In situ new variations in *Puccinia triticina* causing leaf rust on wheat

Subodh Kumar, S.C. Bhardwaj*, O.P. Gangwar, P. Prasad, Charu Lata and Sneha Adhikari

ICAR-Indian Institute of Wheat and Barley Research,
Regional Station, Flowerdale, Shimla-171002 HP (India)

*Subhash.Bhardwaj@icar.gov.in

Abstract

During the periodic purity checks of pathotypes 107-1(45R35=JCGPL) and 20(5R27=SGQPL) of *Puccinia triticina* in national repository at Shimla, India, mixtures of infection types were observed on the differential *Lr3* and *Lr15*, respectively. Single pustule isolations and further pure cultures in both the cases yielded new pathotypes designated as 57(45R39= KGTPL) and 20-2(57R27= SHKPL). Pathotype 57 was one step gain in virulence on *Lr3* in 107-1 whereas 20-2 on *Lr15* in mother culture of pathotype 20. The difference of virulence on one gene to each mother culture and their non occurrence in the field samples is indicative of mutation for virulence *in situ*. To ascertain the novelty of new pathotypes, detailed study on differentials, avirulence/virulence structure, DNA polymorphism using SSR markers, and other related information is presented in this publication. The new cultures are being maintained as PrtI 57 and PrtI 20-2 in the repository.

Keywords *Puccinia triticina*· Wheat leaf rust· Mutation in culture· Virulence· New pathotypes

Introduction

Wheat is the second most important cereal in the world to cater the food and nutrition requirement of majority of the population (Braun et al. 2010). The wheat cultivation faces many challenges among which biotic and abiotic stresses are two main constraints that impede wheat production significantly. Among the biotic threats to wheat, leaf rust caused by *Puccinia triticina* inflicts Eriks. causes more losses than any other rust due to its widespread occurrence (Bhardwaj et al. 2021). Leaf rust occurs in almost all the wheat growing regions of the world. Like other rust pathogens, *P. triticina* is also very dynamic

and evolves frequent variants that render resistant wheat varieties susceptible (McIntosh et al. 1995). Therefore, screening of available wheat germplasm against different pathotypes of *P. triticina* in green house at seedling stage and in field under natural as well as artificial epiphytotic are prerequisites to select rust resistant wheat material (Bhardwaj 2011). Greenhouse evaluation comprises multi pathotype tests whereas in adult plant evaluation, a blend of predominant and virulent pathotypes is used. For undertaking these studies, nucleus inoculum is drawn from the national repository of pathotypes. A repository of wheat rust pathotypes is a collection of live/ cryo-preserved cultures of different isolates identified in a country or more countries. Maintenance of repository involves a lot of efforts, manpower and resources. This collection is a unique treasure for wheat pathology, breeding efforts and ultimately employed for developing rust resistant varieties as well as their deployment.

In India, wheat rust research started in early 1920s. The live culture collection of different wheat, barley, and oat rust pathotypes is being maintained since 1931 at Flowerdale, Shimla, India (Bhardwaj and Singh 2019) and some of the cultures have undergone more than 1415 generations. Similar collections, however with differently evolved pathotypes, are being maintained as active and partly cryo-preserved culture types in Australia, China, Mexico, Denmark, South Africa, and the United States of America (Bhardwaj and Singh 2019). Mostly the genetic or phenotypic changes have not been reported in the pathotypes maintained in these culture collections. The reason may be that all the cultures in various repositories are not multiplied routinely. Multiplication and restoration of cultures is a need based exercise in most of the countries. Secondly, if a culture is found impure, it is replaced through the pure inoculum kept in storage. It may be possible that these biological processes of variation culture collections are going on naturally but have gone unnoticed. Consequently mixtures and variations in pure cultures of pathotypes have not been studied in detail. Variations in color and pathogenicity of wheat rust pathogens *in situ* have been recorded in a few cases. D Oliveira (1939) observed orange colored mutant in race 14 of *P. anomala*. Roberts (1936) recorded a mutation for pathogenicity in a culture of *P. triticina*. Likewise, Gassner and Straib (1932) described a number of recurrent mutations resulting in identical mutants with

more virulence than the race in which they arose. We have also come across mutants for color change earlier in race 107 (Prashar et al. 1996).

Different pathotypes of *Puccinia* spp. are being maintained in our repository for a considerable period; however, evidences for mutability are less common. During routine multiplication and purity check of repository pathotypes, we noticed unusual and discrete mixtures in two pathotypes on differentials. In present study, we characterized two new variants that have appeared one each in the cultures of pathotypes 20, and 107-1 which are being maintained in the repository at ICAR-Indian Institute of Wheat and Barley Research, Regional Station, Flowerdale, Shimla, H.P. India.

Materials and methods

Growing seedlings and inoculation

A mixture of FYM and loam soil (1:1) was sterilized at 60^{o}C for 4 hours and used for sowing wheat material. Plastic pots (4 × 4") filled with the soil were used for growing seedlings of susceptible wheat variety Agra Local. Temperature of seedling chamber was maintained at 20$\pm$2^{o}C. The pots were watered regularly to keep the soil moist. A week-old seedling of Agra Local along with the differential sets (Table1) was inoculated with a lancet needle using urediniospores from 15-20 days old mother cultures. Inoculated pots were kept at 20$\pm$2^{o}C in saturated humidity wooden chambers for 24 hours. The pathotype cultures of national repository were multiplied at an interval of 15-20 days.

Recording of observations and confirming the novelty of pathotypes

The response on wheat differentials was recorded after 14-16 days of inoculation as resistant (0, ;, 12), moderately resistant (2+), moderately susceptible (3) and susceptible 33+, 3+) following Stakman et al. (1962) with some modifications. Cultures showing discrete mixture and different infection types (ITs) than that of the original ones were kept separately. Isolations were taken from off type pustules and pure culture of the same was maintained. Infection types of isolates were confirmed repeatedly on the differentials. The response of differentials to mother cultures, new isolates and other closely related pathotypes were compared under same set of controlled conditions. Based on the distinctness and consistency of infection types, two isolates were confirmed as

new pathotypes. These were designated as per the binomial system of nomenclature being followed in India (Nagarajan et al. 1983) with some modifications (Bhardwaj et al. 2012). To establish the relationship of new pathotypes with the basic races of leaf rust pathogen, the international register of physiologic races of *Puccinia recondita* was considered (Johnston 1961). To facilitate the international communication, the new and closely related pathotypes used in the current study were also evaluated on the 20 North American differentials (Kolmer 2019).

DNA extraction and SSR analysis

For elucidating the genotypic similarity among the 11 pathotypes of *P. triticina,* molecular data were generated using 25 simple sequence repeat (SSR) markers (Prasad et al. 2017; Table 1). For DNA isolation, all the pathotypes were mass multiplied on susceptible wheat variety Agra Local. DNA was extracted from 100 mg of finely ground urediniospores using the modified CTAB method (Kiran et al. 2016). The DNA quantity and quality were checked using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc), and the integrity was confirmed on a 1% agarose gel.

All PCR reactions were carried out in 20 µL volume containing 50 ng template DNA, 200 µM each of the four dNTPs, 1X PCR buffer (10 mM Tris pH 9.0, 50 mM KCl), 1.5 mM $MgCl_2$, 0.5 U *Taq* polymerase (Himedia Laboratory Pvt. Ltd. India), and 10 pmol of both the forward and reverse primers. The reaction programs were set at 94^0C for 4 min, followed by 35 cycles of 30 sec at 94^0C, 30 sec at a primer annealing temperature and 1 min at 72^0C for extension, with a final extension at 72^0C for 10 min in a thermal cycler (Applied Biosystems; Veriti™ 96-Well Thermal Cycler). The PCR product was separated on 3% high-resolution agarose gel (Himedia Laboratory Pvt. Ltd. India) in 1X TAE buffer at 65–70 V for 2-3 h. DNA fragments were visualized under a UV light and photographed using the Gel Documentation System (Bio-Rad Laboratories, Inc.).

The binary (0 for absence and 1 for the presence of an SSR allele) data generated from molecular analysis of each pathotype-primer combination was used to construct dendrogram depicting genetic relationships among the eleven pathotypes of *P. triticina* by using SIMQUAL (Nei and Li 1979) path to obtain similarity coefficient values in

NTSYS-PC software version 2.1 (Rohlf 2000). The generated similarity coefficients (Jaccard 1908) were later utilized to construct tree following an unweighted paired group method of arithmetic averages (UPGMA) algorithm and SAHN clustering.

Results

In 2019, during routine multiplication and purity check on differentials, discrete mixtures were observed in the cultures of two pathotypes (mother cultures) of *P. triticina*. In one pathotype mixture appeared on *Lr3* whereas in other on *Lr15*. Single pustule isolations and derived pure cultures showed novelty. Subsequently, infection types of two new pathotypes were compared with those of respective pure mother cultures, closely related, and predominant pathotypes. Variations occurred in mother cultures are most probably due to single step mutation as new pathotypes differed in the virulence for single gene only. The new cultures are being maintained as PrtI 57 and PrtI 20-2 in the repository since 2019.

Detailed information on the two new pathotypes is given below.

Pathotype 20-2 (57R27)

Pathotype 20 (5R27) produces resistant infection type (;) on *Lr15*. In 2019, while recording purity of pathotype 20 of *P. triticina* in pure culture, an unusual mixture of susceptible pustules (3+) was observed on *Lr15*. Single pustule isolation (3+) was picked up from *Lr15* and pure culture was produced. Repeated testing of this culture along with pathotypes 20 and 20-1 confirmed its novelty and was designated as a new pathotype 20-2 (57R27). The new pathotype was virulent (3+) on *Lr15* whereas mother culture of pathotype 20 and other pathotype in the group 20-1 (93R57) are avirulent (;). Likewise, pathotype 20-1 is virulent on *Lr10* and *Lr26* (3+) whereas pathotypes 20 and 20-2 produced resistant (; and 0;, respectively) response. Pathotypes belonging to 20 group are distinct from the pathotype 10(13R19) and most virulent and predominant pathotypes (Table 2). Avirulence/virulence formula of new pathotype in seedling resistance tests is *Lr3*, *Lr9*, *Lr10*, *Lr17b*, *Lr19*, *Lr23*, *Lr24*, *Lr25*, *Lr26*, *Lr28*, *Lr29*, *Lr32*, *Lr36*, *Lr39*, *Lr42*, *Lr43*, *Lr45*, *Lr46*, *Lr47*, *Lr48*, *Lr49*, *Lr52*, *Lr53*, *Lr58*, *Lr80* / *Lr1*, *Lr2a*, *Lr2b*, *Lr*2c, *Lr11*, *Lr12*, *Lr13*, *Lr14a*, *Lr14b*, *Lr14ab*, *Lr15*, *Lr16*, *Lr17*, *Lr18*, *Lr20*, *Lr21*, *Lr22a*, *Lr22b*, *Lr27*, *Lr30*, *Lr33*, *Lr34*, *Lr35*, *Lr37*, *Lr38*, *Lr40*, *Lr44*, *Lr51*, *Lr57*, *Lr67*.

This variant is not of much epidemiological consequence as many genes occurring in Indian wheat cultivars confer resistance to this pathotype. Moreover, this variant does not occur in field population. The new pathotype (20-2) appears to be a single step virulence gain on *Lr15* in mother culture of pathotype 20. It is probably a natural *in situ* mutation because it occurred as a mixture in mother pathotype 20. Moreover this type of a variant has not been observed in *P. triticina* so far anywhere else in India. It was designated as SHKPL under North American system of race designation and found different to those reported currently from the United States.

Pathotype 57 (45R39)

Pathotype 107-1(45R35) produces resistant (;) infection types on *Lr3* (Democrat). However, in 2018 while observing purity of pathotype 107-1 (4535), an unusual mixture of susceptible pustules (3+) was observed on *Lr3*. Single susceptible pustule isolation was taken from *Lr3* and a pure culture was attained. Evaluation of this culture on differentials along with other closely related, most virulent and predominant pathotypes revealed its distinctness and therefore, inferred as a new pathotypes. When the response of this pathotype was compared with those in the international register, we found it similar to race 57 (45R39). Comparing on Indian differentials (Table 2), it was close to 107 group. However, pathotype 57 is virulent (3+) on *Lr3* whereas pathotypes 107 (45R3) and 107-1 (45R35) are avirulent (;). Both 107-1 and 57 pathotypes were virulent (3+) on *Lr26*, and 107 was avirulent (;). The new pathotype 57 resemble 107-1 but the former has gained virulence on *Lr3*. The new pathotype was designated as 57 (45R39). Avirulence/ virulence formula of new pathotype in seedling resistance evaluation is *Lr1*, *Lr9*, *Lr10*, *Lr19*, *Lr20*, *Lr23*, *Lr24*, *Lr25*, *Lr28 Lr29*, *Lr32*, *Lr39*, *Lr42*, *Lr43*, *Lr45*, *Lr46*, *Lr47*, *Lr53*, *Lr57*, *Lr58*, *Lr80*/ *Lr2a*, *Lr2b*, *Lr2c*, *Lr3*, *Lr11*, *Lr12*, *Lr13*, *Lr14a*, *Lr14b*, *Lr14ab*, *Lr15*, *Lr16*, *Lr17*, *Lr17b*, *Lr18*, *Lr21*, *Lr22a*, *Lr22b*, *Lr26*, *Lr27*, *Lr30*, *Lr33*, *Lr34*, *Lr35*, *Lr36*, *Lr37*, *Lr38*, *Lr40*, *Lr44*, *Lr48*, *Lr49*, *Lr51*, *Lr52*, *Lr67*.

This pathotype can infect the wheat lines carrying only *Lr26*. However, wheat cultivars having *Lr1*, *Lr9*, *Lr10*, *Lr19*, and *Lr24* would confer resistance to this pathotype. Since pathotype 57 was identified from a pure culture of 107-1, it has probably appeared *in situ* due to single step mutation for gain in virulence on *Lr3*. According to North

American system, this pathotype was designated as KGTPL and was different to North American pathotypes of *P. triticina*.

Molecular characterization

Among the 25 SSR primers screened to study the genetic variability, 22 were found polymorphic for 11 *P. triticina* pathotypes (Fig. 1). These primers could amplify 89 alleles with an average of 4.04 alleles per primer. Four SSRs (SSR-P AC-32, SSR-P TATC-40, SSR-P TATTG-60 and SSR-P CCAGAA-48) amplified maximum (6) while SSR-P GCTGTT-60 amplified lowest (2) number of alleles among the polymorphic primers. Eight primers *viz.* SSR-P GT-42, SSR-P TC-32, SSR-P GGT-45, SSR-P GTT-45, SSR-P TGGA-32, SSR-P TCTTT-50, SSR-P TAGCG-40, and SSR-P GCTGTT-60 amplified three alleles each. Primers SSR-P AG-40, SSR-P AGA-48, and SSR-P GTGGA-35 were monomorphic (Table 1).

The UPGMA algorithm and SAHN grouping resulted in two major groups of *P. triticina* pathotypes. The group I and II were further divided into two and three sub-groups, respectively. Sub-group 1a had three pathotypes (10, 20 and 107-1) while sub-group 1b had only one pathotype i.e. 107. Likewise, sub-group 2a, 2b and 2c had 3 (20-1, 57 and 77-5), 3 (20-2, 104-2 and 12-5) and 1 (77-9) pathotypes, respectively. Pathotypes 10 and 20 in sub-group 1a and 20-1 and 57 in sub-group 2a had 91% genetic similarity (Figure 2).

Discussion

New pathotypes in *P. triticina* arise through sexual reproduction. However, in absence of functional alternate hosts (Mehta 1940, 1952) new pathotypes evolve mainly through mutation and in some cases through somatic recombination (Johnson and Newton 1946; Park and Wellings 2012). Mutation is a very common phenomenon of evolving new pathotypes in wheat rust pathogens (Statler 1985). Since wheat rusts produce enormous number of spores and number of mutants arising is very high (Knott 1989). However, chances for the selection of new pathotypes on resistant gene pyramids are very less (Mundt 1990). Mostly mutations in *P. triticina* occur for one step gain in virulence as reported for *Lr19* in India (Bhardwaj et al. 2005) and for *Lr24* in Australia (Park et al. 2002). The phenomenon of mutation or variation in repository of rust

pathotypes is not frequent and has not been studied to a larger extent. Mostly it is considered as contamination of culture in the repository and is replaced from the cryo-preserve/ stored culture. In this study, the changes got picked up due to distinct mixture on one differential each. This starting point led us to unravel the phenomenon of *in situ* variation in *P. triticina*.

There had been a few sporadic cases of recorded variations in color and pathogenicity of wheat rusts. D Oliveira (1939) observed orange colored mutant in race 14 of *P. anomala*. Roberts (1936) mentioned a mutation for pathogenicity in a culture of *P. triticina*. Likewise, Gassner and Straib (1932) also published a number of recurrent mutations and detection of identical mutants having more virulence than the mother race. We have also come across mutant for colour change in four pathotypes earlier (Prashar et al. 1996). These mutants must have resulted due to the solar radiations falling directly on the plants of source pathotypes (Brown and Sharp 1970; Rao and Lele 1962). A change to race 178 in a stored pure culture of 52 of *P. graminis tritici* probably due to mutation has been reported long back (Newton and Johnson 1939). A number of weather factors and chemicals may induce mutation in rust genotype (Volkova et al. 2020).

The similarity based on the SSR genotypes has resulted in two clades which further ramified into 2 and 3 subclades. Some correlation occurred between SSR genotypes similarity and virulence phenotypes. Among 38 isolates of *P. triticina* from Pakistan, 27 SSR groups were observed each with a high degree of heterogeneity and significant correlation with pathogenicity indicating clonal reproduction (Kolmer et al. 2017) as observed in our study. Similarly, a correlation between virulence phenotypes and SSR genotypes was observed in a previous study (Prasad et al. 2017). The virulence phenotypes MBDSS and MCDSS had a definite correlation with SSR genotypes. The phenotypes from Turkey, Europe, Central Asia, the Middle East, North America, and South America, reflecting a possible movement between continents (Kolmer 2019). In China also, two main DNA groups were found in the *P. triticina* population (Zhang et al. 2020). In our case there is *in situ* evolution of pathotypes with gain of virulence on one gene. The observation is interesting and unique as there are counted number of reports on *in situ* changes in pathotypes.

Conclusion

In absence of functional alternate hosts, wheat rusts evolve through mutation, and parasexuality. Wheat rust pathogens produce urediniospores in huge number of which many are mutants. These mutants are the result of forward mutation resulting in gain of virulence on one resistance gene. In some case there are recessive mutations which are rare and result in loss of virulence. Occurrence of mutations in repository is less common. In present manuscript we report *in situ* changes in the mother cultures. In pure cultures of pathotype 107-1(45R35=JCGPL) and 20(5R27=SGQPL) of *P. triticina* mixture of infection types were observed on *Lr3* and *Lr15*, respectively. Single pustule isolations in both the pathotypes yielded two new pathotypes designated as 57 (45R39= KGTPL) and 20-2 (57R27= SHKPL). Pathotype 57 was one step gain in virulence on *Lr3* in 107-1 whereas 20-2 on *Lr15* when compared with pathotype 20. The difference in virulence on one gene to each mother culture and their absence in the field samples led us to claim gain in virulence. These are one of the few records of *in situ* mutations for virulence.

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Author contribution

SCB and SK identified variants, conducted experimentation, tabulation; OPG, PP, CL and SA helped in molecular studies, analysis; SCB wrote the draft, SK, OPG and PP improved the manuscript.

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Declarations

This article does not contain any studies with human or animal subjects

Ethics approval

The authors declare no competing interests

Conflict of interest

The authors declare no competing interests

Data availability

Authors confirm that all relevant data are included in the article and/or its supplementary information files.

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373 **Table 1.** List of SSR markers used for genotypic profiling of the *Puccinia triticina* pathotypes

S.No.	Primer code	Forward primer sequence (5' - 3')	Reverse primer sequence (5' - 3')	Number of amplified alleles
1	SSR-P GT-42	GGGGTGAGTTTCTGTATTGA	CAGAGATCATCGAGGAAAAC	3
2	SSR-P AG-40	CTTTCTTACCCCCACAACTAC	CTCTCTCTCTCTCTCTCTCTCTC	1
3	SSR-P CT-36	ACTCTCAAACTCACTCCCTCT	GACTACACCATTTCAAACCAA	5
4	SSR-P AC-32	ACAAAACAAACAGATCCACTG	ACGTATTTGGTCTTCTTCTCC	6
5	SSR-P TC-32	TAGAATTCTTGGTAGGACGAG	CGGTCAGAGTGTCTGTCAATA	3
6	SSR-P CAA-60	AACTGCGAGGACAACTTTC	CGTCTGCTGAGTTTCTGTATT	4
7	SSR-P AGA-48	CAAACGAAGCAAACTAGAAGA	TGTTGTTGTTGTTGTTGTTGT	1
8	SSR-P GGT-45	GCTGCTTGATGGAGGATG	AACAGCTTCAGCGACCTC	3
9	SSR-P GTT-45	GATGAGGTTGTTGAAGGAGA	ACCAGAACCAACAAAACAAC	3
10	SSR-P CAC-45	GAAGACCATCCTCACGACT	TTCTTCTTGTTGGTTTTTCTG	5
11	SSR-P CAAC- 44	AGCGTAGAGTCAGTCAGTCAG	GCTAATAAGGAGATTGGGTTG	4
12	SSR-P TATC-40	AAGCGTGATCAAGTAGGTTTA	GATGGACAAGTAGAGAGATGG	6
13	SSR-P TCCG-36	TTTTTCTAGATCCACCAACC	TACGAACAGGAGTCCCTCA	5
14	SSR-P AGCC-32	GGGAAAGAAAAACACATCCT	GTCTCTTCGCTGATCTGG	4
15	SSR-P TGGA-32	GCATTTGTTTTTGTTGATTG	AGACACCTCCCCTTAAAAAC	3
16	SSR-P TATTG-60	TCAAACAACTTCATCCTGAAC	ATGTGATATCTTTTGGATTGG	6
17	SSR-P TCTTT-50	GGGTTTATATGGTGGGTGT	GTTGAGTGGGTGAGATGAGTA	3
18	SSR-P TAGCG-40	GCTAACGCTATGCAAAATAGA	CAGTTCAGTACCCACCAGTTA	3
19	SSR-P CCCGT-35	TTTTTGAAGGGCTTGTAGTG	AAAGGGACAGTTATGGGATAG	4
20	SSR-P GTGGA-35	TGTTTGGGAGTGTATGTGTG	GCCGAGTACCACTACCACTA	1
21	SSR-P TGAGGA-48	GTATCGGATGTTGTTGTGAAG	CTACCAAGTCTATCCGTCCTC	4
22	SSR-P ACAAAC-48	ATACATTTTGGTTACCCACCT	TGTGTTTGTTTGTGTTTGTGT	4
23	SSR-P CCAGAA-48	GAAGAACTCGATCCCAGAA	CTGGTTTGTTGTTGTTGTTG	6
24	SSR-P CCGCAC-60	TTTTGGCTGAAGTTCTGAAT	GTTGTTGAGTTGAAGGACAAG	2
25	SSR-P GCTGTT-60	GATGAGCAGCATGAGGAG	CACCAGAACAACATACTCCAT	3

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376 **Table 2.** *In situ* evolved new pathotypes of *Puccinia triticina*

Name of pathotype			Reaction types on differentials							
Vernacular	Binomial	North American	*Lr*1	*Lr*2a	*Lr*3	*Lr*10	*Lr*15	*Lr*20	*Lr*23	*Lr*26
10	13R19	SGHLL	3+	3+	;	;	;	;1	;	0;
20	5R27	SGQPL	3+	3+	;	;	;	3+	;	0;
20-1	93R57	NHKTL	3+	3+	;	3+	;	3+	;1	3+
20-2*	57R27	SHKPL	3+	3+	;1	;	3+	3+	;	0;
107	45R3	JBGPL	;	3+	;1	;	3+	;	;-	;
107-1	45R35	JCGPL	;	3+	;	;	3+	;	;	3+
57*	45R39	KGTPL	;-	3+	3+	;	3+	;-	;-	3+
77-5	121R63-1	THTTM	3+	3+	3+	3+	3+	3+	3+	3+
77-9	121R60-1	MHTKL	3+	;-	3+	3+	3+	3+	3+	3+
104-2	21R55	PHTTL	3+	12+	3+	3+	0;	;1	3+	3+
12-5	29R45	FHTPM	0;	12+	3+	;1	0;	3+	3+	3+

377 *New pathotypes

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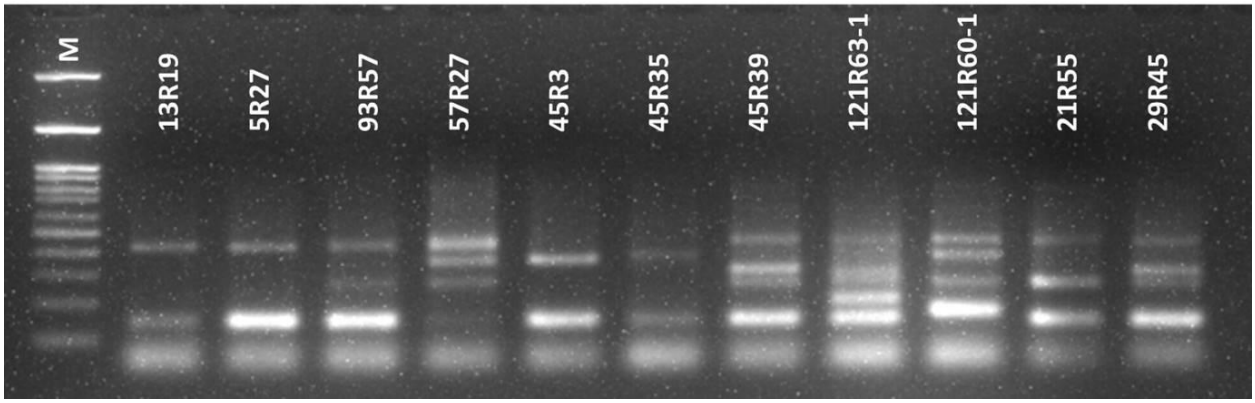
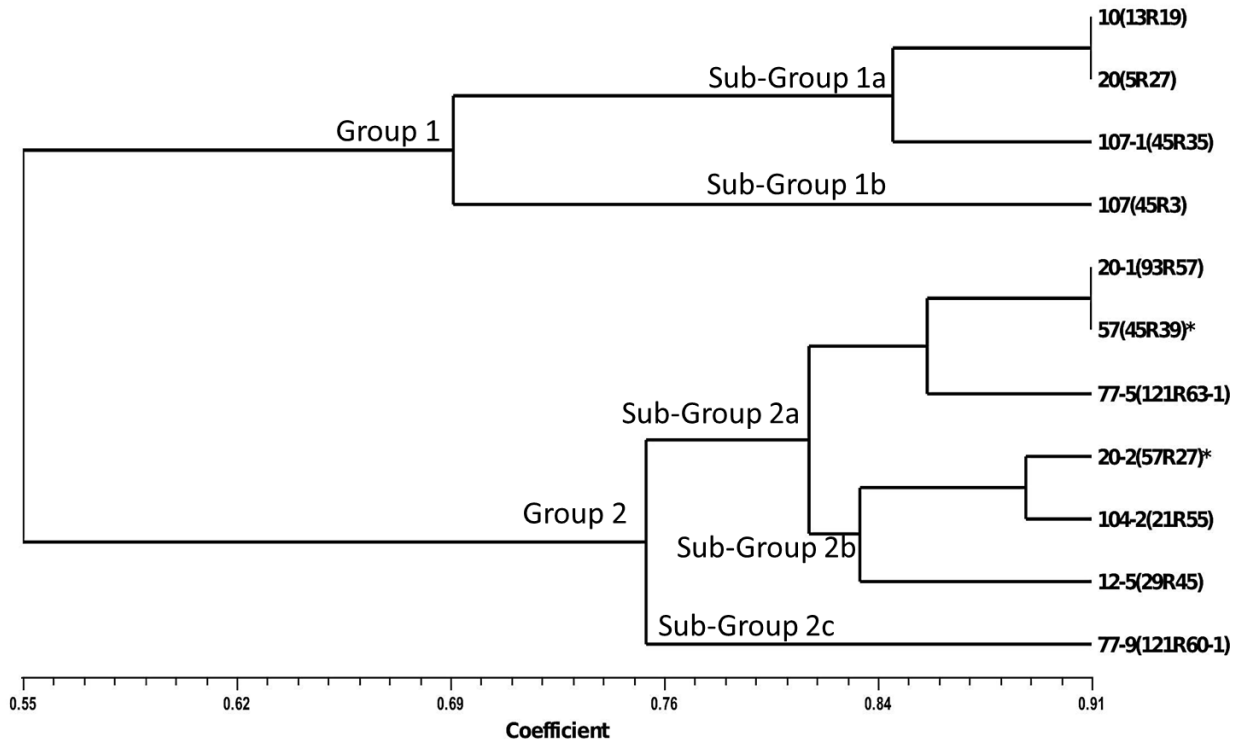


Fig. 1 Allelic pattern among *Puccinia triticina* pathotypes generated with SSR marker TATTG-60



391

392 **Fig. 2** SSR analysis based genotypic similarity among eleven pathotypes of *Puccinia triticina*