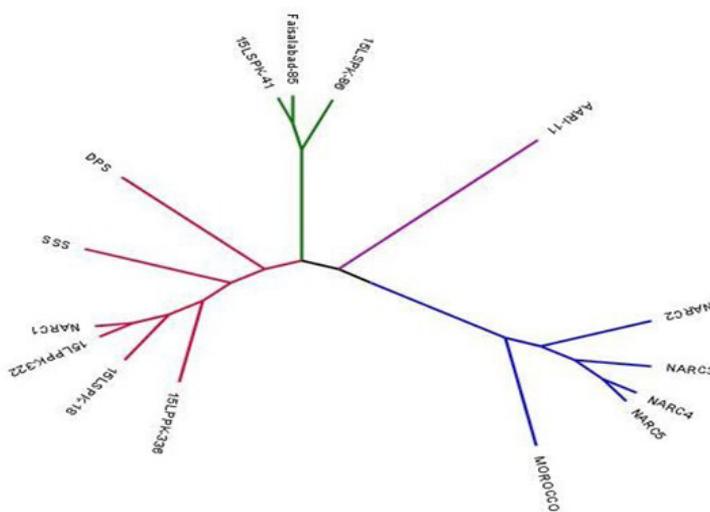

Figure 2. Dendrogram Based on Nei's Genetic distance: Method.

Table 3. Similarity matrix of *Puccinia triticina* isolates.

POP	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	****	0.6909	0.6909	0.6364	0.8545	0.8909	0.6909	0.8182	0.8000	0.6727	0.5636	0.5636	0.5273	0.5273	0.5321
2	0.3697	****	0.7818	0.6909	0.7273	0.6909	0.7091	0.6545	0.6364	0.5455	0.4727	0.4727	0.5091	0.5091	0.5138
3	0.3697	0.2461	****	0.6545	0.7273	0.7273	0.7818	0.6909	0.6364	0.6545	0.5455	0.5091	0.6545	0.5818	0.5872
4	0.4520	0.3697	0.4238	****	0.6727	0.6364	0.5455	0.6000	0.6545	0.5636	0.5273	0.4545	0.5273	0.5273	0.6055
5	0.1572	0.3185	0.3185	0.3964	****	0.8909	0.7273	0.7818	0.8364	0.6727	0.5636	0.5636	0.5273	0.5273	0.5688
6	0.1155	0.3697	0.3185	0.4520	0.1155	****	0.7636	0.8182	0.9091	0.7455	0.5636	0.5273	0.5273	0.4909	0.5321
7	0.3697	0.3438	0.2461	0.6061	0.3185	0.2697	****	0.8000	0.7091	0.7273	0.5818	0.6182	0.6545	0.6545	0.6239
8	0.2007	0.4238	0.3697	0.5108	0.2461	0.2007	0.2231	****	0.8000	0.7818	0.6000	0.5636	0.5636	0.6000	0.6239
9	0.2231	0.4520	0.4520	0.4238	0.3185	0.0953	0.3438	0.2231	****	0.7273	0.5818	0.5091	0.5091	0.4727	0.5505
10	0.3964	0.6061	0.4238	0.5733	0.3964	0.2938	0.3185	0.2461	0.3185	****	0.6727	0.6000	0.6727	0.6364	0.5138
11	0.5733	0.7492	0.6061	0.6400	0.5733	0.5733	0.5416	0.5108	0.5416	0.3964	****	0.7455	0.7455	0.7455	0.6422
12	0.5733	0.7492	0.6751	0.7885	0.5733	0.6400	0.4810	0.5733	0.6751	0.5108	0.2938	****	0.8545	0.8545	0.7156
13	0.6400	0.6751	0.4238	0.6400	0.6400	0.6400	0.4238	0.5733	0.6751	0.3964	0.2938	0.1572	****	0.8909	0.7156
14	0.6400	0.6751	0.5416	0.6400	0.6400	0.7115	0.4238	0.5108	0.7492	0.4520	0.2938	0.1572	0.1155	****	0.7340
15	0.6309	0.6660	0.5324	0.5017	0.5642	0.6309	0.4718	0.4718	0.5970	0.6660	0.4428	0.3346	0.3346	0.3093	****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

DPS, SSS and in group-A3 is AARI11. Two members of the A2 group (SSS and SDP) are closely related to each other. In the same way, the third group contains four isolates (NARC1, 15LPPK-332, 15LSPK-18, and 15LPPK-336). Isolates NARC1 and 15LPPK-322 are closely related to each other followed by the 15LSPK-18 isolate. In group-A1, 15LSPK-41 and Faisalabad-85 are closely related to each other followed by 15LSPK-86. The first group [NARC isolates (B, C, D, E) and Morocco] is a 5 membered group. NARC isolates are closely related to each other but a bit distinct to Morocco (AARI isolate) so Morocco is clustered separately. NARC (D) and (E) isolates are closely related to each other followed by C and then B isolates. It was found that a maximum genetic relationship was found between NARC isolates (D) and (E).

4. Discussion

RAPDs, PCR-based dominant DNA markers, randomly amplify the genomic regions and does not require the previous genome sequence information and can amplify much smaller genomic DNA. RAPDs markers had some concerns regarding the reproducibility and reliability of experiments and environmental factors have influence on RAPDs reaction (Heun et al., 1994). The influence of environmental and experimental conditions on RAPDs (PCR) amplification should be optimized. The RAPDs marker system has been used successfully to characterize molecular variation in other rust fungi (Chen et al., 1995; Hamelin et al., 2000). Besides knowing genetic diversity has important basic and applied implications, including understanding pathogen evolution. It was found that 15ng/µl gave the best results and it was finally used as an optimized DNA concentration for RAPDs (PCR). Likewise, 2.5mM dNTPs, 3mM MgCl2, and 1U Taq DNA polymerase were found optimum for RAPDs (PCR) in a total volume of 25µl reaction mixture.

In this study 15 out of tested 20 RAPDs primers amplified reproducible and polymorphic PCR products. Sedra et al. (1998) reported 123 RAPD primers from them 19 produced reproducible results. El-Rayes (2009) reported 16 out of 40 RAPDs primers that revealed the polymorphic and reproducible results. By screening 15 RAPD primers 105 RAPD loci were identified among 15 *P. triticina* germplasm, out of which 87 produced polymorphic results and the percentage of polymorphism identified was 74.9%, showed a relationship among *P. triticina*. Chen et al. 1995 studied a sample of 115 isolates from the United States and identified six pathotypes and five random amplified polymorphic DNA (RAPD) groups, with a low correlation coefficient found between pathotypes and RAPD groups. Although five RAPDs groups were identified, the genetic diversity level appeared low.

The results showed that almost all the studied isolates were genetically different, all studied *P. triticina* isolates were produced unique band patterns, similarly, high genetic diversity within the population of the leaf rust fungus was described by McDonald and Linde (2002). Out of 15 samples, 4 samples collected from NARC-3, NARC-2, NARC-4, NARC-5 were genetically more similar and closely related from samples collected from a susceptible wheat genotype "Morocco" and widely different from other samples. Similarly, the isolate 15LSPK-41 collected from Seher-06 was similar to samples collected from varieties AARI-11 and Faisalabad-85. The cluster analysis shows a wide distribution of isolated about space and all over wheat-growing areas of Punjab and Sindh.

5. Conclusion

Results suggested that there is genetic similarity between different isolates of Group A found all over Punjab and Sindh showing similar pattern however the isolates of group B are distinctly showing the presence of another population in northern and central Punjab. A narrow genetic base was found between isolates may due to monoculture situation as a result of the prevalence of wheat variety Seher-06 in all wheat-growing areas of Punjab and Sindh.

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